Characterization of NIST Human Mitochondrial DNA SRM-2392 and SRM-2392-I Standard Reference Materials by Next Generation Sequencing

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Abstract

Standard Reference Materials SRM 2392 and 2392-I are intended to provide quality control when amplifying and sequencing human mitochondrial genome sequences. The National Institute of Standards and Technology (NIST) offers these SRMs to laboratories performing DNA-based forensic human identification, molecular diagnosis of mitochondrial diseases, mutation detection, evolutionary anthropology, and genetic genealogy. The entire mtGenome (~16569 bp) of SRM 2392 and 2392-I have previously been characterized at NIST by Sanger sequencing. Herein, we used the sensitivity, specificity, and accuracy offered by next generation sequencing (NGS) to: (1) re-sequence the certified values of the SRM 2392 and 2392-I; (2) confirm Sanger data with a high coverage new sequencing technology; (3) detect lower level heteroplasmies (< 20 %), and thus (4) support mitochondrial sequencing communities in the adoption of NGS methods. To obtain a consensus sequence for the SRMs as well as identify and control any bias, sequencing was performed using two NGS platforms and data was analyzed using different bioinformatics pipelines. Our results confirm five low level heteroplasmy sites that were not previously observed with Sanger sequencing: three sites in the GM09947A template in SRM 2392 and two sites in the HL-60 template in SRM 2392-I.

Keywords: Standard Reference Material (SRM), Next generation sequencing, Mitochondrial DNA, Heteroplasmy

INTRODUCTION

Sequence analysis of the mitochondrial (mt) genome has proven valuable for evolutionary anthropology, genetic genealogy, and human identification in cases where forensic biological evidence contains minute, highly degraded, or no genomic DNA [1-8]. Mitochondrial DNA (mtDNA) has also been an informative marker for diagnoses of a number of diseases [9-12] and detection of pathogenic point mutation [13].

The Applied Genetics group at the National Institute of Standards and Technology (NIST) has produced well characterized human mtDNA Standard Reference Materials (SRMs) [14-16]. SRMs are intended to provide quality control for various disciplines performing polymerase chain reaction (PCR) and sequencing of human mtDNA for forensic identification, genetic structure of population, and molecular diagnosis of mitochondrial diseases. The mtDNA SRMs can also be used for quality assurance when assigning sequence confidence to in-house control materials. This assures that the DNA sequencing methods and interpretations being used are conducted correctly [14-16].

The most widely used method of mtDNA typing focuses only on a portion of the mtGenome. The standard assay is based on Sanger sequencing of the hypervariable regions located in the non-coding displacement loop (D-loop) or control region [17-19]. This region contains a high density of sequence variation; however, under certain circumstances [20], examination of the control region only provides limited information and certain individuals will remain indistinguishable. Sequencing the entire mtGenome provides a higher discrimination power that can allow for further resolution of individuals coming from various populations in addition to those with common HVI/HVII types [21-24]. Although sequencing the entire 16569 bases of human mtGenome can be accomplished with existing Sanger methods [25], the workflow can be labor intensive, expensive, and time consuming.

Recently developed next generation sequencing (NGS) technologies have offered manageable workflow capabilities to mtGenome sequencing, increased the depth of coverage at reduced cost and time, and reliably detected intra-individual mtDNA variation [26-30]. With the introduction of bench top DNA sequencers employing different chemistries and technical approaches [31, 32], NGS technology has impacted mtGenome variant discovery and been adopted in several research laboratories [21, 29, 30, 33-35].

60 The entire mtGenome of the SRM 2392 and 2392-I have been characterized at NIST
61 by Sanger type sequencing [14-16]. An update of the SRM 2392 certificate of analysis was
62 released in 1/12/2009. The updated certificate corrected a sequencing error in the SRM-
63 CHR at position 4929 in the mtGenome. Multiple sequencing experiments confirmed a 'T'
64 nucleotide present at position 4929 and not a 'C' as inferred by the 2003 certificate. In
65 order to support the adoption of NGS technology in **forensics and clinical DNA laboratories**,
66 NIST has recently undertaken steps to re-sequence the certified mtDNA SRMs using
67 orthogonal NGS platforms. The enhanced sensitivity of NGS can increase the information
68 content of NIST SRMs, reveal any sequence variation that is undetectable with Sanger-
69 based mtDNA analysis, and increase the confidence in the accuracy of the materials
70 through investigating suspected sources of bias.

71 Here we present the sequencing results of three mtGenomes obtained from NIST
72 mtDNA SRMs 2392 (CHR and GM09947A) and 2392-I (HL-60). To reliably detect sequence
73 variants arising from the DNA SRM templates and identify any potential bias in a specific
74 NGS chemistry and/or informatics pipelines, samples were prepared using two different
75 library preparation chemistries and were sequenced on two different benchtop NGS
76 platforms, the Ion Torrent Personal Genome Machine (PGM) (Life Technologies, San
77 Francisco, CA) and the Illumina MiSeq (Illumina, San Diego, CA). The results were analyzed
78 with various bioinformatics pipelines and compared to the sequencing data obtained with
79 traditional Sanger typing methodology.

