Analytical validation and performance characteristics of a 48-gene next-generation sequencing panel for detecting potentially actionable genomic alterations in myeloid neoplasms

(Analytical validation of a 48-gene next-generation sequencing panel for myeloid neoplasms)

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Abstract

Identification of genomic mutations by molecular testing plays an important role in diagnosis, prognosis, and treatment of myeloid neoplasms. Next-generation sequencing (NGS) is an efficient method for simultaneous detection of clinically significant genomic mutations with high sensitivity. However, due to lack of standard NGS protocols, the application of NGS for hematologic malignancies into clinical settings remains limited. We report development and validation of a 48-gene NGS panel for molecular profiling of myeloid neoplasms including acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), and myeloproliferative neoplasms (MPN). Target regions were captured by hybridization with complementary biotinylated DNA baits, and NGS was performed on an Illumina NextSeq500 instrument. A bioinformatics pipeline that was developed in-house was used to detect single nucleotide variations (SNVs), insertions/deletions (indels), and *FLT3* internal tandem duplications (*FLT3*-ITD). An analytical validation study was performed on 184 unique specimens for variants with allele frequencies ≥5%. Variants identified by the 48-gene panel were compared to those identified by a 35-gene hematologic neoplasms panel using an additional 137 unique specimens. The developed assay was applied to a large cohort (n=2,053) of patients with suspected myeloid neoplasms. Analytical validation yielded 99.6% sensitivity (95% CI: 98.9-99.9%) and 100% specificity (95% CI: 100%). Concordance of variants detected by the 2 tested panels was 100%. Among patients with suspected myeloid neoplasms (n=2,053), 54.5% patients harbored at least one clinically significant mutation: 77% in AML patients, 48% in MDS, and 45% in MPN. Together, these findings demonstrate that the assay can identify mutations associated with diagnosis, prognosis, and treatment options of myeloid neoplasms.

Introduction

Myeloid neoplasms are a heterogeneous group of malignancies of the hematopoietic stem/progenitor cells. Substantial clinical and genomic overlap exists among different subclasses of myeloid neoplasms that are currently classified by the World Health Organization (WHO): acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), myeloproliferative neoplasms (MPN), and overlap myelodysplastic/myeloproliferative neoplasms (MDS/MPN) [1]. Coupled with clinical, morphologic, and immunophenotypic abnormalities, identification of genetic alterations by molecular testing has an important role in the classification, risk stratification, and management of myeloid neoplasms [2]. For example, mutations in *NPM1*, *CEBPA*, and *RUNX1* can identify specific subclass of AML, and mutations in *FLT3*, *IDH1*, *IDH2*, and *JAK2* can guide targeted therapies for AML [3]. For patients with MDS, identification of mutations in genes including *TET2*, *SF3B1*, *ASXL1* and *TP53* is particularly useful to help establish clonal hematopoiesis to make a definitive diagnosis of MDS [4]. In addition, for MPN, certain mutations such as *JAK2* V617F or exon 12 mutation satisfy diagnostic criteria to help establish a diagnosis of MPN [5]; mutations in *ASXL1*, *SRSF2*, *EZH2*, *IDH1*, and *IDH2* categorize patients to high molecular risk in Primary Myelofibrosis (PMF) [6]; and mutations in *IDH2*, *U2AF1*, *EZH2*, *TP53*, *SH2B3*, and *SF3B1* indicate adverse prognostic value in Essential Thrombocythemia (ET) and Polycythemia Vera (PV) [7, 8].

Historically, single gene testing using Sanger sequencing or real-time PCR have been used to identify genetic alterations in myeloid neoplasms [9]. Although these methods are readily

adaptive and widely used in clinical laboratories, their major limitation is that very few selected mutations can be detected at a time. In recent years, whole genome sequencing (WGS) and whole exome sequencing (WES) studies have been used to identify oncogenic mutations in hundreds of genes, revealing a wide genetic heterogeneity in myeloid neoplasms [9]. Indeed, more molecular genetic markers have been added in the most recent revision of the WHO classification [1]. Since the number of mutations that can guide diagnosis, prognosis, and treatment options are increasing, using single-gene testing for myeloid malignancies is becoming impractical. Although WGS and WES are standard in research settings, targeted next generation sequencing (NGS) panel assays that are composed of genes associated with a disease and recurrently mutated are more commonly used in clinical settings. Compared to WGS or WES, targeted NGS assays are highly sensitive for detecting low-frequency variants and can identify a number of mutations that are critical in diagnosis and risk stratification in a relatively short time [10].

Although an increasing number of hematologic laboratories are in the process of integrating NGS procedures into the diagnostic algorithms of myeloid neoplasms, the application of NGS in clinical settings has certain challenges [11]. For example, many artifacts are known to arise during NGS library preparation, sequencing, and data analysis (eg, read mapping, variant calling) and these may cause challenges in discriminating true genetic alterations from artifacts caused by PCR, sequencing, and post-sequencing steps. In addition, technical difficulties hinder the ability to capture targets with high GC content, such as *CEBPA,* which is associated with poor prognosis of AML, or repetitive genomic regions, such as *FLT3*-ITD, which is associated with poor prognosis of AML in the absence of *MPN1* mutation [10, 12, 13-15]. The lack of uniform practice standards for quality assessment of NGS data also challenges implementation of

NGS in clinics [11]. As such, it is critical to carefully validate each clinical NGS assay for its defined performance requirements for the intended use.

In this study, we report development and validation of a 48-gene NGS panel for the detection of alterations that have a putative role in diagnosis, prognosis, or therapy of myeloid neoplasms. We also report our experience with the first 2,053 clinical specimens of suspected myeloid neoplasms submitted for the 48-gene panel and estimate the frequency of actionable alterations in a clinical laboratory.

Materials and Methods

Gene selection

A total of 48 genes (**S1 Table**) frequently mutated and/or associated with known diagnostic, prognostic, or therapeutic utilities for myeloid neoplasms at the time of test development were selected: 42 genes for AML; 36 genes for MDS; and 26 genes for MPN. Of the 48 genes, 47 were analyzed by NGS: the entire coding regions were covered for 23 genes (*ATM*, *BCOR*, *BCORL1*, *BRAF*, *CDKN2B*, *CEBPA*, *CREBP*[*ATF2*], *CUX1*, *DDX41*, *ETNK1*, *ETV6*, *GATA2*, *HRAS*, *IKZF1*, *KDM6A*, *NF1*, *PHF6*, *PTEN*, *STAG2*, *STAT3*, *STK11*, *TP53*, and *ZRSR2*) and targeted exons were covered for 24 genes (*ASXL1*, *CALR*, *CBL*, *CSF3R*, *DNMT3A*, *EZH2*, *FLT3*, *GATA1*, *IDH1*, *IDH2*, *JAK2*, *KIT*, *KRAS*, *MPL*, *NPM1*, *NRAS*, *PTPN11*, *RUNX1*, *SETBP1*, *SF3B1*, *SRSF2*, *TET2*, *U2AF1*, and *WT1*). *KMT2A* (formerly known as *MLL*) partial tandem duplication (PTD) analysis was supplemented by a long-range PCR method developed in our laboratory using a previously reported primer set, a forward primer on exon 8 and a reverse

primer on exon 2 [16], to detect the 3 most frequent forms of *MLL*-PTD (NM_005933.1: exons 2~8, 2~9, and 2~10) [17].

