APPLICATION NOTE

SeqStudio Genetic Analyzer

MLPA assays on the SeqStudio Genetic Analyzer

In this application note, we demonstrate that:

- The new Applied Biosystems™ SeqStudio™ Genetic Analyzer generates high-quality data from multiplex ligation–dependent probe amplification (MLPA™) assays developed by MRC-Holland.
- The SeqStudio Genetic Analyzer provides fast and efficient results; each run cycle accommodates 4 MLPA reaction samples and can be completed within 45 min.
- The connectivity features allow MLPA data files on the CE platform to be automatically transferred to personal or shared space accounts; with the Thermo Fisher Cloud for example, data files are available for immediate review from any location with Internet access.
- The SeqStudio Genetic Analyzer exhibits high dynamic range and achieves high sizing precision and peak height fidelity of MLPA probe amplicons.
- The fragment analysis data files (.fsa) can be readily imported and analyzed with **Coffalyser.Net** MLPA data analysis software.

Introduction

Multiplex ligation–dependent probe amplification (MLPA) is a widely used molecular biology technique for copy number determination of multiple DNA sequences in the study of human genetic diseases [1]. This technique is based on the ligation and PCR amplification of up to 50 multiplexed pairs of probe oligonucleotides, which hybridize to the loci of interest.

Each oligonucleotide pair is designed to give an amplification product of a specific length; and by using sequence-tagged ends, all ligated probes can be amplified with a single primer pair in a PCR reaction. The forward PCR primer carries an Applied Biosystems™ 6-FAM™ fluorescent label, allowing for the detection and quantification of size-separated probes on an automated capillary electrophoresis (CE) system (Figure 1).

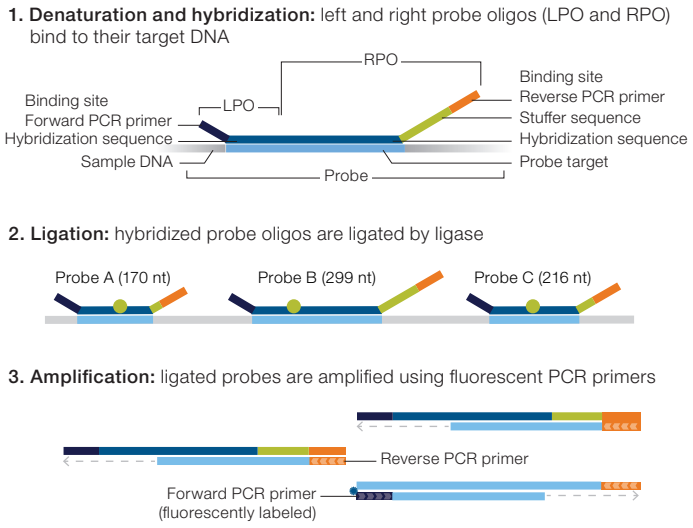

Figure 1. Schematic representation of MLPA technique.

This method has been successfully used for numerous applications in which deletions and duplications of one or more exons are a cause of a disease, like the analysis of the Duchenne muscular dystrophy (*DMD*) and *BRCA1/BRCA2* genes. Furthermore, a variant of the MLPA technique can also be applied for quantitative methylation analysis of various genomic sequences.

MRC-Holland has developed a large portfolio of commercially available MLPA assays for human genetic disease and cancer research. Detailed information on the product range, the MLPA protocol, and the recommended MLPA data analysis software **Coffalyser.Net** can be found at **mlpa.com**. Here we demonstrate the feasibility of using the newly developed SeqStudio instrument as a qualified genetic analyzer platform for MLPA assays.

