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Analysis of TMB and MSI Status with TruSight™ Oncology 500

An optimized solid tumor assay with error-corrected sequencing and an informatic pipeline for the robust analysis of tumor mutational burden (TMB) and microsatellite instability (MSI)

Introduction

Cancer immunotherapy is a powerful strategy that uses the immune system to identify and destroy neoplasms. Successful clinical trials and regulatory approvals have established a variety of treatments for multiple tumor types.[1] Ufortunately only a fraction of patients benefit significantly from immunotherapy, driving a need to develop accurate methods for differentiating responders from nonresponders.[2-4]

Tumor mutational burden (TMB), or the number of nonsynonymous mutations within the coding region of a tumor genome, is an emerging biomarker that correlates with response to immunotherapeutic agents such as checkpoint inhibitors.[2-4] While TMB has historically been assessed by whole-exome sequencing (WES), recent studies have demonstrated that TMB can be effectively estimated using targeted sequencing panels covering 1.1 Mb or more of genomic content,[5,6] thereby providing methods that may be more efficient and compatible with current cancer testing paradigms. While the clinical utility of TMB is being defined, continuing efforts to standardize TMB calling between laboratories and manufacturers are ongoing.

Microsatellite instability (MSI) status is an independent biomarker that is FDA-approved for selection of solid tumors for treatment with checkpoint inhibition[7,8] MSI is traditionally analyzed with PCR (MSI-PCR) and immunohistochemistry. However, NGS allows for the analysis of a greater number of microsatellite loci than MSI-PCR, presenting opportunities to identify new MSI profiles in previously uncharacterized cancer types.[9]

TruSight Oncology 500 is a comprehensive next-generation sequencing (NGS) assay targeting the full coding regions of 523 genes implicated in the pathogenesis of solid tumors. Using enrichment-based library preparation techniques for use with formalin-fixed, paraffin-embedded (FFPE) samples, TruSight Oncology 500 can analyze DNA and RNA from the same sample, detecting single nucleotide variants (SNVs), indels, amplifications, splice variants, and fusions, in a single sequencing run.

Alongside variant detection, TruSight Oncology 500 provides the ability to assess key immunotherapy biomarkers including TMB and MSI. The TruSight Oncology 500 panel contains 1.94 Mb genomic content, though the performance of TMB detection is set by analyzing SNVs and indels in the coding regions, with sophisticated variant calling and germline filtering algorithms for enhanced accuracy (Figure 1, Table 1). This application note demonstrates the use of TruSight Oncology 500 for assessing TMB and MSI status, with high concordance to WES and MSI-PCR.

Table 1: TMB features for TruSight Oncology 500

TMB detection feature	Benefits
Genomic region of > 1.1 Mb[5]	Larger genome footprint confers higher TMB accuracy
Using nonsynonymous and synonymous SNV and indels at 5% limit of detection	Increases TMB sensitivity by utilizing more variants
Unique molecular identifiers	Sequencing error reduction guards against incorrectly high TMB values
Post-processing	FFPE artifact reduction guards against incorrectly high TMB values
Removal of variants in low confidence regions	Protects against false positives resulting in incorrectly high TMB values
Removal of driver mutations	Protects against panel design bias resulting in incorrectly high TMB values
Germline filtering using population databases, followed by VAF filtering	Allows for tumor only workflow

