Journal of
Molecular Pathology

MDPI

Brief Report

Technical Validation of a Fully Integrated NGS Platform in the Real-World Practice of Italian Referral Institutions

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Abstract: Aims: To date, precision medicine has played a pivotal role in the clinical administration of solid-tumor patients. In this scenario, a rapidly increasing number of predictive biomarkers have been approved in diagnostic practice or are currently being investigated in clinical trials. A pitfall in molecular testing is the diagnostic routine sample available to analyze predictive biomarkers; a scant tissue sample often represents the only diagnostical source of nucleic acids with which to conduct molecular analysis. At the sight of these critical issues, next-generation sequencing (NGS) platforms emerged as referral testing strategies for the molecular analysis of predictive biomarkers in routine practice, but the need for highly skilled personnel and extensive working time drastically impacts the widespread diffusion of this technology in diagnostic settings. Here, we technically validate a fully integrated NGS platform on diagnostic routine tissue samples previously tested with an NGS-based diagnostic workflow by a referral institution. Methods: A retrospective series of n = 64 samples (n = 32 DNA, n = 32 RNA samples), previously tested using a customized NGS assay (SiRe™ and SiRe fusion), was retrieved from the internal archive of the University of Naples Federico II. Each sample was tested by adopting an Oncomine Precision Assay (OPA), which is able to detect 2769 molecular actionable alterations [hotspot mutations, copy number variations (CNV) and gene fusions] on fully integrated NGS platforms (Genexus, Thermo Fisher Scientific (Waltham, MA, USA). The concordance rate between these technical approaches was determined. Results: The Genexus system successfully carried out molecular analysis in all instances. A concordance rate of 96.9% (31 out of 32) was observed between the OPA and SiRe™ panels both for DNA- and RNA-based analysis. A negative predictive value of 100% and a positive predictive value of 96.9% (62 out of 64) were assessed. Conclusions: A fully automatized Genexus system combined with OPA (Thermo Fisher Scientific) may be considered a technically valuable, time-saving sequencing platform to test predictive biomarkers in diagnostic routine practice.

Keywords: NGS; predictive biomarkers; diagnostic samples

Citation: De Luca, C.; Pepe, F.; Russo, G.; Nacchio, M.; Pisapia, P.; Russo, M.; Conticelli, F.; Palumbo, L.; Scimone, C.; Cozzolino, D.; et al. Technical Validation of a Fully Integrated NGS Platform in the Real-World Practice of Italian Referral Institutions. *J. Mol. Pathol.* **2023**, *4*, 259–274. https://doi.org/10.3390/jmp4040022

Academic Editor: Sabine Merkelbach-Bruse

Received: 14 September 2023
Revised: 23 October 2023
Accepted: 25 October 2023
Published: 31 October 2023

1. Introduction

In recent decades, personalized medicine has laid the basis for a novel therapeutical option for solid-tumor patients [1,2]. Currently, target therapy is routinely available for the clinical administration of several solid-tumor patients, including metastatic colorectal cancer (mCRC), melanoma (MM), non-small cell lung cancer (NSCLC), gastrointestinal stromal tumor (GIST), and breast cancer (BC) patients [3–9]. In particular, an increasing number of predictive biomarkers are being approved in clinical practice to provide lung

cancer patients diagnosed with the NSCLC type with the best therapeutical option [8,9]. In this evolving scenario, the minimal request in terms of predictive biomarkers to clinically administrate solid-tumor patients has been regulated by international societies [10–14]. The most common diagnostic sample available to approach diagnosis and molecular tests in the advanced tumor stage consists of a "scant sample" with a low abundance of neoplastic cells to successfully carry out mandatory gene testing [15–17]. In this scenario, cytological specimens and small biopsies represent the most common biological source to accurately perform molecular analysis. In addition, cell block (CB), a hybrid preparation where the aspirated material is processed following standardized formalin fixation and paraffin embedding (FFPE), represents an alternative source of neoplastic cells affected by the lowest quality and quantity of nucleic acids adopted in molecular tests [18,19]. Despite tissue specimens being considered the "gold standard" for molecular testing, a non-negligible percentage of patients do not have access to molecular tests due to insufficient diagnostic material [16,17]. In this scenario, liquid biopsy becomes an integrating biological source for successfully performing molecular analysis when tissue is not available. Moreover, circulating tumor DNA (ctDNA) isolated from peripheral blood is a reliable source for detecting target molecular alterations [20,21]. At the sight of these aspects, single plex technology results are inadequate to successfully analyze the minimum gene panel established for each solid tumor. In this heterogeneous landscape of biological sources, next-generation sequencing (NGS) platforms play a crucial role in the molecular analysis of predictive biomarkers [22–24]. This technology allows us to simultaneously analyze very low-frequency clinically relevant biomarkers using very low amounts of nucleic acids in a single run [22,23]. Remarkably, NGS systems are scalable, decreasing reaction costs in accordance with the number of samples processed in each run [24]. On the other hand, an adequate number of samples may be collected in more than 30 days for a non-negligible number of small–medium institutions involved in molecular tests, thereby saving on technical costs. This aspect drastically impacts turnaround time (TAT), resulting in a delay in the clinical administration of tumor patients [24,25]. In this scenario, the Ion Torrent™ Genexus™ Integrated Sequencer (Genexus; Thermo Fisher Scientific, Waltham, MA, USA) was designed to automatically carry out the entire NGS workflow (from tissue and liquid biopsy-derived nucleic acids extraction to data analysis) without other manual operations [26–28]. This technology allows us to successfully carry out the molecular analysis of a small batch of diagnostic specimens [1–8] without impacting the turnaround time (TAT) of the diagnostic workflow. We aimed to evaluate the concordance rate between the Genexus system and Ion Torrent S5™ Plus (Thermo Fisher Scientific, Waltham, MA, USA) on a retrospective series of extracted genomic DNA (gDNA) from solid-tumor patients previously tested in our diagnostic routine.

2. Study Design

A retrospective series of n = 64 previously extracted DNA and RNA specimens from solid-tumor patients (n = 16 CRC, n = 13 NSCLC, n = 2 BC and n = 1 MM and n = 32 NSCLC cases for DNA- and RNA-related molecular analysis, respectively) was retrieved from the internal archive of the predictive molecular pathology laboratory of the University of Naples Federico II. Clinical pathological data are listed in Tables 1 and 2.