SeqStudio Genetic Analyzer

The new SeqStudio Genetic Analyzer builds upon the 30-year history of the Applied Biosystems™ genetic analyzers based on capillary electrophoresis technology. The genetic analyzers made major scientific breakthroughs by enabling mapping and sequencing of numerous small microbial genomes as well as large plant, mammalian, and human genomes. A functional hallmark of our genetic analyzers is their capability to perform in two modes: fluorescent Sanger sequencing and DNA fragment detection by multicolor fluorescent labeling and precision sizing. The SeqStudio Genetic Analyzer has a compact design that fits into tight laboratory spaces and is ideally suited for both 8-well strip tubes as well as standard 96-well PCR plates. The easy-to-use functional core of this instrument utilizes an all-in-one cartridge that integrates 4 capillaries, a universal polymer, an anode buffer, and the fluidics hardware into a simple integrated device (Figure 2). The integrated cartridge gives the user an added flexibility and control to generate data. Runs can be set up using either the onboard computer through an electronic graphical user interface (eGUI) or by using Plate Manager, the stand-alone software that operates within Thermo Fisher Cloud (**thermofisher.com/cloud**), or on a separate computer via a server or USB connection. The enhanced connectivity of the SeqStudio Genetic Analyzer with Thermo Fisher Cloud allows for data sharing, real-time monitoring of runs, and data analysis anytime, anywhere.

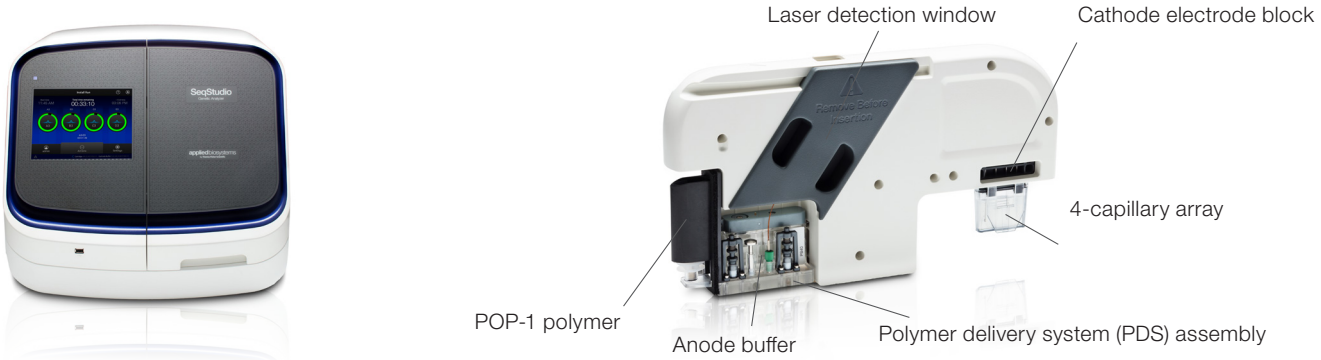

Figure 2. SeqStudio Genetic Analyzer and cartridge.

DNA	MLPA	CE	Data
• Genomic DNA preparation of proband and reference samples • See **mlpa.com** for guidance and detailed protocols	• Select and procure appropriate MLPA assay and reagents kit from MRC-Holland or authorized distributor • See **mlpa.com** for detailed product information and protocol • Overnight hybridization of loci-specific MLPA probes to the sample DNA • Ligation and PCR reaction (~2 hr)	• Prepare a Hi-Di Formamide and size-standard CE mix, and add the PCR amplification product • Capillary electrophoresis on SeqStudio Genetic Analyzer: fragment analysis run (45 min for 4-sample run) • Output is automatically sized in .fsa data files and is ready for fragment analysis	• MLPA data analysis using **Coffalyser.Net** software (free download from **mlpa.com**) • Project setup and import of probes worksheet (**mlpa.com**); add sample files (.fsa) from SeqStudio platform • Fragment analysis and quality review • Compare test sample from proband with reference samples

Figure 3. Overview of the MLPA workflow—from DNA to data.

Materials and methods

Preparing the MLPA reaction

The MLPA reactions were prepared from commercially available SALSA™ MLPA™ probe mixes and annotated DNA samples according to the general MLPA protocol per the manufacturer's instructions (**mlpa.com**).

