Validation and clinical application of a targeted next-generation sequencing gene panel for solid and hematological malignancies

Iván Prieto-Potin [1], **Nerea Carvajal** [1], **Jenifer Plaza-Sánchez** [1], **Rebeca Manso** [1], **Carmen Laura Aúz Alexandre** [1], **Cristina Chamizo** [1], **Sandra Zazo** [1], **Almudena López-Sánchez** [1], **Socorro María Rodríguez-Pinilla** [1], **Laura Camacho** [2], **Raquel Longarón** [2], **Beatriz Bellosillo** [2], **Rosa Somoza** [3], **Javier Hernández-Losa** [3], **Víctor Manuel Fernández-Soria** [4], **Ricardo Ramos-Ruiz** [4], **Ion Cristóbal** [5], **Jesús García-Foncillas** [5], **Federico Rojo** [Corresp. 1]

[1] Department of Pathology, CIBERONC, UAM, Fundación Jiménez Díaz University Hospital Health Research Institute, Madrid, Spain

[2] Department of Pathology, Hospital Del Mar Medical Research Institute, Barcelona, Spain

[3] Department of Pathology, Vall d'Hebron University Hospital, Barcelona, Spain

[4] Genomics Unit, Madrid Science Park, Cantoblanco, Madrid, Spain

[5] Translational Oncology Division, UAM, Fundación Jiménez Díaz University Hospital Health Research Institute, Madrid, Spain

Corresponding Author: Federico Rojo
Email address: frojo@fjd.es

Background: Next-generation sequencing (NGS) has become a high-throughput technology widely integrated in molecular diagnostics laboratories. Among the large diversity of NGS-based panels, the Trusight Tumor 26 (TsT26) permitted the detection of low-frequency variants across 26 genes using the MiSeq platform. **Methods:** We described the clinical implementation of the panel in 399 patients affected of varied tumors types: gastrointestinal (GI, 29%), hematological (18%), lung (13%), gynecological and breast (8% each), among other tumor kind, after performing a previous inter-laboratory validation. **Results:** The panel performance resulted in an overall agreement of 93%. Two thirds of the patients succeeded in the sequencing testing against a third that failed due to unsuccessful quality controls filtering. The major number of detected variants was observed in *TP53* (28%), *KRAS* (16%), *APC* (10%) and *PIK3CA* (8%) genes. Globally, 372 variants were identified in 23 genes, primarily distributed as missense (81%), stop gain (9%) and frameshift (7%) altered sequences and mostly reported as pathogenic (78%) and VUS (19%). The more repeated variant across GI and lung tumors were *KRAS* G12D/V/C. **Conclusions:** Together, an appropriate validation of the TsT26 panel has granted a good application into the clinical routine by providing several relevant and potentially targetable variants across multiple FFPE tumors.

Corresponding author: Federico Rojo, M.D., Ph.D., Department of Pathology, IIS-Fundación Jiménez Díaz, UAM, Madrid 28040, Spain. E-mail: frojo@fjd.es, ORCID: https://orcid.org/0000-0001-9989-0290

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Introduction

Most of the laboratories committed to molecular diagnostics have incorporated next-generation sequencing (NGS) technology permitting a high-throughput sequencing of the genome. In part, due to the necessity of clinicians to gather data of genetic alterations to precisely guide the matching of a specific molecular-based therapy to the appropriate patient (Friedman et al., 2015). On the other hand, the cost-effectivity and multiple advantages of its application among other technologies might explain its extended use (Tan et al., 2018). A simultaneous screening of multiple genes in numerous samples in a single assay better defines this molecular testing standing out from the rest of diagnostic platforms generally analyzing an individual gene of a unique sample. It is a high sensitivity tool requiring small amounts of DNA input, besides of providing variant allele frequencies (Luthra et al., 2015; Surrey et al., 2017).

A crescent number of biomarkers are progressively required to characterize the molecular profile of a specific type of tumor or to administer targeted therapies (Morganti et al., 2018). Hence, platforms analyzing a single marker are becoming less convenient, leaving more room to those yielding results from several markers at once. An NGS-based gene panel test tolerates the

detection of genetic aberrations of different biomarkers sensitive of being targetable by molecular-based drugs (Nagahashi et al., 2018). Accordingly, the development of distinct gene panels confers the opportunity to identify multiple mutations of a concrete tumor type. In lung cancer, alterations in *EGFR, ALK or ROS1* genes are used to guide FDA-approved therapies (Hyman, Taylor & Baselga, 2017). Among the large diversity of NGS-based panel types, the Trusight Tumor 26 (TsT26) from Illumina was released time ago as a small actionable gene panel enabling the identification of low-frequency variants of genes involved in targeted therapy of solid tumors (Dong et al., 2015). The panel included *KRAS, NRAS* and *BRAF* genes that may be used for eligibility of colorectal cancer (CRC) patients against targeted anti-EGFR treatment, as well as to establish prognostication at any stage of the disease (Sepulveda et al., 2017). Several guidelines and recommendations are meant to standardize the implementation of NGS-based panels into the clinics by the consideration of a prior technical validation (Jennings et al., 2017). A NGS-based panel should not be set up into the clinical practice unless an acceptable validation is performed beforehand (Matthijs et al., 2016). In fact, the validation process should be able to document thoroughly how the assay is sufficiently reliable in identifying known mutations detected by diagnostic standards (McCourt et al., 2013).

After multidisciplinary clinical consensus, the need for an NGS-based panel in the medical routine was established for detailed molecular characterization of patients affected of diverse advanced cancer types. A unique molecular testing would bring the possibility of considering either administering targeted therapy or selecting appropriate candidates to participate in early-stage clinical trials from our institution. We postulated that a small gene panel such is the TsT26 panel including several genes implicated in targeted therapy and targets required for the recruitment to specific early-stage clinical trials would be suitable to fulfill our care necessity. In

order to test the panel capacities, the study first aimed to demonstrate whether its use might determine the mutational status of three precise genes, *KRAS*, *NRAS* and *BRAF*, altogether associated with treatment decision-making in CRC. For that purpose, we managed an inter-laboratory validation to incorporate the assay in the medical routine. Between 2015 and 2017, the laboratory subsequently employed the panel in the habitual activity. We have secondly described the successful practicability of the TsT26 panel in 399 patients presenting diverse tumorigenesis and evaluated its utility within the clinical context.

Material and Methods

FFPE tissue collection

The TsT26 panel performance was conducted in three clinical centers: Hospital Del Mar Medical Research Institute (Barcelona, Spain, n=16), Vall d'Hebron University hospital (Barcelona, Spain, n=16) and Fundación Jiménez Díaz University hospital (Madrid, Spain, n=16) with the collaboration of the Madrid Science Park. It overall included archived FFPE material from 48 patients affected of primary colorectal cancer with a prior known mutational status of *KRAS*, *NRAS* and *BRAF* genes. Samples were obtained from MARBiobank (PT17/0015/0002), VHIR-Biobank (PT17/0015/0026) and the Biobank Fundación Jiménez Díaz (PT17/0015/0006), each of them belonging to the Spanish National Biobanks Network. Additional FFPE samples of varied tumor types undertook the TsT26 panel testing at the Fundación Jiménez Díaz University hospital (Madrid, Spain, n=399). Written consent was received from each donor (Data S1 and S2). All investigations followed standard operating procedures with the approval of the Fundación Jiménez

Díaz University hospital Ethic and Scientific Committee (PIC 23-2012) and were conducted in accordance to the principles outlined in the Declaration of Helsinki.

