Clinical Impact of Panel Based Error Corrected Next Generation Sequencing versus Flow Cytometry to Detect Measurable Residual Disease (MRD) in Acute Myeloid Leukemia (AML)

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Abstract:

We accrued 201 patients of adult AML treated with conventional therapy, in morphological remission and evaluated MRD using sensitive error corrected next generation sequencing (NGS-MRD) and multiparameter flow cytometry (FCM-MRD) at the end of induction (PI) and consolidation (PC). Nearly 71% of patients harbored PI NGS-MRD and 40.9% harbored PC NGS-MRD (median VAF 0.76%). Patients harboring NGS-MRD had a significantly higher cumulative incidence of relapse (p=0.003), inferior overall survival (p=0.001) and relapse free survival (p<0.001) as compared to NGS-MRD negative patients. NGS-MRD was predictive of inferior outcome in intermediate cytogenetic risk and demonstrated potential in favorable cytogenetic risk AML. Patients who cleared PI NGS-MRD had a significantly improved survival as compared to patients who became negative subsequently indicating that kinetics of NGS-MRD clearance was of paramount importance. NGS-MRD identified over 80% of cases identified by flow cytometry at PI time point whereas FCM identified 49.3% identified by NGS. Only a fraction of cases were truly missed by NGS as compared to FCM-MRD. NGS-MRD emerged as the most important independent prognostic factor predictive of inferior outcome (p<0.001). We demonstrate a widely applicable, scalable NGS-MRD approach that is clinically informative and advantageous when compared to FCM-MRD in AML treated with conventional therapies.
Introduction

Acute Myeloid Leukemia is a disease characterized by heterogeneous biology resulting in varying clinical outcomes including relapse. ¹, ² There are limited novel treatment options such as targeted therapies using FLT3 or IDH2 inhibitors and most patients are treated based on the presumptive risk of relapse. ³, ⁴ This risk adapted therapy typically considers standard cytogenetic and molecular variables as recommended by the European LeukemiaNet. ⁵ Based on this risk, patients are recommended treatment with conventional (induction and consolidation) regimens or offered intensive therapy such as allogeneic bone marrow transplantation (BMT) after achievement of remission. Amongst these, the largest group remains as intermediate risk AML characterized by non-uniform clinical outcomes.

The monitoring of a patient’s response to chemotherapy, called, measurable residual disease (MRD) is one of the most important predictors of outcome in hematological malignancies. Several investigators have demonstrated the clinical utility of flow cytometry based MRD detection (FCM-MRD) in AML at early chemotherapy time points as well as in a pre-transplant setting.⁶-¹⁴ Although universally applicable, FCM-MRD suffers from suboptimal ability to predict relapse in AML compared to precursor B lineage acute lymphoblastic leukemia. A diverse array of sensitive molecular methods have been used to detect MRD in AML such as real time PCR¹⁵ and droplet digital PCR.¹⁶ These are useful for monitoring of individual gene mutations such as AML with mutated NPM1¹⁷, ¹⁸ and chimeric gene fusions such as RUNX1-RUNX1T1.¹⁹ Next generation sequencing (NGS) is a promising tool for sensitive MRD monitoring and has been used successfully to monitor NPM1²⁰, ²¹, RUNX1²² and FLT3²³ mutations as well as chimeric gene fusions.²⁴, ²⁵ However, these methodologies can be utilized in only a subset of patients that harboured these mutations at diagnosis and this strategy discounts for clonal heterogeneity and evolution which are relevant to the pathogenesis of AML.

DNA based focussed target enrichment strategies (gene panels) are an attractive solution to detect MRD using NGS (NGS-MRD) in a mutation rich disease such as AML.²⁶, ²⁷, ²⁹ However, short read sequencers are inherently prone to base calling errors limiting variant calling at 3-5% variant allele
fraction (VAF).\(^3\) Although acceptable for diagnostic molecular pathology, this is undesirable assay performance for the detection of MRD. Error corrected sequencing involves the physical incorporation of random oligonucleotides or unique molecular identifiers (UMI) at the library preparation stage prior to amplification of DNA. This allows us to tag individual DNA molecules with an unique molecular fingerprint.\(^31,\)\(^32\) Such approaches have been used for myelodysplastic syndromes\(^33\) and for pre transplant MRD monitoring of AML as demonstrated by Thol and colleagues.\(^34\) Thol utilized a sensitive patient specific mutation tracking approach using UMI based MRD detection. Although applicable to a broad spectrum of AML mutations, a tailored approach poses logistical and regulatory hurdles towards prospective MRD testing especially for early MRD timepoints.

In this study, we have evaluated the clinical utility of error corrected NGS to detect MRD in AML using single molecule molecular inversion probes (smMIPS).\(^31,\)\(^35\) Each smMIP contains an 8 bp UMI and binds to a single molecule of DNA. Using consensus sequence-based variant calling we can detect somatic mutations including small indels in a sensitive manner. We demonstrate that error corrected NGS-MRD at early timepoints in therapy is significantly predictive of outcome in patients of AML treated with conventional therapies. Furthermore, we systematically compare multicolour FCM-MRD with error corrected NGS-MRD and assess the clinical utility of these two assays in a cohort of AML.
Methods

1. PATIENTS

a. Patient Accrual and Initial work up: The study was approved by the institutional ethics committee (IEC-III project 163) and participants were accrued after informed consent. A total of 393 adult patients of AML, diagnosed as per 2008 WHO criteria, were accrued in this study over a period of six years (Feb 2013 to May 2019). Cytogenetic (FISH and karyotyping) workup was as previously described.9, 21 Somatic mutations at diagnosis were evaluated using a smMIPS based 50 gene myeloid panel as described previously by our group.36 Of these, the smMIPS MRD panel (see below) was applicable to 83.2% patients [327 out of 393 AMLs, median 2 mutations per case (range 1 – 6 trackable mutations); Supplementary Figure 1]. Of those (n=319) achieving morphological CR, the smMIPS MRD panel was applicable to 266 (83.4%). MRD assessment could be performed in 201 adult patients of AML (enrolment flow chart in supplementary figure 2). A summary of the clinical and laboratory characteristics of these 201 patients can be seen in Table 1.

b. Treatment of AML and MRD Sampling: All patients were treated with conventional “3+7” induction chemotherapy and further treated with high dose cytarabine (HiDAC) or allogeneic bone marrow transplantation (aBMT), if feasible.36 Only 15 patients received aBMT and their outcome was not different from the rest with respect to OS and RFS (p = not significant; supplementary figure 3) and are not considered separately. Sample for FCM-MRD was obtained from the bone marrow at end of induction (PI; n=200) and end of first consolidation cycle (PC, n=98). NGS MRD sample also obtained at the same time points (PI-196; PC-127) from the bone marrow (n=269; PI:181 and PC:88) or peripheral blood (n=51; PI:15 and PC:36).

c. Evaluation of Treatment Outcome: Overall Survival (OS) and Relapse Free Survival (RFS) were calculated as previously described.9, 21, 36 The prognostic impact of NGS and FCM-MRD assays on OS and RFS was computed using the Kaplan-Meier method and
compared using log-rank test. Multivariate analysis was performed using the Cox proportional-hazards regression analysis that considered FCM-MRD and NGS-MRD. Separate models were constructed for post induction and post consolidation MRD time points. Grey test was used to compare the cumulative incidences of relapse (CIR) and non-relapse mortality (NRM) using “cmprsk” module in R. The same module was used to generate representative graphs. Positive predictive value (PPV) and negative predictive value (NPV) were calculated as described.

