Main specifications for the DS3000

■ Main unit specifications

Item	Details
Number of capillaries	4
Capillary length	36 cm
Sample format	8-tube strip × 4
Device control	Touch panel PC
Number of Dyes	6
Application	Sequencing analysis / Fragment analysis
Size	400 (W) × 600 (D) × 600 (H) mm
Weight	45 kg
Performance guarantee temperature	15 – 30˚C
Performance guarantee humidity	20 – 80% RH (no condensation)
Power input	100 – 240 ±10% VAC, 50/60 Hz
Rated power	260 VA
Supported secondary analysis software	• Mutation Surveyor (SoftGenetics, LLC, sold separately) • GeneMarker (SoftGenetics, LLC, sold separately) • GeneMarker HID (SoftGenetics, LLC, sold separately)

■ Run module specifications

Run Module	Application	Polymer type	Contiguous Read Length*1 (bp, QV20 CRL)	Average run time (minutes)
Fast_Sequence36_Polymer7	Sequencing analysis	Polymer7	≥600	≤32
Standard_Sequence36_Polymer7	Sequencing analysis	Polymer7	≥700	≤60
BDx_Fast_Sequence36_Polymer7	BDx sequencing analysis	Polymer7	≥600	≤32
BDx_Standard_Sequence36_Polymer7	BDx sequencing analysis	Polymer7	≥700	≤60

*1 Contiguous Read Length (bp, QV20 CRL) is measured with BigDye® Terminator v3.1. Sequencing Standard Kit (Thermo Fisher Scientific, sold separately)

Run Module	Application	Polymer type	Average run time (minutes)	Sizing precision*2 (bp, 50 – 400 bp)
Fragment_Analysis36_Polymer7	Fragment analysis	Polymer7	≤35	NA
Fragment_Analysis36_Polymer4	Fragment analysis	Polymer4	≤44	<0.16

*2 Sizing precision (bp, 50 - 400 bp) is measured with PowerPlex™ ESI17 Fast Allelic Ladder and WEN ILS 500 ESS. (Promega®, sold separately)

- BigDye is a registered trademark of Thermo Fisher Scientific Inc.
- Promega, PowerPlex is a registered trademark of Promega Corporation.

■ Consumables specifications

Product name	Part number	Details	Remarks
Capillary Cartridge 36 cm	613-0330	1 pcs	Storage temperature: 15 – 30˚C
Buffer	613-0252	Anode Buffer × 2 cartridges Cathode Buffer × 2 cartridges	Storage temperature: 2 – 10˚C
Polymer7	613-0251	4 cartridges	Storage temperature: 2 – 10˚C
Polymer4	613-0250	4 cartridges	Storage temperature: 2 – 10˚C
Septa for Cathode Buffer Cartridge	613-7231	10 pcs	
Retainer for Cathode Buffer Cartridge	613-7233	4 pcs	
Septa for 8 well tubes	613-7230	24 pcs	
Base and Retainer for 8 well tubes	613-7232	4 pcs	
Anode Electrode Assembly	613-7263	1 pcs	

Specifications in this catalog are subject to change with or without notice,
as Hitachi High-Tech Corporation continues to develop the latest technologies and product for its customers.

CAUTION:For correct operation, follow the instruction manual when using the instrument.

NOTICE:The system is For Research Use Only, and is not intended for any animal or human therapeutic or diagnostic use.
Hitachi High-Tech does not guarantee the performance on the document with every possible sample under every possible condition.

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NGS data confirmation using the DS3000 Compact CE Sequencer

Abstract

Due to the ease of the analysis, genomic variations detected by next-generation sequencing (NGS variants) are sometimes confirmed by Sanger sequencing (1). Here, we introduce a practical example of confirming NGS variants using the DS3000 Compact CE Sequencer (DS3000). First, 12 single-nucleotide substitutions were analyzed in genomic DNA extracted from a human colon cancer cell line (HCT116). The DS3000 base caller detected all of the variants. Furthermore, DS3000 paired with Mutation Surveyor (ver. 5.1.2, SoftGenetics, Inc.) successfully quantified the mutation rates and detected nucleotide insertions and deletions. This system is for research use only and is not intended for any animal or human therapeutic or diagnostic use.