Validation specimens

A total of 184 unique specimens (**S2 Table**) were included: 96 whole blood, 20 bone marrow aspirate, 20 cell pellet, 17 FFPE, and 31 extracted DNA. The 184 specimens included 32 commercial controls, 25 from healthy individuals, and 127 clinical specimens previously tested by alternative analytical methods for indications of myeloid neoplasms (n=105), unknown history (n=18), and adjacent normal tissue from FFPE section (n=4). All patient specimens were de-identified before use. Alternative analytical methods included Sanger sequencing, fragment analysis for *FLT3*-ITD, and 4 different CLIA-validated NGS assays developed in our laboratory (see Alternative analytical methods, below). The 32 commercial controls included 6 multiplex mutation controls (5% Tier, Horizon discovery, Lafayette, CO), 19 controls with known mutation(s) within the 48 genes in the panel (Coriell Institute, Camden, NJ), and 7 well-characterized Genome In a Bottle (GIAB) reference specimens (Coriell Institute, Camden, NJ). For the 7 GIAB specimens, high-quality public sequence data were downloaded from http://ftp-trace.ncbi.nlm.nih.gov/giab/ftp/release/ for comparison.

Alternative analytical methods

Sanger sequencing on *CEBPA* or *JAK2* were performed using gene-specific PCR primers for target amplification and incorporation of BigDye Terminator (Applied biosystems, Foster

City, CA, USA). T7 promoter and terminator sequences fused to gene specific primers were used for sequencing on an ABI 3730 fragment analyzer (Applied biosystems, Foster City, CA, USA).

FLT3-ITD fragment analysis was performed by Laboratory for Personalized Molecular Medicine (LabPMM, San Diego, CA) or in-house using a modified protocol based on one reported previously [18]. In brief, *FLT3*-ITD was PCR-amplified with a fluorescence-labeled forward primer, and a non-labeled reverse primer. The PCR products were analyzed using an ABI3730 genetic analyzer (Applied Biosystems, Foster City, CA, USA), and the amplicons with a size greater than that of wild type (324-326 bp) were interpreted as positive for the *FLT3*-ITD.

Four separate CLIA-validated NGS panel assays were used for the accuracy study to cover the variants detected by the 48-gene panel. The first, LeukoVantage v1.0, is a 30-gene NGS test for somatic mutations in myeloid neoplasms using the Truseq Amplicon Cancer Panel kit (Illumina, San Diego, CA). It contains amplicons for hot-spot locations of the following genes: *ASXL1*, *CALR*, *CBL*, *CEBPA*, *CSF3R*, *DDX41*, *DMNT3A*, *EZH2*, *FLT3*, *GATA1*, *IDH1*, *IDH2*, *JAK2*, *KDM6A*, *KIT*, *KRAS*, *MLL*, *MPL*, *NPM1*, *NRAS*, *PTPN11*, *RUNX1*, *SETBP1*, *SF3B1*, *SRSF2*, *TET2*, *TP53*, *U2AF1*, *WT1*, and *ZRSR2* [19]. The second, MyVantage, is a germline mutation panel of 34 cancer predisposition genes, including *ATM* and *PTEN*, and uses RNA bait capture method [20]. The third, Watson Genomics, is a 50-gene NGS test for solid tumor mutation using RNA bait capture-based NGS, and includes *BRAF*, *CDKN2B*, *FLT3*, *HRAS*, *IDH1*, *JAK2*, *KIT*, *KRAS*, *NRAS*, *PTEN*, *PTPN11*, and *TP53*. The fourth, MPN diagnostic cascading reflex test, includes hotspots in *MPN*, *CALR*, *MPL*, and *CSF3R* genes and employs multiplex PCR method and sequencing on Ion Torrent S5 XL (Thermo Fisher Scientific, Markham, ON).

DNA isolation, library preparation, and NGS

Genomic DNA was isolated from whole blood, bone marrow, or cell pellet using Qiagen EZ1 kit or DSP DNA mini kit (Qiagen, Mississauga, ON). FFPE DNA was extracted using QIAAMP DNA FFPE tissue kit (Qiagen, Mississauga, ON), or Arcturus PicoPure kit (Thermo Fisher Scientific, Markham, ON). Isolated genomic DNA was mechanically sheared to an average size of 250 bases using a Covaris instrument LE220 (Covaris Inc., Woburn, MA). The fragmented DNA was enzymatically repaired and end-modified with adenosine (NEBNext® Ultra™ II End Repair/dA-Tailing Module, NEB, Ipswich, MA) and ligated (NEBNext® Ultra ™ II Ligation Module, NEB, Ipswich, MA) with barcoded adapters (Integrated DNA Technologies, Coral, IL). The ligated products were size-selected (AMPure Beads, Agencourt, Beverley, MA) and amplified (GeneRead DNA I Amp Kit, Qiagen, Mississauga, ON); then the regions of interest were captured using biotinylated DNA baits (Integrated DNA Technologies, Coral, IL). The hybridized DNA fragments were enriched with streptavidin-attached magnet beads (Dynabeads M-270, Thermo Fisher Scientific, Markham, ON) and washed under increasing stringency to remove non-targeted DNA sequences (xGen® Lockdown® Reagents, Integrated DNA Technologies, Coral, IL). A second amplification was performed (KAPA HiFi HotStart ReadyMix, Kapabiosystems, Wilmington, MA), followed by bead purification (AMPure Beads, Agencourt, Beverley, MA) to remove all unused primers and nucleotides. The prepared sequencing library was then quantified (Qubit dsDNA HS Assay Kit, Thermo Fisher Scientific, Markham, ON) and sequenced on an Illumina NextSeq 500 sequencer, 2 x 150 cycles (NextSeq 500/550 Mid Output v2 kit, Illumina, San Diego, CA).

Bioinformatics processing

De-multiplexing and conversion of NextSeq500 BCL files were done by using Illumina's bcl2fastq software utility. The raw sequence reads in FASTQ files were then aligned to the Genome Reference Consortium human genome build 37(GRCh37) by using BWA alignment package. Reads were sorted and indexed using SAMtools with subsequent reads duplication removal by Picard Tools. Local realignment and base quality score recalibration were performed using the Genome Analysis Toolkit (GATK). Mapped reads were further filtered by mapping score ≥ 30 ($\geq 99.9\%$ accuracy) and base quality score ≥ 20 ($\geq 99\%$ accuracy) before downstream analysis. This assay covered 98,809 bp from a total of 999 target regions, approximately100 bp for each target, across the 48 genes in the panel. Average and minimum depth of coverage for every region of interest (ROI) and depth of each targeted positions were computed using SAMtools Pysam. SNVs and short indels were called by MuTect2 and LoFreq. *CALR* indels and *FLT3*-ITD were called by PINDEL. Criteria used for specimen and variant quality control are provided in **S3 Table**.

Variant call comparison

An additional 137 unique specimens that were submitted for the 48-gene panel or a 35-gene panel, which was independently developed and analytically validated for hematologic neoplasms (HemeSEQ, med fusion, Lewisville, TX), were subsequently sequenced by the other panel. The 35-gene panel uses Illumina TruSeq custom-amplicon library preparation chemistry, which is sequenced on an Illumina MiSeq. A total of 33,812 bp in 26 genes overlapped between the 2 assays; genes included *ASXL1*, *CALR*, *CBL*, *CSF3R*, *DNMT3A*, *ETV6*, *EZH2*, *FLT3*, *IDH1*,

193 *IDH2*, *JAK2*, *KIT*, *KRAS*, *MPL*, *NPM1*, *NRAS*, *PHF6*, *RUNX1*, *PTEN*, *SETBP1*, *SF3B1*, *SRSF2*,
194 *TET2*, *TP53*, *U2AF1*, and *WT1*. Each of the 137 specimens (22 whole blood and 115 bone
195 marrow) were selected because they harbored at least one pathogenic mutation within the
196 overlapping genes.