2. DETECTION OF MRD USING NGS

a. smMIPS panel: We created a 35 gene panel comprising of a pool of 302 smMIPS (as seen in Supplementary Table 1). In brief, this panel covers regions of 35 commonly mutated genes in AML (ATM, BCOR, DNMT3A, EZH2, FLT3, GATA1, GATA2, IDH1, IDH2, JAK2, KDM6A, KIT, KMT2D, KRAS, NF1, NOTCH1, NOTCH2, NPM1, NRAS, PHF6, PTPN11, RAD21, RUNX1, SETBP1, SF3B1, SH2B3, SMC1A, SRSF2, STAG2, TET2, TP53, U2AF1, WT1, ZRSR2). The panel was rebalanced (Supplementary Figure 4) to ensure uniform capture across regions. Approximately 600ng of genomic DNA was captured, treated with exonucleases and PCR amplified to create a sequencing ready library. Details pertaining to smMIPS design, assay standardization and sequencing are detailed in supplementary methods.

b. Bioinformatics: Reads were demultiplexed, trimmed, paired end assembled and mapped to the human genome (build hg19). Singleton reads (originating from one UMI) were discarded, and consensus family based variant calling performed using tools described in supplementary methods. We then created a site and mutation specific error model to ascertain the relevance of an observed variant at each site. Criteria for variant calling using the smMIPS MRD assay are described in supplementary methods.
c. **MRD for FLT3-internal tandem duplications (ITD):** FLT3-ITD were detected using a novel one-step PCR based NGS assay (see Supplementary Table 3). Variants were detected using a recently described algorithm for the accurate detection of FLT3-ITD.38

d. **Orthogonal detection of MRD in NPM1 mutated AML:** NPM1 mutations were additionally tracked using an ultrasensitive orthogonal NPM1 MRD assay.21

3. **DETECTION OF MRD IN USING MULTICOLOUR FLOW CYTOMETRY (FCM-MRD)**

FCM-MRD was detected using a previously described 10 colour two tube MRD assay.9, 21, 36, 39 This approach uses a combination of leukemia associated immunophenotype and difference from normal approaches to detect MRD in AML.
Results

1. PATIENTS:

The median follow-up of the cohort was 42.3 months. The median OS was 35.9 months (95%CI- 27.2 to 42.8) for the entire cohort. The median RFS was 21.6 months (95%CI- 17.0 to 28.9) months. Additional patient characteristics can be seen in Table 1.

2. NGS BASED AML MRD:

   a. Assay Characteristics:

   We describe an NGS-MRD approach that is applicable to 83.2% of all AML. For patients in morphological CR (n=319) this approach was applicable to 83.4% (n=266). Of these, MRD could be assessed in 201 cases. A co-occurrence plot indicating interactions of mutations tracked by NGS-MRD, prior to therapy, can be seen in Figure 1A. The applicability of this MRD panel, when patients (n=201) are classified by cytogenetic risk is seen in supplementary table 2.

   b. Limit of Detection:

   In a limit of detection experiment (supplementary figure 5), we demonstrated that we could detect leukemic clones till a lower limit of 0.05% (0.03% for NPM1 mutation). Error modelling of normal samples indicated a higher prevalence of C>T and G>A changes consistent with oxidative DNA damage (supplementary figure 6). FLT3-ITD could be detected at a limit of 0.002% VAF (supplementary figure 7). For smMIPS based MRD, samples were sequenced at a median coverage of 14,728x (11,363x consensus coverage) whereas, for FLT3-MRD assay, the median coverage was 1,396,366x. A total of 344 mutations could be detected in 323 samples (Figure 1B,C) with a median VAF of 0.95% [0.76% after exclusion of mutations in DNMT3A, TET2, ASXL1 (DTA) genes]. Amongst positive patients, a median of 2 mutations could be detected per patient (range 1-4) at end of induction.

   c. Clinical Relevance of NGS-MRD:
Nearly 71% (n=139; 70.9%) of patients harboured MRD at the end of induction and 40.9% (n=52) at the end of consolidation. Patients harbouring MRD had a significantly higher CIR as compared to MRD negative patients at PI time point as seen in Figure 2A, Table 2. For patients who were PC NGS-MRD positive a similar observation for CIR was made (Figure 2B, Table 2). The presence of NGS-MRD at the end of induction was associated with inferior OS and RFS (Figure 2C,E) as detailed in Table 3. Similarly, the presence of NGS-MRD (n=52; 40.9%) was predictive of inferior OS and RFS at the end of consolidation (Figure 2D,F; Table 3).

i. **Cytogenetics and NGS-MRD:** We did not observe a significant distribution of MRD results when cases were distributed by cytogenetic risk. However, the presence of NGS-MRD was predictive of inferior outcome in intermediate cytogenetic risk (and a tendency in favorable risk) as seen in supplementary figure 8.

ii. **Change in mutations over MRD time points:** Amongst paired samples (n=122), 83 samples were positive at PI time point, of which 37 became negative at end of consolidation. For 46 patients in whom MRD persisted a change in MRD profile occurred in 18 patients (39.13%). This included a loss of mutation in most cases (n=14) and gain in the rest (supplementary figure 9). There were 5 patients who were negative at PI time point and became positive at end of consolidation. Of these, relapse was seen in two patients.

iii. **Clinical relevance of the kinetics of NGS-MRD clearance:** Patients who cleared NGS-MRD at end of induction (and persisted) had a significantly improved OS [HR- 0.45; 95% CI- 0.22 to 0.9; (p=0.02)] and RFS [HR- 0.49; 95% CI- 0.27 to 0.89; (p=0.01)] as compared to patients who became negative at end of consolidation (supplementary figure 10). Similarly, patients who persistently harboured MRD had a significantly inferior outcome as compared to patients who were MRD negative at both time points.
(supplementary figure 11). There was no genetic difference observed between these two groups (supplementary figure 12).

d. **Orthogonal detection of NGS-MRD in NPM1 mutated AML:**

Orthogonal comparison of MRD detection in NPM1 mutated AML was performed in 75 patients (23.2% of all MRD samples; Supplementary Methods, supplementary figure 13). There was a good correlation observed with NPM1 NGS MRD assay ($R^2=0.71$). The PPV of smMIPS MRD assay for NPM1 mutated AML was 58.5% (95%CI- 52.35 to 64.42%) as compared to a lower PPV of NPM1 NGS MRD assay [50% (95%CI- 45.34 to 54.66%)]. The NPM1 NGS MRD assay had a much lower specificity [11.11%(95%CI- 3.11 to 26.06%)] as compared to smMIPS MRD assay for MRD detection in NPM1 mutated AML [46.58%(95%CI- 34.80 to 58.63%)].

3. **FCM BASED AML- MRD:**

The presence of FCM-MRD was associated with inferior OS, RFS and CIR at the end of induction and consolidation as detailed in Tables 2, 3 and supplementary figure 14.

4. **COMPARISON OF FCM AND NGS MRD:**

On incorporating results combining both the MRD modalities, patients that were positive by both techniques (FCM+ NGS+) had a significantly inferior outcome with respect to OS, RFS and CIR at any MRD time point as compared to patients negative by both modalities as seen in Table 2,3 (Figure 3). A comparison of the baseline mutational profiles between dual PI MRD positive and negative groups revealed a significantly higher ($p=0.04$) prevalence of RUNX1 mutations in the dual MRD positive subset (supplementary figure 15).

a. **Clinico-pathological correlates of FCM MRD Positive NGS MRD Negative AML:**

A total of 20 patients were (FCM+NGS-) at assessed at PI and/or PC time points, of which 4 were detected at subsequent (or preceding) MRD time point. Of the remaining 16, in three patients a paired MRD sample was lacking. Amongst the rest, six patients (out of
13) had relapsed, three died due to non-relapse causes and four were alive at last follow-up (supplementary table 5).

b. **Accuracy of NGS-MRD in comparison with FCM-MRD:**

The PPV and NPV metrics of end of induction NGS-MRD to predict relapse in AML were 70.5% and 57.89% respectively with an accuracy of 66.84%. FCM MRD metrics at end of induction were comparable for PPV (75%), NPV (48.2%) and accuracy to predict relapse (60%). At the PI time point, NGS-MRD correctly identified 80% (68 out of 85) of cases classified as MRD positive by FCM, whereas FCM identified just 68 out of 138 cases (49.3%) identified by NGS. A detailed comparison of PPV, NPM and accuracy of combinations of patients detected between these two assays can be seen in supplementary table 6.

5. **MULTIVARIATE ANALYSIS:**

Both FCM and NGS MRD were important in predicting outcome as seen in Table 3. However, PI NGS-MRD emerged as the most important independent prognostic factor predictive of inferior outcome for OS [HR- 1.94; 95% CI- 1.15 to 3.27; (p<0.0001)] as well as RFS [HR- 2.05; 95% CI- 1.30 to 3.23; (p<0.0001)].
Discussion

Recently, Hourigan and colleagues performed ultra deep sequencing using a 13-gene panel to detect MRD in AML. In a pioneering effort, they demonstrated an advantage of myeloablative conditioning in preventing relapse in an AML cohort based on NGS MRD results. The authors, however, were unable to compare their results with other MRD assessment techniques. Here, we have assessed MRD at serial time points and have compared our results with 10 colour FCM-MRD, which is a widely used technique for the assessment of response to chemotherapy. We find that NGS-MRD is comparable in applicability and adds value, especially when a clear distinction of regenerating myeloid progenitors from leukemic blasts is absent. Advantages over FCM-MRD include potential for multi-institutional standardization, an ability to scale up operations and lack of requirement of expert operators. In our manuscript we demonstrate that NGS-MRD identified over 80% of cases identified by flow cytometry at PI time point. On evaluating 20 cases (from both PI and PC timepoints) missed by NGS-MRD (but detected by FCM), we observed that 20% of these were detected by NGS-MRD at a subsequent or preceding MRD time point. An additional 56.3% (n=9) are alive or have died due to causes other than relapse indicating that these could have been false positives. In that case, only a fraction of cases (n=7) are truly missed by NGS as compared to FCM-MRD. An additional novel insight gained is gained by studying the kinetics of MRD (supplementary figure 10). We demonstrate that patients who became negative at end of induction are likely to have a superior outcome as patients who turn negative at a subsequent time point.