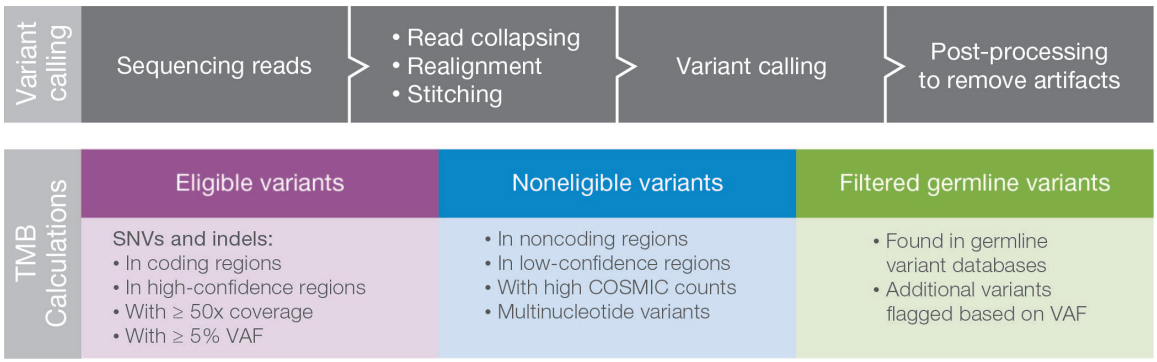

Figure 1: TMB data analysis pipeline— After initial variant calls are processed for error reduction, TMB evaluation involves selection of variants with specific criteria. Additional variants were filtered based on VAF and copy number. TMB = number of eligible somatic mutations per Mb (targeted region defined as high confidence regions with ≥ 50× coverage).

Methods

Assessment of immuno-oncology markers with sequencing of cancer-related genes

To assess the performance of TruSight Oncology 500 for TMB estimation and MSI status, three types of analyses were performed:

1) 1102 lung, melanoma, and colorectal cancer samples from the Cancer Genome Atlas (TCGA) previously assessed for TMB using WES data, were reevaluated by informatically extracting the targeted regions in TruSight Oncology 500, and TruSight Tumor 170, from the WES data *in silico*. From these simulated datasets, TMB was estimated and compared to the original results from the full WES data.

2) Using 95 FFPE tumor samples from various tissues (Table 2), we investigated whether TruSight Oncology 500 analysis of tumor samples alone combined with a computational germline filtering and background noise removal algorithm can measure TMB accurately compared with whole-exome sequencing of tumor-normal pairs.

3) Microsatellite instability (MSI) status was assessed for 92 FFPE tumor samples and compared to a PCR-based assay.

Library preparation and NGS

To compare original data from WES to TruSight Oncology 500, DNA was isolated and aliquoted from 95 FFPE tumor-normal samples (Table 2). For each sample, libraries were prepared using the TruSight Oncology Library Prep Kit[10] followed by enrichment with either the TruSight Oncology 500 enrichment reagents for tumor samples only, or IDT exome enrichment reagents[11] for tumor-normal pairs. All libraries were sequenced on the NextSeq™ or NovaSeq™ Systems.

Table 2: Tumor samples analyzed with Trusight Oncology 500 and WES

Tissue type	No. of samples
Lung	26
Melanoma	11
Colon	30
Endometrium	18
Gastric	10

Data Analysis

TMB in silico analysis was assessed by using WES tumor-normal (T/N) data from a cohort of 1102 lung, melanoma, and colorectal cancer samples from Cancer Genome Atlas (TCGA), and filtering through content from TruSight Oncology 500 and TruSight Tumor 170 panels. The goal of in-silico analysis is to understand the effect of panel size on TMB estimation, with the expectation that smaller panels add more sampling noise for samples with mid-to-low TMB scores.

TMB measurement of real FFPE samples sequenced with the TruSight Oncology 500 assay was performed using an in-house tumor-only pipeline designed to call small nucleotide variants and indels while filtering germline variants and removing technical noise. TMB estimation in FFPE samples requires extremely high specificity in variant calling, which is difficult to achieve in FFPE samples. To overcome such challenges, the TruSight Oncology 500 informatic pipeline uses unique molecular identifiers (UMIs) to reduce sequencing noise during the initial step. Reads from complimentary strands are also collapsed through a duplex collapsing step, which greatly reduces FFPE deamination artifacts. Second, to further reduce FFPE false positives, a likelihood ratio based variant filtering method is used to dynamically adjust calling threshold based on the observed nucleotide change type, strand and error rate in a given sample. The net effect of UMI and likelihood ratio filtering reduces false positives in a typical FFPE sample from ~1500/Mb to less than 5/Mb. Finally, residual germline variants are minimized by employing a filtering strategy using a population database to identify germline variants, and also leveraging copy number and allele frequency information.