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198 Clinical specimens

199 A total of 2,053 consecutive patient specimens submitted for the 48-gene NGS panel
200 were included in this study. Patient results were de-identified before analysis. Based on the
201 clinical information submitted, indications for testing included AML (23.9%, n=490), MDS
202 (49.6%, n=1,018), or MPN (26.5%, n=545). Patient characteristics are presented in **Table 1**. This
203 retrospective study was exempt from Institutional Review Board oversight, as determined by the
204 Western Institutional Review Board.

205 **Table 1. Patient characteristics**

	AML	MDS	MPN	Total
Number of patients	490	1,018	545	2,053
% of total	23.9	49.6	26.5	100.0
Male, %	61.6	61.5	57.8	60.5
Female, %	38.4	38.5	42.2	39.5
Median age (Min, Max)	58 (2, 89)	60 (2, 89)	55.5 (14, 69)	58 (2, 89)

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Variant classification

For the 2,053 clinical cases, clinical interpretation of variants was performed in conjunction with a third-party annotation group (N-of-One, Concord, MA). Detected variants were classified in the following categories based on the 2017 guideline recommendations by AMP/ASCO/CAP [21]: Tier I, strong clinical significance; Tier II, potential clinical significance; Tier III, uncertain clinical significance; and Tier IV, benign or likely benign. A pathologist or a licensed clinical laboratory director reviewed the results and the clinical annotation, edited information as deemed necessary for specific cases and indications, and signed out the results for reporting. For this study, variants were considered clinically significant if they provided prognostic or diagnostic information for the disease or were clinically actionable. Variants were considered clinically actionable when a targeted therapy or an experimental drug was available for the disease or other disease.

Results

Assay performance

The analytical validation study was performed on 184 unique specimens for a total of 427 trials in 20 consecutive sequencing runs. On average, each specimen generated 11.8 million reads; 100% (SD=8.8%) of reads mapped to the reference sequence (hg19); 65% (SD=8.8%) of reads were on-target; and average coverage depth across target regions was 1,767 (SD=875) (**S4**

228 **Table**). Average and median coverage of each target region across all validation specimens are
229 plotted in **Fig 1A**. Of the 184 specimens, 181 (98.3%) specimens exceeded the coverage
230 requirement in 422 trials (98.8%) and 3 specimens (1 whole blood, 1 FFPE, and 1 extracted
231 DNA) failed in 5 trials. This result demonstrated that the developed workflow is robust for
232 routine clinical testing and compatible with different specimen types.

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234 **Fig 1. Target coverage depth.** (A) Mean and median coverage of each target region across all
235 validation trials (n=427). Target regions are sorted by chromosomal location. Targets on X
236 chromosome are marked with a dotted line. (B) NA12878 coverage across all target regions from
237 17 independent setups. Target regions are sorted by % GC (orange dot). Standard deviation of
238 coverage is shown (black bar). (C) Percentage of specimens that passed a base quality Q20
239 coverage of 100X, 250X, or 500X.

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242 We next assessed coverage at each target region as a function of target region % GC
243 using a control specimen NA12878, which was tested 17 times. Throughout the whole % GC
244 spectrum (range: 12%-87%), we observed narrow coverage distribution, even in the extreme end
245 of % GC (**Fig 1B**). In addition, we reviewed specimen pass rates of each target for Q20 coverage
246 of 500X, 250X, or 100X (**Fig 1C**). A limited number of target regions did not achieve 500X
247 coverage in >10% of validation specimens. Those regions included *STAG2* exon 11, 16, and 21,
248 all of which were situated near polyA tracts and represented 0.36% (354/98,809bp) of our target

capture region. All 3 target regions achieved 100X coverage in 100% of validation sets and 250X coverage in >96% of validation sets.

Precision

To test inter-assay precision, 22 specimens that harbored at least one pathogenic mutation by alternative methods and a negative control (NA12878) were analyzed 3 times in 3 independently prepared sequencing runs. A total of 141 variants (98 SNVs, 39 indels, and 4 *FLT3*-ITD) were detected and were 100% concordant among 3 repeated runs (**S5A Table**). As expected, no reportable variant was detected from the negative control (NA12878) after applying variant filtering rules (**S3 Table**). Intra-assay precision was tested on 9 specimens that were tested in triplicate within a run. A total of 40 variants (30 SNVs, 8 indels, and 2 *FLT3*-ITD) were detected and all were concordant among the triplicates (**S5B Table**). In addition, detected variant frequency among replicates was reproducible within coefficient of variation <0.2 in 98.3% and <0.25 in 100% of variants (n=181) tested in the precision studies (**Figs 2A and 2B**). In summary, both intra- and inter-assay variant call concordance was 100%.

Fig 2. (A) Inter-assay precision of 141 variants from 22 specimens repeated 3 times. The mean of the detected variant frequencies (orange dot) with standard deviation (closed vertical bar) and coefficients of variations (blue dot) are shown. (B) Intra-assay precision of 40 variants from 9 specimens replicated 3 times. The mean of the detected variant frequencies (orange dot) with standard deviation (closed vertical bar) and coefficients of variations (blue dot) are shown.

Accuracy

A total of 140 unique specimens harboring variant(s) identified by alternative analytical methods, such as Sanger sequencing, fragment analysis, and other CLIA validated NGS assays, were analyzed for a total of 237 trials (**S6 Table**). The identified 165 unique variants by alternative methods consisted of 97 SNVs, 49 indels (1-33 bp insertions, 1-52 bp deletions), and 19 *FLT3*-ITDs (18-117 bp). From the 237 trials, collectively, 405 variants of various frequencies were analyzed including 229 SNVs, 117 indels, and 59 *FLT3*-ITDs in 29 genes (**Figs 3A, 3B**). All expected SNV and indel variants were accurately detected. If variant frequency was provided by an alternative method (n=106), frequency of detected variant was highly concordant with expected (R^2=0.966, **Fig 3C**). In addition, all expected *FLT3*-ITD variants from 59 trials of 27 unique specimens were detected by this assay (**Table 2**). In 18.5% (5/27) of *FLT3*-ITD positive specimens, additional ITD sizes that were not reported by a fragment analysis method were detected; this result may indicate higher sensitivity of NGS assay for *FLT3*-ITD detection.

Fig 3. (A) Number and type of variant used for accuracy study per gene. (B) Variant frequency distribution used for accuracy study. Variant frequency not provided by alternative method is categorized as undetermined. (C) Variant frequency concordance. Compared only if variant frequency is known by alternative method.