Tumor cell content quality control (QC) 1

FFPE tissue sections (4 µm thick) were obtained for hematoxylin and eosin staining (Dako Coverstainer, Agilent, Santa Clara, CA, USA) to assess tumor cell content (TCC) of at least 30%. A pathologist (SMR-P and FR) examined the tumor cell content, scored the percentage of neoplastic nuclei and encircled the tumor area. When the TCC was below 30%, macrodissection was manually achieved with a scalpel blade (Fig. S1A).

DNA isolation

Consecutive FFPE tissue sections were obtained to extract genomic DNA according to the specimen type. Surgical resections were sectioned 10 µm thick, biopsies 30 to 40 µm, and endoscopies and cytologies 100 µm deep. Isolation was done by using the cobas® DNA Sample Preparation Kit (Roche Diagnostics, Pleasanton, CA, USA). Briefly, xylene deparaffinized FFPE sections were incubated at 56ºC in 22 mg/ml proteinase K lysis/binding buffer, followed by incubation at 90ºC. Isopropanol was added to the mixture and subsequent centrifugation through a glass fiber filter insert column was performed. Released nucleic acids were washed and eluted in a volume of 30 µl. Both concentration and purity were determined by Nanodrop (Thermo Fischer, Waltham, MA, USA) and Qubit 3.0 (Thermo Fisher, Waltham, MA, USA).

TsT26 ΔCq DNA QC2

Extracted DNA was amplified in triplicate by quantitative PCR using the KAPA SYBR FAST master mix (Life technologies, Grand Island, NY) on the Lightcycler® 480 system (Roche Molecular System, Pleasanton, CA, USA). The amount of DNA input was established by

89 comparing the ability of DNA to be amplified in relation to a non-FFPE reference genomic DNA.
90 A ΔCq value was calculated for each sample as follows: ΔCq= mean sample Cq value - mean
91 non-FFPE control Cq value. A mean of ΔCq<6 was considered as appropriate for library
92 preparation despite the instructions of the protocol that recommended a ΔCq<4 (Fig. S1B).

93 TsT26 Library preparation QC3

94 NGS libraries were prepared using the TsT26 panel (Illumina, San Diego, CA, USA) (Table S1),
95 a multiplexing kit of 178 amplicons covering 82 exonic regions across 26 genes (Table 1).
96 Undiluted or diluted DNA, because of the ΔCq score, was used to generate complementary
97 libraries targeting both positive and negative strands for each sample with two distinct
98 oligonucleotide pools, pool A and pool B. Both oligo pools were hybridized to DNA overnight
99 and resulting products were ligated and amplified with adaptors and index sequences (barcodes).
100 Then, the amplified libraries were purified using Agencourt AMPure XP magnetic beads
101 (Beckman-Coulter, Indianapolis, IN, USA). The obtained products were checked for their base
102 pair range using 2% agarose gel electrophoresis along with a 50 bp ladder (Sigma-Aldrich, San
103 Luis, USA) or a 2100 bioanalyzer instrument (Agilent, Santa Clara, CA, USA) (Fig. S1C).
104 Generated libraries in the 300-330 base pair range were considered suitable for sequencing.
105 Library concentration was measured using Qubit 3.0 fluorometer (Thermo Fisher, Waltham, MA,
106 USA) and normalized to 4 nM in elution buffer with Tris.

107 TsT26 high-throughput sequencing

108 Libraries were then diluted to 10 or 12 pM and to 15 or 20 pM and pooled on a v2 300-
109 cycle or v3 600-cycle sequencing kits according to the manufacturer's protocol. Sequencing was

achieved for both pool A and B by loading 600 µl of library mixes. Some runs were loaded along with 1% PhiX.

TsT26 analysis, quality metrics and variant detection

The integrated analysis software (Illumina, San Diego, CA, USA) including image analysis, base calling and assignation of quality scores automatically performed primary analysis. The sequencing analysis viewer software (SAV, Illumina, San Diego, CA, USA) confirmed quality metrics by using interop files along with run info and parameters. A Phred score of Q30 was considered for each run. The MiSeq Reporter software (Illumina, San Diego, CA, USA) included demultiplexing, sequence alignment and variant calling. Successful sequencing runs generated 2 FASTQ files, 2 BAM and BAM-BAI files for each sample pool A and pool B library pair and a single genomic variant call (VCF) file. Integrative Genomics Viewer software (IGV, Broad Institute, CA, USA) enabled to visualize sequenced regions (Thorvaldsdóttir, Robinson & Mesirov, 2013). An exportable excel format was generated for amplicon coverage assessment.

Annotation of detected variants used the Illumina Variant Studio version 2.2 software (Illumina, San Diego, CA, USA). The model only called variants for bases that were covered at 300x or greater for a single amplicon. Every variant with a variant allele frequency (VAF) less than 3% was filtered and excluded before review. Detected variants were marked with a PASS filter flag if satisfying the following criteria: variant must be present in both pools, cumulatively have a depth of 1000x or an average depth of 500x per pool. Those detected variants that did not accomplished this criterion or presented strand bias were further assessed during interpretation. A biologist (NC or SZ or CC) evaluated variants by identifying missense, frameshift, stop gain or loss, inframe insertions or deletions affected sequences. Variant classification employed ClinVar (http://www.ncbi.nlm.nih.gov/clinvar) (Harrison et al., 2016; Landrum et al., 2016), COSMIC

(http://cancer.sanger.ac.uk/cosmic) (Tate et al., 2019) and cbioPortal (http://www.cbioportal.org/) (Cerami et al., 2012; Gao et al., 2013) databases. Additional catalogues such as CIVIC (https://civicdb.org/home) (Griffith et al., 2017), OncoKB (https://oncokb.org/) (Chakravarty, Gao & Phillips, 2019) or the Cancer genome interpreter (https://www.cancergenomeinterpreter.org/analysis) (Tamborero, David Dienstmann et al., 2018) were also accessed for variant interpretation. Pathogenic, likely pathogenic, variant of uncertain significance (VUS) and benign or likely benign variants were reported according to standard guidelines (Richards et al., 2015; Hoskinson, Dubuc & Mason-Suares, 2017). A pathologist (SMR-P and FR) finally authenticated the reported variants.