To calculate positive predictive values (PPV) and negative predictive values (NPV), WES data was used as a standard, with the assumption that WES values were 100%. Sequence data from TruSight Oncology 500 was also used for MSI analysis. For comparison, the same samples were analyzed with a commercially available MSI-PCR assay (Promega). Additionally, MSI was assessed using an internal tumor-only algorithm examining 130 repeat loci covered by TruSight Oncology 500.

Results of *in silico* studies

A recent study reported that TMB can be accurately predicted using NGS to analyze more than 1.1 Mb of genomic content.[5,6] To confirm this, WES data from 1102 TCGA samples were filtered and analyzed *in silico* using content from two sequencing panels with different amounts of targeted content: Trusight Oncology 500 and TruSight Tumor 170 (~0.4 Mb). When using samples with all TMB values, both TruSight Oncology 500 and TruSight Tumor 170 showed high concordance to TMB estimated from WES, with correlation values (R^2) of 0.97 and 0.89, respectively (Figure 2A, 2B). When assessing samples with TMB values less than 30 mutations/Megabase (mut/Mb), TruSight Oncology 500 showed significantly higher concordance with WES TMB estimation (R^2=0.84) than TruSight Tumor 170 (R^2=0.51) (Figure 2C, 2D). These results further support the potential value of using a larger sequencing panel for TMB analysis.

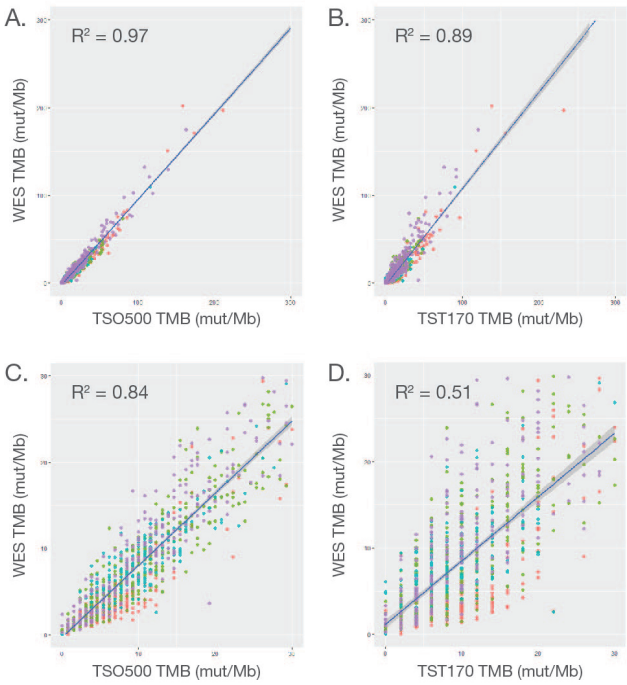

Figure 2: TMB evaluation of a TCGA cohort using targeted panel content— WES data from 1102 TCGA samples were filtered through the content of and TruSight Oncology 500 (TSO500) and TruSight Tumor 170 (TST170) to demonstrate the difference in performance with larger panels, and with samples that have TMB values below a 30 mutations/Megabase (mut/Mb) cutoff. (A) WES vs TSO500 with mut/Mb > 30. (B) WES vs TST170 with mut/Mb > 30. (C) WES vs TSO500 with mut/Mb < 30. (D) WES vs TST170 with mut/Mb < 30. TCGA samples were from 4 tissues types (red = colorectal, green = lung, blue = lung squamous cell, purple = melanoma).

