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Analysis of rare variants with the DS3000 Compact CE Sequencer and Mutation Surveyor

Abstract

Rare variants are mutations that are found in a small subpopulation of specimens. They have been recognized for their usefulness in cancer research, because they can cause tumorigenesis and promote tumor progression. The signals from rare variants are buried behind the signals from wild-type DNA, which comprises the vast majority of DNA in samples. Therefore, the detection of rare variants requires very high sensitivity and accuracy. In general, Sanger sequencing using capillary sequencers cannot detect mutations with frequencies below 25%. Here, we introduce an example in which the sensitivity of detecting rare variants is increased using the DS3000 Compact CE Sequencer (DS3000) paired with Mutation Surveyor (SoftGenetics). This pairing successfully detected low-frequency mutations, including some with a frequency of 5% in several genes, although the sensitivity varied depending on the gene.

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

Mutation Surveyor (SoftGenetics) implements its own base caller to draw electropherograms and perform base calling from raw data. We analyzed the same raw data with Mutation Surveyor and DS3000 to compare the electropherograms (Figure 1). Although there were some differences in the peak shapes and the rendering of background noise, both base callers were able to detect the target mixed base (S, highlighted in Figure 1). Next, we compared the mutation detection rate of each base caller (Figure 2) and found that Mutation Surveyor significantly improved the detection rate of rare variants (<10%). Therefore, we used Mutation Surveyor for the following analyses. Figure 3 summarizes the expected and observed mutation rates for each gene. The sensitivity and percent agreement with the expected mutation rate varied depending on the target gene and allele. Mutations with a frequency of 5% could be detected in *EGFR, GNAS, KRAS,* and *NRAS,* whereas mutations with a frequency less than 20% could not be detected in *BRAF* (V600K). In addition, in *GNAS* (R201C), a false-positive result was obtained in wild-type DNA (highlighted in Figure 3).

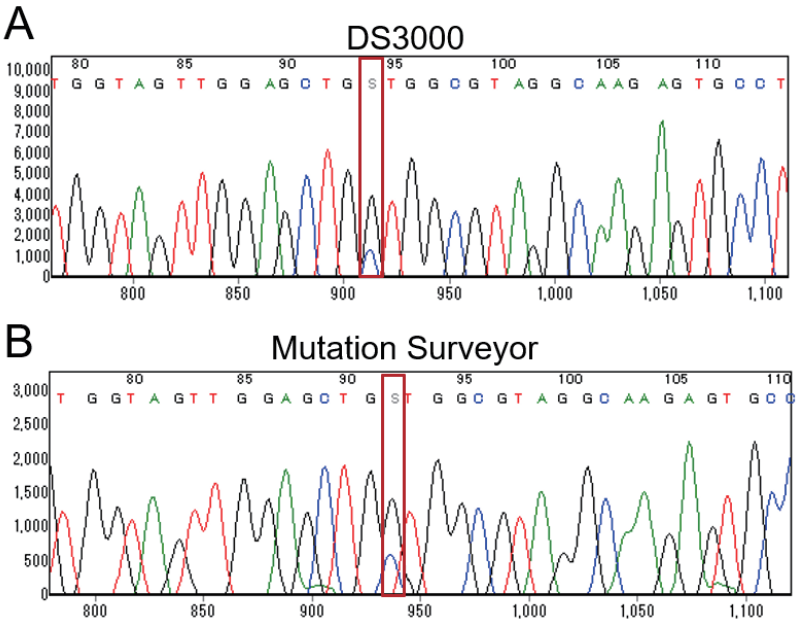

Figure 1:
Comparison of electropherograms
The same raw data for *KRAS* G12A (mutation rate: 20%) were analyzed by DS3000 (A) and Mutation Surveyor (B). Both base callers successfully detected the expected mixed base (S, highlighted by the red box). The x-axis shows the size of the DNA fragment (top) and scan number (bottom), and the y-axis shows the signal intensity (RFU).

