

Ultrasensitive detection of acute myeloid leukemia minimal residual disease using single molecule molecular inversion probes

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SUPPLEMENTARY METHODS:

smMIP Design

A smMIP capture panel was designed against AML-relevant targets (**Table 2, Supplementary Table 2**) using the program MIPgen¹ with the following flags: -max_capture_size 162, -min_capture_size 152, -logistic_priority_score 0.5, -common_snps off, -arm_lengths 18:24,19:23,20:22,21:21, -starting_mip_overlap 1, -tag_sizes 4,4. Manual review of smMIPs with a logistic score of less than 0.6 was then performed, and smMIPs with near-zero logistic scores were manually redesigned. Based on recurrently mutated factors identified in published AML exome sequence data², this capture panel is predicted to identify cancer-associated mutations in ~80% of AML specimens. For this pilot work, dedicated smMIP probes were also designed to target identifying polymorphisms carried in cell lines³ KMH2, RAJI, OCI-AML2, and L1236, as well as several other clinically relevant genes (*ABL1*, *ALK*, *JAK2*, *NT5C2*, and *ROS1*). All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, Iowa), using standard salt purification.

DNA extraction

DNA extraction from all specimens was performed using a QiAgen X-tractor with Reagent Pack DX (Germantown, MD).

smMIP capture protocol

smMIP pools were initially 5' phosphorylated using 1U T4 Polynucleotide Kinase (New England Biolabs, Ipswich, Massachusetts) per 27 pmol probes in 1X T4 Ligase Buffer, by

incubating at 37°C for 45 minutes and 65° for 20 minutes. To perform smMIP capture, we combined 500ng genomic DNA, 20,000 smMIPs per genome (ie, 28.05 μ mol), 2mM MgCl₂ (Thermo Fisher Scientific, Waltham, Massachusetts), 0.5mM NAD (NEB), 25mM KCl (Thermo Fisher Scientific), 0.01% triton-X 100 (Sigma-Aldrich, St. Louis, Missouri), 20mM Tris-HCl pH8.3 (Sigma-Aldrich), 320 pmols dNTPs (NEB), 1 U Q5 polymerase (NEB), and 1 U Ampiligase (Epicentre) in a total reaction volume of 25ul. Reactions were incubated for 95°C for 10 minutes, 60°C for 2.5 hours, then 8 cycles of 95°C for 1 minute, 60°C for 2.5 hours. Reactions were cooled on ice prior the addition of 5 U Exonuclease I (NEB) and 25 U Exonuclease III (NEB) in 1X Ampiligase buffer and incubation at 37°C for 45 minutes followed by 95°C for 2 minutes. PCR amplification and addition of index sequences was performed using 1X iProof High-Fidelity Master Mix with 23.75 pmol each oligonucleotide. PCR was carried out using an initial incubation of 98°C for 30 seconds, followed by 19 cycles of 98°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 2 minutes. After amplification, libraries were purified using 0.8X Agencourt AMPure XP (Beckman Coulter, Pasadena, California). Size selection for 280bp product was performed using a 3% agarose gel and Monarch Gel Purification Kit (NEB).

smMIP rebalancing

Initially, smMIP probes were pooled together at equimolar concentrations, capture performed using NA12878 genomic DNA, and sequenced to determine the relative abundance of reads from each probe. Probes were empirically rebalanced and repooled to achieve better performance uniformity, as well as to remove several probes with consistently poor performance.

Data analysis Pipeline

Reads were demultiplexed using `bcl2fastq-v2.17` (Illumina). `Fastq-mcf 1.04.676` was then used to remove regions from reads corresponding to the smMIP backbone and to remove reads less than 53 bp in length using parameters `-l 53 -k 0 -q 0`. Read pairs were then self-assembled using `pear v0.9.54`. Read sequence identifiers were next renamed to contain a string corresponding to a concatenation of the 4 bp UMID at the 5' end of the assembled read and the 4 bp molecular tag at the 3' end of the assembled read. Reads were then mapped to hg37 using `bwa mem v0.7.125` and `samtools v1.16`. For performance reasons we split mapped reads first by chromosome and subsequently to files corresponding to individual smMIPs, based on their expected start and stop coordinates, and these files were independently processed.

