APPLICATION NOTE

Easy and fast workflow for identifying RNA fusion events for cancer research

Using Illumina RNA Prep with Enrichment for TruSight™ RNA Fusion and TruSight RNA Pan-Cancer Panels

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Introduction

RNA transcripts are dynamically expressed and highly informative about the activity and condition of tissues at the cellular level. Targeted RNA sequencing (RNA-Seq) of tumor samples is a powerful method for quantifying expressed RNA transcripts and identifying gene fusions relevant for cancer research. Cancer cells accumulate numerous genetic changes, but typically only a few changes drive tumor progression.[1–3] Measurement of gene expression patterns using RNA-Seq can help differentiate these driver mutations from passenger mutations.[1–3] Gene fusion detection is important for cancer research, as 20% of all human tumors carry translocations and gene fusions.[4–6] Most gene fusions have a significant impact on tumorigenesis and a strong association with morphological phenotype, making them useful as potential biomarkers.[4–6]

Focusing on a subset of relevant genes enables RNA-Seq on a benchtop sequencing system with high sensitivity and efficient analysis. Enrichment, or hybrid–capture sequencing, offers a robust and comprehensive approach for researchers requiring deeper insights into complex genomic data. This method excels in detecting larger target regions and is highly sensitive, allowing for the identification of a broad range of genetic alterations, including known and unknown fusions, splice variants, and other critical biomarkers. Additionally, enrichment maintains efficacy even with challenging sample types, including formalin-fixed, paraffin-embedded (FFPE) specimens and other compromised DNA sources.

The TruSight RNA Fusion Panel and TruSight RNA Pan-Cancer Panel are used for enrichment-based RNA-Seq to help clinical researchers measure gene expression and detect gene fusions relevant to cancer.[7,8] The TruSight RNA Fusion Panel covers 507 fusion-associated genes and the TruSight RNA Pan-Cancer Panel targets 1385 genes for comprehensive assessment of cancer-related RNA transcripts (Table 1). Both panels accommodate as little as 10 ng of total RNA input for high-quality RNA or 20 ng for RNA from FFPE samples.

Easy and fast workflow

Using Illumina RNA Prep with Enrichment with the TruSight RNA Fusion and TruSight RNA Pan-Cancer oligo panels offers a rapid workflow to get focused results for cancer-related gene expression and fusion detection (Figure 1). The streamlined library preparation protocol features fewer steps, shorter incubation times, and numerous safe stopping points with a total assay time of only nine hours.[9] Illumina RNA Prep with Enrichment uses on-bead tagmentation technology to mediate a uniform tagmentation reaction, eliminating the need for separate fragmentation steps and saving time.[9] This is followed by simplified, single-cycle hybridization for probe panel–based enrichment. Enrichment can be performed at either a one-plex or three-plex format. A limited-cycle PCR program exponentially amplifies the enriched fragments to increase the amount of library. Sequencing is performed on an Illumina benchtop sequencing system and data is analyzed using BaseSpace™ Sequence Hub. This application note provides users with guidance and

