The LymphoTrack® Flex Assay: A High-Throughput NGS Assay for the Assessment of Clonality in Lymphoproliferative Diseases

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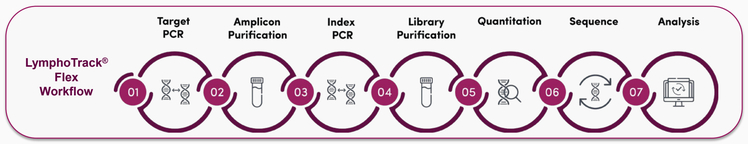
Introduction

Detection of clonal rearrangements within the immunoglobulin (Ig) and T-cell receptor (TCR) genes in clinical specimens is critical in the diagnosis and monitoring of lymphoproliferative disease. Sequence analysis using next-generation sequencing (NGS) based Ig/TCR detection methods can be used in the detection and monitoring of clonal rearrangements, providing comprehensive information on disease state.

We present the LymphoTrack Flex (LTF) NGS Assays[1] for the Illumina NextSeq1000. The LTF IGHV Leader, *IGH* FR1, and *TRG* Assays[1] enable users to process and sequence up to 96 samples per target, increasing throughput while reducing sequencing run costs. The LymphoTrack Enterprise Software[1] (LTE) features compatibility with NextSeq1000 data output and reduced analysis time. In addition to clonality assessment, the increased sequencing read depth allows individual sequence tracking for measurable residual disease (MRD) monitoring.

The LymphoTrack Flex Assays[1] and Enterprise Software[1] provide a cost effective, high-throughput solution for highly sensitive sequence detection for both clonality and MRD applications.

Materials & Methods



LymphoTrack Flex IGHV Leader, *IGH* FR1, and *TRG* Assays[1] employ a two-step PCR procedure: up to 96 samples per target (200ng input) are amplified in the first PCR (PCR1) and purified; sequencing indexes are added in the second PCR (PCR2). Indexed PCR products for each target are pooled and purified, then combined to create 3-target combination libraries. Libraries were sequenced on the NextSeq1000 using P1 600 cycle flowcell. FASTQ files were analyzed by the LymphoTrack Enterprise (LTE) Software v3.0.1, reporting V(D)J DNA sequences, total read counts, and the associated frequencies.

For analytical validation studies (Limit of Blank, Limit of Detection, Linearity, and Precision / Reproducibility), cell line DNA with a known clonal rearrangement (V(D)J sequence) for each target was diluted in polyclonal negative DNA. Testing for the assay was performed using 2 operators and 2 reagent lots; the 3-target combination libraries were sequenced on 2 NextSeq1000 instruments at 2 unique testing sites for a total of 4 sequencing runs.

Study	Dilution Level	N per Target per Run	N per Target	Grand Total (All Targets, All Runs)
Limit of Blank (LoB)	N/A (0%)	20	80	240
Limit of Detection (LoD) / Linearity	0.25%	16	64	192
	0.5%	10	40	120
	1.0%	10	40	120
	1.5%	16	64	192
	5.0%	16	64	192
	10%	4	16	48
Precision / Reproducibility	0.25%, 1.50%, 5.0%	16	64	192

[1]Research Use Only (RUO)

Results

Limit of Blank

The Limit of Blank (LoB) was calculated as the 95th percentile Percent Reads value of the top rank sequence observed over a total of 80 negative samples.

Limit of Blank		
IGHV Leader	*IGH* FR1	*TRG*
0.03%	0.02%	0.40%

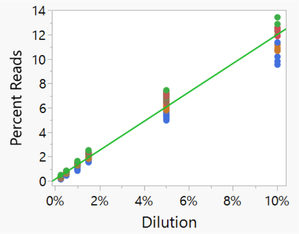
Results

Limit of Detection and Linearity

Limit of Detection (LoD) was determined as the lowest cell line dilution level at which at least 95% of replicates had a top rank Percent Reads value above the LoB value. Linearity was also determined through the R^2 value of the best-fit line for all dilutions per target.

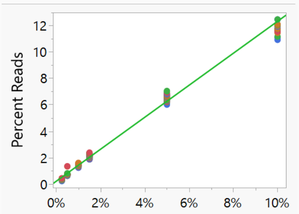
In the B-cell target IGHV Leader, the assay detected rearrangements at 0.25%, with all 64 replicates at the 0.25% level producing a Percent Reads value > 0.03%. The R^2 value was 0.97.

IGHV Leader				
N	DNA Dilution	Mean Percent Reads (%) (SD)	Median Percent Reads (%)	N(%) Top Rank Percent Reads>LoB (Final LoD)
64	0.25%	0.30 (0.07)	0.31	64 (100.0%)
40	0.50%	0.67 (0.11)	0.70	40 (100.0%)
40	1.00%	1.31 (0.18)	1.32	40 (100.0%)
64	1.50%	2.01 (0.25)	2.06	64 (100.0%)
64	5.00%	6.43 (0.70)	6.38	64 (100.0%)
16	10.00%	11.51 (1.14)	11.62	16 (100.0%)



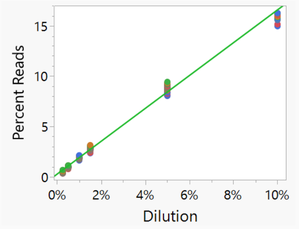
In *IGH* FR1, the assay detected rearrangements at 0.25% demonstrating high sensitivity, with all 64 replicates at the 0.25% level producing a Percent Reads value > 0.02%. The R^2 value was 0.99.