Development of the TruSight Oncology immuno-oncology pipeline for use with FFPE tumor samples

Comparison of WES and TruSight Oncology 500 TMB measurement with FFPE samples

DNA from 95 FFPE tumor samples was profiled using both WES and TruSight Oncology 500 and the correlation of TMB estimation was assessed. Using WES-based TMB estimation as the reference, WES results were filtered through TruSight Oncology 500 content for a simulated comparison. The resulting correlation (R^2=0.93) between expected TMB values from TruSight Oncology 500 and WES is based on the assumption of identical variant calling and perfect germline variant filtering (Figure 3A).When the same WES results were compared to real data obtained from FFPE samples run through the TruSight Oncology 500 workflow and sequenced on the NextSeq System, high concordance (R^2 = 0.92) was also observed (Figure 3B). These results demonstrate high concordance between TruSight Oncology 500 TMB estimation in a tumor-only workflow and WES TMB estimation using tumor-normal paired samples.

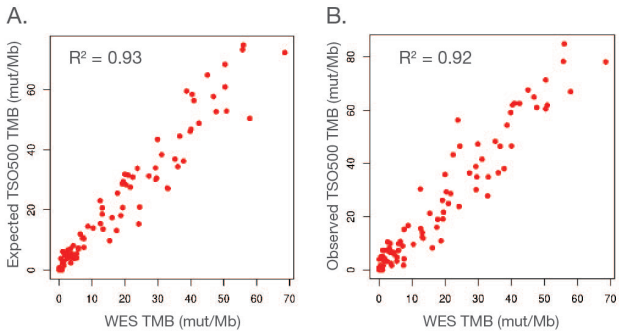

Figure 3: Concordance between WES and TruSight Oncology 500 TMB measurements with FFPE tumor samples—Ninety-five FFPE tumor samples were analyzed with both WES and Trusight Oncology 500 (TSO500). Three outliers with TMB values > 100 mut/MB were removed in these figures for better visualization. (A) WES TMB vs expected TSO500 TMB. Comparison with Expected TSO500 TMB evaluation was done by filtering WES results with TSO500 panel content, assuming identical variant calling and perfect germline variant calling. (B) WES TMB vs observed TSO500 TMB. WES results were compared to TSO500.

FFPE Tumor samples were classified as TMB-high or TMB-low using 10 mut/Mb as the cutoff value. Using the same method of filtering WES results through TruSight Oncology 500 panel content, expected TMB-high and TMB-low classifications were similar to experimentally observed values (Table 3, Table 4).

Table 3: Classification of tumor samples as TMB-high or TMB-low (all samples)

	TMB-High (expected TSO500)	TMB-Low (expected TSO500)
TMB-High (WES)	52	1
TMB-Low (WES)	5	37
	TMB-High (observed TSO500)	**TMB-Low (observed TSO500)**
TMB-High (WES)	51	2
TMB-Low (WES)	4	38

Expected TruSight Tumor 500 (TSO500) TMB evaluation was done by filtering WES results with TSO500 panel content, assuming identical variant calling and perfect germline variant calling. TMB-High classification was assigned to samples with > 10 mut/Mb.

Table 4: Classification of tumor samples as TMB-high or TMB-low (samples < 30 mut/Mb)

	TMB-High (expected TSO500)	TMB-Low (expected TSO500)
TMB-High (WES)	26	1
TMB-Low (WES)	5	37
	TMB-High (observed TSO500)	**TMB-Low (observed TSO500)**
TMB-High (WES)	25	2
TMB-Low (WES)	4	38

Expected TruSight Tumor 500 (TSO500) TMB evaluation was done by filtering WES results with TSO500 panel content, assuming identical variant calling and perfect germline variant calling. TMB-High classification was assigned to samples with > 10 mut/Mb.

Using WES TMB measurements as the standard, we set the values from WES analysis be 100% accurate, and then calculated percent agreement and positive predictive values using WES data as a reference point. Concordance was similarly high between expected and observed TruSight Oncology 500 values even when analysis was limited to samples with < 30 mut/Mb (Table 5).