Table 2. *FLT3*-ITD used for accuracy study

Sample	*FLT3*-ITD length (bp)	Number trial(s)	Detected	Additional *FLT3*-ITD detected (bp)
BM08	36	3	yes	-
BM15	57	3	yes	-
BM17	57	3	yes	-
BM19	60	3	yes	-
CP04	45	1	yes	-
CP17	24	1	yes	-
CP20	21, 27, 36, 117	1	yes	48
WB09	30	11	yes	33, 81
WB32	21	1	yes	-
WB45	96	6	yes	-
WB46	72	3	yes	-
WB47	51, 114	4	yes	-
WB48	63	1	yes	-
WB50	18, 63, 90	4	yes	-
WB51	33, 57	1	yes	-
WB52	21, 51, 90	1	yes	-
WB53	18	1	yes	-
WB54	36	1	yes	24
WB56	24	1	yes	-
WB57	48	1	yes	33
WB58	30	1	yes	-
WB59	36	1	yes	-
WB60	51, 54	1	yes	-
WB61	24	2	yes	-
WB62	66	1	yes	-
WB63	24	1	yes	-
WB64	84	1	yes	27

BM, bone marrow; CP, cell pellet; WB, whole blood

We extended the accuracy study to 7 well-characterized GIAB reference specimens by comparing variant calls made by our pipeline with publicly available data. The variant

297 concordance study was limited to those regions where high-quality public sequence data were
298 available, approximately 62% to 86% of our target regions for each GIAB reference specimen
299 (**Table 3**). Population SNVs that would normally be excluded from reporting were also included
300 for this study. Across the 7 reference specimens, a total of 464,162 bp were analyzed. All 142
301 expected variants were correctly detected (**Table 3**), and no false positives were called, yielding
302 100% sensitivity (95% CI: 97.3-100%) and 100% specificity (95% CI: 100%). Review of the
303 detected variant frequency at the expected homozygous and heterozygous positions revealed the
304 mean observed variant frequency to be 99.9% (N=70, SD=0.1) and 49.6% (N=72, SD=2.6),
305 respectively, demonstrating good agreement with the expected values.

306 **Table 3. Variant call concordance study using Genome in a Bottle reference specimens**

Specimen	Overlapping regions: High confidence sequence from GIAB	Number of variants	% Concordance
NA12878	86,423 bp	23	100
NA24143	62,850 bp	16	100
NA24149	62,924 bp	20	100
NA24385	62,815 bp	22	100
NA24631	63,047 bp	21	100
NA24694	63,070 bp	19	100
NA24695	63,033 bp	21	100
Total	464,162 bp	142	100

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Analytical sensitivity

In order to evaluate the analytic sensitivity of the assay, 5 multiplex mutation control specimens (5% Tier, Horizon discovery) were tested for a total of 16 trials. Collectively, 124 expected variants (104 SNVs and 20 indels) with variant allele frequency (VAF) ranging from 3.8% to 25% were identified (**S7A Table**). All expected variants were detected when expected VAF was either at 5% (71/71) or >5% (35/35). However, 89% (16/18) of expected variants were detected when expected VAF was <5%.

In addition, DNA from each of 5 well-characterized GIAB reference specimens was mixed with NA12878 DNA in a proportion of 10% test specimen and 90% NA12878. From heterozygous or homozygous variants unique to test specimens, a total of 64 variants (63 SNVs and 1 insertion) were created with VAF 10% or 5% (**S7B Table**). A total of 119 variants were tested from the 5 mixed specimens of which 4 specimens were repeated twice. All (50/50) variants were detected at 10% VAF and 95.6% (66/69) variants were detected at 5% VAF.

Similarly, 9 specimens harboring variants (2 SNVs and 10 indels) identified by alternative analytical methods were serially diluted with a GIAB control (NA12878) up to 64-fold, yielding an expected VAF well below 5% (**S7C Table**). All expected variants were detected when expected VAF was >5% (n=45). However, when expected VAF was below 5%, only 41% (2/8 at <3.0%, 5/9 at 3.1-3.4%) of expected variants were detected.

In addition, 3 *FLT3*-ITD positive specimens were serially diluted up to 16-fold with NA12878. We considered *FLT3*-ITD positive when at least one ITD size is called. All dilution series yielded correct ITD calls (**S7D Table**). As the orthogonal method does not provide

absolute *FLT3*-ITD frequency, in order to estimate sensitivity, we simulated expected variant frequency based on the relative fraction of different ITD sizes in a specimen, WB52. WB52 contained 3 different ITD sizes (21b, 51b, and 90b) with relative ITD fraction of 1.4, 75.7, and 23.0%. The major ITD size (51b) was detected from all dilution series including 16-fold dilution with simulated VAF of 4.7% (16-fold dilution of 75.7%). This result demonstrated that *FLT3*-ITD detection sensitivity to be at least 5%.

A summary of analytical sensitivity study results is provided in **Table 4**. Inspection of the discordant variants from this study (12 at ≤3.1% VAF and 3 at 5% VAF) showed that those variants had been detected by a variant caller, but the calls were filtered out because of low frequency (<3%) or low variant count (<25) (**S8 Table**).

Table 4. Summary of analytical sensitivity study result

Study	Expected VAF			Total
	<5%	**5%**	**>5%**	
Multiplex mutation control	89 (16/18)	100 (71/71)	100 (35/35)	98 (122/124)
GIAB dilution	N/A	96 (66/69)	100 (50/50)	97 (116/119)
Positive specimen dilution	41 (7/17)	N/A	100 (58/58)	87 (65/75)
Total	66 (23/35)	98 (137/140)	100 (143/143)	95 (303/318)

Performance is provided as % detected (no. detected/no. expected). N/A, not applicable.

Collectively, in this validation, a total of 154 unique specimens were analyzed for 830 known variants (588 SNVs, 167 indels, and 75 *FLT3*-ITDs) of VAF ≥5%, including results

described in the accuracy section. Almost all variants (99.6%, 827/830) of VAF ≥5% variants were correctly detected, resulting in analytical sensitivity of 99.6% (95% CI: 98.9-99.9%). Analytical sensitivity for various VAFs for each variant type is summarized in **Fig 4** and **S9 Table**. In conclusion, the variant detection limit for this assay is 5% for SNV, indels (including *CALR* 52 bp deletion), and *FLT3*-ITD.

Fig 4. Analytical sensitivity of the assay for various VAFs for each variant type. Analytical sensitivity percentage represents the proportion of detected variants out of all variants tested at a given VAF.

Variant call comparison study

To increase per-sample data comparison efficiency, we compared variants of an additional 137 unique specimens identified by the validated 48-gene NGS panel for myeloid neoplasms to those detected by an independently developed and analytically validated 35-gene NGS panel for hematologic neoplasms (HemeSEQ, med fusion, Lewisville, TX). The 137 specimens were selected because they harbored at least one pathogenic mutation within overlapping genes (n=26) between the 2 assays. From the 137 specimens (141 trials), a total of 1,094 variants (278 unique) of various frequencies were concordantly detected by the 2 assays: 1,007 (219 unique) SNVs and 87 (59 unique) indels (**Fig 5A**). In this study, benign variants (eg, common population SNVs) were also included. In addition, agreement in VAF between the 2

assays was good (R^2=0.986) (**Fig 5B)**. Collectively, a total of 4.8 million individual base calls (33,812 bp X 141 specimens) were compared, including 1,094 variants and resulted in 100% sensitivity (95% CI: 99.7-100%) and 100% specificity (95% CI: 99.9-100%).

Fig 5. Variant call concordance study between the 48-gene panel for myeloid neoplasms and 35-gene panel for hematologic neoplasms. (A) Number and type of variants compared per gene. (B) Scatter plot of VAFs (n=1,094) across 141 trials detected by the 48-gene panel (x-axis) and 35-gene panel (y-axis).

