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Detection of Innate and Artificial Mitochondrial DNA Heteroplasmy by Massively Parallel Sequencing

- Considerations for Analysis -

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Abstract

Detection of Innate and Artificial Mitochondrial DNA Heteroplasmy by Massively Parallel Sequencing

- Considerations for Analysis -

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Background: Mitochondrial heteroplasmy, the co-existence of different mitochondrial polymorphisms within an individual, has various forensic and clinical implications. But there is still no guideline on the application of massively parallel sequencing (MPS) in heteroplasmy detection. I present here some critical issues that should be considered in heteroplasmy studies using MPS.

Methods: Among five samples with known innate heteroplasmies, two pairs of mixture were generated for artificial heteroplasmies with target minor allele frequencies (MAFs) ranging from 1 to 50%. Each sample was amplified by two-amplicon method and sequenced by Ion

Torrent system. The outcomes of two different analysis tools, Torrent Suite Variant Caller (TVC) and mtDNA-Server (mDS), were compared.

Results: All the innate heteroplasmies were detected correctly by both analysis tools. Average MAFs of artificial heteroplasmies correlated well to the target values. The detection rates were almost 90% for high-level heteroplasmies, but decreased for low-level heteroplasmies. TVC generally showed lower detection rates than mDS, which seems to be due to their own computation algorithms which drop out some reference-dominant heteroplasmies. Meanwhile, mDS reported several unintended low-level heteroplasmies which were suggested as nuclear mitochondrial DNA sequences. The average coverage depth of each sample placed on the same chip showed considerable variation. The increase of coverage depth had no effect on the detection rates.

Conclusion: In addition to the general accuracy of the MPS application on detecting heteroplasmy, our study indicates that the understanding of the nature of mitochondrial DNA and analysis algorithm would be crucial for appropriate interpretation of MPS results.

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keywords : **Mitochondrial DNA;**
Heteroplasmy;
DNA Sequence Analysis;
Massively Parallel Sequencing;
Computational Biology

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LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
High set	high coverage set
IGV	Integrative Genomics Viewer
ISPs	Ion Sphere Particles
MAF	minor allele frequency
mDS	mtDNA-Sever
MPS	massively parallel sequencing
NADH	reduced nicotinamide adenine dinucleotide
ND5	mitochondrial NADH dehydrogenase subunit 5
NIST SRM	Standard Reference Materials® provided by National Institute of Standards and Technology
NUMTs	nuclear mitochondrial DNA sequences
PCR	polymerase chain reaction
rCRS	revised Cambridge Reference Sequence
RFLP	restriction fragment length polymorphism
Std set	standard coverage set
TE buffer	tris EDTA buffer
TVC	Torrent Variant Caller (Variant Caller plug-in of Ion Torrent Suite™)

INTRODUCTION

Forensic and clinical meaning of mitochondrial heteroplasmy

Mitochondrial heteroplasmy refers to the co-existence of mitochondrial DNA genomes with different polymorphisms in an individual or a cell, whether the polymorphisms are originated from wild type inheritance or *de novo* mutation. This phenomenon could be found not only in normal samples with inherited heteroplasmy or somatic mutations by aging, but also in pathologic status, such as cancers or hereditary mitochondrial diseases.

Forensic scientists have noticed early the role of mitochondrial heteroplasmy in human identification and familial search. If the subjects of comparison are identical in their haplotypes and heteroplasmy pattern, it gives significant meanings to the case interpretation. The existence of heteroplasmy implies that there has been a recent event of mutation, and the subjects are in a more close relationship within their maternal lineage. If the subjects are the same person, the heteroplasmy gives much higher evidential power to the identification.[1] If the subjects are relatives, they might be in a very close maternal lineage such as mother-child, brothers or sisters. The power of heteroplasmy have been seen in the classic example of Tsar Nicholas II and his brother Georgij Romanov.[2] Of course, the possibility of sample mixture or contamination should be ruled out.

In clinical medicine, mitochondrial heteroplasmy can be used in the assessment of treatment plan and its prognosis.[3] If patients have a heteroplasmic mutation in the mitochondrial region which is critical to certain disease or symptoms, the heteroplasmy level in the affected organ can be related to the penetrance, severity, or expected course of the disease. For an example, 8993T>G mutation is known to cause 20-25% of Leigh syndrome, one of the most famous mitochondrial neuromusculopathy. These patients with mutation load under 30% rarely have related symptoms. As seen here, the organ or cell affected by pathologic heteroplasmy usually maintain their function because of low heteroplasmy level under the threshold or compensation by other mitochondria or cells.

But the mutation load can be shifted over time within an individual or through generation. Heteroplasmic shift induced by mitochondrial dynamics such as vegetative segregation or relaxed replication can result in the congregation of the pathologic mitochondrial genome in an important organ or a few populations of germ cells, therefore affecting not only the patient themselves but also their descendants.[4] Considering that the probability of severe symptoms in the patients with Leigh syndrome becomes much higher when the mutation load goes over 60-70%, their heteroplasmy level should be carefully monitored.

Recently, there are several attempts to manage various types of genetic diseases with mitochondrial origin. For example, defective mitochondrial copies can be detected by prenatal detection[5] or preimplantation diagnosis techniques[6] before the conception of the oocytes or eggs with certain mutation load. In the therapeutic area of mitochondrial disease, some promising strategies such as stem cell therapy,[7] mitochondrial replacement therapy,[8,9] and heteroplasmy shifting therapy[10] are on the way to the preclinical application. The precise detection and measure of mitochondrial heteroplasmy level will be needed to evaluate the effect and promise of each therapy.

Cancer is another clinical field for which heteroplasmic phenomenon could be utilized. There have been several hypotheses that mitochondria would have an important role in carcinogenesis or cancer progression, considering several characteristics related to mitochondria such as Warburg effect, reactive oxygen species production, or impaired gene repair system.[11,12] Many previous studies had shown that frequent mitochondrial mutation and heteroplasmy generation occurs in the tumor, which could be expected through the nature of the tumor such as high proliferation rate, rapid regeneration, and cellular heterogeneity.[12] The genetic difference between normal and tumor tissue could be whether the cause and clue of mitochondrial dysfunction or a consequence of genetic instability of mitochondria, which have different meanings on cancer research in the future. However, there is still no consensus about the role of mitochondria on the tumor behavior, with some limitations such as inadequate analysis of heteroplasmy or unintegrated interpretation of the result data and clinical information.

Detection of mitochondrial heteroplasmy

In the past, Sanger direct sequencing or RFLP methods were used in distinction of homoplasmy and heteroplasmy,[13] which are known to be insufficient to detect the minor alleles with frequencies under about 10 to 20%. With these methods, there is risk of misjudging the low-level heteroplasmies as homoplasmies, which can lead to failure to identify the targeted person or relationship, because the level of heteroplasmy could be variable between the body parts, individuals or family members. In clinical medicine, a patient's clinical phenomenon can be misunderstood, followed by wrong diagnosis. Fortunately, with the development of the MPS method, both the accurate detection and measurement of low-level heteroplasmy has become feasible, at least theoretically.[14] However, the reliability and the accuracy of each system should be evaluated separately because they have different characteristics generating their own pros and cons.[15]

Considering many ongoing efforts to reveal the role and function of mitochondria and its genome, it could be expected that there would be increased need for mitochondrial DNA research in the near future. But although many researchers have reported successful detection results of low-level heteroplasmy using MPS,[16-22] there is no consensus about the criteria for determining mitochondrial heteroplasmy so far; whether the cut-off value should be applied to the frequency or read coverage of minor alleles, and if so, how the value could be determined; whether secondary confirmation through duplicate sequencing tests or other analysis tools would be necessary for the procurement of reliability; what kind of computation models would be appropriate to determine the status of heteroplasmy; and so on. This indicates that the reliability of the detection results of mixed alleles obtained from MPS techniques might be controversial yet. Still, some recent articles have regarded the nucleotide positions with MAF up to 15% as 'homoplasmy'.[23,24]

There is more classic problem in analyzing mitochondrial heteroplasmy. Several types of false positives pretend to be mitochondrial heteroplasmy, such as NUMTs, sample contamination or mixture, repetitive codons, and even technical error. Various overcoming strategies are being suggested and tried in the steps of amplification or data analysis. As their name indicates, NUMTs are parts of nuclear genome which have same or at least similar sequences with mitochondrial genome.[25] They could be amplified together with mitochondrial genome by the primers bound to the sequence homologous between nuclear and mitochondrial genomes, and pretend as mitochondrial heteroplasmy. Historically, many researchers have mistaken NUMTs for novel mitochondrial mutations.[26] The two amplicon method has been widely used to prevent amplification of NUMTs, especially for MPS.[14] There are sets of primers previously suggested for this method[27,28] and one of them was adopted in this study.

In addition, it should not be overlooked that there are barriers against proper analysis of the MPS data. Despite several bioinformatics tools and pipelines of no cost being suggested and launched,[16,24,29-32] they usually require specific operation systems such as Unix or Linux, knowledge about programming languages or structures, or additional compilation of numerous result files and spreadsheet works. Although there are some commercial programs with more integrated functions and user-friendly interfaces,[33] their costs to purchase and maintenance might be a burden to a small group of researchers. It can be expected that considerable need exists for easily accessible and reliable analysis tools for detecting low-level heteroplasmies, but as of yet, there are no perfect choices. Even after the analysis tools and pipelines are determined and the sequencing results are acquired, understanding of the actual meaning of the result data is required for proper interpretation.