Conclusion

In this report, we described a practical example of how Mutation Surveyor can improve the detection of low-frequency mutations. The pairing of DS3000 with Mutation Surveyor significantly improved the detection rate (Figures 2 and 3), and successfully detecting rare variants with a frequency of 5% in some genes. However, in some other genes, such as *BRAF* (V600K), mutations with a frequency less than 20% could not be detected. In addition, a false-positive result was observed for *GNAS* (R201C). These variations may have been caused by a combination of factors, such as the difficulty of handling the diluted DNA, amplification bias during PCR and cycle sequencing reactions, and sensitivity differences among fluorescent dyes. To obtain more reliable results, the number of experimental replicates as well as the timing of repetitions*1 should be carefully considered.

*1: It is possible to address the cause of experimental errors by considering the timing of the preparation of replicates. Examples are described below.

1. Biological replicates

Sampling from the target cell population should be performed multiple times.

2. Technical replicates

Samples collected from the same cell population should be divided into multiple tubes during the experiment.

3. PCR replicates

Samples should be collected in multiple tubes immediately prior to PCR to examine PCR amplification bias. Since the amplification of DNA with a small number of molecules, such as low-frequency mutations, is prone to bias, verification by PCR replicates is

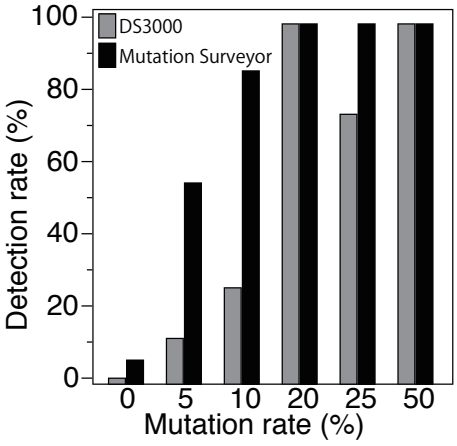

Figure 2: Detection rate with each base caller

Reference standards harboring known mutations were diluted with wild-type DNA to prepare samples containing the mutations at a range of frequencies, as shown on the x-axis. The y-axis shows the detection rate, *i.e.,* the percentage of successfully detected mutations among the expected mutations, at each mutation rate (n > 7).

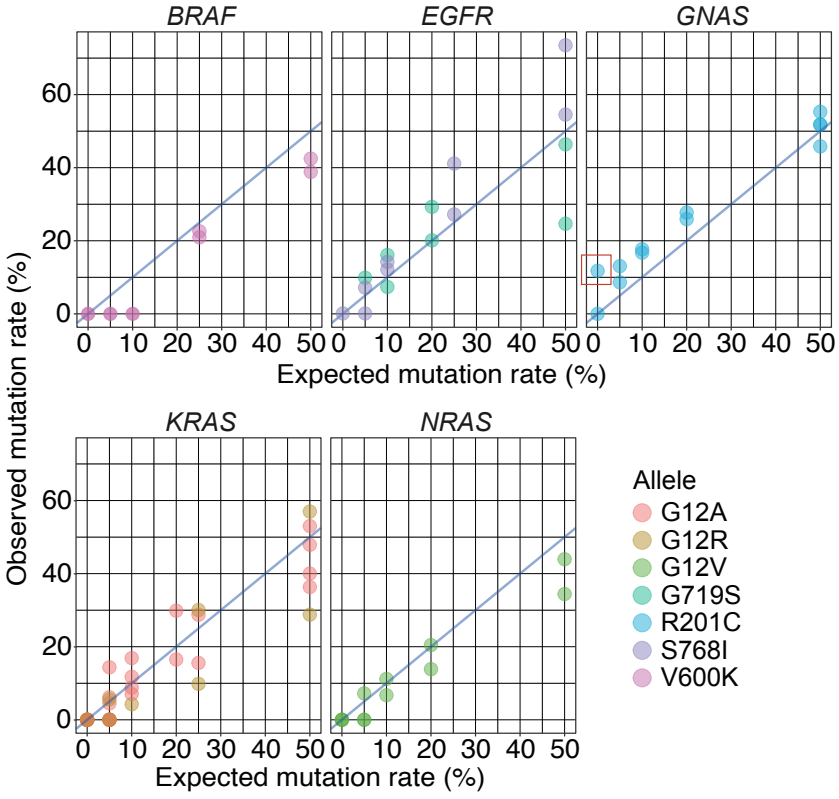

Figure 3: Comparison of the observed mutation rates

Blue lines indicate a 100% correlation between the observed and expected values. A red box highlights the false-positive result observed in *GNAS*.

