HITACHI
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Example of MLPA® analysis using a DS3000 Compact CE Sequencer

Overview

The MLPA® method (Reagent Manufacturer: MRC Holland, Reagent Distributor in Japan: FALCO biosystems Ltd.) is widely used to analyze copy number variation (deletions/duplications: CNV), known SNP, and methylation status. Sequences that specifically hybridize to the target gene are designed within the MLPA® probe, and mutations are analyzed by capillary electrophoresis through hybridization, ligation, and PCR. An example of MLPA® analysis using the DS3000 Compact CE Sequencer (hereafter referred to as the DS3000) is presented here. Hematopoietic tumor-related genes were analyzed using the JAK2 p.V617F 50 % reference standard (Horizon Discovery), which is a reference sample containing 50 % of JAK2 V617F mutation. Myeloproliferative neoplasms, one of the hematopoietic tumors, include chronic myelogenous leukemia, chronic neutrophilic leukemia, polycythemia vera (PV), primary myelofibrosis (PMF), essential thrombocythemia (ET), chronic eosinophilic leukemia, and unclassifiable myeloproliferative neoplasms. Of these, more than 95 % of PV cases and approximately half of ET and PMF cases have JAK2 mutations (Reference 1). Analysis using DS3000 detected the expected duplication of genes IKZF1, CDK6, RELN, MET, DPP6 and DCC and mutations in JAK2. This product is for research use only and is not approved or certified as a medical device under the Pharmaceuticals and Medical Device Act. Also, it is not for diagnostic or procedural use.

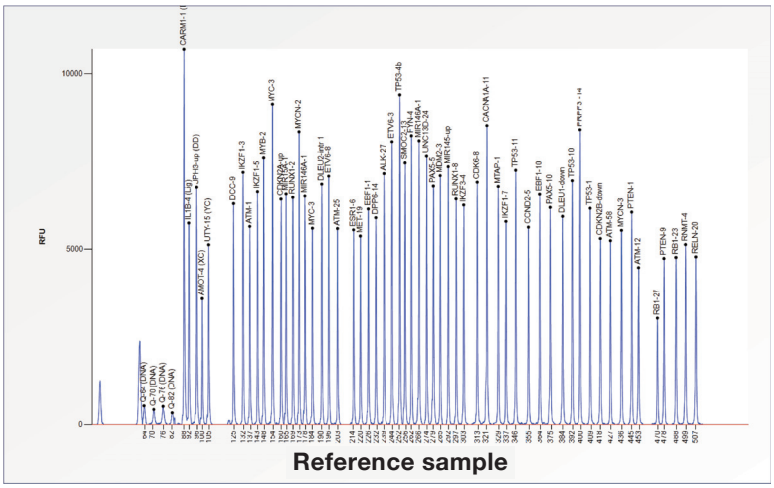
DS3000
Compact CE Sequencer

Results

The JAK2 p.V617F 50 % reference standard (Horizon Discovery) was prepared according to the MLPA® General Protocol (Reference 2) and electrophoresed with Polymer4 using the DS3000. The obtained fsa file was analyzed with Coffalyser.Net™ (MRC Holland). The "Fragment Run Separation Score (FRSS)", which indicates the quality of electrophoresis, and the "Fragment MLPA Reaction Score (FMRS)", which indicates the quality of the MLPA® reaction, were all 100 %, confirming that there were no problems in terms of data quality. The mutation was confirmed by the detection of a signal in JAK2 p.V617F 50 % reference standard (Horizon Discovery) using the JAK2 probe, which shows a signal only the gene contains a point mutation (JAK2 p.V617F) (Figures 1 and 2). In addition, the signal intensities of IKZF1, CDK6, RELN, MET, DPP6, and DCC were all approximately 1.5 times greater than that of the reference sample, confirming the existence of duplications (Figure 2). These mutations and duplications in the JAK2 p.V617F 50 % reference standard (Horizon Discovery) were as expected (Reference 3).

Conclusion

The DS3000 was capable of detecting all expected genetic mutations and duplications with MLPA® application targeting JAK2 mutations in hematopoietic tumors. In addition, the DS3000 also detected all expected mutations and methylation associated with oligodendroglioma, EGFR, and dMMR using MLPA® and MS-MLPA® applications. See “References” for details. We therefore confirm that DS3000 can be used for MLPA® applications.



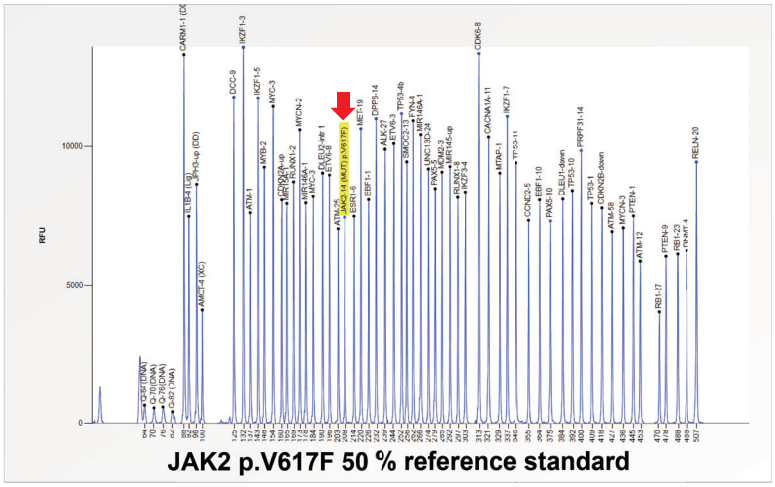


Figure 1: Electropherograms of P377-A3, JAK2 p.V617F 50 % reference standard produced with Coffalyser.Net™ (X-axis: Base length, Y-axis: Signal intensity)

Electropherograms are shown for the reference sample 2800M Control DNA (Promega®) (top) and the JAK2 p.V617F 50 % reference standard (bottom). In the bottom figure, a signal that was detected in JAK2 is highlighted in yellow to indicate a mutation.