DS3000
Compact CE Sequencer

Results

Single-nucleotide substitutions

Twelve single-nucleotide substitutions were randomly selected from the NGS variants of HCT116 that were detected in reference (2) (Table 1). The sequencing platform used was HiSeq® 2000 (Illumina).

First, homozygous substitutions were analyzed. The DS3000 base caller detected all five single-nucleotide substitutions (Table 1, ID: 1-5). Next, seven heterozygous mutations were analyzed (Table 1, ID: 6-12). Heterozygous single-nucleotide substitutions are detected as two peaks overlapping at a specific site. The DS3000 base caller identified all of the heterozygous single-nucleotide substitutions (Table 1). Figure 1 shows an example of an electropherogram obtained with DS3000 (Table 1, ID: 9). The overlapping of cytosine and thymine peaks formed a mixed base (Y). The results were concordant with those from NGS (Figure 1A). Next, the heterozygous (Table 1, ID: 6-12) mutation rates were determined with Mutation Surveyor. DS3000 is equipped with two types of sequencing assays: fast sequencing and standard sequencing. In general, fast sequencing is used to obtain read lengths as long as possible within a short period, while standard sequencing, which takes longer, is used to obtain longer read lengths. Figure 2 shows no significant difference in the results between fast and standard sequencing.

Table 1: Results of single-nucleotide substitutions

ID	Zygosity	Gene	Wild type	Hiseq® 2000	DS3000
1	Homozygote	KLHL34	C	T	T
2	Homozygote	LHFPL1	C	T	T
3	Homozygote	LMBRD2	G	C	C
4	Homozygote	USP9Y	A	G	G
5	Homozygote	USP9Y	G	A	A
6	Heterozygote	KRAS	C	C, T	Y
7	Heterozygote	MYCBP2	C	C, T	Y
8	Heterozygote	MYCBP2	A	A, G	R
9	Heterozygote	MYCBP2	C	C, T	Y
10	Heterozygote	PARK2	T	A, T	W
11	Heterozygote	PIK3CA	A	A, G	R
12	Heterozygote	PINK1	G	A, G	R

Y: C or T. R: A or G. W: A or T.

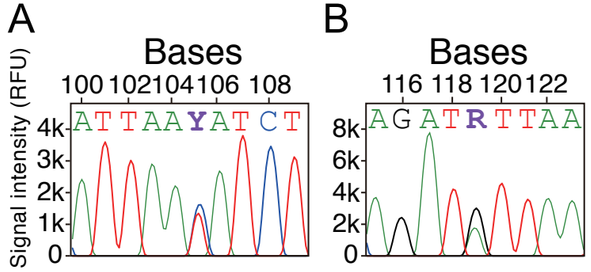

Figure 1. Electropherogram of heterozygous single-nucleotide substitutions observed in MYCBP2 (Table 1, ID: 9).

A. Electropherogram of the forward strand.
The cytosine and thymine peaks, and the mixed base (Y) identified by NGS are observed. B. Electropherogram of the reverse strand.
This is the complementary strand in Table 1 and Figure 1A.
Peaks of adenine and guanine and the mixed base (R) are observed.

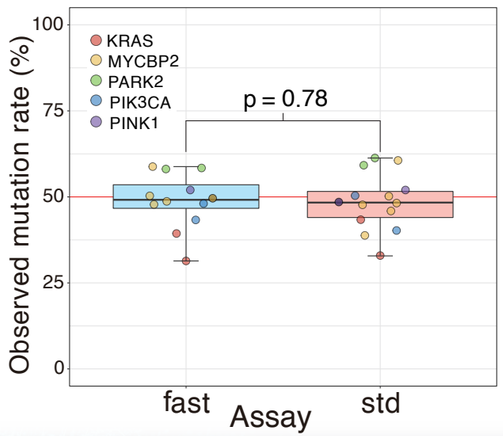

Figure 2. Determination of the mutation rates using DS3000 and Mutation Surveyor.

The mutation rates are distributed at around 50%.
This indicates that each mutant allele is heterozygous.
There was no significant difference between fast sequencing (fast) and standard sequencing (std).
The p-value was obtained from a two-tailed t-test.

