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Evaluation of the TruSight Tumor 170 Assay and Its Value in Clinical Diagnostics

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Abstract: Background: Parallel sequencing technologies have become integrated into clinical practice. This study evaluated the TruSight Tumor 170 assay for the simultaneous detection of somatic gene mutations (SNPs and indels), gene fusions and CNVs, and its implementation into routine diagnostics. Methods: Forty-four formalin-fixed, paraffin-embedded tissue samples analyzed previously with validated methods were evaluated with the TruSight Tumor 170 assay (Illumina). For data analysis the TruSight Tumor 170 app, the BaseSpace Variant Interpreter (Illumina), and the Molecular Health Guide Software (Molecular Health) were used. Results: All somatic gene mutations were identified when covered by the assay. Two high-level *MET* amplifications were detected by CNV analysis. Focal *MET* amplifications with a copy number below 10 were not reliably detected at the DNA-level. Twenty-one of 31 fusions and splice variants were confirmed with the assay on the RNA-level. The remaining eight aberrations were incorrect by previous methods. In two cases, no splicing was observed. Conclusions: The TruSight Tumor 170 gives reliable results even if low DNA and RNA concentrations are applied in comparison to other methods and can be used in a routine workflow to detect somatic gene mutations, gene fusions, and splice variants. However, we were not able to detect most focal gene amplifications/deletions.

Keywords: TruSight Tumor 170; NGS; FFPE; tumor

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1. Introduction

In the last decade, high-quality molecular analysis of formalin-fixed, paraffin-embedded (FFPE) tissue has become crucial for personalized treatment strategies in routine clinical practice [1]. Targeted parallel sequencing not only provides a high through-put, fast, and cost effective technology, but also offers a more comprehensive and accurate approach for genome wide analysis and the detection of somatic mutations [2,3]. Many institutions use amplicon-based parallel sequencing approaches for the detection of somatic gene mutations. With this method, target regions are enriched by multiplex PCR. One advantage of this method is that only low amounts of DNA are needed and further, that it is suitable for chemically modified and fragmented DNA from FFPE tissue [4,5]. Besides, amplicon-based approaches are time saving and cost effective for the detection of single nucleotide variants (SNVs), insertions, deletions (indels), or duplications on the DNA-level [6]. The detection of copy number variations (CNVs) is more problematic and the analysis of structural variants is not possible on the DNA-level [7]. Thus, amplicon-based panels cannot detect all relevant genomic alterations like SNVs, indels, CNV, or gene fusions in one assay. Another disadvantage of amplicon-based panels are the generation of artifacts. Artefacts can be due to fixation processes, polymerase errors in synthesis processes, or can emerge from random DNA mismatches. Additionally, PCR duplicate reads are present in these panels

since target enrichment is PCR-based. The poorer the DNA quality the higher the amount of PCR duplicates, leading to sequencing artefacts [8].

Fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) still represent standard technologies for detecting chromosomal aberrations such as copy number variations and gene fusions in routine clinical practice [9–11]. However, to utilize small biopsies more efficiently for genetically guided treatment decisions in the clinic, larger panels and new technologies are being developed to analyze all diagnostic and therapy relevant genes and gene aberrations in one assay.

In this study, we evaluated the TruSight Tumor 170 assay on FFPE tumor samples with a variety of known genetic aberrations for the simultaneous detection of somatic gene mutations (SNPs and indels), gene fusions, and CNVs and its implementation into routine diagnostics. All samples were previously analyzed with validated methods including amplicon-based sequencing, targeted RNAseq, FISH, and IHC. The TruSight Tumor 170 assay is a hybrid capture-based parallel sequencing approach for simultaneous DNA and RNA analysis. In this approach, biotinylated capture probes hybridize to target regions and are enriched by streptavidin magnetic beads for the detection of somatic gene mutations including single nucleotide variants (SNVs) and small insertions or deletions (indels) in 151 genes, gene fusions, and splice variants in 55 genes and copy number variations (CNVs) in 59 cancer-associated genes. For data analysis the TruSight Tumor 170 app, the BaseSpace Variant Interpreter (Illumina, San Diego, CA, USA) and the Molecular Health Guide Software (Molecular Health, Heidelberg, Germany) were used.

2. Materials and Methods

2.1. Samples

A collection of 44 samples was analyzed. The 42 formalin-fixed and paraffin embedded (FFPE) tumor samples with known genetic aberrations including somatic gene mutations (SNPs and indels), CNVs, gene fusions, and splice variants and two FFPE control samples (Quantitative Multiplex Reference Standard (FFPE) from Horizon Discovery, Cambridge, United Kingdom were included in the cohort. All samples were prepared routinely as FFPE according to local practice. The FFPE tumor samples were selected from the registry of the Institute of Pathology of the University Hospital Cologne, Germany. FFPE tissue samples were obtained as part of routine clinical care under approved ethical protocols complied with the Ethics Committee of the Medical Faculty of the University of Cologne, Germany. The study was approved by the same Ethics Committee (Ethics-No. 13-091, BioMaSOTA, approval in May 2016) and written informed consent was obtained from all patients before enrollment into the study. SNVs, indels, and small duplications were previously detected with a customized GeneRead DNAseq Targeted Panel V2 and the GeneRead DNAseq Panel PCR Kit V2 (Qiagen) or an Ion AmpliSeq Custom DNA Panel (Thermo Fisher Scientific, Waltham, MA, USA) and the Ion AmpliSeq Library Kit 2.0 (ThermoFisher Scientific) as previously described [12]. *MET* amplifications as well as fusions in *ROS1*, *BRAF*, *NTRK1*, *ALK*, *FGFR2*, *PAK3*, and *RET* were either detected by FISH (ZytoVision, Bremerhaven, Germany) or with the FusionPlex Lung Panel (Invitae, San Francisco, CA, USA) following manufactures instructions. The *EGFR* exon 1–8 deletion was detected with the FusionPlex Lung Panel (Invitae). The *BRCA1* exon 17 deletion was previously detected by Multiplex Ligation-dependent Probe Amplification (MLPA) from MRC Holland (Amsterdam, The Netherlands).