Each sample was previously tested by adopting a customized NGS assay (SiRe™ and SiRe fusion) that covers n = 568 clinically relevant alterations in *BRAF*, *EGFR*, *KRAS*, *NRAS*, *PIK3CA*, *c-KIT*, *PDGFRA* and *ALK*, *ROS1*, *RET*, and *NTRK* gene fusions, as well as MET exon 14 skipping alterations, which is routinely employed in the molecular testing of solid-tumor patients [29]. The Oncomine Precision Assay (OPA), able to detect 2769 molecular actionable alterations [hotspot mutations, copy number variations (CNV) and gene fusions], was combined with the Genexus (Thermo Fisher Scientific) platform to assess the molecular profile of selected samples [26,27]. The concordance rate of the OPA in the Genexus system with SiRe™ on the S5 Plus platform was investigated. All information regarding human material were managed using anonymous numerical codes, and all samples were handled

in compliance with the Helsinki Declaration (http://www.wma.net/en/30publications/10policies/b3/, accessed on 1 September 2023).

Table 1. Clinical characteristics of archival cases and corresponding requests on DNA-based molecular alterations.

ID	Sex	Age	Sample Type	Tumor	N.C.	DNA Amount (ng/μL)	DIN	Clinical Request
DNA 1 *	M	78	Resection	CRC	70.0%	11.8	NA	RAS, BRAF
DNA 2 *	M	78	Resection	CRC	70.0%	47.7	NA	RAS, BRAF
DNA 3	M	89	Biopsy	CRC	50.0%	12.9	NA	RAS, BRAF
DNA 4	F	68	Resection	NSCLC	70.0%	54.1	6.8	EGFR, KRAS, BRAF
DNA 5	M	73	Resection	CRC	50.0%	60.0	NA	RAS, BRAF
DNA 6	M	53	Biopsy	NSCLC	30.0%	6.0	5.6	EGFR, KRAS, BRAF
DNA 7	M	66	Resection	CRC	40.0%	35.6	NA	RAS, BRAF
DNA 8	F	78	Resection	CRC	40.0%	20.2	NA	RAS, BRAF
DNA 9	F	67	Resection	NSCLC	60.0%	5.02	3.1	EGFR, KRAS, BRAF
DNA 10	F	51	Resection	CRC	30.0%	23.5	NA	RAS, BRAF
DNA 11	M	50	Resection	CRC	80.0%	39.1	NA	c-KIT, PDGFRA
DNA 12	F	50	Biopsy	NSCLC	50.0%	9.8	1.6	EGFR, KRAS, BRAF
DNA 13	M	70	Biopsy	NSCLC	20.0%	15.9	3.7	EGFR, KRAS, BRAF
DNA 14	F	59	Resection	NSCLC	40.0%	47.3	6.5	EGFR, KRAS, BRAF
DNA 15	M	66	Biopsy	NSCLC	30.0%	2.8	3.3	EGFR, KRAS, BRAF
DNA 16	M	56	Resection	CRC	50.0%	55.0	NA	RAS, BRAF
DNA 17	M	66	Resection	NSCLC	60.0%	115.0	4.9	EGFR, KRAS, BRAF
DNA 18	F	51	Biopsy	CRC	50.0%	37.0	NA	RAS, BRAF
DNA 19	F	41	Biopsy	BC	30.0%	35.1	3.7	PIK3CA
DNA 20	F	82	Biopsy	CRC	30.0%	29.8	NA	RAS, BRAF
DNA 21	M	67	Biopsy	CRC	50.0%	27.2	NA	RAS, BRAF
DNA 22	M	82	Resection	NSCLC	80.0%	39.9	6.9	EGFR, KRAS, BRAF
DNA 23	M	74	Resection	NSCLC	70.0%	45.5	4.3	EGFR, KRAS, BRAF
DNA 24	M	74	Resection	CRC	40.0%	2.2	NA	RAS, BRAF
DNA 25	F	44	Biopsy	CRC	40.0%	7.3	NA	RAS, BRAF
DNA 26	F	69	Biopsy	NSCLC	60.0%	14.8	4.7	EGFR, KRAS, BRAF
DNA 27	M	54	Resection	CRC	30.0%	22.6	NA	RAS, BRAF
DNA 28	F	74	Resection	MM	90.0%	11.4	NA	BRAF, NRAS
DNA 29	F	63	Biopsy	NSCLC	40.0%	8.5	6.2	EGFR, KRAS, BRAF
DNA 30	M	56	Resection	NSCLC	50.0%	3.9	4.5	EGFR, KRAS, BRAF
DNA 31	F	52	Resection	CRC	60.0%	37.9	NA	RAS, BRAF
DNA 32	F	45	Resection	BC	60.0%	25.2	NA	PIK3CA

* Same patient, different lesions. Abbreviations: BC (Breast Cancer); BRAF (Murine Sarcoma Viral Oncogene Homolog B); c-KIT (KIT Proto-Oncogene); CRC (Colorectal Cancer); DNA (Deoxyribonucleic Acid); EGFR (Epidermal Growth Factor Receptor); F (Female); ID (Identifier); KRAS (Kirsten Rat Sarcoma Viral Oncogene Homolog); M (Male); MM (Malignant Melanoma); NA (Not Assessable N.C. (Neoplastic Cellularity); NSCLC (Non-Small-Cell Lung Cancer); PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha); RAS (Rat Sarcoma Viral Oncogene Homolog).

Table 2. Clinical characteristics of archival cases and corresponding requests on RNA-based molecular alterations.