KRAS and *NRAS* pyrosequencing

Pyrosequencing was determined for *KRAS* and *NRAS* genes was determined using the CE-IVD therascreen KRAS, NRAS and RAS Extension pyro kits (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Briefly, 10 ng/ul DNA templates were amplified in a SimpliAmp™ thermal cycler (Applied Biosystems, Foster City, CA, USA) targeting codons 12/13, 59, 61, 117 and 146. Amplicons were then immobilized on Streptavidin Sepharose®High Performance beads (GE Healthcare, Little Chalfont, UK). The obtained single-stranded DNA was prepared with the corresponding sequencing primers to DNA annealing. Further pyrosequencing run and analysis was carried out on the Pyromark Q24 system along with the software version 2.0 KRAS plug-in report (Qiagen, Hilden, Germany). Mutation thresholds were identified in relation to the manufacturer's limit of detection (>LOD + 3%). Both unmethylated control DNA and non-template control were included in every run for comparison and background levels screening.

BRAF cobas assay and direct sequencing

The CE-IVD cobas® 4800 BRAF V600 Mutation Test (Roche Diagnostics, Pleasanton, CA, USA) was used to identify BRAF V600E mutation by real time PCR technology, in agreement with the manufacturer's protocol. Extracted DNA was diluted to a concentration of 5 ng/ul and further amplified with specific fluorescent dye-labeled Taqman probes targeting the exon 15 region and binding to either wild-type or V600E sequences. Detection of fluorescence was performed on the cobas z 480 Analyzer and mutational status was reported as mutation detected or not detected with the cobas 4800 software (Roche Diagnostics, Pleasanton, CA, USA).

The mutational status of *BRAF* was also determined by direct sequencing. Primers were designed with the Primer Express software (Applied Biosystems, Foster City, CA, USA) using *BRAF* sequences NG-007873.3: *BRAF*-Fw: 5′-CTCTTACCTAAACTCTTCATAATGCTTGC-3′ and BRAF-Rv: 5′-CAGCATCTCAGGGCCAAAAA-3′. Amplification conditions included initial denaturation 10 min at 95ºC, 40 cycles of 1min at 95ºC, 1 min at 55ºC, 1 min at 72ºC, and a final step of 10 min at 72ºC. Amplicons were processed for DNA sequencing using the ABI-PRISM Big Dye version 3.1 (Applied Biosystems Foster City, CA, USA). Sequencing data were generated using the ABI-Prism 3730 XL DNA analyzer (Applied Biosystems Foster City, CA, USA) (Bessa et al., 2008).

Statistical analysis

We hypothesized that the expected difference of the detected variants found between the conventional methods and the NGS-based panel was less than 10% for the group of samples with mutations in *BRAF*, *KRAS* or *NRAS* genes. By using the PS program (Dupont & Plummer, 1990), the minimal sample needed to detect this difference was 44 cases with a power of 0,90 and two-sided error alpha of 0,05. Concordance data was analyzed using the SPSS version 21.0 software

for Windows (IBM, New York, NY, USA) and GraphPad Prism version 5.0 software (GraphPad Software, Inc., La Jolla, CA), as previously described (Van Stralen et al., 2009). Descriptive data were expressed as the mean and 95% confidence interval (CI).

Results

Inter-laboratory performance of the TsT26 panel

Each center participating in the validation procedure tested every set of the selected samples to identify altered variants in *KRAS, NRAS* and *BRAF* genes against a gold standard platform. Twenty-nine variants were found. The same samples were run through the TsT26 panel in the MiSeq platform. Sample 1-5 variant G12A was not detected and samples 3-4 and 3-11 were identified as variant detected, respectively as G12S and G12V (Table S2). So, NGS analysis finally identified thirty variants. Center 1 was able to detect 25 variants whereas centers 2 and 3 both distinguished 29 detected variants (Fig. 1A). Performance of the TsT26 panel was calculated as the whole data produced by each center. That is 48 samples run in triplicate or 144 outcomes considered for the agreement analysis between results found with the TsT26 panel and the reference gold standard, as pointed out in Table 2.

High-throughput sequencing quality metrics of the panel performance

Nine runs employing v2 300-cycle sequencing chemistry were done during the validation process. Quality metrics including cluster density and cluster passing filter were found slightly increased in comparison to the manufacturer's guidance as depicted in Fig. 1B. From every detected variant discovered through NGS testing, each gene presented a read depth higher than 1000x and a variant allele frequency (VAF) greater than 5% (Figs. 1C and 1D).

Patient characteristics and clinical practicability of the TsT26 panel

A set of 399 patients was included in this study. Sex and age data were available for respectively 386 and 365 patients. Regarding the varied tumor types, the most abundant consisted in gastrointestinal (GI), hematological, lung, gynecological, and breast samples, whereas melanoma, head and neck, genitourinary, central nervous system (CNS), and other histological cancer types were limited in number (Table S3). Overall consisted in biopsy specimens, surgical resections, endoscopies and cytologies (Table 3). From the entire data set, 40% was from external origin.

Two thirds of the total samples succeeded for the sequencing testing against a third that failed due to unsuccessful quality controls filtering (Fig. 2A and 2B). Quality controls included a first TCC management, then a second assessment of DNA quality and a final quantitation of libraries preparation. Unfulfilling any of these quality controls lead to sequencing failure (Fig. 2C).

DNA quality assessment for high-throughput sequencing by quantitative PCR

The majority of the samples presenting a $\Delta Cq<4$ resulted in a successful NGS sequencing. Five samples were sequenced despite of showing $\Delta Cq>6$ upon explicit clinical request. Several samples exhibiting a ΔCq value between 4 and 6 were able to generate valuable libraries. Consequently, we extended the cut-off value of the ΔCq to 6. Thirty-seven samples did not undergo DNA quality assessment, 5% of them resulting in a NGS fail, 3% in detected variants and 1% in not detected variant (Table 4).

Variant detection and high-throughput sequencing quality metrics during TsT26 panel implementation

Detected variants were identified in 74% (194 samples) of the successful sequenced samples whereas not detected variants or a wild type genotype was found in 26% (69 samples)

(Table S4). The major number of detected variants was observed in *TP53* (28%), *KRAS* (16%), *APC* (10%) and *PIK3CA* (8%) genes. In contrast, a lower amount of detected variants was encountered in *MET* (5%), *BRAF* (4%), *SMAD4* (3%), as well as in *KIT*, *PTEN*, *NRAS*, *CTNNB1*, *FBXW7*, *CDH1*, *HER2*, (2% each) and *GNAS*, *MAP2K1*, *STK11*, *EGFR*, *PDGFRA*, *MSH6*, *FGFR2*, *GNAQ*, *SRC* (1% each). No variants were detected in *AKT1*, *ALK* and *FOXL2* genes (Fig. 3A).

Thirty-seven runs were done during the clinical implementation, 17 employing v2 300-cycle and 20 using v3 600-cycle sequencing chemistries. Although certain runs experienced underclustering, the cluster density mean was found within the optimal range recommended by the manufacturer between 1000 and 1400 clusters per mm^2 (K/mm^2). Accordingly, these runs showed a high percentage value of cluster passing filter that lead to an elevated mean of this parameter (Fig. 3B). A read depth greater than 1000x was observed for each gene, except in two skin melanomas presenting a reduced depth value that subsequently required further corroboration by additional molecular testing. As well, every detected variant demonstrated a VAF superior than 3%. The *MET* gene showed the highest VAF mean in comparison to the other studied genes (Figs. 3C and 3D).