Long-term test reproducibility

To evaluate long-term reproducibility, we examined variant calls for a multiplex mutation control (HD728) that was repeatedly tested, 119 times over 10 months, as a mutation positive control. The mutation control contained 7 alterations of ≥5% VAF (6 SNVs and 1 deletion, **Table S7A**). All alterations were successfully detected in all repeat tests, including 5 alterations occurring at 5% VAF. Detected VAF remained stable over the extended time period (**Fig 6**) with coefficient of variation less than 0.1x (range: 0.04-0.1x).

Fig 6. Detected variant frequency of a mutation-positive control (HD728) from 119 repeat tests over 10 months. Expected variant frequency is indicated along with variant name.

Detection of clinically actionable alternations

Retrospective analysis was performed on the mutation profiling of 2,053 consecutive, de-identified unique patient specimens submitted for the 48-gene NGS panel. In total, 98.3% (2,018/2,053) of specimens were successfully tested upon initial testing and the remaining 35 specimens passed QC criteria upon repeat test. Based on the clinical information submitted, indications for testing included 490 AML (23.9%), 1,018 MDS (49.6%) and 545 MPN (26.5%). At least one pathogenic mutation was detected in 55.6% (1,142/2,053) of patient specimens. AML patients had the highest positive rate (81.2%, 398/490), followed by MDS (49.0%, 499/1,018) and MPN (45.0%, 245/545). A median of 2 mutations (range 1-12) were detected per positive patient: 3 (1-12) in AML, 2 (1-9) in MDS, and 2 (1-7) in MPN. Collectively 2,799 pathogenic mutations (Tier I or Tier II) were found in 44 genes (**Fig 7**). The most frequently mutated genes, which were found in at least 10% of patients for each indication, were *TET2*, *ASXL1*, *TP53*, *FLT3*, *NPM1*, *DNMT3A*, *IDH2*, *RUNX1* and *NRAS* in AML; *TET2*, *SF3B1*, and *ASXL1* in MDS; and *JAK2*, *TET2*, and *ASXL1* in MPN (**S1 Fig)**. The genomic alterations identified across 2,023 specimens are depicted in **Fig 8**.

Fig 7. Pathogenic mutations (Tier I or Tier II) detected in 44 genes from the total cohort of 2,053 patients by the 48-gene NGS panel for myeloid neoplasms.

Fig 8. Identified Tier I and Tier II mutations in 44 genes from the total cohort of 2,053 patients detected by the 48-gene NGS panel for myeloid neoplasms. Each column in the x=axis represents a patient. Only patients with at least 1 mutation (n=1,142) are shown. The percent of patients with Tier I and Tier II mutations in the indicated gene is presented.

Based on functionally related categories (**Fig 8**), genes involved in epigenetics (*ASXL1*, *BCOR*, *BCROL1*, *DNMT3A*, *EZH2*, *IDH1*, *IDH2*, *KDM6A*, and *TET2*) were the most frequently mutated group, detected in 43% (n=875/2,053) of patients. Genes involved in signal transduction (*BRAF*, *CALR*, *CBL*, *CSF3R*, *FLT3*, *JAK2*, *KIT*, *KRAS*, *MPL*, *NF1*, *NRAS*, *PTPN11*, and *STAT3*), RNA splicing (*SF3B1*, *SRSF2*, *U2AF1*, and *ZRSR2*), and transcription factor (*CEBPA*, *ETV6*, *GATA1*, *GATA2*, *IKZF1*, *KMT2A*, *PHF6*, *RUNX1*, *SETBP1*, and *WT1*) accounted for 27% (n=574/2,053), 22% (n=444/2,053), and 13% (n=272/2,053) of patients, respectively. These findings are consistent with the mutational frequency of gene groups in myeloid neoplasms as reported in literature [22-26].

In 41.7% (856/2,053) of patients, at least 1 actionable mutation was identified (**Fig 9**): 27.5% (n=565) of patients harbored mutations for which a targeted therapy is available either in the disease (n=126) or another disease (n=439); and 40.1% (n=823) patients contained mutations for which an experimental drug is available. For this study, variants were considered clinically actionable when a targeted therapy or an experimental drug was available for the disease or other disease. In addition, 34.6% of patients (n=711) had mutations with prognostic significance, and 36.4% (n=748) of patients had mutations with diagnostic significance. In total, the assay

identified clinically significant mutations in 51.7% (1,062/2,053) of patients. Mutations were considered clinically significant if they provided prognostic or diagnostic information for the disease or were clinically actionable.

Discussion

Molecular profiling can help diagnose, classify, and guide treatment of myeloid neoplasms [27-28]. In this study, we reported development and validation of a 48-gene NGS panel for molecular profiling of myeloid neoplasms. The assay demonstrated good inter- and intra-assay precision for SNVs, indels including *CALR* 52 bp deletion, and *FLT3*-ITDs (**Fig 2**, **S5A and S5B Tables**). In addition, the assay detected 827 of 830 variants with VAF ≥5% reported by alternative analytical methods for a sensitivity of 99.6% (95% CI: 98.9-99.9%) (**Fig 4**, **S8 Table**). False positive calls with low frequency in low complexity sequence regions are a relatively common phenomenon in NGS [11]. The possibility of detecting this type of event has been reduced by using bioinformatic filtering process established based on the results of a training set of 100 unique normal patient specimens (data not shown). The specificity of this assay appears to approach 100% as no false positive call was made in 7 GIAB accuracy studies across 0.4 million bp (**Table 3**) and in a cross-platform study using 137 unique specimens across 4.8 million bp (**Fig 5**).

In clinical settings, specimen types submitted for an assay may vary. Thus, an ideal assay must be able to deal with a wide range of specimen types appropriate for the assay. Overall, our assay produced robust results (98.8% pass rate) for all specimen types (**Table S2**), suggesting

that DNA recovered from various specimen types can be successfully and accurately sequenced by this protocol. In NGS, the limitation of a target enrichment method often leads to low coverage in genes with high GC content, and this may cause sub-optimal assay accuracy. Supplementary single-gene assays are recommended to improve low coverage targets in some clinical assays [29]. In this study, we demonstrated a broad reportable range of the 48-gene panel even in extreme % GC spectrum (**Fig 1B**). For example, despite the very high GC content of *CEBPA* gene (up to 87% GC within a 100-bp window), hybridization enrichment coupled with careful bait design achieved an excellent result: median depth of 1,918X. During our development, direct comparison of capture technologies demonstrated superior performance of DNA bait over RNA bait for targets with high GC contents (**S2 Fig**). During our validation, we showed that VAF of SNVs and indels detected by our assay were highly concordant with ones observed by reference methods (**Fig 3C**, **Fig 5B**), indicating our assay is accurate in variant quantification. Long-term test reproducibility of an analytical method is critical in clinical settings following initial validation; consistent variant allele frequencies over a 10-month period in a positive control specimen highlighted the long-term reproducibility of our assay (**Fig 6**). *FLT3*-ITD is inherently difficult to detect using NGS approaches [15, 30]. In our validation studies, we also identified *FLT3*-ITDs of varying lengths (range: 18-117 bp) with 100% sensitivity and specificity (**Table 2**). In some trials, additional ITD size(s) were detected, and we reasoned that our assay is more sensitive than the method compared, fragment analysis.