ID	Sex	Age	Sample Type	Tumor	N.C.	Clinical Request
RNA 1	M	56	Resection	NSCLC	60.0%	ALK, ROS1, RET, MET, NTRK
RNA 2	F	58	Biopsy	NSCLC	70.0%	ALK, ROS1, RET, MET, NTRK
RNA 3	M	77	Biopsy	NSCLC	25.0%	ALK, ROS1, RET, MET, NTRK
RNA 4	M	79	Resection	NSCLC	70.0%	ALK, ROS1, RET, MET, NTRK
RNA 5	M	79	Biopsy	NSCLC	30.0%	ALK, ROS1, RET, MET, NTRK
RNA 6	M	59	Biopsy	NSCLC	30.0%	ALK, ROS1, RET, MET, NTRK
RNA 7	F	70	Biopsy	NSCLC	50.0%	ALK, ROS1, RET, MET, NTRK
RNA 8	M	62	Biopsy	NSCLC	25.0%	ALK, ROS1, RET, MET, NTRK
RNA 9	M	61	Biopsy	NSCLC	40.0%	ALK, ROS1, RET, MET, NTRK
RNA 10	M	66	Resection	NSCLC	60.0%	ALK, ROS1, RET, MET, NTRK
RNA 11	M	68	Biopsy	NSCLC	40.0%	ALK, ROS1, RET, MET, NTRK
RNA 12	M	64	Biopsy	NSCLC	50.0%	ALK, ROS1, RET, MET, NTRK
RNA 13	F	65	Biopsy	NSCLC	60.0%	ALK, ROS1, RET, MET, NTRK
RNA 14	M	58	Biopsy	NSCLC	20.0%	ALK, ROS1, RET, MET, NTRK
RNA 15	F	79	Biopsy	NSCLC	50.0%	ALK, ROS1, RET, MET, NTRK
RNA 16	M	52	Biopsy	NSCLC	50.0%	ALK, ROS1, RET, MET, NTRK
RNA 17	M	67	Resection	NSCLC	60.0%	ALK, ROS1, RET, MET, NTRK
RNA 18	M	87	Biopsy	NSCLC	40.0%	ALK, ROS1, RET, MET, NTRK
RNA 19	M	25	Biopsy	NSCLC	60.0%	ALK, ROS1, RET, MET, NTRK
RNA 20	F	60	Biopsy	NSCLC	30.0%	ALK, ROS1, RET, MET, NTRK
RNA 21	M	60	Resection	NSCLC	60.0%	ALK, ROS1, RET, MET, NTRK
RNA 22	F	36	Biopsy	NSCLC	30.0%	ALK, ROS1, RET, MET, NTRK
RNA 23	M	66	Biopsy	NSCLC	60.0%	ALK, ROS1, RET, MET, NTRK
RNA 24	F	47	Biopsy	NSCLC	50.0%	ALK, ROS1, RET, MET, NTRK
RNA 25	M	67	Biopsy	NSCLC	30.0%	ALK, ROS1, RET, MET, NTRK
RNA 26	F	64	Biopsy	NSCLC	10.0%	ALK, ROS1, RET, MET, NTRK
RNA 27	M	54	Biopsy	NSCLC	40.0%	ALK, ROS1, RET, MET, NTRK
RNA 28	F	37	Biopsy	NSCLC	50.0%	ALK, ROS1, RET, MET, NTRK
RNA 29	M	79	Biopsy	NSCLC	50.0%	ALK, ROS1, RET, MET, NTRK
RNA 30	F	71	Biopsy	NSCLC	30.0%	ALK, ROS1, RET, MET, NTRK
RNA 31	M	68	Biopsy	NSCLC	50.0%	ALK, ROS1, RET, MET, NTRK
RNA 32	F	72	Biopsy	NSCLC	70.0%	ALK, ROS1, RET, MET, NTRK

Abbreviations: ALK (Anaplastic Lymphoma Kinase); F (Female); ID (Identifier); M (Male); MET (Tyrosine-Protein Kinase Met); N.C. (Neoplastic Cellularity); NSCLC (Non-Small-Cell Lung Cancer); NTRK (Neurotrophic Tyrosine Receptor Kinase); RET (RET Proto-Oncogene); RNA (Ribonucleic Acid); ROS1 (Proto-Oncogene Tyrosine-Protein Kinase ROS).

3. Material and Methods

3.1. Routine Sample Processing Strategy

Nucleic acids were previously purified from $n = 4$ representative slides of neoplastic area (>10%). Specifically, a QIAamp DNA Mini Kit (Qiagen, Crawley, West Sussex, UK) was utilised following manufacturer instructions. DNA quantification was successfully carried out in all cases, adopting a Qubit fluorimeter (Thermo Fisher) or a TapeStation

4200 microfluidic platform (Agilent Technologies, Santa Clara, CA, USA) following manufacturer instructions. In the instance of an inadequate amount of nucleic acids, we maximized for volume input. Conversely, RNA volume was maximized for cDNA synthesis. Selected samples were routinely analyzed with SiRe™ and SiRe fusion panels using the Ion S5™ Plus software (Thermo Fisher Scientific) to assess mutational status in clinically relevant biomarkers for NSCLC patients [29,30]. Briefly, 15 µL of extracted DNA/cDNA was dispensed into the Ion Kit-Chef system (Thermo Fisher Scientific) for library preparation. A total of n = 8 samples was simultaneously processed following previously validated thermal conditions. After pooling, a templating procedure was carried out for n = 16 libraries by using the Ion 510™, Ion 520™ and Ion 530™ Kit-Chef (Thermo Fisher Scientific) according to manufacturer instructions on a 520 chip (Thermo Fisher Scientific). Data were inspected by adopting designed bed files on proprietary Torrent Suite software [v.5.0.2]. In detail, variant inspection was performed with a variant caller plug-in (v.5.0.2.1), which is able to filter variants with $\geq$5× allele coverage and a quality score $\geq$20, within an amplicon that covered at least 500× alleles.

3.2. Genexus Analysis

A series of n = 64 extracted gDNA and gRNA samples from solid-tumor patients was retrospectively tested in the Genexus (Thermo Fisher Scientific) system. The platform enables entire NGS workflows (from library preparation to data interpretation) within 24 h. The OPA assay includes the most clinically relevant actionable genes (*EGFR*, *BRAF*, *KRAS*, *ALK*, *ROS1*, NTRK, and *RET*) for NSCLC patients [27,28]. Briefly, samples were created on a dedicated server and assigned to a new run. The Genexus platform was loaded with OPA primers, strip solutions, strip reagents, and supplies according to manufacturer instructions. A total of 10 ng was required by the OPA assay on the Genexus platform. Accordingly, each sample was diluted and immediately dispensed on a 96-well plate, following manufacturer instructions. Finally, nucleic acids were sequenced on a GX5TM chip that allows for the simultaneous processing of n = 8 samples in a single line with an OPA assay. Data analysis was performed using proprietary Genexus software (1.0). Particularly, detected alterations were annotated by adopting Oncomine Knowledgebase Reporter Software (Oncomine Reporter 5.0). In addition, BAM files were also visually inspected with the Golden Helix Genome Browser v.2.0.7 (Bozeman, MT, USA) in hotspot regions in EGFR, KRAS, and BRAF lung cancer-addicted molecular alterations.

4. Results

4.1. Hotspot Mutations

Overall, the Genexus system successfully carried out molecular analysis in all DNA series. In detail, a median number of total reads, mapped reads, mean read length, percent reads on target, mean depth, uniformity of amplicon coverage of 1,134,878.2 (ranging from 424,900.0 to 1,791,041.0), 1,074,345.7 (ranging from 365,139.0 to 1,756,414.0), 90.9 bp (ranging from 71 to 103 bp), 88.3% (ranging from 77.7 to 93.7%), 3602.9 (ranging from 994.00 to 6097.0) and 98.2% (ranging from 96.7 to 99.4%) were detected, respectively (Table 3).

Table 3. Technical parameters from DNA-based analysis by using S5 Plus (Ion Reporter 5.2.0.1) and Genexus systems.

