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Development of a Comprehensive *IGH* NGS Assay for Detecting Suspected B-Cell Clonality

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Abstracts

Introduction: PCR-based methods targeting immunoglobulin heavy chain (*IGH*) frameworks 1, 2, and 3 (FR1, FR2 and FR3) are the current gold standard for first-line clonality testing in suspected B-cell proliferations. Recently, next-generation sequencing (NGS) based approaches for testing immune receptor genes have been suggested to improve sensitivity and to identify the specific V-J DNA sequence required to track clones in follow-up testing. Here we report the development of a comprehensive NGS-based *IGH* assay which allows detection of all three FRs, simultaneously, in a single Illumina® MiSeq® run.

Methods: The LymphoTrack® *IGH* (FR1/FR2/FR3) Assay targets the FR1/J, FR2/J and FR3/J regions. The proprietary V and J consensus primers were designed and adapted to enable the PCR products to be sequenced on the MiSeq® platform. For each *IGH* FR, a set of 24 master mixes are provided, each designed with a different index, allowing analysis of 22 patient samples plus positive and negative controls. Single-step multiplexed PCR was followed by amplicon purification using the AMPure® XP PCR system. Purified equimolar amounts of amplicons from different samples and FRs were pooled to form a library. The harmonized and quantified library was sequenced using the MiSeq® v2 Reagent kit (500 cycles). LymphoTrack product data output from the MiSeq® were analyzed using Invivoscribe's proprietary bioinformatics software, which analyzes and sorts sequences by both index and FR, to generate frequency distributions of V-J rearrangements and to determine the somatic hypermutation (SHM) rate of the *IGHV* region based on the sequence of FR1/J amplicons.

Results: The ability to detect FR1, FR2 and FR3 regions was verified by testing different known V-J rearrangement B cell line DNA. Cell line DNA, serially diluted into tonsil DNA, demonstrated the limit of detection (LoD) at 5% dilution using 50 ng DNA input. The assay achieved excellent linearity (R2 > 0.95) and reproducibility (<25% CV), demonstrated by 2 operators, 2 MiSeqs and 2 lots of PCR master mixes. Preliminary testing of genomic DNA from peripheral blood (PB), bone marrow (BM) aspirates, and formalin-fixed paraffin-embedded tissue (FFPE) demonstrated that the clonality detection missed by one FR could be detected by another FR, thus increasing the overall detection rate.

Conclusions: A comprehensive NGS-based *IGH* assay has been developed for the Illumina® MiSeq® platform that identifies clonal *IGH* V-J rearrangements and DNA sequences. This LymphoTrack Assay has demonstrated that combining FR1, FR2 and FR3 helps to decrease the false-negative rate due to somatic hypermutation in primer binding sites of the involved V_H gene segments.

Materials and Methods

- The LymphoTrack® *IGH* (FR1/FR2/FR3) Assay targets the FR1/J, FR2/J and FR3/J regions. The proprietary V and J consensus primers were designed and adapted to enable the PCR products to be sequenced on the MiSeq® platform.

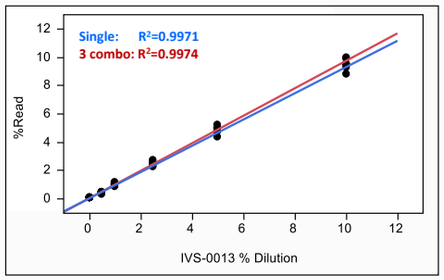
- For each *IGH* FR, a set of 24 master mixes are provided, each designed with a different index, allowing analysis of 22 patient samples plus positive and negative controls.
- Single-step multiplexed PCR was followed by amplicon purification using the AMPure XP PCR system. Purified equimolar amounts of amplicons from different samples and FRs were pooled to form a library. The harmonized and quantified library generated with the LymphoTrack Assay was sequenced using the MiSeq® v2 Reagent kit (500 cycles). LymphoTrack Assay output fastq data were analyzed using Invivoscribe proprietary bioinformatics software, which can sort the sequences by both index and FR, to generate frequency distributions of V-J rearrangements and to determine the somatic hypermutation (SHM) rate of the *IGHV* region based on the sequence of FR1/J amplicons.

- The analytical validation panel consists of tonsil DNA (IVS-0000) and a dilution series of 10%, 5%, 2.5% and 1.0%, cell line DNA (IVS-0013) diluted into tonsil DNA.
- For each FR, LoD and linearity were validated in both single FR and 3 combined FRs MiSeq® runs.
- Precision and reproducibility were validated for each FR. A total of combined 96 data points were generated from 8 MiSeq® runs (2 operators x 2 runs x 2 days) for each FR.
- DNA from different clinical samples was tested by both the capillary-based IdentiClone® *IGH* Clonality Assay and the NGS-based LymphoTrack® *IGH* (FR1/FR2/FR3) Assay.
- The statistical program JMP was used to perform the statistical analyses.