Bearing in mind these situations, I present in this study the detection and analysis results of innate and artificially mixed mitochondrial heteroplasmies using the Ion Torrent system to confirm its reliability and accuracy. There are several previous studies that have used this type of controlled samples to show the accuracy of heteroplasmy detection by different MPS systems or techniques.[18,19,22,34,35] This study has different experiment design from them, with the combination of the MPS system and mixture ratios. This study focuses on the evaluation of point heteroplasmy, considering the characteristics of the Ion Torrent system and the relative importance of point heteroplasmy in the area mentioned above.

For a robust analysis, two different variant calling tools were applied to the same raw data. Through comparing these results, several issues that should be considered in the analysis of mitochondrial heteroplasmy using MPS were revealed. This will be discussed also, including differentiation of false positives and the effect of different analysis algorithms.

MATERIALS AND METHODS

Sample selection

There is a dataset of the whole mitochondrial genome sequences of over two hundred Korean subjects using the Ion Torrent System, and part of these results were reported previously.[31] Based on these MPS data, I selected samples for this study.

First, I chose the original samples that were confirmed to have innate point heteroplasmies, in the purpose to confirm the reproducibility of heteroplasmy detection. For straightforward analysis, samples having insertion or deletion against the revised Cambridge Reference Sequence (rCRS) were excluded.

Next, as I know the whole mitochondrial sequence of each sample, the numbers of discordant nucleotide positions were counted for every case of mixture pairs. These positions would be observed as 'heteroplasmy' when the pairs were mixed. The pairs with the largest numbers of discrepant positions were selected.

Finally, 5 samples with various levels of MAFs, ranged from 24 to 45%, were selected and labeled as A to E. Among them, 4 samples were mixed to generate 2 pairs with artificial heteroplasmies. As a control, AmpFlSTR® Control DNA 9947A (Lot No. 1104081; Applied Biosystems, Warrington, UK) was used. 9947A was previously reported to have heteroplasmy at 2 nucleotide positions.[34,37]

DNA extraction, mixing and amplification

All samples were stored as blood, so DNA was extracted using the Maxwell® 16 Blood DNA Purification Kit and Maxwell® 16 instrument (Promega, Madison, WI, USA), according to the manufacturer's protocol. Mitochondrial copy numbers were quantified with PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, Austin, TX, USA), according to the manufacturer's protocol. Partial sequence of mitochondrial ND5 gene was used for the standard of qPCR, as suggested by Kavlick et al.[38] The original stock of ND5 gene was serially diluted to 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 copies per 2 ㎕. For every dilution process, TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) was used.

The extracted DNAs were diluted to have 1,000,000 mitochondrial gene copies per 1 ㎕. Then each pair was mixed to 5 different ratios of 50:50, 20:80, 10:90, 2.5:97.5, and 1:99. Sample A of A-B pair and sample C of C-D pair were minor components, so their proportions in the mixtures were 50%, 20%, 10%, 2.5%, and 1.0%, respectively. The numbers of discrepant positions were 30 for A-B and 35 for C-D, excluding the innate heteroplasmies of original samples.

Two amplicon method which produces 2 overlapping 8 kb-length amplicons covering the whole mitochondrial genome was adopted to prevent NUMTs.[27] The sequences of each primer were as follows (5'

→ 3').

Forward A : AAA TCT TAC CCC GCC TGT TT
Reverse A : AAT TAG GCT GTG GGT GGT TG
Forward B : GCC ATA CTA GTC TTT GCC GC
Reverse B : GGC AGG TCA ATT TCA CTG GT

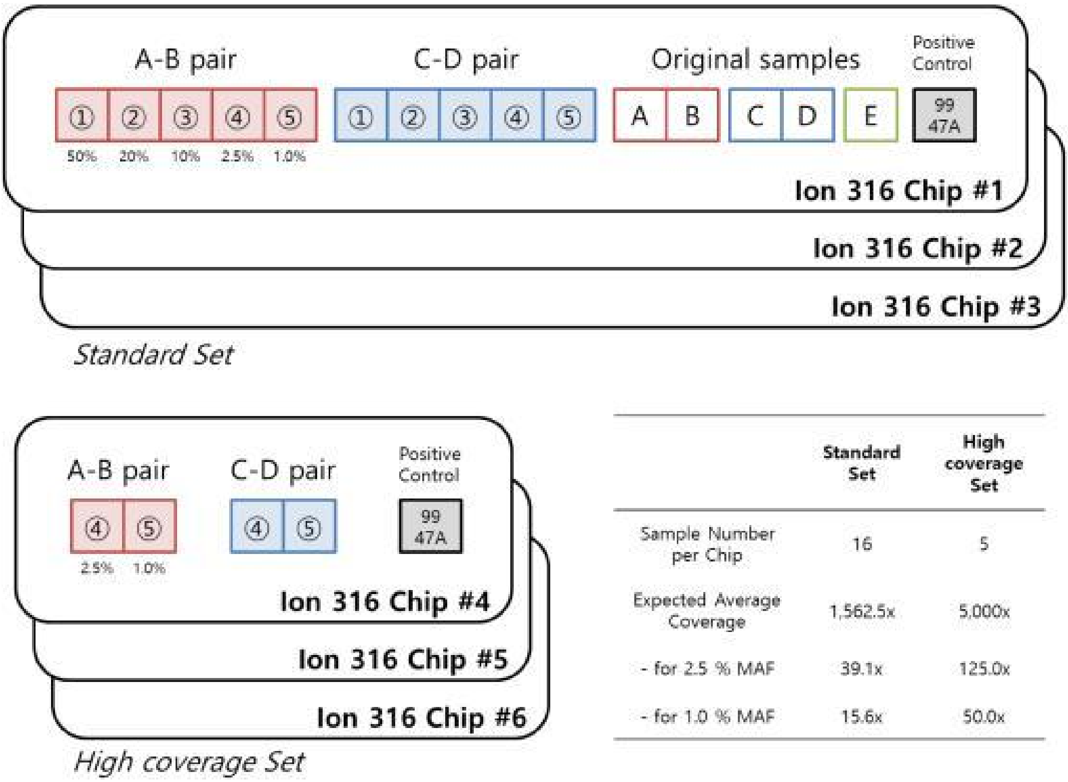
For each amplification, 2.5 unit of TaKaRa LA Taq DNA polymerase (Takara Bio Inc., Shiga, Japan), each 2.5 ㎕ of forward and reverse primers with the concentration of 10 μM, and 3 ㎕ of prepared DNA were used according to the manufacturer's guide. Thermal cycling condition was denaturation for 2 min at 94 ℃, amplification for 30 cycles of 10 sec at 98 ℃, 30 sec at 60 ℃, 15 min at 68 ℃, final extension for 10 min at 72 ℃, and hold at 4 ℃. All samples were processed in triplicate from the amplification step and the size of each amplified product was confirmed by gel electrophoresis (without gel extraction).

The PCR products were purified with HiYield^{TM} Gel/PCR DNA mini Kit (Real Biotech Corporation, Taipei, Taiwan) or Wizard® SV Gel and PCR Clean-Up System (Promega) and quantified by NanoDrop spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA). Two amplicons of each sample were pooled 20 ㎕ equally and the final concentration was 50 ng/㎕.

Library preparation and sequencing

The PCR products were sheared to about 200 bp using the Ion Xpress^{TM} Plus Fragment Library Kit (Thermo Fisher Scientific). The fragments were ligated with the Ion Xpress^{TM} Barcode Adapters Kit (Thermo Fisher Scientific) and purified using the AMPure® XP beads (Beckman Coulter, CA, USA), according to the manufacturer's protocols. They were quantified by the Ion Library Quantitation Kit (Thermo Fisher Scientific) and all the libraries were pooled in equal concentrations of 26 pM. The libraries were used as a template for emulsion PCR, and template-positive ISPs were enriched according to the manufacturer's protocol. Samples were loaded on Ion 316 chips (Thermo Fisher Scientific) as described below and Figure 1, and sequenced by Ion Torrent PGM^{TM} platform (Thermo Fisher Scientific). Every sequencing process included negative controls.

Total 6 chips were used. Three chips were allocated to standard coverage set ('Std set') and other 3 chips to high coverage set ('High set') for triplicate experiment. Sixteen samples were placed on each chip of Std set, which were composed of 5 original samples, 10 mixed samples, and 1 control. In case of High set, 5 samples were placed on each chip, which were composed of 4 mixed samples of 2.5% and 1.0% MAFs from each pair and 1 control. The expected average coverages were 1,562.5× for Std set and 5,000× for High set.



	Standard Set	High coverage Set
Sample Number per Chip	16	5
Expected Average Coverage	1,562.5x	5,000x
- for 2.5 % MAF	39.1x	125.0x
- for 1.0 % MAF	15.6x	50.0x

Figure 1. Experiment Design including Sample Allocation.

Data analysis

The sequenced data were analyzed with Ion Torrent Suite^{TM} Software version 5.0 and Variant Caller plug-in (TVC) version 5.0-13/e975447 (Thermo Fisher Scientific) using the rCRS as reference. The default setting of 'Germline-Low Stringency' was used for the overall parameters, which is described as 'optimized for allele frequencies >10%, high sensitivity, and minimal false negative calls' in the manual. The called variants and their frequencies were generally consistent across the modified thresholds of MAF which ranged from 0.1 to 0.01. Other parameters were not adjusted from their default values.

