NGS Characterization of Multiple Immune Receptors from a Single Multiplex PCR Reaction

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INTRODUCTION

B cell repertoire analysis by next-generation sequencing (NGS) has shown utility in the field of hematological oncology research. Some advantages provided by NGS-based techniques include a lower limit-of-detection and simpler paths to standardization compared to flow-based methods, and the elimination of specifically designed primers often required for qPCR-based methods. Owing to primer-primer interactions and incompatibility of reaction conditions, most current multiplex PCR assays require separate PCR reactions to survey each immunoglobulin chain (IGH, IGK, IGL), leading to a longer time-to-answer, and increased sample usage, for samples in which no marker is initially detected. We have developed an assay for immune receptor analysis based on Ion AmpliSeq™ technology to circumvent these issues, allowing the effective use of up to thousands of primers in a single reaction. **This highly multiplexed, BCR Pan-Clonality NGS assay provides for efficient detection of IGH, IGK, and IGL chain rearrangements in a single reaction.**

PANEL DESIGN

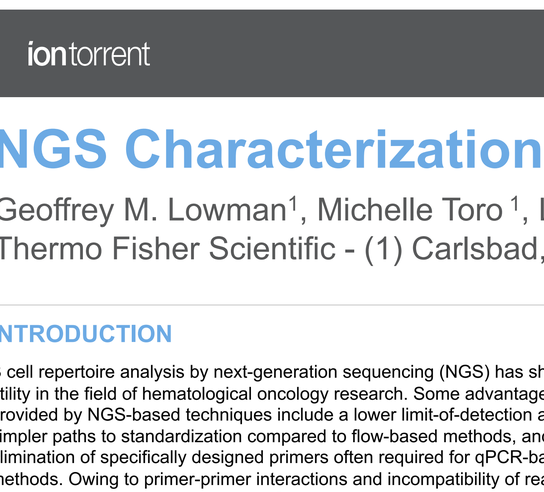

Figure 1. (A) BCR Pan-Clonality Assay contains FR3-J targeting primers for IGH, IGK, IGL, as well as KDE/Cint in a **single library preparation reaction. (B)** Schematic of two additional BCR IGH assays that target the distal region of FR3 and joining region (FR3(d)-J) as well as an FR2-J assay.

MATERIALS AND METHODS

The Pan-Clonality assay includes a single primer panel targeting the framework heavy-3 (FR3) portion of the variable gene and the joining gene region of and light-chain loci (IGH, IGK, IGL) for all alleles found within the IMGT database, enabling readout of the complementary-determining region 3 (CDR3) sequence of each immunoglobulin chain. To maximize sensitivity, we included primers to amplify IGK loci rearrangements involving Kappa deletion and C intron elements. To evaluate performance, we conducted clonality assessment and limit-of-detection testing.

Testing used gDNA from research samples representing common B cell malignancies, including B cell lines (ATCC, DSMZ) and clinical research samples (Cureline). We included samples derived from peripheral blood, bone marrow, and FFPE-preserved tissues. Sequencing was performed on the Ion GeneStudio™ S5 sequencer and analysis using Ion Reporter™ software 5.16.

Clonality Assessment (cell lines): Clonality assessment provides a means to identify the dominating clone and determine the CDR3 sequence of the clone of interest. 27 B cell lines derived from a variety of B cell malignancies (including B-ALL, CLL, Multiple Myeloma, Non-Hodgkin's Lymphoma) were profiled in a background (1:100 dilution) of PBL gDNA using the Pan-Clonality (IGH/K/L) assay and the associated reflex assays (FR3(d)-J and FR2-J). Table 1 presents the results of the cell line clonality assessment, with the green boxes in the table reporting positive detection of clonality in the sample and the number of each rearrangement receptors detected. Positive detection of at least one rearrangement (IGH, IGK, IGL, KDE/Cint) was found in 25/27 cell lines tested (93%) using the Pan-Clonality (IGH/K/L) assay (red box in Table 1).

