Detect low-level somatic mutations in FFPE samples using an extended RAS research assay

Optimized performance using Sanger sequencing

In this report, we demonstrate:

- Use of an extended RAS research assay targeting the 8 most frequently studied *KRAS* and *NRAS* hotspot regions
- Gold-standard Sanger sequencing performance using the extended RAS research assay on the Applied Biosystems™ SeqStudio™ Genetic Analyzer—the newest capillary electrophoresis instrument in our portfolio
- Minor variant detection using the extended RAS research assay showing comparable performance on both capillary electrophoresis instruments—the SeqStudio and Applied Biosystems™ 3500xL Genetic Analyzers

Sanger sequencing is often used in oncology research applications for molecular profiling of cancers. Applied Biosystems™ Minor Variant Finder (MVF) Software now enables low-level variant detection in Sanger sequencing traces. This is especially important in detecting somatic mutations.

RAS mutational testing is frequently performed by clinical researchers due to the strong correlation between *RAS* mutational profiles of some cancers (such as colorectal) and their anti–epidermal growth factor receptor (EGFR) response. The activated MAPK pathway in tumors harboring *RAS* mutations is more likely to confer resistance to EGFR inhibitors [1].

Here are the highlights of the ready-to-use Sanger sequencing–based extended RAS research assay designed for sensitive identification of low-level mutations (down to 5%) in formalin-fixed, paraffin-embedded (FFPE) samples.

Extended RAS research assay highlights

- Limit of detection (LOD) at 5%
- Extended RAS coverage (8 frequently studied hotspot regions of *KRAS* and *NRAS* genes)
- Fast turnaround time (e.g., <6 hours/6 samples for the 3500xL Genetic Analyzer) with minimal hands-on time
- Offers accurate and complete bidirectional sequence information
- Ready-to-use plates
- Low DNA input requirement (as little as 1 ng/reaction)

Compatible instrumentation:

- SeqStudio Genetic Analyzer
- Applied Biosystems™ 3500 and 3500xL Genetic Analyzers

"The new SeqStudio Genetic Analyzer is fast, intuitive, and simple to use. Compared to the qPCR platform, the RAS panel on SeqStudio system allows comprehensive analysis with detailed sequence information, starting with minimal low-quality FFPE DNA input."

—Dr. Luca Quagliata, Sr. Director
University Hospital of Basel

Benefits of using Sanger sequencing coupled with MVF Software:

LOD at 5%: Sanger sequencing enables variant detection down to 5% when combined with MVF Software. A value of 5% is often the recommended cut-off criteria used by clinical researchers.

Extended RAS coverage: We have developed the extended RAS research assay for Sanger sequencing that targets 8 hotspot regions of *KRAS* and *NRAS* genes (codons 12–13 of exon 2, 59–61 of exon 3, 117 and 146 of exon 4). Most of the currently available qPCR-based or pyrosequencing methods do not cover all of these hotspot codons; using this Sanger sequencing–based extended RAS research assay, any variant along the entire amplicon can also be detected.

Fast turnaround time: Sanger sequencing offers fast identification of known somatic mutations. This solution is ideal for low-throughput laboratories that are seeking fast turnround time in a cost-effective manner (<6 hr/sample using the SeqStudio Genetic Analyzer, <5 hr/sample using the 3500 genetic Analyzer, and <4 hr/sample using the 3500xL Genetic Analyzer). In fact, the turnaround time on the 3500xL platform with a full 96-well plate is <6 hr/6 samples. Additionally, the data analysis—interpretation and report generation using MVF Software—is fast, smooth, and simple, as compared to the more tedious peak analysis and interpretation of results produced by pyrosequencing. Next-generation sequencing (NGS) is ideal for high-throughput applications where more targets and samples can be multiplexed and screened in a fast and cost-effective manner.

Accurate and complete bidirectional sequence information: Sanger sequencing enables identification of all required hotspot variants and many others along the entire amplicon length within the context of its sequence. Most qPCR-based methods only provide a "yes" or "no" answer for the hotspot mutation—they do not accurately identify the particular variant or provide sequence information. The specific allele and additional sequence information are not only important in detecting *de novo* mutations, but also can have functional impact from certain variations. There is evidence, for example, that different amino acid substitutions at any one *KRAS* hotspot region can have differential oncogenic potencies as well as distinct functional consequences [2].