Coverage by amplicon was calculated by obtaining the mean of each amplicon covering each exonic region. The mean coverage of *AKT1* exon 2, *STK11* exons 1 and 6 did not satisfied the minimum coverage of 1000x required by the panel. In addition, *EGFR* ex21, *STK11* exons 4, 8 and *TP53* exon 11 presented the same condition although this was compensated by cumulatively counting the coverage of the second pool (Fig. S2).

Detected variants analysis by tumor type

In GI tumors, detected variants were more frequently observed in *KRAS* (23%), *TP53* (22%), *APC* (16%) and *PIK3CA* (8%) genes. Besides, detected variants were identified in the same genes in gynecological tumors, whereas in lung there were more recurrently perceived variants in only *TP53* (33%) and *KRAS* (21%) genes. In contrast, detected variants in hematological malignancies were merely seen in the *KRAS* gene (Fig. 4). In melanoma, 46% of the detected variants were found in *BRAF* and 15% in *NRAS* genes. In breast, *TP53* and *PIK3CA* genes presented a major amount of affected variants in comparison to other genes. Whereas genitourinary, head and neck, and SNC tumor types exhibited at least one detected variant per concerned gene except in *TP53*, *PIK3CA* and *MET* (Fig. S3).

Three hundred and seventy-two detected variants were identified in 23 genes of the TsT26 panel. The dominant type of detected variants consisted in missense altered sequences (81%); followed by stop gain (9%) and frameshift (7%) affected sequences. Minor alterations corresponded to inframe deletions (2%), splice region variants (1%), inframe insertions (1%) and start lost (1%). Mostly detected variants were reported as pathogenic (78%) or likely pathogenic (1%), whereas 19% of variants were classified as VUS and 2% as benign or likely benign. The more repeated variants across tumor types were *KRAS* G12D, G12V and *MET* E168D in GI tumors, *TP53* R248W in hematological malignancies, *KRAS* G12C and G12V in lung, *PIK3CA* H1047R/L in breast and *BRAF* V600E in both melanoma and head and neck cancers (Table S5).

Gene mutation frequencies by histological tumor type

We further compared mutation frequencies of the genes presenting detected variants to those encountered at the TGCA database in each histological tumor type. Similarity was observed when relating mutations frequencies of genes contained in the TsT26 panel to those from the TCGA set for most of the tumors included in the study. For instance, *KRAS, TP53, APC, PIK3CA,*

SMAD4, FBXW7 and *BRAF* genes presented higher mutation frequencies in CRC. Additional genes prevalence in other tumor types can be seen in Figs. S4 and S5. Hepatocarcinoma, cervical and mesothelioma tumor types did not present sufficient cases to perform comparisons.

Subsequent clinical decision-making to high-throughput sequencing

After reviewing the medical records of the 194 patients presenting detected variants in diverse genes after application of the TsT26 panel, we were able to associate a subsequent clinical action to a reported detected molecular alteration (Table S6). Arising from the 372 detected variants found, 37% were considered clinically relevant and a treatment decision was attempted on 13% of them. Considering patients, only 14% of them received a targeted therapy based on the detected variant found by the TsT26 panel (Table S7).

Discussion

In this study, we have conducted an inter-laboratory validation of the TsT26 panel into the clinical routine based on the reproducibility of detecting alterations in three genes of potential therapy interest. We obtained robust data regarding the detection of relevant and likely targetable variants across multiple tumors from 399 patients, despite a large number of samples that failed strict quality assessments. The reporting of detected variants was supported by adequate sequencing metrics and subsequent clinical decision-making when indicated.

The TsT26 panel incorporated rigorous pre-analytical requirements to obtain a favorable sequencing outcome. Samples were primarily evaluated for their TCC. Although a minimum of 30% of TCC was set up as the cut-off value to contemplate, a large number of samples with a lower TCC were selected to initiate the panel testing. In part, because the standard reference of the laboratory was established in a 10% value as the minimum TCC, however most of these samples

underwent tissue macrodissection. Secondly, DNA quality was assessed by qRT-PCR. Indeed, this estimation is considered a better indicator of amplifiable material than other common methods such can be fluorometric and spectrophotometric evaluations, the latter usually overestimating the amount of double stranded DNA (Deans et al., 2017). Final quality control measured library adequacy for ultimate sequencing. Despite of showing a good DNA quality demonstrated by a favorable qRT-PCR assessment, several samples failed in the library generation procedure by unfulfilling an appropriate right base pair size. Because of the severe quality control undergone before initiating the sequencing justified that a third of the global set of samples failed the TsT26 testing.

Our data revealed a similar prevalence of detected variants in GI, lung and melanoma tumors with previous published NGS results. In 52 colorectal tumors, *KRAS*, *TP53* and *APC* were the genes affected with a major number of detected variants using the same panel TsT26 (Giardina et al., 2018). The GI tumor type presented the same genes as more frequently altered. In other 45 lung adenocarcinomas, *TP53, KRAS* and *PIK3CA* showed the more elevated percentages of detected variants per gene using the ion torrent AmpliSeq Cancer Hotspot v2 assay (Tsongalis et al., 2014). Likewise, *TP53*, *KRAS* and *APC* presented more somatic alterations in the set of lung samples, whereas *PIK3CA* was represented in a much lower proportion. Others confirmed the elevated quantity of mutations in the *KRAS* gene despite the use of a limited sample size (Patel et al., 2017) or a much larger data set (Legras et al., 2018). An implementation study employing a customized Ampliseq NGS panel including 35 genes reported *BRAF*, *TERT* and *NRAS* as the more prevalent mutated genes in a set of 100 primary melanoma samples (De Unamuno Bustos et al., 2017). Although the TsT26 panel lacks from the study of the gene *TERT*, *BRAF* and *NRAS* were

the more frequently mutated genes in melanoma samples, as supported by others (Fisher et al., 2016; Giardina et al., 2018).