RESULTS

Cell Line	IGH	IGK	IGL	KDE/Cint	IGH FR3(d)-J	IGH FR2-J
WSU-NHL	-	IGKV1-16 IGKV1-17	IGLV3-25	IGKdel-IGKCint	-	-
CA46	IGHV5-10-1**	IGKV2-28	-	-	IGHV5-51	IGHV5-51
Toledo	-	IGKV1-33	IGLV3-21 IGLV2-14	IGKdel-IGKCint	-	-
GA-10	IGHV4-34**	IGKV2-28	-	-	IGHV4-34	IGHV4-34
Daudi	-	-	-	IGKdel-IGKCint	IGHV3-74	IGHV3-74
U266B1	-	-	IGLV2-8	IGKdel-IGKCint	-	-
GM14952	IGHV3-48 IGHV1-46	-	IGLV2-14 IGLV2-23	IGKdel-IGKCint	IGHV4-39 IGHV1-46	IGHV4-39 IGHV3-74
Ramos	IGHV3-33 IGHV4-28*	-	IGLV2-23 IGLV2-18	-	IGHV4-34	IGHV4-34
RL	-	-	-	IGKdel-IGKCint	IGHV3-23	IGHV3-23
HS611.T	IGHV4-39	IGKV3-20 IGKV1-39	-	IGKdel-IGKCint	IGHV4-4	-
SU-DHL-6	-	-	-	-	-	-
BDCM	IGHV3-23	-	IGLV4-60	IGKV2-28-IGKdel IGKdel-IGKCint	IGHV3-23	-
SU-DHL-8	-	-	IGLV1-36	-	-	IGHV3-23
GM04154	IGHV3-23	-	IGLVI-70	-	IGHV3-23	IGHV3-23
IM9	-	-	IGKV1-16	-	IGHV3-9	-
MM.1R	IGHV3-30	-	IGLV2-18	IGKdel-IGKCint	IGHV3-30	-
NALM-1	IGHV3-9	IGKV2D-29	-	-	IGHV3-9	IGHV3-9
DS-1B	-	-	-	-	-	-
HT	-	IGKV3-11	-	-	IGHV3-53*	-
JVM-2	IGHV3-9	-	IGLV2-14	-	IGHV3-9	IGHV3-9
LP1	-	-	IGLV3-21	IGKV2-30-IGKdel	IGHV3-30	IGHV3-30
JM1	-	-	IGLV3-10 IGLV3-1	IGKdel-IGKCint	-	-
Pfeiffer	IGHV3-66	-	-	-	IGHV3-11	IGHV3-7
MC116	-	-	IGLV2-14	IGKdel-IGKCint	-	-
TMM	IGHV1-24	-	-	IGKV2-30-IGKdel	IGHV1-24	-
NU-DUL-1	-	IGKV1-17 IGKV1-39	IGLVI-70 IGLV4-60	-	-	-
BCP-1	IGHV3-23**	-	-	-	IGHV3-23	IGHV3-23

** Detection of rearrangement at low frequency indicating potential of*
*** Analysis reports single clone with multiple entries due to possible SHM*

Table 1. Detection of clonality in cell line samples diluted 1:100 in PBL gDNA using the BCR Pan-Clonality Assay – columns outlined in red, as well as the FR3(d)-J and FR2-J assays. Green boxes indicate positive detection with the variable gene identity of the rearrangements detected. The BCR Pan-Clonality Assay shows an overall 93% positive detection rate.

Sample	IGH	IGK	IGL	KDE/Cint
CLL_1 (BMA)	IGHV3-11	-	IGLV3-21 IGLV3-12 IGLV2-14	-
CLL_1 (PBMC)	IGHV3-11	-	IGLV3-21 IGLV3-12 IGLV2-14	-
CLL_2 (BMMC)	IGHV3-73	IGKV2D-26 IGKV1D-8	IGLV2-11	IGKdel-IGKCint
CLL_2 (PBMC)	IGHV3-73	IGKV2D-26 IGKV1D-8	IGLV2-11	IGKdel-IGKCint
Multiple Myeloma_2 (BMA)	-	IGKV1-33 IGKV1-16	-	IGKdel-IGKCint
CLL_3 (PBMC)	IGHV3-11	-	-	-
DLBCL_13 (FFPE)	-	-	-	IGKV3-20-IGKdel
DLBCL_15 (FFPE)	-	-	IGLV4-60	IGKV2-30-IGKdel
Multiple Myeloma_1 (PBMC)	-	IGKV2-30 IGKV2-28	-	-
Multiple Myeloma_1 (BMA)	-	IGKV2-30 IGKV2-28	-	-
Multiple Myeloma_1 (BMMC)	-	IGKV1-33 IGKV4-1	-	-
Multiple Myeloma_3 (BMA)	-	IGKV1-33	IGLV3-25	-
Multiple Myeloma_4 (BMA)	-	-	-	-
Multiple Myeloma_5 (BMA)	IGHV3-11	IGKV3-11	-	-
Multiple Myeloma_6 (BMA)	IGHV4-61 IGHV4-28	IGKV4-1	-	IGKdel-IGKCint
Multiple Myeloma_7 (BMA)	IGHV3-11	-	-	-
Multiple Myeloma_8 (BMA)	IGHV3-9 IGHV3-23	IGKV3-20	-	-
Multiple Myeloma_9 (BMA)	-	IGKV1-33	IGLV3-25	-
Multiple Myeloma_10 (BMA)	IGHV4-28	IGKV2-28	-	-
Multiple Myeloma_11 (BMA)	IGHV3-7 IGHV4-28	IGKV2-30	-	IGKdel-IGKCint