Materials and Methods

Sample preparation

Reference standards obtained from Horizon Discovery, Ltd., were used in this report. These standards were derived from the genomic DNA of a cell line in which mutations were introduced by genome editing, and both the mutated site and the frequency are known. The reference standards were diluted with wild-type DNA to prepare samples containing the mutated alleles at a range of frequencies. The samples used in this report are summarized in Table 1. PCR was performed using the diluted DNA (Tables 2 – 4), and purified with the DNA Clean & Concentrator® Kit (Zymo Research). Amplicons were eluted with 10 μL of EB, and quantified with QuBit (Invitrogen). The cycle sequencing reaction was then performed using 10 ng of the amplicons (Tables 5 and 6). The reaction products were purified by ethanol precipitation, and dissolved in 10 μL of Hi-Di™ formamide (Applied Biosystems®) according to the manufacturer's instructions. Sequencing analysis was performed on DS3000 (Table 7). Wild-type DNA was electrophoresed in parallel as a negative control. Electrophoresis data (.ab1 file) were analyzed by Mutation Surveyor (ver. 5.1.2, SoftGenetics).

Data analysis

Mutation analysis using Mutation Surveyor requires the electrophoresis data of wild-type DNA as a control. When analyzing rare variants, it is strongly recommended to perform sequencing analysis of both forward and reverse strands. If possible, electrophoresis data of the positive control, *i.e.,* a sample with a known percentage of mutations, should also be considered to make the analysis more accurate. An example of the operations to be performed for analysis in Mutation Surveyor is described below.

Settings before loading files

First, select "Process", then "Settings" from the home screen to access the Mutation Project Settings. Select the " Input" and "Display" tabs, and set the configurations shown in Figure 4.

Settings for loading files

Select "Open Files" from the home screen. It is recommended to obtain the reference files in the same run as the sample files.

Settings for the detection process

Configure the "Mutation Quantifier" as shown in Figure 6. If there is a positive control, register it in the "Quantification Group Editor" as shown in Figure 7.

Table 1: List of reference standards

Catalog ID	Gene	Mutation	Allele	Amplicon size (bp)	Primer ID	PCR condition
HD239	*BRAF*	V600K	GT/AA	513	1	1
HD253	*EGFR*	G719S	G/A	408	2	2
HD261	*EGFR*	S768I	G/T	373	3	3
HD674	*GNAS*	R201C	C/T	246	4	4
HD265	*KRAS*	G12A	G/C	246	4	4
HD287	*KRAS*	G12R	G/C	385	5	5
HD302	*NRAS*	G12V	G/T	351	6	6

Table 2: PCR primers and annealing temperature

Primer ID	Forward Primer	Reverse Primer	Annealing temperature (˚C)
1	CTGGGCCTACATTGCTAAAAT	AGTTGAGACCTTCAATGACTTTCTA	54
2	GCTGAGGTGACCCTTGTCTC	GTCAATGGCCCCTTTCATAA	56
3	CTCTCCCACTGCATCTGTCA	ACACACCAGTTGAGCAGGTA	56
4	GGTGGAGTATTTGATAGTGTATTAACC	AGAATGGTCCTGCACCAGTAA	54
5	TAAGGATGGGGGTTGCTAGA	TGGGTAAAGATGATCCGACA	54
6	GGACTCTGAGCCCTCTTTCC	CACAGCATCCTACCGTTGAA	56

Table 3: PCR conditions

Temperature (˚C)	Time (s)	Cycle
98	30	×35
See Table 2	30	
72	30	
70	300	×1
4	Hold	

Table 4: PCR mixture

Reagent	Volume (μL)
TaKaRa Ex Taq HS (5 U/μL)	0.25
10× Ex Taq Buffer (Mg^{2+} plus)	5
dNTP mixture (2.5 mM each)	4
Forward primer (10 μM)	2
Reverse primer (10 μM)	2
DNA (10 ng/μL)	1
PCR-grade water	35.75