2.2. Nucleic Acid Extraction

Sections were cut to 10-µm thick from FFPE tissue blocks for DNA and RNA extraction. To distinguish tumor from normal tissue a senior pathologist marked the tumor area on a hematoxylin–eosin (H&E) stained slide. Depending on the size of the biopsy one of the following extraction systems was used:

For samples with larger tumor areas the Maxwell 16 (Promega, Fitchburg, WI, USA) was used for DNA extraction and the Maxwell RSC (Promega) was used for RNA extrac-

tion. For DNA extraction, the tumor areas were macrodissected after deparaffinized from unstained slides using the marked hematoxylin–eosin (H&E) stained slide as a reference and processed with the Maxwell 16 FFPE Plus Tissue LEV DNA Purification Kit (Promega). For RNA extraction, the Maxwell RSC RNA FFPE Kit (Promega) was used following the manufacturer's protocol.

For samples with small tumor areas the truXTRAC FFPE total Nucleic Acid Kit (Covaris, Woburn, MA, USA) was used according to the manufacturer's instructions.

DNA and RNA concentrations were measured with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific) using the Qubit dsDNA HS Assay Kit or Qubit RNA HS Assay Kit (Thermo Fisher Scientific), respectively. For quality control of the DNA samples, the Illumina FFPE QC Kit for TruSeq Amplicon (Illumina, San Diego, CA, USA) was used. For quality control of RNA samples, the DV200 value was measured using the Fragment Analyzer (Agilent, Santa Clara, CA, USA) with the DNF-472 High Sensitivity RNA Kit (15 nt) (Agilent).

2.3. TruSight Tumor 170 Assay

A mass of 9–120 ng of DNA was sheared on the Covaris E220 Focused-ultrasonicator to a fragment size of 150 bp using the 8 microTUBE–50 Strip AFA Fiber V2 (Covaris) following the manufacturer's instructions. The treatment time was optimized for FFPE material. The treatment settings were the following: Peak Incident Power (W): 75; Duty Factor: 15%; Cycles per Burst: 500; Treatment Time(s): 360; Temperature (°C): 7; Water Level: 6. A mass of 1–85 ng of RNA was used. For DNA and RNA library preparation and enrichment the TruSight Tumor 170 assay (Illumina) was used following manufacturer's instructions. Post-enriched libraries were quantified, pooled, and sequenced on a NextSeq 500 (Illumina).

The quality of the NextSeq 500 (Illumina) sequencing runs were assessed with the Illumina Sequencing Analysis Viewer (Illumina). Sequencing data was analyzed with BaseSpace TruSight Tumor 170 app Version 1.0.2. and BaseSpace Variant Interpreter (Illumina). For all DNA samples, the Median Insert Size ($\geq$79 bp), the percentage of exonic bases that have an equal or greater coverage than 100X (PCT Exon Bases 100X ($\geq$0.95)), the median of absolute deviation from the median of the normalized count of each CNV target (Coverage MAD ($\leq$0.2)) and the median of the raw read count of each CNV target region (BinCount CNV Targets ($\geq$1)) were evaluated according to the TruSight Tumor 170 v1.0.2 Local App User Guide (Illumina). For all RNA samples, the Median Insert Size ($\geq$63 bp), the median of the CV value of target region coverage across all genes with coverage above 1000 (Median CV Coverage 1000X) and the percentage of reads that map to target regions out of total passing filter reads (PCT On Target Reads) were noted according to the TruSight Tumor 170 v1.0.2 Local App User Guide (Illumina). The total mean coverage and the mean coverage of *MET* were calculated with GATK 3.8 DepthOfCoverage (Broad Institute, Cambridge, MA, USA).

Additionally, the Variant Call Format (VCF) files were analyzed with the Molecular Health Guide Software Version 5.0.2 (Molecular Health, Heidelberg, Germany) for variant filtering and annotation.

3. Results

In this study, a collection of 44 samples was analyzed with the TruSight Tumor 170 assay, 42 FFPE samples of different tumor types with known genetic aberrations including somatic gene mutations (SNVs and indels), gene fusions, splice variants, and CNVs and two control samples. DNA and RNA were extracted successfully. DNA concentrations varied between 9–120 ng per sample. Forty-three of 44 DNA libraries were analyzable (Table 1). For sample 9, no sequencing reads were obtained even though the final library concentration was sufficient and all quality criteria were passed. All analyzable samples passed the quality thresholds of Median Insert Size ($\geq$79 bp) and the percentage of exonic bases that have an equal or greater coverage than 100X (PCT Exon Bases 100X ($\geq$0.95)), which give a higher confidence in small variant calling [13]. Only sample 28 was below the

threshold of the PCT Exon Bases 100X with a value of 70.7. The passed quality thresholds of the median of absolute deviation from the median of the normalized count of each CNV target (Coverage MAD ($\leq$0.2)) and the median of the raw read count of each CNV target region (BinCount CNV Targets ($\geq$1)) lead to a higher confidence in copy number variant calling. In our study, all samples passed these quality settings (Table 1).

Table 1. Results and quality scores of the 44 DNA samples analyzed with the TruSight Tumor 170 assay.

No.	Tumor Type	TCC (%)	DNA Input (ng)	Median Insert Size ($\geq$79 bp)	PCT Exon Bases 100X ($\geq$95)	Cov. MAD [($\leq$0.2)]	Bin Count CNV Targets ($\geq$1)	Expected Somatic Gene Mutations	Expected CNVs	Results DNA Illumina
1	NSCLC, Squamous	40	120	130	99.72	0.12	32.14	*KEAP1*: c.743C>G p.A248G; *PIK3CA*: c.1633G>A p.E545K; *TP53*: c.892G>T p.E298*		√ (*KEAP1* not in panel)
2	NSCLC, Adeno	50	120	125	99.73	0.09	23.6	*KEAP1*: c.1576G>C p.D526H; *KRAS*: c.35G>T p.G12V; *TP53*: c.475G>C p.A159P		√ (*KEAP1* not in panel)
3	NSCLC, Adeno	60	120	89	99.76	0.1	8.35	*TP53*: c.848G>C p.R283P	*MET* amplification (4.87 Copies)	(√) (*MET* amplification not detected)
4	NSCLC, Adeno	50	95.5	127	99.77	0.09	19.42	*TP53*: c.713G>A p.C238Y		√
5	NSCLC, Adeno	80	120	107	99.74	0.11	14.84	n/a		n/a
6	NSCLC, Adeno	40	117	124	99.77	0.06	22.61	n/a		n/a
7	NSCLC, Adeno	50	120	125	99.75	0.08	26.92	n/a		n/a
8	NSCLC, Adeno	20	120	134	99.64	0.08	24.6	*TP53*: c.614A>G p.Y205C; *MET*: c.[2942-20_2942-7del]; [2942-14_2942-4del]	*MET* amplification (6.87 Copies)	(√) (*MET* amplification not detected, MET deletion not detected on DNA-level)
9	NSCLC, Adeno	60	120	125	0	0	0	*MET*: c.3082_3082+23del	*MET* amplification (4.77 Copies)	n.n.
10	NSCLC, Adeno	80	120	119	99.66	0.16	19.39	*EGFR*: c.2309_2310ins23 p.D770Efs*4; *KEAP1*: c.340G>T p.G114W; *PTEN*: c.640C>T p.Q214*; *TP53*: c.746G>T p.R249M	*MET* amplification (Copies 3.20)	(√) (*KEAP1* not in panel, *MET* amplification not detected)
11	NSCLC, Adeno	50	120	138	99.61	0.08	25.37	*EGFR*: c.2235_2249del p.K746_A750del; *EGFR*: c.2369C>T p.T790M; *TP53*: c.661G>T p.E221*	*MET* amplification (Copies 4.47)	(√) (*MET* amplification not detected)
12	NSCLC, Adeno	50	55.9	126	99.32	0.19	9.1	*BRAF*: c.1780G>A p.D594N; *EGFR*: c.2300_2308dup p.A767_V769dup	*MET* amplification (Copies 3.85)	(√) (*MET* amplification not detected)
13	Chordoma	80	120	125	99.73	0.08	22.03	n/a		n/a
14	NSCLC, Adeno	70	90.2	131	99.69	0.13	17.84	n/a		n/a