For reads corresponding to each smMIP, UMIDs represented in only one sequence read were initially discarded. Remaining reads were grouped based on whether their UMID was contained in two, or more than two independent reads. Single nucleotide polymorphisms and indels were called on these read groups using the `mpileup2snp` and `mpileup2indel` commands of `VarScan.v2.3.77`, respectively, using a “majority rule” approach. For UMID groups containing only two reads, variant calling was performed using the flags `--min-coverage 2 --min-var-freq 1.0 --min-freq-for-hom 1.0 --strand-filter 0 --p-value 1 --output-vcf 1` in order to specify that 50% or more of the reads must contain a given variant in order to be called, whereas for UMID groups comprised of three or more reads parameters were set to `--min-coverage 3 --min-var-freq 0.5 --min-freq-for-hom 0.5 --strand-filter 0 --p-value 1 --output-vcf 1` in order to specify that both reads must contain a given variant in order to be called.

Variant calls from each individual smMIP were then combined into a single file, including provisions to cumulatively add read counts in regions overlapped by multiple smMIPs. Variant files were annotated using ANNOVAR⁸ (last accessed 2015-06-17).

In summary, the analysis pipeline expresses variant calls in terms of the number of unique smMIP capture events which are consistent with a given variant over the total number of smMIP capture events overlying that site.

Source code for the smMIP analysis pipeline is available from:

`ssh://git@bitbucket.org/uwlabmed/smmips_analysis.git`

Error model

We produced a site- and mutation-specific error model in order to assess the significance of observed variation at each site in the smMIP panel. We sequenced DNA extracted from cell line NA12878 and bone marrow specimens from four healthy donors to serve as a baseline for measuring error rates. To determine the variant allele frequency threshold which defined heterozygous sites, we intersected variant calls for NA12878 from the smMIP assay with those from “platinum genome” datasets⁹, and subsequently selected a minimum variant allele frequency threshold of 0.2 for defining heterozygosity. We therefore masked sites with variant allele frequency of 0.2 or greater, and considered all remaining variation to be errors. We empirically determined that our error distribution were best modeled as a β distribution. Thus, for each base position and each possible nucleotide substitution error, we fit a β distribution based on the mean fraction of capture events consistent with the error and the standard deviation of this value across multiple samples. For sites and/or substitutions where no variation was detected in one or more of the baseline specimens, we assumed a fixed error rate of 1 in 15,000 consensus smMIP

reads, which can be considered a conservative threshold given the measured performance of the assay, and an appropriate β distribution was modeled. Subsequently, for each variant encountered experimentally, a site- and position-specific p -value was ascribed to each variant called using these pre-computed β distributions, and sites with $p > 0.005$ were excluded as potential artifacts.

Comparison of conventional deep sequencing to smMIP capture

We utilized sequencing data from smMIP capture to compare error rate differences between conventional deep sequencing strategies and smMIP capture. To evaluate the effects of smMIP mediated error correction, standard smMIP analysis was implemented as above. To determine the error rate of conventional sequence analysis, the same sequence data were aligned to the human genome as above, with variant calling performed using VarScan's `mpileup2snp` and `mpileup2indel` functions with flags set to `--min-reads2 2 --min-coverage 2 --min-var-freq 0.000000000001 --strand-filter 0 --p-value 1` for evaluating standard variant detection and `--min-reads2 2 --min-coverage 2 --min-var-freq 0.000000000001 --strand-filter 0 --p-value 1` for ultrasensitive variant detection.

SUPPLEMENTARY REFERENCES:

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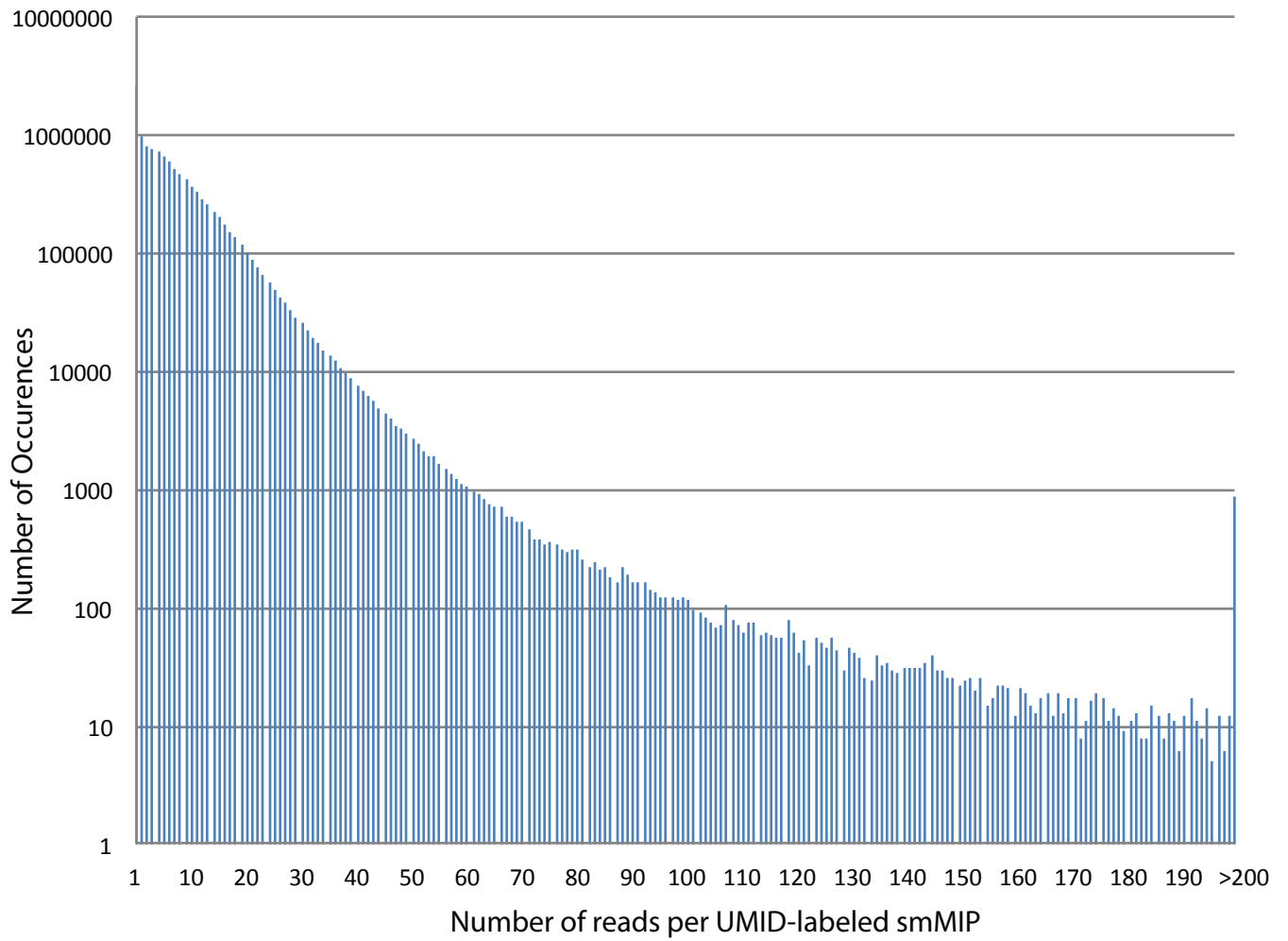


Figure S1. Distribution of reads across uniquely identifiable smMIP probes.