Previously Jongen-Lavrencic have demonstrated clinical utility of NGS to detect MRD in AML by using computational error correction to mitigate sequencing errors. Such an approach although easy to implement discounts for batch effects and variability that occurs because of library clustering and batch dependent PCR artefacts. In that context, to the best of our knowledge, this is the first study to determine the clinical importance of (error corrected, panel based) NGS-MRD in AML treated with conventional therapies. Although our NGS-MRD strategy works in a majority of AML, we were curious about the genetic basis of cases (n=65 out of 393) in which this strategy did not work. The
cytogenetic and mutational landscape can be seen in supplementary figure 16. Nearly half of these patients did not harbour any mutation at diagnosis (n=29; 44.6%). Insight into rest of the cases revealed ASXL2 as a recurrently mutated gene (n=8, 12.3%). Incorporation of ASXL2 in future iterations of our panel will as well as other UMI based RNA sequencing approaches to monitor chimeric gene fusions will help in increasing the breadth of our approach.

Consistent with previous reports, we find that in some patients, mutations in DTA genes are present at high VAF at MRD time points (Figure 1) indicating an origin from an ancestral clone possibly originating from clonal haematopoiesis. We are in agreement with Thol and colleagues who indicated that mutations in TET2 could reliably be used as MRD markers, at least in a fraction of cases. Unlike amplicon based approaches, the advantage of a smMIPS based capture includes a stable panel which can be used across a spectrum of cases and no susceptibility to allelic skew or PCR induced errors prior to incorporation of the UMI barcode. Disadvantages of smMIPs include poor performance for GC rich genes such as CEBPA gene and inability to capture low yield or poor quality of DNA (a problem not infrequently seen with MRD samples). The library preparation process, is relatively low cost in nature and the overall process has a realistic, turnaround time of five to seven days. Our observation is that sensitivity in the clinic for most mutations is close to 0.1% VAF. A lower can be obtained for complex indels such as NPM1 and FLT3-ITD. Improvements with sensitivity may be possible through duplex sequencing based methods albeit at a much higher cost of sequencing.

Lastly, based on this data we find that mutations in NPM1, FLT3, NRAS, KIT, IDH1, IDH2, WT1, RUNX1, GATA2, U2AF1 and PHF6 were most helpful in making an positive NGS-MRD call. (supplementary figure 17).

To conclude, we demonstrate that panel based error corrected NGS-MRD is clinically relevant and possibly advantageous when compared to FCM-MRD based AML MRD.
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NP designed the study, conducted research, analyzed, interpreted the data, and wrote the manuscript.

AFS, CK, RS, PB, SR, SJ, SC, RK conducted research conducted research and analyzed data. DS, SG, GC, ND, PT, PGS, SG conducted research and analyzed data. BB, HM, NK, MS recruited patients and analyzed data.

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Agreement to Share Publication-Related Data and Data Sharing Statement:

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References

1. Estey E, Dohner H. Acute myeloid leukaemia. *Lancet (London, England)* 2006 Nov 25; 368(9550): 1894-1907.

2. Papaemmanuil E, Gerstung M, Bullinger L, Gaidzik VI, Paschka P, Roberts ND, et al. Genomic Classification and Prognosis in Acute Myeloid Leukemia. *N Engl J Med* 2016 Jun 9; 374(23): 2209-2221.

3. Stone RM, Mandrekar SJ, Sanford BL, Laumann K, Geyer S, Bloomfield CD, et al. Midostaurin plus Chemotherapy for Acute Myeloid Leukemia with a FLT3 Mutation. *N Engl J Med* 2017 Aug 3; 377(5): 454-464.

4. Stein EM, DiNardo CD, Pollyea DA, Fathi AT, Roboz GJ, Altman JK, et al. Enasidenib in mutant IDH2 relapsed or refractory acute myeloid leukemia. *Blood* 2017 Aug 10; 130(6): 722-731.

5. Dohner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Buchner T, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. *Blood* 2017 Jan 26; 129(4): 424-447.

6. Kern W, Bacher U, Haferlach C, Schnittger S, Haferlach T. The role of multiparameter flow cytometry for disease monitoring in AML. *Best practice & research Clinical haematology* 2010 Sep; 23(3): 379-390.

7. Walter RB, Gooley TA, Wood BL, Milano F, Fang M, Sorror ML, et al. Impact of pretransplantation minimal residual disease, as detected by multiparametric flow cytometry, on outcome of myeloablative hematopoietic cell transplantation for acute myeloid leukemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2011 Mar 20; 29(9): 1190-1197.

8. Loken MR, Alonzo TA, Pardo L, Gerbing RB, Raimondi SC, Hirsch BA, et al. Residual disease detected by multidimensional flow cytometry signifies high relapse risk in patients with de novo acute myeloid leukemia: a report from Children's Oncology Group. *Blood* 2012 Aug 23; 120(8): 1581-1588.

9. Patkar N, Kakirde C, Bhanse P, Joshi S, Chaudhary S, Badrinath Y, et al. Utility of Immunophenotypic Measurable Residual Disease in Adult Acute Myeloid Leukemia-Real-World Context. *Front Oncol* 2019; 9: 450.

10. San Miguel JF, Vidriales MB, Lopez-Berges C, Diaz-Mediavilla J, Gutierrez N, Canizo C, et al. Early immunophenotypical evaluation of minimal residual disease in acute myeloid leukemia identifies different patient risk groups and may contribute to postinduction treatment stratification. *Blood* 2001 Sep 15; 98(6): 1746-1751.

11. Freeman SD, Virgo P, Couzens S, Grimwade D, Russell N, Hills RK. Prognostic relevance of treatment response measured by flow cytometric residual disease detection in older patients with acute myeloid leukemia. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2013 2013//; 31.

12. Buccisano F, Maurillo L, Spagnoli A, Del Principe MI, Fraboni D, Panetta P, et al. Cytogenetic and molecular diagnostic characterization combined to postconsolidation minimal residual
disease assessment by flow cytometry improves risk stratification in adult acute myeloid leukemia. *Blood* 2010 Sep 30; **116**(13): 2295-2303.

13. Venditti A, Buccisano F, Del Poeta G, Maurillo L, Tamburini A, Cox C, *et al.* Level of minimal residual disease after consolidation therapy predicts outcome in acute myeloid leukemia. *Blood* 2000 Dec 1; **96**(12): 3948-3952.

14. Terwijn M, Putten WLJ, Kelder A, Velden VHJ, Brooimans RA, Pabst T. High prognostic impact of flow cytometric minimal residual disease detection in acute myeloid leukemia: data from the HOVON/SAKK AML 42A study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2013//; **31**.

15. Gabert J, Beillard E, van der Velden VH, Bi W, Grimwade D, Pallisgaard N, *et al.* Standardization and quality control studies of 'real-time' quantitative reverse transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia - a Europe Against Cancer program. *Leukemia* 2003 Dec; **17**(12): 2318-2357.

16. Mencia-Trinchant N, Hu Y, Alas MA, Ali F, Wouters BJ, Lee S, *et al.* Minimal Residual Disease Monitoring of Acute Myeloid Leukemia by Massively Multiplex Digital PCR in Patients with NPM1 Mutations. *J Mol Diagn* 2017 Jul; **19**(4): 537-548.

17. Ivey A, Hills RK, Simpson MA, Jovanovic JV, Gilkes A, Grech A, *et al.* Assessment of Minimal Residual Disease in Standard-Risk AML. *N Engl J Med* 2016 Feb 4; **374**(5): 422-433.

18. Dillon R, Hills R, Freeman S, Potter N, Jovanovic J, Ivey A, *et al.* Molecular MRD status and outcome after transplantation in NPM1-mutated AML. *Blood* 2020 Feb 27; **135**(9): 680-688.