Moreover, Sanger sequencing allows users to visually inspect the actual sequences, and accept or reject variant candidates using both forward and reverse strands as double confirmation, giving more confidence in the results.

Ready-to-use plates: Primers for the extended RAS assay are preloaded on 96-well plates, thus only the PCR mix and templates need to be added. One plate can accommodate five specimens and one control for both forward and reverse sequencing (Table 1). To simplify and accelerate the process further, the assay was optimized for the Applied Biosystems™ BigDye™ Direct Cycle Sequencing Kit and the BigDye XTerminator™ Purification Kit (Figure 1). Using these kits, the same plate can migrate through the entire workflow, including electrophoresis, without transferring any of the samples or reagents out of the plate, which minimizes the risk of sample mix-up or contamination.

Figure 1. The extended RAS research assay combines a 96-well plate preloaded with dried-down primer pairs with the BigDye Direct Cycle Sequencing Kit and the BigDye XTerminator Purification Kit.

Table 1. Plate layout of the extended RAS research assay with five samples and one control.

	Control*		Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
	1	2	3	4	5	6	7	8	9	10	11	12
A	T1	T1	T1	T1	T1	T1	T1	T1	T1	T1	T1	T1
B	T2	T2	T2	T2	T2	T2	T2	T2	T2	T2	T2	T2
C	T3	T3	T3	T3	T3	T3	T3	T3	T3	T3	T3	T3
D	T4	T4	T4	T4	T4	T4	T4	T4	T4	T4	T4	T4
E	T5	T5	T5	T5	T5	T5	T5	T5	T5	T5	T5	T5
F	T6	T6	T6	T6	T6	T6	T6	T6	T6	T6	T6	T6
G	T7	T7	T7	T7	T7	T7	T7	T7	T7	T7	T7	T7
H	T8	T8	T8	T8	T8	T8	T8	T8	T8	T8	T8	T8

* The Control DNA CEPH 1347-02 supplied in the BigDye Direct Cycle Sequencing Kit can be used as the control for reference sequence.
"T" stands for hotspot target.

Low DNA input requirement: Using the Sanger sequencing–based approach, variants can be detected from as little as 1 ng of FFPE DNA per reaction. Other qPCR–based and pyrosequencing methods (often even with less coverage) usually require a higher amount of input DNA (>10 ng per reaction).

A simplified workflow for the extended RAS research assay

The extended RAS research assay was designed and optimized to sequence short amplicons (124–181 bp) of 8 hotspot regions of *KRAS* and *NRAS* genes amplified from DNA that is extracted from FFPE specimens of varied quality (Table 2). The streamlined workflow starts with an Applied Biosystems™ MicroAmp™ Optical 96-Well Reaction Plate preloaded with the 8 primer pairs. Only the PCR master mix and templates need to be added (Figure 2).

For each of the 8 amplicons, DNA Reference Standards (Horizon Discovery, Cambridge, UK) were used as positive controls. To assess the performance and LOD of the extended RAS research assay on the SeqStudio Genetic Analyzer as well as the 3500xL Genetic Analyzer instruments, we prepared dilution mixes by combining CEPH 1347-02 wild-type control DNA with Reference Standards at the allelic frequency of 50% to create pools with 10% and 5% minor allele frequencies. The DNA concentration of the Reference Standards and the CEPH DNA was measured by the Applied Biosystems™ QuantStudio™ 3D Digital PCR System before preparing 1 ng/µL Reference Standards, CEPH DNA, and dilution mixtures.

Table 2. Detailed information about the hotspot targets (T) of the extended RAS research assay and Reference Standards.

Row	Target	Gene	Exon	Hotspot codon	Genotype	Forward variant	Reference Standard
A	T1	*KRAS*	2	12–13	G12A	C →G	HD265
B	T2	*KRAS*	3	59–61	A59T	C →T	HD694
C	T3	*KRAS*	4	117	K117N	T →G	HD758
D	T4	*KRAS*	4	146	A146T	C →T	HD299
E	T5	*NRAS*	2	12–13	G12A	C →T	HD695
F	T6	*NRAS*	3	59–61	A59T	C →T	HD697
G	T7	*NRAS*	4	117	K117N	C →A	HD696
H	T8	*NRAS*	4	146	A146T	C →T	HD757

Note: **Row:** Primer pair location in the extended RAS research plate.

Genotype: Genotypes of the Horizon Reference Standards.

Forward variant: Variant shown in the forward electropherogram.