Table 5: Cycle sequencing reaction

Temperature (˚C)	Time (s)	Cycle
96	60	×1
96	10	×25
54	5	
60	240	
4	Hold	

Table 6: Reaction mixture for cycle sequencing

Reagent	Volume (μL)
PCR amplicon (5 – 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 7: Conditions for the electrophoresis

Polymer	Module	Run time	Run voltage
Polymer 7	AB_Seq_36_Std	ca.60 min	7.5 kV

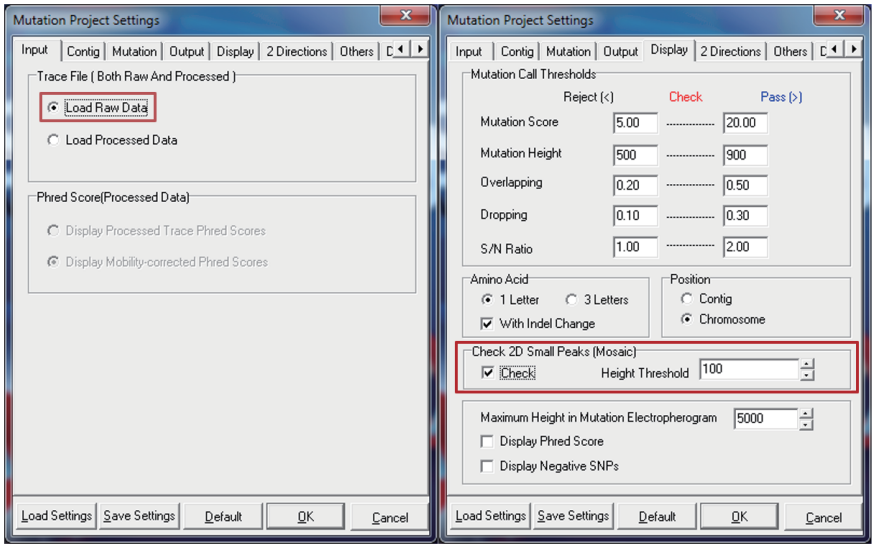

Figure 4 : Settings before loading files

Selecting "Load Raw Data" will allow you to analyze the raw data using the base caller implemented in Mutation Surveyor®.