Table 1. *Cont.*

No.	Tumor Type	TCC (%)	DNA Input (ng)	Median Insert Size (≥79 bp)	PCT Exon Bases 100X (≥95)	Cov. MAD [(≤0.2)]	Bin Count CNV Targets (≥1)	Expected Somatic Gene Mutations	Expected CNVs	Results DNA Illumina
15	NSCLC, Adeno	40	52.8	101	99.25	0.19	3.91	n/a		n/a
16	Pancreas	70	120	132	99.78	0.16	19.29	*ATM*: c.2494C>T p.R832C		√
17	NSCLC, Adeno	15	8.6	115	95.55	0.13	1.47	n/a	*MET* amplification (3.85 Copies)	X *MET* amplification not detected on DNA-level
18	NSCLC, Adeno	40	43.4	116	99.22	0.07	6.45	*TP53*: c.473G>T p.R158L	*MET* amplification (9.23 Copies)	(√) (*MET* amplification not detected)
19	NSCLC, Adeno	70	120	127	99.77	0.11	29.91	*DDR2*: c.1189A>G p.N397D; *KRAS*: c.35G>T p.G12V; *TP53*: c.722C>A p.S241Y	*MET* amplification (7.50 Copies)	√
20	Pancreas	50	120	121	99.78	0.09	20.04	*BRCA2*: c.10095delCinsGAATTATAT p.S3366Nfs*4		√
21	Ovary	70	90	115	99.75	0.1	19.37	*BRCA2*: c.3975_3978dup p.A1327Cfs*4, BRCA2: c.682-9_682-3delinsTTTTGG		X *BRCA2* deletion not detected on DNA-level
22	NSCLC, Adeno	90	120	101	76.85	0.14	1.14	*MET*: c.2942-19_2942-9del		X *MET* deletion not detected on DNA-level
23	NSCLC, Adeno	50	120	129	98.74	0.08	5.15	*TP53*: c.637C>T p.R213*; *MET*: c.2942-27_2942-5del		(√) (*MET* deletion not detected on DNA-level)
24	NSCLC, Adeno	50	120	137	99.25	0.1	6.98	*MET*: c.2942-28_2942-2del		X *MET* deletion not detected on DNA-level
25	NSCLC, Adeno	70	120	131	99.32	0.1	6.37	*MET*: c.3070_3082+22del		√
26	NSCLC, Adeno	20	120	123	97.6	0.07	3.25	*MET*: c.3073_3082+21del		√
27	NSCLC, Adeno	25	120	96	99.43	0.11	4.58	*PIK3CA*: c.3145G>C p.G1049R; *MET* c.3076_3082+4del		√
28	Rhabdomyosarcoma	25	120	110	70.7	0.16	1.08	n/a		n/a
29	Cholangiocellular Carcinoma	50	120	113	99.32	0.14	6.78	n/a		n/a
30	NSCLC, Adeno	15	120	150	99.57	0.1	12.3	*MET*: c.3334C>T p.H1112Y		√
31	NSCLC, Adeno	30	120	148	99.58	0.12	13.48	*DDR2*: c.2321G>T p.G774V; *TP53*: c.818G>A p.R273H	*MET* amplification (11.72 copies)	√
32	NSCLC, Adeno	70	120	123	99.67	0.1	11.74	*KRAS*: c.182A>T p.Q61L		√

Table 1. *Cont.*

No.	Tumor Type	TCC (%)	DNA Input (ng)	Median Insert Size (≥79 bp)	PCT Exon Bases 100X (≥95)	Cov. MAD [(≤0.2)]	Bin Count CNV Targets (≥1)	Expected Somatic Gene Mutations	Expected CNVs	Results DNA Illumina
33	Glioblastoma	70	120	136	99.61	0.13	14.04	n/a		n/a
34	Thyroid	50	120	139	99.62	0.09	12.35	n/a		n/a
35	Melanoma	80	120	144	99.6	0.14	11.83	*PTEN*: c.112C>T p.P38S		√
36	Melanoma	50	52.8	121	96.99	0.09	3.43	n/a		n/a
37	Melanoma	60	120	114	98.83	0.11	4.95	n/a		n/a
38	Melanoma	40	44	123	99.09	0.1	6.21	n/a		n/a
39	Breast	90	120	115	99.52	0.12	8.91	n/a		n/a
40	Ovary	50	120	120	99.39	0.2	8.11	n/a		n/a
41	NSCLC, Adeno	60	120	121	99.48	0.2	9.11	*ROS1*: c.5858G>T p.S1953I; *TP53*: c.463_468delACCCGC p.T155_R156del	*MET* amplification (3.25 Copies)	(√) (*MET* amplification not detected on DNA-level)
42	NSCLC, Adeno	70	120	130	98.79	0.19	7.35	n/a		n/a
43	Control sample 1	-	120	138	99.74	0.13	25.49	See Table 2		√
44	Control sample 2	-	120	133	99.72	0.13	25.97	See Table 2		√

NSCLC: Non-small cell lung cancer; Cov.: Coverage; n/a: Not applicable; n.n.: Not analyzable; TCC: Tumor cell content; Green: Correct; Orange: Partially correct; Red: Not detected.