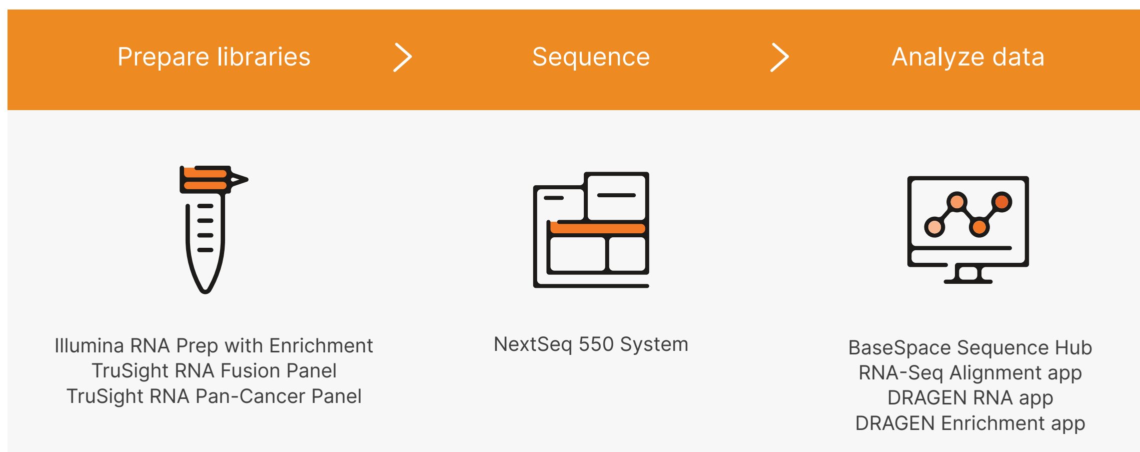

Figure 1: **TruSight RNA panels workflow using Illumina RNA Prep with Enrichment**—Targeted RNA-Seq for cancer research with the TruSight RNA Fusion and TruSight RNA Pan-Cancer panels and Illumina RNA Prep with Enrichment follows a streamlined comprehensive workflow including library prep, sequencing, and data analysis.

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Table 1: TruSight RNA panel content

Panel name	No. of probes	No. of target genes	No. of targeted exonic regions
TruSight RNA Fusion Panel	21,283	507	7690
TruSight RNA Pan-Cancer Panel	57,010	1385	21,043

performance data for use of Illumina RNA Prep with Enrichment with the TruSight RNA Fusion and TruSight RNA Pan-Cancer panels to identify cancer-related RNA gene expression and fusion events.

Methods

Library preparation

TruSight RNA Fusion Panel (Illumina, Catalog no. 20046101) and TruSight RNA Pan-Cancer Panel (Illumina, Catalog no. 20046104) performance with Illumina RNA Prep with Enrichment (Illumina, Catalog no. 20040536) was tested using both high-quality RNA (Table 2) and FFPE RNA (Table 3) sample types, at both one-plex and three-plex enrichment.

High-quality RNA samples used for the one-plex enrichment study were Universal Human Reference (UHR) RNA (Thermo Fisher Scientific, Catalog no. QS0639), total RNA from the K562 human leukemia cell line (ATCC, Catalog no. CCL-243), and Human Brain Reference RNA (HBRR) (Thermo Fisher Scientific, Catalog no. QS0611) at both 10 ng and 100 ng inputs. FFPE samples used for the one-plex enrichment study were the “FFPE-A” colon cancer sample, “FFPE-B” lung cancer sample, “FFPE-C” unknown cancer primary sample, and “FFPE-D” lymphoma sample at 20 ng inputs. At least three replicate libraries were generated from each sample type and used to form one-plex enrichment reactions. Both the TruSight RNA Fusion and TruSight RNA Pan-Cancer oligo panels were tested for each sample type at one-plex enrichment.

RNA samples used for the three-plex enrichment study were high-quality Universal Human Reference RNA and K562 total RNA at 10 ng inputs, and the “FFPE-E” colon cancer sample at 20 ng input. Three replicate libraries were generated from each sample type and used to form three-plex enrichment reactions. Both the TruSight RNA Fusion and TruSight RNA Pan-Cancer oligo panels were tested for each sample type at three-plex enrichment.

Table 2: High-quality RNA samples

Description	Note	Input quantity
Universal Human Reference RNA	Derived from a proprietary mixture of 10 human cell lines	10 ng, 100 ng
K562 total RNA	Derived from a chronic myelogenous leukemia cell line	10 ng, 100 ng
Human Brain Reference RNA	Derived from a multidonor pool of human brain tissues	10 ng, 100 ng

Table 3: FFPE RNA samples

Sample	Tissue	Diagnosis	DV_{200}[a]	Input quantity
FFPE-A	Cell pellet	Colon cancer	81.9	20 ng
FFPE-B	Lung	Lung cancer	45.5	20 ng
FFPE-C	Unknown	Unknown or unspecified cancer primary	36.5	20 ng
FFPE-D	Unknown	Lymphoma	56.1	20 ng
FFPE-E	Unknown	Colon cancer	59.7	20 ng

a. DV_{200} is the percentage of RNA fragments > 200 nucleotides. DV_{200} values provided from the sample respository.