Abundant studies have characterized a prior validation to implement an NGS-based panel commonly employed in the assessment of targeted therapies in solid tumors (Tsongalis et al., 2014; Csernak et al., 2017; Kou et al., 2017; Luthra et al., 2017; Lee et al., 2018; Maxwell et al., 2018; Sussman et al., 2018; Williams, H.L., Walsh, K., Diamond, A., Oniscu, A., Deans, 2018). Likewise, other authors corroborated its use on pediatric hematological malignancies (Kluk et al., 2016) or myeloid neoplasms (Maes et al., 2017). On the contrary, other investigation has directly focused on a concrete cancer type such as in non-small cell lung cancer (NSCLC) (Legras et al., 2018). Although several studies validated NGS-based panels on both solid tumors and hematological malignancies (Cottrell et al., 2014; Garcia et al., 2017), only a few reports aimed to demonstrate that the TsT26 panel is a validated method to implement into the clinics in a considerable number of varied tumor tissues (Fisher et al., 2016; Giardina et al., 2018). In addition, this panel has also been used to validate other molecular testing platform in 90 NSCLC tumor samples (Quinn et al., 2015). Indeed, the more validated NGS studies following its application in the everyday practice, the better the way to integrate the NGS technology into the clinics. Although the TsT26 panel was indicated for the analysis of solid tumors, we also underwent extra solid tumors types and hematological malignancies samples across the panel. This is certainly not the more appropriate panel to test hematological malignancies. Particular customized panels are exclusively designed for that purpose such as the personalized panel including 48 genes in T-cell lymphomas (Manso et al., 2018). Despite of that, detected variants were found in the *TP53* gene allowing a concrete clinical decision-making and prognostication of several subgroups of lymphomas (Xu-Monette et al., 2012).

Other limitations may be recognized in the present study. Even though the validation analysis exhibited good concordance regarding *KRAS*, *NRAS* and *BRAF* genes, supplementary verification considering the rest of the targeted genes would probably demand additional authentication. Another restraint concerns the kind of genetic aberration that the panel is able to recognize. Essentially, the panel merely detects either single or multiple nucleotide variants in a restricted number of genes and cannot identify gene fusions. Moreover, concrete exonic regions of the genes *AKT1*, *STK11*, *EGFR* and *TP53* were not adequately covered, thus slightly constraining the limited sum of gene regions analyzed by the panel.

Despite of incorporating a limited quantity of genes, most of them were tightly linked to potential FDA-approved clinical actionability, such as *BRAF* mutations V600 in melanoma regarding dabrafenib, trametinib or vemurafenib treatments, and *EGFR* tyrosine-kinase domain mutations in NSCLC for afatinib, erlotinib, gefitinib and osimertinib therapies (Hovelson et al., 2015; Paasinen-Sohns et al., 2017). Likewise, *PIK3CA* mutations in patients with hormone receptor positive and ERBB2-negative advanced breast cancer who previously received endocrine therapy for alpelisib-fulvestrant (André et al., 2019). As well, *KIT* mutations in GIST for regorafenib, sunitinib and imatinib drugs (Demetri et al., 2012). Novel NGS-targeted panels may include a much larger number of genes to be tested as demonstrated by a panel targeting 170 genes that proved to bring relevant clinical information in diffuse gliomas by improving both diagnosis and prognostication (Na et al., 2019). Other panels can detect further alterations such as fusions and copy number variations in combination with point mutations in an elevated number of genes (Luthra et al., 2017). In fact, eligibility of the NGS-based panel remains of vital importance according to the kind of alterations that need to be target.

Conclusions

The inter-laboratory validation has permitted the effective practice of the NGS-based panel testing in 399 samples of diverse tumorigenesis. Although the panel testing required passing strict quality controls, two thirds of the samples were able to be sequenced and from the 372 detected variants an 80% of them were reported as clinically relevant. Thus, demonstrating the utility of the TsT26 panel as a suitable diagnostic tool applied in the clinical routine.

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Figure 1

Sequencing quality metrics of the Trusight® Tumor 26 panel during the validation procedure of the mutational status of KRAS, NRAS and BRAF genes.

(A) Reproducibility of detected variants by the gold standard method against the Trusight® Tumor 26 panel. Data are shown as percentage. (B) Cluster density and cluster passing filter quality metrics respectively expressed in cluster per mm2 and percentage. (C) and (D) Read depth of detected variants is expressed in kilo base pair and variant allele frequency of each gene is shown as a percentage value. Data are represented as box and whisker plots with median and IQR.

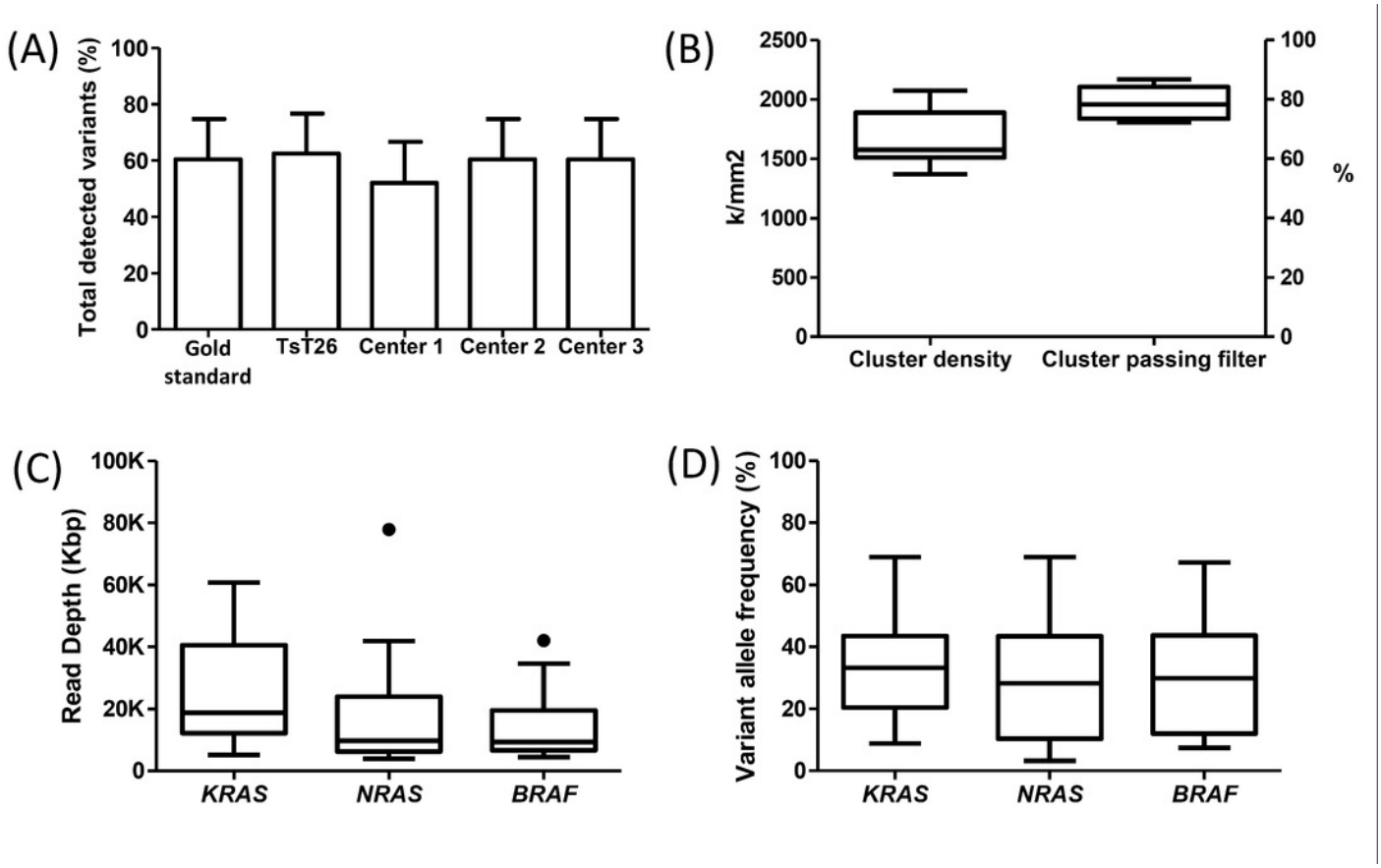
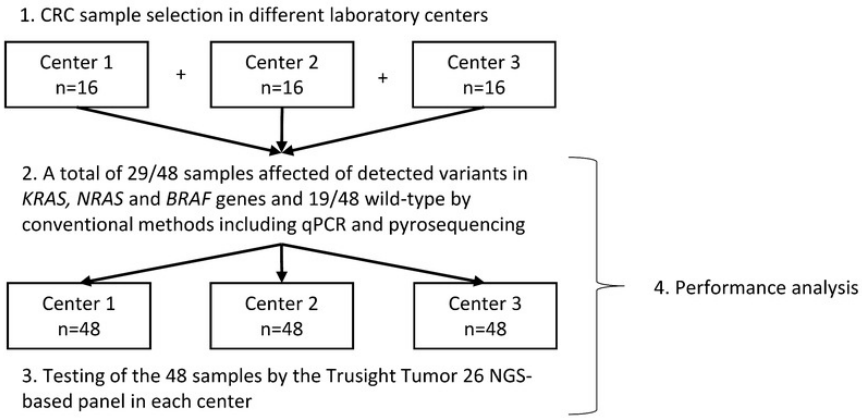


