Development and Validation of a NOTCH Custom NGS Assay for Identifying NOTCH1 Mutations in Chronic Lymphocytic Leukemia and Other Lymphoid Malignancies

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Introduction

NOTCH1 is known to be activated by oncogenic mutations in both hematologic and solid tumors including chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL), T cell acute lymphoblastic leukemia (T-ALL), non-small cell lung cancer (NSCLC) and breast cancer. In CLL, NOTCH1 mutations were detected in ~12% of patients at the time of diagnosis and at a higher rate during disease progression to Richter's transformation. As such, NOTCH1 may be a valuable biomarker of therapeutic efficacy, or clinical candidacy, for novel anti-cancer agents or regimens. For prospective evaluation of NOTCH1's utility as a biomarker, it is critical to have a sufficiently sensitive, specific, and comprehensive assay to detect NOTCH1 mutations in clinical specimens. Compared to Sanger sequencing, a targeted Next Generation DNA Sequencing (NGS) assay is more comprehensive, sensitive, and specific, and is therefore better suited for clinical applications with limited specimen availability, provided a NOTCH1 NGS assay can be designed and validated to address the challenges in gene size and sequence presented by the NOTCH1 gene. Here we present development and validation of our NOTCH1 NGS assay using cell lines and clinical samples (both blood and FFPE).

Results

Assay performance

The primers targeting NOTCH1 ROI were divided into three pools, 49 pairs in Pool1, 46 pairs in Pool2 and 26 pairs in Pool3. The normalized NOTCH1 ROI coverage for all the Pool1, Pool2 and Pool3 libraries from the fresh samples, the contrived FFPE samples, and the clinical FFPE samples were comparable (Figure 1A-C). In all the sequencing runs performed in the validation studies, the Average Library Coverage for the libraries from the fresh samples (cell line or blood) or the contrived FFPE samples were very similar, ranging from 924 to 4361 for Pool1 libraries, 588 to 2940 for Pool2 libraries, or 775 to 3461 for Pool3 libraries. For the libraries from clinical FFPE samples, the Average Library Coverage for the Pool1 or Pool2 libraries were similar to those from the fresh or the contrived samples, ranging 543 to 2870 for Pool1 libraries, or 341 to 2344 for Pool2 libraries. The Average Library Coverage of the Pool3 libraries from the clinical FFPE samples, in general, was relatively lower and covered a wider range than those from the fresh or the contrived samples, ranging from 3 to 2311. The lower coverage may be due to the variable quality of clinical FFPE samples and the inefficient amplification of the poor quality FFPE DNA with Pool3 primers. Pool3 primers were designed to generate longer amplicons than Pool1 and Pool2 primers in order to capture longer indels.

In the 13 batches of library preparations that included the Positive Control, all seven expected mutations in the Positive Control were reproducibly detected; the CV of the allele frequency for each variant was below 17% (Table 3).

