illumina®

Optimizing RNA input to detect gene fusions with TruSight™ Oncology 500 High-Throughput

TruSight Oncology 500 High-Throughput and the NovaSeq™ 6000 System provide a sensitive solution for detecting gene fusions.

Introduction

Gene fusions, known to drive tumorigenesis across many tumor types, are important cancer-related biomarkers included in key guidelines and clinical studies. Currently, more than 20,000 gene fusions have been discovered across 33 cancer types.[1] Many of these oncogenic fusion genes, such as *NTRK*, *ALK*, and *RET*, have multiple fusion partners, making it imperative that tumor profiling assays detect these genes agnostic of the fusion partner. To meet this challenge, Illumina offers TruSight Oncology 500 and TruSight Oncology 500 High-Throughput. These assays provide a proven next-generation sequencing (NGS) solution, using both RNA and DNA, for accurate analysis of multiple tumor types and biomarkers, including gene fusions, in a single assay.

Fusion-agnostic strategy

TruSight Oncology 500 and TruSight Oncology 500 High-Throughput take advantage of a hybrid-capture strategy to capture target fragments. This strategy uses probes that align to the gene of interest with sequencing proceeding in the 3' and 5' directions, capturing any adjacent sequence. Using this method, it's possible to identify and characterize novel and known fusions agnostic of the fusion partner without additional confirmation steps (Figure 1). In addition, hybrid-capture offers the sensitivity needed when working with degraded samples, such as FFPE tissue.

Other assays employ an amplicon-based approach that uses primer pairs to target a specific gene, or sequence, of interest. However, amplicon assays can be prone to dropouts when mutations occur at primer binding sites, yielding unreliable results and often requiring confirmatory testing.

In addition, while effective for identifying known fusions, the primer-pair design of traditional amplicon methods prevents them from amplifying novel events (Figure 1). Some amplicon assay providers have developed an indirect approach to uncovering novel fusions that relies on detecting a 5'/3' sequencing coverage imbalance. This method increases the likelihood of identifying a novel fusion, but requires a confirmatory test, such as fluorescent *in situ* hybridization (FISH), adding time and expense to a study. Furthermore, the fusion partner will remain uncharacterized.

Assay differences

TruSight Oncology 500 and TruSight Oncology 500 High-Throughput provide the same content and use the same hybrid-capture strategy for tumor profiling. The differences lie in the intended sequencing platforms, sample throughput, some reagents, minor workflow changes, and secondary analysis (Table 1).

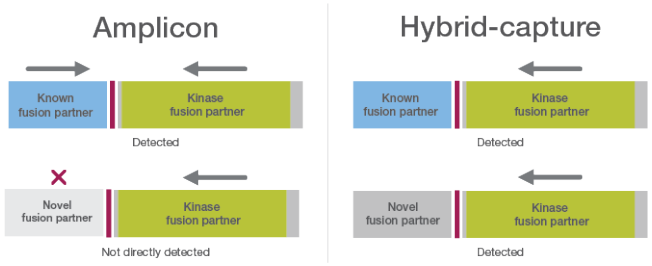

Figure 1: The hybrid-capture strategy detects novel fusions missed by an amplicon-based approach—An amplicon-based can use an indirect method based on 5'/3' imbalance to detect a novel fusion. This approach typically requires a confirmatory test and does not characterize the novel fusion partner.

Table 1: Differences between TruSight Oncology 500 and TruSight Oncology 500 High-Throughput