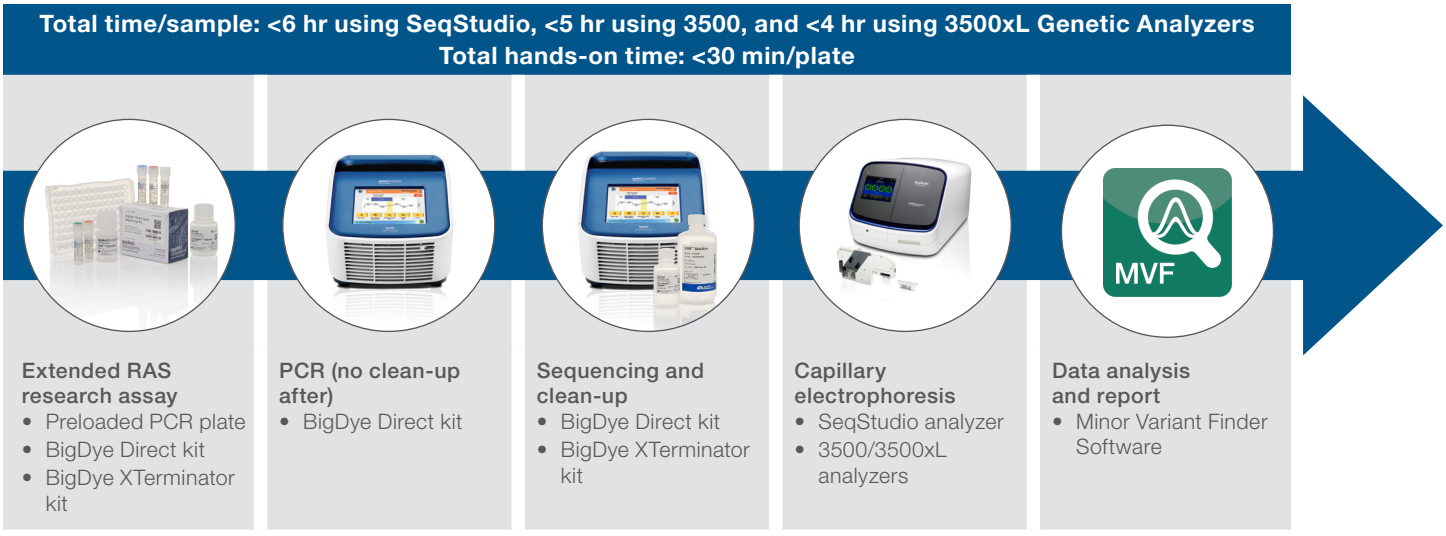

Figure 2. A simple and fast workflow of the extended RAS research assay.

The PCR and sequencing reactions were performed in the Applied Biosystems™ Veriti™ Thermal Cycler. Use of the BigDye Direct Cycle Sequencing Kit eliminates the PCR clean-up step, improves 5´ sequence quality, and simplifies cycle sequencing by using M13 sequencing primers. Lastly, the BigDye XTerminator Purification Kit facilitates sequencing reaction clean-up. Test samples and wild-type CEPH 1347-02 DNA were sequenced in both forward and reverse directions, and processed under similar conditions throughout the entire workflow on the same 96-well plate. Plates were electrophoresed on SeqStudio and 3500xL Genetic Analyzers, and .ab1 files were analyzed using MVF Software.

Sanger sequencing convenience with the SeqStudio Genetic Analyzer

The SeqStudio Genetic Analyzer builds upon the 30-year history of the Applied Biosystems™ genetic analyzers based on capillary electrophoresis technology. The instrument 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 3). The integrated cartridge gives the user 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 with an Internet connection.