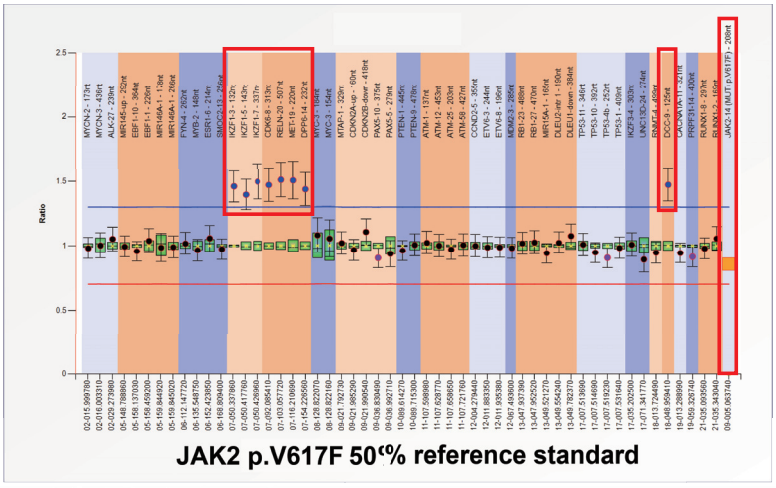


Figure 2: Ratio chart of the P377-A3, JAK2 p.V617F 50 % reference standard produced with Coffalyser.Net™ (X-axis: Individual genes, Y-axis: Ratio of signal intensity relative to reference sample)

For JAK2, which is a mutation-specific probe, the orange box indicates that mutation was detected. As expected, the signal ratios of IKZF1, CDK6, RELN, MET, DPP6, and DCC in the JAK2 p.V617F 50 % reference standard were all approximately 1.5, indicating that a duplication was detected (Reference 3).

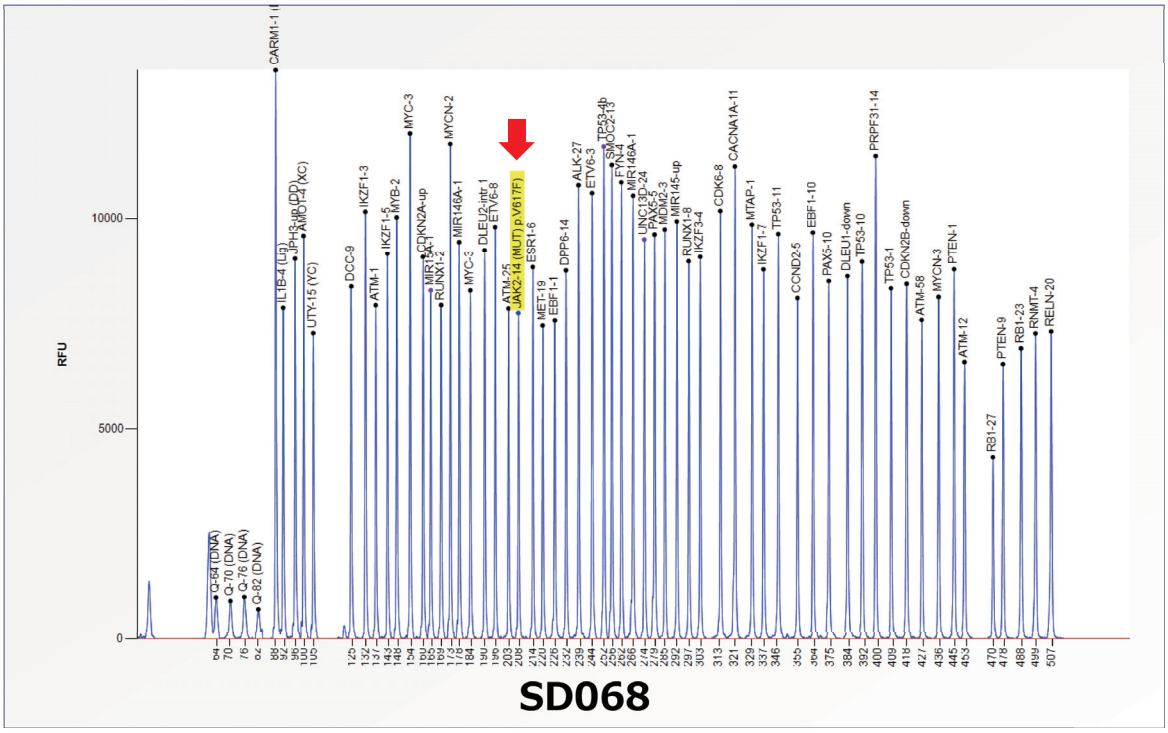


Figure 3: Electropherograms of P377-A3, SD068-S01 produced with Coffalyser.Net™ (X-axis: Base length, Y-axis: Signal intensity)

A signal that was detected in JAK2 is highlighted in yellow to indicate a mutation.