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Nucleotide insertions and deletions

Heterozygous insertions and deletions result in the overlapping of peaks at all bases after the mutation point. This makes it difficult to identify mutant alleles (Figure 3). The pairing of DS3000 and Mutation Surveyor makes it possible to detect nucleotide insertions and deletions. Please note that wild-type genomic DNA data are required for the analysis. To ensure that the data quality is consistent, it is recommended to obtain the wild-type genomic DNA data from the same sample injection as the mutant. The NGS data indicated a cytosine-thymine-thymine (CTT) deletion in CTNNB1 and a guanine (G) insertion in PLA2G15 (Table 2). Figure 3 shows that each of these was correctly detected.

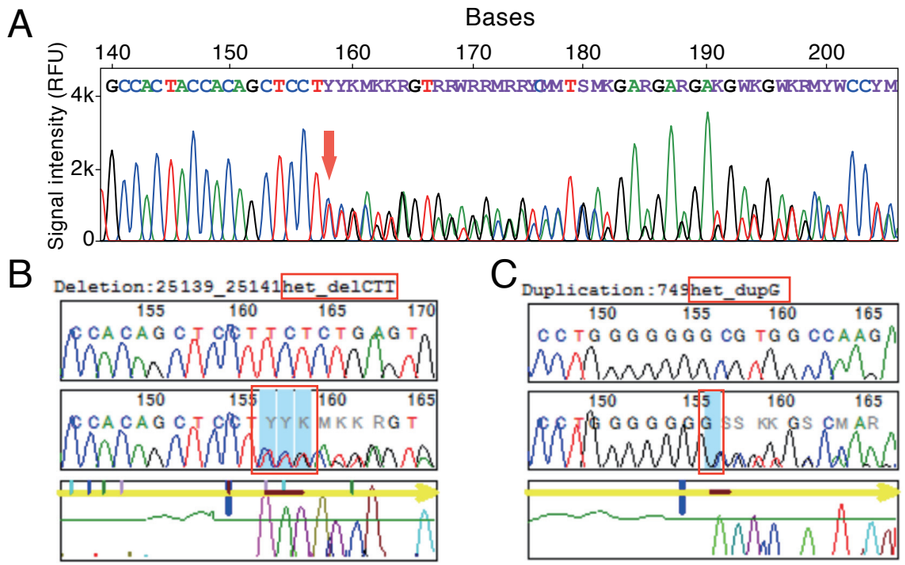

Figure 3. Analysis of nucleotide insertions and deletions.

A. Electropherogram of the allele (Table 2, ID: 13) in which the heterozygous deletion of cytosine-thymine-thymine (CTT) occurs.
The arrow indicates the point at which the peaks start to overlap. Mixed bases are continuously called in the subsequent region.
B. A part of a report generated by Mutation Surveyor. The electropherogram shown in A was analyzed.
Electropherogram of the allele in which the deletion of CTT occurs in heterozygosity (Table 2, ID: 13). The deletion of CTT was detected (red box in the figure).
C. Analysis of the allele (Table 2, ID: 14) in which the heterozygous guanine insertion occurs. Guanine insertions are shown in Mutation Surveyor.
Electropherograms of the wild type (top) and mutant (middle), and the difference between the wild type and mutant (bottom) are shown.

Conclusion

DS3000 detected all of the NGS variants. By pairing it with Mutation Surveyor, it is possible to quantify the mutation rates and identify nucleotide insertions and deletions. When confirming NGS variants, it is recommended to check not only the called results but also the quality of the peak shape.

The quality of the sequence data greatly influences the analysis of mutant alleles. To obtain more reliable data, please consider the following points.

1. DS3000 allows you to set a threshold for calling mixed bases. If the threshold is too high, heterozygous single-nucleotide substitutions may not be detected. Please refer to the DS3000 instruction manual to set the threshold. Please note that the Mutation Surveyor base caller is not affected by the settings on DS3000.
2. If the signal intensity is too low, the mutant allele may not be detected. Please adjust the sample concentration, injection conditions, and purification method to obtain an intensity of approximately 1,000 RFU or higher.
3. Due to the nature of electrophoresis, the resolution decreases as the nucleotide length increases, and the peak shapes may be distorted by dye blobs at around 60 to 120 bp.

Therefore, it is recommended to design primers which 3' end locates approximately 200 to 300 bp away from the nucleotide of interest.