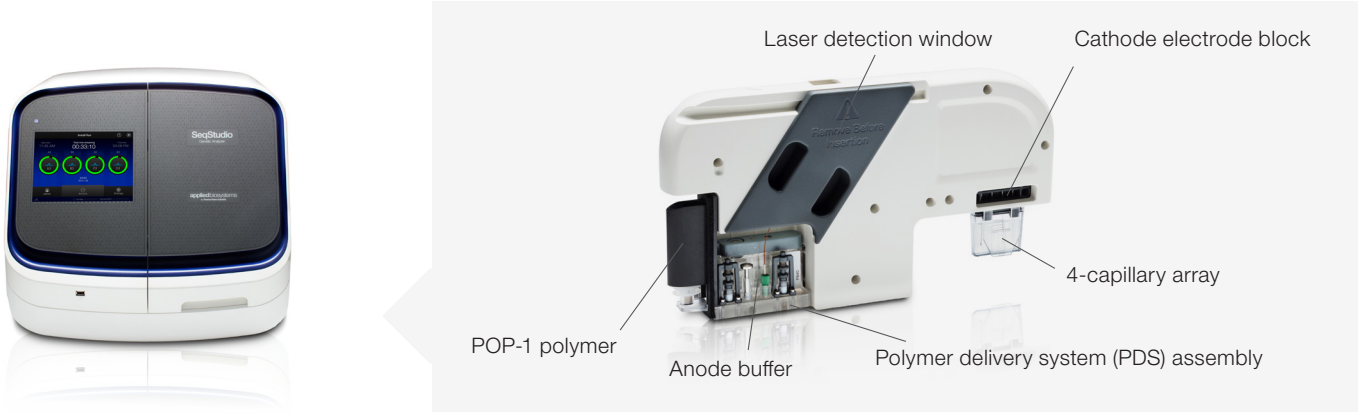

Figure 3. SeqStudio Genetic Analyzer and cartridge.

Easy data analysis using MVF Software

- Detects minor variants down to 5%
- Fast and easy interpretation and analysis workflow
- Identifies variant candidates for expert review and reporting

Analysis of allele frequencies was performed using MVF Software—a user-friendly desktop software specifically designed for the detection, display, and reporting of single-nucleotide variants (SNVs) in Sanger sequencing traces with a detection level as low as 5%. On a test set of 632,452 base positions, it exhibits a 5% LOD with 95.3% sensitivity and 99.83% specificity. The sophisticated algorithm filters out systematic noise components in bidirectional traces, and highlights and presents genuine somatic variant candidates for review and reporting. The identity and minor allele frequency of *NRAS*-specific variants were confirmed through analysis by MVF Software (Figure 4). MVF Software can also readily align sequences with the human reference genome and .vcf files from NGS experiments, providing a smooth workflow for NGS confirmation with annotations in the single-nucleotide polymorphism (SNP) database (dbSNP).