Figure 1A. Normalized NOTCH1 ROI coverage for Pool1 libraries

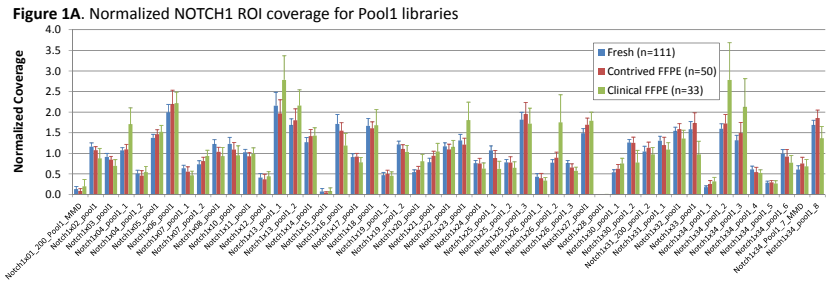


Figure 1B. Normalized NOTCH1 ROI coverage for Pool2 libraries

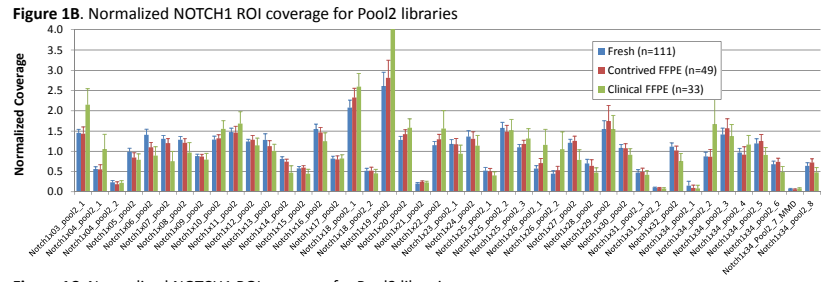
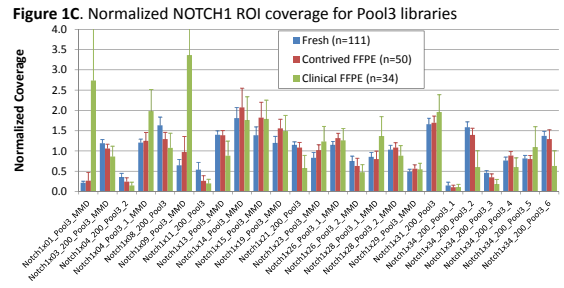


Figure 1C. Normalized NOTCH1 ROI coverage for Pool3 libraries



Accuracy

NOTCH1 L1600P Genomic DNA Reference Standards from Horizon Diagnostics, with frequencies at 50%, 20%, 10%, and 5% were used to determine the accuracy of variant frequency measured by this NOTCH NGS assay. The results showed that the variant frequencies reported by the NGS assay were within 20% variation from those in the standard (Figure 2).

DNA samples extracted from the four control cell lines (ML-1, REC-1, ALL-SIL and HPB-ALL, in Table 1) and a mixture of ML-1 and HPB-ALL (Mix-MH), as well as the corresponding contrived FFPE samples (Table 1, and FFPE-Mix-MH), were evaluated by both Sanger sequencing and NOTCH1 NGS assay. The allele frequency for 30 out of 32 (94%) mutations was concordant with those estimated by the Sanger (Figure 3). The allele frequencies for a 12bp insertion in sample HPB-ALL and FFPE-HPB-ALL were consistently lower, indicating that the estimated allele frequency of insertions or deletions called by the pipeline may be lower than the true frequency of the variant.

Figure 2. Concordance of allele frequency determined by ddPCR vs by NGS

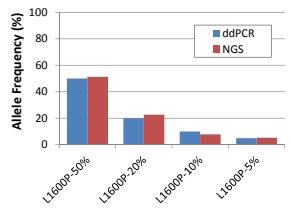
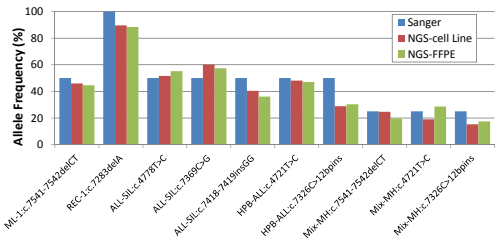


Figure 3. Concordance of allele frequency determined by Sanger vs by NGS



LOD

To determine the LOD of the NOTCH1 NGS assay, serially diluted mixtures of cell lines ML-1 and HPB-ALL (Mix-MH-dilu-2), or corresponding contrived FFPE samples FFPE-ML-1 and FFPE-HPB-ALL (FFPE-Mix-MH-dilu-2) were analyzed. These mixtures contained mutations at an expected allele frequency of 5-6%. Each of sample was sequenced 20 times by at least two operators in three batches. Each of the first and the second batches contained a negative control and 9 replicates, and was split into two sequencing runs; the third batch contained a negative control and 2 replicates in one sequencing run. The study showed that 3/3 mutations in Mix-MH-dilu-2 were detected in 20/20 replicates (Table 4). Two mutations (the SBS and the deletion) in FFPE-Mix-MH-dilu-2 were detected in 20/20 replicates; the insertion was detected in 19/20 replicates. Therefore, the NOTCH1 NGS assay can detect mutations present at 5-6% allele frequency with at least 95% confidence.