80 **This work focuses on the use of NGS methods to assign high confidence base calls in**
81 **the SRM 2392 series of materials. The purpose of the material is to provide support for**
82 **laboratories validating and implementing mtDNA testing using NGS technologies. The**
83 **intention is not to recommend a sequencing platform, bioinformatics software, or a specific**
84 **threshold for identification of base variants.**

MATERIALS AND METHODS

SRM Samples

SRM 2392 became available to the public in December 1999 and is packaged in a single box containing three components: (1) extracted DNA from cell culture line CHR; (2) extracted DNA from cell culture line GM09947A; and (3) cloned HV1 region of the DNA template designated CHR containing a C-stretch which covered base pairs 16133 through 16569 and 1 through 40. CHR is an Epstein-Barr virus-immortalized human white blood cells prepared by the American Type Culture Collection (ATCC; Rockville, MD) [15]. GM09947A cell culture line is a human lymphoid cell line that was transformed with the Epstein-Barr virus and immortalized as a cell culture line by Life Technologies, Inc. (Grand Island, NY) [15]. Both cell culture lines were obtained from normal individuals [15]. SRM 2392-I, first released in 2003, contains one DNA template and is certified for the sequences of the human mtDNA from a promyelocytic cell line (HL-60) derived from the peripheral blood leukocytes from an individual with acute promyelocytic leukemia [14]. The DNA from CHR was extracted using a procedure that enhanced the concentration of mtDNA and reduced, versus fully eliminating, the nuclear DNA. The DNA isolated from GM09947A and HL-60 is the total extracted DNA including nuclear DNA [14-16]. To verify the primary DNA sequence for the mtGenomes of NIST SRM 2392 and 2392-I (NIST, Gaithersburg MD, USA), the materials were sequenced on the Ion Torrent Personal Genome Machine (PGM) (Life Technologies, San Francisco, CA) and Illumina MiSeq (Illumina, San Diego, CA) platforms. SRM 2392 CHR, SRM 2392 GM09947A, and SRM 2392-I will be referred to in the text as SRM-CHR, SRM-9947A, and SRM-HL-60, respectively.

PCR amplification of mtDNA

MtGenomes were first amplified according to the Human mtDNA Genome Guide for the Illumina Sequencing Platform (Part # 15037958 Rev. B, Illumina) [36]. Two separate PCRs were amplified that produced two long amplicons (9.1 kb and 11.2 kb) spanning the entire 16569 bases of the human mtDNA genome [36, 37]. The TaKaRa LA Taq kit (Takara Clontech, Mountain View CA, USA) was used to amplify the mtGenome of the SRM components. Thermal cycling parameters were: initial denaturing for 5 minutes at 94 °C then 30 cycles of (98 °C for 15 s, 68 °C for 10 s [ramp rate 0.2 °C/s], 60 °C for 15 s, then 68

°C for 11 minutes), followed by a final extension at 72 °C for 10 minutes and 4 °C hold. Reaction conditions were: 1 X LA PCR Buffer II (with 25 mM MgCl₂), 400 nM dNTPs, 400 nM each primer, 2.5 Units TaKaRa LA Taq, and 1 ng of genomic template DNA. PCR products were quantified on an Agilent Bioanalyzer 2100 (Agilent, Santa Clara CA, USA) system with the DNA 12,000 Kit. Based on the quantitation results, amplicons were adjusted to a concentration of 0.2 ng/μL with molecular biology grade water, and PCR products for each sample were combined on an equal-volume basis. To assess the reproducibility and precision of the PGM system, two PCR replicates were performed and sequenced for each SRM sample, with each replicate incorporating different barcode sequences.

Library Building and Sequencing on Ion PGM Torrent Platform

A total of approximately 300 ng of input DNA was used for automated library preparation using the AB Library Builder System (ThermoFisher Scientific), Ion Plus Fragment Library Kit for AB Library Builder System (ThermoFisher Scientific) and IonXpress Barcode Adapters 1-16 Kit (ThermoFisher Scientific) according to the manufacturer's recommended procedure [38]. Size selection was performed off-platform with the Agencourt Ampure XP (Beckman Coulter, Brea CA) system. Size selected library was quantitated using the Ion Library TaqMan Quantitation Kit (ThermoFisher Scientific) on an ABI 7900HT Fast Real-Time PCR System (ThermoFisher Scientific) [39], and libraries were diluted to a concentration of 20 pM for subsequent pooling and sequencing template preparation. Templated Ion Sphere Particles were prepared and loaded into an Ion 318 Chip v2 BC (ThermoFisher Scientific) by the Ion Chef System (ThermoFisher Scientific) using the Ion PGM Hi-Q Chef Kit (ThermoFisher Scientific) [40]. Sequencing was performed on the PGM using 500 flows (200 bp read length).

