Validation of a Next Generation Sequencing Clinical Assay for Detection of Immunoglobulin Heavy Chain Clonality and Somatic Hypermutation in Chronic Lymphocytic Leukemia

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Xin-Xing Tan • Tricia Peters • Meredith Berry • Kristyn Jeter • Brigitte Lovell • Victor Venegas • Yanglong Mou • Brad Thomas • Vince Funari • Shari Brown • Josette William
NeoGenomics Laboratories

Introduction

Background: Next-generation sequencing (NGS) provides a powerful high-throughput approach to identify and track clonal B-cell immunoglobulin (IG) heavy chain clonality and to assess somatic hypermutation (SHM) status in a massively parallel manner. The NGS-based clonality/SHM testing demonstrated superior performance over the conventional capillary electrophoresis (CE) methods in characterization of B-cell neoplasms. However, its broad utilization in clinical diagnostics requires extensive validation of assay as well as standardization of the data interpretation.

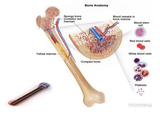
Methods: In this study, we present an NGS assay for characterization of B-cell malignancies, its validation, and standardized result interpretation. For superior sensitivity, this assay was designed to simultaneously target the Leader, FR1, FR2, and FR3 regions of the IGH gene to identify clonal IGH V_H-J_H rearrangement, the associated DNA sequences, and to assess the status of somatic hypermutation within the rearranged genes. The assay was also designed to target the IGK gene to identify clonal IGK V_K-J_K, V_K-K_{de}, and INTR-K_{de} rearrangement. Bioinformatics and immunoinformatics analysis were performed using LymphoTrack software, IMGH V-Quest, and ARResT/AssingSubsets. The analysis results were reviewed and interpreted by pathologists.

Results: To implement this assay for clinical diagnostics in B-cell malignancies such as CLL, B-All, DLBCL and MM, *etc.*, we performed an analytical and clinical assay validation to establish the assay accuracy, specificity, sensitivity, repeatability, and reproducibility, with both pre-characterized reference controls and clinical specimens included. Over forty DNA samples from clinical peripheral blood and bone marrow aspirate specimens were collected and assessed by the NGS assay for IG clonality and SHM evaluation. Triplicates were included and testing were performed at different times and by different operators to assess the assay precision. Assay sensitivity as low as 2.5% for clonality and 2% for SHM were observed for baseline clonality with as low as 0.001% for tracking MRD. Near-perfect assay specificity (100%) and precision (100%) were observed at these sensitivity levels. The validated assay was further qualified by ERIC (the European Research Initiative on CLL) with a certificate granted to standardize the data interpretation of this assay for testing in chronic lymphocytic leukemia. Under the ERIC standards our laboratory was able to assess and interpret precisely numerous rare and analytically challenging cases or cases difficult to categorize in CLL such as borderline SHM at mutation rate of 2.08% and classification of productive clone into major CLL stereotyped subsets based on thorough analysis of the VH CDR3 sequence, etc.

Conclusions: A NGS IG clonality/SHM assay was analytically and clinically validated in NeoGenomics' CLIA-certified and CAP-accredited laboratory under medical oversight, with a demonstrated rigor of the test by its high accuracy, sensitivity, specificity and robust reproducibility. The clinical diagnostic testing results with this assay is interpreted in accordance with ERIC standards for reliable clinical reporting.