19. Rucker FG, Agrawal M, Corbacioglu A, Weber D, Kapp-Schwoerer S, Gaidzik VI, *et al.* Measurable residual disease monitoring in acute myeloid leukemia with t(8;21)(q22;q22.1): results from the AML Study Group. *Blood* 2019 Nov 7; **134**(19): 1608-1618.

20. Salipante SJ, Fromm JR, Shendure J, Wood BL, Wu D. Detection of minimal residual disease in NPM1-mutated acute myeloid leukemia by next-generation sequencing. *Mod Pathol* 2014 Nov; **27**(11): 1438-1446.

21. Patkar N, Kodgule R, Kakirde C, Raval G, Bhanshe P, Joshi S, *et al.* Clinical impact of measurable residual disease monitoring by ultradeep next generation sequencing in NPM1 mutated acute myeloid leukemia. *Oncotarget* 2018 Nov 27; **9**(93): 36613-36624.

22. Kohlmann A, Nadarajah N, Alpermann T, Grossmann V, Schindela S, Dicker F, *et al.* Monitoring of residual disease by next-generation deep-sequencing of RUNX1 mutations can identify acute myeloid leukemia patients with resistant disease. *Leukemia* 2014 Jan; **28**(1): 129-137.

23. Levis MJ, Perl AE, Altman JK, Gocke CD, Bahceci E, Hill J, *et al.* A next-generation sequencing-based assay for minimal residual disease assessment in AML patients with FLT3-ITD mutations. *Blood Adv* 2018 Apr 24; **2**(8): 825-831.

24. Dillon LW, Hayati S, Roloff GW, Tunc I, Pirooznia M, Mitrofanova A, *et al.* Targeted RNA-sequencing for the quantification of measurable residual disease in acute myeloid leukemia. *Haematologica* 2019 Feb; **104**(2): 297-304.
25. Patkar N, Bhanshe P, Rajpal S, Joshi S, Chaudhary S, Chatterjee G, et al. NARASIMHA: Novel Assay based on Targeted RNA Sequencing to Identify ChiMeric Gene Fusions in Hematological Malignancies. *Blood Cancer J* 2020 May 5; 10(5): 50.

26. Kim T, Moon JH, Ahn JS, Kim YK, Lee SS, Ahn SY, et al. Next-generation sequencing-based posttransplant monitoring of acute myeloid leukemia identifies patients at high risk of relapse. *Blood* 2018 Oct 11; 132(15): 1604-1613.

27. Jongen-Lavrencic M, Grob T, Hanekamp D, Kavelaars FG, Al Hinai A, Zeilemaker A, et al. Molecular Minimal Residual Disease in Acute Myeloid Leukemia. *N Engl J Med* 2018 Mar 29; 378(13): 1189-1199.

28. Press RD, Eickelberg G, Froman A, Yang F, Stentz A, Flatley EM, et al. Next-generation sequencing-defined minimal residual disease before stem cell transplantation predicts acute myeloid leukemia relapse. *American journal of hematology* 2019 Aug; 94(8): 902-912.

29. Klco JM, Miller CA, Griffith M, Petti A, Spencer DH, Ketkar-Kulkarni S, et al. Association Between Mutation Clearance After Induction Therapy and Outcomes in Acute Myeloid Leukemia. *Jama* 2015 Aug 25; 314(8): 811-822.

30. Schmitt MW, Kennedy SR, Salk JJ, Fox EJ, Hiatt JB, Loeb LA. Detection of ultra-rare mutations by next-generation sequencing. *Proceedings of the National Academy of Sciences of the United States of America* 2012 Sep 4; 109(36): 14508-14513.

31. Hiatt JB, Pritchard CC, Salipante SJ, O’Roak BJ, Shendure J. Single molecule molecular inversion probes for targeted, high-accuracy detection of low-frequency variation. *Genome Res* 2013 May; 23(5): 843-854.

32. Yoest JM, Shirai CL, Duncavage EJ. Sequencing-Based Measurable Residual Disease Testing in Acute Myeloid Leukemia. *Front Cell Dev Biol* 2020; 8: 249.

33. Duncavage EJ, Jacoby MA, Chang GS, Miller CA, Edwin N, Shao J, et al. Mutation Clearance after Transplantation for Myelodysplastic Syndrome. *N Engl J Med* 2018 Sep 13; 379(11): 1028-1041.

34. Thol F, Gabdoulline R, Liebich A, Klement P, Schiller J, Kandziora C, et al. Measurable residual disease monitoring by NGS before allogeneic hematopoietic cell transplantation in AML. *Blood* 2018 Oct 18; 132(16): 1703-1713.

35. Waalkes A, Penewit K, Wood BL, Wu D, Salipante SJ. Ultrasensitive detection of acute myeloid leukemia minimal residual disease using single molecule molecular inversion probes. *Haematologica* 2017 Sep; 102(9): 1549-1557.

36. Patkar N, Shaikh AF, Kairde C, Nathany S, Ramesh H, Bhanshe P, et al. A novel machine-learning-derived genetic score correlates with measurable residual disease and is highly predictive of outcome in acute myeloid leukemia with mutated NPM1. *Blood cancer journal* 2019 Oct 1; 9(10): 79.

37. Scrucca L, Santucci A, Aversa F. Competing risk analysis using R: an easy guide for clinicians. *Bone marrow transplantation* 2007 Aug; 40(4): 381-387.
38. Blatte TJ, Schmalbrock LK, Skambraks S, Lux S, Cocciardi S, Dolnik A, et al. getITD for FLT3-ITD-based MRD monitoring in AML. *Leukemia* 2019 Oct; 33(10): 2535-2539.

39. Shaikh AF, Kakirde C, Dhamne C, Bhanse P, Joshi S, Chaudhary S, et al. Machine learning derived genomics driven prognostication for acute myeloid leukemia with RUNX1-RUNX1T1. *Leuk Lymphoma* 2020 Aug 5: 1-7.

40. Hourigan CS, Dillon LW, Gui G, Logan BR, Fei M, Ghannam J, et al. Impact of Conditioning Intensity of Allogeneic Transplantation for Acute Myeloid Leukemia With Genomic Evidence of Residual Disease. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* 2020 Apr 20; 38(12): 1273-1283.

41. Schuurhuis GJ, Heuser M, Freeman S, Béné MC, Buccisano F, Cloos J, et al. Minimal/measurable residual disease in AML: a consensus document from the European LeukemiaNet MRD Working Party. *Blood* 2018 Mar 22; 131(12): 1275-1291.
Figure legends

Figure 1: Somatic mutations in AML detected at diagnosis and during therapy. Figure 1A: The interaction of mutations at baseline is demonstrated here using Fisher’s Exact test. Co-occurrence is indicated in grey colour and mutual exclusivity is indicated in red. Figure 1B: The total number of mutations detected per patient and the number of such patients in the cohort is displayed. The total number of mutations in DNMT3A-TET2-ASXL1 genes is indicated here as a fraction. Figure 1C: Variant allele frequencies of mutations detected at MRD time points for patients of AML in morphological remission. The bars indicate median values with interquartile ranges.

Figure 2: Clinical relevance of error corrected NGS-MRD. Presence of NGS MRD at post induction (A) and post consolidation time points (B) is associated with a higher cumulative incidence of relapse (CIR). Kaplan Meyer plots indicate the clinical relevance of NGS-MRD with respect to OS and RFS at post induction (C,E) and post consolidation time points (D,F).