Table 2. Results of the two control samples. The *EGFR* p.T790M mutation in control sample 1 was only observed in the bam file by the IGV.

Gene	Variant	Expected Allelic Frequency (%)	Control Sample 1		Control Sample 2	
			Allelic Frequency (%)	Coverage	Allelic Frequency (%)	Coverage
BRAF	p.V600E	10.7	9.84	2013	10.88	2113
cKIT	p.D816V	10.0	18.50	1135	23.19	1186
EGFR	p.E746-A750del	1.9	1.88	5783	1.43	5678
EGFR	p.L858R	2.8	3.17	6243	3.48	6476
EGFR	p.T790M	0.9	1.04 (IGV)	6699 (IGV)	1.46	6316
EGFR	p.G719S	24.5	24.08	5354	22.67	5523
KRAS	p.G13D	15.0	14.88	1526	16.16	1547
KRAS	p.G12D	6.3	6.52	1502	7.41	1555
NRAS	p.Q61K	12.5	15.53	1951	13.98	1940
PIK3CA	p.H1047R	17.5	19.05	1454	16.27	1352
PIK3CA	p.E545K	8.8	24.65	706	22.00	710

%: Percentage; IGV: Integrative Genomics Viewer.

On the DNA-level, the 42 FFPE tumor samples had 43 previously known mutations including SNPs and small insertions/deletions/duplications in a variety of genes (Table 1). Thirty-three of these mutations were detected in the DNA sequencing data. Three *KEAP1* mutations could not be confirmed as *KEAP1* is not covered by the TruSight Tumor 170 assay. Five of the eight *MET* deletions resulting in exon 14 skipping and one *BRCA2* deletion were not detected on the DNA-level as these mutations were intronic deletions. Three *MET* exon 14 skipping deletions that were crossing the exon/intron border were called on the DNA- and RNA-level (Figure 1).

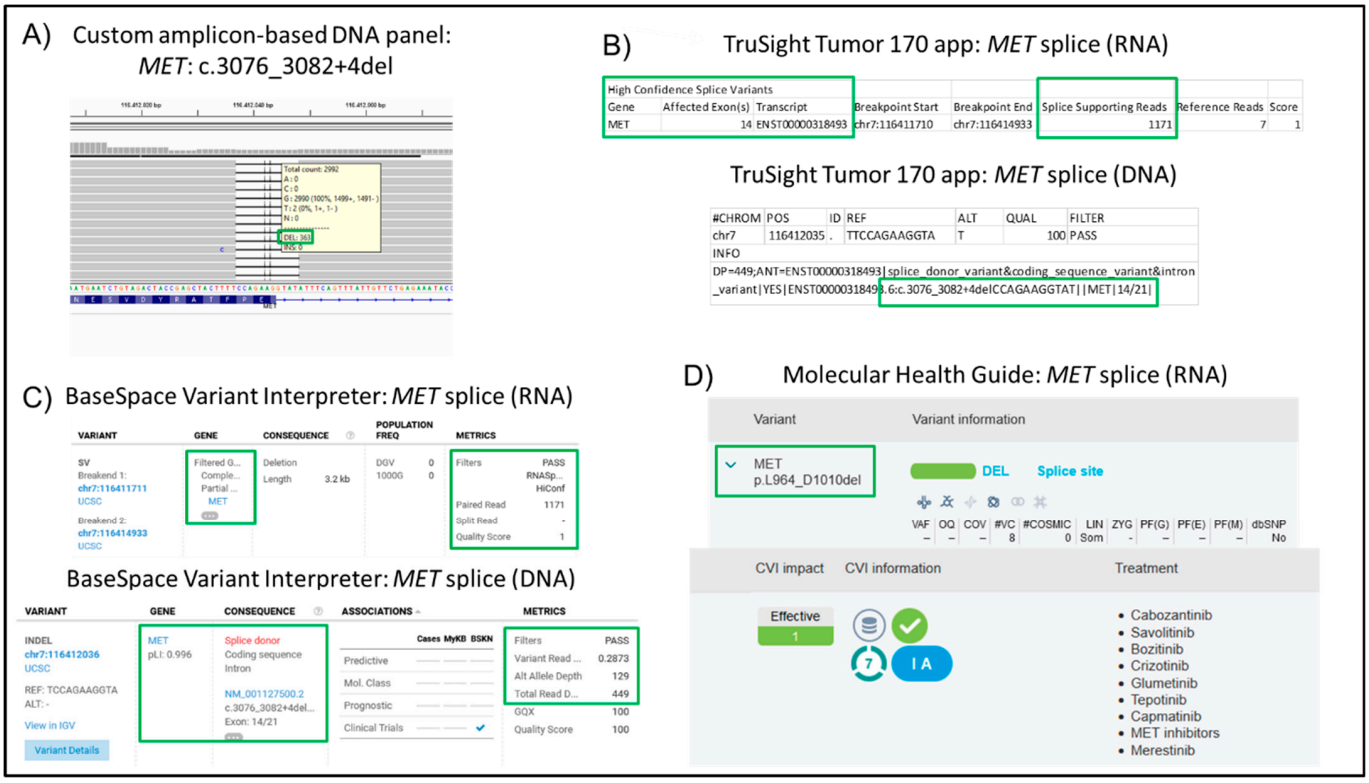

Figure 1. Sample 27 with *MET* exon 14 skipping mutation. Sequencing was performed with a custom amplicon-based DNA panel and the TruSight Tumor 170 assay. The custom amplicon-based DNA panel was analyzed with an in-house developed pipeline as previously described [6] and visualized with the Integrative Genomics Viewer (Broad Institute of MIT and Harvard, Cambridge, MA, USA) (**A**). The TruSight Tumor 170 assay data was evaluated with the TruSight Tumor 170 app (**B**) on BaseSpace Sequence Hub and the BaseSpace Variant Interpreter (**C**) as well as the Molecular Health Guide Software (**D**). The mutation and the quality criteria are highlighted with a green rectangle for each software.

The Quantitative Multiplex Reference Standard (FFPE) was tested twice as the control sample. In both samples, all 12 mutations covering the genes *BRAF*, *KIT*, *EGFR*, *KRAS*, *NRAS*, and *PIK3CA* with expected allelic frequencies from 0.9–24.5% were detected with the TruSight Tumor 170 assay (Table 2). However, the *EGFR* mutation p.T790M with an expected allelic frequency of 0.9% was only called in one of the samples with the TruSight Tumor 170 app and was visible in the BaseSpace Variant Interpreter (Illumina) and the Molecular Health Guide Software (Molecular Health). In the other sample, the mutation was not called by the software and was only present in the bam file and visible in the Integrative Genomics Viewer (IGV) (Broad Institute of MIT and Harvard, Cambridge, MA, USA) with an allelic fraction of 1.04%.