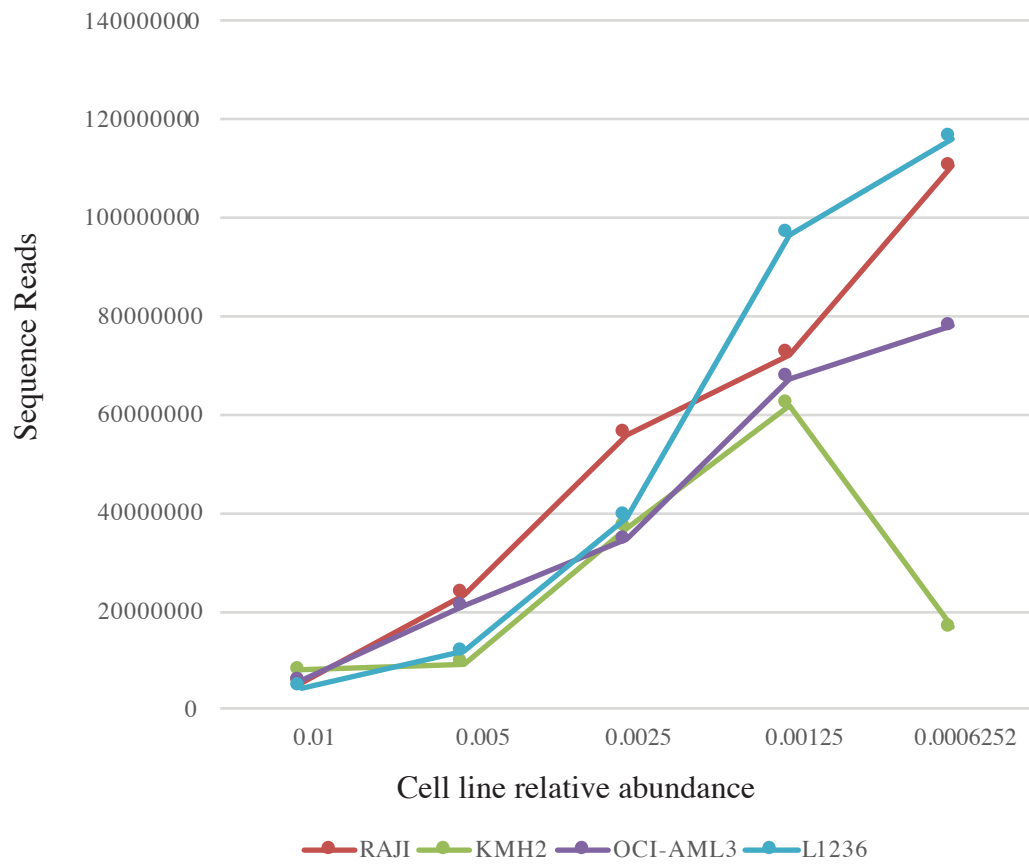


Figure S2. Total number of sequence reads required to detect mutations at different levels of relative abundance

Table S1. Additional patient testing results

| Patient | Day from initial diagnosis | Days post transplant | Bone marrow blast percentage | White blood cell count (Thousand/ μ L) | Hemoglobin (g/dL) | Hematocrit (%) | Platelet count (Thousand/ μ L) | Cytogenetics | FISH | Chimerism |
|---------|----------------------------|------------------------------|--|--|-------------------|----------------------------|------------------------------------|---|--|---|
| 1 | 83 | Not performed | Less than 1% blasts | N/A* | N/A | N/A | N/A | N/A | N/A | N/A |
| | 196 | Not performed | N/A | 0.9 (27% monocytes, Absolute neutrophil count 100) | 10 | 30 | 87 | N/A | N/A | N/A |
| | 329 | Not performed | 90% blasts by morphology | 1.8 (Absolute neutrophil count 1.2) | 9.6 | 26 | 34 | N/A | N/A | N/A |
| | 412 | Not performed | N/A | 4.1 | 9.4 | 28 | 65 | N/A | N/A | N/A |
| | 531 | Not performed | esetimate 90% blasts | 1 | 10 | 30 (MCV 87.6 fL, RDW 14%) | 17 | From day 430, ISCN Diagnosis: 48,XY,+8,+22[7]/46,XY[13] | From day 430, nuc ish(RUNX1T1x3,RUNX1x2)[37/200] | N/A |
| 2 | 0 | -97 | 2% blasts, diagnosed as negative with no significant morphologic evidence of residual acute monocytic leukemia | 1.52 | 12 | N/A | 138 | 46,XY [28] | Prior testing, normal AML (5, 7, 8, 20q, MLL) and MDS (chr 5, 7, 8, 13q, 20q) FISH panel | N/A |
| | 69 | 28 | Blasts not increased | 5.06 | 10.3 | N/A | 229 | 46,XY [1], 46,XX [19] | N/A | T cells 39% donor; NK cells 93% donor; no data for myeloid fraction myeloid 100% donor; T cells 50% donor |
| | 125 | 84 | Less than 5% blasts; no circulating blasts | 4.59 | 9.3 | 26 (MCV 83 fL) | 205 | 46,XX [20] | N/A | 99% donor, 1% host |
| | 393 | 352 | Blasts not increased | 11.21 | 10.5 | N/A | 193 | 46,XX [20] | N/A | N/A |
| | 397 | 356 | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| | 461 | 420 | Less than 5% blasts; no circulating blasts | 10.12 (1.52 eosinophils) | 13.7 | N/A | 157 | 46,XX [20] | N/A | N/A |
| | 579 | 538 | Blasts not increased | 17.01 | 16.1 | N/A | 232 | N/A | N/A | T cells 71% donor; myeloid 100% donor |
| 746 | 705 | "florid relapse, 80% blasts" | N/A | N/A | N/A | N/A | N/A | N/A | N/A | |
| 3 | 0 | -115 | N/A | 1.0 | 8.7 | 24 (MCV 87 fL, RDW 14.3%) | 32 | 46,XX [20] | Negative for deletion 7q/monosomy 7, t(8;21), t(9;22), MLL, t(15;17), inv(16)/t(16;16) | N/A |
| | 94 | -21 | N/A | N/A | N/A | N/A | N/A | 46,XX [20] | N/A | N/A |
| | 143 | 28 | No blasts seen | 5.29 | 9.3 | N/A (RDW 20%) | 331 | 46,XX [20] | N/A | N/A |
| | 269 | 154 | No blasts seen | 2.36 | 9.7 | 30 (MCV 87 fL, RDW 15.4%) | 32 | N/A | N/A | 100% donor |
| | 346 | 231 | No blasts seen | 3.2 | 13.4 | 40 (MCV 108 fL, RDW 19.1%) | 70 | 46,XX [20] | N/A | N/A |
| | 437 | 322 | No blasts seen | 1.21 | 11.5 | 34 (MCV 114 fL, RDW 14.6%) | 97 | 46,XX[20] | N/A | 100% donor |
| | 542 | 427 | No blasts seen | 2.35 | 11.5 | 34 (MCV 113 fL, RDW 18.1%) | 38 | N/A | N/A | N/A |
| | 584 | 469 | N/A | 2.17 | 11.3 | 34 (MCV 117 fL, RDW 14.3%) | 49 | N/A | N/A | N/A |
| | 599 | 484 | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| | 269 | -917 | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| 4 | 1083 | -103 | Blasts not increased | 2.67 | 10.8 | 32 (MCV 98 fL, RDW 13.7%) | 56 | 46,XY [20] | nuc ish(EGR1,D5S23)x2[199/200]_(D7Z1,D7S486)x2[199/200]_(D8Z2x2)[200/200]_(D20S108x2)[200/200] | N/A |
| | 1214 | 28 | Blasts not increased | 6.92 | 11.8 | 35 (MCV 103 fL, RDW 18.9%) | 186 | 46,XX [20] | Normal AML IFISH panel: absence of t(8;21), MLL, t(15;17), inversion or rearrangement CBFb | N/A |
| | 1556 | 370 | Blasts not increased | 6.88 | 14.4 | 35 (MCV 104 fL, RDW 14.6%) | 282 | 46,XX [20] | N/A | N/A |
| | | | | | | | | | | |