Table 5: Predictive value and percent agreement between TruSight Oncology 500 and WES TMB measurements

	All samples		Samples with TMB < 30 mut/Mb	
	TSO500 TMB (Expected)	TSO500 TMB (Observed)	TSO500 TMB (Expected)	TSO500 TMB (Observed)
PPA	98.10%	96.20%	96.30%	92.60%
NPA	88.10%	90.50%	88.10%	90.50%
PPV	91.20%	92.70%	83.90%	86.20%
NPV	97.30%	95.00%	97.30%	95.00%

Expected TruSight Tumor 500 (TSO500) TMB evaluation was done by filtering WES results with TSO500 panel content, assuming identical variant calling and perfect germline variant calling. TMB-High classification was assigned to samples with > 10 mut/Mb.

PPA = positive percent agreement (with WES TMB measurement)

NPA = negative percent agreement (with WES TMB measurement)

PPV = positive predictive value (percentage of positive results that are in agreement with WES)

NPV = negative predictive value(percentage of negative results that are in agreement with WES)

Analysis of MSI status

To demonstrate TruSight Oncology 500 performance with MSI status analysis, the Promega MSI Analysis System was used as a comparator assay. The PCR-based assay assessed five specific MSI marker alleles resulting in a qualitative result of either a MSI-high or MSI-stable phenotype, while TruSight Oncology 500 analyzed 130 MSI marker sites to calculate a quantitative score. Results from the assays were highly concordant. One sample classified as MSI-stable by the PCR assay yielded a higher quantitative score by analysis with TruSight Oncology 500 (Figure 4A).

MSI results were also compared to TMB calculations from TruSight Oncology 500, demonstrating that MSI-high samples generally have higher TMB for specific tissue types (Figure 4B). The level of concordance between TMB and MSI analysis is consistent with previous reports of high proportions of MSI-high tumors also exhibiting high TMB in some tumor types (colon, endometrium), while other tissues have been reported to have high TMB status simultaneously with low MSI status (lung, melanoma).[12] These results demonstrate that TruSight Oncology 500 can be used to assess both biomarkers from the same sample, as may be desired for specific tumor types.

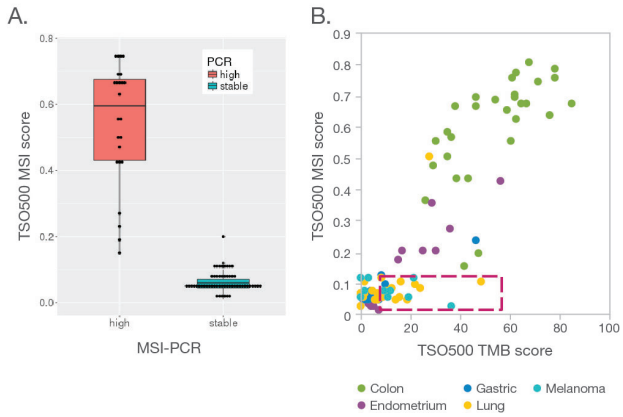

Figure 4: MSI status analysis with TruSight Oncology 500 (TSO500)—(A) Ninety-five FFPE tumor samples were analyzed with both TSO500 and the Promega MSI Analysis System (MSI-PCR). (B) TSO500 analysis of TMB plotted against TSO500 analysis of MSI status. Blue dotted line indicates samples that had high TMB scores concurrently with low MSI scores.

Reproducibility

To demonstrate reproducibility of TruSight Oncology 500 performance, samples from four FFPE tissues and four cell lines were analyzed. Three operators processed twelve replicates of each sample using an independent sequencing instrument and independent reagent lot. Because accuracy is a concern with low TMB values, samples with a wide range of expected values were selected. Low variations were observed between replicates and between operators, at both high and low TMB values (Figure 5).