Also, mtDNA-Sever1) (mDS) version 1.0.6 was used for the variant detection. mDS is a free web-based mitochondrial analysis tool that provides reports of heteroplasmy and haplotyping status of uploaded samples.[16] Their parameters are fixed as presented in the user manual, for example, the threshold of MAF is 1%.

Integrative Genomics Viewer2) (IGV) was also used for the visual inspection of the interested positions. Additional analysis of the result files was performed with Microsoft Excel (Microsoft Corporation, Redmond, WA, USA).

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1) https://mtdna-server.uibk.ac.at/
2) http://software.broadinstitute.org/software/igv/home

Ethics statement

The study was approved by the institutional review board of Seoul National University Hospital with confirmation of the informed consents (IRB No. C-1403-104-567).

RESULTS

Coverage depths

In TVC, the average coverage depth of each sample was reported as 1,369× in Std set and 4,517× in High set, which was similar with expectation. However, there were considerable imbalances between the samples placed on each chip. In the Std set, the maximum average value was up to 5.66-fold of the minimum within the same chip, and they were ranged from 644 to 4,122× (Figure 2). In the High set, the maximum was up to 8.34-fold of the minimum, and they were ranged from 1,034 to 8,620×.

Meanwhile, mDS reported the average coverage depth as 1,275× in the Std set and 4,213× in the High set, which were about 7% less than that of TVC. This tendency was observed throughout all the individual samples and positions, which implies that mDS accepts less number of reads for analysis than TVC does.

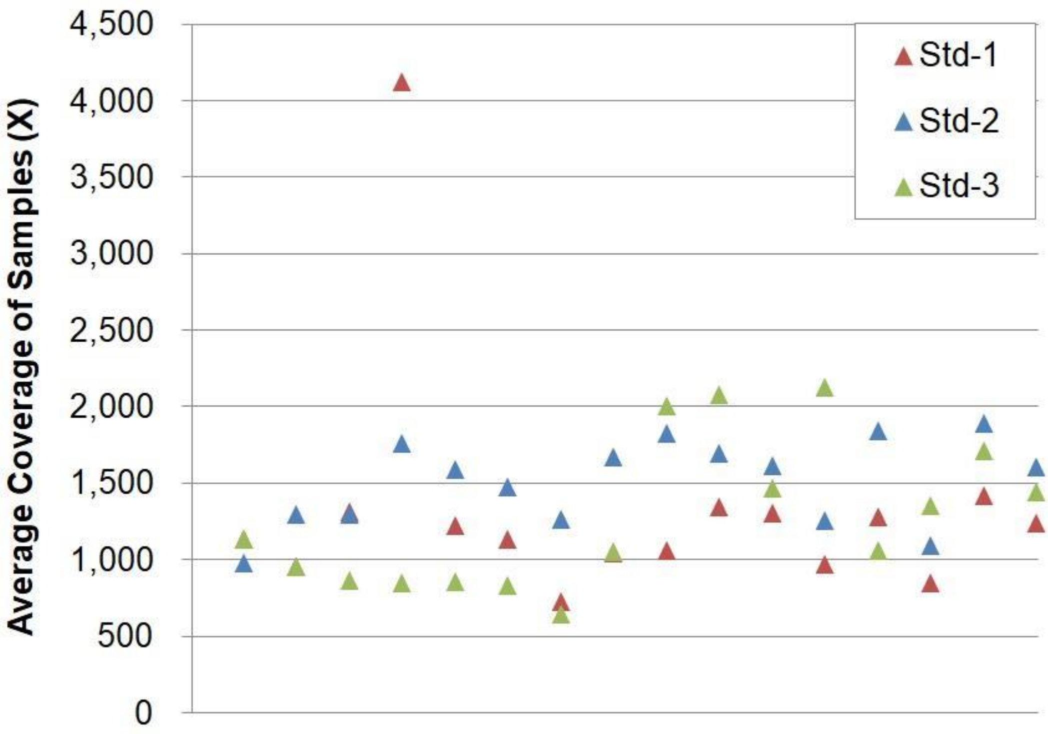


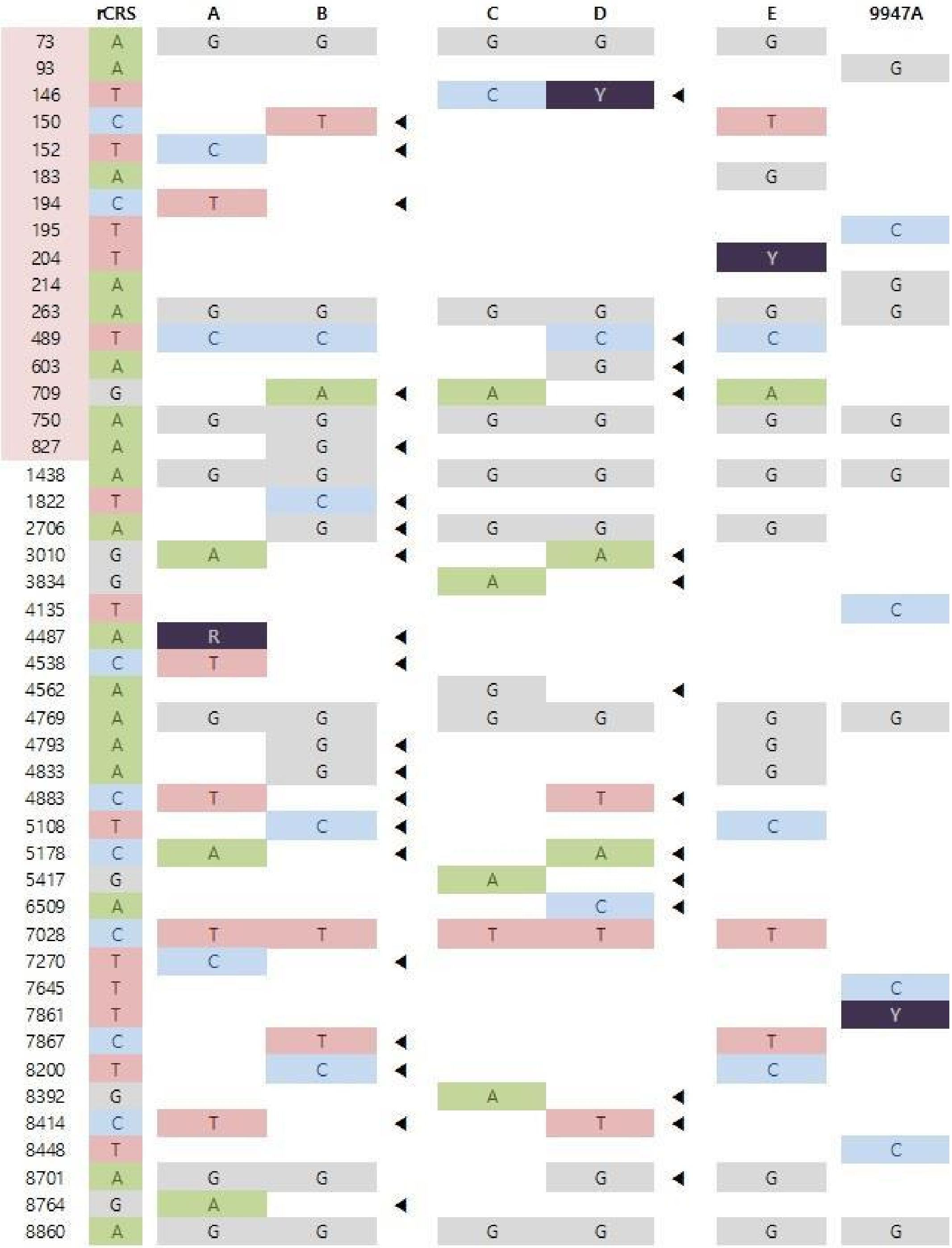
Figure 2. Average coverage depths of the Std set. Although most of them were around the expected value of 1,500×, there were notable variations.

Innate heteroplasmy in 5 original and 1 control samples

Whole mitochondrial sequence of the 5 original and 1 control samples are presented in Figure 3, compared with rCRS. All the variants called by TVC were identical to our previous results as presented in Table 1, which could be easily expected because the same analysis method was used in both studies.[36] The observed MAFs by TVC and mDS were both very close to the previous results. However, in the case of A4487R of sample A, the gaps of the average MAFs between mDS and TVC were larger than that of other innate heteroplasmies. This seems to be originated from the calculation error of mDS, as explained in Table 2.