* Not Available

| | | | | | |
|----------------------|---|----------|----------|----------|---|
| SMCIA/NM_006306_0466 | X | 53440108 | 53440127 | 53439971 | 53439992 CTCAACAAGGAACTGGCCTCAANNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTNNNNATCGATGCCCCCTGCCAC |
| SMCIA/NM_006306_0467 | X | 53440156 | 53440175 | 53440286 | 53440307 GCTTGTCTACTCTCGCCAGNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTNNNNAGCCAAGAGGTAGCTTGGCC |
| SMCIA/NM_006306_0468 | X | 53440265 | 53440285 | 53440406 | 53440426 CTTCACCTTCAGACCCAGCCNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTNNNNCTTCACCATTTCTTCTTTC |
| SMCIA/NM_006306_0469 | X | 53441797 | 53441814 | 53441663 | 53441686 TTGGCTATGTGTTTTTCCAGACNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTNNNNCTTCGTAGTACAAGATC |
| SMCIA/NM_006306_0470 | X | 53441704 | 53441721 | 53441832 | 53441855 GCATGAGTTGGCAAGGGTCAGCCNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTNNNNACCTGGAAACGAGGAA |
| SMCIA/NM_006306_0471 | X | 53441811 | 53441828 | 53441939 | 53441962 CACGGCAAGGTACGGTCTCAGNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTNNNNAAGAACCTCTACAAGAC |
| SMCIA/NM_006306_0472 | X | 53442056 | 53442073 | 53441912 | 53441935 TTGTAGTGGGTGAGGTGGCCAGNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTNNNNAACCGAACCTGGCGGG |
| SMCIA/NM_006306_0473 | X | 53441999 | 53442016 | 53442132 | 53442155 GACGAGGCAGTAGAGGGAAAGTCANNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTNNNNGGTTGGCAGCTGGCTTGC |
| SMCIA/NM_006306_0474 | X | 53449533 | 53449550 | 53449399 | 53449422 CGGCCCTGGCCAGTCCCGCCTGNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTNNNNATGGGGTTCTCTGAAACT |
| SMCIA/NM_006306_0475 | X | 53449580 | 53449599 | 53449448 | 53449469 CACCCGATCATTGGACCAATNNNNCTTCAGCTTCCCGATATCCGACGGTAGTGTNNNNCTCGGGCTACGGCGGGCC |