Parameter	TruSight Oncology 500	TruSight Oncology 500 High-Throughput
Sequencing system		
System	NextSeq 550 and NextSeq 550Dx[a] Systems	NovaSeq 6000 System; compatible with NextSeq 550 and 550Dx[a] Systems
Run cycle	2 × 101 bp, 2 × 8 bp (indexing)	2 × 101 bp, 2 × 10 bp (indexing)
Throughput	up to 8 samples per run	16-192 samples per run
Reagents		
Library prep indexes	SUA1 (Short Universal Adapters 1), UMI1 (Unique Molecular Identifier 1), UPxx (Unique Index Primer)	IDT for Illumina UMI DNA Index Anchors–Set A and Set B
Enrichment buffer	TCA1 (Target Capture Additives 1) and TCB1 (Target Capture Buffer 1)	EHB (Enrichment Hybridization Buffer)
Workflow		
Recommended RNA input amount	40 ng	40-80 ng
End-repair/A-tailing temperature	30°C/72°C	30°C/65°C
Library prep SPRI elution volume	27.5 µl	22.5 µl
Hybridization reaction	20 µl library + 15 µl TCB1 + 10 µl TCA1 + 5 µl probes	20 µl library + 25 µl EHB + 5 µl probes
Variant calling	Local Docker or on-instrument with Local Run Manager (LRM)	Local Docker

a. NextSeq 550Dx System in Research Mode

RNA input amounts

Both assays achieve highly sensitive results; however, a lower count of unique reads covering a gene fusion has been observed when using TruSight Oncology 500 High-Throughput. While 40 ng RNA can be sufficient for both assays in specific situations (Table 2), Illumina recommends using 40-80 ng RNA with TruSight Oncology 500 High-Throughput. It is highly recommended to use 80 ng RNA when working with low-quality RNA.

Although the update from the NextSeq™ System to the NovaSeq™ System is significant, this change is not causing the difference in RNA input performance. Both assays were run on both sequencing systems, producing concordant results between platforms for detecting RNA fusions. These studies rule out the sequencing platform as the source of the performance difference.

To ensure higher levels of multiplexing, TruSight Oncology 500 High Throughput has adopted a more diverse set of indexes, allowing for sequencing of up to 192 samples (DNA + RNA) in a single run. To achieve this, the length of the index sequences was increased and corresponding buffers and UMI index anchors were revised. During development of TruSight Oncology 500 High Throughput, the input requirement was increased to ensure that all relevant fusions were detected using the modified library preparation chemistry.

Optimizing results

Even with the decrease in read coverage, TruSight Oncology 500 High-Throughput detects gene fusions with high analytical sensitivity. To achieve the best results, it is important to optimize RNA input amounts, using a range from 40-80 ng, depending on RNA sample quality. This technical note demonstrates the impact of RNA inputs ranging from 20-80 ng on the number of sequencing reads produced when using TruSight Oncology 500 High-Throughput on the NovaSeq 6000 System.

Methods

TruSight Oncology 500 vs TruSight Oncology 500 High-Throughput

Analytical performance between TruSight Oncology 500 and TruSight Oncology 500 High-Throughput was compared using an in-house control sample containing a mix of pre-fragmented cell lines with multiple RNA variants to mimic clinical FFPE RNA samples. TruSight Oncology 500 was sequenced on the NextSeq 550 System. TruSight Oncology 500 High-Throughput was sequenced on the NovaSeq 6000 System.

RNA titration for TruSight Oncology 500 High-Throughput

Sample preparation

RNA was isolated from formalin-fixed paraffin-embedded (FFPE) tissue samples using the QIAGEN AllPrep DNA/RNA Mini Kit (QIAGEN, Catalog no. 80204). RNA amounts of 20 ng, 40 ng, 60 ng, or 80 ng from the same parent same were added to 8.5 µl buffer and used in library preparation with TruSight Oncology 500 High-Throughput, following the provided assay protocol.

Sequencing

Prepared libraries were sequenced on a NovaSeq 6000 S2 flow cell at a read length of 2 × 101 bp at full multiplexing capacity.

Analysis

Data was processed locally using the TruSight Oncology 500 v2.1 Local App. For RNA fusions, the count of unique supporting reads for the fusion event was considered, with a minimum of 5 unique supporting reads required to make a positive fusion call.