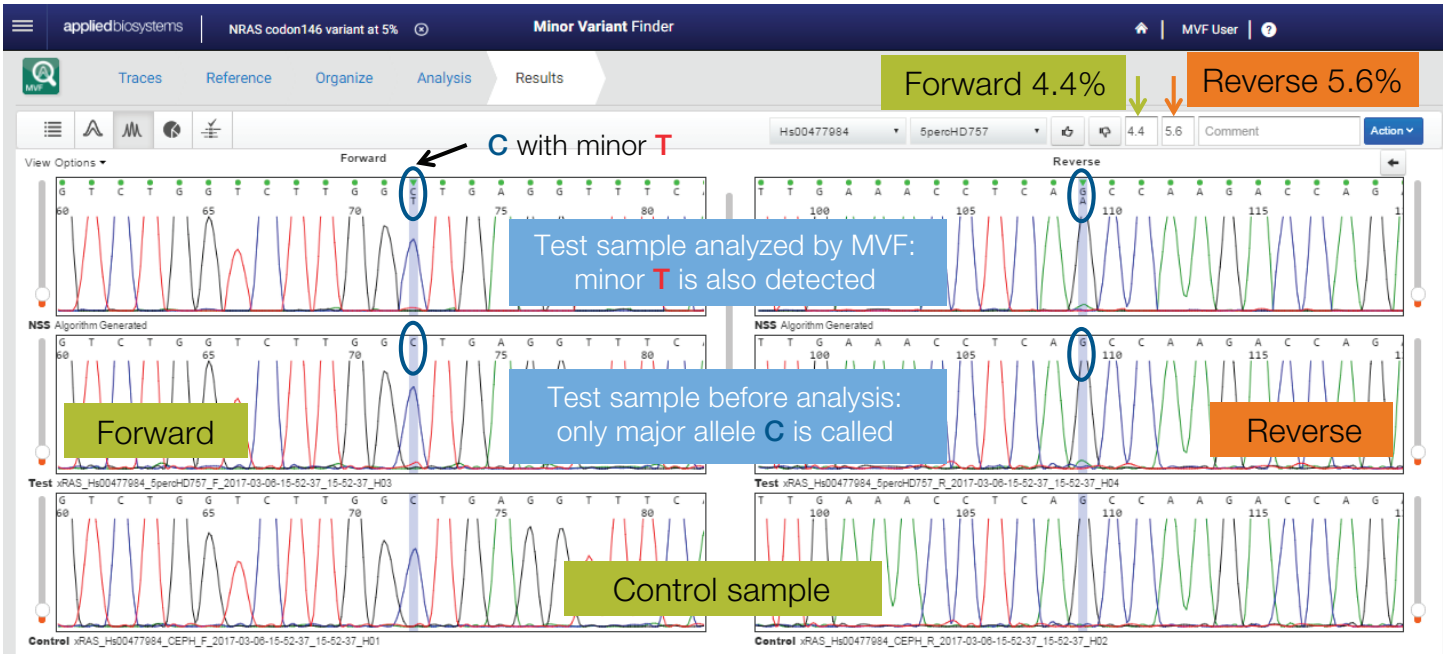

Figure 4. Representative electropherogram view in MVF Software. Minor variant "T" (or the corresponding "A" in the reverse sequence) of *NRAS* codon 146, which is barely visible in the test sample before analysis (electropherograms in the middle), is detected at ~5% level when analyzed by MVF (top electropherograms). Bottom electropherograms are sequences generated using the CEPH wild-type reference control DNA with 100% "C" allele processed on the same 96-well plate.

Results

All expected variants from the 8 DNA Reference Standards were successfully identified at 50%, 10%, and 5% variant allele frequencies using MVF Software. Results from the SeqStudio and 3500xL Genetic Analyzers were in concordance, and the identified variant allele frequencies were highly correlated (Figure 5). Additionally, variants were successfully detected in both forward and reverse directions from 1 ng DNA template per reaction. We recommend a minimum of 1 ng of input DNA per reaction for high-quality data, because based on our findings, the overall sequencing quality can be compromised and the allele ratios appear more variable below 1 ng/reaction.

Third-party customer laboratory testing

The performance of the extended RAS research assay on the new SeqStudio instrument was also tested in the laboratory of Dr Luca Quagliata, Institute for Medical Genetics and Pathology at the University of Basel, Switzerland.

Twenty-two FFPE DNA samples derived from colon cancer biopsies were selected for the study. All samples were previously sequenced on the Ion S5™ XL System using the Ion Torrent™ Oncomine™ Solid Tumor DNA Kit. FFPE sample preparation, FFPE DNA extraction (digestion with proteinase K), and the entire extended RAS workflow, including electrophoresis on a SeqStudio Genetic Analyzer prototype instrument, were performed in Dr. Quagliata's laboratory. Concentrations of the FFPE DNA samples were measured using the Invitrogen™ Qubit™ 3.0 Fluorometer. The PCR and sequencing reactions were performed in the Veriti Thermal Cycler.