Table S3. Quantitation of mutations in cell line dilution experiments

| Measured % cell line | RAJI | | | KMH2 | | | | | OCI-AML3 | | | L1236 | | | Average | Standard Deviation | CV |
|----------------------|---|---|--|---|---|---|---------------------------------------|---|--------------------------------------|---|---|--|-------------------------------------|------------|------------|--------------------|----|
| | MYC:NM_002467 :exon2:c.G62C:p.S21T & MYC:NM_002467 :exon2:c.G162C | TP53:NM_001126 115:exon3:c.T304 C:p.Y102H | TP53:NM_001126 115:exon2:c.G242 A:p.R81Q | MTOR:NM_00495 8:exon48:c.C6695 T:p.T2232I | EGFR:NM_00522 8:exon28:c.G3471 C:p.W1157C | NOTCH2:NM_001200001:exon11:c.G1759A:p.G587S | RAF1:NM_002880 :exon5:c.T436A:p.F146I | KMT2A:NM_001197104:exon12:c.A4546T:p.T1516S | NRAS:NM_00252 4:exon3:c.A182T:p.Q61L | NPM1:NM_19918 5:exon10:c.772_773insTCTG | JAK2:NM_004972 :exon16:c.T2025G;p.C675W | ERBB3:NM_001982:exon22:c.A2663 G:p.Y888C | CDK4:NM_00007 5:exon2:c.G97C:p.A33P | | | | |
| 0.010000 | 0.001308356 | 0.005150916 | 0.006203331 | 0.01611829 | 0.017857143 | 0.010043042 | 0.011557572 | 0.007343722 | 0.010436588 | 0.002083333 | 0.020689655 | 0.010339734 | 0.007518797 | 0.00974234 | 0.00557926 | 0.57268196 | |
| 0.005000 | 0.000593522 | 0.002692273 | 0.003042473 | 0.007425455 | 0.006674757 | 0.004013761 | 0.004760368 | 0.003758833 | 0.005638064 | 0.003289474 | 0.005649718 | 0.003215434 | 0.003888723 | 0.0042033 | 0.00174876 | 0.41604431 | |
| 0.002500 | 0.000353899 | 0.001251924 | 0.001185352 | 0.002309913 | 0.005064573 | 0.002257336 | 0.002570263 | 0.002278356 | 0.00282516 | 0.001477105 | 0.003533569 | 0.000862813 | 0.00128522 | 0.00209658 | 0.00120507 | 0.57477951 | |
| 0.0012500 | 3.70E-05 | 0.000576181 | 0.000855237 | 0.001604033 | 0.002554931 | 0.001005892 | 0.001030822 | 0.001051156 | 0.000900231 | 0.000979192 | 0.003514938 | 0.000475285 | 0.000797978 | 1.18E-03 | 0.0008827 | 0.74596425 | |
| 0.0006250 | 3.43E-05 | 0.000558116 | 0.000770614 | 0.000899483 | 0.000825764 | 0.000771159 | 0.000762571 | 0.000331895 | 0.000924214 | 0 | 0.002150538 | 0.000367647 | 0.000874661 | 7.13E-04 | 0.00051526 | 0.72251585 | |

Table S4. Quantitation of mutations in patient specimens

| Mutation (or NPM1NGS result) | Day from initial Diagnosis | | | | |
|---|----------------------------|--------------|------|------|------|
| | 83 | 196 | 329 | 412 | 531 |
| NPM1NGS variant allele frequency | 0.001 | 0 | 0.25 | 0.12 | 0.49 |
| NPM1-NM_199185:exon10:c.772_773insTCTG | Not Detected | Not Detected | 0.31 | 0.15 | 0.55 |
| NRAS-NM_002524:exon2:c.G35A:p.G12D | 0.001 | Not Detected | 0.25 | 0.11 | 0.5 |
| WT1-NM_000378:exon8:c.G1339A:p.D447N | Not Detected | Not Detected | 0.23 | 0.14 | 0.72 |
| SMC1A-NM_006306:exon2:c.T198G:p.H66Q | 0.002 | 0.006 | 0.53 | 0.26 | 0.99 |