Table 4. Results for LOD and intra-run reproducibility study

Sample Name	Sample Type	Mutated cDNA	Mutated Amino Acid	Mutation Frequency (%)	Read Depth	Detected/ Tested
Mix-MH-dilu-2	Fresh Cell Line DNA	exon34:c.7541-7542delCT	p.P2514fs	5.8 ± 1.2	766 ± 91	20/20
		exon34:c.7326C>GGGCCGTGGACG	p.D2442fs	3.3 ± 0.5	1346 ± 273	20/20
		exon26:c.4721T>C	p.L1574P	4.2 ± 0.6	1064 ± 231	20/20
FFPE-Mix-MH-dilu-2	FFPE	exon34:c.7541-7542delCT	p.P2514fs	4.2 ± 0.7	734 ± 96	20/20
		exon34:c.7326C>GGGCCGTGGACG	p.D2442fs	4.6 ± 0.9	794 ± 262	19/20
		exon26:c.4721T>C	p.L1574P	6.7 ± 1.3	1137 ± 199	20/20

Intra-Run Reproducibility

As shown in Table 4, all of the three mutations (one SBS, one 2bp del and one 12bp ins) were detected in at least triplicates of the mixtures of cell line DNA or contrived FFPE DNA, the CV for the same mutation call is below 7% (data not shown).

Inter-Run Reproducibility

The experiments were designed to reproducibly detect each type of the variant (i.e., SBS, deletion, insertion) in both the fresh and FFPE samples containing the variants near the 5% LOD. The samples from the serially diluted mixture of the four cell line samples with NOTCH1 activating mutations (Mix4, Mix4-dilu-1) and the serially diluted mixture of the four corresponding contrived FFPE samples (FFPE-Mix4, FFPE-Mix4-dilu-1) were assayed in three separate runs using different barcodes on different days by at least 2 different operators. All seven mutations with expected allele frequency above 5% LOD in Mix4 or Mix4-dilu-1 were detected in 3/3 runs (Figure 4). All seven mutations with expected allele frequency above 5% LOD in FFPE-Mix4 or FFPE-Mix4-dilu-1 were detected in 3/3 runs (Figure 5). The CV for the allele frequencies (above 5% LOD) of the mutations reproducibly detected were all below 30% .

Figure 4. Inter-run reproducibility with cell line DNA samples

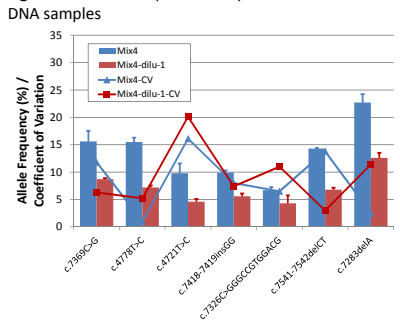
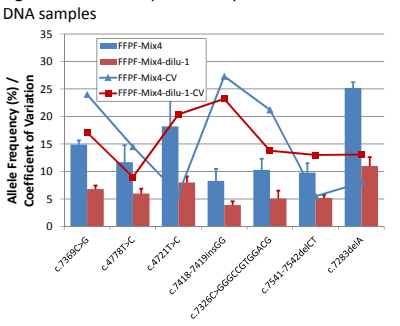


Figure 5. Inter-run reproducibility with contrived FFPE DNA samples



Materials and Methods

The 7.8 kb region of interest (ROI) includes the coding exons NOTCH1 (34 exons) and the intronic sequence encompassing the essential splicing sites. Three pools of the primers to sequence the ROI were designed primarily by Ion AmpliSeq Designer, supplemented with MolecularMD's proprietary primer design method to ensure 100% coverage of the ROI and all of the known critical hotspots. HapMap sample GM12878 was used as the Negative Control. A mixture of DNA from GM12878 and four human cancer cell lines was used as the Positive Control, in which seven NOTCH1 activating mutations were expected to be detected: three single base substitution (SBS) mutations, two insertions (2bp and 12bp), and 2 deletions (1bp and 2bp) with mutation frequency close to 5% (limit of detection).