In our study of 2,053 clinical patients, 55.6% had at least one pathogenic variant and 51.7% harbored clinically significant mutations with prognostic, diagnostic, or therapeutic relevance (**Fig 9**). The clinical utility of our assay is underscored by our ability to identify clinically significant variants in specific diseases. For example, from our cohort of 490 patients

478 with indications of AML, *TET2* had the highest mutation rate (17% of patients) followed by
479 *ASXL1*, *TP53*, *FLT3*, *NPM1*, *SRSF2*, *DNMT3A*, *IDH2*, *RUNX1* and *NRAS* (10~15%),
480 frequencies similar to literature [26]. Among those frequently mutated genes, *ASXL1*, *RUNX1*,
481 *TP53*, and *FLT3*-ITD mutations have been associated with poor prognosis, whereas *NPM1*
482 mutations in the absence of *FLT3*-ITD have been associated with favorable outcomes in AML
483 [31]. In the 2016 revision of the WHO classification, AML with an *NPM1* mutation is
484 recognized as a subtype of AML; and AML with an *RUNX1* mutation has been added as a
485 provisional category of AML [1]. In addition, FDA-approved targeted therapies for AML are
486 available for *FLT3*-ITD and *IDH2*, as well as *IDH1* [32] which was mutated in 5% of patients in
487 our study.

488 From our cohort of 1,018 patients with indications of MDS, *TET2*, *SF3B1*, and *ASXL1*
489 were the most frequently mutated genes (in >10% of patients), followed by *SRSF2*, *TP53*,
490 *DNMT3A*, and *RUNX1* (in >5% of patients), similar to Haferlach and colleague's study on the
491 mutational profiles of 944 patients with MDS [25]. Among those genes, *ASXL1* and *TP53*
492 mutations have been associated with poor prognosis [33], whereas *SF3B1* mutation in MDS
493 patients with ringed sideroblasts has been associated with favorable prognosis [34]. All of the
494 frequently mutated genes have been associated with clonal hematopoiesis, and support the
495 diagnosis of several different myeloid malignancies, including MDS, when found in combination
496 with other diagnostic features [35, 36]. While there are currently no therapies directly targeting
497 mutated genes in MDS, hematologic malignancies harboring *SF3B1* and *SRSF2* mutations have
498 been reported to be sensitive to splicing factor 3B subunit 1 (SF3b155) inhibitors, which are in
499 clinical development [37, 38].

Certain mutations in the MPN-associated genes satisfy subclassification of the disease. In our study of 545 patients with indications of MPN, *JAK2* was the most frequently mutated gene (23% of patients) followed by *TET2* (12%) and *ASXL1* (10%), frequencies similar to literature [39, 40]. *CALR*, *CSF3R*, and *MPL* were mutated in 4%, 3%, and 1% patients, respectively. Presence of a mutation in *JAK2*, *CALR*, and *MPL* is among the major criteria for the diagnosis of myelofibrosis (MF) or essential thrombocythemia (ET), while presence of *JAK2* V617 or exon 12 mutations is among the major criteria for the diagnosis of polycythemia vera (PV) [1, 41, 42]. Activating *CSF3R* mutations have been found in the majority of chronic neutrophilic leukemias (CNLs) [43]. FDA-approved therapies for *JAK2* mutations in PV and MF are available [44]. In addition, mutations in *CALR* exon 9, *CSF3R*, and *MPL* have been shown to be sensitive to Jak inhibitors [45-47].

For *BCR-ABL1*-negative MPN, common mutations of *JAK2*, *CALR*, and *MPL* genes are often examined as diagnostic targets using a cascade single-gene assay [42]. In our cohort of 545 patients with indications of MPN, 159 patients (29%) were positive for those 3 genes. Detection of an additional clinically significant mutation is more common in an NGS panel assay than single-gene tests owing to the multiplicity of genes tested. An additional 86 patients (16%) who were negative for those 3 genes were positive for pathogenic mutations in other genes in the panel. Of the 86 patients, 23 had mutations in genes that can aid diagnosis when found in combination with other diagnostic features (*CBL*, *NRAS*, and *PTPN11*) [1, 8, 36], 43 had mutations in genes with poor prognosis (*ASXL1*, *EZH2*, *IDH2*, *NRAS*, *SETBP1*, *SRSF2*, *TP53*, and *U2AF1*), and 29 had mutations in genes for which a therapy is available (*BRAF*, *CSF3R*, *IDH2*, *KRAS*, *NF1*, *NRAS*, *PTPN11*, and *STAG2*). In addition, of the 159 patients who were positive for those 3 genes, 84 patients had additional mutation(s) in 16 other genes. Among the

16 genes, 6 genes (*IDH1*, *IDH2*, *KRAS*, *NF1*, *NRAS*, and *STAG2*) are linked to available therapies in other diseases [27, 48]. Combinations of Jak inhibitors with other targeted therapies may be relevant for those patients who harbor additional mutations.

In contrast to single-gene assays, NGS allows assessment of co-occurring mutations that might have heterogeneity of response to targeted therapy and survival. According to the recent National Comprehensive Cancer Network guidelines, AML patients with *FLT3*-ITD and *NPM1* double mutation (AML *FLT3*-ITD+/*NPM1*+) are categorized as favorable and intermediate risk levels, depending on the allelic ratio of *FLT3*-ITD, for whom allogenic stem cell transplantation (allo-HSCT) is not obligated. Loghavi and colleagues reported that AML *FLT3*-ITD+/*NPM1*+ patients with a *DNMT3A* mutation had shorter event-free survival compared to those in other mutation groups [49]. Similarly, recent studies have suggested that AML patients with concomitant *DNMT3A* R882+/*FLT3*-ITD+/*NPM1*+ mutations had a very poor prognosis, and allo-HSCT could moderately improve their survival [50, 51]. Our study cohort included 21 AML *FLT3*-ITD+/*NPM1*+ patients, 4 of whom had a *DNMT3A* R882 mutation. In addition, Ardestani and colleagues reported that *DNMT3A* R882 mutations alone do not affect the clinical outcomes of AML patients, but when accompanied by *FLT3*-ITD mutations, overall survival was reduced, even after allo-HSCT [52]. In our study, 5 of 26 *DNMT3A* R882-positive AML patients had *FLT3*-ITD. These results support the clinical utility of our assay for detecting mutations that can alter prognostic and therapeutic significance when they occur in combination.

Conclusions

We have developed and validated a 48-gene NGS assay that can detect SNVs, indels, and *FLT3*-ITD with high sensitivity and specificity. The assay detects variants with clinical significance from a substantial proportion of patients tested. The developed assay may be used to guide more precise and targeted therapeutic strategies, possibly leading better treatment outcomes for patients with myeloid neoplasms.

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References

1. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Clara DB, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127(20): 2391-2405.