Figure 3: Comparison between FCM and NGS-MRD. The clinical relevance detection of MRD during complete remission when measured by FCM or error corrected NGS at post induction (A,B) and post consolidation time points (C,D).
| Parameter | Observation (%) |
|-----------|-----------------|
| **Demographics:** | |
| Age | Range: 18-63 years  
Median: 36 years |
| Sex | Male : Female : 1.48 : 1 |
| **Clinical Characteristics:** | |
| Total Number of Patients Accrued | 201 |
| **Remission Characteristics:** | |
| Complete remission (CR) | 31 |
| CR with incomplete hematologic recovery (CRi) | 170 |
| **Bone Marrow Transplantation:** | |
| Patients who underwent BMT | 15 |
| **Laboratory Characteristics:** | |
| Blood Counts at Presentation | |
| 1. More than 50,000/mm$^3$ | 49 |
| 2. Less than 50,000/mm$^3$ | 152 |
| Classification according to Cytogenetic Risk: | |
| Favorable Risk | 48 (23.9%) |
| Intermediate Risk | 136 (67.7%) |
| Poor Risk | 17 (8.5%) |
| **Post Induction Flow MRD (n=200):** | |
| MRD Positive | 88 (44.0%) |
| MRD Negative | 112 (56.0%) |
| **Post Consolidation Flow MRD (n=98):** | |
| MRD Positive | 21 (21.4%) |
| MRD Negative | 77 (78.6%) |
| **Post Induction NGS MRD (n=196):** | |
| MRD Positive | 139 (70.9%) |
| MRD Negative | 57 (29.1%) |
| **Post Consolidation NGS MRD (n=127):** | |
| MRD Positive | 52 (40.9%) |
| MRD Negative | 75 (59.1%) |
| **Comparative Analysis of FCM MRD and NGS MRD (Post Induction; n=195)** | |
| NGS MRD+ FCM MRD+ | 68 (34.9%) |
| NGS MRD+ FCM MRD- | 70 (35.9%) |
| NGS MRD- FCM MRD+ | 17 (8.7%) |
| NGS MRD- FCM MRD- | 40 (20.5%) |
| **Comparative Analysis of FCM MRD and NGS MRD (Post Consolidation; n=87)** | |
| NGS MRD+ FCM MRD+ | 09 (10.3%) |
| NGS MRD+ FCM MRD- | 28 (32.2%) |
| NGS MRD- FCM MRD+ | 08 (9.2%) |
| NGS MRD- FCM MRD- | 42 (48.3%) |

Table 1: Summary of clinical, laboratory and MRD characteristics of patients accrued in this study.
### Table 2: Prognostic influence of NGS-MRD, FCM-MRD and a combination of these modalities on the cumulative incidence of relapse (CIR).

| Post Induction NGS MRD | Cumulative Incidence of Relapse (CIR) | Non-Relapse Mortality (NRM) |
|------------------------|---------------------------------------|-----------------------------|
|                        | HR                                    | 3-year CIR (95% CI)         | P-value |
| MRD Negative           | 1                                     | 25.7 % (14.9 to 37.9)       | 0.003   |
| MRD Positive           | 8.81                                  | 47.5 % (38.7 to 55.8)       | 0.333   |

| Post Consolidation NGS MRD | Cumulative Incidence of Relapse | Non-Relapse Mortality |
|----------------------------|--------------------------------|-----------------------|
|                            | HR                                    | 3-year CIR (95% CI) | P-value |
| MRD Negative               | 1                                     | 36.2 % (25.1 to 47.4) | 0.03    |
| MRD Positive               | 4.79                                  | 52.8 % (37.8 to 65.7) | 0.39    |

| Post Induction FCM MRD | Cumulative Incidence of Relapse | Non-Relapse Mortality |
|------------------------|--------------------------------|-----------------------|
|                        | HR                                    | 3-year CIR (95% CI) | P-value |
| MRD Negative           | 1                                     | 33.6 % (24.7 to 42.7) | 0.08    |
| MRD Positive           | 6.97                                  | 49.8 % (38.6 to 59.9) | 0.98    |

| Post Consolidation FCM MRD | Cumulative Incidence of Relapse | Non-Relapse Mortality |
|----------------------------|--------------------------------|-----------------------|
|                            | HR                                    | 3-year CIR (95% CI) | P-value |
| MRD Negative               | 1                                     | 35.9 % (25.1 to 46.9) | 0.01    |
| MRD Positive               | 6.03                                  | 61.9 % (36.3 to 79.7) | 0.22    |

| Comparative Analysis of FCM MRD and NGS MRD (Post Induction) | Cumulative Incidence of Relapse | Non-Relapse Mortality |
|-----------------------------------|--------------------------------|-----------------------|
| NGS MRD- FCM MRD-                 | HR                                    | 3-year CIR (95% CI) | P-value |
|                                   | 1                                     | 18.7 % (8.0 to 32.7) | 0.003   |
| NGS MRD+ FCM MRD-                 | -                                     | 41.9 % (29.6 to 53.7) | 0.425   |
| NGS MRD- FCM MRD+                 | -                                     | 41.2 % (17.7 to 63.6) | 0.632   |
| NGS MRD+ FCM MRD+                 | 14.01                                 | 52.5 % (39.6 to 63.9) | 0.36    |

| Comparative Analysis of FCM MRD and NGS MRD (Post Consolidation) | Cumulative Incidence of Relapse | Non-Relapse Mortality |
|---------------------------------------------------------------|--------------------------------|-----------------------|
| NGS MRD- FCM MRD-                                             | HR                                    | 3-year CIR (95% CI) | P-value |
|                                   | 1                                     | 24.8 % (12.6 to 39.0) | 0.0003  |
| NGS MRD+ FCM MRD-                                             | -                                     | 50.0 % (29.9 to 67.1) | 0.398   |
| NGS MRD- FCM MRD+                                             | -                                     | 87.5 % (17.2 to 98.9) | 0.834   |
| NGS MRD+ FCM MRD+                                             | 18.81                                 | 55.5 % (14.7 to 83.5) | 0.36    |

| Dual Timepoint NGS MRD | Cumulative Incidence of Relapse | Non-Relapse Mortality |
|------------------------|--------------------------------|-----------------------|
|                        | HR                                    | 3-year CIR (95% CI) | P-value |
| MRD Negative           | 1                                     | 27.8 % (13.5 to 44.0) | 0.04    |
| Either MRD Positive    | -                                     | 44.7 % (28.6 to 59.7) | 0.834   |
| MRD Positive           | 6.46                                  | 52.9 % (37.0 to 66.6) | 0.36    |
# Univariate Cox Analysis

