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Development and Analytical Validation of a Next Generation Sequencing Assay to evaluate T-Cell Diversity in a CAP-accredited, CLIA-Certified laboratory

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Introduction

The field of immuno-oncology (IO) is rapidly advancing our understanding of cancer biology and improving outcomes for a variety of tumor types. Novel NGS approaches enable sensitive evaluation of the highly diverse T-Cell Receptor (TCR) repertoire, emerging as a valuable tool for a variety of uses such as IO biomarker discovery, research and identification of biomarkers associated minimal residual disease, and chimeric antigen receptor T-cell analysis. Here we describe the analytical validation of our production workflow for T-Cell repertoire analysis utilizing the Oncomine™ TCR Beta short read (TCRb-SR) assay in a CAP/CLIA laboratory. The Oncomine™ TCRb-SR assay is an Ampliseq-based method that targets the highly variable CDR3 region of TCRb utilizing either DNA or RNA as input, with sample-to-result in 2 days.

The Oncomine™ TCRB-SR Assay

The Oncomine™ TCRB-SR assay identifies unique T-cell clones through AmpliSeq multiplex PCR targeting of the diverse complementarity-determining region 3 (CDR3) of the TCR gene locus in genomic DNA or RNA, utilizing forward primers targeting the framework 3 (FR3) V-region and reverse primers targeting FR3 J-region as illustrated in figure 1.

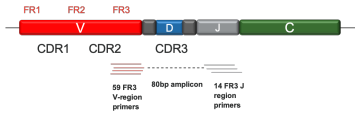

Figure 1: TCRB-SR Assay Design

Materials and Methods

The assay was validated using peripheral blood mononuclear cells and buffy coat specimens. DNA and RNA were extracted using the MagMAX™ DNA Multi-Sample Ultra 2.0 kit and the MagMAX™ *mirVana*™ Total RNA Isolation kit on the King Fisher™ Flex. Libraries were prepared using a minimum of 25ng RNA or 100ng DNA following TCRb kit instructions, followed by templating and sequencing using Ion Chef and S5 systems. Raw data was processed using Torrent Suite Software (v5.10) followed by alignment to the IMGT database of variable, diversity, and joining genes using Ion Reporter (v5.10). All bioinformatic parameters were locked and remained unchanged throughout validation. Analytical sensitivity of the assay was determined by serially diluting Jurkat cell line DNA/RNA with DNA/RNA from donor peripheral blood leukocytes (PBL) to a target clone frequency of 10^{-6}. Analytical accuracy was assessed via sample exchange with an offsite research laboratory (n=81) running a TCRb-SR workflow that was benchmarked to 5'RACE, utilizing a Pearson correlation to compare the $\log_{10}$ of V-gene usage across the two laboratories. Precision was determined via repeatability (3 replicates with 1 technologist within 24 hours) and reproducibility (4 replicates with 2 technologists prepared on different days across different sequencers), with precision measured by Pearson correlation of $\log_{10}$ of V-gene usage across replicates.

Laboratory Workflow

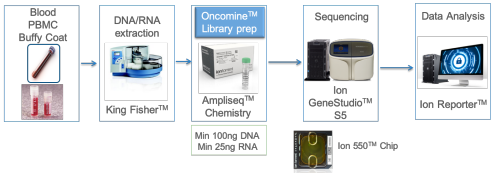

Figure 2: The Oncomine™ TCRB-SR Assay workflow with sample-to-result in 2 days

Analytical Sensitivity

The analytical sensitivity of the TCRB-SR assay was determined using dilution studies of Jurkat cell line gDNA and RNA. The presence of a single T cell clone in Jurkat cells enables precise control of dilution studies to evaluate the sensitivity of clone detection via the TCRB SR assay. Figure 3 depicts two spectratyping plots generated from TCRB short read gDNA sequencing performed at LTCSL:

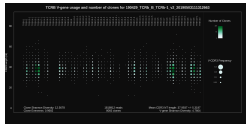

Figure 3A: Diverse TCRB Library (PBL)

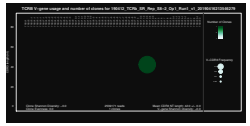

Figure 3B: Jurkat gDNA Library

To evaluate analytical sensitivity of clone detection in the background of a diverse library, Jurkat gDNA/RNA was spiked into peripheral blood leukocyte gDNA/RNA at spike-in levels ranging from 10^{-1} to 10^{-6}. Peripheral blood leukocyte gDNA/RNA was used as the background for spike in studies due to its high T-Cell diversity. Analytical sensitivity studies were designed to evaluate both the overall analytical sensitivity of the assay in terms of clone frequency, as well as the minimum nucleic acid input required to reach a given sensitivity. Our results enable applications such as repertoire profiling, rare clone detection, and minimal residual disease research. Figure 4 demonstrates the relationship between dilution levels of the gDNA dilution series, and table 1 summarizes sensitivity performance for each dilution level for both gDNA and RNA.