DNA Analysis Technical Parameters—S5 Plus (SiRe™ Panel) vs. Genexus (OPA Panel)							
ID	**Platform**	**Total Reads**	**Mean Read Length**	**Mapped Reads**	**On Target Reads**	**Mean Depth**	**Uniformity**
DNA 1 *	S5 Plus	254,212	126	253,622	94.6%	5712	100%
	Genexus	872,831	76	736,530	77.7%	2044	99.1%
DNA 2 *	S5 Plus	215,464	128	215,047	92.6%	4740	100%
	Genexus	732,691	84	663,064	83.9%	2034	98.8%

Table 3. *Cont.*

DNA Analysis Technical Parameters—S5 Plus (SiRe™ Panel) vs. Genexus (OPA Panel)							
ID	**Platform**	**Total Reads**	**Mean Read Length**	**Mapped Reads**	**On Target Reads**	**Mean Depth**	**Uniformity**
DNA 3	S5 Plus	298,541	135	297,999	93.9%	6662	100%
	Genexus	1,143,038	91	1,076,855	88.8%	3528	98.1%
DNA 4	S5 Plus	524,926	155	523,086	92.3%	11,489	100%
	Genexus	1,419,289	101	1,393,603	92.9%	5210	98.1%
DNA 5	S5 Plus	361,148	137	360,373	91.3%	7830	100%
	Genexus	1,094,620	98	1,064,051	91.5%	3810	98.6%
DNA 6	S5 Plus	314,176	128	313,706	99.2%	7406	100%
	Genexus	1,090,358	98	1,049,935	90.8%	3837	99.0%
DNA 7	S5 Plus	635,201	142	634,226	92.1%	13,911	100%
	Genexus	1,002,231	92	946,318	88.9%	3150	98.9%
DNA 8	S5 Plus	524,182	131	523,608	93.0%	11,591	100%
	Genexus	1,262,760	95	1,208,543	90.9%	4176	98.9%
DNA 9	S5 Plus	942,781	161	940,605	94.6%	21,192	100%
	Genexus	1,791,041	97	1,756,414	93,0%	6097	97.9%
DNA 10	S5 Plus	393,979	126	393,371	89.5%	8381	100%
	Genexus	989,635	60	717,385	64.9%	1459	98.9%
DNA 11	S5 Plus	451,494	139	450,779	94.4%	10,127	100%
	Genexus	776,893	78	679,358	80.4%	1863	96.7%
DNA 12	S5 Plus	88,915	129	88,784	98.0%	2072	92.9%
	Genexus	1,297,992	91	1,263,558	92.7%	3996	93.9%
DNA 13	S5 Plus	296,845	143	296,434	96.2%	6790	100%
	Genexus	1,196,122	99	1,174,442	92.7%	4258	98.5%
DNA 14	S5 Plus	37,206	133	37,173	95.2%	842.7	97.6%
	Genexus	1,125,616	97	1,093,531	91.8%	3824	98.6%
DNA 15	S5 Plus	782,397	150	780,894	95.2%	17,703	100%
	Genexus	1,465,786	92	1,423,741	91.9%	4574	95.3%
DNA 16	S5 Plus	378,978	140	378,373	93.3%	8402	100%
	Genexus	1,084,647	87	1,012,693	87.6%	3054	98.2%
DNA 17	S5 Plus	520,304	135	519,653	91.5%	11,317	100%
	Genexus	1,048,030	98	1,016,324	91.4%	3617	98.8%
DNA 18	S5 Plus	49,127	138	49,055	95.3%	1113	97.6%
	Genexus	1,294,194	97	1,256,161	91.9%	4435	98.9%
DNA 19	S5 Plus	486,407	147	485,652	96.6%	11,165	97.6%
	Genexus	1,343,529	97	1,311,776	92.3%	4658	99.4%
DNA 20	S5 Plus	346,019	131	345,464	97.4%	8010	97.6%
	Genexus	974,476	71	759,420	75.7%	2023	98.8%
DNA 21	S5 Plus	67,488	130	67,417	95.9%	1540	97.6%
	Genexus	1,150,249	90	1,094,010	90.3%	3519	98.8%
DNA 22	S5 Plus	52,080	170	51,956	90.4%	1119	100%
	Genexus	14,94,337	100	1,470,085	92.3%	5451	97.9%
DNA 23	S5 Plus	614,960	141	613,813	96.2%	14,059	97.6%
	Genexus	1,574,234	91	1,510,266	91.2%	4865	97.7%
DNA 24	S5 Plus	188,967	136	188,623	98.1%	4407	97.6%
	Genexus	1,093,646	103	1,071,141	92.2%	4072	99.1%

Table 3. *Cont.*

DNA Analysis Technical Parameters—S5 Plus (SiRe™ Panel) vs. Genexus (OPA Panel)							
ID	**Platform**	**Total Reads**	**Mean Read Length**	**Mapped Reads**	**On Target Reads**	**Mean Depth**	**Uniformity**
DNA 25	S5 Plus	140,163	145	139,930	95.5%	3183	97.6%
	Genexus	949,852	94	911,448	90,0%	3064	99.4%
DNA 26	S5 Plus	40,233	142	40,180	96.7%	925.4	97.6%
	Genexus	1,497,022	99	1,476,425	93.7%	5365	98.3%
DNA 27	S5 Plus	153,378	133	153,236	96.0%	3501	97.6%
	Genexus	1,059,772	95	1,021,186	90.2%	3498	98.7%
DNA 28	S5 Plus	155,154	118	154,695	96.5%	3553	92.8%
	Genexus	424,900	75	365,139	79.3%	994	97.4%
DNA 29	S5 Plus	358,001	160	356,995	95.2%	8095	100%
	Genexus	1,165,795	98	1,134,969	92.2%	4075	98.4%
DNA 30	S5 Plus	275,579	149	274,340	98.4%	6428	100%
	Genexus	1,080,846	92	1,034,348	90.3%	3392	98.4%
DNA 31	S5 Plus	259,364	130	258,623	92.6%	5702	100%
	Genexus	1,109,488	92	1,054,465	89.9%	3457	98.9%
DNA 32	S5 Plus	263,420	126	262,682	93.4%	5841	97.6%
	Genexus	710,181	82	631,880	82.5%	1893	96.7%

* Same patient with different lesions. Abbreviations: DNA (Deoxyribonucleic Acid); ID (Identifier).