Preparing the sample plate for capillary electrophoresis

Typically, an electrophoresis sample mix is prepared by combining Applied Biosystems™ Hi-Di™ Formamide (use 12 µL x (number of samples + 1)) with the Applied Biosystems™ GeneScan™ 500 Size Standard (labeled with either ROX™ dye or LIZ™ dye; use 0.3 µL x (number of samples + 1)). Then 12.3 µL of the formamide–size standard mix is dispensed into the CE injection plate, and 0.8 µL of the PCR reaction is added. The injection plate is sealed and heated at 86°C for 3 min in a thermal cycler, followed by cooling for 2 min at 4°C. The plate is spun briefly to remove any air bubbles and placed in the sample holder of the SeqStudio Genetic Analyzer for capillary electrophoresis.

Setting up the SeqStudio analyzer for a CE run

The CE sample sheet and run conditions were completed either with the eGUI on the instrument or using the Plate Manager software on a PC, followed by transfer of the sample sheet to the instrument via the Thermo Fisher Cloud or a USB drive. The FragAnalysis run module was used and dye set DS-30 (D) was selected since the size standard was labeled with ROX dye and the MLPA samples were labeled with 6-FAM dye. The MLPA samples are typically electrophoresed with either the GeneScan 500 ROX Size Standard for use with dye set "D" or with the LIZ dye–labeled GeneScan 500 LIZ Size Standard for use with dye set "G5". Suitable size standards from other vendors with appropriate fluorescent labels are also an option, provided that the resulting sizing values of the MLPA peaks are matching the bin settings of the analysis template (see next page).

MLPA data analysis using Coffalyser.Net software

Upon completion of a CE run, the SeqStudio Genetic Analyzer performs fragment sizing and generates fragment analysis data files (.fsa) that can be exported to the cloud or a network server, or stored locally for manual export via a USB port. The data files are then imported into an MLPA data analysis project created by Coffalyser.Net software (available as a free download from **mlpa.com**). Note: In the instrument settings of this software, "3500" was chosen as a proxy. Future versions of Coffalyser.Net will have a specific setting for the SeqStudio Genetic Analyzer.

Results

Detecting a major deletion using MLPA

To demonstrate the feasibility of MLPA analysis using the SeqStudio Genetic Analyzer, an MLPA assay targeting a well-characterized deletion in the human hemoglobin alpha (*HBA*) gene was used. The Southeast Asian double alpha-globin gene deletion (– –SEA) in the *HBA* gene consists of a 19.3 kb deletion of the alpha-globin gene cluster that includes the complete *HBA1* and *HBA2* genes, and is found primarily in Southeast Asia [2]. DNA research samples from a heterozygous deletion carrier ("sample") and DNA from three normal diploid research samples (one of which is shown as "reference") were analyzed by MLPA using the SALSA™ MLPA™ probemix P140 HBA. Figure 4A shows the annotated MLPA peaks in the electropherogram of the reference and test samples. Note that the probe signals for the deletion region are diminished by about half of the peak height compared to the reference sample. A quantitative analysis with a ratio chart of the same probemix is shown in Figure 4B. Each dot represents a probe ratio between test and reference sample. Note the 21 consecutive data points (shown in red) below the 0.7 ratio mark, indicating and delineating the large deletion in the test sample.