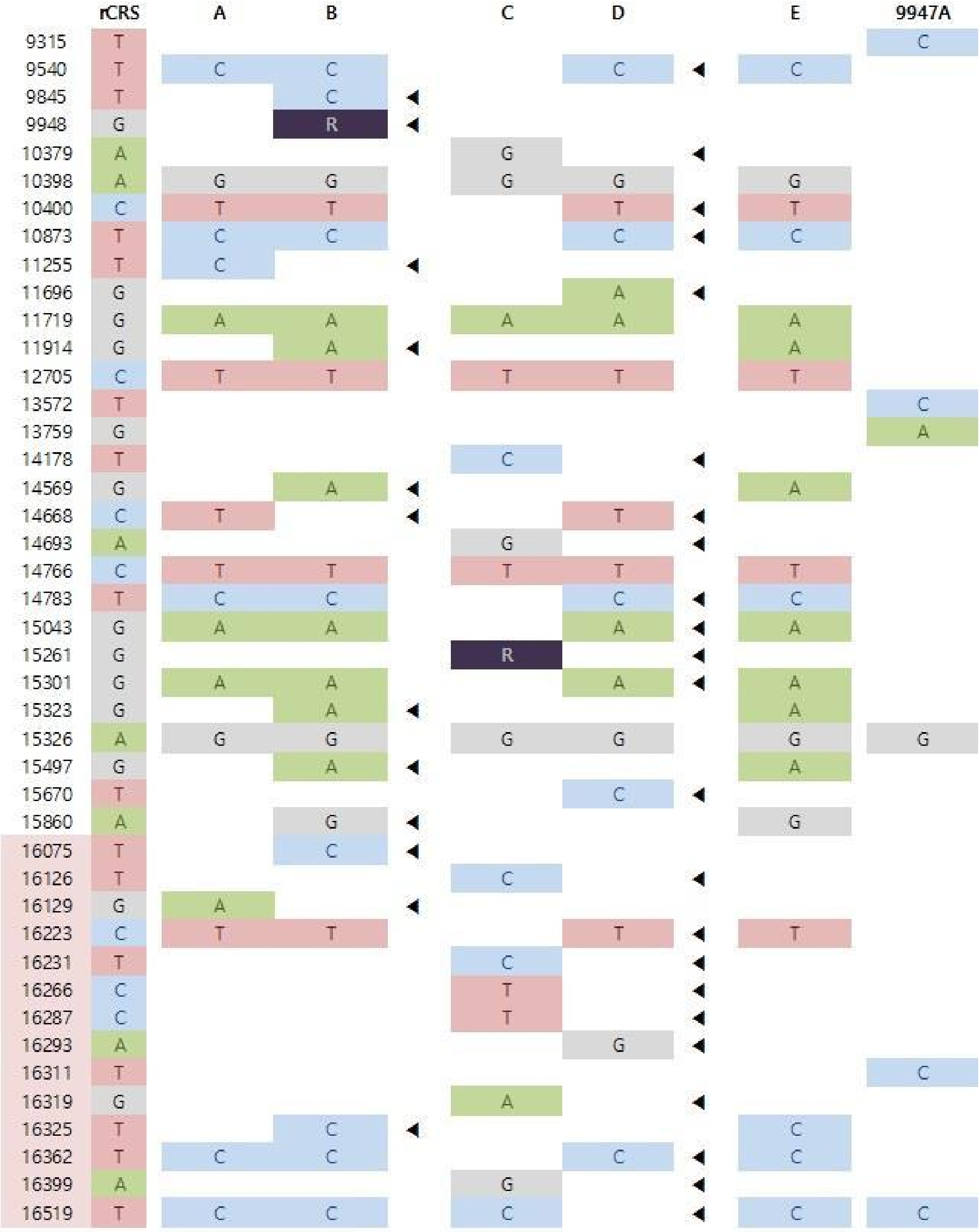
Meanwhile, a few variants were not reported by mDS as presented in Table 3. The homoplasmic variants of T489C and T10873C were frequently dropped-out from several samples but these events were not regular and the reason is also not clear. Though the coverage depths of these positions were generally lower than average values, they were still high enough to be analyzed. The visual inspection of the reads of these positions also showed no specific differences.

In case of A2706G in sample C, TVC reported it as homoplasmic variant, while mDS included it in heteroplasmy report because of minor allele A with very low MAF as 2.3% and 2.1%. It seems to be due to different strategy of reporting homoplasmy and heteroplasmy between TVC and mDS, which can be overcame easily by the researcher with careful reading of the results.



	rCRS	A	B		C	D		E	9947A
73	A	G	G		G	G		G	
93	A								G
146	T				C	Y	◀		
150	C		T	◀				T	
152	T	C		◀					
183	A							G	
194	C	T		◀					
195	T								C
204	T							Y	
214	A								G
263	A	G	G		G	G		G	G
489	T	C	C			C	◀	C	
603	A					G	◀		
709	G		A	◀	A		◀	A	
750	A	G	G		G	G		G	G
827	A		G	◀					
1438	A	G	G		G	G		G	G
1822	T		C	◀					
2706	A		G	◀	G	G		G	
3010	G	A		◀		A	◀		
3834	G				A		◀		
4135	T								C
4487	A	R		◀					
4538	C	T		◀					
4562	A				G		◀		
4769	A	G	G		G	G		G	G
4793	A		G	◀				G	
4833	A		G	◀				G	
4883	C	T		◀		T	◀		
5108	T		C	◀				C	
5178	C	A		◀		A	◀		
5417	G				A		◀		
6509	A					C	◀		
7028	C	T	T		T	T		T	
7270	T	C		◀					
7645	T								C
7861	T								Y
7867	C		T	◀				T	
8200	T		C	◀				C	
8392	G				A		◀		
8414	C	T		◀		T	◀		
8448	T								C
8701	A	G	G			G	◀	G	
8764	G	A		◀					
8860	A	G	G		G	G		G	G

Figure 3. Whole sequence of the mitochondrial genome of the original and control samples. The control region is highlighted by pink color and the discrepant positions are tagged by triangle (◀).



	rCRS	A	B		C	D		E	9947A
9315	T								C
9540	T	C	C			C	◀	C	
9845	T		C	◀					
9948	G		R	◀					
10379	A				G		◀		
10398	A	G	G		G	G		G	
10400	C	T	T			T	◀	T	
10873	T	C	C			C	◀	C	
11255	T	C		◀					
11696	G					A	◀		
11719	G	A	A		A	A		A	
11914	G		A	◀				A	
12705	C	T	T		T	T		T	
13572	T								C
13759	G								A
14178	T				C		◀		
14569	G		A	◀				A	
14668	C	T		◀		T	◀		
14693	A				G		◀		
14766	C	T	T		T	T		T	
14783	T	C	C			C	◀	C	
15043	G	A	A			A	◀	A	
15261	G				R		◀		
15301	G	A	A			A	◀	A	
15323	G		A	◀				A	
15326	A	G	G		G	G		G	G
15497	G		A	◀				A	
15670	T					C	◀		
15860	A		G	◀				G	
16075	T		C	◀					
16126	T				C		◀		
16129	G	A		◀					
16223	C	T	T			T	◀	T	
16231	T				C		◀		
16266	C				T		◀		
16287	C				T		◀		
16293	A					G	◀		
16311	T								C
16319	G				A		◀		
16325	T		C	◀				C	
16362	T	C	C			C	◀	C	
16399	A				G		◀		
16519	T	C	C		C		◀	C	C

Figure 3. *(continued)*

Table 1. Innate Heteroplasmies and Variants in 5 Original Samples

Sample Name	Innate Heteroplasmies						No. of Variants in TVC
	Hetero plasmy	Major Allele	Minor Allele	Previous MAF	Average MAF in TVC	Average MAF in mDS	
A	A4487R	A	G	45.0%	43.1%	37.5%	35
B	G9948R	A	G	40.0%	40.5%	38.8%	41
C	G15261R	G	A	28.0%	29.2%	29.9%	29
D	T146Y	T	C	38.0%	38.7%	37.8%	33
E	T204Y	T	C	24.0%	24.2%	24.3%	38

* MAF, minor allele frequency; TVC, Torrent Variant Caller; mDS, mtDNA-Server

Table 2. Detailed Heteroplasmy Level in np 4487 of Sample A

Analysis Tool	Sample ID	Forward Strand		Reverse Strand		Reported Heteroplasmy Level in mDS[a]	Re-calculated Heteroplasmy Level for Allele G[b]
		Coverage Depth (×)	Minor Allele & its MAF	Coverage Depth (×)	Minor Allele & its MAF		
mDS	A_1	455	A (39.1%)	598	G (41.3%)	40.4%	49.4%
	A_2	293	A (30.7%)	427	G (40.8%)	36.7%	51.0%
	A_3	258	A (31.4%)	300	G (39.0%)	35.5%	51.4%
	Average	335.3		441.7		37.5%	50.6%
TVC	A_1	679	G (43.4%)	635	G (41.6%)	42.5%	42.5%
	A_2	476	G (44.5%)	439	G (40.3%)	42.5%	42.5%
	A_3	362	G (48.3%)	306	G (39.2%)	44.2%	44.2%
	Average	505.7		460.7		43.1%	43.1%

* mDS, mtDNA-Server; TVC, Torrent Variant Caller; MAF, minor allele frequency

a. The following Formula A gives the same values as reported by mDS.

Formula A:

$$\frac{Coverage\ of\ Forward \times MAF\ of\ Forward + Coverage\ of\ Reverse \times MAF\ of\ Reverse}{Coverage\ of\ Forward + Coverage\ of\ Reverse}$$

This does not consider the possibility that minor alleles of forward strand and reverse strand could be different, which is already classified as 'Type 3 heteroplasmy' by mDS. Even for these cases, the heteroplasmy level should be calculated for either side, not mixing up them.

b. These were calculated using the following formula B. Note that allele G was considered as major allele in forward strands by mDS. If the heteroplasmy level is to be reported for the specific allele X, the Formula B will be as follows:

Formula B:

$$\frac{Coverage\ of\ Forward \times Forward\ Frequency\ of\ allele\ X + Coverage\ of\ Reverse \times Reverse\ Frequency\ of\ allele\ X}{Coverage\ of\ Forward + Coverage\ of\ Reverse}$$

Of course, the heteroplasmy level calculated by Formula A and Formula B will be equal if minor alleles of forward and reverse strand are identical.