Three libraries were prepared for each DNA sample (10 ng double-stranded DNA input per library) with Ion AmpliSeq Library Kit, using three separate pools of primers. Each library was uniquely barcoded. The libraries were amplified on the OneTouch system using OneTouch OT2 200 Kit, enriched on the Ion One Touch ES, and sequenced on the Ion PGM using the Ion PGM Sequencing 200 Kit v2 and a 318 chip v2. The sequencing was performed in batches, with no more than 30 libraries and two chips per batch. A positive control, a negative control and a no-template control were included in each batch. The sequencing data were analyzed with Torrent Suite 3.4.2 and MolecularMD's proprietary analysis pipeline.

A series of experiments was conducted to validate the following five parameters: 1) The accuracy of mutant allele frequency reported by the NGS assay; 2) the limit of detection (LOD) of SBS and indels; 3) the intra-run reproducibility; 4) the inter-run reproducibility; and 5) the analytical sensitivity and specificity. The samples tested including cell line DNA samples and contrived FFPE DNA samples generated from those cell lines (Table 1), and clinical samples, including 26 blood specimens and 27 FFPE specimens (Table 2).

Table 1. Summary of cell line DNA/contrived FFPE samples

Cell Line / FFPE ID	NOTCH1 Activating Mutation	
	cDNA	Amino Acid
GM12878 / FFPE-GM12878	Wild type	Wild type
ML-1/ FFPE-ML-1	exon34:c.7541-7542delCT	p.P2514fs
REC-1 / FFPE-REC-1	exon34:c.7283delA	p.H2428fs
ALL-SIL / FFPE-ALL-SIL	exon26:c.4778T>C	p.L1593P
	exon34:c.7369C>G	p.L2457V
	exon34:c.7418-7419insGG	p.P2474fs
HPB-ALL / FFPE- HPB-ALL	exon26:c.4721T>C	p.L1574P
	exon34:c.7326C>GGGCCGTGGACG	p.D2442fs
NOTCH1 L1600P* Genomic DNA Reference Standard, 50%, 20%, 10%, 5% (Horizon Diagnostics)	Exon26: c.4799T>C	p.L1600>P

Table 2. Summary of clinical specimens

Specimen Type	Clinical Origin	Number	Supplier
Blood	Normal	4	MolecularMD
Blood	CLL	25	Bioserve, Conversant Bio, OHSU, OncoMed
Blood	DLBCL	1	OncoMed
FFPE	Normal lymph Node	4	MolecularMD
FFPE	CLL	11	AdeptBio, Asterand
FFPE	DLBCL	9	Asterand, OncoMed
FFPE	ALCL	4	AdeptBio
FFPE	MCL	3	OncoMed

Table 3. Summary of performance of the Positive Control (n=13)

Mutated cDNA	Mutated Amino Acid	Mutation Frequency Expected (%)	Mutation Frequency Detected (%)	Read Depth	CV for Variant Frequency (%)
exon34:c.7369C>G	p.L2457V	6.25	8.18 ± 0.88	1484 ± 381	16.79
exon26:c.4778T>C	p.L1593P	6.25	7.30 ± 0.50	2766 ± 592	12.42
exon26:c.4721T>C	p.L1574P	6.25	4.91 ± 0.82	1088 ± 310	10.72
exon34:c.7418-7419insGG	p.P2474fs	6.25	4.94 ± 0.61	1800 ± 284	14.42
exon34:c.7326C>GGGCCGTGGACG	p.D2442fs	6.25	3.93 ± 0.57	1455 ± 372	12.97
exon34:c.7541-7542delCT	p.P2514fs	6.25	7.28 ± 1.22	795 ± 189	6.88
exon34:c.7283delA	p.H2428fs	12.50	11.49 ± 1.49	1291 ± 316	16.61