|                                | Overall Survival (OS) | Relapse Free Survival (RFS) |
|--------------------------------|-----------------------|----------------------------|
| **Post Induction FCM MRD**     |                       |                            |
| MRD Negative                   | 1                     | 1                          |
| MRD Positive                   | 2.1 (1.40 to 3.13)    | 1.8 (1.26 to 2.60)         |
| **Post Induction FCM MRD**     |                       |                            |
| MRD Negative                   | Mean OS: 58.0 months; | Mean RFS: 47.0 months;     |
|                                | 95% CI (51.2 to 64.8 months), | 95% CI (40.2 to 53.8 months), |
|                                | Median OS: Not Reached | Median RFS: 32.2 months;   |
|                                |                       | 95% CI (22.4 to 40.0 months) |
| MRD Positive                   | Mean OS: 36.3 months; | Mean RFS: 28.4 months;     |
|                                | 95% CI (29.6 to 43.0 months), | 95% CI (22.6 to 34.3 months), |
|                                | Median OS: 18.4 months; | Median RFS: 15.4 months;   |
|                                | 95% CI (15.1 to 33.9 months) | 95% CI (12.7 to 20.1 months) |
| **Post Consolidation FCM MRD** |                       |                            |
| MRD Negative                   | 1.9 (0.90 to 3.91)    | 2.4 (1.17 to 4.81)         |
| **Post Consolidation FCM MRD** |                       |                            |
| MRD Negative                   | Mean OS: 52.6 months; | Mean RFS: 45.4 months;     |
|                                | 95% CI (44.6 to 60.7 months), | 95% CI (37.5 to 53.3 months), |
|                                | Median OS: Not Reached | Median RFS: 31.9 months;   |
|                                |                       | 95% CI (19.1 to 38.9 months) |
| MRD Positive                   | Mean OS: 28.7 months; | Mean RFS: 19.4 months;     |
|                                | 95% CI (18.7 to 38.7 months), | 95% CI (11.6 to 27.2 months), |
|                                | Median OS: 16.5 months; | Median RFS: 11.8 months;   |
|                                | 95% CI (11.2 to 33.9 months) | 95% CI (9.6 to 20.1 months) |
| **Post Induction NGS MRD**     |                       |                            |
| MRD Negative                   | 2.2 (1.47 to 3.42)    | 2.3 (1.58 to 3.31)         |
| **Post Induction NGS MRD**     |                       |                            |
| MRD Negative                   | Mean OS: 63.2 months; | Mean OS: 54.9 months;      |
|                                | 95% CI (54.4 to 72.0 months), | 95% CI (45.7 to 64.2 months), |
|                                | Median OS: Not Reached | Median OS: Not Reached      |
| MRD Positive                   | Mean OS: 32.9 months; | Mean OS: 26.1 months;      |
|                                | 95% CI (28.9 to 36.9 months), | 95% CI (22.4 to 29.6 months), |
|                                | Median OS: 27.0 months; | Median OS: 16.7 months;    |
|                                | 95% CI (17.9 to 42.8 months) | 95% CI (14.5 to 22.4 months) |
| **Post Consolidation NGS MRD** |                       |                            |
| MRD Negative                   | 1                     | 1                          |
| **Post Consolidation NGS MRD** |                       |                            |
| MRD Negative                   | Mean OS: 63.2 months; | Mean OS: 54.9 months;      |
|                                | 95% CI (54.4 to 72.0 months), | 95% CI (45.7 to 64.2 months), |
|                                | Median OS: Not Reached | Median OS: Not Reached      |
| MRD Positive                   | Mean OS: 32.9 months; | Mean OS: 26.1 months;      |
|                                | 95% CI (28.9 to 36.9 months), | 95% CI (22.4 to 29.6 months), |
|                                | Median OS: 27.0 months; | Median OS: 16.7 months;    |
|                                | 95% CI (17.9 to 42.8 months) | 95% CI (14.5 to 22.4 months) |
| MRD Positive | 1.9 (1.14 to 3.22) | 1.9 (1.18 to 3.06) |
| Post Consolidation | Overall Survival (OS) | Relapse Free Survival (RFS) |
| NGS MRD | HR (95% CI) | P | HR (95% CI) | P |
| MRD Negative | Mean OS: 55.5 months; 95% CI (47.2 to 63.7 months), Median OS: Not Reached | 0.008 | Mean OS: 46.9 months; 95% CI (38.8 to 54.9 months), Median OS: 33.0 months; 95% CI (21.4 to 42.0 months) | 0.004 |
| MRD Positive | Mean OS: 30.1 months; 95% CI (23.7 to 36.5 months), Median OS: 18.1 months; 95% CI (16.1 to 36.5 months) | Mean OS: 24.6 months; 95% CI (18.7 to 30.6 months), Median OS: 15.4 months; 95% CI (11.2 to 17.5 months) |
| Dual Timepoint | Overall Survival (OS) | Relapse Free Survival (RFS) |
| NGS MRD | HR (95% CI) | P | HR (95% CI) | P |
| MRD Negative | 1 | 0.03 | 1 | 0.01 |
| Either MRD Positive | 1.2 (0.66 to 2.28) | 1.2 (0.71 to 2.21) |
| MRD Positive | 2.5 (1.33 to 4.61) | 2.5 (1.45 to 4.48) |
| Comparative Analysis of FCM MRD and NGS MRD (Post Induction) | Overall Survival (OS) | Relapse Free Survival (RFS) |
| NGS MRD | HR (95% CI) | P | HR (95% CI) | P |
| NGS MRD- FCM MRD- | 1 | 0.0002 | 1 | 0.0001 |
| NGS MRD+ FCM MRD- | 1.6 (0.96 to 2.61) | 1.4 (0.87 to 2.12) |
| NGS MRD- FCM MRD+ | 1.3 (0.58 to 2.74) | 1.3 (0.63 to 2.52) |
| NGS MRD+ FCM MRD+ | 4.7 (2.71 to 8.00) | 4.0 (2.51 to 6.47) |
| Comparative Analysis of FCM MRD and NGS MRD (Post Induction) | Overall Survival (OS) | Relapse Free Survival (RFS) |
| NGS MRD- FCM MRD- | Mean OS: 71.5 months; 95% CI (62.4 to 80.7 months), Median OS: Not Reached | Mean OS: 63.7 months; 95% CI (53.5 to 73.8 months), Median OS: Not Reached |
| NGS MRD+ FCM MRD- | Mean OS: 37.7 months; 95% CI (31.8 to 43.5 months), Median OS: 42.8 months; 95% CI (19.8 to 42.8 months) | Mean OS: 29.2 months; 95% CI (23.8 to 34.5 months), Median OS: 19.1 months; 95% CI (14.9 to 33.1 months) |
Table 3: Difference in OS and RFS between FCM-MRD, NGS-MRD and comparative analysis between the two modalities at post Induction and post consolidation timepoints. OS: Overall Survival, RFS: Relapse Free Survival, CI: confidence interval.

| Comparison | Overall Survival (OS) | Relapse Free Survival (RFS) |
|------------|-----------------------|-----------------------------|
| NGS MRD- FCM MRD+ | Mean OS: 32.7 months; 95% CI (22.2 to 43.0 months), Median OS: 21.0 months; 95% CI (14.1 to 33.8 months) | Mean OS: 27.0 months; 95% CI (17.5 to 36.5 months), Median OS: 19.6 months; 95% CI (11.8 to 33.0 months) |
| NGS MRD+ FCM MRD+ | Mean OS: 28.7 months; 95% CI (23.4 to 34.0 months), Median OS: 17.5 months; 95% CI (13.5 to 35.9 months) | Mean OS: 18.4 months; 95% CI (18.4 to 27.7 months), Median OS: 15.4 months; 95% CI (11.7 to 18.9 months) |

Comparative Analysis of FCM MRD and NGS MRD (Post Consolidation)

| Comparison | Overall Survival (OS) | Relapse Free Survival (RFS) |
|------------|-----------------------|-----------------------------|
| NGS MRD- FCM MRD- | Mean OS: 33.5 months; 95% CI (29.2 to 37.8 months), Median OS: Not Reached | Mean OS: 30.9 months; 95% CI (26.6 to 35.1 months), Median OS: 24.9 months; 95% CI (8.3 to 41.5 months) |
| NGS MRD+ FCM MRD- | Mean OS: 34.6 months; 95% CI (26.1 to 43.2 months), Median OS: 20.5 months; 95% CI (17.3 to 36.5 months) | Mean OS: 28.3 months; 95% CI (20.1 to 36.4 months), Median OS: 16.7 months; 95% CI (15.1 to 35.5 months) |
| NGS MRD- FCM MRD+ | Mean OS: 43.9 months; 95% CI (20.7 to 367.1 months), Median OS: 21.0 months; 95% CI (11.7 to 33.9 months) | Mean OS: 24.9 months; 95% CI (8.3 to 41.5 months), Median OS: 14.1 months; 95% CI (10.1 to 32.6 months) |
| NGS MRD+ FCM MRD+ | Mean OS: 18.2 months; 95% CI (8.04 to 28.4 months), Median OS: 12.6 months; 95% CI (5.60 to 27.0 months) | Mean OS: 13.4 months; 95% CI (5.1 to 21.8 months), Median OS: 9.6 months; 95% CI (4.7 to 15.4 months) |

Multivariate Cox Analysis

| Timepoint | Overall Survival (OS) | Relapse Free Survival (RFS) |
|-----------|-----------------------|-----------------------------|
| Post Induction Timepoint | HR (95% CI) | P | HR (95% CI) | P |
| NGS MRD Positive | 1.94 (1.15 to 3.27) | <0.000 | 2.05 (1.30 to 3.23) | <0.000 |
| FCM MRD Positive | 1.88 (1.21 to 2.84) | 1 | 1.61 (1.12 to 2.31) | 1 |
| Post Consolidation Timepoint | Overall Survival (OS) | Relapse Free Survival (RFS) |
|---------------------------|-----------------------|-----------------------------|
| NGS MRD Positive | 1.82 (1.09 to 3.29) | 0.02 | 1.82 (1.06 to 3.11) | 0.002 |
| FCM MRD Positive | 2.03 (1.05 to 3.94) | 0.002 | 2.46 (1.34 to 4.47) | 0.002 |
Supplementary Methods
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1. Detection of AML MRD using smMIPS

Supplementary Figure 1: Above oncoplot (on 393 patients) demonstrates the frequency of somatic mutations at diagnosis in AML detected using a 50 gene smMIPS myeloid panel. This genomic landscape formed the basis for designing the NGS MRD panel.

a. Design of smMIPS:

We reviewed somatic mutations in 393 patients of adult AML (Supplementary Figure 1) diagnosed at the Tata Memorial Centre as well as mutations in TCGA AML cohort (www.cbioportal.org). Based on this data we designed a 35 gene myeloid hotspot panel comprising of 302 single molecule molecular inversion probes (smMIPS). The genes covered by this “hot-spot” panel can be seen in Supplementary Table 1. The panel comprised of a main panel and an add on module which covered uncommon mutations. The latter panel was added if mutations (covered by addon panel) were present at diagnosis. smMIPS were designed using MIPgen\(^1\) software with the parameters -max_capture_size 162, -min_capture_size 152, -logistic_priority_score 0.5. Extension arm length parameters were set between 18-21 and ligation arm length between 21-24. Each smMIP was designed to include a four
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basepair (bp) unique molecular identifier (UMI) at each end (total of 8bp degenerate nucleotide sequence per smMIP).

Recruitment of Patients in NGS-MRD Study

Supplementary Figure 2: Flow chart indicates recruitment of patients in AML NGS-MRD study.

