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Input Recommendations for TMB, MSI, and Small Variant Analysis with TruSight™ Oncology 500

Robust performance with FFPE-extracted DNA across input amounts, tissue types, and DNA quality levels.

Introduction

TruSight Oncology 500 is a next-generation sequencing (NGS) assay that analyzes 523 cancer-relevant genes from DNA, and 55 genes from RNA*. Using proven enrichment chemistry, TruSight Oncology 500 enables robust measurement of tumor mutational burden (TMB), microsatellite instability (MSI), and small variants in DNA extracted from formalin-fixed paraffin-embedded (FFPE) tumor samples. While not the focus of this application note, fusions and splice variants can also be detected from FFPE-extracted RNA within the same workflow.

Although the recommended input is 40 ng DNA and RNA*, yields from FFPE samples can vary widely. This application note evaluates TMB, MSI and small variant detection performance of TruSight Oncology 500 across various tumor types with a range of input amounts and DNA quality. Notably, this evaluation suggests that decreasing the DNA input amount from 40 to 20 ng does not significantly affect TMB, MSI and small variant detection.

Methods

FFPE-based tumor tissue samples were procured from third-party vendors. For genomic DNA extraction, the AllPrep DNA/RNA FFPE Kit (Qiagen) was used. To assess MSI, TMB, and small variants at DNA input ranges below and above the recommended level of 40 ng DNA, two sets of samples were used (Table 1). The first set included five tumor types, and each sample was analyzed with input amounts at 10, 20, 30 and 40 ng DNA input. The second set included 11 tumor types, each analyzed at 40, 120 and 500 ng of DNA input. DNA quality was assessed by comparing the DNA amplification potential to that of a non-FFPE reference gDNA to generate a ΔCq value. Samples that amplified at later cycles than control DNA were of lower quality than samples with ΔCq values close to zero.

DNA libraries were prepared according to the TruSight Oncology 500 Reference Guide.[1] Sequencing was performed on the NextSeq™ 550Dx System in research mode using high-output reagents with eight libraries per flow cell at 2×101 bp read length. The resulting sequencing data was analyzed using the TruSight Oncology 500 Local App[2] to identify small variants, and calculate percent unstable MSI sites and TMB scores. More details on the methods used to measure TMB and MSI, along with performance data, are described in an accompanying application note.[3]

MSI analysis with varied DNA inputs

TruSight Oncology 500 uses 125 homopolymer microsatellite loci to evaluate MSI status. For a site to be included in the evaluation of the MSI status of a sample, a minimum number of 60 reads spanning the microsatellite region must be observed. Microsatellite unstable sites are scored by dividing the number of unstable sites by the total number of sites assessed (percent unstable sites). To observe the effect of DNA input, FFPE-extracted DNA was titrated from 40 ng to 10 ng. The percentage of unstable sites remained unchanged when input was lowered from the recommended 40 ng to 20 ng (Figure 1A).

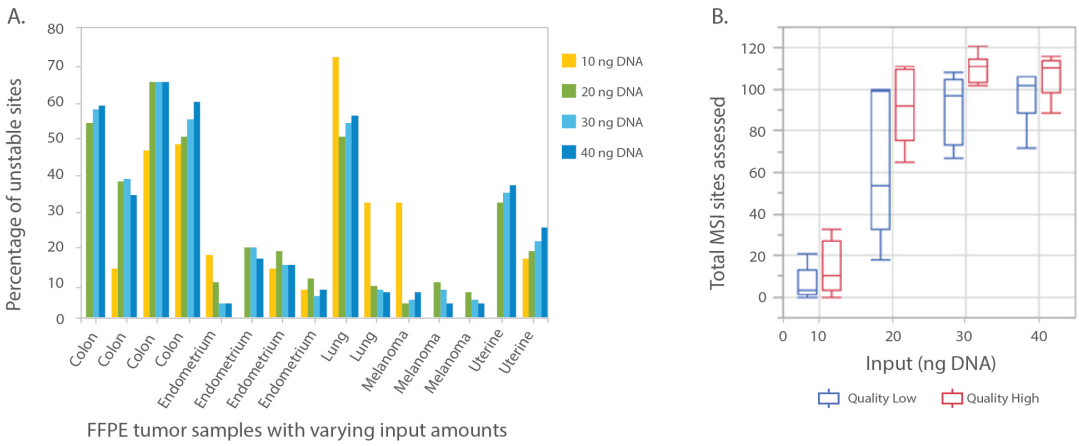

Figure 1: MSI analysis with FFPE-extracted DNA below recommended input levels—(A) Percentage of unstable sites from tumor samples with 40-10 ng input DNA. (B) Total eligible MSI sites assessed from tumor samples of lower quality (blue, ΔCq between 3-6, n=7) and higher quality (red, ΔCq < 3, n=8) at input levels between 40-10 ng.