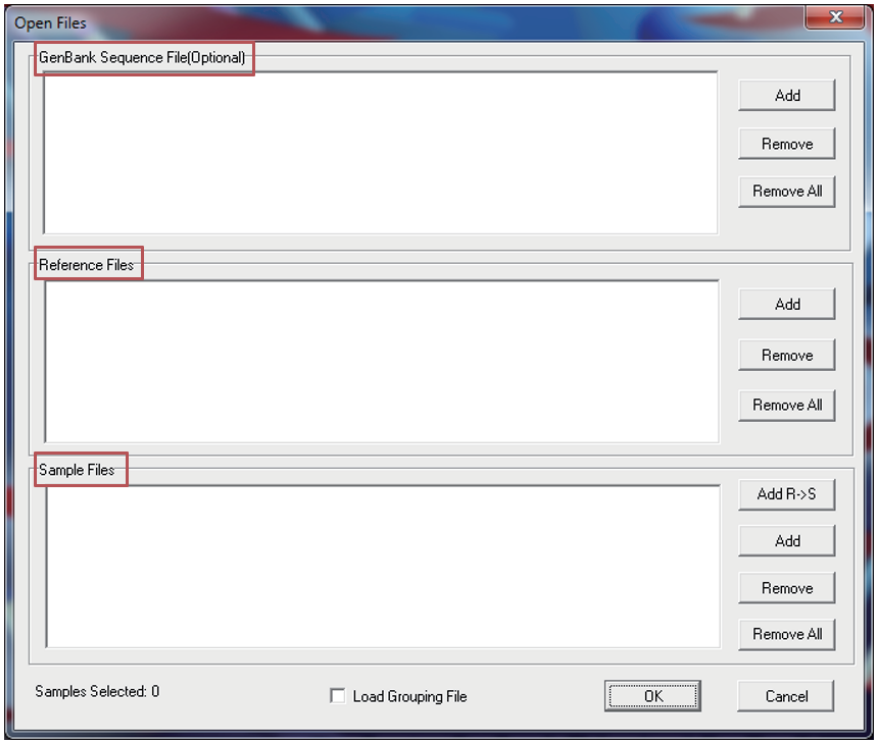

Figure 5 : Settings to load the files

GenBank Reference Files: GenBank files (.gbk).
Reference Files: Negative control (sample with 0% mutation, such as wild-type DNA).
It is strongly recommended to obtain reference data on the same run with Sample Files.
Sample Files: Sequence data (.ab1) of interest. If you import a negative control registered in the Reference Files in this section, you can easily compare the electropherograms in the subsequent analysis.

