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Detecting B-Cell Clonality in Clinical Samples using a Comprehensive NGS LymphoTrack Dx® *IGH* FR1/2/3 Assay

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Abstract

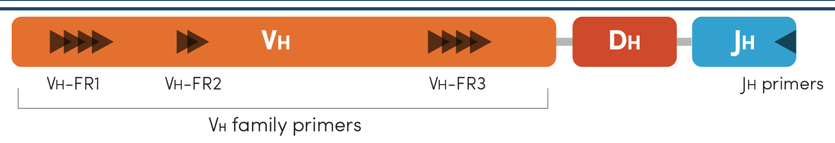
Introduction: PCR-based methods targeting immunoglobulin heavy chain (*IGH*) frameworks 1, 2, 3 (FR1, FR2, FR3), and joining region (JH) are the current gold standard for clonality testing in suspected B-cell proliferations. Recently, next-generation sequencing (NGS) based approaches for immune receptor genes have been suggested to improve sensitivity and identify the specific V-(D-)J DNA sequence required to track clones in follow-up testing. We developed the comprehensive LymphoTrack Dx® *IGH* FR1/2/3 Assay for both the Illumina® MiSeq® and Thermo Fisher Scientific® Ion PGM™ platforms, which detect the vast majority of rearrangements in a single NGS run. Here, we report a pilot study on detecting suspected B-cell clonality using the LymphoTrack Dx® *IGH* FR1/2/3 Assay.

Methods: The LymphoTrack Dx® *IGH* FR1/2/3 Assay was been developed for both the MiSeq® and Ion PGM™ platforms. The proprietary V and J consensus primers were designed and adapted to enable the PCR products to be sequenced directly on either the MiSeq® or Ion PGM™ platform. Each MiSeq® *IGH* FR master mix uses 1 of 24 different indices, allowing analysis of 22 samples with 2 controls. Each Ion PGM™ *IGH* FR master mix uses 1 of 12 different indices, allowing analysis of 10 samples with 2 controls. One-step PCR was followed by amplicon purification. Purified equimolar amounts of amplicons were pooled to form a library, which was loaded onto the MiSeq® or Ion PGM™. The sequencing data was analyzed using Invivoscribe® LymphoTrack Dx® bioinformatics software, which sorts the sequences by both index and FR, generates frequency distributions, and determines the somatic hypermutation (SHM) rate of FR1 amplicons.

Results: The analytical performance of the LymphoTrack Dx® *IGH* FR1/2/3 Assay on both platforms was evaluated using contrived samples with known V-J rearrangements. Both NGS assays demonstrated excellent linearity (R^2>0.96), clonality sensitivity (2.5%), and reproducibility (<20% CV). The clinical performance of the *IGH* Assay was evaluated with over 40 clinical samples that have been tested by control methods. Results demonstrated that clonality detection missed by one FR was detected by another FR, increasing the overall detection rate. Both the MiSeq® and Ion PGM™ NGS assays demonstrated excellent concordance in detecting clonality. In addition, data generated with the MiSeq® and the Ion PGM™ assays were concordant.

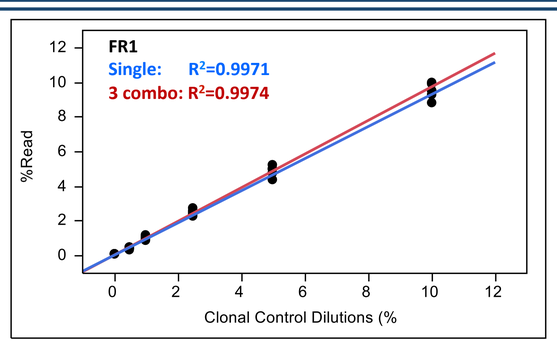
Conclusions: A comprehensive LymphoTrack Dx® *IGH* FR1/2/3 Assay was developed for both MiSeq® and Ion PGM™ platforms that identifies clonal *IGH* V-J rearrangements and DNA sequences. Assay performance demonstrated that combining FR1, FR2, and FR3 helps to decrease the false-negative rate due to failure in amplification of any individual targeted FR region. The inter-assay concordance in clonality detection using assays developed for the MiSeq® and Ion PGM™ platforms was excellent.