Remarkably, *n* = 29 out of 32 (90.6%) patients [*n* = 16 CRC, *n* = 10 NSCLC, *n* = 2 BC and *n* = 1 MM] showed molecular alterations covered by OPA reference genes. Of note, 24 out of 29 (82.7%) cases highlighted clinically relevant molecular alterations referenced by the SiRe™ panel. In particular, *n* = 3 out 29 *EGFR* mutations [*n* = 1 exon 19 c.2300_2308dup p.A767_V769dup; *n* = 1 exon 21 c.2573T>G p.L858R and a concomitant *EGFR* exon 20 c.2369C>T p.T790M+ exon 21 c.2573T>G p.L858R]; *n* = 13 out of 29 *KRAS* molecular alterations [*n* = 3 exon 2 c.35G>A p.G12D; *n* = 2 exon 2 c.34G>T p.G12C; *n* = 2 exon 2 c.35G>A p.G12V; *n* = 1 exon 2 c.38G>A p.G13D; *n* = 1 exon 3 c.182A>T p.Q61L]; *n* = 1 exon 3 c.181C>A p.Q61K; *n* = 1 exon 4 c.436G>A p.A146T and *n* = 2 concomitant KRAS exon 2 c.35G>A p.G12D+ c.38G>A p.G13D; *KRAS* exon 2 c.38G>A p.G13D+ c.38_39delinsAA p.G13E]; *n* = 3 out of 29 *BRAF* mutations [*n* = 2 exon 15 c.1799T>A p.V600E and *n* = 1 exon 15 c.1801A>G p.K601E]; *n* = 4 out of 29 *PIK3CA* hotspot mutations [*n* = 2 exon 9 c.1633G>A p.E545K and *n* = 2 exon 20 c.3140A>G p.H1047R]; *n* = 3 out 29 *NRAS* mutations [*n* = 2 exon 3 c.181C>A p.Q61K and *n* = 1 exon 3 c.182A>G p.Q61R]; and *n* = 1 out of 29 c-KIT molecular alterations [exon 11 c.1727T>C p.L576P] were detected (Table 4).

Table 4. Comparison of DNA-related molecular alterations between S5 Plus and Genexus platforms.

ID	S5Plus (SiRe™ Panel)	Genexus (OPA Panel)
DNA 1 *	KRAS p.G12C 27.6% PIK3CA p.H1047R 35.0%	KRAS p.G12C 32.9% PIK3CA p.H1047R 33.2%
DNA 2 *	KRAS p.G12C 37.2% PIK3CA p.H1047R 42.2%	KRAS p.G12C 32.7% PIK3CA p.H1047R 36.4%
DNA 3	KRAS p.G12D 20.7%	KRAS p.G12D 18.9%
DNA 4	EGFR p.L858R 27.7%	EGFR p.L858R 18.9%
DNA 5	KRAS p.G12V 34.5%	KRAS p.G12V 33.0%
DNA 6	WT	WT
DNA 7	KRAS p.G12D 57.2%	KRAS p.G12D 60.8%

Table 4. *Cont.*

ID	S5Plus (SiRe™ Panel)	Genexus (OPA Panel)
DNA 8	KRAS p.Q61K 16.8%	KRAS p.Q61K 19.3%
DNA 9	WT	WT
DNA 10	KRAS p.G12D 50.6%	KRAS p.G12D 55.3%
DNA 11	c-KIT p.L576P 68.0%	c-KIT p.L576P 63.8%
DNA 12	EGFR p.A767_V769dup 67.2%	EGFR p.A767_V769dup 72.8%
DNA 13	WT	WT
DNA 14	WT	WT
DNA 15	BRAF p.K601E 16.3%	BRAF p.K601E 16.1%
DNA 16	KRAS p.G12D 9.3% KRAS p.G13D 14.1%	KRAS p.G12D 8.2% KRAS p.G13D 12.1%
DNA 17	KRAS p.Q61L 32.7%	KRAS p.Q61L 36.3%
DNA 18	NRAS p.Q61K 19.3%	NRAS p.Q61K 18.2%
DNA 19	PIK3CA E545K 0.8% **	PIK3CA E545K 7.2%
DNA 20	BRAF p.V600E 30.5%	BRAF p.V600E 30.0%
DNA 21	NRAS p.Q61K 46.7%	NRAS p.Q61K 36.2%
DNA 22	KRAS p.G13D 47.4% *** KRAS p.G13E 47.9% ***	KRAS p.G13D 41.9% *** KRAS p.G13E 42.0% ***
DNA 23	WT	WT
DNA 24	KRAS p.A146T 30.80%	KRAS p.A146T 26.4%
DNA 25	WT	WT
DNA 26	BRAF p.V600E 27.3%	BRAF p.V600E 30.3%
DNA 27	KRAS p.G13D 14.9%	KRAS p.G13D 12.2%
DNA 28	NRAS p.Q61R 34.3%	NRAS p.Q61R 28.2%
DNA 29	EGFR p.L858R 9.7% EGFR p.T790M 9.5%	EGFR p.L858R 9.3% EGFR p.T790M 11.0%
DNA 30	WT	WT
DNA 31	KRAS p.G12V 51.2% PIK3CA p.E545K 32.2%	KRAS p.G12V 59.2% PIK3CA p.E545K 31.0%
DNA 32	WT	WT

* Different lesion of same patient. ** Below 5%; *** Concomitant SNV. Abbreviations: BRAF (Murine Sarcoma Viral Oncogene Homolog B); c-KIT (KIT Proto-Oncogene); DNA (Deoxyribonucleic Acid); EGFR (Epidermal Growth Factor Receptor); ID (Identifier); KRAS (Kirsten Rat Sarcoma Virus); PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha); RAS (Rat Sarcoma Virus); WT (Wild-Type).

No significant variations in accordance with histological groups, mutation type and mutant allele fraction levels between Genexus and the previously tested samples on the S5 platform were identified. In addition, the OPA assay also identified n = 16 out of 32 (50.0%) DNA-based molecular alterations in other genes not covered by the SiRe panel. Moreover, 12 out of 16, 1 out of 16, and 1 out of 16 highlighted *TP53*, *CTNNB1* and *MTOR* hotspot molecular alterations, respectively. Moreover, concomitant *TP53* (exon 7 p.G279E plus exon 5 p.V197M) and *TP53* (exon 4 p.R175H) in association with *CTNNB1* (exon 3 p.S45F) hotspot mutations were identified in ID#2 and ID#16 cases (Table 5).

The molecular profile detected by OPA on the Genexus platform matched with the Sire panel on the S5 Plus system in 31 out of 32 patients (96.9%). Remarkably, positive results previously identified adopting the SiRe panel were confirmed in 23 out of 24 (95.8%) patients. Particularly, ID#19 showed an exon 9 *PIK3CA* p.E545K hotspot mutation not observed by using the S5 system with a standardized clinical cut-off (MAF = $\geq$5.0%) (Figure 1).