Initial data analysis was performed with Torrent Server Suite software version 4.4.3 (ThermoFisher Scientific) to generate FASTQ files which were then analyzed for variants relative to the *Homo sapiens* mitochondrion reference genome (NCBI Reference Sequence NC_012920.1) using different bioinformatic pipelines.

Library Building and Sequencing on Illumina MiSeq Platform

For Illumina MiSeq sequencing, PCR products were diluted to a nominal concentration of 0.2 ng/ μ L in molecular biology grade water. Libraries were generated using the Nextera XT DNA Library Preparation Kit with the Nextera XT Index Kit V2 Set A (Illumina) according to the manufacturer's recommended procedure [36]. Libraries were quantitated with the ABI Prism Library Quantitation Kit for Illumina Platforms (Kapa Biosystems, Wilmington MA, USA). Based on the results of the quantitation, libraries were adjusted to a concentration of 20 pM then pooled and sequenced on the MiSeq using a MiSeq Reagent Kit v3 (600 Cycles) (Illumina).

Data Analysis

Identification of heteroplasmic and homoplasmic sites from the NGS data was analyzed using CLC bio Genomics Workbench version 9.0 (Qiagen) [41], Galaxy [42-45], and GeneMarker® HTS (GM-HTS) (SoftGenetics, Inc.) [46, 47]. Detailed analysis parameters for CLC and Galaxy software pipelines are shown in Figures S1 and S2, respectively.

For the detection of point heteroplasms in GM-HTS, reads in FASTQ format were aligned to the entire circularized mtDNA revised Cambridge Reference Sequence rCRS [48], using the following alignment options: built in motif file, default values for both identity and soft clipping based on the sequencer used. Variant filter settings were as follows: variant percentage $\geq$ (20 %, 10 %, 5 %, and 1 %), variant allele coverage $\geq$ 40, total coverage $\geq$ 200, allele score difference $\leq$ 10, and allele balance ratio for SNPs ($\leq$ 2.5) and indels ($\leq$ 5.0).

Heteroplasmy Verification using STRait Razor

To further verify the accuracy of detection of heteroplasmy calls obtained from CLC Bio, Galaxy and GM-HTS, FASTQ files generated with the PGM and MiSeq were directly analyzed with the STR Allele Identification Tool – Razor (STRAit Razor) [49, 50]. Sequence reads containing both leading and trailing flanking regions (Table S1) directly adjacent to the identified length and point heteroplasmy were extracted from the NGS-based mtDNA SRM raw data by modifying the STRait Razor configuration file with mtDNA recognition sequences flanking the regions of interest. The filtered reads were evaluated and counted.

The frequency of heteroplasmy was calculated by dividing the number of filtered reads containing the variant by the total number of filtered reads.

RESULTS AND DISCUSSION

MtGenome Depth of Coverage for the Three SRM Samples Across the PGM and MiSeq Platforms

As has been previously observed [51-53], mitochondrial sequencing results in a varying range of depth of coverage depending on the platform, degree of multiplexing, and alignment method used. As observed from reads aligned in CLC software, the average depth of sequencing coverage on the MiSeq for SRM-CHR, SRM-9947A, and SRM-HL-60 was 16k (range 3,758-41,065), 17k (range 3,088-46,408), and 21k (range 3,504-55,548), respectively. Samples sequenced on the PGM had a lower average depth of coverage than those sequenced on the MiSeq. For example, samples sequenced on the PGM and aligned in CLC yielded an average read depth of 4k, range of: 732-10,941 for SRM-CHR, 791-10,667 for SRM-9947A, and 755-10,581 for SRM-HL-60. This coverage variation is attributed to the higher total sequence output of the MiSeq platform as compared to the Ion Torrent PGM [51].

mtDNA SRM Homoplasmic Variants

Variants, positions across the mtGenome where the primary base differs from the reference rCRS, were analyzed for each of the SRM mtDNA sequences analyzed. Table 1 shows all the variants found in SRM-CHR, SRM-9947A, and SRM-HL-60 mtDNA sequences generated on both the MiSeq and PGM platforms. Also, Table 1 shows that the majority of the variants are homoplasmic (> 98 %), previously characterized with the Sanger Sequencing method, and reported in the published certified reference materials for the mtDNA SRMs [14-16].

We detected 33, 17, and 32 homoplasmic variants in SRM-CHR, SRM-9947A, and SRM-HL-60 NGS data, respectively. The consensus data from the different NGS platforms for the mtDNA SRMs was in concordance with certified values determined by the Sanger Sequencing typing method for all homoplasmic variants.