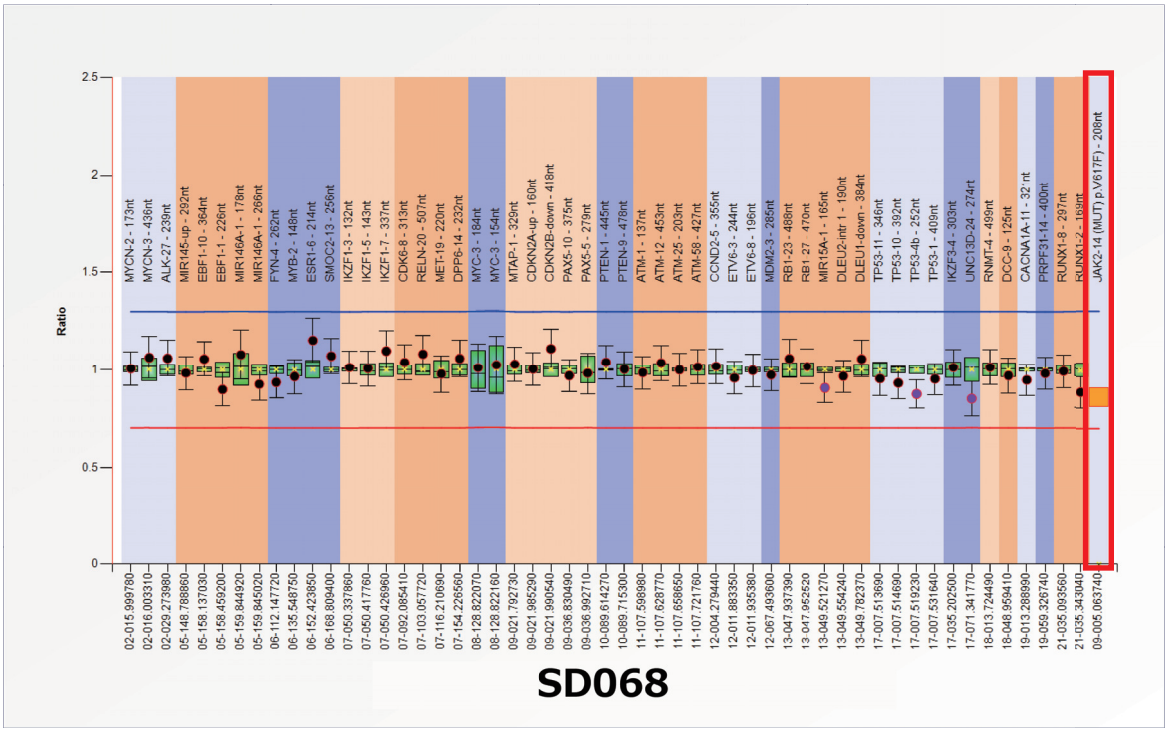


Figure 4: Ratio chart of P377-A3, SD068-S01 produced with Coffalyser.Net™ (X-axis: Individual genes, Y-axis: Ratio of signal intensity relative to reference sample)

SD068 is an artificial sample that was designed to detect mutations in JAK2. As expected, a mutation in JAK2 was detected (indicated by the orange box) (Reference 6).

Experiment

1. Sample preparation

Samples were prepared according to the MLPA® General Protocol (Reference 2). SALSA® MLPA® Probemix P377-A3 Hematologic Malignancies was used to detect the JAK2 mutation in hematopoietic tumors.

1.1. Sample selection

The samples used are listed in Table 1. Four types of samples are used: JAK2 p.V617F 50 % reference standard (Horizon Discovery); 2800M Control DNA (Promega®) was used as a reference sample for no point mutations; SALSA® Binning DNA SD068-S01 (MRC Holland), which is an artificial DNA that was used as a positive control to detect peak mutation; and TE buffer without DNA was used as a negative control.

1.2. Hybridization

DNA at a concentration of 50 ng per 5 μL was denatured at 98 ˚C for 5 minutes and cooled to room temperature. Next, the hybridization mixture shown in Table 2 was prepared and 3 μL of the mixture was gently pipetted into 5 μL aliquots of DNA. The resulting mixture was then denatured at 95 ˚C for 1 minute and hybridized at 60 ˚C for 20 hours.

1.3. Ligation

The ligation mixture shown in Table 3 was prepared. After cooling, 8 μL of the hybridized DNA solution was cooled to 54 ˚C and 32 μL of the ligation mixture was added to a tube by gentle pipetting. The resulting mixture was then ligated on a thermal cycler at 54 ˚C for 15 minutes followed by inactivation of the ligase by heating at 98 ˚C for 5 minutes.

1.4. PCR

The PCR mixture was prepared as shown in Table 4. After cooling 40 μL of the ligated DNA solution to room temperature, 10 μL of the polymerase mixture was added by gentle pipetting. The PCR was then performed using the conditions shown in Table 5.

2. Capillary electrophoresis

Performed according to the MLPA® General Protocol (Reference 2).

To 0.7 μL of PCR product, 9.0 μL of Hi-Di™ Formamide (Thermo Fisher Scientific) and 0.2 μL of GeneScan™ 500 LIZ™ dye Size Standard (Thermo Fisher Scientific) were added. The PCR product was denatured at 86 ˚C for 3 minutes and then cooled to 4 ˚C for 2 minutes. The sample was electrophoresed using the DS3000 under the conditions shown in Table 6.

3. Analysis with Coffalyser.Net™

The fsa file from the DS3000 was processed Coffalyser.Net™ (MRC Holland) according to the CRM Coffalyser.Net™ Reference Manual (Reference 4).

3.1. Checking data quality

The quality of the data was checked using the "Fragment Run Separation Score (FRSS)", which indicates the quality of electrophoresis using a size standard that is electrophoresed with the sample, and the "Fragment MLPA Reaction Score (FMRS)", which indicates the quality of the MLPA® reaction.

3.2. Checking results

The obtained data were checked for any mutations and duplications. A representative example of one of the outputs of Coffalyser.Net™ is shown in Figure 1-10. Figure 1 shows the "Electropherograms" screen with mutations highlighted in yellow. In the JAK2 p.V617F 50 % reference standard (Horizon Discovery), JAK2 was highlighted in yellow, whereas no signal was highlighted in yellow in the reference sample. Figure 2 shows the results for the JAK2 p.V617F 50 % reference standard (Horizon Discovery) in a "ratio chart" screen, which shows the ratio of signal intensity against the reference sample. An orange box was displayed at the rightmost JAK2; this box is displayed when a signal is detected in the mutation-specific probe, i.e., when a mutation is present. In addition, the signal intensities of IKZF1, CDK6, RELN, MET, DPP6, and DCC had ratios of approximately 1.5.