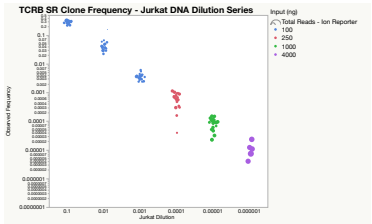

Figure 3: Jurkat gDNA frequency by dilution level

Analytical Sensitivity (cont.)

Dilution Level	gDNA				RNA			
	Input	TP	FN	Sens.	Input	TP	TN	Sens.
10^{-1}	100ng	20	0	100%	25ng	22	0	100%
10^{-2}	100ng	20	0	100%	25ng	22	0	100%
10^{-3}	100ng	20	0	100%	25ng	21	0	100%
10^{-4}	250ng	20	0	100%	25ng	22	0	100%
10^{-5}	1ug	19	1	95%	25ng	21	1	95%
10^{-6}	4x1ug	5	0	100%	100ng	35	1	97%

Table 1: Analytical Sensitivity Performance

Accuracy and Interfering Substances

TCRb repertoire concordance as determined by TCRb-SR was compared to immune repertoire data generated at an offside R&D laboratory that was blinded to expected results. The R&D laboratory was selected to use as the accuracy benchmark due to their extensive expertise developing, verifying, and validating TCRB assays. In addition, the offsite laboratory has performed orthogonal concordance studies comparing their TCRB workflow to results generated using mRNA based 5'RACE sequencing with high correlation. Analytical specificity (interfering substances) was assessed in this experiment as well by evaluation of assay performance in clinical research specimens that mimic the intended use case – fresh and cryopreserved specimens prepared from whole blood. For a total of 81 clinical research samples (50 gDNA and 31 RNA), the same extracted material was used for immune repertoire profiling via TCRB-SR at each site, with either 100ng gDNA or 25ng RNA used for library preparation. To quantitate assay accuracy, V-gene frequency data was obtained from Ion Reporter and compared for each sample pairwise using a Pearson correlation of $\log_{10}$(V gene frequency). Accuracy performance is summarized in table 2.

Analyte	Assay Input	N	Accuracy	95% Confidence Interval
gDNA	100ng	50	**0.96**	0.96-0.97
RNA	25ng	31	**0.96**	0.94-0.98

Table 2: TCRB SR Accuracy Performance

Precision

The precision of TCRb repertoire sequencing was evaluated by performing repeatability and reproducibility studies. To evaluate the variability in TCRb repertoire within a run (repeatability), 20 samples were run in triplicate by a single operator within a 24-hour period using 100ng input for gDNA or 25ng input for RNA. Results were compared between technical replicates by $\log_{10}$ Pearson correlation of V gene frequency. To evaluate the variability in TCRb repertoire across runs (reproducibility), 19 samples were run four times by a two operators on different days using 25ng input. Each operator prepared libraries on different days, used different sequencing barcodes, and used different sequencing instruments for reproducibility studies.
To quantitate assay reproducibility, V-gene frequency data was obtained from Ion Reporter and compared for each technical replicate pairwise using a Pearson correlation of $\log_{10}$(V gene frequency).

Precision (cont.)

Study	Analyte	Assay Input	N	Precision	95% Confidence Interval
Repeatability	gDNA	100ng	10	**0.98**	0.97-0.98
Reproducibility	gDNA	100ng	10	**0.97**	0.96-0.97
Repeatability	RNA	25ng	10	**0.98**	0.97-0.99
Reproducibility	RNA	25ng	9	**0.95**	0.93-0.97

Table 3: Assay Precision Performance

Assay Performance Summary

Validation Item	Analyte	Performance
Analytical Sensitivity	gDNA	>99% to an absolute clone frequency of 10^{-6}
	RNA	>97% to an absolute clone frequency of 10^{-6}
Accuracy	gDNA	0.96 (95%CI 0.96-0.97)
	RNA	0.96 (95%CI 0.94-0.98)
Intra-Run Precision (Repeatability)	gDNA	0.98 (95%CI 0.97-0.98)
	RNA	0.98 (95%CI 0.97-0.99)
Inter-Run Precision (Reproducibility)	gDNA	0.97 (95% CI 0.96-0.97)
	RNA	0.95 (95%CI 0.93-0.97)

Table 4: Analytical Performance of the Oncomine™ TCRB SR assay

Conclusions

This analytical validation was carried out in a CAP-accredited, CLIA-certified laboratory and was designed in accordance with CAP molecular pathology next generation sequencing requirements. We demonstrate highly sensitive clone detection in the background of a diverse T-cell repertoire via analytical sensitivity studies, and show highly accurate and reproducible assessment of T-cell landscape in a given specimen. In sum, we demonstrate accurate and precise profiling using as little as 100ng gDNA or 25ng RNA, with sample-to-result in 2 days.

Acknowledgements

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