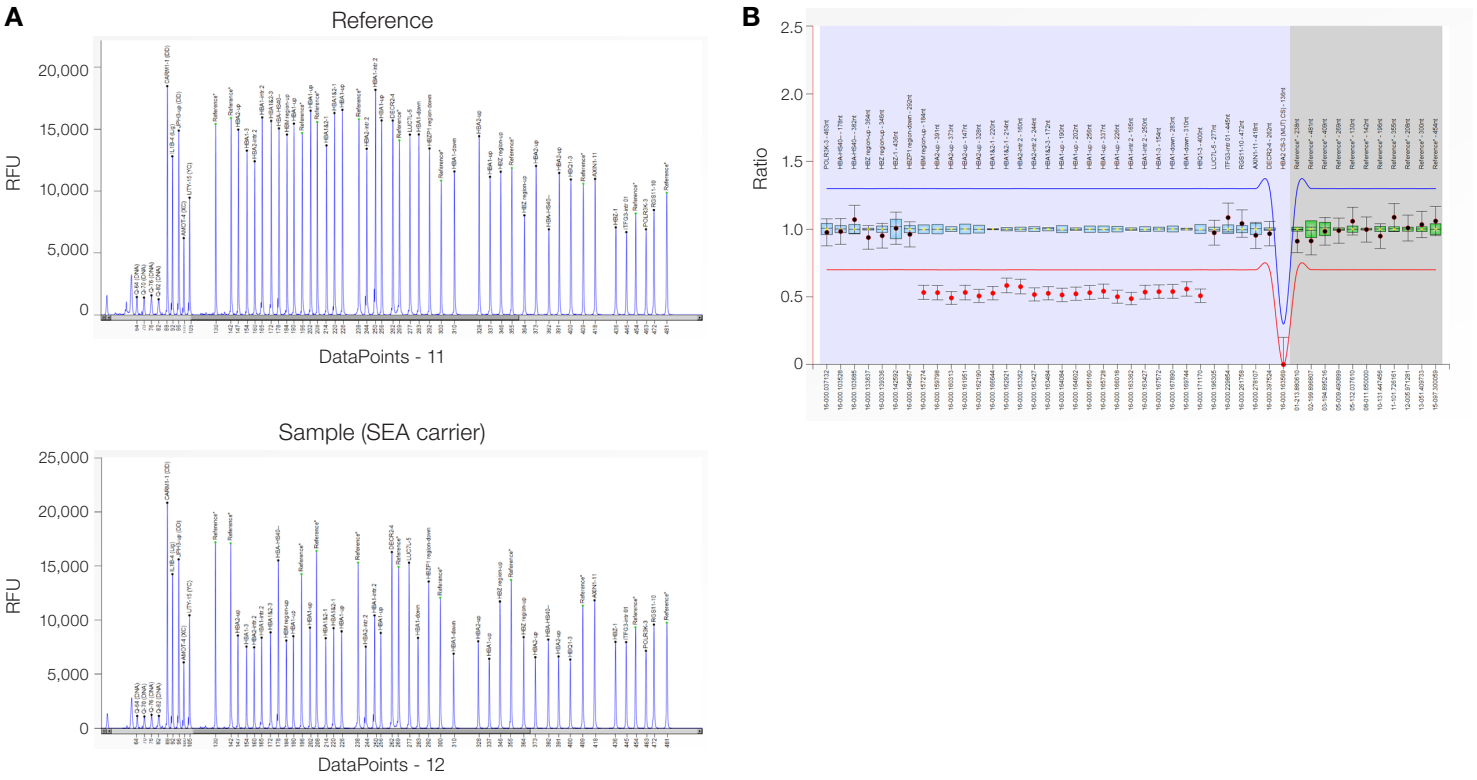

Figure 4. Detecting and analyzing a well-characterized deletion by MLPA. (A) Peak profile of MLPA assay P140 HBA for a reference sample (top) and the SEA carrier (bottom). Note the decrease in the peak heights of the probes detecting the HBA loci in the latter. The numbers on the x-axis indicate the relative size in nucleotides (nt) of the MLPA probe amplicon; the y-axis indicates the peak height of the target by relative fluorescence (RFU). **(B)** Ratio chart (SEA carrier) generated by Coffalyser.net MLPA analysis software. A ratio of 1 signifies a normal copy number; a probe ratio of 0.5 indicates a heterozygous deletion.