Sequence Heteroplasmy Discovery in the NIST Human mtDNA SRM-9947A and SRM-HL-60

Heteroplasmy is the co-existence of multiple types of mtDNA sequences within the same cell, tissue, or individual [54, 55]. Heteroplasmy can be observed as: point heteroplasmy (PHP) and/or length heteroplasmy (LHP). PHP is the presence of mixture of two DNA bases at a single position. LHP is the presence of at least two populations of mtDNA sequences that differ in the number of nucleotides and thus their length [55-58]. LHP occurs in the C-stretches of HV I (16184-16193), II (303-315), and III (568-573) regions as well as in the AC repeat region (515-524) of the HVIII [58-60]. Both types of heteroplasmic variation have been previously observed within mtDNA SRMs by Sanger sequencing [14-16].

Due to the higher sensitivity and coverage at each mtDNA site, an increase in the number of heteroplasmy positions were further detected using the NGS technologies. To have confidence in the heteroplasmy call, only variants which were observed across all NGS sequencing experiments on different platforms, with the different bioinformatics pipelines, and further confirmed with STRait-razor method [49, 50] were considered to be novel heteroplasmic sites.

Previously with Sanger sequencing, one instance of PHP was observed in SRM-CHR at position 64 (C/T) (herein Reference/Variant). When SRM-CHR was sequenced on the NGS platforms, no further heteroplasmy sites were detected across the platforms, PCR replicates, and pipeline analyses (Table 1A).

Three new PHP sites were identified from the PGM and MiSeq data of SRM-9947A. Table 1B depicts the newly characterized positions at 1393 (G/A), 3242 (G/A), and 7861 (T/C). A previous study also detected the 1393 and 7861 sites from the SRM-9947A mtDNA in data generated on the Roche 454 GS Junior sequencer [34]. The authors validated sites 1393 and 7861 using single base extension assays with both capillary electrophoresis and MALDI-TOF. Also, an early study published by the Applied Genetics Group at NIST on characterizing NIST standard reference materials by NGS also detected both 1393 and 7861 across different NGS platforms including HiSeq 2000 (Illumina) as well as SOLiD 5500 system [61].

Table 1C shows two novel PHP sites observed in SRM-HL-60: 2445 (T/C) and 5149 (C/T). The PHP at 12071 (T/C) was characterized previously [14].

Five novel heteroplasmies discovered in the human mtDNA SRM-9947A and SRM-HL-60 were not reliably detected by the Sanger sequencing studies performed at NIST and thus were not reported in the NIST SRM 2392 and 2392-I certificates. However, 64 (C/T) in SRM-CHR and 12071 (T/C) in SRM-HL-60 were further confirmed and quantitatively evaluated with this NGS study. Together these results show that the NGS system is extremely sensitive, increases the amount of information from the mtDNA SRMs, and allows detection of rare variants in comparison to Sanger sequencing.

A.

Nucleotide Position	rCRS Reference	SRM-CHR Sanger Call	SRM-CHR PGM Consensus	SRM-CHR MiSeq Consensus
64	C	C/T	C/T	C/T
73	A	G	G	G
195	T	C	C	C
204	T	C	C	C
207	G	A	A	A
263	A	G	G	G
<u>309.1</u>		<u>C</u>		
<u>315.1</u>		<u>C</u>		
709	G	A	A	A
750	A	G	G	G
1438	A	G	G	G
1719	G	A	A	A
2706	A	G	G	G
4769	A	G	G	G
4929	C	T	T	T
5186	A	G	G	G
6221	T	C	C	C
6371	C	T	T	T
6791	A	G	G	G
7028	C	T	T	T
8503	T	C	C	C
8860	A	G	G	G
11719	G	A	A	A
11878	T	C	C	C
12612	A	G	G	G
12705	C	T	T	T
13708	G	A	A	A
13966	A	G	G	G
14470	T	C	C	C
14766	C	T	T	T
15326	A	G	G	G
16183	A	C	C	C
16189	T	C	C	C
<u>16193.1</u>		<u>C</u>		
16223	C	T	T	T
16278	C	T	T	T
16519	T	C	C	C

B.

Nucleotide Position	rCRS Reference Sequence	SRM-9947A Sanger Call	SRM-9947A PGM Consensus	SRM-9947A MiSeq Consensus
93	A	G	G	G
195	T	C	C	C
214	A	G	G	G
263	A	G	G	G
<u>309.1</u>		<u>C</u>		
<u>309.2</u>		<u>C</u>		
<u>315.1</u>		<u>C</u>		
750	A	G	G	G
1393	G	G	G/A	G/A
1438	A	G	G	G
3242	G	G	G/A	G/A
4135	T	C	C	C
4769	A	G	G	G
7645	T	C	C	C
7861	T	C	T/C	T/C
8448	T	C	C	C
8860	A	G	G	G
9315	T	C	C	C
13572	T	C	C	C
13759	G	A	A	A
15326	A	G	G	G
16311	T	C	C	C
16519	T	C	C	C

C.