4. To obtain results with higher reliability, please consider sequencing both forward and reverse strands.

Materials and Methods

Sample preparation

Fourteen point mutations were randomly selected from reference (2) (Table 2). The sequencing platform used was HiSeq® 2000 (Illumina). The mutations consisted of 12 single-nucleotide substitutions (seven homozygous and five heterozygous), one heterozygous base insertion, and one deletion. The region containing each allele was amplified by PCR according to Tables 3-5. PCR amplicons were purified using the DNA Clean & Concentrator™ Kit (Zymo Research). Amplicons were eluted with 10 μL of Elution Buffer and quantified using QuBit® (Thermo Fisher Scientific). Cycle sequencing reactions were then performed using 10 ng of amplicon (Tables 6 and 7). The resulting products were purified by ethanol precipitation and dissolved in 10 μL of HiDi™ formamide (Applied Biosystems™) according to the manufacturer's instructions. Sequencing analysis was performed on DS3000 (Table 8). The obtained results (ab1 file) were analyzed with Mutation Surveyor (ver. 5.1.2, SoftGenetics, Inc.).

Data Analysis

First, the electrophoresis data were loaded into "Open Files" under "Files" (Figure 4). The "Reference Files" area is for the wild-type data, and the "Sample Files" area is for the data to be analyzed. Next, Mutation Quantifier was set according to Figure 5. The electropherograms and results shown in Figure 3 were obtained by selecting the mutation and clicking "Clinical report" from the "Display" menu.

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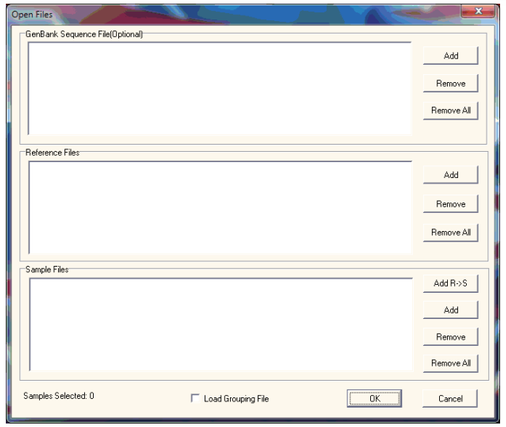

Figure 4. Mutation Surveyor settings 1.
The "Open Files" dialog is shown.
Data of the wild type were loaded into "Reference files", and data to be analyzed were loaded into "Sample Files".

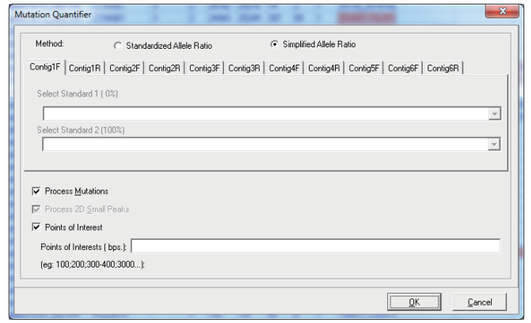

Figure 5. Mutation Surveyor settings 2.
An example of the Mutation Quantifier settings is shown.

Table 2. List of mutations analyzed in this report

ID	Gene	Position	Wild type	Observed mutation	Zygosity
1	KLHL34	chrX:21675124	C	T	Homozygote
2	LHFPL1	chrX:111914255	C	T	Homozygote
3	LMBRD2	chr5:36136519	G	C	Homozygote
4	USP9Y	chrY:14821418	A	G	Homozygote
5	USP9Y	chrY:14885820	G	A	Homozygote
6	KRAS	chr12:25398281	C	T	Heterozygote
7	MYCBP2	chr13:77713342	C	T	Heterozygote
8	MYCBP2	chr13:77751942	A	G	Heterozygote
9	MYCBP2	chr13:77764421	C	T	Heterozygote
10	PARK2	chr6:162394383	T	A	Heterozygote
11	PIK3CA	chr3:178952085	A	G	Heterozygote
12	PINK1	chr1:20977047	G	A	Heterozygote
13	CTNNB1	chr3:41266133	CCTT	C	Heterozygote
14	PLA2G15	chr16:68289854	T	TG	Heterozygote