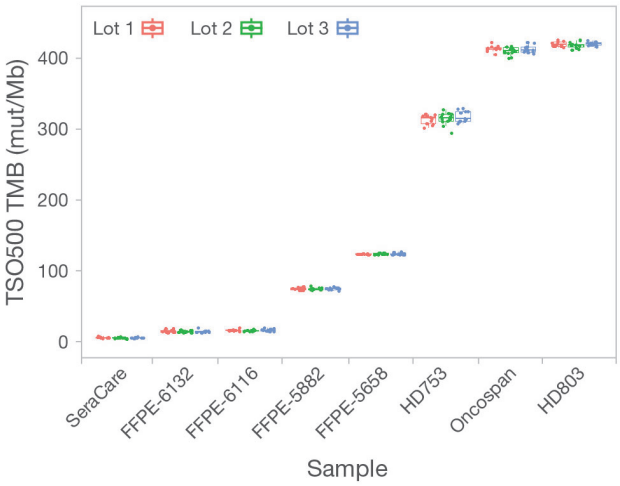

Figure 5: TMB estimates are reproducible from TruSight Oncology 500—Eight unique samples were analyzed from FFPE tissues or cell lines. Each sample was analyzed by three independent operators, sequencing instruments, and reagent lots. A total of 36 replicates are shown per sample, with 12 replicates per lot (reagent lot/operator/instrument).

MSI analysis of FFPE tissues and cell lines with both stable and unstable classifications also demonstrated low variability between different operators using independent reagent lots and instruments (Figure 6). Together these results demonstrate that the TruSight Oncology 500 assay is highly reproducible for both TMB and MSI analysis.

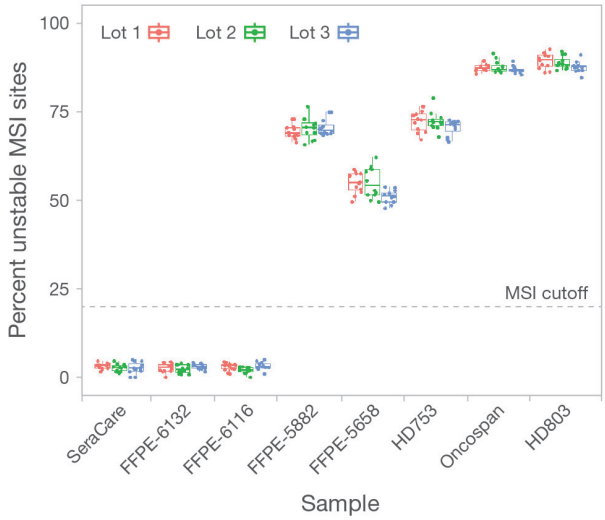

Figure 6: MSI estimates are reproducible from TruSight Oncology 500— Eight unique samples were analyzed from FFPE tissues or cell lines. Each sample was analyzed by three independent operators, sequencing instruments, and reagent lots. A total of 36 replicates are shown per sample, with 12 replicates per lot (reagent lot/operator/instrument).

Summary

The comprehensive nature and novel algorithms of TruSight Oncology 500 provide assessment of important cancer-related genes while also enabling evaluation of important immunotherapy biomarkers such as TMB and MSI status. Recent studies have demonstrated that high TMB and positive MSI status identify patient populations that benefit from immunotherapy.[2-4,7,8] Because the financial cost of WES may be prohibitive when developing a personalized medicine approach, there is interest in obtaining accurate assessment of TMB with less sequencing. In this application note we described how TMB is detected using TruSight Oncology and evaluated its TMB detection performance. Results demonstrate that TruSight Oncology 500 demonstrates high concordance with WES for accurate assessment of TMB. Furthermore, evaluation of MSI status showed high concordance with a PCR-based assay. TruSight Oncology 500 is a high-performance assay that allows the user to evaluate TMB, MSI, and somatic variants from the same sample and workflow.

Learn more

For more information about TruSight Oncology 500, visit www.illumina.com/tso500

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