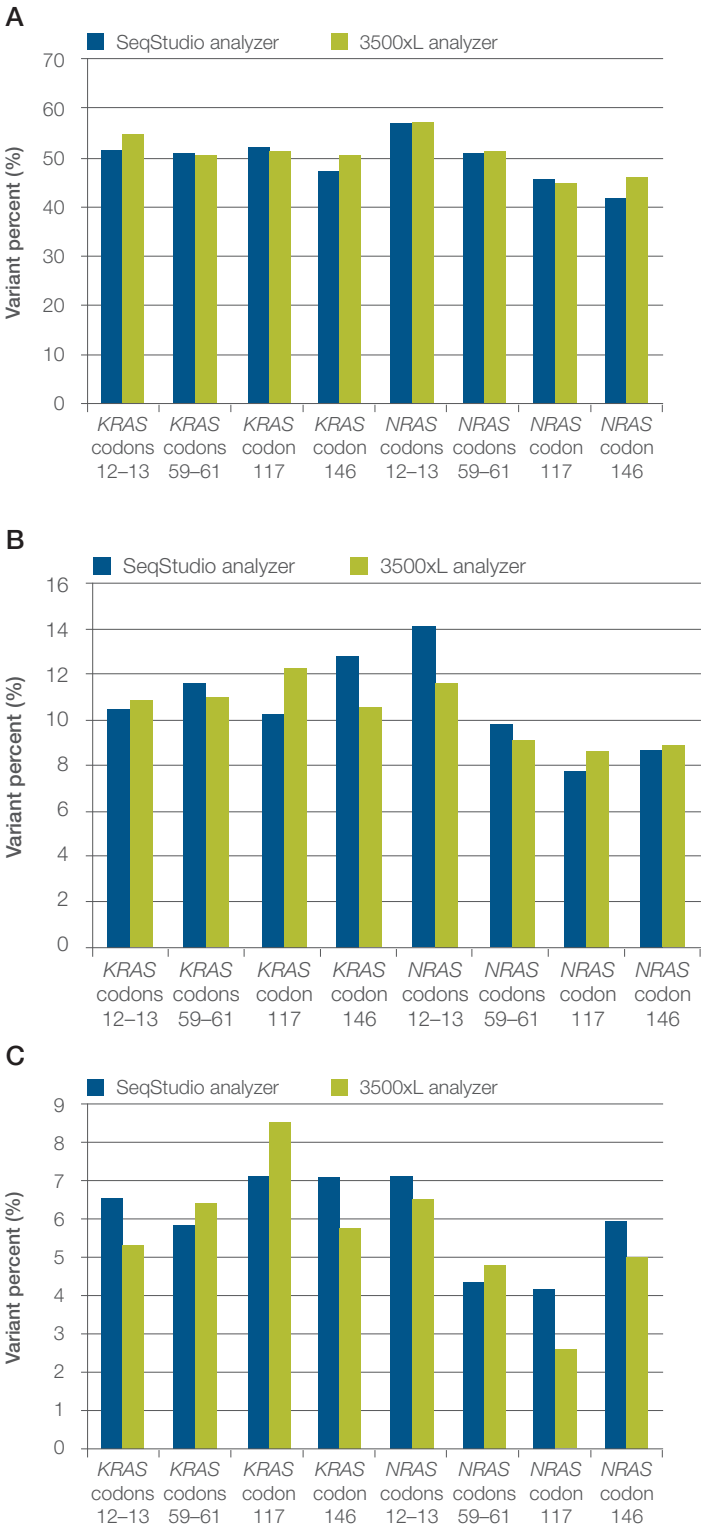

Figure 5. Performance of the extended RAS research assay on the SeqStudio and 3500xL Genetic Analyzers using Reference Standards with (A) 50%, (B) 10%, and (C) 5% allele frequencies, and 1 ng/reaction input DNA. Variant percent (%) is shown on the y-axis. Variant percent represents the average of the percent detected in the forward and the percent detected in the corresponding reverse sequence for a particular hotspot variant. It is the peak height percentage for a particular variant that is compared to the reference peak at the same position. Variant percent is calculated using the following formula: 100 x (variant base peak height)/(primary + variant base peak heights).

Results from colon cancer biopsy research samples

Variants were successfully detected even from research samples where less than 1 ng of input DNA per reaction was used. DNA input per reaction was above 1 ng for 16 samples; however, only limited amount of microdissected FFPE sections were available for 6 research samples where DNA concentrations were below 1 ng. For 1 sample, the input amount was as low as 0.094 ng per reaction. Results from the SeqStudio Genetic Analyzer were in concordance with the data generated by Ion Torrent™ NGS (Figure 6).