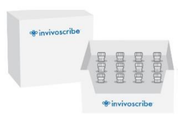
Assay Workflow & Specifications

1A. Specimen Collection



1B. DNA Extraction

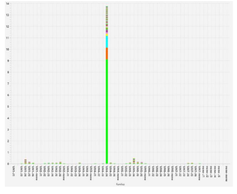
1C. NGS Library Preparation



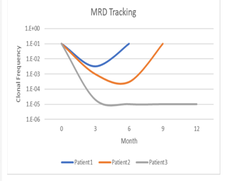
1D. Sequencing

1E. Data Analysis for Diagnostic Clone Detection & MRD Clone Tracking

Rank	Sequence	Length	Merge count	V-gene	J-gene	% total reads	Cumulative %	Mutation rate to partial V-gene (%)	In-frame (Y/N)	No Stop Codon (Y/N)	V-coverage	CDR3 Seq
1	TTCTCGTGGTGC	455	21172	IGHV4-59_05	IGHJ4_02	15.06	15.06	11.26	Y	Y	98.63	GCGAGACGGAG
2	CTGCTACTGAC	460	57	IGHV3-7D_10	IGHJ6_02	0.04	15.10	1.99	Y	Y	98.67	GCACGGGCGGA
3	CTCGCCCTCCTC	454	46	IGHV5-a_03	IGHJ5_02	0.03	15.13	3.40	Y	Y	100.00	GCGAGACATGG
4	CTGCTACTGACG	472	44	IGHV3-7D_12	IGHJ6_03	0.03	15.16	4.65	Y	Y	99.00	not found
5	GTTCCTCTTTGT	470	43	IGHV1-69_01	IGHJ4_02	0.03	15.19	0.00	Y	Y	98.99	GCGAGCGGCCG
6	GTTCCTCTTTGT	470	43	IGHV1-69_01	IGHJ4_02	0.03	15.23	0.00	Y	Y	98.31	GCGGTCGCTCG
7	CTCGCCCTCCTC	463	43	IGHV5-51_01	IGHJ4_02	0.03	15.26	0.00	Y	Y	100.00	GCGAGACATGT
8	CTCGCCCTCCTC	460	43	IGHV5-a_03	IGHJ4_02	0.03	15.29	3.72	Y	Y	99.32	GCGAGAAAGTC
9	CTCGCCCTCCTC	463	42	IGHV5-a_03	IGHJ6_02	0.03	15.32	5.10	Y	Y	100.00	GCGAGACAAGG
10	CTTGGCCTCCTC	376	41	IGHV5-a_04	IGHJ3_02	0.03	15.35	0.00	n/a	N	42.52	GCGAGATATTAC



Diagnostic clone detection



MRD clone tracking

Table 1. Diagnostics base-line and MRD tracking assay specifications

Specifications	Parameters
Diagnostics DNA Input	50 ng per library
Diagnostics Library Replicate	1x
Diagnostics Reads Requirement	50,000 per library
Diagnostics Clonality Limit of Detection	2.5%
Diagnostics SHMM Limit of Detection	2%
MRD DNA Input	700 ng per library
MRD Library Replicate	5x
MRD Reads Requirement	700,000 per replicate
MRD Limit of Detection	0.001%

Figure 1. Workflow of the NGS IG clonality/SHM & MRD assay. Human specimen went though DNA extraction, library preparation using the Invivoscribe LymphoTrack kits, and sequenced on Illumina MiSeq with read length of 2x300bp. Raw sequencing data was then analyzed to identify clonality and somatic hypermutation with the Invivoscribe LymphoTrack software, followed with result interpretation using IMGH V-Quest, and ARResT/AssingSubsets.

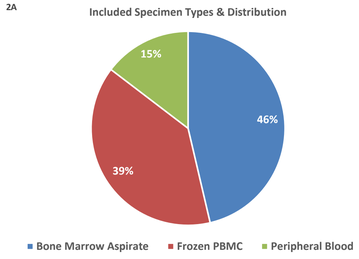
Table 1. Diagnostics base-line and MRD tracking assay specification. The table listed the assay limit of detection and their requirement in DNA input and data amount.

Assay Validation for Diagnostic Base-Line Clonality/SHM Evaluation

Table 2. A representative subset of clinical patient samples included in the assay validation