Results

Comparing TruSight Oncology 500 to TruSight Oncology 500 High-Throughput

Both TruSight Oncology 500 and TruSight Oncology 500 High-Throughput detected RNA fusions and splice variants in a control sample known to contain multiple RNA variants (Table 2). In most cases, the fusion or splice variant was detected above the threshold cutoff (5 unique supporting reads for fusions and 10 unique supporting reads for splice variants) for calling the variant.

Table 2: A comparison of the number of unique supporting sequencing reads generated across assays

	TruSight Oncology 500	TruSight Oncology 500 High-Throughput			
Fusion	40 ng	20 ng	40 ng	60 ng	80 ng
ABL1-BCR	106	18	42	43	74
ALK-EML4	16	0	7	7	8
ALK-TRMT61B	8	0	5	3	7
COL14A1-FGFR2	607	91	224	449	511
ERG-TMPRSS2	662	108	228	338	416
PTPN3-ALK	23	3	8	15	11
RET-CCDC6	98	16	52	90	78
ROS1;GOPC-SLC34A2	215	36	84	99	117
TACC3-FGFR3	162	61	135	192	111
RPS6KB1-VMP1	257	75	183	200	234
Splice variant					
MET exon 14 skipping	207	32	75	92	138

Comparison performed using an in-house control sample. TruSight Oncology 500 was sequenced on the NextSeq 550 System. TruSight Oncology 500 High-Throughput was sequenced on the NovaSeq 6000 System.

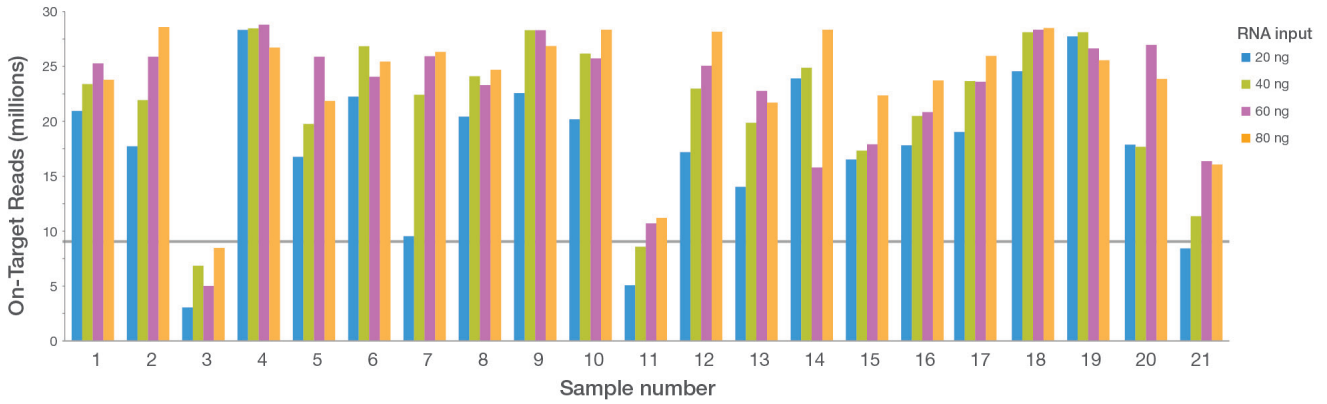

Figure 2: Total On-Target Reads increase with input RNA amounts—For most samples, an increase was in Total On-Target Reads, which measures the number of reads specific to a region, as the amount of input RNA increased. The solid line represents 9 million reads, which is the recommended QC performance metric.

RNA titration for TruSight Oncology 500 High-Throughput

Quality control

Post sequencing, all samples used in this study passed the following quality control (QC) standards: Median CV Gene 500× ≥ 0.93, Median Insert Size ≥ 80 bp, and Total On-Target Reads ≥ 9 million. These metrics were not heavily impacted by the decrease in the input requirement; however, in most cases, the number of Total On-Target Reads, which measures the number of reads specific to the region of interest, increased as the amount of input RNA increased (Figure 2).