The results of SALSA® Binning DNA SD068-S01 (MRC Holland) electrophoresed at the same time are shown in Figures 3 and 4.

For information on MLPA® General Protocol (Reference 2), Coffalyser.Net™ and CRM Coffalyser.Net™ Reference Manual (Reference 4), please visit the MRC Holland home page at https://www.mrcholland.com/.

Table 1. Samples for P377-A3 analysis

Reagent	Sample number	Type
2800M Control DNA (Promega®)	3	Reference sample
JAK2 V617F Reference Standard, 50 % (Horizon Discovery)	1	Test sample
SALSA® Binning DNA SD068-S01 (MRC Holland)	1	Positive control
TE buffer	1	Negative control

Table 2. Hybridization master mix

Reagent	Volume (μL)
MLPA buffer	1.5
SALSA® Probemix	1.5
Total	3.0

Table 3. Ligase-65 master mix

Reagent	Volume (μL)
Ligase buffer A	3
Ligase buffer B	3
Ligase-65	1
PCR grade water	25
Total	32

Table 4. Polymerase master mix

Reagent	Volume (μL)
SALSA® PCR primer mix	2.0
SALSA® polymerase	0.5
PCR grade water	7.5
Total	10

Table 5. PCR conditions

Temperature	Time	Cycle
95 ℃	30 s	×35
60 ℃	30 s	
72 ℃	60 s	
72 ℃	20 min	×1
15 ℃	hold	

Table 6. Electrophoresis conditions for P377-A3 analysis

Polymer	Polymer4
Size Standard	GeneScan™ 500 LIZ™ dye Size Standard (Thermo Fisher Scientific)
Dye Set	AB 5-dye
Matrix Standard	DS-33 Matrix Standard Kit (Dye Set G5) (Thermo Fisher Scientific)
Assay	AB_5Dye_LIZ500(75-500)_36_P4
Injection Voltage/Time	1.6 kV/9 s
Run Voltage	13 kV
Run Time	1930 s

Reference

We confirmed that the following probemixes (i.e., other than P377-A3) can also be analyzed using the DS3000:

- SALSA® MLPA® Probemix P088-D1 Oligodendroglioma 1p-19q can be used to analyze oligodendroglioma, a type of brain tumor.
- SALSA® MLPA® Probemix P315-C1 EGFR can be used to analyze EGFR, which is frequently mutated in lung, colorectal and breast cancers.
- SALSA® MLPA® Probemix ME011-D1 Mismatch Repair Genes can be used to analyze mismatch repair defects (dMMR).

SALSA® Binning DNA (MRC Holland) corresponding to each probemix was analyzed as a mutated sample. As a result, expected mutations of the Binning DNA were correctly detected in all probemixes. The samples used are listed in Tables 7 to 9. The samples were also electrophoresed under the conditions shown in Table 6 or Table 10, and the electrophoresis results are shown in Figures 5 to 10.

ME011-D1 is an MS-MLPA® application that can simultaneously detect copy number variations, methylation status, and point mutations (Reference 5). After hybridization, the methylation-sensitive restriction enzyme HhaI is added along with ligase to the reaction. Methylated DNA is not digested by this restriction enzyme, so the signal is detected by electrophoresis. BRAF exon 15 and MLH1 exon 1 signals are detected for SD086 (Figure 9-D), but they were not detected in the reference sample (Figure 9-B). Note that like P377-A3, the probemixes P088-D1 and P315-C1 are also used for general MLPA® application.

Table 7. Samples for P088-D1 analysis

Reagent	Sample number	Type
2800M Control DNA (Promega®)	3	Reference sample
SALSA® Binning DNA SD079-S01 (MRC Holland)	1	Test sample
TE buffer	1	Negative control

Table 8. Samples in P315-C1 analysis

Reagent	Sample number	Type
2800M Control DNA (Promega®)	3	Reference sample
SALSA® Binning DNA SD006-S01 (MRC Holland)	1	Test sample
TE buffer	1	Negative control

Table 9. Samples for ME011-D1 analysis

Reagent	Sample number	Type
2800M Control DNA (Promega®)	3	Reference sample
SALSA® Binning DNA SD086-S01 (MRC Holland)	1	Test sample
TE buffer	1	Negative control