Libraries were prepared by following the Illumina RNA Prep with Enrichment (L) Tagmentation reference guide (Illumina, Document no. 1000000124435 v03) using the TruSight RNA Fusion and TruSight RNA Pan-Cancer panels for the enrichment steps.

Sequencing

Prepared libraries were diluted to a final loading concentration of 1.8 pM, according to the NextSeq™ 550 System Denature and Dilute Libraries Guide (Illumina, Document no. 15048776 v18) and sequenced on the NextSeq 550 System at 2 × 74 bp read length using the NextSeq 500/550 High Output Kit v2.5 (150 cycles) (Illumina, Catalog no. 20024907) at a depth of ≥ 3M reads per sample. Note that 3M reads per sample was the minimum sequencing depth chosen for this study to examine metrics at the lower edge of performance. Many customer applications benefit from greater sequencing depth, up to 30M reads per sample.

Data analysis

Transcript coverage and alignment results were generated using the Illumina RNA-Seq Alignment app v2.0.2 on BaseSpace Sequence Hub. Expression analysis results were generated using the DRAGEN™ RNA app v4.2.4. Padded read enrichment (PRE) and padded unique read enrichment (PURE) results were generated using the DRAGEN Enrichment app v4.2.4. Fusion detection was checked using both the RNA-Seq Alignment app v2.0.2 and DRAGEN RNA app v4.2.4. Data were downsampled to 1M reads per sample (for the one-plex enrichment) or to 4M reads per sample (for the three-plex enrichment) to normalize for loading differences.

Results

Depletion of abundant transcripts

Outputs from the RNA-Seq Alignment app include an estimate of the proportion of RNA from highly abundant transcript species like ribosomal RNA (rRNA), which are typically of low information value. These abundant transcript species are depleted within the Illumina RNA Prep with Enrichment workflow during the enrichment phase. With both one-plex and three-plex preparation, abundant transcript species are reduced to less than 20% of reads and, frequently, to less than 10% of reads (Figure 2).