Figure 2

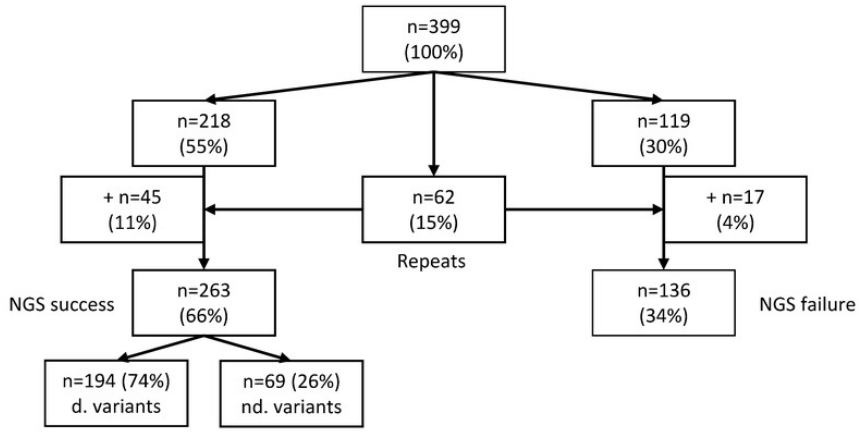
Practicability of the Trusight® Tumor 26 panel.

(A) Study design of the panel performance. (B) Flow diagram depicting the number of FFPE samples that either succeded or failed to NGS testing. (C) Workflow followed by each of the 399 FFPE samples included in TsT26 panel study. Samples underwent diverse quality controls (QC). QC1 referred to the tumor cell content; a cut off value was established in 30%. Note that samples between 10-30% with no possibility of macrodissection underwent direct DNA isolation. QC2 indicated the quality of the sample in comparison to a fresh commercial preserved sample; a ΔCq value less than 6 was acceptable to continue the library preparation. QC3 determined the fragmentation of the library, library products of less than 300 bp were not considered for sequencing (An invalid run was also used as quality control).

(A)


1. CRC sample selection in different laboratory centers
Center 1 n=16
+
Center 2 n=16
+
Center 3 n=16
2. A total of 29/48 samples affected of detected variants in KRAS, NRAS and BRAF genes and 19/48 wild-type by conventional methods including qPCR and pyrosequencing
Center 1 n=48
Center 2 n=48
Center 3 n=48
3. Testing of the 48 samples by the Trusight Tumor 26 NGS-based panel in each center
4. Performance analysis

(B)


n=399 (100%)
n=218 (55%)
n=119 (30%)
+ n=45 (11%)
n=62 (15%)
Repeats
+ n=17 (4%)
NGS success
n=263 (66%)
n=136 (34%)
NGS failure
n=194 (74%) d. variants
n=69 (26%) nd. variants

(C)

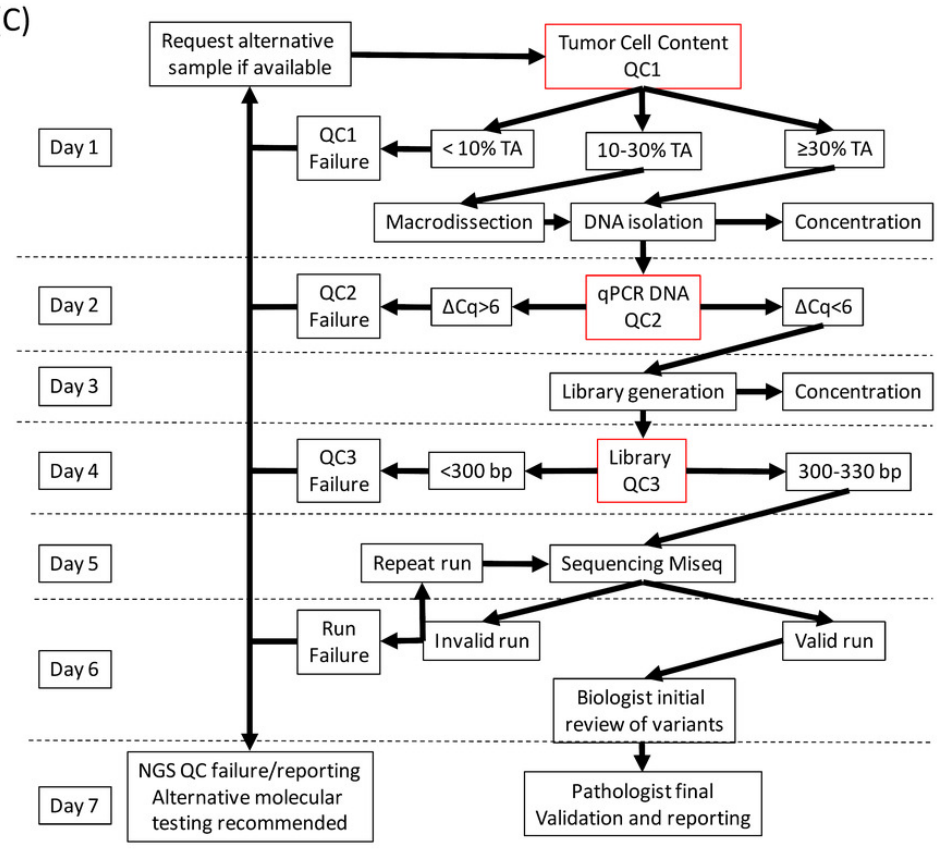

Request alternative sample if available
Tumor Cell Content QC1
Day 1
QC1 Failure
< 10% TA
10-30% TA
≥30% TA
Macrodissection
DNA isolation
Concentration
Day 2
QC2 Failure
ΔCq>6
qPCR DNA QC2
ΔCq<6
Day 3
Library generation
Concentration
Day 4
QC3 Failure
<300 bp
Library QC3
300-330 bp
Day 5
Repeat run
Sequencing Miseq
Day 6
Run Failure
Invalid run
Valid run
Biologist initial review of variants
Day 7
NGS QC failure/reporting Alternative molecular testing recommended
Pathologist final Validation and reporting

Figure 3

Sequencing quality metrics of the Trusight® Tumor 26 panel during clinical implementation.

