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

Carina Heydt, Roberto Pappesch, Katrin Stecker, Martin März, and Sabine Merkelbach-Bruse

Institute of Pathology, University Hospital Cologne, Kerpener Str. 62, 50937 Cologne, Germany; roberto.pappesch@uk-koeln.de (R.P.); sabine.merkelbach-bruse@uk-koeln.de (S.M.-B.)
Molecular Health GmbH, Kurfürsten-Anlage 21, 69115 Heidelberg, Germany; katrin.stecker@molecularhealth.com (K.S.); martin.maerz@molecularhealth.com (M.M.)
* Correspondence: carina.heydt@uk-koeln.de
† These authors contributed equally to this work.

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

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), 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 Multiple 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 (≤0.2)) and the median of the raw read count of each CNV target region (BinCount CNV Targets (≥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 (≥79 bp) | PCT Exon Bases 100X (≥95) | Coverage MAD (≤0.2) | Bin Count CNV Targets (≥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; PTEN: 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.718G>A p.C238Y |               |                 |
| 5   | NSCLC, Adeno  | 80      | 120            | 107                         | 99.74                     | 0.11              | 14.84                     |                       | n/a           |                 |
| 6   | NSCLC, Adeno  | 40      | 117            | 124                         | 99.77                     | 0.06              | 22.61                     |                       | n/a           |                 |
| 7   | NSCLC, Adeno  | 50      | 120            | 125                         | 99.75                     | 0.08              | 26.92                     |                       | n/a           |                 |
| 8   | NSCLC, Adeno  | 20      | 120            | 134                         | 99.64                     | 0.08              | 24.6                      |                       | MET amplification (4.77 Copies) | ✓/ (MET amplification not detected) |
| 9   | NSCLC, Adeno  | 60      | 120            | 125                         | 0                         | 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.2305_2308ins23 p.Q769_G774insC; KEAP1: c.340G>T p.Q114W; PTEN: c.460C>T p.Q154*; 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_2240del 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.D594E; 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           |                 |
| 14  | NSCLC, Adeno  | 70      | 90.2           | 131                         | 99.69                     | 0.13              | 17.84                     |                       | n/a           |                 |
| No. | Tumor Type | TCC (%) | DNA Input (ng) | Median Insert Size (≥79 bp) | PCT Exon Bases 100X (≥95) | Cov. MAD [1<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            | n/a                 |
| 16  | Pancreas    | 70      | 120           | 132                         | 99.78                     | 0.16            | 19.29                       | ATM: c.2494C>T p.R832C (MET amplification not detected on DNA-level) |
| 17  | NSCLC, Adeno | 15      | 8.6           | 115                         | 95.55                     | 0.13            | 1.47                        | n/a                             | n/a            | ✓                   |
| 18  | NSCLC, Adeno | 40      | 43.4          | 116                         | 99.22                     | 0.07            | 6.45                        | TP53: c.473G>T p.R158L (MET amplification (3.85 Copies)) |
| 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                         | 99.76                     | 0.09            | 20.04                       | BRCA2: c.1009G>C p.A334D |
| 21  | Ovary       | 70      | 90            | 115                         | 99.75                     | 0.1             | 19.37                       | X BRCA2 deletion not detected on DNA-level; MET deletion not detected on DNA-level |
| 22  | NSCLC, Adeno | 90      | 120           | 101                         | 76.85                     | 0.14            | 1.14                        | MET: c.2942-19_2942-9del (MET amplification not detected on DNA-level) |
| 23  | NSCLC, Adeno | 50      | 120           | 129                         | 98.74                     | 0.08            | 5.15                        | TP53: c.3145G>C p.G1049R; MET c.3076_3082+4del (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 (MET deletion not detected on DNA-level) |
| 25  | NSCLC, Adeno | 70      | 120           | 131                         | 99.32                     | 0.1             | 6.37                        | MET: c.3073_3082-22del (MET deletion not detected on DNA-level) |
| 26  | NSCLC, Adeno | 20      | 120           | 123                         | 97.6                      | 0.07            | 3.25                        | MET: c.3073_3082-21del (MET deletion not detected on DNA-level) |
| 27  | NSCLC, Adeno | 25      | 120           | 96                          | 99.43                     | 0.11            | 4.58                        | PIK3CA: c.134C>G p.G446S; MET c.3076_3082+4del (MET deletion not detected on DNA-level) |
| 28  | Rhabdomyosarcoma | 120 | 110          | 70.7                         | 0.16                      | 1.08            | n/a                         | n/a                             |
| 29  | Cholangio cellular Carcinoma | 50 | 120          | 113                         | 99.32                     | 0.14            | 6.78                        | n/a                             |
| 30  | NSCLC, Adeno | 15      | 120           | 150                         | 99.57                     | 0.1             | 12.3                        | MET: c.3334C>T p.H1112Y (MET amplification (11.72 copies)) |
| 31  | NSCLC, Adeno | 30      | 120           | 148                         | 99.58                     | 0.12            | 13.48                       | DDR2: c.2221G>T p.G741V; 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 (MET deletion not detected on DNA-level) |
Table 1. Cont.

| No. | Tumor Type       | TCC (%) | DNA Input (ng) | Median Insert Size (≥ 79 bp) | PCT Exon Bases 100X (≥ 95) | Cov. MAD [C<0.81] | 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           | n/a                 |
| 34  | Thyroid          | 50      | 120           | 139                         | 99.62                       | 0.09               | 12.35                       | n/a                             | 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           | n/a                 |
| 37  | Melanoma         | 60      | 120           | 114                         | 98.83                       | 0.11               | 4.95                        | n/a                             | n/a           | n/a                 |
| 38  | Melanoma         | 40      | 44            | 123                         | 99.09                       | 0.1                | 6.21                        | n/a                             | n/a           | n/a                 |
| 39  | Breast           | 90      | 120           | 115                         | 99.52                       | 0.12               | 8.91                        | PTEN: c.112C>T p.P38S (√)       |               |                     |
| 40  | Ovary            | 50      | 120           | 120                         | 99.39                       | 0.2                | 8.11                        | n/a                             | 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           | 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               | 25.07                       | 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).
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). 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                                                   | 2340.00             | 3558.57              |
| 10  | MET amplification (Copies 4.47; Ratio 1.28; low-level) | no                                                   | 2935.49             | 3621.15              |
| 11  | MET amplification (Copies 3.85; Ratio 1.17; low-level) | no                                                   | 1186.01             | 1932.84              |
| 12  | MET amplification (Copies 3.85; 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 (≥63 bp) and the median of the CV value of target region coverage across all genes with coverage above 1000 (Median CV Coverage 1000X) (≤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 | RNA Results | 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    | ✓            | ROS1 FISH false positive |
| 5   | NSCLC, Adeno    | 80      | 85             | 81                          | 0.63                           | 83.79               | ROS1 translocation    | ✓            |          |
| 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    | ✓            |          |
| 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 (KITA1A549-BRAF) | **√**                 |
| 14  | NSCLC, Adeno   | 30      | 5.8            | 113                        | 0.53                             | 82.19               | NTRK1 translocation (EPLS1L1-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) | **√** (RNAseq false positive for ROS1) |
| 17  | NSCLC, Adeno   | 15      | 1.3            | 112                        | 0.5                              | 80.8                | ROS1 translocation (SLC34A2-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               | BRAF: c.6829-9_6829-8delinsTTTTGG | **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) | **√** (RNAseq false positive for 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.

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 where the TruSight Tumor 170 assay showed the highest reliability for gene fusion detection among the 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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