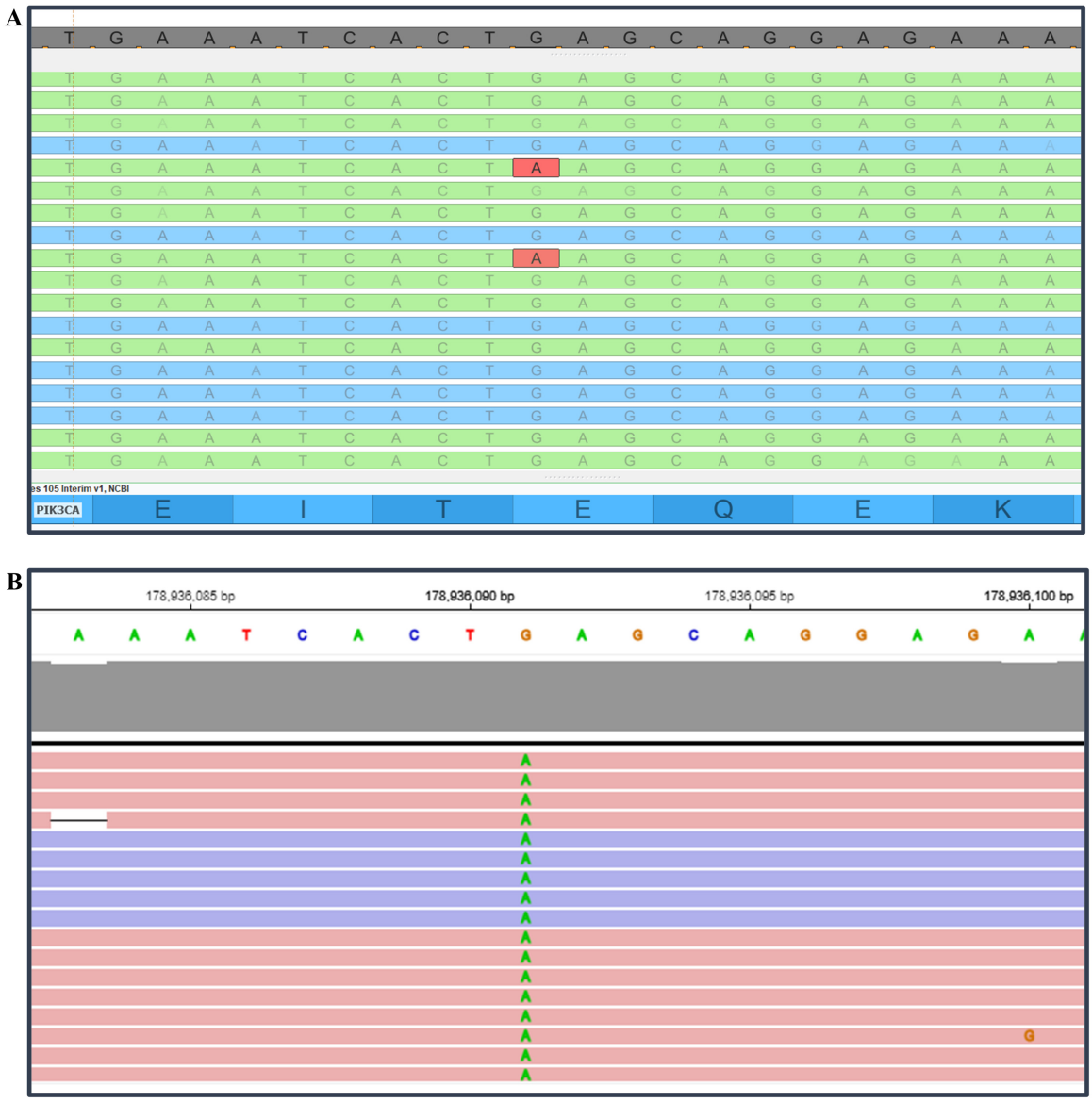

Figure 1. PIK3CA p.E545K hotspot mutations manually inspected with Golden Helix Genome Browser v.2.0.7 (Bozeman, MT, USA) (**A**) and automatically annotated on proprietary Genexus software (**B**).

Table 5. Expanded list of molecular alterations covered by OPA on the Genexus platform.

ID	Other Mutations (OPA Panel)
DNA 1 *	**MTOR** p.R2217W 4.5%
DNA 2 *	**TP53** p.G279E 4.8% **TP53** p.V197M 4.0%
DNA 7	**TP53** p.H179Y 75.8%
DNA 9	**TP53** p.R273H 35.0%
DNA 12	**TP53** p.V197M 77.7%
DNA 14	**TP53** p.R273H 10.0%
DNA 16	**CTNNB1** p.S45F 41.1% **TP53** p.R175H 13.2%

Table 5. *Cont.*

ID	Other Mutations (OPA Panel)
DNA 18	**TP53** p.Y220C 19.7%
DNA 19	**TP53** p.L194F 9.9%
DNA 20	**TP53** p.P151S 54.7%
DNA 21	**TP53** p.K132R 51.4%
DNA 23	**TP53** p.C238S 25.3%
DNA 27	**CTNNB1** p.S45F 21.8%
DNA 30	**TP53** p.H179Y 24.6%
DNA 31	**TP53** p.Y220C 56.1%
DNA 32	**TP53** p.E285K 4.8%

* Same patient, different lesion. Abbreviations: CTNNB1 (Catenin Beta 1); DNA (Deoxyribonucleic Acid); ID (Identifier); MTOR (Mammalian Target Of Rapamycin); TP53 (Tumor Protein P53).

4.2. Fusions Rearrangements

Regarding RNA samples, the Genexus platform successfully analyzed all retrieved cases. Briefly, a median number of total reads, mapped reads and mean read length of 1,721,491.0 (ranging from 1,471,817.00 to 2,462,555.00), 158,230.4 (ranging from 37,387.0 to 1,029,745.00), 98.8 bp (ranging from 91 to 104 bp) were identified, respectively (Table 6).

Table 6. Technical parameters from RNA-based analysis by using S5 Plus and Genexus systems.

RNA Analysis Technical Parameters—S5 Plus (SiRe Fusion Panel) vs. Genexus (OPA Panel)				
ID	**Platform**	**Total Reads**	**Mean Read Length**	**Mapped Reads**
RNA 1	S5 Plus	503,832	92	489,474
	Genexus	2,355,408	99	170,105
RNA 2	S5 Plus	829,380	124	823,978
	Genexus	1,748,261	99	140,327
RNA 3	S5 Plus	641,591	89	348,169
	Genexus	2,462,555	104	54,529
RNA 4	S5 Plus	254,394	93	242,076
	Genexus	1,667,488	100	37,387
RNA 5	S5 Plus	234,803	67	176,276
	Genexus	1,755,508	91	111,713
RNA 6	S5 Plus	357,284	89	319,350
	Genexus	1,542,252	101	72,995
RNA 7	S5 Plus	1,070,656	111	1,067,615
	Genexus	1,571,469	100	150,711
RNA 8	S5 Plus	535,701	103	526,127
	Genexus	1,737,696	96	1,029,745
RNA 9	S5 Plus	494,550	87	421,901
	Genexus	1,634,624	103	72,104
RNA 10	S5 Plus	161,964	100	153,003
	Genexus	1,815,512	96	51,505
RNA 11	S5 Plus	190,170	98	187,044
	Genexus	1,597,727	98	386,493
RNA 12	S5 Plus	677,654	91	513,093
	Genexus	1,554,237	101	171,919