Table 3. Homoplasmic Variants dropped-out in mDS reports

Variants	Sample ID	Coverage Depths (×) in TVC[a]
T489C	A_1	542
	B_1	296
	B_2	563
	B_3	354
	D_3	416
	E_3	350
A2706G	C_2	792
	C_3	677
G8392A	C_1	314
	C_2	284
	C_3	209
T10873C	A_1	167
	B_1	149
	D_1	736
	E_1	175
C14766T	B_1	211

* mDS, mtDNA-Server; TVC, Torrent Variant Caller

a. Because mDS did not provide individual coverage depth of each nucleotide position, the values of TVC were adopted here.

If the minor alleles are identical to previously reported NUMTs, the heteroplasmy report of mDS provides the information of NUMTs based on the publication of Dayama et al.[39] In this study, mDS reported 232 low-level heteroplasmies which were not reported by TVC, as presented in Table 4. Among them, 73.7% were from sample C and all but one from sample D were reported as NUMTs before. Moreover, 214 (92.2%) of them was located within the range of about 700 bps started from np 2499 which is one end of the primer binding sites (Figure 4). The MAFs of the suspected NUMTs from sample C tended to be decreased as the proportion of sample C lowered in C-D mixed pair, as shown in Figure 5. This trend supported the possibility of the existence of NUMTs in sample C (refer to next section for mixed samples).

Meanwhile, the results of the control sample 9947A in this study were partially discordant with previous studies. Two heteroplasmies of 9947A had been observed at np 1393 and np 7861 through several methods, with MAFs of about 10 to 20%.[34,37] But in this study, TVC reported only np 7861 as a variant with average MAF of 18.1%, while mDS reported only np 1393 as NUMTs with average MAF of 2.5%. Based on the visual inspection and the detailed data from both analysis tools, it seems to be due to strand imbalance or low detection rate of low-level heteroplasmy, which will be further described in the following section. Also, the different source of control DNA could be the reason for different sequencing results.

Table 4. Summary of Additional Variants reported by mDS

Sample	np Range	No. of NUMTs/Variants	Average MAF (Range)
A	2523-3017	12/12	1.33% (0.96% - 2.67%)
B	2625	1/1	0.83% (-)
C	2523-7158	171/171	2.01% (0.77% - 5.74%)
D	204-14924	10/11	1.03% (0.74% - 1.96%)
E	2523-11116	37/37	1.41% (0.79% - 3.32%)

* np, nucleotide position; mDS, mtDNA-Server; MAF, minor allele frequency; NUMTs, nuclear mitochondrial DNA segments

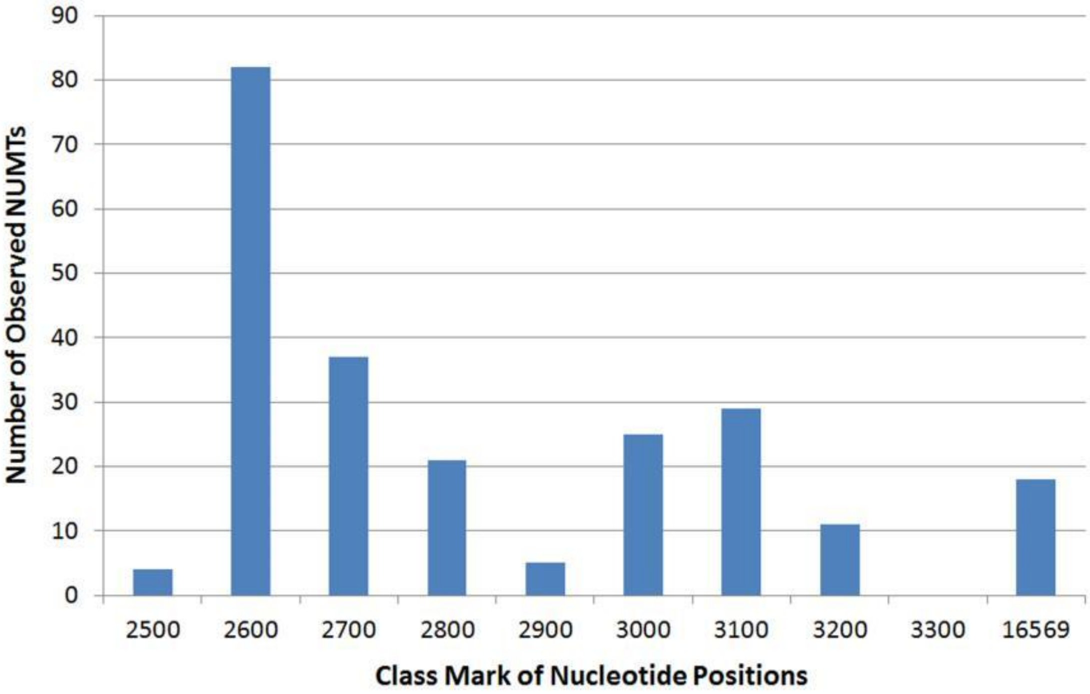


Figure 4. Histogram of observed NUMTs throughout 5 original samples. Considering that one of the primer binding sites was ended right before np 2499, it could be assumed that there were homology sequences for this primer on nuclear DNA, generating NUMTs.

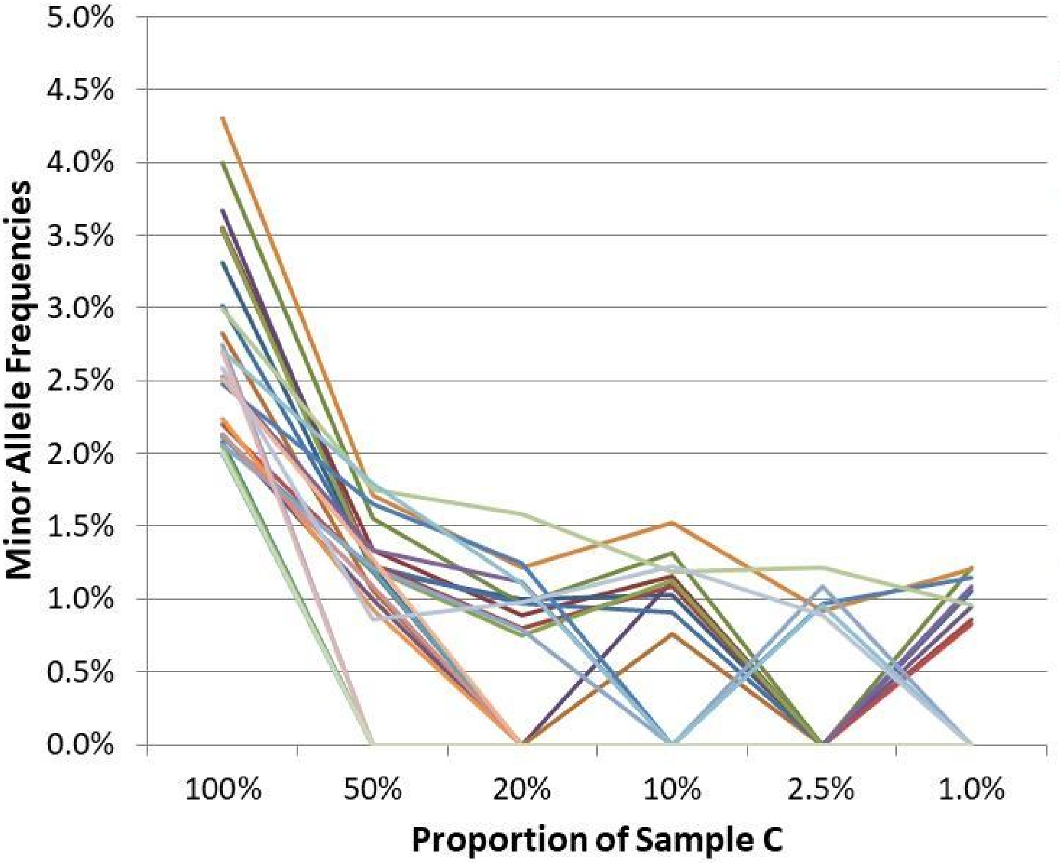


Figure 5. The MAFs of suspected NUMTs reported by mDS from sample C. Each line indicates a nucleotide position within the range of 700 bps from np 2499. Also, those with MAFs over 2.0% in original sample C were selectively presented in this graph, because most of the others were disappeared in mixed samples due to extremely low MAFs.

Artificial heteroplasmy in 10 mixed samples

Total 1,035 heteroplasmic positions, including 975 artificially mixed and 60 innate, were generated by triplicated experiments for 10 mixed samples. Average MAFs were proportionately well correlated to the targeted MAFs, but were generally lower than their targeted values, except for the 2.5% and 1.0% heteroplasmies reported in TVC (Figure 6). The average MAFs of 1.0% heteroplasmies were even higher than the targeted values in TVC, which seems due to the extremely low detection rate and erroneous read counts of minor alleles.

For targeted MAFs of 50%, 20%, 10%, 2.5%, and 1.0%, the detection rates of TVC were 100%, 87.2%, 58.5%, 32.8% and 3.6%, while those of mDS were 94.9%, 84.6%, 79.0%, 56.9% and 10.8%, respectively (Figure 7). The detection rates were close to 90% for high-level heteroplasmies ≥ 20%, but they decreased for low-level heteroplasmies ≤ 10%. Also, TVC and mDS showed similar results for high-level heteroplasmies, but mDS showed generally higher detection rates than TVC for low-level heteroplasmies. At first, these results were thought to be due to different MAF thresholds between these programs. The threshold of MAF is fixed to 1% in mDS, while TVC provides parameter customization with recommending its range between 1% and 20%. However, some selected MAF thresholds within this range showed very similar results (data not shown).