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Table 3. Primer sequences

ID	Forward	Reverse
1	ACAGAGTTGCTGGAGCGTGT	CCTCCTCCTCTTCCTCCAAC
2	TGGACAAAGTGAAGGGGGTG	GTGGAAGAATGTGGGCGCTA
3	ACTTGCTTACCTCCATGGCA	AGAAACAGTGTCTCCTGTGTT
4	GGTCTCTGCAAGATGTTTTGTCC	CCACACTTAGCCCACAGTCA
5	CTCTGTGCTCCTCAGGCAAA	ACCTCAGGTACAATGTTGGCA
6	AGAATGGTCCTGCACCAGTAA	TGATTGAATTTTGTAAGGTATTTTGAA
7	TGTGGGTTACCTCAGAACTGA	TGCAGACTTTCAGACCTTGCT
8	TGGTAAATCCTGATACACAACCCA	ACCTTTGGATGGTTTTGTTAAAGGT
9	GCTGAATGCTTCAAATAATTTCTCCC	AAAAGAGTGAATCATTTTCATTGACAT
10	CTTACCTCACGTCCGTGGAG	CTGGGAAAGGTTTGATGCTGA
11	ATGATGCTTGGCTCTGGAAT	ATGCTGTTCATGGATTGTGC
12	GCTTCCCTTCCTGTTGCAGA	CTCCCACCCTCACCATTCAC
13	TGGGTCATATCACAGATTCTTTTT	TCAAAACTGCATTCTGACTTTCA
14	TGAAAACGGGCCCTACTTCC	GCCCAGGAGGACACTTAGGA

Table 4. PCR conditions

Temp. (°C)	Time (s)	Cycle
98	30	×36
98	10	
65	30	
72	30	
70	300	×1
4	hold	

Table 5. PCR mixture

Reagents	Volume (μL)
Phusion™ Hot Start II DNA Polymerase (Thermo Fisher Scientific, 2U/μL)	0.2
5×Phusion™ HF Buffer	4
dNTP Mixture (2.5 mM each)	1.6
Forward primer (10 μM)	1
Reverse primer (10 μM)	1
DNA (10 ng/μL)	1
PCR grade water	11.2

Table 6. Cycle sequencing mixture

Temp. (°C)	Time (s)	Cycle
96	60	×1
96	10	×25
54	5	
60	240	
4	hold	

Table 7. Cycle sequencing conditions

Reagent	Volume (μL)
PCR amplicon (5 to 10 ng/μL)	2
BigDye® Terminator v3.1 Ready Reaction Mix	4
Primer (10 μM)	0.64
BigDye® Terminator v1.1 & v3.1 5×Sequencing Buffer	2
PCR grade water	11.36

Table 8. Sample injection and electrophoresis conditions

Assay	Sample injection		Electrophoresis	
	Voltage	Time	Voltage	Time
AB_Seq_36_Fast	1.2 kV	4 s	14 kV	900 s
AB_Seq_36_Std	1.2 kV	4 s	7.5 kV	2,360 s

Table 9. List of GenBank Sequence files

ID	Gene	GenBank file
1	KLHL34	https://www.ncbi.nlm.nih.gov/nuccore/NM_153270.3
2	LHFPL1	https://www.ncbi.nlm.nih.gov/nuccore/NM_178175.4
3	LMBRD2	https://www.ncbi.nlm.nih.gov/nuccore/NM_001007527.2
4	USP9Y	https://www.ncbi.nlm.nih.gov/nuccore/NM_004654.4
5	USP9Y	
6	KRAS	https://www.ncbi.nlm.nih.gov/nuccore/NG_007524.2
7	MYCBP2	https://www.ncbi.nlm.nih.gov/nuccore/NM_015057.5
8	MYCBP2	
9	MYCBP2	
10	PARK2	https://www.ncbi.nlm.nih.gov/nuccore/NM_004562.3
11	PIK3CA	https://www.ncbi.nlm.nih.gov/nuccore/NG_012113.2
12	PINK1	https://www.ncbi.nlm.nih.gov/nuccore/NM_032409.3
13	CTNNB1	https://www.ncbi.nlm.nih.gov/nuccore/NG_013302.2
14	PLA2G15	https://www.ncbi.nlm.nih.gov/nuccore/NM_012320.4

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(1) Mu W., Lu H-M., Chen J., Li S. & Elliott A. Sanger confirmation is required to achieve optimal sensitivity and specificity in next-generation sequencing panel testing. J. Mol. Diagn. 18, 923-932 (2016)
(2) Mouradov, D. et al. Colorectal cancer cell lines are representative models of the main molecular subtypes of primary cancer Cancer Res. 74, 3238-3247 (2014)