(A) Total detected variants per gene type identified in the 399 samples tested. (B) Cluster density and cluster passing filter quality metrics respectively expressed in cluster per mm2 and percentage. (C) and (D) Read depth of detected variants is expressed in kilo base pair and variant allele frequency of each gene is shown as a percentage value. Data are represented as box and whisker plots with median and IQR.

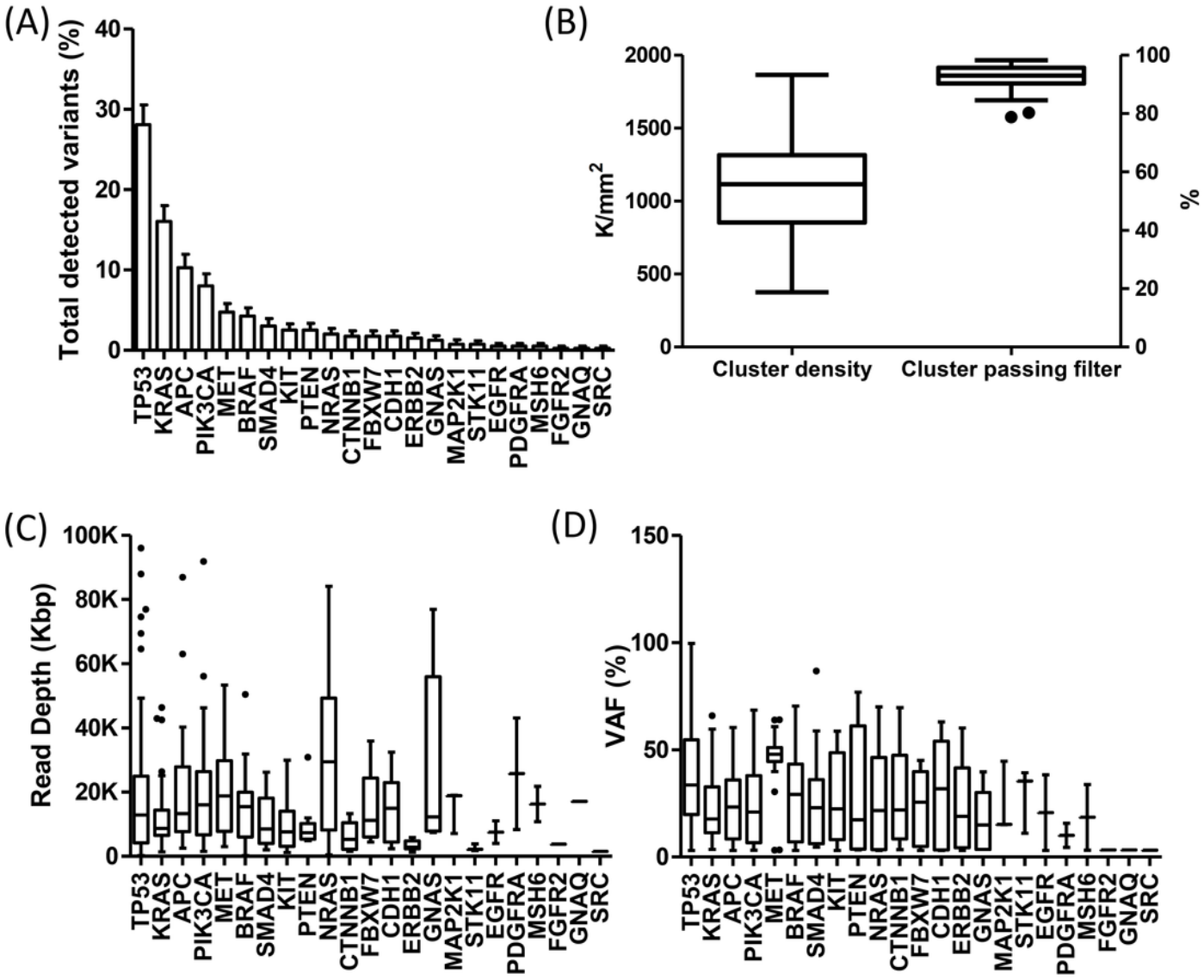

(A)
Total detected variants (%)
40
30
20
10
0
TP53
KRAS
APC
PIK3CA
MET
BRAF
SMAD4
KIT
PTEN
NRAS
CTNNB1
FBXW7
CDH1
ERBB2
GNAS
MAP2K1
STK11
EGFR
PDGFRA
MSH6
FGFR2
GNAQ
SRC
(B)
K/mm2
2000
1500
1000
500
0
100
80
60
40
20
0
%
Cluster density
Cluster passing filter
(C)
Read Depth (Kbp)
100K
80K
60K
40K
20K
0
(D)
VAF (%)
150
100
50
0

Figure 4

Detected variant frequencies across tumor type.

(A) Gastrointestinal. (B) Hematological malignancies. (C) Lung. (D) Gynecological. Columns represent samples and rows genes indicated by percentage of samples with detected variants in a precise gene. Detected variants are shown by grey squares whereas more than one detected variant is depicted by black squares.