Analytical Sensitivity and Specificity

Fifty-three clinical specimens (26 patient blood samples and 27 patient FFPE samples , Table 2) were blinded and analyzed. Due to the low positive rate of the clinical samples with NOTCH1 activating mutations and the confined types of hematological cancers targeted by the OncoMed trial, additional contrived samples were also employed to mimic blood and FFPE specimens for the purposes of this validation study. The contrived samples contain NOTCH1 activating mutations with the allele frequencies close to 5% LOD.

Among the 53 clinical specimens, 14 NOTCH1 activating mutations, including SBS and deletion, were detected by the NOTCH NGS assay (Table 5; 10 in blood samples, 4 in CLL FFPE samples). In addition, 2 NOTCH1 inactivating mutations were detected (1 each in blood and FFPE samples), and 5 NOTCH1 SBS mutations classified as potentially benign or unknown were detected (all in FFPE samples). All but one of these 21 mutations were verified as true-positive by Sanger sequencing, with enrichment as needed (representative traces shown in Figure 6); a single activating mutation from one FFPE specimen was not confirmed due to the low quality of this sample; this unconfirmed mutation was counted as a false positive. Among the contrived samples, all of the 28 expected NOTCH1 activating mutations were identified in blood samples, and all of the 42 expected NOTCH1 activating mutations were identified in FFPE samples (with allele frequency close to LOD).

For blood samples, because all expected mutations were detected, and all detected mutations were confirmed by Sanger, both the false negative rate and false positive rate is 0; consequently, both the analytical sensitivity and specificity are 100% for detection of NOTCH1 activating mutations in blood samples. For FFPE samples, all of the expected mutation were detected, and all but one of the detected mutations were confirmed by Sanger. Thus the false negative rate is 0, whereas the false positive rate is 2.2% (1/(42+4)), so the analytical sensitivity is 100%, and the specificity is 97.8% for detection of NOTCH1 activating mutations FFPE samples.