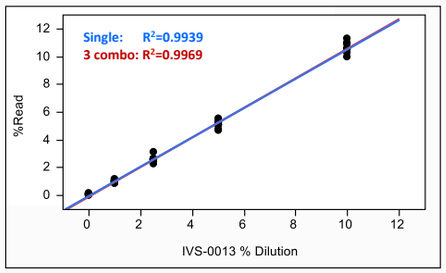
Results: Analytical Validations

•LoD and linearity validation

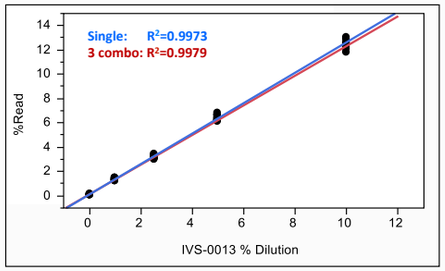
IGH FR1: Single versus 3 Combo (FR1+FR2+FR3)



IGH FR2: Single versus 3 Combo (FR1+FR2+FR3)



IGH FR3: Single versus 3 Combo (FR1+FR2+FR3)



- **Precision and reproducibility validation**

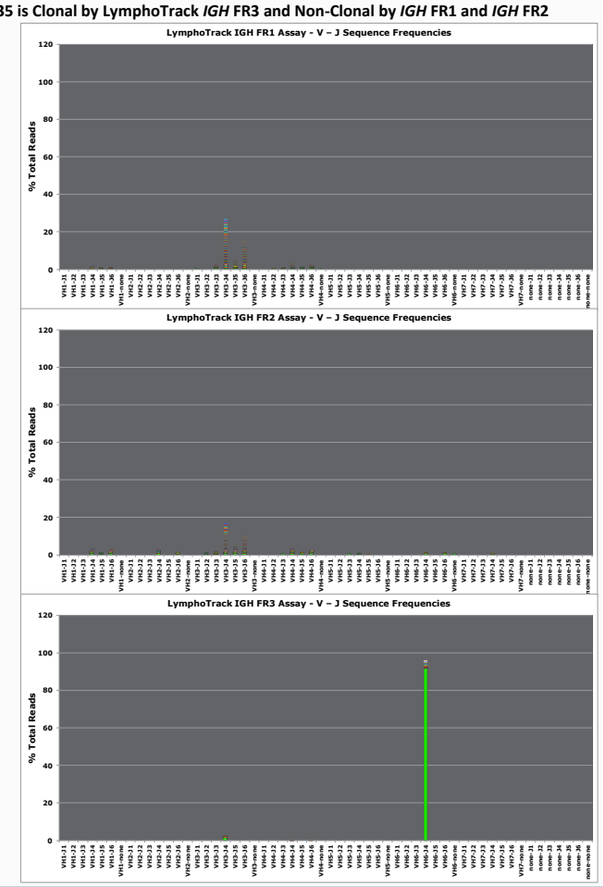
IVS-0013 %Dilutions	N	*IGH* FR1			*IGH* FR2			*IGH* FR3		
		Size (bp)	Mean % Reads	CV%	Size (bp)	Mean % Reads	CV%	Size (bp)	Mean % Reads	CV%
10	32	295	5.08	17.5	243	15.74	9.9	104	12.99	6.2
5	48	295	2.58	19.4	243	7.72	8.2	104	7.04	8.4
2.5	48	295	1.28	14.1	243	3.93	9.2	104	3.50	8.0
1	48	295	0.50	29.3	243	1.52	12.5	104	1.43	16.1
Tonsil	16	varies	0.04	34.8	varies	0.06	26.5	104	0.07	28.6

Results: Clinical Study

- **Clinical samples tested with the IdentiClone *IGH* Assay and the LymphoTrack *IGH* (FR1/FR2/FR3) Assay**

Sample	Sample ID	Sample Type	IdentiClone *IGH* (Capillary Electrophoresis)	LymphoTrack *IGH* FR1	LymphoTrack *IGH* FR2	LymphoTrack *IGH* FR3
1	P221	PB	Clonal	Clonal	Clonal	Clonal
2	P222	FFPE	Clonal	Clonal	Clonal	Clonal
3	P224	PB	Clonal	Clonal	Clonal	Clonal
4	P228	PB	Clonal	Clonal	Clonal	Clonal
5	P232	FFPE	Clonal	Clonal	Clonal	Clonal
6	P235	PB	Clonal	Clonal	Clonal	Clonal
7	P236	PB	Clonal	Clonal	Clonal	Clonal
8	**P253**	**FFPE**	**Clonal**	**Clonal**	**Non-Clonal**	**Non-Clonal**
9	P262	FFPE	Non-Clonal	Non-Clonal	Non-Clonal	Non-Clonal
10	**CE-0035**	**PB**	**Non-Clonal**	**Non-Clonal**	**Non-Clonal**	**Clonal**

- **Sample CE-0035 is Clonal by LymphoTrack *IGH* FR3 and Non-Clonal by *IGH* FR1 and *IGH* FR2**



Conclusions

A comprehensive NGS-based *IGH* assay has been developed for the Illumina® MiSeq® platform that identifies clonal *IGH* V-J rearrangements and DNA sequences. The LymphoTrack® Assay has demonstrated that combining FR1, FR2 and FR3 helps to decrease the false-negative rate due to somatic hypermutation in primer binding sites of the involved V_H gene segments.