Of the 11 previously known *MET* amplifications determined by FISH, only two high-level *MET* amplifications were called by CNV analysis (Tables 1 and 3, Figure 2). Sample 31 had a copy number of 11.7 and a ratio of *MET* signals divided by centromer7 signals (*MET*/CEN7 ratio) of 3.46 passing both thresholds for high-level *MET* amplification. Sample 19 was determined high-level *MET* amplified by a copy number of 7.5. The *MET*/CEN7 ratio was below the threshold for high-level *MET* amplification in this sample. Seven low-level as well as two high-level *MET* amplifications were not detected. One of the low-level samples (sample 9) was lost during bead normalization of the library and no reads were called for this sample. The two high-level *MET* amplified samples (sample 8 and 18) were positive by MET/CEN7, had copy number scores between 6 and 10 and were still not detected by CNV analysis with the TruSight Tumor 170 assay (Tables 1 and 3).

In Table 3, the total mean coverage and the mean coverage of *MET* of the 11 samples with previously known *MET* amplifications determined by FISH are shown as well as two samples without *MET* amplification. In samples 19 and 31, the *MET* amplification was detected, here an increase in the mean coverage of *MET* in comparison to the total mean coverage can be seen.

Table 3. Total mean coverage and mean coverage of *MET* of the 11 *MET* amplified samples as well as the two samples without *MET* amplification.

No.	*MET* Amplification Status	*MET* Amplification Detected by TruSight Tumor 170 Assay	Total Mean Coverage	Mean Coverage of *MET*
3	*MET* amplification (4.87 Copies; Ratio 1.87; low-level)	no	1048.70	1615.26
4	no *MET* amplification	no	2297.26	2637.08
5	no *MET* amplification	no	1767.57	2006.12
8	*MET* amplification (6.87 Copies; Ratio 3.30; high-level)	no	2877.43	3911.24
9	*MET* amplification (4.77 Copies; Ratio 1.4; low-level)	no	0	0
10	*MET* amplification (Copies 3.20; Ratio 1.28; low-level)	no	2340.00	3558.57
11	*MET* amplification (Copies 4.47; Ratio 1.35; low-level)	no	2935.49	3621.15
12	*MET* amplification (Copies 3.85; Ratio 1.17; low-level)	no	1186.01	1932.84
17	*MET* amplification (3.85 Copies; Ratio 1.04; low-level)	no	418.03	436.77
18	*MET* amplification (9.23 Copies; Ratio 3.28; high-level)	no	846.52	1281.82
19	*MET* amplification (7.50 Copies; Ratio 1.55; high-level)	yes	3639.3	7673.86
31	*MET* amplification (11.72 copies; Ratio 3.46; high-level)	yes	1815.54	5022.72
41	*MET* amplification (3.25 Copies; Ratio 1.12; low-level)	no	1357.40	1716.84

RNA concentrations of the 44 analyzed samples varied between 1 and 85 ng. All 44 RNA libraries were analyzable (Table 4) and passed the quality thresholds of Median Insert Size ($\geq$63 bp) and the median of the CV value of target region coverage across all genes with coverage above 1000 (Median CV Coverage 1000X) ($\leq$88), which gives a higher confidence in fusion calling [13] (Table 4).

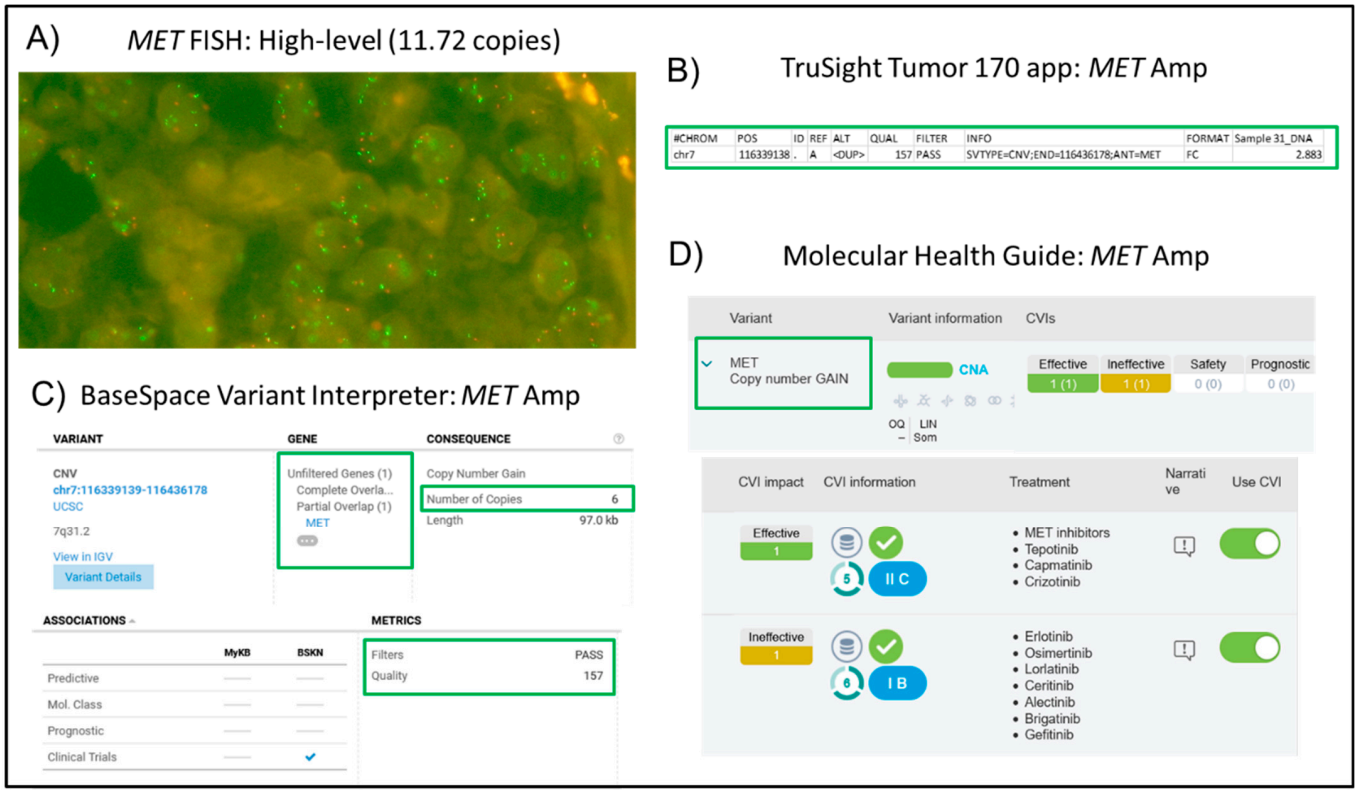

Figure 2. Sample 31 with a *MET* amplification determined by *MET* FISH (**A**), sequenced with the Table 170. app (**B**) on BaseSpace Sequence Hub and the BaseSpace Variant Interpreter (**C**) as well as the Molecular Health Guide Software (**D**). The amplification and the quality criteria are highlighted with a green rectangle for each software.