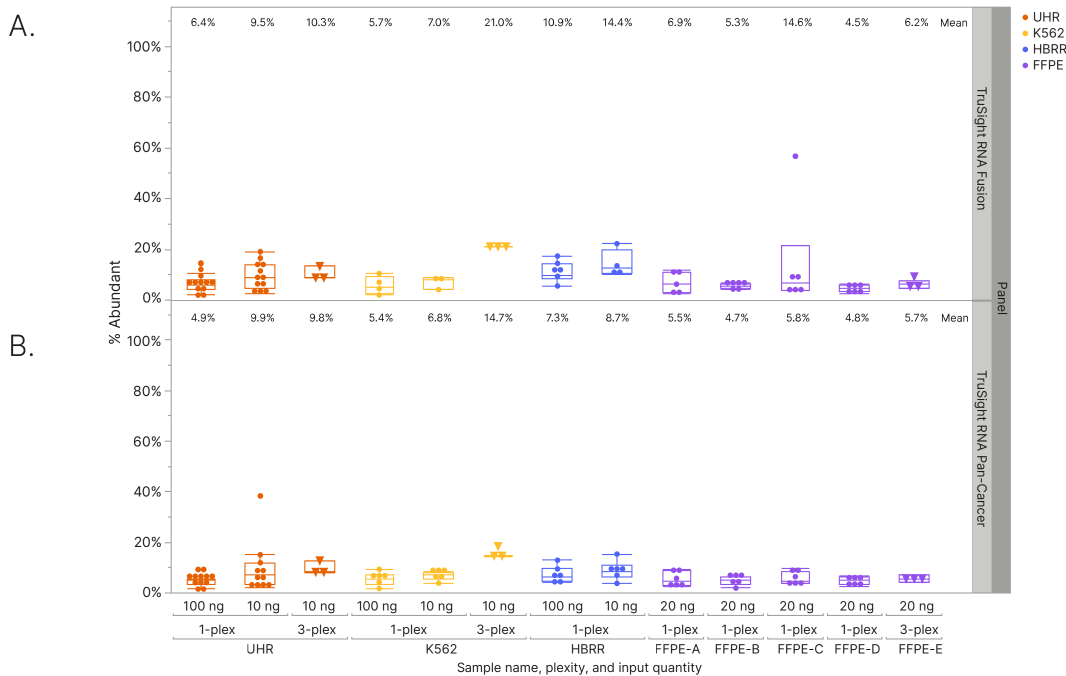

Figure 2: Effective depletion of abundant RNA species using Illumina RNA Prep with Enrichment—Using the (A) TruSight RNA Fusion and (B) TruSight RNA Pan-Cancer oligo panels for enrichment, less than 20% of RNA transcripts sequenced were rRNA or other highly abundant transcripts. UHR, Universal Human Reference RNA; HBRR, Human Brain Reference RNA.

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Percent duplicates

The percent of duplicate reads represents the percent of total reads that correspond to duplicates of unique sample fragments (frequently PCR duplicates). Lower percent duplicate values are generally preferred and show efficient use of sequencing bandwidth. In this study, reads were normalized to 3M reads per sample, at which point lower percent duplicate values indicate increased unique read content. Here, duplication rates correlate with input RNA sample quality and with oligo panel size (Figure 3). The smaller TruSight RNA Fusion panel produces more duplicates per sample than the TruSight RNA Pan-Cancer panel because the later has more unique targets that can be captured. For one-plex enriched libraries, the high-quality RNA samples exhibit lower percent duplicates than most FFPE samples and this is due to input sample quality. For three-plex enriched libraries, UHR samples show a mean duplication rate of ≤ 32% whereas the K562 cell line and FFPE-E samples show higher duplication rates, indicating less unique read content in the final library.

Read enrichment

High read enrichment values demonstrate that panel probes efficiently captured target libraries during enrichment, resulting in reduced sequencing of unwanted off-target regions. Two measures of enrichment are PRE and PURE. PRE refers to the percent of reads that map to panel-targeted regions or their immediate vicinity (padded regions), including duplicate reads. The PURE metric excludes duplicate reads and may appear lower when the proportion of duplicate reads is high (Figure 4). PRE is considered a more useful metric for some RNA-Seq applications.

For the one-plex enrichment study, enrichment across the high-quality RNA samples was generally > 70% by both metrics, indicating successful enrichment. This was true for both high- and low-input samples. Enrichment efficacy of FFPE samples was high for all samples for metric PRE and highly variable for metric PURE, but on average > 50%. This variability is correlated with the number of duplicate reads per sample as indicated by the color gradient on the plot (Figure 4). For the three-plex enrichment study,