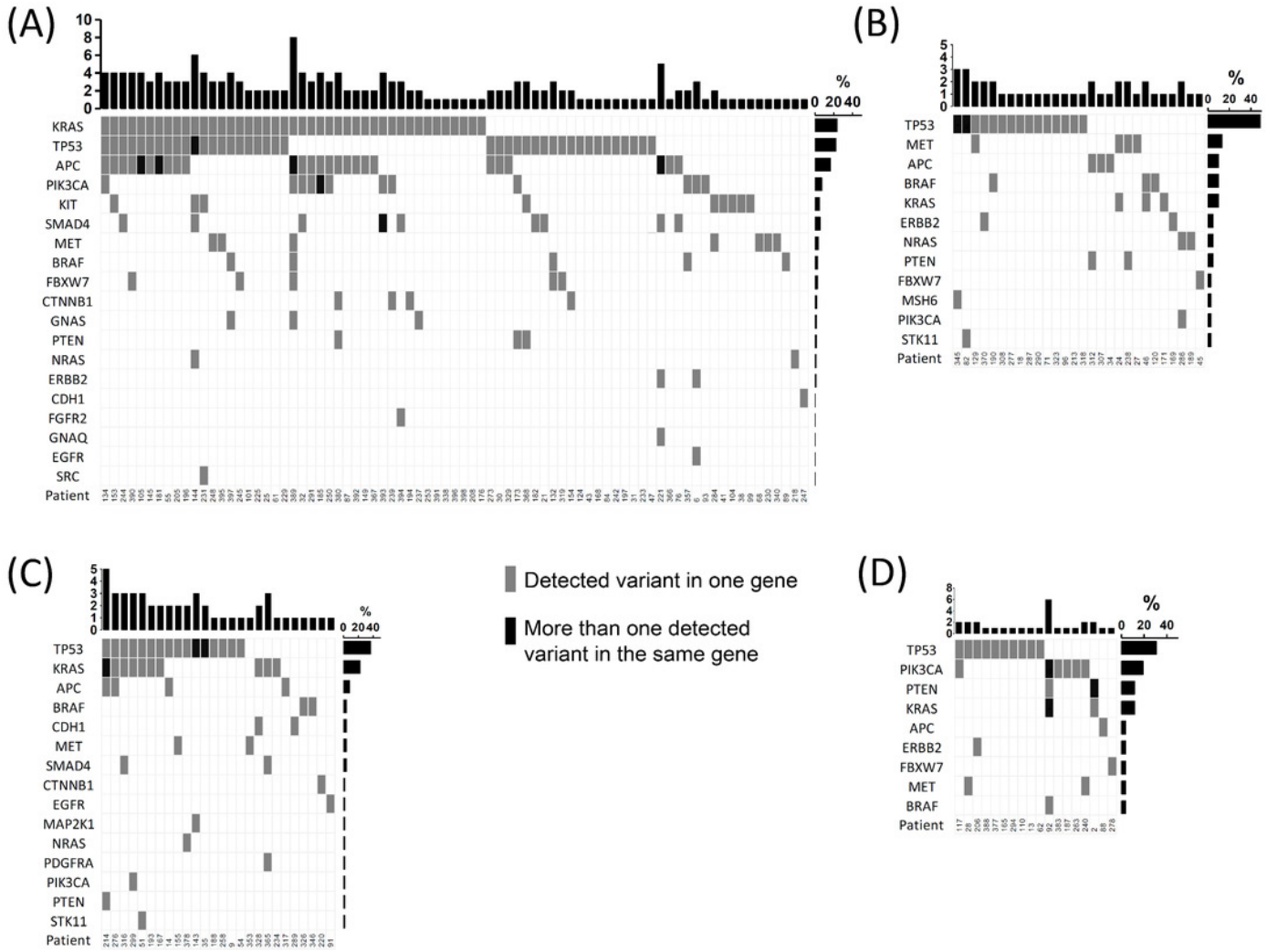


Table 1(on next page)

Trusight®Tumor 26assay exon coverage by amplicons (82 exons from 26 genes were covered by 178 amplicons).

Gene symbol	Accession Number	Exons covered	Number of amplicons to exon coverage
AKT1	NG_012188.1	2	1
ALK	NG_009445.1	23	1
APC	NG_008481.4	15*	14
BRAF	NG_007873.3	11,15	3
CDH1	NG_008021.1	8,9,12	6
CTNNB1	NG_013302.2	2	2
EGFR	NG_007726.3	18,19,20,21	7
ERBB2	NG_007503.1	20	2
FBXW7	NG_029466.2	7,8,9,10,11	13
FGFR2	NG_012449.2	6	2
FOXL2	NG_012454.1	1	1
GNAQ	NG_027904.2	4,5,6	6
GNAS	NG_016194.2	6,8	2
KIT	NG_007456.1	9,11,13,17,18	9
KRAS	NG_007524.1	1,2,3,4	8
MAP2K1	NG_008305.1	2	1
MET	NG_008996.1	1,4,13,15,16,17,18,20	22
MSH6	NG_007111.1	5	3
NRAS	NG_007572.1	1,2,3,4	8
PDGFRA	NG_009250.1	11,13,17	5
PIK3CA	NG_012113.2	1,2,7,9,20	15
PTEN	NG_007466.2	$1^{\#}$,2,3,4,$5^{\#}$,$6^{\#}$,7,9	17
SMAD4	NG_013013.2	8,11	5
SRC	NG_023033.1	10	2
STK11	NG_007460.2	1,4,6,8	7
TP53	NG_017013.2	$2^{\&}$,$3^{\&}$,$4^{\&}$,$5^{\&}$,$6^{\&}$,7,$8^{\&}$,$9^{\&}$,10,11	16

*exon 15 of the *APC* gene was split into 3 regions and each covered respectively by 2, 2 and 10 amplicons. #exons 1, 5 and 6 of the *PTEN* gene were split into 2 regions each and separately covered by 2, 2 and 2 amplicons. &exons 2, 3 and 4 of the *TP53* gene were together covered by 6 amplicons; as well as exons 5 and 6, 8 and 9, respectively by 4 and 3 amplicons.

Table 2(on next page)

TsT26 panel performance by determining the mutational status ofKRAS, NRASandBRAFgenes

TsT26	Gold standard		
	Detected variant	Not detected variant	Total
Detected variant	80	3	83
Not detected variant	7	54	61
Total	87	57	144
Positive agreement	92% (80/87, 95%CI=84-97)		
Negative agreement	95% (54/57, 95% CI=85-99)		
Overall agreement	93% (134/144, 95% CI=88-97)		
Positive predictive value	96% (80/83, 95% CI=90-99)		
Negative predictive value	88% (54/61, 95%CI=79-94)		

20

21

22

23

24

Table 3(on next page)

Clinical and patient characteristics

Characteristics	Number of patients	Cytology	Resection	Endoscopy	Biopsy
Sex, no. (%)*					
Female	188(49)				
Male	198(51)				
Mean age, y.o. (95%CI)*	59(58-61)				
Tumor type, no. (%)					
Gastrointestinal	115(29)	0	35(30)	23(20)	57(50)
Hematologic	73(18)	0	12(16)	0	61(84)
Lung	51(13)	13(25)	6(12)	0	32(63)
Gynecologic	38(8)	0	14(34)	1(3)	23(63)
Breast	33(8)	2(6)	9(27)	1(3)	21(64)
Genitourinary	20(5)	0	9(47)	0	11(53)
Head and Neck	19(5)	0	6(32)	0	13(68)
Melanoma	15(4)	1(7)	3(20)	0	11(73)
Central Nervous System	10(3)	0	3(30)	0	7(70)
Other solid tumor	25(6)	1(4)	3(12)	0	21(84)

*Sex and age data was not available for every patient included in the study

Table 4(on next page)

DNA quality assessment by quantitative PCR

	ΔCt<4	4<ΔCt<6	ΔCt>6
Detected variant	159 (40%)	19 (5%)	3 (1%)
Not detected variant	59 (15%)	5 (1%)	2 (1%)
NGS fail	26 (6%)	27 (27%)	62 (15%)