* The products to evaluate DNA and RNA variants consist of the TruSight Oncology 500 DNA panel and the TruSight Tumor 170 RNA panel.

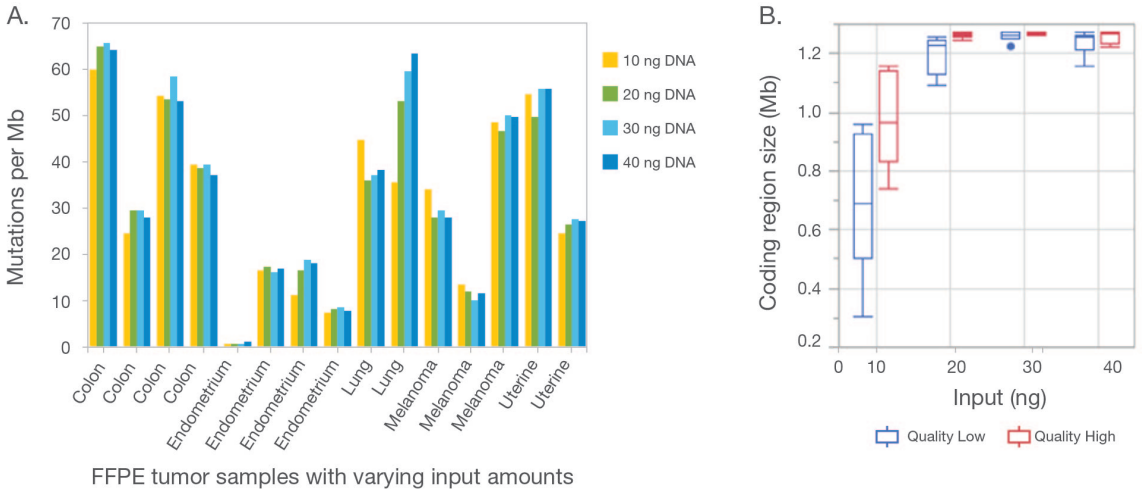

Figure 2: TMB estimation with FFPE-extracted DNA below recommended input levels—(A) TMB values from tumor samples with 40-10 ng input DNA. (B) Eligible mean coding region size from tumor samples of lower quality (blue, ΔCq between 3-6, n=7) and higher quality (red, ΔCq < 3, n=8) at input levels between 40-10 ng DNA.

Using 10 ng of DNA input did yield altered MSI results for many samples, partly due to a dramatic decrease in the total number of microsatellites assessed (data not shown).

To demonstrate the impact of both reduced input and lower-quality DNA on MSI detection, samples were grouped according to their quality value. A cutoff ΔCq value of 3 was used to distinguish higher- and lower-quality samples. Samples were plotted by their quality versus the mean total assessed sites per input amount (Figure 1B). Regardless of sample quality, the addition of only 10 ng of DNA resulted in a large reduction in sites assessed. However, lower-quality samples yielded better results with increased input amounts, as demonstrated by the reduced variability seen at 30 and 40 ng compared to 20 ng. Total microsatellite sites assessed in higher-quality samples appear to plateau near 30 ng, indicating no significant improvement with increased inputs. Additional samples analyzed with up to 500 ng input displayed similar results with stable MSI scores and no significant improvement in the percent of sites assessed (data not shown). Importantly, no deleterious effects with inputs up to 500 ng were observed (data not shown).

TMB estimation with varied DNA inputs

The effective coding region used for TMB analysis is calculated for each sample and requires a minimum coverage of 50× for inclusion. TMB is measured as the number of mutations (SNVs or indels) per Mb of coding region assessed. To observe the effect of low input on TMB estimation, FFPE-extracted DNA was titrated from 40 ng to 10 ng. Similar to the trend found with MSI analysis, TMB performance was consistent between 40-20 ng DNA input, while 10 ng input yielded increased variability in resulting TMB values (Figure 2A). Variability in TMB estimation correlated with a decrease in the size of eligible coding region when input amounts were reduced (Figure 2B). This reduction was most pronounced in lower-quality samples with greater variability at 10 and 20 ng input.

Table 1: FFPE samples analyzed

DNA input range	No. of samples	Tumor types
10-40 ng	15	Colon, Endometrium, Lung, Melanoma, Uterine
40-500 ng	12	Breast, Kidney, Prostate, Stomach, Uterus, Cervix, Skin, Colon, Bladder, Lung, Lymph node

To evaluate the effects of input amounts above the recommended 40 ng, the second set of FFPE samples were analyzed with 40, 120, and 500 ng input. No significant change in TMB estimation occurred at higher input levels (Table 2).