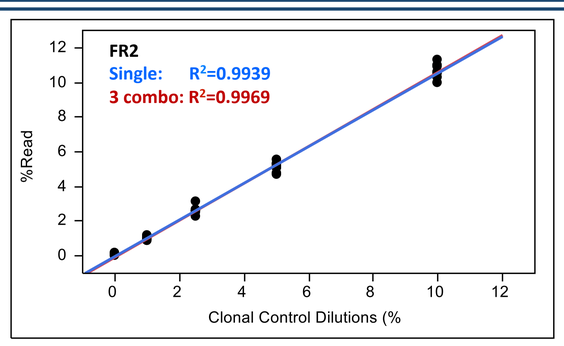
Materials and Methods

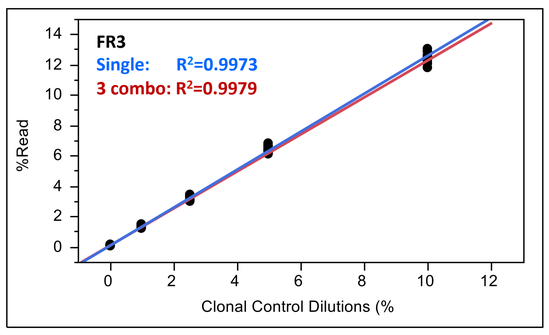


- The CE-marked LymphoTrack Dx® *IGH* FR1/2/3 Assays for the MiSeq® and Ion PGM™ were manufactured under cGMP standards and QC tested under a QSR-compliant regulatory system prior to use.
- Limit of detection (LoD), linearity, precision and reproducibility (P/R) were calculated using clonal control DNA diluted in wild-type polyclonal (tonsil) DNA.
- DNA from a variety of samples (35 from peripheral blood, 4 from bone marrow aspirates, and 1 from lymph node) were extracted using common extraction methods by collaborators.
- Libraries were prepared with amplicons generated by the LymphoTrack Dx® *IGH* FR1/2/3 Assay optimized for each NGS platform.
- Libraries were either sequenced for each FR individually or for all FRs (*IGH* FR1/2/3) combined.
- LymphoTrack Dx® Software - MiSeq® and LymphoTrack Dx® Software - PGM™ analyzed FASTQ data from the MiSeq® and the Ion PGM™, respectively.
- All statistical analyses were performed in JMP®.

MiSeq® *IGH* Results: LoD, Linearity, Precision and Reproducibility

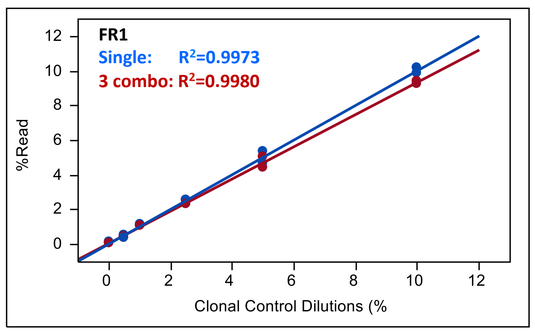


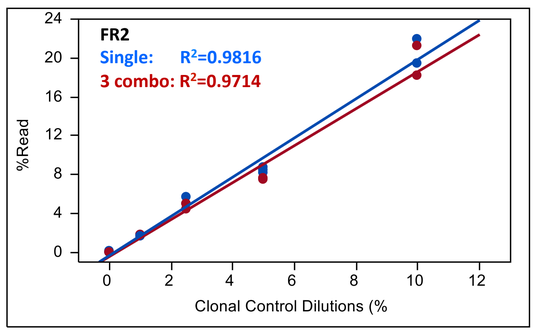


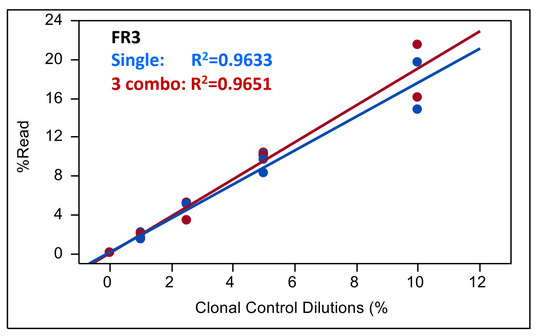


Clonal Control Dilutions (%)	N	MiSeq® *IGH* FR1			MiSeq® *IGH* FR2			MiSeq® *IGH* FR3		
		Size (bp)	Mean % Reads	CV%	Size (bp)	Mean % Reads	CV%	Size (bp)	Mean % Reads	CV%
10	32	295	5.08	17.5	243	15.74	9.9	104	12.99	6.2
5	48	295	2.58	19.4	243	7.72	8.2	104	7.04	8.4
2.5	48	295	1.28	14.1	243	3.93	9.2	104	3.50	8.0
1	48	295	0.50	29.3	243	1.52	12.5	104	1.43	16.1
0	16	varies	0.04	34.8	varies	0.06	26.5	104	0.07	28.6