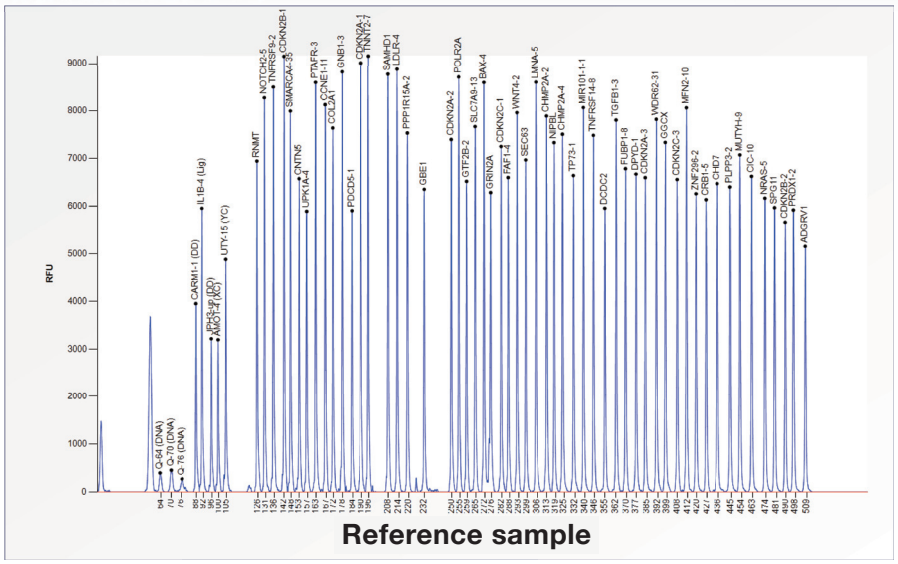
Notes

- In the analysis of ME011-D1, the warning message "Percentage noise peaks" was displayed with the data after using the conditions shown Table 6. This warning occurs if there is a lot of noise, and the overall signal intensity was high at the time of analysis; correspondingly, the noise was also high. Therefore, the sample injection condition was changed from 1.6 kV / 9 s to 1.2 kV / 4 s (Table 10). This modification resulted in lower overall signal and noise intensities, the warning disappeared, and the sample could be analyzed correctly. The signal intensity obtained by probes may be increased depending on the type of probemix used or sample to be analyzed; as a result, the noise may also be higher than the detection threshold. In such cases, consider changing sample injection conditions.
- Before starting MLPA® analysis with the DS3000, please perform a trial run using known DNA samples. If necessary, change sample injection conditions, etc. For details on how to change the sample injection conditions, please refer to the DS3000 instruction manual.
- In the analysis of P377-A3, SALSA® Binning DNA was used as a positive control. However, SALSA® Binning DNA was also used originally as an indicator for adjusting Bin in Coffalyser.Net™ (Reference 2).

Table 10. Electrophoresis conditions for ME011-D1 analysis

(Changed injection voltage from existing assay to 1.2 kV / 4 s)

Polymer	Polymer4
Size Standard	GeneScan™ 500 LIZ™ dye Size Standard (Thermo Fisher Scientific)
Dye Set	AB-5dye
Matrix Standard	DS-33 Matrix Standard Kit (Dye Set G5) (Thermo Fisher Scientific)
Assay (Original)	AB_5Dye_LIZ500(75-500)_36_P4
Injection Voltage/Time	1.2 kV/4 s
Run Voltage	13 kV
Run Time	1930 s



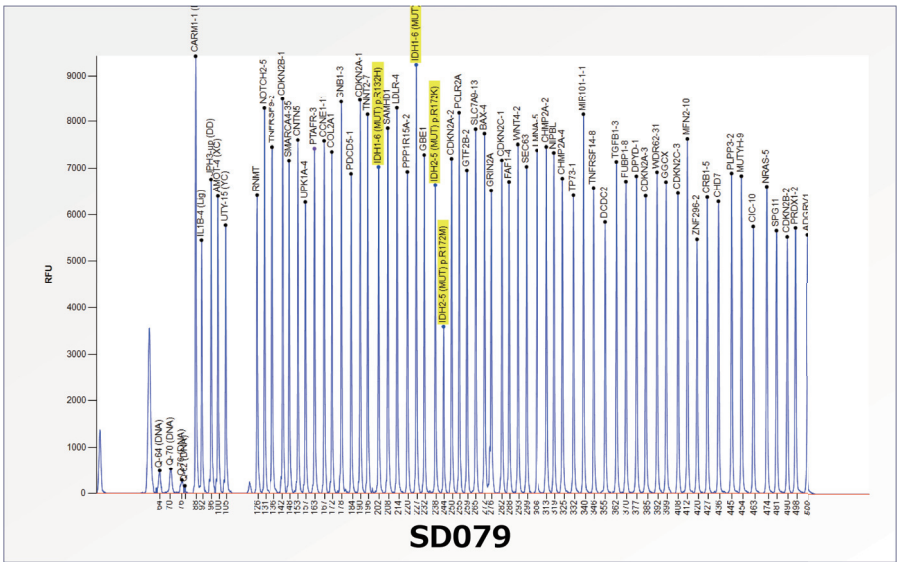


Figure 5: Electropherograms of P088-D1, SD079-S01 produced with Coffalyser.Net™ (X-axis: Base length, Y-axis: Signal intensity)

Electropherograms are shown for the reference sample 2800M Control DNA (Promega®) (top) and SD079 (bottom). In the bottom figure, signals that were detected in IDH1 exon 6 (p.R132H, p.R132C) and in IDH2 exon 5 (p.R172K, p.R172M) are highlighted in yellow to indicate mutations.

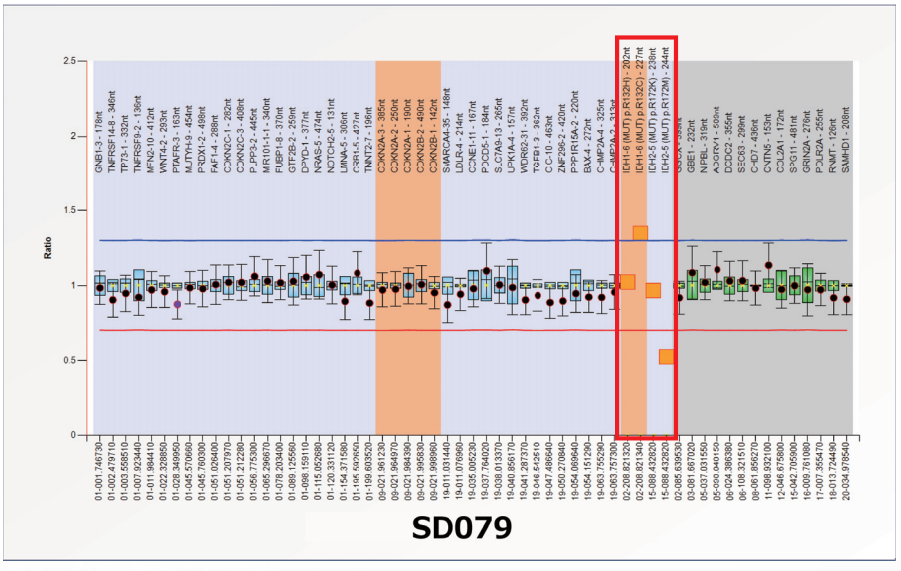


Figure 6: Ratio chart of P088-D1, SD079-S01 produced with Coffalyser.Net™ (X-axis: Individual genes, Y-axis: Ratio of signal intensity relative to reference sample)

SD079 is an artificial sample that is designed to detect mutations in IDH1 exon 6 (p.R132H, p.R132C) and IDH2 exon 5 (p.R172K, p.R172M). As expected, all four mutations were detected (indicated by the orange boxes) (Reference 7).