Table 4. Results and quality scores of the 44 RNA samples analyzed with the TruSight Tumor 170 assay.

No.	Tumor Type	TCC (%)	RNA Input (ng)	Median Insert Size (≥63 bp)	Median CV Coverage 1000X (≤0.88)	PCR On Target Reads	Expected Variants RNA	Results RNA Illumina
1	NSCLC, Squamous	40	85	127	0.53	81.89	n/a	n/a
2	NSCLC, Adeno	30	85	113	0.55	82.62	n/a	n/a
3	NSCLC, Adeno	50	85	78	0.74	91.25	n/a	n/a
4	NSCLC, Adeno	50	25.5	96	0.71	83.31	*ROS1* translocation	√
5	NSCLC, Adeno	80	85	81	0.63	83.79	*ROS1* translocation	√ (*ROS1* FISH false positive)
6	NSCLC, Adeno	10	27.2	98	0.58	80.05	*ROS1* translocation	√
7	NSCLC, Adeno	50	85	125	0.49	87.98	*ROS1* translocation (*WNK1-ROS1*)	√
8	NSCLC, Adeno	20	7.1	113	0.5	81.91	*MET*: c.[2942-20_2942-7del];[2942-14_2942-4del]	√
9	NSCLC, Adeno	60	85	103	0.6	80.17	*MET*: c.3082_3082+23del	√
10	NSCLC, Adeno	80	85	111	0.56	88.61	n/a	n/a
11	NSCLC, Adeno	15	12.8	114	0.61	81.44	n/a	n/a
12	NSCLC, Adeno	30	29.8	117	0.57	83.23	*ROS1* translocation	√ (*ROS1* FISH false positive)

Table 4. *Cont.*

No.	Tumor Type	TCC (%)	RNA Input (ng)	Median Insert Size (≥63 bp)	Median CV Coverage 1000X (≤0.88)	PCR On Target Reads	Expected Variants RNA	Results RNA Illumina
13	Chordoma	80	85	116	0.54	83.66	*BRAF* translocation (*KIAA1549-BRAF*)	√
14	NSCLC, Adeno	30	5.8	113	0.53	82.19	*NTRK1* translocation (*EPS15L1-NTRK1*)	√
15	NSCLC, Adeno	40	5	89	0.53	78.81	*ALK* translocation (*EML4-ALK*)	√
16	Pancreas	70	85	132	0.5	89.05	*FGFR2* translocation (*FGFR2-KIAA1598*)	√
17	NSCLC, Adeno	15	1.3	112	0.5	80.8	*ROS1* translocation (*SLC34A2-ROS1*)	√ (RNAseq false positive for *ROS1*)
18	NSCLC, Adeno	10	0.8	97	0.64	76.71	n/a	n/a
19	NSCLC, Adeno	70	85	121	0.5	84.4	n/a	n/a
20	Pancreas	30	85	96	0.61	80.51	n/a	n/a
21	Ovary	60	85	116	0.54	85.33	*BRCA2*: c.682-9_682-3delinsTTTTGG	X No splicing effect detected
22	NSCLC, Adeno	90	85	97	0.68	82.55	*MET*: c.2942-19_2942-9del	√
23	NSCLC, Adeno	40	85	109	0.66	83.22	*MET*: c.2942-27_2942-5del	√
24	NSCLC, Adeno	50	85	107	0.64	88.87	*MET*: c.2942-28_2942-2del	√
25	NSCLC, Adeno	70	85	100	0.66	87.07	*MET*: c.3070_3082+22del	√
26	NSCLC, Adeno	20	85	98	0.67	88.17	*MET*: c.3073_3082+21del	√
27	NSCLC, Adeno	25	85	96	0.69	84.72	*MET* c.3076_3082+4del	√
28	Rhabdomyosarcoma	25	85	107	0.68	89.44	*PAK3* translocation (*PAK3-FOXO1*)	√
29	Cholangiocellular Carcinoma	40	85	150	0.58	86.69	*FGFR2* translocation	√
30	NSCLC, Adeno	15	37.4	111	0.78	89.81	n/a	n/a
31	NSCLC, Adeno	30	85	142	0.59	93.5	n/a	n/a
32	NSCLC, Adeno	70	85	113	0.62	84.39	n/a	n/a
33	Glioblastoma	70	85	135	0.55	90.73	*EGFR* (Exon 1)–*EGFR* (Exon 8) deletion	√
34	Thyroid	50	85	146	0.53	91.06	*RET* translocation (*NCOA4*-RET)	√
35	Melanoma	70	85	150	0.58	86.69	*BRAF* translocation (*NRF1-BRAF*)	√
36	Melanoma	50	85	141	0.64	87.23	*BRAF* translocation	√ (RNAseq false positive for *BRAF*)
37	Melanoma	70	76.5	131	0.55	82.26	*BRAF* translocation	√ (RNAseq false positive for *BRAF*)
38	Melanoma	10	8.5	116	0.68	76.48	*BRAF* translocation	√ (RNAseq false positive for *BRAF*)
39	Breast	90	67.7	104	0.62	73.52	*BRCA1* (Exon 17) deletion	X Deletion not detected
40	Ovary	50	85	135	0.55	82.91	ALK immuno+, *ALK*-FISH negative	√ (ALK IHC false positive)
41	NSCLC, Adeno	60	85	123	0.51	80.1	*ROS1* translocation	√ (*ROS1* FISH false positive)
42	NSCLC, Adeno	70	85	134	0.6	82.54	*ROS1* translocation	√
43	Control sample 1	-	85	153	0.43	89.09	n/a	n/a
44	Control sample 2	-	85	136	0.49	87.93	n/a	n/a

NSCLC: Non-small cell lung cancer; n/a: Not applicable; TCC: Tumor cell content; Green: Correct; Red: Not detected.