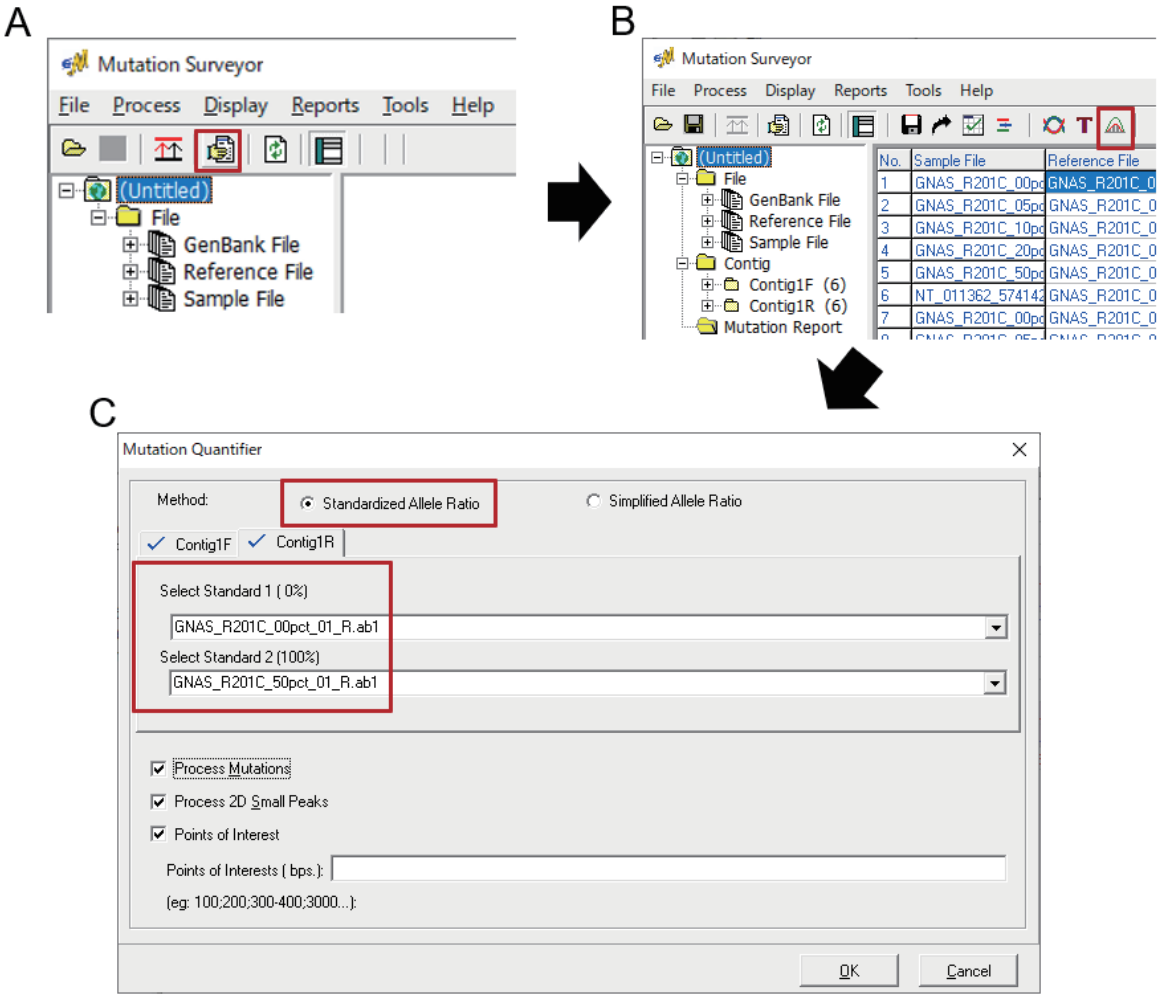

Figure 6: Settings after loading the files

Select “Run” from the home screen (A). Next, launch “Mutation Quantifier” (B), and configure each setting as described in (C).
Load the negative control in “Select Standard 1 (0%)” and the positive control (sequence data with a known mutation rate) in “Select Standard 2 (100%)”.
When the mutation rate of the positive control is below 100%, register the value as shown in Figure 7.

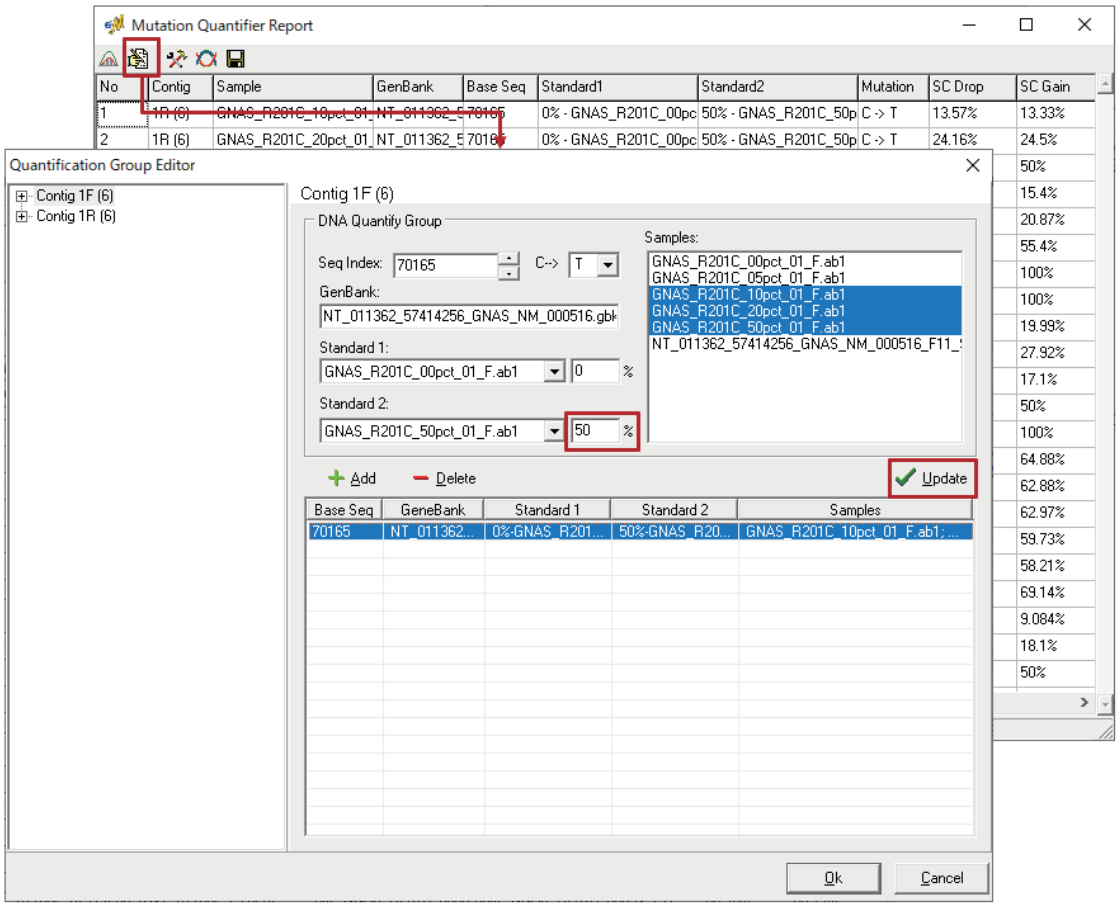

Figure 7：Registration of the positive control

When the mutation rate of the positive control is below 100%, register the rate in “Standard2” under “Quantification Group Editor”, then run “Update”.

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

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

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

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

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