CLINICAL ASSESSMENT OF **CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)** SAMPLES FOR SOMATIC HYPERMUTATION STATUS BY NEXT-GENERATION SEQUENCING AND SANGER SEQUENCING

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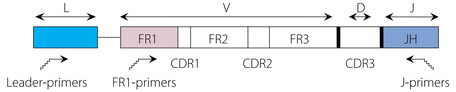
Introduction

- Somatic hypermutation (SHM), is an important process to increase the affinity of immunoglobulin molecules. The presence of ≥ 2% SHM is an important prognostic factor for CLL samples.
- The current method used to determine SHM status requires two steps: a PCR/capillary electrophoresis (CE) step for *IGH* FR1 to detect clonality, followed by a Sanger sequencing step. This multistep approach is labor intensive and time consuming.
- Next-generation sequencing (NGS) based LymphoTrack® Dx SHM assays (MiSeq® *IGH* FR1, MiSeq Leader and PGM *IGH* FR1) have been developed to address these limitations.
- The clinical performance of LymphoTrack® Dx *IGH* assays to detect SHM status was assessed with 50 anonymous, blinded CLL samples (17 BM and 33 PB) in comparison with the traditional PCR/Sanger method.

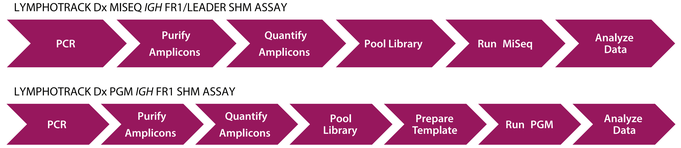
Materials and Methods

FIGURE 1. PRIMER DESIGN



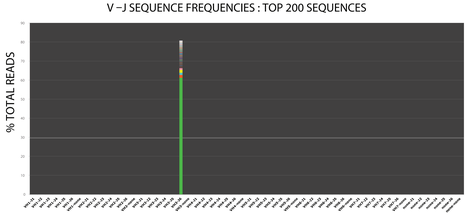
LymphoTrack® Dx SHM assays have been developed for both the MiSeq and PGM NGS platforms. Both assays and NGS platforms were used in this study. The MiSeq SHM Assay employs two master mixes. One amplifies genomic DNA between the upstream leader (VHL) region and the downstream joining (J) region of the *IGH* gene. The other amplifies from the framework1 (FR1) to J region. Amplicon products from VHL/J primers span the entire variable (V) region. Amplicon products from FR1/J primers encompass portions of the FR1 region to the downstream J region. The PGM Assay only employs FR1/J primers. The proprietary V and J consensus primers were designed and adapted to enable the PCR products to be sequenced on either the MiSeq or PGM platform. The *IGH* Leader and *IGH* FR1 assays for MiSeq use 24 indices allowing analysis of 22 patient samples. *IGH* FR1 assay for PGM uses 12 indices allowing analysis of 10 patient samples. Turnaround time for the FR1 analysis of 22 patient samples on either platform is 3 days. Analysis for the *IGH* Leader is 4 days due to the increased sequencing length of the amplicon.

FIGURE 2. WORKFLOW



Genomic DNA (50 ng) was amplified with consensus primers using a single multiplex PCR. Amplicons were purified using the Agencourt® AMPure XP PCR Purification system before pooling of the libraries. Amplicon libraries were quantified before being loaded onto the MiSeq or PGM. MiSeq data was generated using the MiSeq v2 Reagent kit (500 cycles) for FR1 or the MiSeq v3 Reagent kit (600 cycles) for the Leader assay. PGM data was generated using the Ion PGM Template OT2 400 kit and Sequencing 400 kit. *IGH* Leader was not analyzed on the PGM due to length limitations of the PGM platform.

FIGURE 3. LYMPHOTRACK Dx SHM SOFTWARE



Rank	Sequence	Length	Raw 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
1	GCCTCTGGATTCA…	290	354037	IGHV3-43_01	IGHJ6_02	61.69	61.69	5.24	Y	Y	97.82
2	GCCTCTGGATTCA…	287	8147	IGHV3-43_01	IGHJ6_02	1.42	63.10	5.24	Y	Y	97.82
3	GCCTCTGGATTCA…	290	6798	IGHV3-43_01	IGHJ6_02	1.18	64.29	5.68	Y	Y	97.82
4	GCCTCTGGATTCA…	290	6602	IGHV3-43_01	IGHJ6_02	1.15	65.44	5.68	Y	Y	97.82
5	GCCTCTGGATTCA…	290	4082	IGHV3-43_01	IGHJ6_02	0.71	66.15	5.24	Y	Y	97.82
6	GCCTCTGGATTCA…	290	2364	IGHV3-43_01	IGHJ6_02	0.41	66.56	5.68	Y	Y	97.82
7	GCCTCTGGATTCC…	290	1672	IGHV3-43_01	IGHJ6_02	0.29	66.85	5.68	Y	Y	97.82
8	GCCTCTGGATTCA…	290	1494	IGHV3-43_01	IGHJ6_02	0.26	67.11	5.68	Y	Y	97.82
9	GCCTCTGGATTCA…	290	1361	IGHV3-43_01	IGHJ6_02	0.24	67.35	5.24	Y	Y	97.38
10	GCCTCTGGATTCA…	290	1314	IGHV3-43_01	IGHJ6_02	0.23	67.58	5.68	Y	Y	97.82