2. Tremblay D, Sokol K, Bhalla S, Rampal R, Mascarenhas JO. Implications of mutation profiling in myeloid malignancies-Part 1: Myelodysplastic syndromes and acute myeloid leukemia. Oncology (Williston Park). 2018;32(4): e38-e44.
3. Döhner H, Weisdorf DJ, Bloomfield CD. Acute Myeloid Leukemia. N Engl J Med. 2015;373(12): 1136-1152.
4. Kennedy JA, Ebert BL. Clinical Implications of Genetic Mutations in Myelodysplastic Syndrome. J Clin Oncol. 2017;35(9): 968-974.
5. Barbui T, Thiele J, Gisslinger H, Kvasnicka HM, Vannucchi AM, Guglielmelli P, et al. The 2016 WHO classification and diagnostic criteria for myeloproliferative neoplasms: document summary and in-depth discussion. Blood Cancer J. 2018;8(2): 15.
6. Vannucchi AM, Lasho TL, Guglielmelli P, Biamonte F, Pardanani A, Pereira A, et al. Mutations and prognosis in primary myelofibrosis. Leukemia. 2013;27(9): 1861-1869.
7. Tefferi A, Lasho TL, Guglielmelli P, Finke CM, Rotunno G, Elala Y, et al. Targeted deep sequencing in polycythemia vera and essential thrombocythemia. Blood Adv. 2016;1(1): 21-30.
8. McClure RF, Ewalt MD, Crow J, Temple-Smolkin RL, Pullambhatla M, Sargent R, et al. Clinical Significance of DNA Variants in Chronic Myeloid Neoplasms: A Report of the Association for Molecular Pathology. J Mol Diagn. 2018;20(6): 717-737.
9. Kuo FC, Mar BG, Lindsley RC, Lindeman NI. The relative utilities of genome-wide, gene panel, and individual gene sequencing in clinical practice. Blood. 2017;130(4): 433-439.

10. Duncavage EJ, Tandon B. The utility of next-generation sequencing in diagnosis and monitoring of acute myeloid leukemia and myelodysplastic syndromes. Int J Lab Hematol. 2015;37(Suppl 1): 115-121.
11. Bacher U, Shumilov E, Flach J, Porret N, Joncourt R, Wiedemann G, et al. Challenges in the introduction of next-generation sequencing (NGS) for diagnostics of myeloid malignancies into clinical routine use. Blood Cancer J. 2018;8(11): 113.
12. Ng CWS, Kosmo B, Lee PL, Lee CK, Guo J, Chen J, et al. CEBPA mutational analysis in acute myeloid leukaemia by a laboratory-developed next-generation sequencing assay. Journal of Clinical Pathology. 2018;71: 522-531.
13. Griffith M, Miller CA, Griffith OL, Krysiak K, Skidmore ZL, Ramu A, et al. Optimizing cancer genome sequencing and analysis. Cell Syst. 2015;1(3): 210-223.
14. Spencer DH, Abel HJ, Lockwood CM, Payton JE, Szankasi P, Kelley TW, et al. Detection of FLT3 internal tandem duplication in targeted, short-read-length, next-generation sequencing data. J Mol Diagn. 2013;15: 81-93.
15. Au CH, Wa A, Ho DN, Chan TL, Ma ES. Clinical evaluation of panel testing by next-generation sequencing (NGS) for gene mutations in myeloid neoplasms. Diagn Pathol. 2016;11: 11. doi: 10.1186/s13000-016-0456-8.
16. Strout MP, Marcucci G, Bloomfield CD, Caligiuri MA. The partial tandem duplication of ALL1 (MLL) is consistently generated by Alu-mediated homologous recombination in acute myeloid leukemia. Proc Natl Acad Sci U S A. 1998;95(5): 2390-2395.
17. Steudel C, Wermke M, Schaich M, Schäkel U, Illmer T, Ehninger G, et al. Comparative analysis of MLL partial tandem duplication and FLT3 internal tandem duplication

603 mutations in 956 adult patients with acute myeloid leukemia. Genes Chromosomes
604 Cancer. 2003;37: 237-251
605 18. Zwaan CM, Meshinchi S, Radich JP, Veerman AJ, Huismans DR, Munske L, et al. FLT3
606 internal tandem duplication in 234 children with acute myeloid leukemia: prognostic
607 significance and relation to cellular drug resistance. Blood. 2003; 102: 2387-2394.
608 19. Leisch M, Jansko B, Zaborsky N, Greil R, Pleyer L. Next Generation Sequencing in
609 AML-On the Way to Becoming a New Standard for Treatment Initiation and/or
610 Modulation? Cancers (Basel). 2019;11(2): 252. doi: 10.3390/cancers11020252.
611 20. Rosenthal SH, Sun W, Zhang K, Liu Y, Nguyen Q, Gerasimova A, et al., Development
612 and Validation of a 34-Gene Inherited Cancer Predisposition Panel Using Next-
613 Generation Sequencing. BioMed Research International. 2020;
614 doi.org/10.1155/2020/3289023.
615 21. Li MM, Datto M, Duncavage EJ, Kulkarni S, Lindeman NI. Standards and Guidelines for
616 the Interpretation and Reporting of Sequence Variants in Cancer: A Joint Consensus
617 Recommendation of the Association for Molecular Pathology, American Society of
618 Clinical Oncology, and College of American Pathologists. J Mol Diagn. 2017;19(1): 4-
619 23.
620 22. Carbonell D, Suárez-González J, Chicano M, Andrés-Zayas C, Triviño JC, Rodríguez-
621 Macías G, et al. Next-Generation Sequencing Improves Diagnosis, Prognosis and
622 Clinical Management of Myeloid Neoplasms. Cancers (Basel). 2019;11(9): 1364. doi:
623 10.3390/cancers11091364.

624 23. Papaemmanuil E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND, et al.
625 Genomic Classification and Prognosis in Acute Myeloid Leukemia. N Engl J Med.
626 2016;374(23): 2209-2221.
627 24. Lars Bullinger, Konstanze Döhner, Hartmut Döhner. Genomics of Acute Myeloid
628 Leukemia Diagnosis and Pathways. Journal of Clinical Oncology. 2017; 35(9): 934-946.
629 25. Haferlach T, Nagata Y, Grossmann V, Okuno Y, Bacher U, Nagae G, et al. Landscape of
630 genetic lesions in 944 patients with myelodysplastic syndromes. Leukemia. 2014;28(2):
631 241-247.
632 26. The Cancer Genome Atlas Research Network. Genomic and Epigenomic Landscapes of
633 Adult De Novo Acute Myeloid Leukemia. N Engl J Med. 2013; 368: 2059-2074.
634 27. Tremblay D, Sokol K, Bhalla S, Rampal R, Mascarenhas JO. Implications of Mutation
635 Profiling in Myeloid Malignancies-PART 1: Myelodysplastic Syndromes and Acute
636 Myeloid Leukemia. Oncology (Williston Park). 2018;32(4): e38-e44.
637 28. Shumilov E, Flach J, Kohlmann A, Banz Y, Bonadies N, Fiedler M, et al. Current status
638 and trends in the diagnostics of AML and MDS. Blood Rev. 2018 Nov;32(6):508-519.
639 29. Bean LJH, Funke B, Carlston CM, Gannon JL, Kantarci S, Krock BL, et al. Diagnostic
640 gene sequencing panels: from design to report—a technical standard of the American
641 College of Medical Genetics and Genomics (ACMG). Genet Med (2019)
642 doi:10.1038/s41436-019-0666-z.
643 30. Garcia EP, Minkovsky A, Jia Y, Ducar MD, Shivdasani P, Gong X, et al. Validation of
644 OncoPanel: A Targeted Next-Generation Sequencing Assay for the Detection of Somatic
645 Variants in Cancer. Arch Pathol Lab Med. 2017;141(6): 751-758.