On the RNA-level the 42 different FFPE samples had 31 fusions and splice variants. Only 21 of these were confirmed with the TruSight Tumor 170 assay (Table 4, Figure 3). All *MET* exon 14 skipping mutations were detected on the RNA-level, including those that

were not called on the DNA-level. The large *EGFR* exon 1–exon 8 deletion was identified on the RNA-level. This deletion was not called by CNV analysis on the DNA-level. Of the ten samples that were previously incorrectly analyzed, eight were fusion positive. After further evaluation by ROS1 IHC and other methods, three *ROS1* fusions of the remaining aberrations were false positive by *ROS1* FISH, these samples had isolated 3′ extra green signals in the *ROS1* FISH. This was confirmed with ROS1 IHC. Three *BRAF* and one *ROS1* fusions were false positive by RNAseq with the FusionPlex Lung panel from Invitae (San Francisco, CA, USA). They showed low fusion supporting reads in the results with the FusionPlex Lung panel. This was also confirmed with *BRAF* and *ROS1* FISH. One *ALK* fusion was false positive by ALK IHC and could not be confirmed by *ALK* FISH and the TruSight Tumor 170 assay. The other two incorrect samples were samples 21 and 39. The known *BRCA2* intronic deletion in sample 21 did not lead to a splicing effect on the RNA-level in this analysis. This variant was classified on the DNA-level as a likely pathogenic variant in the splice site of *BRCA2* exon 9. The *BRCA1* exon 17 deletion was also not visible on the RNA-level. Multiplex Ligation-dependent Probe Amplification (MLPA) on the DNA-level previously classified this variant. On the DNA-level with the TruSight Tumor 170 assay this variant was also not detected.

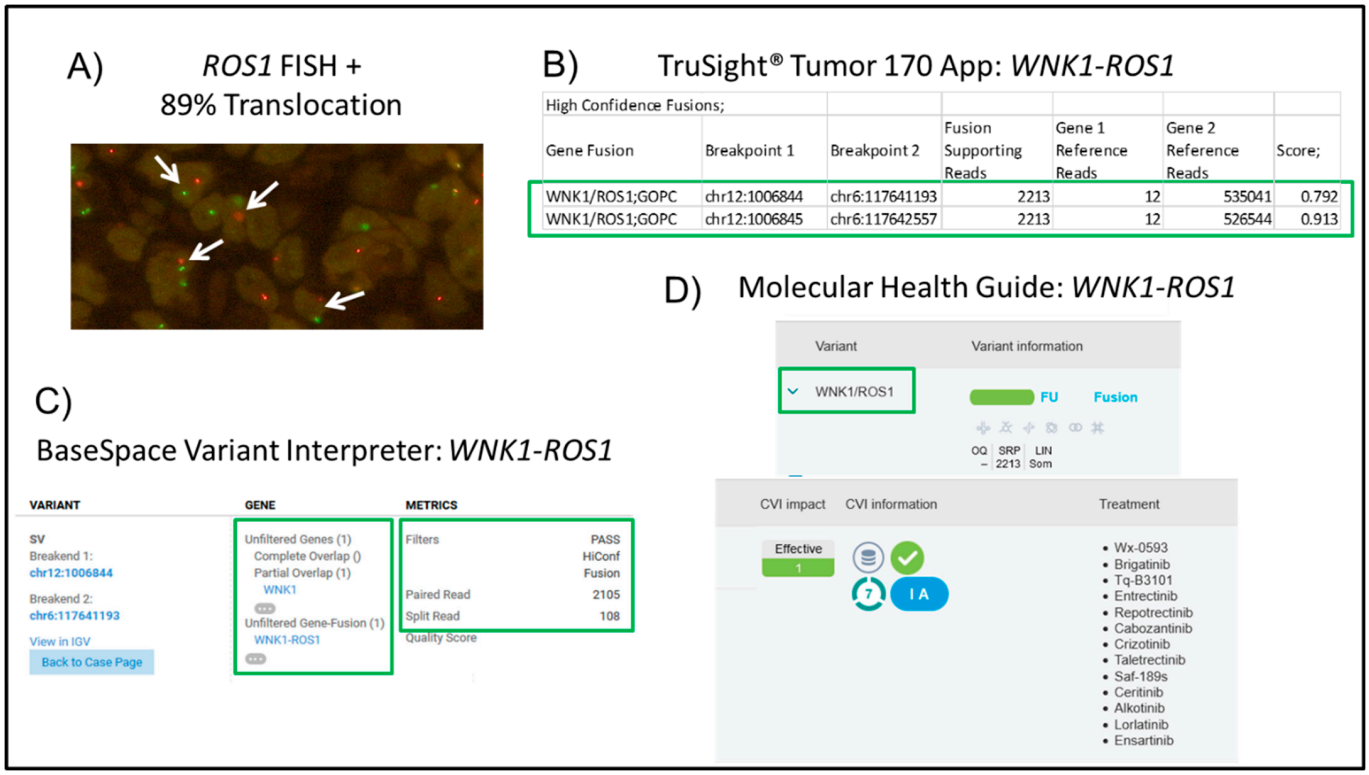

Figure 3. Sample 7 with a *ROS1* translocation detected by *ROS1* FISH (**A**), sequenced with the TruSight Tumor assay and analyzed with the TruSight Tumor 170 app (**B**) on BaseSpace Sequence Hub and the BaseSpace Variant Interpreter (**C**) as well as the Molecular Health Guide Software (**D**). The amplification and the quality criteria are highlighted with a green rectangle for each software.

In all of the samples, RNA concentration was sufficient to perform fusion detection and no fusion was missed.

4. Discussion

Parallel sequencing methods are increasingly used in clinical routine diagnostics for the simultaneous investigation of multiple genes. Additionally, the amount of tissue as well as the time required to complete diagnostic tests become more and more limited. Thus,

larger panels, enabling the simultaneous analysis of all diagnostic and therapy relevant genes and gene aberrations in one single assay are needed.

In this study, we analyzed a cohort of 44 FFPE samples with the TruSight Tumor 170 assay for its ability to reliably detect a variety of known genetic aberrations including somatic gene mutations (SNVs and indels), gene fusions, splice variants, and CNVs. The TruSight Tumor 170 assay worked well even with low DNA and RNA concentrations of challenging FFPE samples especially for the detection of somatic gene mutations, gene fusions, and splice variants.