Nucleotide Position	rCRS Reference Sequence	SRM-HL-60 Sanger Call	SRM-HL-60 PGM Consensus	SRM-HL-60 MiSeq Consensus
73	A	G	G	G
150	C	T	T	T
152	T	C	C	C
263	A	G	G	G
295	C	T	T	T
<u>315.1</u>		<u>C</u>		
489	T	C	C	C
750	A	G	G	G
1438	A	G	G	G
2445	T	T	T/C	T/C
2706	A	G	G	G
4216	T	C	C	C
4769	A	G	G	G
5149	C	C	C/T	C/T
5228	C	G	G	G
5633	C	T	T	T
7028	C	T	T	T
7476	C	T	T	T
8860	A	G	G	G
10172	G	A	A	A
10398	A	G	G	G
11251	A	G	G	G
11719	G	A	A	A
12071	T	T/C	T/C	T/C
12612	A	G	G	G
13708	G	A	A	A
14569	G	A	A	A
14766	C	T	T	T
15257	G	A	A	A
15326	A	G	G	G
15452	C	A	A	A
15812	G	A	A	A
16069	C	T	T	T
16193	C	T	T	T
16278	C	T	T	T
16362	T	C	C	C

Table 1. Variants reported as differences relative to the rCRS.

A. SRM-CHR; B. SRM-9947A; and C. SRM-HL-60. Novel heteroplasmic sites identified by NGS (in bold and highlighted in grey) and that have not been reliably detected using Sanger sequencing are called in the following manner (Nucleotide position where mixed bases are seen followed by the Reference Base/Variant Base). Those sites were: 1393 G/A; 3242 G/A; and 7861 T/C in SRM-9947A, and 2445 T/C and 5149 C/T in SRM-HL-60. Heteroplasmy sites previously published in the SRMs certificates, 64 (C/T) in SRM-CHR and 12,071 (T/C) in SRM-HL-60, are highlighted in bold font. Insertions of C residues in the HV1 and HV2 regions are underlined and in italics.

Different Threshold Cutoff Application

Several pilot analyses were conducted in order to identify the most effective minimum variant frequency setting for use in analysis with CLC bio, Galaxy, and GM-HTS. Software was configured with cutoffs at 20 %, 10 %, 5 %, and 1 %. The observed heteroplasmy sites were initially detected at different cutoff levels (Table 2). When a 20 % variant allele frequency cut-off was applied to CLC, Galaxy, and GM-HTS workflows to analyze the mtDNA SRM data generated on both the PGM and MiSeq, several heteroplasmy sites were called: 64 (C/T) in SRM-CHR, 7861 (T/C) in SRM-9947A, and 12071 (T/C) in SRM-HL-60 (Table 2). As mentioned previously, only 64 (C/T) and 12071 (T/C) had been detected in Sanger sequencing studies with heteroplasmy detection thresholds roughly greater than or equal to 10 %. When the threshold was set to 10 %, 1393 (G/A) was called in SRM-9947A. Upon applying a 5 % threshold, 2445 (T/C) and 5149 (C/T) were called in the SRM-HL-60.

A substantial number of calls were reported as variants in the SRM sequences when a 1 % point heteroplasmy filter was applied. At this low cutoff level, the distinction between true mtDNA variants and noise was challenging. Most of the variant calls observed in the MiSeq data were not concordant with those generated on PGM and vice versa (data not shown). However, of all the calls at this very low cutoff level, one particular position of interest 3242 site in SRM-9947A shows what appears to be a low-level heteroplasmy (G/A) and was reproducible across platforms, among PCR replicates, and with the different bioinformatics pipelines. The 3242 was not detected with Sanger studies nor reported in the certificate as a variant.

Threshold	SRM-CHR	SRM-9947A	SRM-HL-60
20%	64 C/T	7861 T/C	12071 T/C
10%	64 C/T	1393 G/A	12071 T/C
		7861 T/C	
5%	64 C/T	1393 G/A	2445 T/C
		7861 T/C	5149 C/T
			12071 T/C
1%	64 C/T	1393 G/A	2445 T/C
		3242 G/A	5149 C/T
		7861 T/C	12071 T/C

Table 2. Detection of PHP sites in each of the mtDNA SRM material as a function of allele calling thresholds.

To be included in the table, PHP sites must be detected on both the MiSeq and PGM platforms, in both PCR replicates, and across the different bioinformatics pipelines. The 64 C/T and 12071 T/C are listed in the current SRM certificates and were previously detected by Sanger sequencing.

Frequency of Heteroplasmy Sites

We calculated the frequency of heteroplasmic sites by each method to further characterize the reference materials. Frequency was calculated as the number of reads containing the base call divided by the total number of reads covering that position. Quantitative values for the heteroplasmic sites are presented in Table 3.