Table 6. *Cont.*

RNA Analysis Technical Parameters—S5 Plus (SiRe Fusion Panel) vs. Genexus (OPA Panel)				
ID	**Platform**	**Total Reads**	**Mean Read Length**	**Mapped Reads**
RNA 13	S5 Plus	765,186	129	753,177
	Genexus	1,777,747	100	178,846
RNA 14	S5 Plus	222,717	103	217,972
	Genexus	1,503,566	102	48,005
RNA 15	S5 Plus	490,208	125	483,482
	Genexus	1,523,971	99	61,024
RNA 16	S5 Plus	20,405	91	17,060
	Genexus	1,878,041	97	42,572
RNA 17	S5 Plus	367,743	117	346,142
	Genexus	1,769,313	97	80,920
RNA 18	S5 Plus	191,027	99	189,336
	Genexus	1,513,615	97	365,130
RNA 19	S5 Plus	240,954	126	239,481
	Genexus	1,744,270	100	133,226
RNA 20	S5 Plus	203,214	86	195,547
	Genexus	1,284,559	94	173,554
RNA 21	S5 Plus	195,912	91	185,689
	Genexus	1,940,917	96	60,947
RNA 22	S5 Plus	464,854	119	462,638
	Genexus	1,715,374	98	294,552
RNA 23	S5 Plus	258,734	93	251,939
	Genexus	1,644,449	99	141,394
RNA 24	S5 Plus	287,598	104	284,682
	Genexus	1,573,653	103	68,184
RNA 25	S5 Plus	297,871	114	294,124
	Genexus	1,587,686	99	111,160
RNA 26	S5 Plus	428,858	118	426,903
	Genexus	1,682,103	100	185,977
RNA 27	S5 Plus	173,120	98	171,187
	Genexus	1,471,817	98	252,247
RNA 28	S5 Plus	187,176	145	185,591
	Genexus	1,903,859	98	126,388
RNA 29	S5 Plus	311,784	84	262,726
	Genexus	1,839,064	102	45,998
RNA 30	S5 Plus	416,422	93	393,110
	Genexus	1,727,113	101	57,972
RNA 31	S5 Plus	240,891	112	239,186
	Genexus	1,598,494	99	133,522
RNA 32	S5 Plus	156,106	63	97,917
	Genexus	1,965,363	93	52,222

Abbreviations: ID (Identifier); RNA (Ribonucleic Acid).

Of note, 10 out of 32 (31.2%) patients highlighted aberrant transcripts by using the Genexus platform. Among them, 5 out of 10 and 2 out of 10 patients showed *ALK* and *RET* rearrangements, respectively. Moreover, three patients were positive for *ROS1*, *NTRK*

aberrant transcripts and MET Δ 14 skipping mutations, respectively (Table 7). Interestingly, rearranged genes were identified by OPA on the Genexus platform in 9 out of 10 (90.0%) retrieved cases, showing a concordance rate of 96.9% (31 out of 32 cases) with the SiRe panel in the S5 system. Particularly, ID#1 was positive for a *NTRK3–KANK1* fusion transcript not previously detected with the SiRe panel on the S5 platform. No significant variations were observed in accordance with histological groups, rearranged genes, fusion partners, and mapped read levels between Genexus and previously tested samples on the S5 platform.

Table 7. Comparison of RNA-related molecular alterations between S5 Plus and Genexus platforms.

ID	S5Plus (SiRe Fusion Panel)	Genexus (OPA Panel)
RNA 1	**No Fusion**	**NTRK3** (ex14)—KANK1 (ex3) 1571 reads *
RNA 2	**No Fusion**	**No Fusion**
RNA 3	**No Fusion**	**No Fusion**
RNA 4	**No Fusion**	**No Fusion**
RNA 5	**No Fusion**	**No Fusion**
RNA 6	**No Fusion**	**No Fusion**
RNA 7	**ALK** (ex20)—EML4 (ex6) 601 reads	**ALK** (ex20)—EML4 (ex6) 353 reads
RNA 8	**No Fusion**	**No Fusion**
RNA 9	**No Fusion**	**No Fusion**
RNA 10	**No Fusion**	**No Fusion**
RNA 11	**No Fusion**	**No Fusion**
RNA 12	**No Fusion**	**No Fusion**
RNA 13	**ALK** (ex20)—unknown partner 149 reads	**ALK** (ex20)—DCTN1 (ex26) 2268 reads
RNA 14	**No Fusion**	**No Fusion**
RNA 15	**No Fusion**	**No Fusion**
RNA 16	**No Fusion**	**No Fusion**
RNA 17	**No Fusion**	**No Fusion**
RNA 18	**No Fusion**	**No Fusion**
RNA 19	**ROS1** (ex34)—CD74 (ex6) 2208 reads	**ROS1** (ex34)—CD74 (ex6) 1992 reads
RNA 20	**ALK** (ex20)—EML4 (ex6) 43 reads	**ALK** (ex20)—EML4 (ex6) 1040 reads
RNA 21	**No Fusion**	**No Fusion**
RNA 22	**ALK** (ex20)—EML4 (ex13) 11,335 reads	**ALK** (ex20)—EML4 (ex13) 7212 reads
RNA 23	**No Fusion**	**No Fusion**
RNA 24	**RET** (ex12)—KIF5B (ex15) 4063 reads	**RET** (ex12)—KIF5B (ex15) 2417 reads
RNA 25	**MET** (ex13)—MET (ex15) 46,929 reads	**MET** (ex13)—MET (ex15) 9638 reads
RNA 26	**No Fusion**	**No Fusion**
RNA 27	**No Fusion**	**No Fusion**
RNA 28	**ALK** (ex20)—EML4 (ex20) 6293 reads	**ALK** (ex20)—EML4 (ex20) 1140 reads
RNA 29	**No Fusion**	**No Fusion**
RNA 30	**No Fusion**	**No Fusion**
RNA 31	**No Fusion**	**No Fusion**
RNA 32	**RET** (ex12)—CCDC6 (ex1) 494 reads	**RET** (ex12)—CCDC6 (ex1) 172 reads

* Not covered from SiRe Fusion Panel. Abbreviations: ALK (Anaplastic Lymphoma Kinase); CCDC6 (Coiled-Coil Domain-Containing Protein 6); CD74 (HLA Class II Histocompatibility Antigen Gamma Chain); DCTN1 (Dynactin Subunit 1); EML4 (Echinoderm Microtubule-Associated Protein-Like 4); EX (Exon); ID (Identifier); KANK1 (KN Motif And Ankyrin Repeat Domains 1); KIF5B (Kinesin Family Member 5B); MET (Tyrosine-Protein Kinase Met); NTRK (Neurotrophic Tyrosine Receptor Kinase); RET (RET Proto-Oncogene); RNA (Ribonucleic Acid); ROS1 (Proto-Oncogene Tyrosine-Protein Kinase ROS).