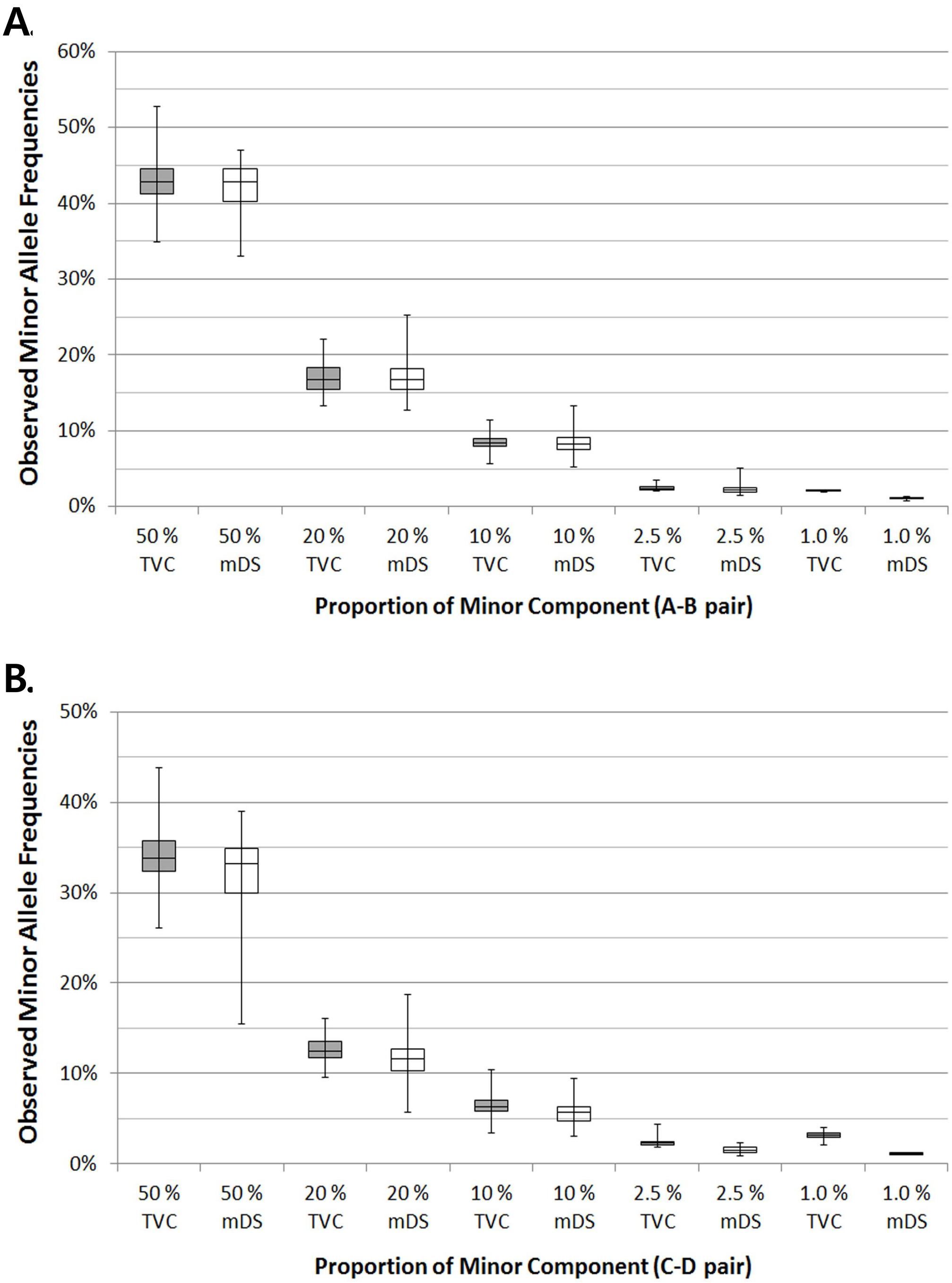


Figure 6. Observed MAFs of the mixed pairs A–B (**A**) and C–D (**B**). The MAF distribution of TVC is represented as gray boxes and mDS as white boxes. Both analysis tools showed generally lower MAFs than expected without significant differences.

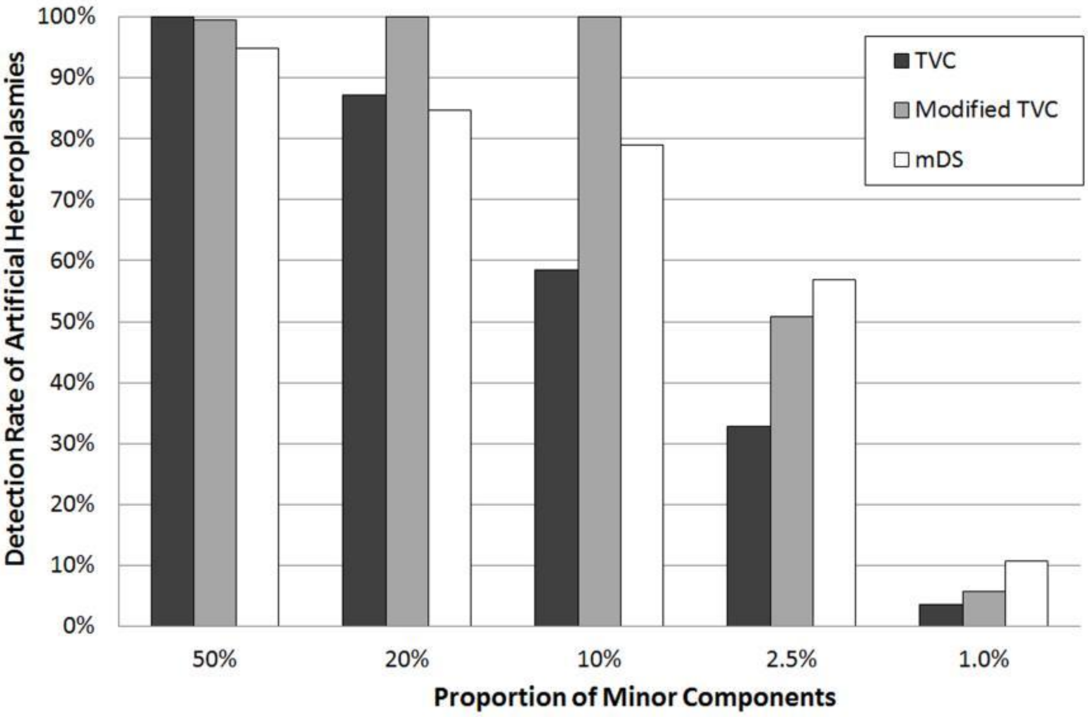


Figure 7. Detection rates of artificial heteroplasmies according to the analysis tools. TVC with reference modification shows the highest detection rates for high-level heteroplasmies, while mDS shows better results for low-level heteroplasmies.

Interestingly, there were great differences in the detection rates between reference-dominant heteroplasmies, the heteroplasmies with major allele identical to rCRS, and variant-dominant heteroplasmies, the heteroplasmies with major allele different from rCRS. Table 5 summarizes these findings. In TVC, the reference-dominant heteroplasmies showed less than half detection rate compared to variant-dominant. These differences were not related with the levels of total coverage depths, minor allele coverage depths, or MAFs and not observed in the results of mDS.

To confirm that the differences were originated from the algorithm of TVC dealing with reference sequence and variants, some modification was tried to the rCRS applied in TVC (Figure 8). It was hypothesized that if the rCRS sequences of heteroplasmic positions were replaced by minor allele sequence, the reference-dominant and variant-dominant heteroplasmies would be treated equivalently and the differences in detection rates would be disappeared. As a result, these modifications were successful and the detection rates were markedly improved, especially for the heteroplasmies $\geq$ 10%, as shown in Figure 7.

Table 5. Detection Results of Artificial Heteroplasmies[a]

	Total	Reference -dominant	Variant -dominant
No. of Heteroplasmies	1035	435	600
- By TVC[b]	583 (56.3%)	152 (34.9%)	431 (71.8%)
- By mDS[b]	684 (66.1%)	277 (63.7%)	407 (67.8%)
Coverage Depth (Average ± SD, ×)	1289.41 ± 713.63	1292.72 ± 733.11	1287.00 ± 699.77
Coverage Depth Range (×)	89-3769	173-3408	89-3769

* TVC, Torrent Variant Caller; mDS, mtDNA-Server; SD, Standard Deviation

a. Numbers of heteroplasmic positions in this table include 60 innate heteroplasmies of total.

b. The percentages in the brackets indicate the detection rates.

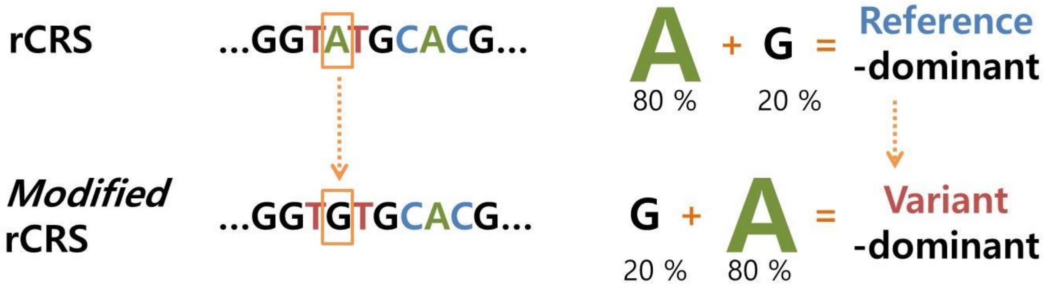


Figure 8. An example of the rCRS modification. For the nucleotide positions of artificial heteroplasmy, the reference sequence was replaced with the sequence of the minor component of each mixed pair, such as sample A for A-B pair and sample C for C-D pair. With this modification, the reference-dominant alleles could be changed to variant-dominant alleles.