There was agreement across all NGS platforms and bioinformatics pipelines including STRait Razor as to the identification and quantitation of mixed bases at each of the studied heteroplasmic positions, with the exception of 7861 site in SRM-9947A (Table 3B). We observed variation in the frequency of this specific heteroplasmy with the reads generated on the PGM platform. MiSeq reads contained variant allele C in proportion of ~88 % of total reads covering that position and a lower frequency when the same region was sequenced on the PGM platform (Table 3B). As seen in the Integrative Genomics Viewer (IGV) [62], the heteroplasmy site 7861 is located in a region surrounding a stretch of three contiguous C residues at positions 7862 through 7864 (Figure 1). Many PGM reads were interpreted by the software as a false deletion at position 7861. The terminator-free sequencing chemistry utilized by Ion Torrent is known to have difficulty assigning integer

base call values around homopolymer stretches (> 3 bases), a situation that applies to 7861 [63, 64]. Only PGM reads with mapping and base quality ≥ 20 were retained by the bioinformatics pipelines (CLC and Galaxy) to compute the variant allele frequency ("C") at 7861. This filtering step of low quality reads caused a decrease in the average read coverage per 7861 site, and thus a lower frequency level of "C". The pattern of heteroplasmy at 7861 was also evaluated in GM-HTS. PGM reads contained proportions of ~ 83 % variant allele C, 13 % of allele T, and 4 % deletion of total reads covering that position. CLC and Galaxy analyses generated a lower variant "C" frequency of 68.5 % and 78.5 %, respectively. Therefore, the variation in frequency calling of this site is attributed to chemistry related limitations as well as different parameter settings in each software (e.g. Q-score filtering, C-stretch resolution).

The algorithm of STRait Razor does not take into consideration the quality of bases sequenced. The frequency of all the heteroplasmy sites sequenced on PGM and MiSeq and computed from Strait Razor output was in concordance with the frequencies calculated by CLC, Galaxy, and GM-HTS except for the 7861 site (Table 3). Although the allele frequency at 7861 site computed by STRait Razor was similar to that of MiSeq (~ 88 %), it should be noted that manual inspection of the FASTQ files of the PGM reads containing 7861 confirmed lower base quality reads as compared to those sequenced on MiSeq.

A	SRM-CHR	
	Position 64	
Platforms & Analysis	REF C%	VAR T%
PGM (CLC)	69	31
MiSeq (CLC)	68	32
PGM (Galaxy)	67.5	32.5
MiSeq (Galaxy)	69	31
PGM (GM-HTS)	70	30
MiSeq (GM-HTS)	70	30
PGM (STRait Razor)	67	33
MiSeq (STRait Razor)	69	31

B	SRM-9947 A					
	Position 1393		Position 3242		Position 7861	
Platforms & Analysis	REF G%	VAR A%	REF G%	VAR A%	REF T%	VAR C%
PGM (CLC)	84.5	15.5	97	3	31.5	68.5
MiSeq (CLC)	82	18	96	4	12	88
PGM (Galaxy)	84.5	15.5	97	3	21.5	78.5
MiSeq (Galaxy)	82	18	95	5	11	89
PGM (GM-HTS)	85	15.0	97	3	13	83
MiSeq (GM-HTS)	83	17.0	97	3	12	88
PGM (STRait Razor)	84	16	97	3	12	88
MiSeq (STRait Razor)	83	17	96	4	11	89

C	SRM-HL-60					
	Position 2445		Position 5149		Position 12071	
Platforms & Analysis	REF T%	VAR C%	REF C%	VAR T%	REF T%	VAR C%
PGM (CLC)	92.5	7.5	91.5	8.5	50	50
MiSeq (CLC)	91	9	94	6	50	50
PGM (Galaxy)	92.5	7.5	91.5	8.5	49.5	50.5
MiSeq (Galaxy)	91	9	93	7	49	51
PGM (GM-HTS)	93	7	93	7	54	46
MiSeq (GM-HTS)	93	6	94	6	52	48
PGM (STRait Razor)	93	7	92	8	50	50
MiSeq (STRait Razor)	92	8	91	9	49	51

Table 3: Values for all point heteroplasmies in mtDNA SRMs generated on PGM and MiSeq NGS platforms using four different bioinformatics software (CLC bio, Galaxy, GM-HTS, and STRait Razor). (A) SRM-CHR; (B) SRM-9947A; (C) SRM-HL-60. The variant frequencies for PHP in PGM reads depicted in the table are the average of the variant frequency levels found in both PCR 1 and PCR2 reads. The variation observed in the quantitation of mixed bases at 7861 site (SRM-9947A) with the reads generated on the PGM platform is highlighted in bold font.

Figure 1. Visualization of BAM files of the

SRM-9947A in IGV before performing any quality-filtering. Sequence pattern of reads containing 7861 position generated on PGM (left panel) and MiSeq (right panel). The 7861 site resides immediately before a homopolymer tract consisting of > 3 identical bases of Cs (red box). The PGM sequencing reads showed a tendency to be miscalled as seen with the deletion pattern and at the 7861 site (left panel). However, low number of false deletions were detected with the MiSeq data (right panel).