5. Discussion

In the era of personalized medicine, the rapidly increasing number of predictive biomarkers approved in clinical practice has revolutionized the treatment strategy for solid-tumor patients [1,2,9]. Although there is a widespread diffusion of single-gene testing platforms in the vast majority of laboratories involved in molecular tests, low multiplexing biomarker analysis discourages their implementation as pivotal diagnostic platforms in clinical practice [23,24]. As regards NGS techniques, they allow us to simultaneously cover clinically relevant molecular alterations from a plethora of diagnostic routine specimens, saving technical costs and maintaining adequate TAT [31]. Moreover, NGS platforms may also benefit from automatized technical procedures that allow for accurate and reproducible analysis, resulting in low bench-working time [31]. The Genexus system consists of a scalable, versatile, and fully automatized sequencer that is able to carry out each technical procedure without manual operations [32]. This system is built to integrate analytical procedures (nucleic acid extraction, library preparation, template generation, sequencing) with data analysis by adopting pre-customized pipeline analysis. Accordingly, automatized data analysis carried out by proprietary software supports healthcare professional figures involved in molecular testing. This approach allows us to save time by accurately interpreting molecular records, in comparison with semi-automatized procedures. As regards the NGS-based multiplexing strategy, it is considered a reliable technical approach that is able to decrease technical costs in molecular tests. Here, we have validated the Genexus system in our diagnostic routine by comparing its analytical performance in a retrospective series of clinical cases previously analyzed with a custom NGS panel in the S5 system. As expected, all diagnostic specimens (n = 64) were successfully analyzed by using this fully automatized system. Overall, a concordance rate of 96.9% (62 out of 64) was reached by adopting the Sire panel in the S5 system as the reference standard. Interestingly, molecular analysis was unmatched with previously archived data in only two cases (DNA-ID#19 and RNA-ID#1). Of note, sample DNA-ID#19 derived from a BC patient had a positive result for *PIK3CA* exon 9 p.E545K hotspot alteration in the Genexus system, with a mutant allele fraction (MAF) of 7.2%. Following the manufacturer's clinical cut-off (MAF $\geq$ 5%), previous analysis did not show any clinically relevant molecular alteration. By conducting a visual inspection of raw data, the same alteration at 0.9% was detected. This event may occur in residual scant samples where mutated alleles may encounter decreasing VAF levels [33]. Similarly, RNA-ID#1 showed *NTRK3* (ex14)—*KANK1* (ex3), an aberrant transcript not previously detected with the standard reference approach. In this case, *NTRK3* was not covered by reference range of the SiRe fusion panel.

In a non-negligible percentage of cases, synchronous lesions may be observed in CRC patients. In this scenario, NGS may be considered an affordable technical strategy to comprehensively conduct the molecular assessment of CRC patients where heterogeneous specimens are clinically available [28]. DNA-ID#11 and DNA-ID#2 represent synchronous lesions of a CRC elected to molecular testing. Interestingly, both S5 and Genexus systems revealed *KRAS* exon 2 p.G12C and *PIK3CA* exon 20 p.H1047R hotspot mutations, demonstrating a common origin of these lesions. Moreover, NGS systems overcome technical issues from the analysis of "complex" molecular alteration. Case DNA-ID#22 confirmed two concomitant *KRAS* exon 2 hotspot mutations (p.G13D+p.G13E) on the Genexus platform, previously detected by reference technology. Although this study provides encouraging results for the implementation of the Genexus system in the clinical routine setting of solid-tumor patients, some limitations may be identified. Firstly, this technical report aims to compare the analytical parameters of two NGS-based technologies using a series of diagnostic routine specimens without any clinical considerations. Secondly, this retrospective study is based on the analysis of a small group of cases retrieved from the internal archive of the University of Naples Federico II. All these crucial points warrant further analysis, but this preliminary data may suggest that a fully automatized Genexus system integrated with commercially available OPA (Thermo Fisher Scientific) represents a

technically affordable, time-saving sequencing platform that enables us to analyze clinically relevant molecular alterations in diagnostic routine specimens.

Author Contributions: Conceptualization, C.D.L., F.P., G.T. and U.M.; methodology, C.D.L., F.P., G.R., M.N., P.P., M.R., F.C., L.P., C.S., D.C., G.G., A.I., G.T. and U.M.; software, C.D.L., F.P., G.T. and U.M.; validation, C.D.L., F.P., G.R., M.N., P.P., M.R., F.C., L.P., C.S., D.C., G.G., A.I., G.T. and U.M.; formal analysis, C.D.L., F.P., G.R., M.N., P.P., M.R., F.C., L.P., C.S., D.C., G.G., A.I., G.T. and U.M.; data curation, C.D.L., F.P., G.T. and U.M.; writing—original draft preparation, C.D.L. and F.P.; writing—review and editing, G.T. and U.M.; visualization, C.D.L., F.P., G.R., M.N., P.P., M.R., F.C., L.P., C.S., D.C., G.G., A.I., G.T. and U.M.; supervision, G.T. and U.M.; project administration, G.T. and U.M. All authors have read and agreed to the published version of the manuscript.

Funding: 1. Monitoraggio ambientale, studio ed approfondimento della salute della popolazione residente in aree a rischio—In attuazione della D.G.R. Campanian.180/2019. 2. POR Campania FESR 2014–2020 Progetto "Sviluppo di Approcci Terapeutici Innovativi per patologie Neoplastiche resistenti ai trattamenti—SATIN". 3. This work has been partly supported by a grant from the Italian Health Ministry's research program (ID: NET-2016-02363853). National Center for Gene Therapy and Drugs based on RNA Technology MUR-CN3 CUP E63C22000940007 to DS.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Written informed consent has been obtained from the patient(s) to publish this paper.

Data Availability Statement: Not applicable.

Conflicts of Interest: Pasquale Pisapia has received personal fees as speaker bureau from Novartis for work performed outside of the curr ent study. Umberto Malapelle has received personal fees (as consultant and/or speaker bureau) from Boehringer Ingelheim, Roche, MSD, Amgen, Thermo Fisher Scientific, Eli Lilly, Diaceutics, GSK, Merck and AstraZeneca, Janssen, Diatech, Novartis and Hedera unrelated to the current work. Giancarlo Troncone reports personal fees (as speaker bureau or advisor) from Roche, MSD, Pfizer, Boehringer Ingelheim, Eli Lilly, BMS, GSK, Menarini, AstraZeneca, Amgen and Bayer, unrelated to the current work. The remaining authors declare no conflict of interest.

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