Detecting a major duplication using MLPA

To demonstrate detection of a duplication event in the human genome, we have analyzed a DNA sample from a proband that is known to carry a duplication of exons 2–30 in the *DMD* gene and compared it to a normal reference DNA sample. The SALSA MLPA probemix P034 DMD-1 was used for this experiment. Figure 5A shows the annotated electropherograms containing the MLPA peak patterns, and Figure 5B the corresponding ratio chart showing the ratios of the probes targeting the duplicated regions in the *DMD* gene. The duplication region is seen above the blue threshold line and is indicated by an increase in the probe ratio from 1 to 1.5, corresponding to a copy number increase from 2 to 3.

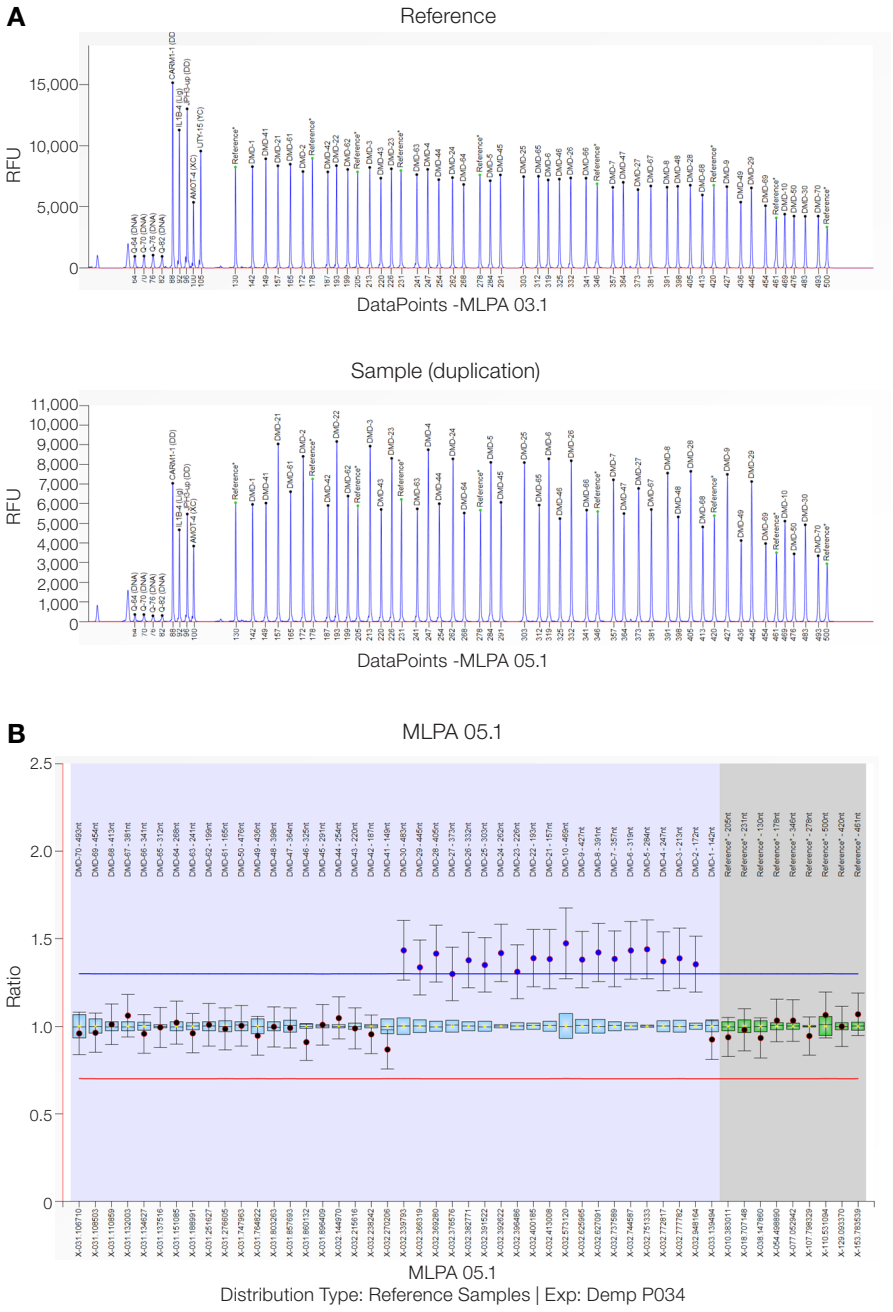

Figure 5. Detecting a major duplication region in the *DMD* gene. (A) Peak profile of MLPA assay P034 DMD-1 of the proband carrying a duplication (bottom) and a reference sample (top). **(B)** Ratio chart of the duplication carrier showing a probe ratio of ~1.5, indicating a copy number increase.

Detecting deletions in the *BRCA1* gene

The *BRCA1* and *BRCA2* genes are frequently mutated in hereditary breast and ovarian cancers. A deletion, when present in coding sequences of a gene, often leads to a disruption in protein function. The SALSA MLPA probemix P002 BRCA1 is widely used by clinical researchers to screen for deletions and/or duplications in the human *BRCA1* gene (Figure 6).