Table 2: TMB estimation with input amounts above 40 ng DNA

Tumor Type	Mutations per Mb		
	40 ng	120 ng	500 ng
Bladder	9.5	10.2	9.5
Breast	1.6	1.6	1.6
Cervical	7.9	7.1	7.1
Colon	1.6	0.0	1.6
Kidney	5.5	6.3	6.3
Lung	6.3	6.3	4.7
Lymph Node	9.5	11.8	9.5
Prostate	0.8	1.6	0.0
Skin	0.8	2.4	0.8
Stomach	4.0	3.9	5.5
Uterine	18.2	22.1	22.1
Uterine	7.1	7.9	7.9

Table 3: Small variant detection at varying DNA input levels

			Variant allele frequency			
Sample source	Gene	Variant	10 ng	20 ng	30 ng	40 ng
Colon	*APC*	p.R1114*	0.2818	0.2381	0.2938	0.3062
Colon	*APC*	p.R1450*	0.2869	0.3051	0.2968	0.3057
Colon	*BRAF*	p.V600E	0.1711	0.1833	0.2044	0.176
Colon	*BRAF*	p.V600E	0.4694	0.1477	0.2827	0.2628
Lung	*BRAF*	p.G643G	0.5667	0.4286	0.4484	0.3755
Melanoma	*BRAF*	p.V600K	0.8088	0.7965	0.7894	0.8117
Melanoma	*CSDE1:NRAS*	p.Q61R	0.3871	0.3211	0.3551	0.3853
Endometrium	*CTNNB1*	p.T41A	ND	ND	0.0133	0.0097
Endometrium	*CTNNB1*	p.G34R	0.0966	0.0426	0.0861	0.0612
Endometrium	*FGFR2*	p.S252W	0.4845	0.4686	0.4082	0.4249
Colon	*FLT3*	p.T227M	0.4217	0.5000	0.4332	0.4696
Colon	*KRAS*	p.A146T	0.3636	0.3477	0.3074	0.3557
Colon	*KRAS*	p.G12C	0.4468	0.4662	0.435	0.4392
Endometrium	*MAP2K2*	p.I220I	0.531	0.4964	0.4406	0.4645
Endometrium	*PIK3CA*	p.Q546R	0.1379	0.1513	0.1252	0.1333
Uterine	*PIK3CA*	p.H1047R	0.6923	0.3662	0.4057	0.4625
Endometrium	*PTEN*	p.R233*	0.1745	0.1126	0.1291	0.1192
Colon	*RNF43:TSPOAP1-AS1*	p.G659fs*41	0.0484	0.1091	0.0984	0.0645
Melanoma	*TP53*	p.Q136*	0.3684	0.3194	0.324	0.3168

ND = not detected. Variants marked with asterisks indicate mutations resulting in premature stop codons.

Small variant calling with varied DNA inputs

TruSight Oncology 500 reports small DNA variants with 95% analytical sensitivity down to 5% variant allele frequency (VAF) at recommended inputs of 40 ng. To evaluate the performance of TruSight Oncology 500 in detecting small variants at lower input amounts, variant calling from the Catalogue Of Somatic Mutations In Cancer (COSMIC)[4] variants were assessed from the same samples used for the TMB and MSI detection studies. Reported VAFs for input amounts ranging from 10 to 40 ng are robust across all inputs tested (Table 3). While calling is unaffected at 120 and 500 ng (data not shown), input reductions do affect the detection of variants below the of 5% VAF limit of detection. This is seen in the inability to call *CTNNB1* p.T41A variant at input quantities of 10 and 20 ng.

Learn More

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

Summary

The TruSight Oncology 500 assay is designed to provide optimal performance using 40 ng DNA extracted from FFPE samples. This application note demonstrates that in a limited sample set, comparable performance for TMB, MSI, and small variant detection is achievable across of a range of tumor types and input levels. Using input amounts above the recommended level also had negligible effects on MSI scores, assessed coding region size, and TMB values. Performance was most impacted when lower-quality DNA (ΔCq values from 3-6) was analyzed at input levels below 30 ng. Under these conditions, impacted results correlate to reductions in total microsatellite sites assessed and eligible TMB coding region evaluated. Samples with reduced input are more likely to yield lower overall coverage and reduced reliability in calling variants at the detection limits. Therefore, care should be taken when interpreting MSI and TMB scores with samples approaching these lower limits due to reductions in input quantities.

References

1. Illumina (2018) TruSight Oncology 500 Reference Guide. Accessed January 24, 2019.
2. Illumina (2018) TruSight Oncology 500 Local App User Guide. Accessed January 24, 2019.
3. Illumina (2018) Analysis of TMB and MSI Status with TruSight Oncology 500. Accessed January 24, 2019.
4. COSMIC Catalogue of Somatic Mutations in Cancer. cancer.sanger.ac.uk/cosmic. Accessed March 5, 2019.

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