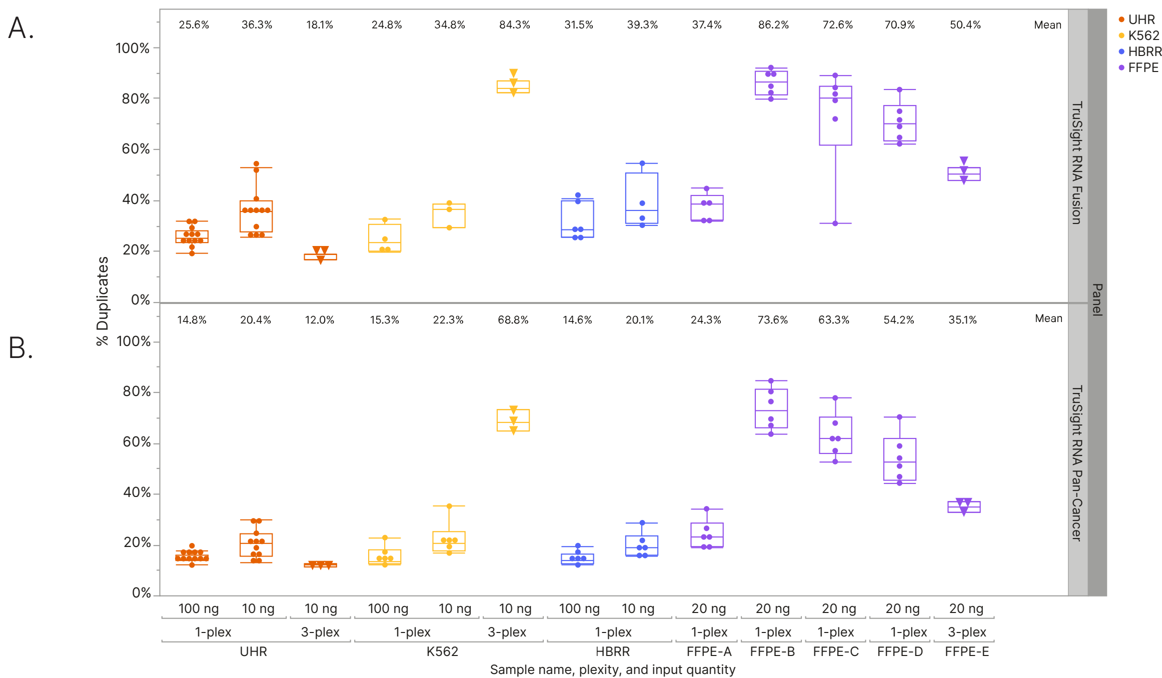

Figure 3: Percent duplicates using TruSight RNA panels and Illumina RNA Prep with Enrichment—Using the (A) TruSight RNA Fusion and (B) TruSight RNA Pan-Cancer oligo panels for enrichment, duplication rates correlate with input RNA sample quality and with oligo panel size.

PRE for high-quality RNA is > 70% and for FFPE is > 90%, suggesting sufficient enrichment with both TruSight RNA Fusion and TruSight RNA Pan-Cancer panels (Figure 4).

Gene fusion detection

TruSight RNA Fusion and TruSight RNA Pan-Cancer panels enabled reliable gene fusion detection (Table 4). The K562 cell line RNA sample containing the *BCR-ABL1* fusion was used as the applicable sample type to test detection when using Illumina RNA Prep with Enrichment. Results showed a 100% call rate for *BCR-ABL1* gene fusion in the K562 cell line across six replicates (10 ng and 100 ng, one-plex enrichment) and three replicates (10 ng, three-plex enrichment) when analyzed via both DRAGEN RNA app v4.2.4 and BaseSpace Sequence Hub RNA-Seq Alignment app v2.0.2.

Exon coverage

Data analysis with the DRAGEN Enrichment app revealed that Illumina RNA Prep with Enrichment resulted in > 85% of the bases covered aligning to coding sequence and untranslated regions (UTR) of RNA. These results demonstrate high capture efficiencies that focus sequencing efforts on the high-value content of RNA coding regions (Figure 5).