Relationship of Coverage Depth and Detection Rate of Low-level Heteroplasmies

To observe the effect of coverage depths on the detection rates of low-level heteroplasmies, different numbers of samples were placed on the Ion 316 chips between Std set and High set (Figure 1). In the High set, the overall coverage depths were expected to be higher than in the Std set, by allocating fewer samples. But as mentioned above, there were imbalances in the coverage depths of individual samples placed on the same chips. As the result of these imbalances, some samples of the High set had similar or even lower average coverage depths than some of the Std set. To observe the relationship of coverage depth and detection rate, the samples were ordered according to their actual average coverage depths, regardless of the type of set. There was no correlation between average coverage depths and detection rates per samples, as shown in Figure 9. The modification of reference sequence or minimum allele frequency threshold had no effect on these tendencies.

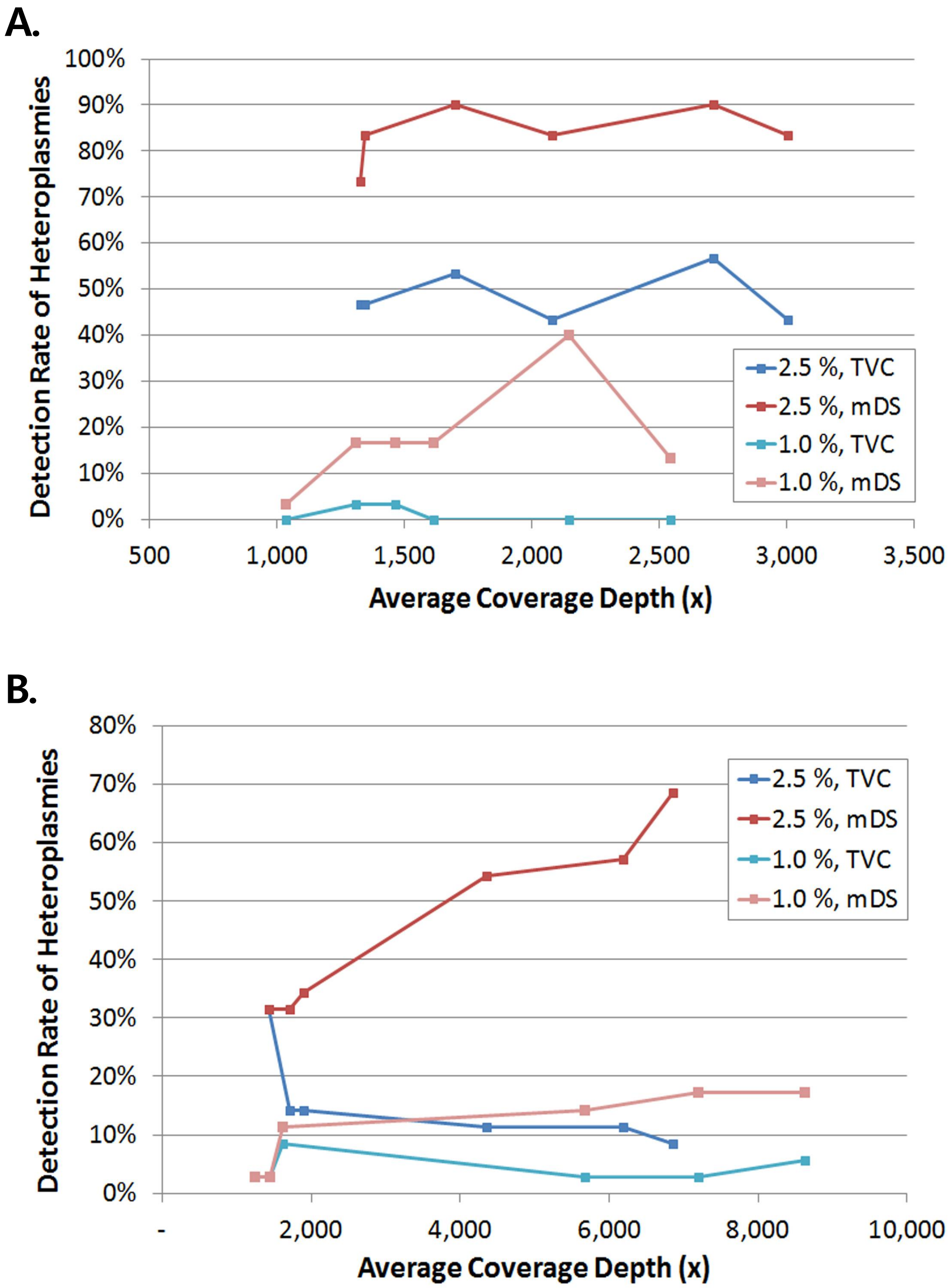


Figure 9. Average coverage depth and detection rate of artificial heteroplasmies in A-B pairs (A) and C-D pairs (B). There was no overt relationship between these parameters. The overall detection rates were higher in mDS than in TVC.

DISCUSSION

In this study, 5 blood samples of Korean with known innate heteroplasmy were selected and 2 pairs of extracted DNA samples were mixed in 5 serial ratios to confirm the accuracy of Ion Torrent platform in measurement of heteroplasmy level. Seneca et al. also made artificial mixtures for heteroplasmy analysis with Ion Torrent system,[18] but they observed only 11 heteroplasmic positions and focused on the determination of the detection limit for heteroplasmy. Also, they analyzed their data only with TVC which is originally developed for nuclear mutations rather than mitochondrial heteroplasmy. In the present study, over 30 heteroplasmic positions per each mixed pair were artificially generated, and the sequenced data were analyzed with both TVC and mDS.

For high-level heteroplasmies with MAFs over 10%, both TVC and mDS showed remarkable detection rates. When compared with our previous study,[36] the sequencing result was very concordant under the same experiment and analysis pipelines and even under another type of analysis tool. Also, the observed MAFs showed considerable correlations with expected MAFs in both mix pairs. However, the differences were more distinct in C-D pair than A-B pair, which suggests the effect of NUMTs detected in sample C, as discussed later.

The detection rates tend to be decreased with the level of heteroplasmies, which should be considered when low-level heteroplasmies with MAFs under 10% are the target of study. As shown in this study, despite the great improvement of MPS techniques, many researchers still recommend setting the detection threshold of mitochondrial heteroplasmy to 10%, especially for the daily practice or casework. As sensitivity and false positive rate are interrelated, more false-positives from various resources should be ruled out to detect more low-level heteroplasmies.

While mDS reported heteroplasmy with remarkable detection rate and detailed information, TVC generated clearly filtered and well-organized results. But its algorithm did not appear to be optimized for mitochondrial genome or heteroplasmy. After a simple modification of the reference sequence, the detection rates of TVC for mitochondrial heteroplasmy were improved to comparable level with mDS.

The sequencing results of mitochondrial heteroplasmy are very similar to those of nuclear somatic mutation. But the important difference is that the mutated sequences are ‘abnormal’ against the reference, while mitochondrial heteroplasmy is a ‘normal’ phenomenon which could be observed in any person, organ, or nucleotide position. Many programs for variant calling, including TVC, were designed focusing on the nuclear mutation. They are usually based on the concept of ‘normal’ reference and ‘abnormal’ variants. If this concept is reflected in the variant detection criteria or computation algorithm of quality scores, different performances in variant detection between the types of dominant allele are inevitable. This study revealed that this difference actually exists, and significantly affects the detection rate of heteroplasmy.

From the fact that the quality score is one of the most important criteria to call a variant in TVC, it can be supposed that this difference has originated from the computation strategy for the quality score. According to their manual, the quality score for each variant position is computed by posterior probability which is applied differently depending on the type of call. For a variant call, they would adopt the posterior probability that the variant allele frequency of the sample is greater than the pre-set threshold of minimum allele frequency. For a reference call, the posterior probability that the variant allele frequency is under the threshold would be used. In contrast, the log likelihood ratio model adopted by mDS categorizes the alleles to major or minor by frequency level, regardless of their coincidence to the reference sequence. This will be more reasonable strategy for the mitochondrial genome.

It could be assumed that the computations by TVC would overlook some low-level heteroplasmies having reference major allele and variant minor allele, as observed in this study. As seen in Figure 7, reference modification by replacement of the interested sequence actually improved the detection rates greatly. However, its usefulness is restricted to only a few situations when the minor allele sequences are known, or at least predictable. Meanwhile, the modified results of

TVC paradoxically show its well-established criteria for the discrimination of false signals. Also, TVC detected considerable numbers of low-level variants, even though their level was under the pre-set thresholds of minimum allele frequency for SNPs.