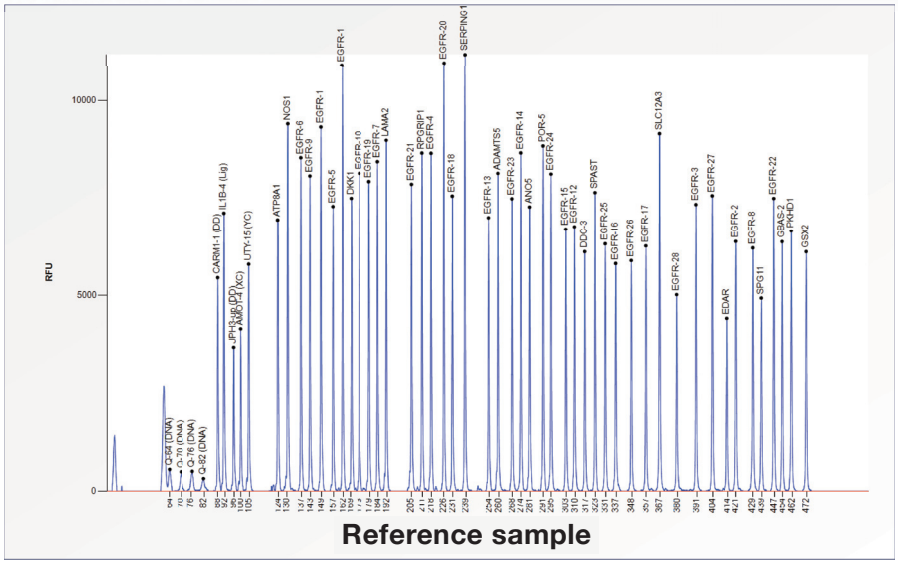
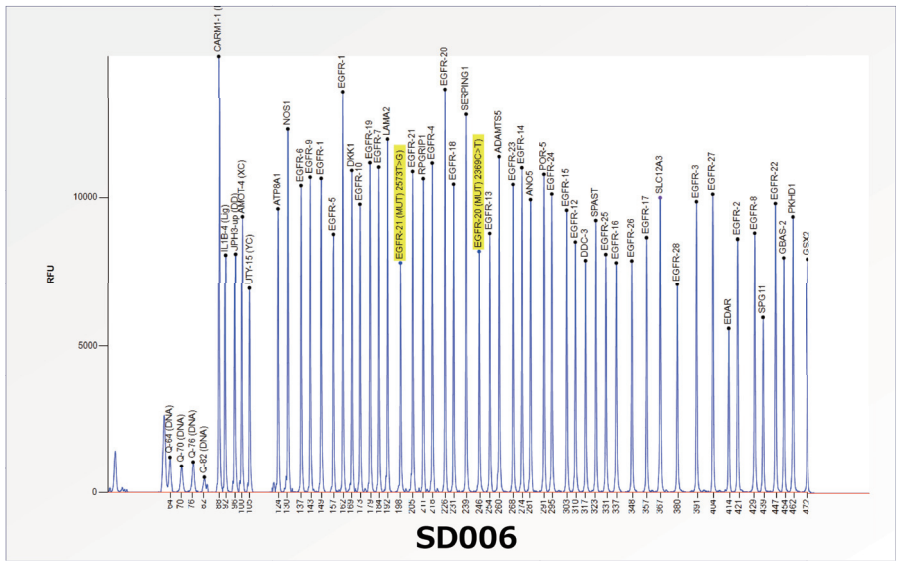


Figure 7：Electropherograms of P315-C1, SD006-S01 produced with Coffalyser.Net™ (X-axis：Base length, Y-axis：Signal intensity)

Electropherograms are shown for the reference sample 2800M Control DNA (Promega®) (top) and SD006 (bottom). In the bottom figure, signals that were detected in EGFR exon 20 and EGFR exon 21 are highlighted in yellow to indicate mutations.



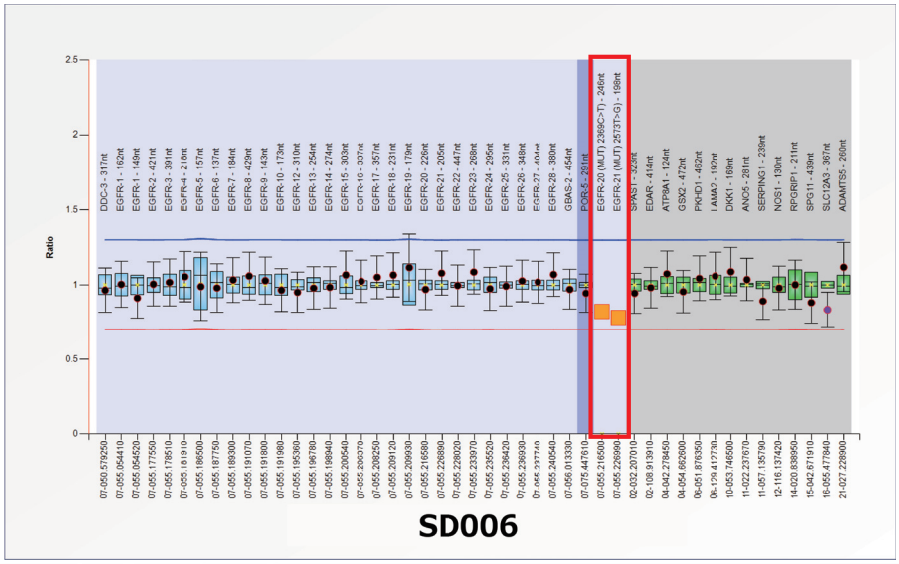


Figure 8：Ratio chart of P315-C1, SD006-S01 produced with Coffalyser.Net™ (X-axis：Individual genes, Y-axis：Ratio of signal intensity relative to reference sample)

SD006 is an artificial sample that was designed to detect mutations in EGFR exon 20 and EGFR exon 21. As expected, two mutations were detected (indicated by the orange boxes) (Reference 8).