| Mutation (or NPM1NGS result) | Day from initial Diagnosis | | | | | | | | |
|---|----------------------------|--------------|--------------|--------------|--------------|--------------|--------------|-------------|--|
| | 0 | 69 | 125 | 393 | 397 | 461 | 579 | 746 | |
| NPM1NGS variant allele frequency | 0.05998 | 0.00041 | 0.00122 | 0.00149 | 0.00124 | Not Detected | Not Detected | 0.474 | |
| NPM1-NM_199185:exon10:c.772_773insTCTG | 0.058527376 | 0.000320376 | 0.001493358 | 0.001774308 | 0.001229875 | Not Detected | Not Detected | 0.614019117 | |
| WT1-NM_001198551:c.134-57C>T | 0.428050052 | 0.027350814 | 0.030102547 | 0.026605853 | 0.007879186 | 0.001394422 | 0.001300672 | 0.915941058 | |
| TP53-NM_001126115:c.-112A>G | 0.514041838 | 0.050551731 | 0.036071537 | 0.035118979 | 0.015493942 | 0.009309029 | 0.039795388 | 0.485174459 | |
| TP53-NM_001126115:c.-107T>C | 0.488877095 | 0.025558804 | 0.026826311 | 0.022991148 | 0.0069315 | 0.001168497 | 0.001300503 | 0.452075576 | |
| TP53-NM_001126118:c.-182T>A | Not Detected | Not Detected | Not Detected | Not Detected | Not Detected | Not Detected | 0.118644068 | 0.040236696 | |
| TP53-NM_001126118:c.-185C>A | Not Detected | Not Detected | Not Detected | Not Detected | Not Detected | Not Detected | 0.056497175 | 0.040236696 | |
| WT1-NM_001198551.1:c.10+79C>T | 0.314771198 | 0.004808909 | 0.002511805 | 0.002229965 | 0.00241955 | 0.00175312 | 0.002473717 | 0.136546185 | |

| Mutation (or NPM1NGS result) | Day from initial Diagnosis | | | | | | | | | |
|---|----------------------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|--------------|
| | 0 | 94 | 143 | 269 | 346 | 437 | 542 | 584 | 599 | |
| NPM1NGS variant allele frequency | 0.000922 | 0.000077 | Not Detected | Not Detected | Not Detected | Not Detected | Not Detected | Not Detected | Not Detected | Not Detected |
| NPM1-NM_199185:exon10:c.772_773insTCTG | 0.001369394 | 0.000347705 | Not Detected | Not Detected | Not Detected | Not Detected | Not Detected | Not Detected | Not Detected | Not Detected |
| WT1-NM_001198551:c.10+67T>C | 0.033406917 | 0.032224889 | 0.000995644 | Not Detected | 0.001707456 | 0.00067659 | 0.004513043 | 0.001269036 | Not Detected | Not Detected |
| STAG2-NM_006603:exon16:c.T1616G:p.V539G | 0.02950905 | 0.00094697 | 0.022482014 | Not Detected | 0.00204499 | 0.001409443 | 0.00425448 | 0.018535286 | Not Detected | Not Detected |
| RAD21-NM_006265:exon8:c.C839G:p.S280X | 0.013621312 | 0.000200401 | 0.000421905 | Not Detected | 0.000470588 | 0.000386026 | 0.000531124 | 0.001582636 | 0.000842105 | Not Detected |
| PTPN11-NM_002834:exon4:c.T495A:p.S165S | 0.013432836 | 0.009255558 | 0.016997167 | 0.009584665 | 0.021341463 | 0.002717391 | 0.000640369 | 0.005813953 | 0.008230453 | Not Detected |
| KRAS-NM_004985:exon2:c.G34A:p.G12S | Not Detected | Not Detected | Not Detected | Not Detected | Not Detected | Not Detected | Not Detected | 4.61135E-05 | 0.00051595 | Not Detected |
| IDH1-NM_001282386:exon4:c.C394A:p.R132S | Not Detected | Not Detected | Not Detected | Not Detected | 0.001100537 | 0.001453992 | 0.000937082 | 0.001924481 | 0.001413957 | Not Detected |