A typical MiSeq *IGH* FR1 result from a BM sample showing evidence for clonality with one sequence at 61.69% of the total reads and mutation rate at 5.24%. Only the top 10 sequences are presented here. FASTQ data from either MiSeq or PGM can be analyzed by LymphoTrack® Dx Software running on a Windows PC. LymphoTrack® Dx Software generates frequency distributions, DNA sequences, V-J assignment and usage, and SHM status. The SHM status is determined two ways: 1) alignment statistics obtained from the BLAST algorithm for the top clonal read for the calculation of mismatches and gaps to the V-gene reference sequence; 2) the mutation rate is calculated as the sum of mismatches and gaps divided by the V-gene length.

Results

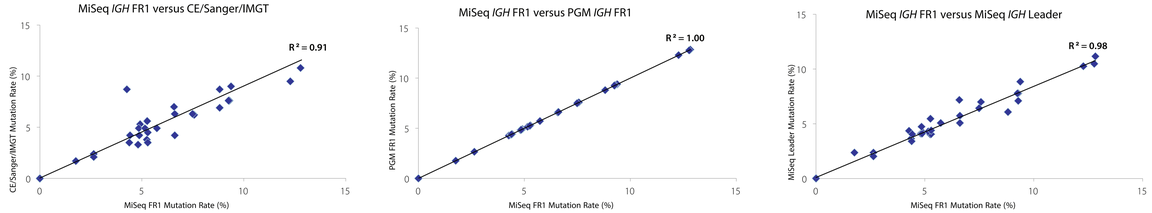
- The standard CE/Sanger method was able to detect 47 out of 50 samples with 30 (64%) samples exhibiting SHM rate ≥ 2.0 and 17 (36%) samples exhibiting SHM rate < 2.0. Three samples were not evaluable and were excluded for comparison in Table 1.
- The LymphoTrack Dx *IGH* FR1 Assay - MiSeq was able to detect 50 out of 50 samples with 31 (62%) samples exhibiting SHM rate ≥ 2.0 and 19 (38%) samples exhibiting SHM rate < 2.0.
- The LymphoTrack Dx *IGH* Leader Assay MiSeq was able to detect 50 out of 50 samples with 32 (64%) samples exhibiting SHM rate ≥ 2.0 and 18 (36%) samples exhibiting SHM rate < 2.0.
- The LymphoTrack Dx *IGH* FR1 Assay - PGM was able to detect 50 out of 50 samples with 31 (62%) samples exhibiting SHM rate ≥ 2.0 and 19 (38%) samples exhibiting SHM rate < 2.0.

TABLE 1. SENSITIVITY, SPECIFICITY AND PREDICTIVE VALUES OF THE THREE LYMPHOTRACK Dx ASSAYS FOR DETERMINING SHM STATUS[A]

Test	No. of samples with indicated result[b]				Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	S+T+	S+T-	S-T+	S-T-				
MiSeq FR1	30 (9 BM and 21PB)	0	0	17 (6 BM and 11PB)	100	100	100	100
MiSeq Leader	30 (9 BM and 21 PB)	0	1[c] (PB)	16 (6 BM and 10 PB)	100	94	97	100
PGM FR1	30 (9 BM and 21 PB)	0	0	17 (6 BM and 11 PB)	100	100	100	100

[a] S, standard (CE/Sanger for FR1); T, NGS test; +, positive (SHM ≥ 2.0%); -, negative (SHM < 2.0); PPV, positive predictive value; NPV, negative predictive value.
[b] Three samples were not evaluable by standard Sanger method.
[c] Additional mutations occur upstream of FR1 primer increasing the SHM rate from 1.8% to 2.4%.

FIGURE 4. SHM CONCORDANCE



- A comprehensive NGS assay has been developed for both MiSeq and PGM platforms that identifies clonal *IGH* V-J rearrangements, associated specific V-J region DNA sequences and determines the SHM status in CLL specimens as well as likelihood of expression with the determination of the sequence being in frame, the absence of stop codons and the degree of V coverage.
- These NGS assays have demonstrated excellent clinical concordance for determining SHM status as compared to the standard PCR/Sanger sequencing method.

Conclusions

- A comprehensive NGS assay has been developed for both MiSeq and PGM platforms that identifies clonal *IGH* V-J rearrangements, associated specific V-J region DNA sequences and determines the SHM status in CLL specimens as well as likelihood of expression with the determination of the sequence being in frame, the absence of stop codons and the degree of V coverage.
- These NGS assays have demonstrated excellent clinical concordance for determining SHM status as compared to the standard PCR/Sanger sequencing method.