Table 2. Detection of clonality in clinical research samples (MM – Multiple Myeloma, CLL – Chronic Lymphocytic Leukemia, B-ALL – B cell Acute Lymphoblastic Leukemia, DLBCL – Diffuse Large B cell Lymphoma) using the Pan-Clonality (IGH/K/L) assay. Green boxes indicate positive detection with the rearrangements detected.

Clonality Assessment (clinical research samples) 20 clinical research samples from a variety of B cell malignancies (including B-ALL, CLL, Multiple Myeloma, and Diffuse Large B Cell Lymphoma) were profiled using the Pan-Clonality (IGH/K/L) assay. Table 2 presents the results of the clinical research sample clonality assessment. Positive detection of at least one rearrangement (IGH, IGK, IGL, KDE/Cint) was found in 19/20 cell lines tested (95%) using the Pan-Clonality (IGH/K/L) assay.

Samples	Total Tested	Total positive (Heavy Chain) [%]	Total positive (Light Chains) [%]	Total positive (Heavy+Light) [%]
Cell Lines	27	13 [48%]	22 [81%]	25 [93%]
Clinical Research Samples (MM, CLL, DLBCL)	20	11 [55%]	19 [95%]	19 [95%]

Samples	Total Tested	Total positive FR3(d)-J [%]	Total positive FR2-J [%]	Total positive (Pan-Clonality, FR2, FR3(d) [%]
Cell Lines	27	**18** **[67%]**	13 [48%]	25 [93%]

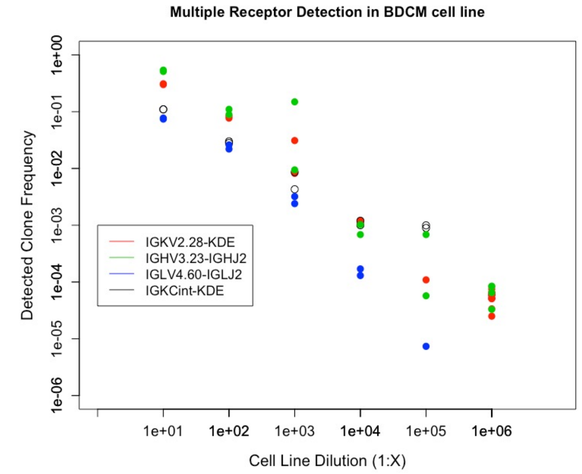

Figure 2. Linearity/Limit-of-detection evaluation of the single reaction Pan-Clonality (IGH/K/L) assay using the BDCM cell line. Cell line gDNA was serially diluted in PBL gDNA from 1:10 to 1:10^6. Each of four rearrangements for BDCM detected by the Pan-Clonality assay shown in Table 1 show linear response to dilution and two of the four rearrangements are detected at 1:10^6.

Linearity/Limit-of-detection (BDCM cell line) Linearity of response of detection of a cell line spike-in (BDCM) in a background of PBL gDNA is shown in Figure 2. Using only a single library reaction, the Pan-Clonality (IGH/K/L) assay detects 4 rearrangements in the BDCM cell line. All four rearrangements detected by the assay respond linearly to cell line dilution and two of the 4 rearrangements are detected at a dilution level of 1:10^6.

CONCLUSIONS

Ion AmpliSeq library technology enables an assay for profiling B cell receptor heavy and light chains in a single library reaction. Combining receptors in a single reaction allows for a higher success rate in clonality detection while maintaining the ability to detect rare clones (down to 1:10^6). We expect this assay to simplify the workflow for clonality assessment and rare clone detection in B cell malignancy research.

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