All point heteroplasmies analyzed from the mtDNA SRMs' output data generated on both NGS platforms were compared with those previously obtained in Sanger sequencing data. Clear double peaks were observed in all Sanger sequencing reads for PHP ≥ 20 %, such as 64 (C/T) (Figure S3) in SRM-CHR and 12,071 (C/T) (data not shown) in SRM-HL-60. Sanger sequencing has several limitations due to its restricted sensitivity and its inability to accurately detect and quantitate low-level heteroplasmy. As shown in Figure 2, 1393 heteroplasmy site in SRM-9947A was not always detected by Sanger Sequencing. The mixed bases (G/A) at 1393 were ambiguous and not reproducible in all reads. Three out of six Sanger reads reveal low level heteroplasmy (Figure 2A). However, the site was detected from the NGS data generated on both PGM and MiSeq, showing an agreement across platforms (Figure 2B). The 3242 site was not reported as a variant from the rCRS with Sanger studies and the 7861 site was reported in the certificate as a homoplasmic variant (7861 C). Upon revisiting the Sanger sequence electropherograms for SRM-9947A (Figure S4), one out of three reads show a mixed base at 3242 (Figure S4) and a mixture of T and C

334 bases was indeed present at 7861 (Figure S4), but was not considered due to lack of
335 reproducibility.

Figure 2. Comparison between data generated with Sanger Sequencing and NGS platforms.

Panel A. shows the level of heteroplasmy at 1393 in SRM-9947A was not always detectable and reproducible in all Sanger reads. The mixed bases obtained from Sanger Sequencing reads are noted in red asterisk on the side. Only 3 out of six reads show the mixed bases at 1393 position (red asterisk) in SRM-9947A. The 3 other reads showed no evidence of heteroplasmy. Panel B. shows allele frequency observed by both NGS platforms for each base (G or A) at the heteroplasmic position 1393 in SRM-9947A.

336 In SRM-CHR (Table 1A and S2), an insertion of a C at position 309.1 in HV2 as well
337 as the transition of T to C at position 16189 in HV1 region produced two instances of LHP.
338 The insertions of two Cs at 309.1 and 309.2 relative to the reference sequence lead to a
339 length heteroplasmic situation in the HV2 of SRM-9947A (Table 1B and S2). In the SRM-
340 HL-60, a C insertion at 315.1 did not cause a length heteroplasmy (Table 1C).

341 All C-insertions in the HV1 and/or HV2 regions of the SRMs (Table 1) were not
342 properly called according to forensic nomenclature rules [65] using CLC and Galaxy
343 software packages.

344 In order to resolve, fully capture, and accurately confirm insertions and LHP calls in
345 the homopolymeric regions from the NGS data of the mtDNA SRMs, we used STRait Razor.

This step further provided information not possible with Sanger Sequencing, which produced uninterpretable sequences downstream of LHP [57].

All C-insertions in each SRM were determined and yielded full concordance with those obtained with Sanger and published in the certificates [14, 16]. However, we were able to further resolve the LHP found in HV1 and HV2 regions with NGS (Table S2). We only examined the sequence calls that were detected in the reads generated from both PGM and MiSeq for concordance. Any pattern that was called only on one platform was discarded (Table S2).

In SRM-CHR, the length types seen with Sanger, an i.e. C₁₀ in HV1 and C₈-T-C₆ in HV2, were concordant to the ones generated on the NGS platforms. However, we further noticed additional patterns of the Poly-C tract with SRM-CHR NGS data, 4 in the HV1 C-stretch (C₈, C₉, C₁₁, C₁₂) and 2 in the HV2 region (C₇-T-C₆; C₉-T-C₆) (Table S2).

Besides the prominent LHP (C₉-T-C₆) seen in the HV2 C-stretch of SRM-9947A, NGS revealed another two low LHP levels (C₈-T-C₆; C₁₀-T-C₆) (Table S2).

To get an estimate of the frequency of each LHP, we divided the number of reads for a given length by the total number of detected tracts generated by this method (Table S2). Although, length variants sequenced on PGM have same pattern to those generated on MiSeq, we noticed that proportions of LHP within each SRM are different across the platforms and don't agree on reporting the dominant length variant (Table S2). This difference strongly depends on the sequencing technology and chemistry used especially when sequencing along long stretches of homopolymers [51, 63, 64].

The C-insertions in the HV1 and/or HV2 regions were also assessed using GM-HTS. The GM-HTS package has been reported to provide an improved alignment and reporting of length variants [46]. The software eliminates poor alignment by applying a built-in or user-customized motif file to the aligned reads [46, 47]. All C-insertions in reads generated on MiSeq platform were called properly with the mtDNA typing nomenclature. When analyzing PGM reads, a minimal manual interpretation was required to call the 16193.1C insertion in SRM-CHR (data not shown).

Quality Control Measures

In this study low frequency heteroplasmy variants were difficult to detect in Sanger data (Figure S3 and S4) and were only reproducibly seen with the NGS-based studies. However, low-level mixed mtDNA bases detected can be misinterpreted due to the presence of Nuclear mitochondrial Sequences (NumtS), misalignment issues especially around homopolymer regions, sequencing errors, polymorphisms in the primer region, or errors induced during the PCR process that are propagated with each PCR cycle and becomes difficult to distinguish from true low level PHP.