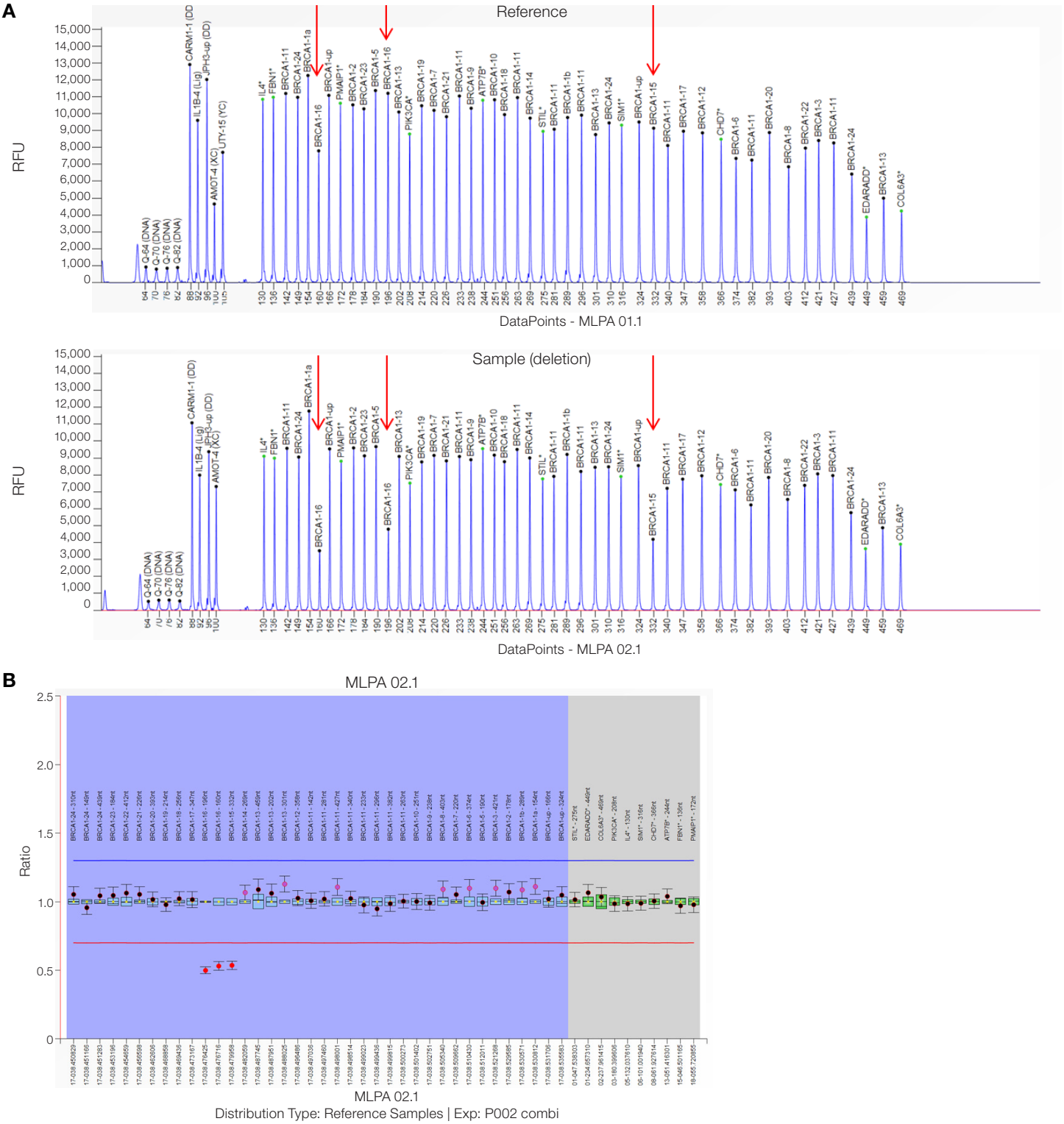

Figure 6. Deletion in the *BRCA1* gene detected by MLPA. (A) Peak profile of MLPA assay P002 BRCA1 of Coriell DNA sample 18949 with a heterozygous *BRCA1* deletion in exons 15 and 16 (bottom) and a reference sample (top). **(B)** Ratio chart of the Coriell sample showing a probe ratio of 0.5, indicating the heterozygous deletion.

MLPA profile comparison between 3130*xl* Genetic Analyzer and SeqStudio Genetic Analyzer

MLPA assays can be analyzed on a variety of capillary electrophoresis instruments, including the widely used Applied Biosystems™ 3130*xl* Genetic Analyzer. To compare the electrophoretic patterns between the 3130*xl* and the SeqStudio Genetic Analyzers, the MLPA products from the samples described above were analyzed by each of these genetic analyzers side by side. The results are shown in Figure 7.

The profile on the 3130*xl* instrument may differ slightly from that of the SeqStudio Genetic Analyzer because of a difference in capillary length, polymer, run voltage, and run time, which may lead to slightly different sizing values. However, this difference in appearance and sizing values has no impact on the comparative data analysis of MLPA assays because the reference samples are run under the same respective conditions.