IGH FR1				
N	DNA Dilution	Mean Percent Reads (%) (SD)	Median Percent Reads (%)	N(%) Top Rank Percent Reads>LoB (Final LoD)
64	0.25%	0.33 (0.04)	0.33	64 (100.0%)
40	0.50%	0.73 (0.14)	0.71	40 (100.0%)
40	1.00%	1.40 (0.09)	1.40	40 (100.0%)
64	1.50%	2.10 (0.13)	2.12	64 (100.0%)
64	5.00%	6.53 (0.24)	6.56	64 (100.0%)
16	10.00%	11.74 (0.46)	11.76	16 (100.0%)



In the T-cell target *TRG*, the assay detected all replicates with rearrangements at 0.5%. At the 0.25% level, the clonal rearrangement was detected in 92% of the replicates. The R^2 was 0.99.

TRG				
N	DNA Dilution	Mean Percent Reads (%) (SD)	Median Percent Reads (%)	N(%) Top Rank Percent Reads>LoB (Final LoD)
64	0.25%	0.48 (0.06)	0.49	59 (92.1%)
40	0.50%	0.97 (0.08)	0.97	40 (100.0%)
40	1.00%	1.90 (0.11)	1.90	40 (100.0%)
64	1.50%	2.82 (0.15)	2.82	64 (100.0%)
64	5.00%	8.78 (0.32)	8.79	64 (100.0%)
16	10.00%	15.85 (0.39)	15.89	16 (100.0%)



Precision and Reproducibility

To evaluate Precision, Percent Reads values of the top V(D)J sequence result as reported by the LTE Software were analyzed for the 0.25%, 1.50%, and 5.0% dilution levels in each target. Overall %CV of Percent Reads values was <25%, with the highest variation occurring in the lowest dilution level of IGHV Leader, which is the longest amplicon product of the three targets. To evaluate Reproducibility, Percent Reads values were analyzed for sources of variation. 'Between Run', 'Between Operator,' and 'Within Run' demonstrated the greatest variation.

				Percent Reads Values Statistics			
Target	Top V(D)J Result	DNA Dilution	N	Mean Value	Median Value	SD	%CV
IGHV Leader	IGHV1-46_03 / IGHJ4_02	0.25%	64	0.31	0.31	0.08	24.9
		1.50%	64	2.02	1.98	0.28	14.1
		5.00%	64	6.33	6.29	0.83	13.1
IGH FR1	IGHV1-46_03 / IGHJ4_02	0.25%	64	0.34	0.33	0.05	15.1
		1.50%	64	2.11	2.08	0.16	7.5
		5.00%	64	6.53	6.42	0.32	4.9
TRG	TRGV11_01 / TRGJ1_02/TRGF2_01	0.25%	64	0.49	0.49	0.07	14.1
		1.50%	64	2.82	2.83	0.17	5.9
		5.00%	64	8.79	8.79	0.36	4.1

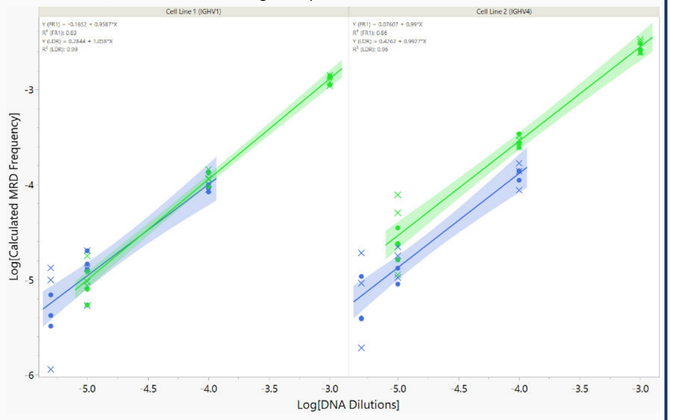
Results

MRD Level Detection

To test MRD detection, DNA from two ALL-positive cell lines (IGHV1 and IGHV4) were diluted in healthy pooled peripheral blood (PB) DNA and healthy pooled bone marrow (BM) DNA.

Samples were tested using the LymphoTrack Flex Assay[1] at 2000ng in triplicate and sequenced on the NextSeq1000. FASTQ files were analyzed using the MRD functionality in the LymphoTrack Enterprise (LTE) Software.[1] To estimate cell equivalents, the LymphoQuant® Internal Control[1] was spiked into each sample.

Both cell lines were detected in both sample types. The log calculated frequencies of each dilution were plotted below, with solid dots denoting BM and Xs denoting PB. A best-fit line was plotted for each target (*IGH* FR1 in blue, IGHV Leader in green).



	Cell Line 1 (IGHV1)		Cell Line 2 (IGHV4)	
	IGH FR1	IGHV Leader	*IGH* FR1	IGHV Leader
R^2	0.83	0.99	0.86	0.96
Slope	0.95	1.05	0.99	0.99

At 2000ng input, the IGHV Leader Assay achieved a limit of detection of 1 x 10^{-5} (corresponding to approximately 3 clonal equivalents), while the *IGH* FR1 Assay demonstrated a limit of detection of 5 x 10^{-6} (approximately 2 clonal cell equivalents).

Conclusions

- The LymphoTrack Flex IGHV Leader, *IGH* FR1, and *TRG* Assays[1] demonstrate high analytical sensitivity and accuracy in clonal sequence detection.
- Standardized, automation-compatible workflows across all three targets streamline laboratory processes and improve reproducibility. When paired with the Dockerized LymphoTrack Enterprise Software,[1] results are automatically analyzed to provide detailed clonal sequence and frequency data with minimal hands-on time.
- The LymphoTrack Flex Assays[1] demonstrate MRD-level sensitivity at higher DNA inputs and deliver results in under 50 hours, making them an optimal solution for high-throughput laboratories seeking scalable, automated, and cost-efficient NGS-based clonality assessment.

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