Thirty-four of the 43 previously known somatic gene mutations were detected. The missed mutations were either not covered by the TruSight Tumor 170 assay or were intronic deletions that were not detected on the DNA-level. A major limitation of the TruSight Tumor 170 assay is that on the DNA-level no mutations are called in the intronic splice site regions of the targeted genes. The regions are covered by capture probe, but the variant caller of the TruSight Tumor 170 app filters out the intronic mutations. Therefore, important mutations might be missed if they are not visible on the RNA-level and also the nomenclature of the exact mutation cannot be made on the RNA-level. Thus, these mutations were also not present in the BaseSpace Variant Interpreter app and the Molecular Health Guide software on DNA-level as both programs used the VCF-file. Ideally, the TruSight Tumor 170 app should be adjusted for this, as also stated in a previous study [14] and the company should state more clearly what the assay can and cannot detect. Therefore, only on the RNA-level splicing effects of the intronic *MET* deletions could be observed without classification of the exact mutation. Deletions, however, that were crossing the exon/intron border were called on the DNA-level.

The Quantitative Multiplex Reference Standard (FFPE) was tested twice as the control sample. In both samples, all 12 mutations were detected. However, in one control sample the *EGFR* mutation p.T790M was only visible in the IGV with an allelic fraction of 1.04% and not called by the TruSight Tumor app, as filter criteria were not passed. Considering the results, we could detect all variants down to 1.43% allelic fraction, however, these results have to be confirmed in a larger cohort in the future. A previous study showed in a larger cohort of 234 samples that the assay could detect somatic mutations in FFPE samples with a 5% mutant allele frequency with a sensitivity and specificity of greater than 95%. Additionally, they stated, that variants with lower allele frequency could be reported in a specific clinical setting with confidence after DNA quality evaluation [14], which we also saw. This was also confirmed by another study where different variant callers were compared [15].

The analysis of parallel sequencing data can be challenging especially in smaller labs were no bioinformaticians are available. Larger panels like the TruSight Tumor 170 assay result in many somatic gene mutations, which have to be filtered and annotated correctly. As Karimnezhad et al. [15] highlighted, different single nucleotide variant calling pipelines can produce divergent and false positive results. Here, we used the BaseSpace TruSight Tumor 170 app for variant calling and the BaseSpace Variant Interpreter (Illumina) and the Molecular Health Guide Software (Molecular Health) for variant filtering and annotation from the generated VCF-file. Both options can be used by smaller labs with little or no bioinformatic knowledge. However, the BaseSpace Variant Interpreter does not give detailed information on biomarker protein functionalities, clinical relevance, treatment options, and clinical trials, which is essential in a clinical setting. For this, the Molecular Health Guide Software was used and showed reliable information.

Another limitation of the TruSight Tumor 170 assay is the detection of CNVs. Previous studies have shown that the detection of CNVs by parallel sequencing in FFPE material can be challenging, especially in samples with focal gene amplifications, low tumor purity, in highly degraded samples and in samples with chromosomal deletion [12,14,16]. In our study, seven samples had low-level *MET* amplifications and four were high-level *MET* amplified as determined by *MET* FISH [17]. Only two of these 11 *MET* amplified samples were positive by the TruSight Tumor 170 assay even though all samples were passing the

quality threshold of the assay. These two samples had a *MET* high-level amplification determined by *MET* FISH with a copy number >7.5. However, sample 18, which passed two of the criteria by Schildhaus et al. [17] for *MET*-FISH analysis, a copy number of 9.23 and a MET/CEN7 of 3.28, was not detected by CNV analysis. The same effect had been shown by Heydt et al. [12], especially in samples with low-level and intermediate-level *MET* amplifications.

On the RNA-level, 21 of 31 fusions and splice variants were confirmed. A big advantage of the TruSight Tumor 170 assay is that therapy relevant splicing effects like MET exon 14 skipping and the EGFR exon 1–8 deletion can be detected. The large *EGFR* deletion would have been missed by DNA-based amplicon sequencing though. Further, the effect of rare mutations on exon splicing can be analyzed at the same time if the variant was found on the DNA-level.

However, ten variants could not be confirmed by the TruSight Tumor 170 assay on the RNA-level. Eight of these were fusions incorrectly identified by FISH, RNAseq, or IHC. Three samples were false positive by *ROS1* FISH, these samples had isolated 3′ extra green signals in the *ROS1* FISH and although a chromosomal break occurred, it seemed that no functional fusion product was generated. This has also been seen in another published study [18] and was confirmed by ROS1 IHC in our study. Three other samples were false positive by RNAseq, showing low fusion supporting reads in the results, which were confirmed by FISH and one positive ALK IHC could not be confirmed by *ALK* FISH and the TruSight Tumor 170 assay. Thus, different variables can lead to false positive results across methodologies. Therefore, the ongoing evaluation of new assays like the TruSight Tumor 170 assay can discover and eliminate patterns that are leading to false positive results.

In our study, no false positive fusions or splice variants were called. This was also shown in another gene fusion detection study were the TruSight Tumor 170 assay showed the highest reliability for gene fusion detection among five other assays and identified all gene fusions in cell line samples and FFPE samples. Additionally, the assay showed the smallest number of false positive results [19]. We also demonstrated in our study that fusions can be detected with the TruSight Tumor 170 assay in samples with a very small amount of RNA. One *ROS1*, one *ALK*, and one *NTRK1* fusion each were correctly identified with as little as 1.3–5.8 ng RNA per sample. This was also shown in a further study, where a *ROS1* fusion was only called with the TruSight Tumor 170 assay and two other parallel sequencing assays failed to detect the fusion [18].

Another important part in the accurate detection of all relevant gene aberrations by parallel sequencing and other molecular methods in general is the retrieval of enough DNA and RNA from small FFPE biopsies. An additional RNA extraction is often not possible, therefore combined automated DNA and RNA extractions with enough DNA and RNA yield are still needed and have to be developed for clinical laboratories [14].

5. Conclusions

In summary, the TruSight Tumor 170 assay works well even with very low DNA and RNA concentrations in comparison to other methods and can be used in a routine workflow to reliably detect small deletions, gene fusions, and splice variants. However, we were not able to detect most focal gene amplifications/deletions. The bioinformatic pipeline also needs some improvements to detect variants, which are localized in intronic sequences. In combination with the Molecular Health Guide Software, the assay can be used for the credible delivery of clinical interpretations.

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Data Availability Statement: The raw data presented in this study are available on request from the corresponding author.

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