Supplementary Figure 3: Survival estimates for overall survival (A) and relapse free survival (B) of allogeneic bone marrow transplantation in this patient cohort.

b. Rebalancing smMIPS to ensure uniform capture:

Following initial equimolar pooling and analysis of read depths, the smMIPS pool underwent two rounds of rebalancing. A balanced version can be seen in Supplementary Figure 2.
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Supplementary Figure 4: Unique Capture Events per smMIP for three normal specimens.

a. **Sequencing:**

Initial standardization and balancing experiments using NA12878 control DNA were performed on a MiSeq (standard V2 flow cell 150PE chemistry). Once the assay was standardized, error modelling, limit of detection, and MRD detection experiments were performed on multiple S4 flow cells of a NovaSeq 6000 using 150PE chemistry.

b. **Data Analysis:**

The bioinformatics approach was similar to that published by Waalkes et al\(^2\) with a few modifications. Demultiplexing was performed using bcl2fastq-v2.17. Adapter sequences, smMIPs backbone and reads less than 53bp were trimmed using fastq-mcf tool of ea-utils (https://expressionanalysis.github.io/ea-utils/). Paired end assembly was carried out using PEAR (v0.9.8).\(^3\) A custom script was used to trim, concatenate the 4bp UMI at the 5’ and 3’ end of assembled read and add it to the read header. Reads were mapped to the human genome (build hg19) using bwa-0.7.12\(^4\) and pre-processed using SAMtools-1.1.\(^5\) For computational efficiency, mapped reads were split by chromosome and into files sourced from reads mapping to individual smMIPS based on their genomic start and stop coordinates. Each of these files is processed individually for downstream variant calling. All reads originating from a single UMI were discarded (singleton reads). The rest of the reads (two or more UMI) were used to create a consensus .sam file using CallMolecularConsensusReads function of fgbio-0.4.0 with the following parameters --error-rate-post-umi=30 --min-reads=2 --min-input-base-quality 20 (https://github.com/fulcrumgenomics/fgbio). These reads were converted to fastq using picard-2.17.2 (http://broadinstitute.github.io/picard/) and mapped, sorted and indexed using bwa and samtools as described above. A .mpileup was created using
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Samtools-1.1 and sequence variants detected using a bespoke variant caller. ([https://bitbucket.org/uwlabmed/smmps_analysis/src/master/](https://bitbucket.org/uwlabmed/smmps_analysis/src/master/)). Variant calls from individual smMIPs were combined into a single .vcf file and annotated using ANNOVAR.6

**Supplementary Table 1:** List of genes and their loci sequenced using the smMIPS AML MRD panel.

| Sr. Nos | Gene Name | Region | RefSeqID |
|---------|-----------|--------|----------|
| 1       | ASXL1     | Exon 13 | NM_015338 |
| 2       | ATM       | Exon 37 | NM_000051 |
| 3       | BCR       | Exon 4, 10, 11 | NM_001123383 |
| 4       | DNM73A    | Exon 23 | NM_022552 |
| 5       | EZH2      | Exon 14-18 | NM_152988.2 |
| 6       | FLT3      | Exon 20, 14-15* | NM_004119 |
| 7       | GATA1     | Exon 2   | NM_002049 |
| 8       | GATA2     | Exon 3, 4 | NM_032638 |
| 9       | IDH1      | Exon 4   | NM_005896 |
| 10      | IDH2      | Exon 4, Exon 4 (R172K) | NM_002168 |
| 11      | JAK2      | Exon 14  | NM_004972 |
| 12      | KDM6A     | Exon 17, 20 | NM_021140 |
| 13      | KIT       | Exon 8-11, 17 | NM_000222 |
| 14      | KMT2D     | Exon 34, 39 | NM_003482 |
| 15      | KRAS      | Exon 2-4, 5 | NM_004985 |
| 16      | NF1       | Exon 24, 47 | NM_01042492 |
| 17      | NOTCH1    | Exon 27, 34 | NM_017617 |
| 18      | NOTCH2    | Exon 34  | NM_024408 |
| 19      | NPM1      | Exon 11  | NM_002520 |
| 20      | NRAS      | Exon 2-3  | NM_002524 |
| 21      | PHEF6     | Exon 2, 3-9 | NM_032458 |
| 22      | PTPN11    | Exon 3, 13-14 | NM_002834 |
| 23      | RAD21     | Exon 5, 11 | NM_006265 |
| 24      | RUNX1     | Exon 4-8  | NM_001754 |
| 25      | SETBP1    | Exon 43   | NM_015559 |
| 26      | SF3B1     | Exon 14-16 | NM_012433 |
| 27      | SH2B3     | Exon 3, 6  | NM_005475 |
| 28      | SMC1A     | Exon 2, 11 | NM_006306 |
| 29      | SRSF2     | Exon 1    | NM_003016 |
| 30      | STAG2     | Exon 7-8, 19, 24, 27-29 | NM_006603 |
| 31      | TET2      | Exon 3-11 | NM_001127208 |
| 32      | TP53      | All Exons | NM_001126114 |
| 33      | U2AF1     | Exon 2, 6, 7 | NM_006758 |
| 34      | WT1       | Exon 6-8, 11 | NM_024426 |
| 35      | ZRSR2     | Exon 10   | NM_005089 |

**Supplementary Table 2:** Applicability of NGS-MRD panel in AML patients stratified by cytogenetic risk and number of mutations seen in DTA genes

| Favourable Risk | Intermediate Risk | Poor Risk |
|-----------------|-------------------|-----------|
| Total Number of Patients | Mutations in DTA Genes | Total Number of Patients | Mutations in DTA Genes | Total Number of Patients | Mutations in DTA Genes |
| 1 Mutation      | 26                | 32        | 1        | 7         | 0         |
| 2 Mutations     | 13                | 0         | 33       | 5         | 3         | 0         |
| 3 Mutations     | 9                 | 1         | 48       | 11        | 6         | 0         |
| 4 Mutations     | 0                 | 0         | 18       | 9         | 1         | 0         |
| >5 Mutations    | 0                 | 0         | 5        | 4         | 0         | 0         |

**Supplementary Table 3:** List of mutations and their loci sequenced using the smMIPS AML MRD panel.

| Sr. Nos | Gene Name | Region | RefSeqID |
|---------|-----------|--------|----------|
| 1       | ASXL1     | Exon 13 | NM_015338 |
| 2       | ATM       | Exon 37 | NM_000051 |
| 3       | BCR       | Exon 4, 10, 11 | NM_001123383 |
| 4       | DNM73A    | Exon 23 | NM_022552 |
| 5       | EZH2      | Exon 14-18 | NM_152988.2 |
| 6       | FLT3      | Exon 20, 14-15* | NM_004119 |
| 7       | GATA1     | Exon 2   | NM_002049 |
| 8       | GATA2     | Exon 3, 4 | NM_032638 |
| 9       | IDH1      | Exon 4   | NM_005896 |
| 10      | IDH2      | Exon 4, Exon 4 (R172K) | NM_002168 |
| 11      | JAK2      | Exon 14  | NM_004972 |
| 12      | KDM6A     | Exon 17, 20 | NM_021140 |
| 13      | KIT       | Exon 8-11, 17 | NM_000222 |
| 14      | KMT2D     | Exon 34, 39 | NM_003482 |
| 15      | KRAS      | Exon 2-4, 5 | NM_004985 |
| 16      | NF1       | Exon 24, 47 | NM_01042492 |
| 17      | NOTCH1    | Exon 27, 34 | NM_017617 |
| 18      | NOTCH2    | Exon 34  | NM_024408 |
| 19      | NPM1      | Exon 11  | NM_002520 |
| 20      | NRAS      | Exon 2-3  | NM_002524 |
| 21      | PHEF6     | Exon 2, 3-9 | NM_032458 |
| 22      | PTPN11    | Exon 3, 13-14 | NM_002834 |
| 23      | RAD21     | Exon 5, 11 | NM_006265 |
| 24      | RUNX1     | Exon 4-8  | NM_001754 |
| 25      | SETBP1    | Exon 43   | NM_015559 |
| 26      | SF3B1     | Exon 14-16 | NM_012433 |
| 27      | SH2B3     | Exon 3, 6  | NM_005475 |
| 28      | SMC1A     | Exon 2, 11 | NM_006306 |
| 29      | SRSF2     | Exon 1    | NM_003016 |
| 30      | STAG2     | Exon 7-8, 19, 24, 27-29 | NM_006603 |
| 31      | TET2      | Exon 3-11 | NM_001127208 |
| 32      | TP53      | All Exons | NM_001126114 |
| 33      | U2AF1     | Exon 2, 6, 7 | NM_006758 |
| 34      | WT1       | Exon 6-8, 11 | NM_024426 |
| 35      | ZRSR2     | Exon 10   | NM_005089 |