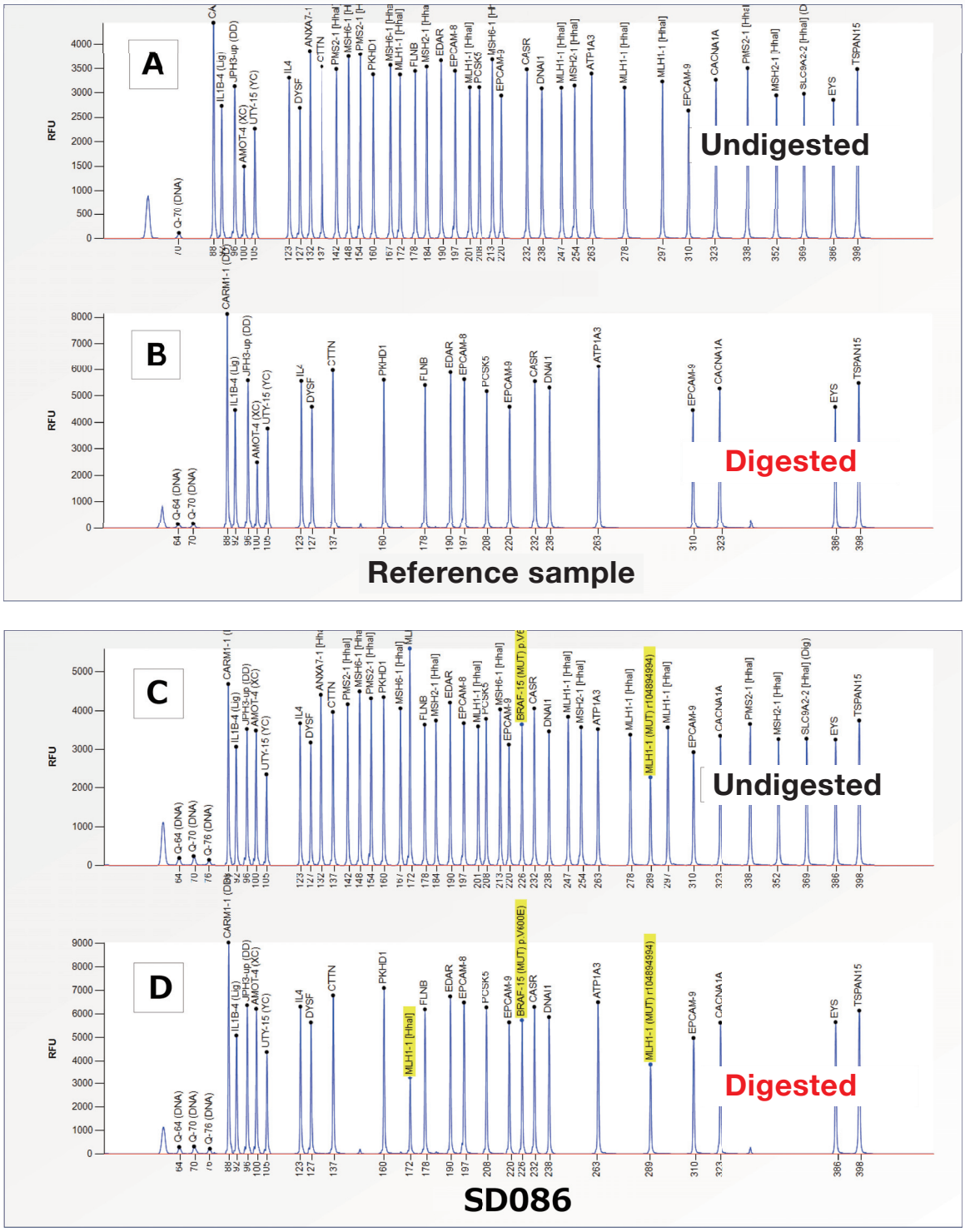


Figure 9: Electropherograms of ME011-D01, SD086-S01 produced with Coffalyser.Net™ (X-axis: Base length, Y-axis: Signal intensity)

Electropherograms are shown for the reference sample 2800M Control DNA (Promega®) (A and B) and SD086 (C and D). The restriction enzyme HhaI targets GCGC sites of the MLH1, MSH2, PMS2 and MSH6 genes. In C, which shows the undigested SD086 sample, signals were detected and highlighted in yellow in BRAF exon 15, a point mutation-specific probe, and MLH1 exon 1, an SNP-specific probe. Furthermore, in D, which shows the SD086 sample digested by restriction enzymes, BRAF exon 15 was undigested, and signal was detected. MLH1 exon 1 was also undigested, and signals were detected at the 172 nt and the 289 nt. The 172 nt signal is expected because the SNP detected by the 289 nt SNP-specific probe is located at the HhaI restriction enzyme recognition site of the 172 nt methylation-specific probe (Reference 9).