Sample #	Specimen Type	Indications	Expected Clonality Status	Expected Clone
1	Peripheral Blood	B-ALL	Detected	V1-18, MUTATED
2	Bone Marrow Aspirate	CLL	Detected	V4-39, MUTATED
3	Bone Marrow Aspirate	B-ALL	Not Detected	N/A
4	Bone Marrow Aspirate	CLL	Detected	V2-70, Unmutated
5	Bone Marrow Aspirate	DLBCL	Detected	V3-74, MUTATED
6	Peripheral Blood	CLL	Detected	V4-34, MUTATED
7	Bone Marrow Aspirate	CLL	Detected	V1-46, MUTATED
8	Bone Marrow Aspirate	B-ALL	Detected	IgKappa V5-2
9	Bone Marrow Aspirate	ALL without remission	Detected	V2-26, Unmutated
10	Peripheral Blood	ALL	Detected	V5-51, MUTATED
11	Bone Marrow Aspirate	ALL	Detected	1) V3-11, UNmutated 2) V3-23, Unmutated
12	Bone Marrow Aspirate	B-ALL	Detected	V3-53, Unmutated
13	Bone Marrow Aspirate	CLL	Detected	V3-11, Unmutated
14	Bone Marrow Aspirate	DLBCL	Detected	V3-21, Unmutated
15	Frozen PBMC	B-ALL with hyperdiploidy	Detected	V4-34_10.6%, Unmuated
…	…	…	…	…

2A



2B

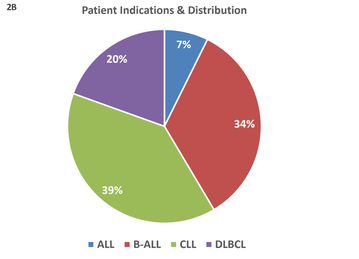


Table 3. Assay performance in validation

Specifications	Clonality	SHM
Assay Accuracy	100%	100%
Assay Specificity	100%	100%
Assay Sensitivity	100%	100%
Assay Repeatability	100%	100%
Assay Reproducibility Across Times	100%	100%
Assay Reproducibility Across Operators	100%	100%
Assay Reproducibility Across Instruments	100%	100%

Limit of Detection determination

Expected %Clone	% Time with Clone Detected
10.0%	100%
5.0%	100%
4.0%	100%
3.0%	100%
2.5%	100%
1.0%	0%
0%	0%

Table 2. Clinical patient samples included in the clonality/SHM assay validation. Table 2 listed the representative subset of forty five clinical samples in total included in the validation. All the included samples were pre-characterized using the validated molecular and/or FLOW methods and their clonality and SHM status are known. **Figure 2A & 2B** are the specimen type distribution and indication distribution of the forty five patient samples included in the validation.

Figure 2. Assay performance in validation and limit of detection (LoD) determination. A cohort of forty five clinical samples were included and assay accuracy, specificity, and sensitivity were determined as the percentage of true positives and true negative calls divided by the total number of evaluated calls, the percentage of true negative calls divided by true negative calls an false positive calls, and the percentage of true positive calls divided by true positive calls and false negative calls, respectively. A total of fifteen clinical samples were processed in three replicates in the same run to determine the assay repeatability. This same sample set was processed by the same operator at different time, by a different operator, and on a different instrument to establish the assay reproducibility across times, operators, and instrument. A serial dilutions was prepared and assessed multiple times to determine the assay LoD.

Assay Validation for MRD Tracking

Table 4. A representative subset of clinical patient samples included in the assay validation