NumtS are DNA tracts located on nuclear chromosomes that are homologous to regions in the mtDNA genome [66]. A combination of several steps aided in limiting NumtS amplification and thoroughly characterizing true low-level PHP. The DNA samples were initially enriched for mtDNA by amplifying the mtDNA genome using long-range PCR primer approach that is designed to avoid nuclear genome amplification [58, 67]. The risk of co-amplifying the NumtS was very minimal since none of the primer sets overlap with NumtS sequence tracts [68] that span a near or perfect range to that of the PCR amplicons. Stringent alignment algorithms and parameters were used in both CLC and Galaxy (Figure S1 and S2) that discarded reads that did not map uniquely to the mtGenome. Also, raw sequencing reads from both PGM and MiSeq platforms were mapped in Galaxy against the human genome build hg19 (with *Homo sapiens* mitochondrion sequence “ChrM” replaced by the rCRS, NC_012920.1) using the BWA-MEM alignment algorithm [42-45]. After selection for the reads that mapped uniquely to the mtGenome, we then filtered out all non-mitochondrial hits, and then assessed point heteroplasmy following the workflow in Figure S2. We did not see any discrepancies between mapping to the hg19 or to rCRS. There was concordance in the calling and the frequency of the low mtDNA heteroplasmic variants.

To control for possible polymerase induced errors, amplification of each SRM material was performed twice before sequencing on the PGM platform and only heteroplasmies reproducible among PCR replicates were considered. Furthermore, a close examination revealed that none of the mtDNA heteroplasmy sites were residing within the primer binding sites ruling out false positive variants due to a PCR primer successfully binding over a variant.

One site, 16183 in SRM-CHR, was flagged as a potential point heteroplasmy “A/C” when both PGM and MiSeq reads were analyzed with the CLC and Galaxy pipelines. The

16183 position resides immediately before the HV1 C-stretch region “16184-16193”. The HV1 C-stretch in SRM-CHR has been shown with Sanger data [14-16] and our NGS study to harbor LHP (insertion variation) (Table 1A and Table S2). To confirm if 16183 is a true heteroplasmy, we further investigated the site using the stringent STRait Razor software tool. Substantial numbers of reads detected by STRait Razor showed either to have insertions or deletions of Cs between 16182-16194 further confirming that this HV1 region exhibits length heteroplasmy. Due to misalignment issues caused by alignment algorithms when dealing with indels, the 16183 has been erroneously called heteroplasmy and was not reported in the list (Table 1A) as a heteroplasmy site (A/C) but rather as homoplasmy “C” variant, as it has been detected with Sanger data.

Reproducibility of NGS for mtDNA Heteroplasmy Detection

In order to account for platform bias [69-71] and provide confidence in our heteroplasmy calls, we compared sequencing data generated on the PGM platform to that generated on the MiSeq. As described above, we reported base calls (Table 1) that we observed to be fully concordant between the two different NGS platforms using all the bioinformatics pipelines including STRait Razor. Analysis of the results from two replicate PCR runs carried out on PGM for each SRM template further revealed the confidence, reproducibility and accuracy of the mtDNA heteroplasmy sites as well as the other variants. These sites were also seen in additional mtDNA SRM NGS data generated using several amplification approaches (“12-amplicon” [72] and “3-amplicon” [73] approaches), library preparation methods, and sequencing platforms (Life Technologies’ SOLiD 5500 and HiSeq (Illumina)) (data was not shown). This rules out potential amplification and/or platform-specific bias and strongly suggests that all mixed mtDNA data observed at given nucleotide positions and mentioned in this report are representative of true biological variation in these reference materials.

CONCLUSION

In this study, sequencing the mtGenome of the three mtDNA SRM templates using PGM and MiSeq NGS chemistries allowed us to simultaneously (*i*) characterize the complete nucleotide sequence for the existing mtDNA SRMs so that our data can provide a

positive control and Certified Reference Materials for laboratories wishing to implement and validate any NGS technology, (ii) uncover and annotate additional low level heteroplasmic variants in SRM-9947A and SRM-HL-60, and (iii) quantify the mtDNA heteroplasmy levels at each position using multiple software packages.

Overall, the results of this study provide concordant mtDNA base calls with Sanger data. The majority of the NGS-Sanger discrepancies are related to the low level heteroplasmies and increased sensitivity of the new technology. Evaluation of raw FASTQ reads with multiple software tools help in distinguishing true heteroplasmy calls, interpreting erroneous ones caused by alignment artifacts especially around the indel regions, and investigating discrepancies in variant frequencies as seen with 7861 (SRM-9947A). The PHP sites detected on both the MiSeq and PGM platforms, in both PCR replicates, and across the different bioinformatics pipelines will be added with their quantitative values as *information values* in the updated SRM certificates.

Conflict of interest

The authors declare no conflict of interest.

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