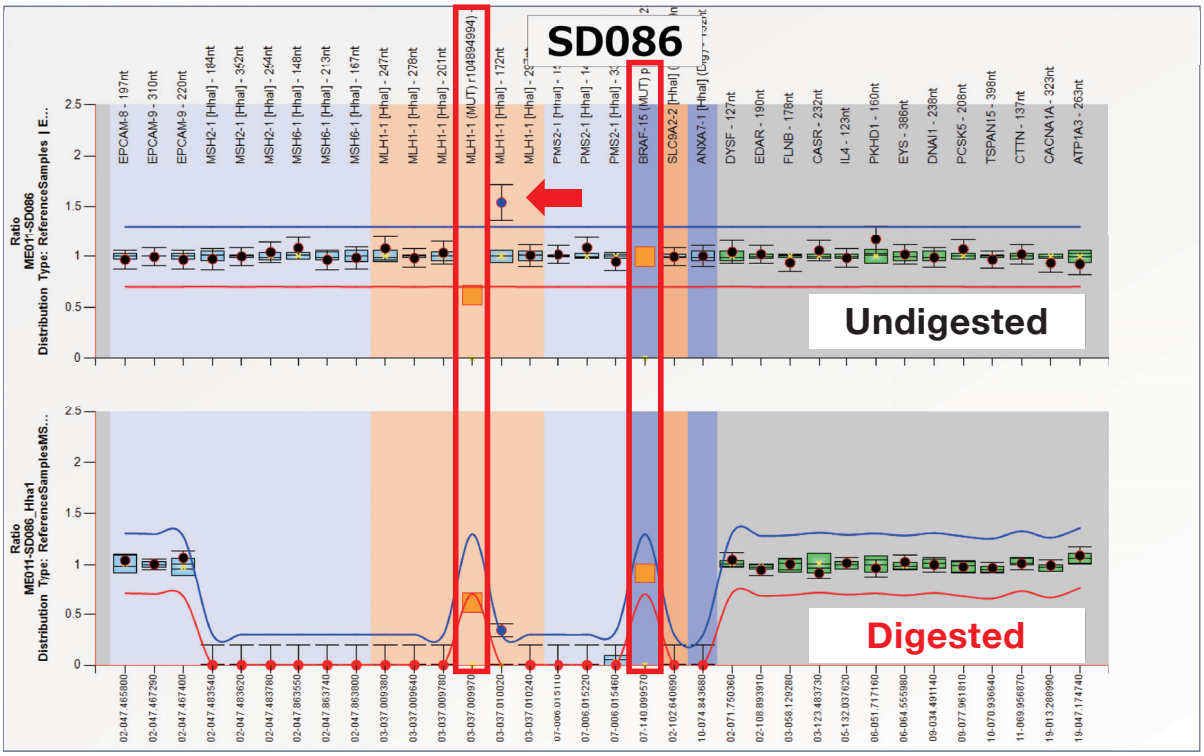


Figure 10：Ratio chart of ME011-D01, SD086-S01 produced with Coffalyser.Net™ (X-axis：Individual genes, Y-axis：Ratio of signal intensity relative to reference sample)

SD086 is an artificial sample that is designed to detect mutations in BRAF exon 15 and MLH1 exon 1. As expected, two mutations were detected (indicated by the orange boxes). Also, in the chart showing the genes that were not digested by restriction enzyme, the signal of the 172 nt MLH1 methylation-specific probe was high. This is because the target sequence of the 289 nt probe overlaps significantly with the target sequence of the 172 nt probe; as a result, an increase in the signal ratio of 1.3–1.65 is expected in SD086 (Reference 9).

References

(1) The Japanese Society of Hematology Practical Guidelines for Hematological Malignancies 2018 Revised Edition
http://www.jshem.or.jp/gui-hemali/table.html
(2) MRC Holland MLPA® General Protocol Version 008, 06 May 2022
(3) MRC Holland "List of verified positive samples that can be used with (digital)MLPA probemixes"
(P377-A: Horizon Discovery JAK2 p.V617F 50 % reference standard/ Heterozygous duplication affecting the probes for IKZF1, CDK6, RELN, MET, DPP6 and DCC. Positive for the JAK2 p.V617F mutation.)
https://support.mrcholland.com/kb/articles/list-of-verified-positive-samples-that-can-be-used-with-digital-mlpa-probemixes
(4) MRC Holland CRM Coffalyser.Net™ Reference Manual For Coffalyser.Net™ version 220513.1739 Version 02, June 2022
(5) MRC Holland MS-MLPA® General Protocol Version 012, 06 May 2022
(6) MRC Holland Product Description SALSA® Binning DNA SD068-S01 Version S01-02, 03 January 2018
(7) MRC Holland Product Description SALSA® Binning DNA SD079-S01 Version S01-02, 14 December 2020
(8) MRC Holland Product Description SALSA® Binning DNA SD006-S01 Version S01-05, 21 August 2020
(9) MRC Holland Product Description SALSA® Binning DNA SD086-S01 Version S01-02, 01 July 2022

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	Maximum number of uses	Remarks
Capillary Cartridge 36 cm	613-0330	1 pcs	300 injections/unit	Storage temperature: 15 – 30˚C
Buffer	613-0252	Anode Buffer × 2 cartridges Cathode Buffer × 2 cartridges	80 injections/unit	Storage temperature: 2 – 10˚C
Polymer7	613-0251	4 cartridges	16 injections/unit	Storage temperature: 2 – 10˚C
	613-0291	4 cartridges	24 injections/unit	Storage temperature: 2 – 10˚C
Polymer4	613-0250	4 cartridges	16 injections/unit	
	613-0290	4 cartridges	24 injections/unit	
Septa for Cathode Buffer Cartridge	613-7231	10 pcs	Single-use	
Retainer for Cathode Buffer Cartridge	613-7233	4 pcs	–	
Septa for 8 well tubes	613-7230	24 pcs	Single-use	
Base and Retainer for 8 well tubes	613-7232	4 pcs	–	
Anode Electrode Assembly	613-7263	1 pc	–	

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.

NOTICE:The system is For Research Use Only, and is not intended for any animal or human therapeutic or diagnostic use.
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HTB-DS-E013 2023.4