Sample #	Identified Clone ID	V-gene	Target	% Clone	Dilute to	Negative to
M01	N200328-HNE-00010_O1T1_IGK_Rank1	V4-1	IGK	93.5	0.001%	N200328-HNE-00016_O1T1_IGK_Rank1, N200328-HNE-00030_O1T1_IGK_Rank1, N200328-HNE-00066_O1T1_IGK_Rank1
M02	N200328-HNE-00016_O1T1_IGK_Rank1	V4-1	IGK	3.3	0.001%	N200328-HNE-00010_O1T1_IGK_Rank1, N200328-HNE-00030_O1T1_IGK_Rank1, N200328-HNE-00066_O1T1_IGK_Rank1
M03	N200328-HNE-00001_O1T1_FR1_Rank1	V1-18	IGH_FR1	73.5	0.001%	N200328-HNE-00009_O1T1_FR1_Rank1, N200328-HNE-00017_O1T1_FR1_Rank1, N200328-HNE-00025_O1T1_FR1_Rank1
M04	N200328-HNE-00009_O1T1_FR1_Rank1	V2-70	IGH_FR1	72.3	0.001%	N200328-HNE-00001_O1T1_FR1_Rank1, N200328-HNE-00017_O1T1_FR1_Rank1, N200328-HNE-00025_O1T1_FR1_Rank1
M05	N200328-HNE-00022_O1T1_FR2_Rank1	V3-74	IGH_FR2	90.2	0.001%	N200328-HNE-00032_O1T1_FR2_Rank1, N200328-HNE-00033_O1T1_FR2_Rank1, N200328-HNE-00034_O1T1_FR2_Rank1
M06	N200328-HNE-00032_O1T1_FR2_Rank1	V3-49	IGH_FR2	34.8	0.001%	N200328-HNE-00022_O1T1_FR2_Rank1, N200328-HNE-00033_O1T1_FR2_Rank1, N200328-HNE-00034_O1T1_FR2_Rank1
M07	N200328-HNE-00018_O1T1_FR3_Rank1	V4-34	IGH_FR3	98.6	0.001%	N200328-HNE-00024_O1T1_FR3_Rank1, N200328-HNE-00029_O1T1_FR3_Rank1, N200328-HNE-00035_O1T1_FR3_Rank1
M08	N200328-HNE-00024_O1T1_FR3_Rank1	V5-a	IGH_FR3	91.5	0.001%	N200328-HNE-00018_O1T1_FR3_Rank1, N200328-HNE-00029_O1T1_FR3_Rank1, N200328-HNE-00035_O1T1_FR3_Rank1
M09	N200328-HNE-00019_O1T1_LEADER_Rank1	V1-46	IGH_LEADER	15.5	0.001%	N200328-HNE-00014_O1T1_LEADER_Rank1, N200328-HNE-00070_O1T1_LEADER_Rank1, N200328-HNE-00074_O1T1_LEADER_Rank1
M10	N200328-HNE-00014_O1T1_LEADER_Rank1	V1-3	IGH_LEADER	58.8	0.001%	N200328-HNE-00019_O1T1_LEADER_Rank1, N200328-HNE-00070_O1T1_LEADER_Rank1, N200328-HNE-00074_O1T1_LEADER_Rank1
…	…	…	…	…		…

Table 4. Clinical patient samples included in the MRD assay validation. Table 4 listed the representative subset of twenty four clinical samples in total included in the validation. All the included samples were pre-characterized and tested in the previous clonality/SHM validation with known clones as biomarkers.

Table 5. Assay performance in validation

Specifications	Acceptance Criteria	Measured Results
Assay Accuracy	90.0%	97.2%
Assay Specificity	90.0%	99.4%
Assay Sensitivity	90.0%	90.9%
Assay Reproducibility Across Times	90.0%	96.6%
Assay Reproducibility Across Operators	90.0%	96.8%

Table 5. Assay performance in validation. The twenty four clinical samples with known clonality and clone percentage were each diluted to 0.001%. The dilutions were processed following the MRD testing protocol to determine the assay accuracy, specificity, sensitivity, and reproducibility. Assay accuracy, specificity, and sensitivity were calculated as the percentage of true positives and true negative calls divided by the total number of evaluated calls, the percentage of true negative calls divided by true negative calls an false positive calls, and the percentage of true positive calls divided by true positive calls and false negative calls, respectively.

Key Conclusions

- An NGS assay for characterization of B-cell malignancies was analytically and clinically validated in NeoGenomics' CLIA-certified and CAP-accredited laboratory under medical oversight.
- The assay was designed to simultaneously target the Leader, FR1, FR2, and FR3 regions of the IGH gene and was designed also to target the IGK gene to identify clonal rearrangement
- The assay was validated for diagnostics base-line clonal rearrangement detection and somatic hypermutation, and also validated for MRD tracking, both with superior accuracy, sensitivity, specificity and robust reproducibility.
- The validated assay was further qualified by EIRC (the European Research Initiative on CLL) with a certificate granted to standardize the data interpretation of this assay for testing in chronic lymphocytic leukemia

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