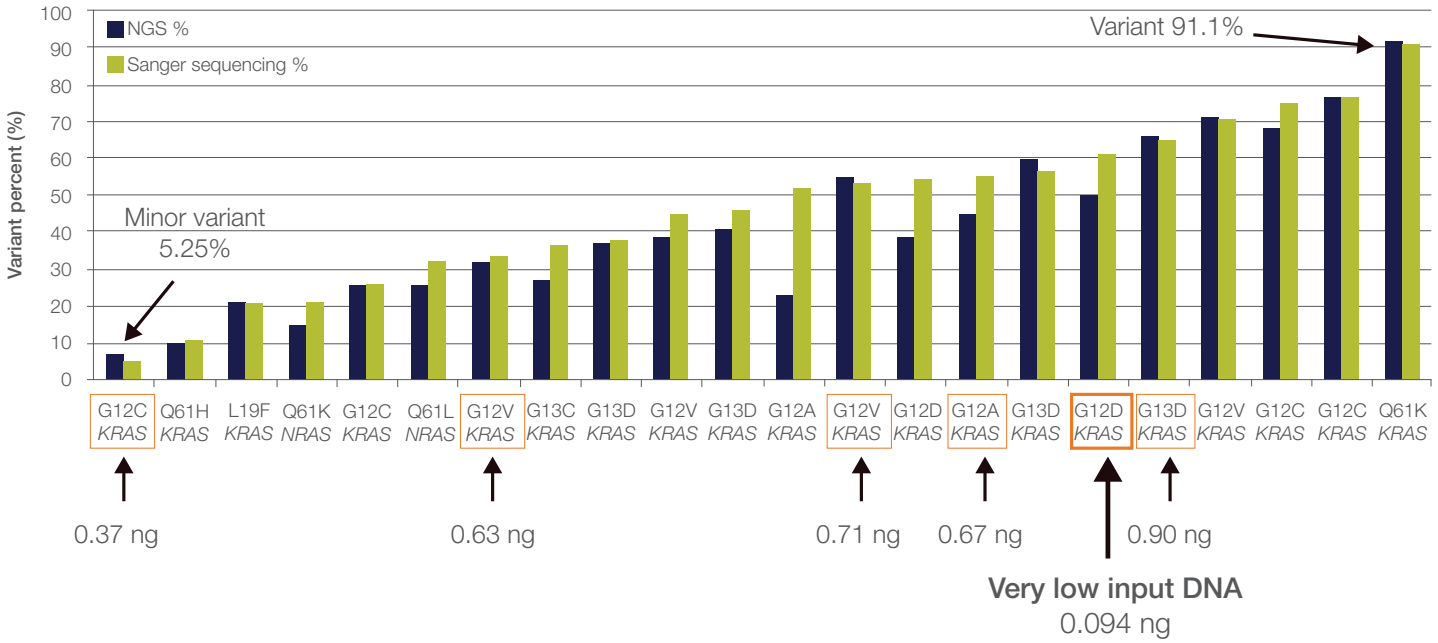

Figure 6. Performance of the extended RAS research assay on the SeqStudio Genetic Analyzer using low input DNA. The entire workflow of the extended RAS research assay comprising 96-well plates was processed at the University of Basel, Switzerland. Variant percent values calculated by MVF Software using .ab1 files from the SeqStudio instrument were compared to variant percent values measured by Ion Torrent NGS, and sorted by Sanger sequencing variant percent values from the lowest (5.25%) to highest (91.1%). Variant percent values from Sanger sequencing represent the average of the variant percent values detected in the forward and reverse sequences.

Conclusions

We have introduced the extended RAS research assay for Sanger sequencing, targeting the 8 most frequently studied *KRAS* and *NRAS* hotspot regions. The assay was optimized for limited amounts of FFPE DNA (down to 1 ng/reaction) and for detection of low-level minor variants (down to 5%) using MVF Software. The robust and simple workflow is further streamlined by a 96-well preloaded plate and offers fast turnaround time (e.g., <6 hr/6 samples for the 3500xL Genetic Analyzer) at a low cost per sample. Moreover, we demonstrate the gold-standard performance of Sanger sequencing using the extended RAS research assay on our new capillary electrophoresis instrument, the SeqStudio Genetic Analyzer, which offers attractive benefits, such as minimized hands-on time with an all-in-one cartridge and an easy-to-use touch-screen interface. We show that the performance of minor variant detection using the extended RAS research assay is comparable on SeqStudio and 3500xL capillary electrophoresis instruments.

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Ordering information

Product	Quantity	Cat. No.
SeqStudio Genetic Analyzer	1 instrument	A34274
SeqStudio Secondary Analysis Software	1 each	4443764
SeqStudio Cartridge v1	500 reactions	A33671
SeqStudio Cartridge v2	1,000 reactions	A41331
SeqStudio Starter Kit	1 kit	A35000
SeqStudio Full-Day SmartStart Training	1 each	A34684
BigDye Direct Cycle Sequencing Kit	1,000 reactions	4458688
BigDye XTerminator Purification Kit	1,000 preps	4376487
Qubit 3.0 Fluorometer	1 each	Q33216
QuantStudio 3D Digital PCR Instrument	1 instrument	4489084
QuantStudio 3D Digital PCR 20K Chip Kit v2 and Master Mix	8 x 12 chips	A26317
Veriti Thermal Cycler	1 instrument	4375786

References

1. Sorich MJ, Wiese MD, Rowland A et al. CS (2015) Extended RAS mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: a meta-analysis of randomized, controlled trials. *Ann Oncol* 26:13–21.
2. Ihle NT, Byers LA, Kim ES et al. (2012) Effect of *KRAS* oncogene substitutions on protein behavior: implications for signaling and clinical outcome. *J Natl Cancer Inst* 104: 228–239.

Find out more about the SeqStudio Genetic Analyzer at
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