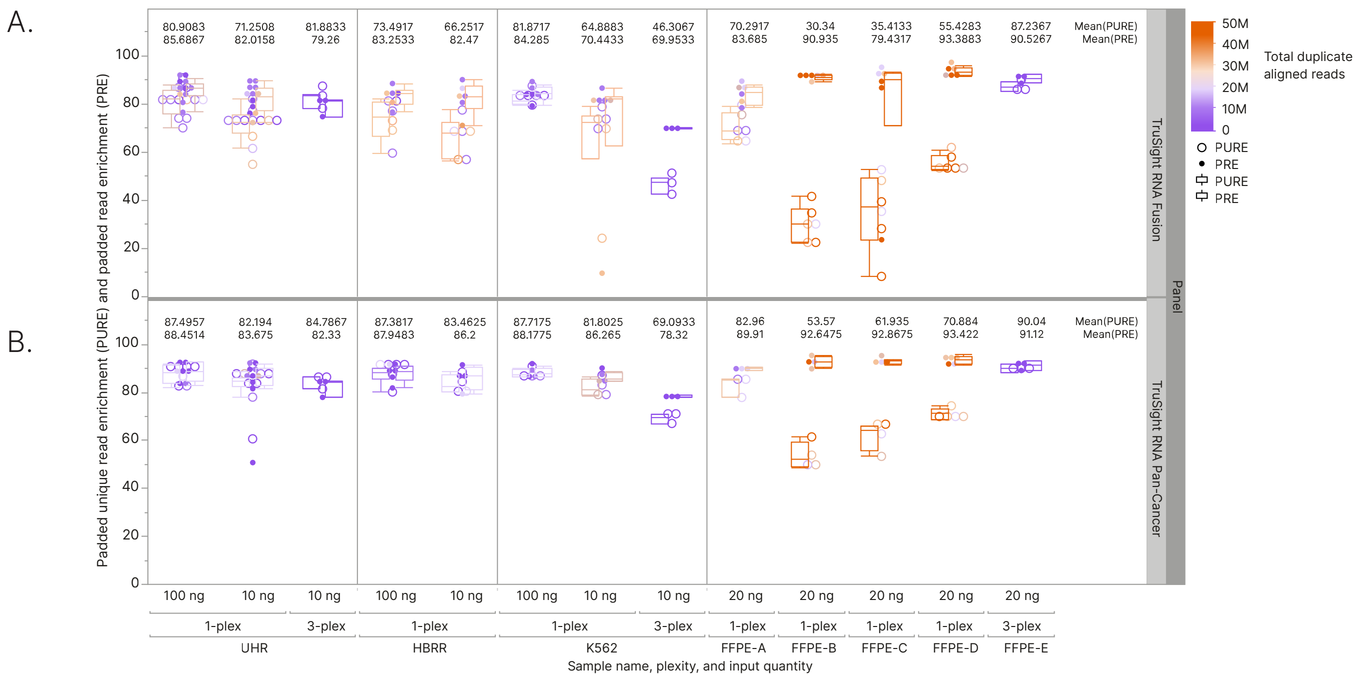

Figure 4: High enrichment efficiency using TruSight RNA panels and Illumina RNA Prep with Enrichment— Enrichment using the (A) TruSight RNA Fusion and (B) TruSight RNA Pan-Cancer oligo panels showed high efficiency across sample types with PRE, a metric that includes duplicates. PURE, a metric that excludes duplicates, is not as useful for RNA-Seq applications. Samples with high duplicate rates show high variability in PURE. Here, the duplicate read count is color-coded from 0 (purple) to 50M (orange). DRAGEN Enrichment analysis is executed without removing duplicate reads (ie, the default option "Mark Duplicates" box should be unchecked in the app). To produce enrichment analysis for the TruSight RNA Fusion and TruSight RNA Pan-Cancer panels using the DRAGEN Enrichment app, the *.bed file must be manually uploaded for each panel within BaseSpace Sequence Hub.