Table 5. NOTCH1 mutations detected in clinical specimens

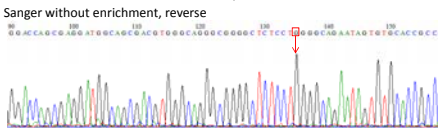
Sample ID	Sample Type	Clinical Origin	Mutated cDNA	Mutated Amino Acid	Mutation Type*	Mutation frequency (%)	Read Depth	Sanger Confirmation
MMD-6-0-130	Blood	DLBCL	exon34:c.7444delC	p.L2482X	A	78.5	1761	Yes
MMD-6-0-131	Blood	CLL	exon34:c.7541-7542delCT	p.P2514fs	A	6.3	711	Yes
MMD-6-0-132	Blood	CLL	exon34:c.7541-7542delCT	p.P2514fs	A	10	430	Yes
MMD-6-0-133	Blood	CLL	exon34:c.7541-7542delCT	p.P2514fs	A	11.9	776	Yes
MMD-6-0-134	Blood	CLL	exon34:c.7541-7542delCT	p.P2514fs	A	32	538	Yes
MMD-6-0-135	Blood	CLL	exon34:c.7541-7542delCT	p.P2514fs	A	42.6	537	Yes
MMD-6-0-126	Blood	CLL	exon34:c.7375C>T	p.Q2459X	A	3.1	1571	Yes
MMD-6-0-125	Blood	CLL	exon34:c.7541-7542delCT	p.P2514fs	A	1.6	520	Yes
MMD-6-0-128	Blood	CLL	exon34:c.4168C>A	p.P1390T	A	18.7	268	Yes
MMD-6-0-129	Blood	CLL	exon23:c.3836G>A	p.R1279H	A	55.8	276	Yes
MMD-6-0-124	Blood	CLL	exon11:c.1862G>A	p.R621H	I	50.7	1314	Yes
MMD-2-5-045	FFPE	CLL/SLL-Axillary Lymph Node	exon34:c.7541-7542delCT	p.P2514fs	A	33.5	287	Yes
MMD-2-5-044	FFPE	CLL/SLL-Neck Lymph Node	exon34:c.7541-7542delCT	p.P2514fs	A	7	132	No
MMD-2-5-043	FFPE	CLL/SLL-Cervical Lymph Node	exon34:c.7541-7542delCT	p.P2514fs	A	24.4	311	Yes
MMD-2-5-038	FFPE	CLL/SLL-Cervical Lymph Node	exon34:c.7541-7542delCT	p.P2514fs	A	60	225	Yes
MMD-2-5-049	FFPE	CLL-Tonsil	exon13:c.2033A>G	p.N678S	I	53.1	4296	Yes
			eoxn21:c.3395G>A	p.R1132H	B/U	52.4	1715	Yes
MMD-2-5-051	FFPE	ALCL-Axillary Lymph Node	exon25:c.4129C>T	p.P1377S	B/U	68.5	330	Yes
MMD-2-4-041	FFPE	Non-Hodgkin Lymphoma-Stomach	exon25:c.4129C>T	p.P1377S	B/U	30	957	Yes
MMD-2-5-055	FFPE	Mantle Cell Lymphoma-Lymph node	exon25:c.4129C>T	p.P1377S	B/U	15.4	403	Yes
MMD-2-5-056	FFPE	Mantle Cell Lymphoma-Lymph node	exon25:c.4129C>T	p.P1377S	B/U	13.2	129	Yes

* A: activating; I: inactivating; B/U: potential benign or uncertain

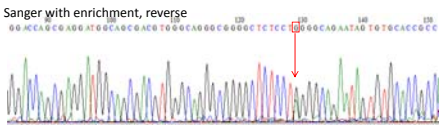
Figure 6. Representative Sanger sequencing traces confirming activating mutations detected by the NOTCH1 NGS assay

MMD-6-0-126, exon34:c.7375C>T, p.Gln2459X, 3.12%

Sanger without enrichment, reverse



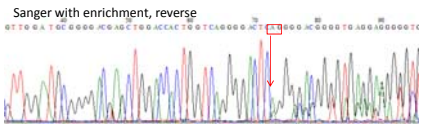
Sanger with enrichment, reverse



MMD-6-0-125, exon34:c.7541-7542delCT, p.P2514fs, 1.6%

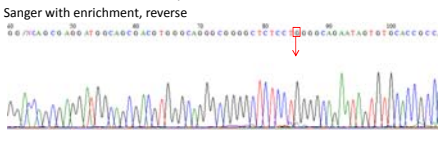
Sanger without enrichment, reverse

Sanger with enrichment, reverse



GM12878, exon34:c.7375, p.Q2459

Sanger with enrichment, reverse



Conclusions

This study demonstrates that, using only 30ng total dsDNA input, the Ion Torrent PGM MolecularMD NOTCH1 NGS assay is capable of detecting NOTCH1 activating mutations, including SBS and small indels, with 5% LOD, ≥95% intra-run reproducibility (precision), 100% inter-run reproducibility, and with ≥95% analytical sensitivity and specificity. The assay has a reportable analytical range of 5% to 100% mutation allele frequency. The performance of this assay represents an achievement because of the complexity and large size of the coverage required for the NOTCH1 gene.

This NGS assay is suitable for use with DNA extracted from both clinical blood and FFPE tissue samples.

This custom panel is tailored to the demands of a targeted drug trial, and was designed to identify mutations in genes and regions most relevant to the interpretation of trial results.

A custom NGS panel is scalable, in terms of ROI, size, and number of samples to be tested, and can be employed to maximize the breadth and depth of profiling information generated from a given tissue specimen.

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