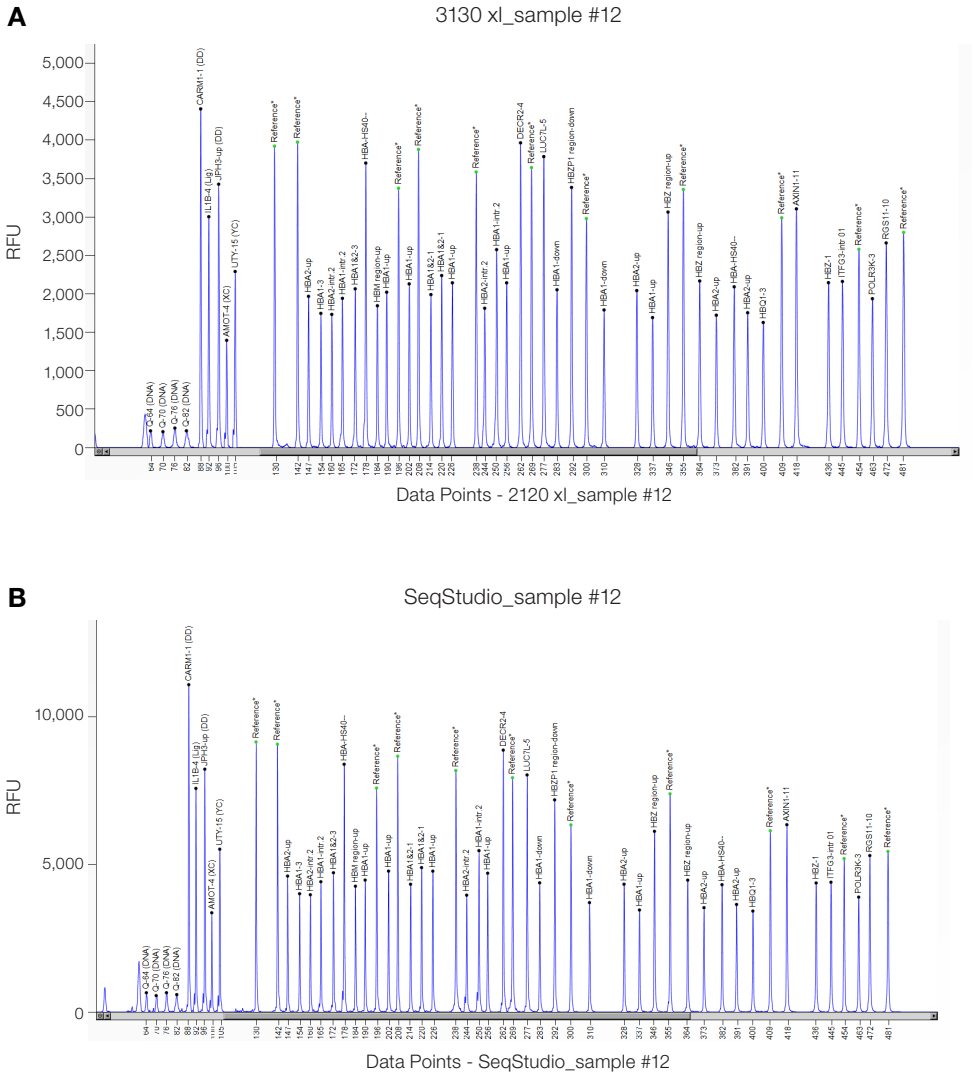

Figure 7. Comparison of MLPA peak patterns generated on (A) the 3130*xl* Genetic Analyzer and (B) the SeqStudio Genetic Analyzer, as visualized in Coffalyser.Net software.

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Conclusions

The SeqStudio Genetic Analyzer is a new addition to the Applied Biosystems™ family of capillary electrophoresis instruments featuring Sanger sequencing and fragment analysis capability.

Here we have demonstrated that the platform is suitable for generating reliable data derived from MLPA assay reactions. The primary fragment analysis data files (.fsa) generated by the instrument can be readily imported into MLPA-specific **Coffalyser.Net** software for MLPA data analysis. The SeqStudio Genetic Analyzer is an affordable, easy-to-use, and low-maintenance instrument, and an essential asset for any genetic analysis laboratory that is currently running or planning to use MLPA or other fragment analysis and sequencing assays for genetic disease research.

Ordering information

Product	Quantity	Cat. No.
GeneScan 500 ROX dye Size Standard	800 reactions	401734
GeneScan 500 LIZ dye Size Standard	800 reactions	4322682
Hi-Di Formamide	25 mL	4311320
SeqStudio Genetic Analyzer System with SmartStart		A35644
GeneMapper Software 6		A38892
SeqStudio Cartridge v1	500 reactions	A33671
SeqStudio Cartridge v2	1,000 reactions	A41331
SeqStudio Starter Kit		A35000

For MLPA products and **Coffalyser.Net** software, go to **mlpa.com**

References

1. Schouten JP et al. (2002) Relative quantification of 40 nucleic acid sequences by multiples ligation-dependent amplification. *Nucleic Acids Res* 30(12):e57.
2. Nicholis RD et al. (1987) Recombination at the human alpha-globin gene cluster: sequence features and topological constraints. *Cell* 49(3):369–378.

Find out more about the SeqStudio Genetic Analyzer at
fishersci.com/seqstudio or **fishersci.ca/seqstudio**

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