646 31. Grossmann V, Schnittger S, Kohlmann A, Eder C, Roller A, Dicker F, et al. A novel
647 hierarchical prognostic model of AML solely based on molecular mutations. Blood.
648 2012;120(15): 2963-2972.
649 32. Lai C, Doucette K, Norsworthy K. Recent drug approvals for acute myeloid leukemia. J
650 Hematol Oncol. 2019;12(1): 100. doi: 10.1186/s13045-019-0774-x.
651 33. Hou HA, Tsai CH, Lin CC, Chou WC, Kuo YY, Liu CY, et al. Incorporation of
652 mutations in five genes in the revised International Prognostic Scoring System can
653 improve risk stratification in the patients with myelodysplastic syndrome. Blood Cancer
654 J. 2018;8(4): 39. doi: 10.1038/s41408-018-0074-7.
655 34. Migdady Y, Barnard J, Ali NA, Steensma DP, DeZern A, Roboz G, et al. Clinical
656 Outcomes with Ring Sideroblasts and SF3B1 Mutations in Myelodysplastic Syndromes:
657 MDS Clinical Research Consortium Analysis, Clinical Lymphoma Myeloma and
658 Leukemia. 2018;18(8): 528-532.
659 35. Kwok B, Hall JM, Witte JS, Xu Y, Reddy P, Lin K, et al. MDS-associated somatic
660 mutations and clonal hematopoiesis are common in idiopathic cytopenias of
661 undetermined significance. Blood. 2015;126(21): 2355-2361.
662 36. Malcovati L, Gallì A, Travaglino E, Ambaglio I, Rizzo E, Molteni E, et al. Clinical
663 significance of somatic mutation in unexplained blood cytopenia. Blood. 2017;129(25):
664 3371-3378.
665 37. Cazzola M, Rossi M, Malcovati L. Biologic and clinical significance of somatic
666 mutations of SF3B1 in myeloid and lymphoid neoplasms. Blood. 2013;121(2): 260-269.
667 38. Obeng EA, Stewart C, Abdel-Wahab O. Altered RNA Processing in Cancer Pathogenesis
668 and Therapy. Cancer Discov. 2019;9(11): 1493-1510.

669 39. Song J, Hussaini M, Zhang H, Shao H, Qin D, Zhang X, et al. Comparison of the
670 Mutational Profiles of Primary Myelofibrosis, Polycythemia Vera, and Essential
671 Thrombocytosis. Am J Clin Pathol. 2017;147(5): 444-452.
672 40. Schischlik F, Kralovics R. Mutations in myeloproliferative neoplasms – their significance
673 and clinical use. Expert Review of Hematology. 2017;10(11): 961-973.
674 41. Rumi E, Cazzola M. Diagnosis, risk stratification, and response evaluation in classical
675 myeloproliferative neoplasms. Blood. 2017;129(6): 680–692.
676 42. Mejía-Ochoa M, Acevedo Toro PA, Cardona-Arias JA. Systematization of analytical
677 studies of polycythemia vera, essential thrombocythemia and primary myelofibrosis, and
678 a meta-analysis of the frequency of JAK2, CALR and MPL mutations: 2000–2018. BMC
679 Cancer. 2019;19: 590. doi: 10.1186/s12885-019-5764-4.
680 43. Maxson JE, Gotlib J, Pollyea DA, Fleischman AG, Agarwal A, Eide CA, et al.
681 Oncogenic CSF3R mutations in chronic neutrophilic leukemia and atypical CML. N Engl
682 J Med. 2013; 368(19): 1781-1790.
683 44. Mascarenhas J, Hoffman R. Ruxolitinib: the first FDA approved therapy for the treatment
684 of myelofibrosis. Clin Cancer Res. 2012;18(11): 3008-3014.
685 45. Tefferi A, Pardanani A. Myeloproliferative Neoplasms: A Contemporary Review. JAMA
686 Oncol. 2015;1(1): 97-105.
687 46. Schieber M, Crispino JD, Stein B. Myelofibrosis in 2019: moving beyond JAK2
688 inhibition. Blood Cancer J. 2019;9(9): 74. doi: 10.1038/s41408-019-0236-2.
689 47. Vainchenker W, Leroy E, Gilles L, Marty C, Plo I, Constantinescu SN. JAK inhibitors
690 for the treatment of myeloproliferative neoplasms and other disorders. F1000Res. 2018;7:
691 82. doi: 10.12688/f1000research.13167.1.

692 48. Economides MP, Verstovsek S, Pemmaraju N. Novel Therapies in Myeloproliferative
693 Neoplasms (MPN): Beyond JAK Inhibitors. Curr Hematol Malig Rep. 2019 Oct;14(5):
694 460-468.
695 49. Loghavi S, Zuo Z, Ravandi F, Kantarjian HM, Bueso-Ramos C, Zhang L, et al. Clinical
696 features of de novo acute myeloid leukemia with concurrent DNMT3A, FLT3 and NPM1
697 mutations. J Hematol Oncol. 2014;7: 74. doi: 10.1186/s13045-014-0074-4.
698 50. Huang Y, Hu J, Lu T, Luo Y, Shi J, Wu W, et al. Acute myeloid leukemia patient with
699 FLT3-ITD and NPM1 double mutation should undergo allogeneic hematopoietic stem
700 cell transplantation in CR1 for better prognosis. Cancer Manag Res. 2019;11: 4129-4142.
701 51. Su L. The Impact of DNMT3A/FLT3-ITD/NPM1 on Patients with Acute Myeloid
702 Leukemia after Allogeneic Hematopoietic Stem Cell Transplantation. Turk J Haematol.
703 2019;36(1): 64-66.
704 52. Ardestani MT, Kazemi A, Chahardouli B, Mohammadi S, Nikbakht M, Rostami S, et al.
705 FLT3-ITD Compared with DNMT3A R882 Mutation Is a More Powerful Independent
706 Inferior Prognostic Factor in Adult Acute Myeloid Leukemia Patients After Allogeneic
707 Hematopoietic Stem Cell Transplantation: A Retrospective Cohort Study. Turk J
708 Haematol. 2018;35(3): 158–167.

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711 # Supporting information

712 **S1 Table. Genes included in the 48-gene NGS panel.**

713 **S2 Table. Specimens used for validation studies.**

714 **S3 Table. Criteria used for specimen and variant quality control.**

715 **S4 Table. Summary of sequencing metrics for validation specimens (n=428,184 unique).**

716 **S5A Table. Inter-assay precision study variant concordance.**

717 **S6 Table. Specimens used for accuracy study.**

718 **S7A Table. Variant call concordance study using multiplex mutation control specimens.**

719 **S7B Table. GIAB reference specimen dilution study.**

720 **S7C Table. Positive specimen dilution study.**

721 **S7D Table. *FLT3-ITD* positive specimen dilution study.**

722 **S8 Table. Summary of discordant variant.**

723 **S9 Table. Analytical sensitivity for various % VAF for each variant type.**

724 **S1 Fig. % patient with mutation per gene for AML (A), MDS (B), and MPN (C).** Pathogenic (Tier I

725 and Tier II) mutations were included**.**

726 **S2 Fig. Integrative Genomics Viewer (IGV) for *CEBPA* (A) and *CUX1* (B) comparing target**

727 **capture performance between RNA bait (top panel) and DNA bait (bottom panel).** Even coverage

728 distribution was achieved using DNA bait whereas low to no coverage was observed using RNA bait for

729 high % GC targets. % GC in 100bp window is color-coded.