Ion PGM™ *IGH* Results: LoD, Linearity, Precision and Reproducibility







Clonal Control Dilutions (%)	N	Ion PGM™ *IGH* FR1			Ion PGM™ *IGH* FR2			Ion PGM™ *IGH* FR3		
		Size (bp)	Mean % Reads	CV%	Size (bp)	Mean % Reads	CV%	Size (bp)	Mean % Reads	CV%
10	32	295	10.86	4.7	243	19.81	6.9	104	17.98	19.6
5	32	295	5.20	6.7	243	8.80	13.0	104	10.70	9.3
1	16	295	0.97	12.4	243	1.91	14.2	104	2.14	13.1
0	16	varies	0.057	35.1	varies	0.05	45.9	varies	0.08	37.5

Results: Clinical Study between *IGH* MiSeq® and Ion PGM™ Assays

	MiSeq® *IGH* Assays				Ion PGM™ *IGH* Assays			
	FR1	FR2	FR3	FR1/2/3	FR1	FR2	FR3	FR1/2/3
Clonal (%)	14/40 (35%)	13/40 (33%)	12/40 (30%)	15/40 (38%)	13/40 (33%)	13/40 (33%)	15/40 (38%)	15/40 (38%)
Non-Clonal (%)	25/40 (63%)	25/40 (63%)	25/40 (63%)	23/40 (58%)	24/40 (60%)	27/40 (68%)	24/40 (60%)	22/40 (55%)

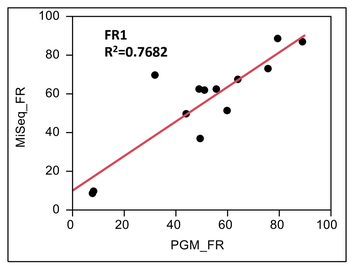
		Ion PGM™ FR1	
		Clonal	Non-Clonal
MiSeq® FR1	Clonal	13	1
	Non-Clonal	0	23

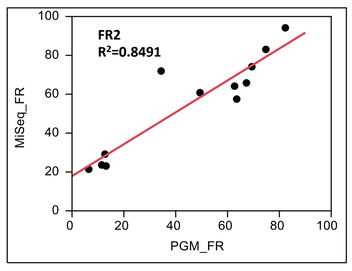
		Ion PGM™ FR2	
		Clonal	Non-Clonal
MiSeq® FR2	Clonal	12	1
	Non-Clonal	1	24

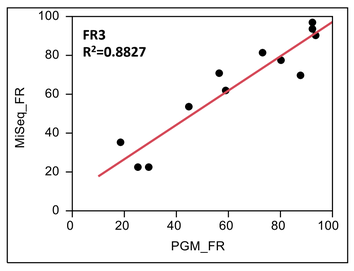
		Ion PGM™ FR3	
		Clonal	Non-Clonal
MiSeq® FR3	Clonal	12	0
	Non-Clonal	2	23

		Ion PGM™ FR1/2/3	
		Clonal	Non-Clonal
MiSeq® FR1/2/3	Clonal	14	1
	Non-Clonal	1	21

	FR1	FR2	FR3	FR1/2/3
Concordance (%)	97	95	95	95
Sensitivity (%)	100	92	86	93
Specificity (%)	96	96	100	95
PPV (%)	93	92	100	93
NPV (%)	100	96	92	95







Conclusions

A comprehensive NGS-based LymphoTrack Dx® *IGH* FR1/2/3 Assay was developed for both the Illumina® MiSeq® and Thermo Fisher Scientific® Ion PGM™ platforms. These assays identify clonal *IGH* V-J rearrangements and the specific clonal DNA sequences, critical for determining the SHM rate and tracking residual disease. Excellent concordance was demonstrated between these assays.

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