RNA input amounts

This study focused on a subset of RNA fusions with clinical relevance. It is important to note that the protocol recommends using 40-80 ng input RNA with TruSight Oncology 500 High-Throughput to obtain optimal results. Many fusions were detected at a level passing the threshold cutoff (minimum of 5 reads) with as little as 20 ng input RNA, while all passed with the recommended 80 ng input (Table 3). Fusions were detected agnostic of the fusion partner; targeted RNA sequence is bolded. This allows researchers using the TruSight Oncology 500 assays to generate data on all fusion partners in a given sample. Two targeted splice variants were also successfully detected as part of the study (Table 4).

Table 3: Fusion detection at varying RNA input levels

Fusion	No. unique supporting reads					Tissue
	Detected	20 ng	40 ng	60 ng	80 ng	
BRCA2-*NRXN3*	√	6	33	60	84	Bone
FLT3-*GTF2F2*	√	0	9	10	14	Bone
FLT3-*LCP1*	√	0	12	32	47	Bone
FLT3-*SMOX*	√	21	50	72	54	Bone
FLT3-*VWA8*	√	12	29	51	69	Bone
EGFR-*METTL1*	√	29	25	84	71	Brain
EGFR-*RAB3IP*	√	7	5	9	19	Brain
MYC-*MRPL13*	√	7	27	35	52	Breast
MYC-*STK3*	√	7	11	39	28	Breast
CCDC170-***ESR1***	√	27	122	59	168	Kidney
ALK-*EML4*	√	11	15	21	40	Lung
ALK-*MRPL33; BRE*	√	0	2	16	6	Lung
ROS1;*GOPC-CD74*	√	0	0	5	7	Lung
ROS1;*GOPC-CD74*	√	45	104	92	141	Lung
ROS1;*GOPC-ENC1*	√	23	32	53	93	Lung
ROS1;*GOPC-HEXB*	√	0	0	0	7	Lung
ROS1;*GOPC-ARSI*	√	2	5	7	4	Lung
ALK-*BRE*	√	19	75	112	128	Sarcoma
EWSR1-*ATF1*	√	6	19	30	32	Sarcoma
EWSR1-*CBY1*	√	17	44	30	97	Sarcoma
NTRK3-*SEMA6A*	√	10	7	16	25	Skin
RET-*NCOA4*	√	53	74	78	154	Thyroid
*ANKUB1;RNF13-****ETV5****;DGKG*	√	11	29	45	72	Uterus
BRCA1-*MPP2*	√	5	25	28	29	Unknown

Data generated using RNA isolated from FFPE samples of the indicated tissue type and sequenced using TruSight Oncology 500 High-Throughput on the NovaSeq 6000 System. Target RNA sequence is bolded. Duplicate fusions are from unique samples.

Table 4: Splice variant detection at varying input levels

Splice variant	No. unique supporting reads					Tissue
	Detected	20 ng	40 ng	60 ng	80 ng	
EGFR v3	√	343	567	884	937	Brain
EGFR v3	√	802	1249	1614	2049	Brain
ARv7	√	11	26	38	46	Breast

Data generated using RNA isolated from FFPE samples of the indicated tissue type and sequenced using TruSight Oncology 500 High-Throughput on the NovaSeq 6000 System. Duplicate fusions are from unique samples.

Summary

TruSight Oncology 500 High-Throughput and the NovaSeq 6000 System offer a sensitive solution for gene fusion detection. While it is in the best interest of each lab to test varying levels of RNA to optimize results, Illumina recommends using 40-80 ng RNA to detect variants expressed at mid to high levels.* When sufficient RNA is available, 80 ng input helps maximize analytical sensitivity for fusions present at very low concentrations.

References

1. Hu X, Wang Q, Tang M, et al. TumorFusions: an integrative resource for cancer-associated transcript fusions. *Nucleic Acids Res*. 2018; 46:D1144-D1149.

* In-house assay validation was performed using 80 ng RNA input.

Illumina • 1.800.809.4566 toll-free (US) • +1.858.202.4566 tel • techsupport@illumina.com • www.illumina.com