Open-source programs and their developers have been contributing to the progress of algorithms to analyze MPS data. There are several programs for mitochondrial genome, and some of them are specialized for heteroplasmy detection.[16,29,30,32] Most are Unix-based programs developed by small non-commercial groups of researchers. This unfamiliar operating system could be challenging for the researchers who lack knowledge of computer science or bioinformatics. The developers might have difficulties in maintenance, such as updates for coding or system errors and algorithms. Besides, each module might have been developed for a specific group, adopting different algorithms and reporting items according to their own purposes. For example, MToolBox, known to be a specialized tool for heteroplasmy analysis,[29] warns in their manual that they provide a heteroplasmy report only for variants found against the reference sequence chosen for read mapping.[40] There are increasing needs for easily accessible and integrated mitochondrial analysis tools, as interest in mitochondrial heteroplasmy is increasing in not only forensic science and classical clinical medicine,[41] but also developing areas of laboratory medicine[5] and biotechnology.[7-9] Fortunately, new programs like mDS have adopted previously known algorithms with modification, introducing more user-friendly interfaces, and are continuously being developed.[16] Also, mDS automatically provides the information about NUMTs for each variant, which can help researchers interested in NUMTs detection. But the difficult decision whether a variant is NUMTs or not is still on the researcher himself.

As presented in Figure 4, mDS reported very low-level heteroplasmies congregated in the front sequence of an amplicon, supposing them as NUMTs. This indicates the possibility of sharing homologous sequences between the primer binding site of mitochondrial DNA and multiple sites of nuclear DNA, resulting in the binding of primer to the nuclear DNA and generating NUMTs.[25] For example, several nuclear sequences highly similar to the sequence of the primers used in this study were found by BLAST search[3] on the reference human genome (data not shown). Mitochondrial DNA has tens to hundreds of times more chance of binding with primer than nuclear DNA does because of their large copy number, which explains the low proportion of NUMTs. Therefore, these heteroplasmies reported by mDS had considerable possibility of NUMTs amplified by unintentional binding of primers to nuclear homologous sequences, rather than 'actual' mitochondrial heteroplasmies.

Because the length of nuclear genome is absolutely and relatively long compared to that of mitochondrial genome, the perfect primer for universal mitochondrial population without nuclear homology is

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3) https://blast.ncbi.nlm.nih.gov/Blast.cgi

difficult to design. Unfortunately, there are no established criteria yet for discriminating NUMTs from mitochondrial heteroplasmy or technical error. So researchers should keep in mind that the NUMTs could be included in the pool of PCR product even the long amplicon method was adopted.[42] NUMTs should be filtered out by bioinformatical approaches, using appropriate thresholds of quality score, minimum MAF and strand bias, and comparing the sequences with previously reported lists of NUMTs. Confirmation by other analysis tools or MPS systems specialized for mitochondrial genome could be also considered.[14]

The actual heteroplasmy and noise signal could be more easily discriminated if the sequence of minor allele or the location of heteroplasmy is known in advance. For example, in familial search, if the information of inherited heteroplasmy has been obtained from the family members of maternal lineage, the researcher can directly target the interested region or compare the suspected low signals. In clinical practices targeting known mutations or variants, it would be more straightforward. But without clues for the composition and distribution of minor components, the possibility of the presence of NUMTs or contamination should always be kept aware of, especially for the low-level heteroplasmies under 5%.

Meanwhile, the actual heteroplasmy and situational mixture might be discriminated by comparing the pattern of heteroplasmies throughout the sequenced genome. Innate heteroplasmies tend to appear in small number, leading to only little effect in determination of haplogroup. For example, in the previous study in Korean, only 7 of 186 individuals showed more than 2 point heteroplasmies and 5 heteroplasmies in an individual were the maximum.[36] Also, the levels of heteroplasmies observed in each individual showed considerable difference. The pattern of situational mixture could be referred to that of artificial heteroplasmy generated in this study. The number of discrepant position of all pairs generated in this study ranged from 7 to 42, with an average of 30 (data not shown). The levels of these artificial heteroplasmies were consistent to the mixture ratio as described above.

Although there is no consensus for heteroplasmy analysis yet, about 10 to 20× coverage depths are regarded as a minimum threshold of minor allele in general. Also, about 1,000 to 2,000× of coverage depths per position are thought to be appropriate even for low-level heteroplasmy, considering both the data capacity of each MPS run and the financial limitation. The generally recommended sample numbers for the Ion Torrent chip models satisfies these suggestions.[24] Increase of the coverage depths more than these level seems to be not effective, as shown in this study.

It should be noted that the actual coverage depth could not always be reached the expected value for two reasons. First, as known before, there is significant variation in coverage depth per position within the mitochondrial genome. So when the heteroplasmy is located in the region of low coverage depth, minor allele sequence may be unable to be detected or discriminated from noise signal. Also, as observed in this study, the coverage imbalances between the samples placed on a chip could be another important factor. So the expectation of the coverage depth should consider not only the level and location of the targeted heteroplasmy, but also the unexpected variation of entire coverage depths which could be several-folds between samples. More study about the coverage imbalance itself and the strategy of balance enhancement is needed in the future.

Also, interestingly, the sequencing results of 9947A in this study showed different heteroplasmy profiles from previous reports.[34,37] 9947A, extracted liquid DNA from human lymphoblastoid cell line GM009947A, is one of the Standard Reference Materials certified by the National Institute of Standards and Technology, and is called as NIST SRM 2392 component B. It is known to have two heteroplasmy positions, np 1393 and 7861, but only part of them was reported in this study. In addition to the differences of the detection algorithm of each tool as described above, the effect of mitochondrial segregation during repetitive cell culture could be an important factor in this result. During mitosis, the mitochondria in a cell are randomly allocated to daughter cells. This might result in a shift in the distribution of mutation or heteroplasmy.[3] This indicates that the detailed sequence information of reference materials, including heteroplasmy status, should be monitored and informed periodically, according to the cell line generation and the lot number.

Finally, the following suggestions could be derived from this study. Coverage depths over about 1,500× seem to be sufficient to obtain reliable detection results of heteroplasmy, while the level of MAF itself is the most important factor for the detection rate. Both the location and level of the heteroplasmy and the effects of coverage imbalances between samples sharing data capacity should be considered in the expectation of coverage depths. The possibility of NUMTs should be kept in mind for the heteroplasmies lower than 5%, especially when they appear unexpectedly. If possible, securing the candidate sequences of the minor component would be helpful in heteroplasmy analysis. The selection of the analysis tools for heteroplasmy detection and the interpretation of their results should be based on the understanding of the different purposes and meanings of the computation algorithms and parameter settings. Secondary analysis tools might be helpful in confirming the results, especially if they use different computation algorithms.

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요약(국문초록)

배경: 미토콘드리아 유전자의 이형세포질성은 한 개체 내에 서로 다른 염기서열이 동시에 존재하는 현상을 말하며, 법의학과 임상의학 영역 모두에서 중요한 의미를 갖고 있다. 이형세포질성의 탐지에 차세대 염기서열 분석법을 활용하기 위해 여러 연구들이 진행되고 있지만, 명확한 가이드라인은 아직 확립되어 있지 않다. 이 연구에서는 차세대 염기서열 분석법을 이용하여 선천적으로 존재하거나 인위적으로 만들어진 이형세포질성을 탐지하고 그 활용 가능성을 알아보았다. 또한 이 과정에서 이러한 형태의 연구를 할 때 반드시 고려해야 할 사항들을 확인하였다.

방법: 선천적 이형세포질성을 갖고 있는 5개의 시료를 선별한 뒤 2쌍의 시료를 혼합하여 인위적인 이형세포질성을 만들었다. 혼합 비율은 1%에서 50%까지 다양하게 구성하였다. 모든 시료는 two-amplicon 방법으로 증폭한 뒤 Ion Torrent system을 이용하여 염기서열 정보를 얻었다. 결과 데이터는 두 가지 프로그램, 즉 Torrent Suite Variant Caller(TVC)와 mtDNA-Server(mDS)를 이용하여 분석하였다.

결과: 선천적 이형세포질성은 분석 프로그램에 관계없이 모두 동일하게 탐지되었으며, 기존 결과와도 일치하였다. 인위적 이형세포질성의 결과값 역시 각 시료의 실제 혼합비율과 유사하게 측정되었다. 혼합비율이 높은 경우(10-50% 사이) 탐지율은 90%에 가까웠지만, 낮은 경우(10% 미만)에는 급격히 감소하는 것을 볼 수 있었다. 각 분석 프로그램의 결과를 비교하였을 때 TVC의 탐지율이 mDS에 비해 전반적으로 낮게 나타났는데, 이것은 TVC가 미토콘드리아 유전자를 핵 유전자와 동일한 알고리즘으로 분석하기 때문인 것으로 판단되었다. 즉 기준 염기서열에 낮은 비율의 변이가 섞인 경우, 반대의 경우에 비해 낮은 가중치를 부여하기 때

문에 최종 단계까지 보고되지 않고 탈락하는 것이다. 또한 mDS 분석 결과에서는 예상치 못한 다수의 낮은 이형세포질성이 탐지되었는데, 이들의 위치나 양상은 기존에 보고된 핵 유사 염기서열(NUMTs)과 일치하는 것으로 보였다. 한편 각 chip에서 함께 분석된 시료들은 모두 동일한 농도였음에도 불구하고 coverage depth가 상당한 차이를 보였다. 다만 각 시료의 이형세포질성 탐지율은 coverage depth와 관계가 없었다.

결론: 본 연구에서는 차세대 염기서열 분석법이 높은 정확도로 미토콘드리아 이형세포질성을 탐지할 수 있음을 확인하였다. 그러나 그 결과를 올바르게 해석하기 위해서는 핵 유전자와 구분되는 미토콘드리아 유전자의 고유한 특성과 각 연구자가 사용하려는 분석 프로그램의 기본적인 원리에 대해 반드시 이해할 필요가 있다는 것을 알 수 있었다.

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주요어 : 미토콘드리아 유전자; 이형세포질성; 유전자 염기서열 분석; 차세대 염기서열 분석법; 생물정보학

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