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Table 4: Gene fusion detection using TruSight RNA panels and Illumina RNA Prep with Enrichment

Fusion (source)	Panel	Enrichment	RNA input	Detection RNA-Seq Align v2.0.2	Detection DRAGEN RNA v4.2.4
BCR-ABL1 (K562)	TruSight RNA Fusion	1-plex	10 ng	6/6 (100%)	6/6 (100%)
			100 ng	6/6 (100%)	6/6 (100%)
		3-plex	10 ng	3/3 (100%)	3/3 (100%)
	TruSight RNA Pan-Cancer	1-plex	10 ng	6/6 (100%)	6/6 (100%)
			100 ng	6/6 (100%)	6/6 (100%)
		3-plex	10 ng	3/3 (100%)	3/3 (100%)

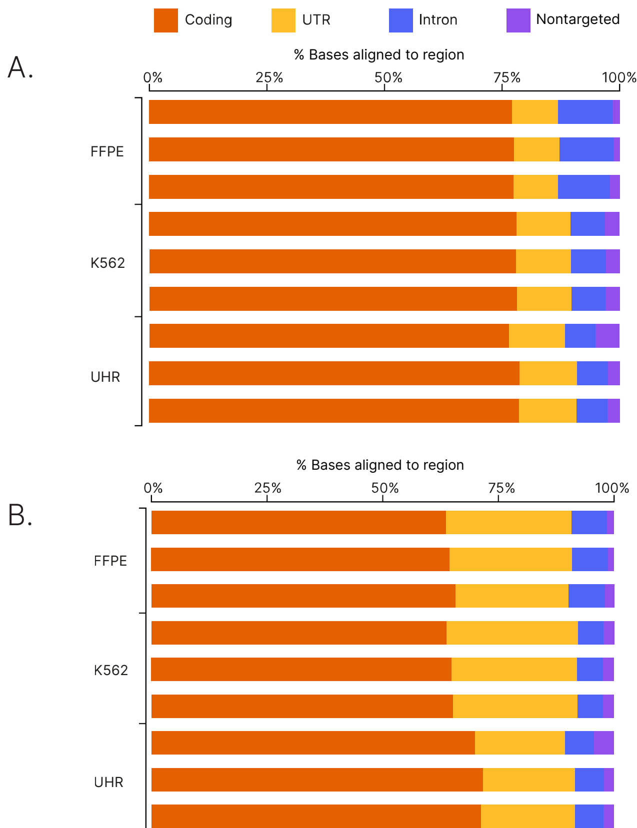

Figure 5: Coverage of coding regions with TruSight RNA panels and Illumina RNA Prep with Enrichment—Using the (A) TruSight RNA Fusion and (B) TruSight RNA Pan-Cancer oligo panels for enrichment results in high-capture efficiencies that align to coding sequence and untranslated regions (UTR).

Summary

By focusing on key genes of interest, targeted RNA sequencing enables researchers to study high-value content enriched with cancer-associated transcripts. Illumina RNA Prep with Enrichment and the TruSight RNA Fusion Panel or TruSight RNA Pan-Cancer Panel demonstrate highly reproducible results with the analytical sensitivity needed to detect rare transcripts and fusions. This workflow delivered robust performance for both panels, even with lower RNA input levels or lower quality RNA. Cancer researchers can benefit from a fast and easy workflow for targeted RNA analysis.

Learn more

TruSight RNA Fusion Panel

TruSight RNA Pan-Cancer Panel

Illumina RNA Prep with Enrichment

Ordering information

Product	Catalog no.
TruSight RNA Fusion Oligo Panel	20046101
TruSight RNA Pan-Cancer Oligo Panel	20046104
Illumina RNA Prep with Enrichment, (L) Tagmentation (16 samples)	20040536
Illumina RNA Prep with Enrichment, (L) Tagmentation (96 samples)	20040537
Illumina DNA/RNA UD Indexes Set A, Tagmentation (96 indexes, 96 samples)	20091654
Illumina DNA/RNA UD Indexes Set B, Tagmentation (96 indexes, 96 samples)	20091656
Illumina DNA/RNA UD Indexes Set C, Tagmentation (96 indexes, 96 samples)	20091658
Illumina DNA/RNA UD Indexes Set D, Tagmentation (96 indexes, 96 samples)	20091660

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