**c. Limit of Detection Experiment:**

In order to determine the limit of detection, we performed an experiment where OCIAML3 cell line [harbouring DNM73A (p.R882C), NRAS (p.Q61L) and NPM1 (Type A mutation)] was
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serially diluted in a normal bone marrow (BM). Similarly, baseline DNA from five AML samples [each harbouring NRAS (p.G12D), IDH1 (p.R132G), IDH2 (p.R140Q), IDH2 (p.R172K) and NPM1 (type A)] were serially diluted in normal BM as seen in Supplementary Figure 3. The expected variant allele frequency (VAF) for that mutation was calculated from the original VAF found in undiluted sample. The range of the expected VAFs was from 1.25% to 0.02%. We could successfully detect VAFs in nearly all cases up to a lower limit of approximately 0.05%. The limit of detection of NGS-MRD assay was thus at 0.05% for all mutations and 0.03% for the NPM1 mutation. A lower LOD threshold was acceptable for NPM1 mutation because of the uniqueness of the indel mutation and a previous observation (based on a limit of blank study) that complex 4bp indels (seen in NPM1) are not observed for short read sequencing data.7
Supplementary Figure 5: Serial dilutions of OCIAML3 and AML DNA in normal bone marrow. The expected VAF for a mutation was calculated from the original VAF found in undiluted samples.
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d. **Error Modelling:**

A site and mutation specific error model was setup to ascertain the occurrence of variations observed in the smMIP MRD panel. We sequenced a NA12878 and four normal bone marrow controls to measure sequencing errors using smMIPS as described by Waalkes and colleagues. As mentioned by Waalkes we discovered a reduction in error rates using UMI based sequencing over standard NGS based sequencing (data not shown). As described, we fitted a $\beta$ distribution for each base position and probable base substitution error. We observed a higher frequency of C>T and G>A changes consistent with oxidative DNA damage (Supplementary Figure 4) occurring in template DNA before sequencing. Where no variation was detected a 1:15,000 error rate was presumed and a $\beta$ distribution was modelled. For each variation observed, a site-specific $p$-value was annotated using these pre-calculated $\beta$ distributions. Sites with $p>0.005$ were excluded as artefacts.

**Supplementary Figure 6:** Frequencies of errors observed using a site and mutation specific model.

e. **Criteria for variant calling using smMIPS MRD assay:**

1. Variants filtered by focussing on exonic regions (including splicing variants if any) followed by population frequency (<0.01) filtering.
2. The variant must have been detected at baseline.
3. Background error modelling at that site must have a $P$ value <0.005.
4. A minimum of 10 alternate variant reads must be present for an SNV.
5. A minimum of 3 alternate variant reads must be present for an indel.
6. The highest VAF was taken as MRD.
7. If the highest VAF was in a variant associated with $DNMT3A$, $TET2$ or $ASXL1$ (DTA mutation) it was ignored.
2. Detection of FLT3-MRD using a one-step PCR assay:

   a. Assay Design:

   We observed that we could not monitor FLT3-ITD using smMIPs. To overcome this issue, we designed a one-step PCR assay that incorporated locus specific primers, dual indices and Illumina adapters in a single step (Supplementary Table 2). The primers were designed to amplify common internal tandem duplication site (chr13: 28608024 - 28608353). The assay was setup using 600ng of genomic DNA as template. The below primers (50nM each) were added to 12.5µL of HotStarTaq Master Mix (Qiagen, Hilden, Germany) to setup a 25 µL reaction. Amplification was carried out under the following conditions; (Denaturation: 95⁰C-15 minutes; Denaturation: 95⁰C-60 seconds; Annealing 61⁰C-60 seconds; Extension 72⁰C-60 seconds; 35 cycles; final extension 72⁰C-45 seconds). The final library was size selected using Agencourt AMPure XP beads (Beckman Coulter Inc., California, USA) and sequenced on an Illumina MiSeq (V2, 250PE chemistry). Each sample was allotted 1.1 million reads.

| FLT3-ITD primers    | Sequences                                                                 |
|---------------------|---------------------------------------------------------------------------|
| Forward Primer*     | 5’AATGATACGGCGACCACCGAGATCTACACXXXXXXXXXXTATGGTGCCTGTAGCAATTATGATGAAAGCCAGCTA |
| Reverse primer*     | 5’CAAGCAGAGACGGCATACGCAGATXXXXXXXXXXAGTCAGTCAGTCTTTCAGCATTTTACGGGAACC     |
| Read 1 Primer       | 5’- TATGGTGCCGCTGACTGCAATTTAGTATGAAAGCCAGCTA-3’                          |
| Read 2 primer       | 5’ AGTCAGTCAGTCCTTACCATTTGACGGCAACC-3’                                  |
| Index Primer        | 5’- GGTTGGCGCGTCAAAATGCTGAAGACTGACTGACTGACT-3’                           |

Supplementary Table 3: Adapter tagged FLT3-ITD primers *-XXXXXXXXXX represents 10 bp sample specific index sequence.

   b. Data Analysis and Limit of Detection:

   We adopted a recently described algorithm for accurate detection of FLT3-ITD using next generation sequencing. We could demonstrate good correlation (Supplementary Figure 5, inset) between conventional and NGS testing for accurate detection of ITD length in 71 FLT3-ITD positive AML. We could validate this assay till a maximum ITD length of 100bp.

   c. Limit of Detection of FLT3 NGS MRD assay:

   In order to determine the limit of detection of this assay, we diluted a FLT3-ITD (30bp ITD) positive sample into normal BM. All dilutions were performed in triplicates (Supplementary Figure 5). We could successfully detect this mutation till a lower limit of 0.002% VAF, as shown in Supplementary Figure 5. Based on this data, we established the limit of detection of the FLT3-ITD NGS MRD assay at 0.002. All FLT3-ITD clones >1% VAF were tracked.
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Supplementary Figure 7: Correlation of FLT3-ITD length by capillary (conventional testing) electrophoresis as compared to NGS assay (Inset). Serial dilution of a FLT3-ITD positive sample in normal DNA demonstrates the limit of detection (in triplicates) to be 0.002%.

| Cytogenetic Risk    | Post Induction FCM MRD | Post Induction NGS MRD |
|---------------------|------------------------|------------------------|
|                     | MRD Positive | MRD Negative | MRD Positive | MRD Negative |
| Favorable           | 21 (23.86%) | 27 (24.11%) | 30 (21.58%) | 17 (29.82%) |
| Intermediate        | 55 (62.50%) | 80 (71.43%) | 99 (71.22%) | 34 (59.65%) |
| Poor Risk           | 12 (13.64%) | 5 (4.46%) | 10 (7.19%) | 6 (10.53%) |
| Total               | 88          | 112         | 139         | 57          |

Supplementary Table 4: Distribution of MRD results measured by FCM and NGS into cytogenetic risk categories. FCM: Flow Cytometry; NGS: Next generation sequencing. Chi Squared test did not reveal a statistical significance of MRD results distributed by cytogenetic risk groups.
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**Supplementary Figure 8**: Clinical relevance of NGS-MRD and their influence on overall survival (OS) and relapse free survival (RFS) when patients are grouped by cytogenetic risk categories.

**Supplementary Figure 9**: Change in mutations over MRD time points amongst paired PI and PC samples.
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Supplementary Figure 10: Clinical relevance of kinetics of NGS-MRD clearance and their influence on overall survival (OS) and relapse free survival (RFS).

Supplementary Figure 11: Clinical relevance paired MRD sampling and influence on overall survival (OS) and relapse free survival (RFS). Patients who persistently harboured MRD had a significantly inferior OS [HR- 2.48; 95% CI- 1.33 to 4.61; (p=0.03)] and RFS [HR- 2.55; 95% CI- 1.45 to 4.48; (p=0.01)] as compared to patients who were MRD negative at both time points.
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Supplementary Figure 12: Comparative oncoplot (A) of patients who were NGS-MRD positive at both time points as compared to patients who were persistently MRD negative. Commonly occurring mutations are highlighted here. Forest plot (B) fails to demonstrate a genetic difference between these two groups.
3. Orthogonal MRD detection of *NPM1* mutations using *NPM1* MRD assay.

Orthogonal Comparision of MRD results of *NPM1* Mutated AML

**Supplementary Figure 13:** Cross validation of smMIPS MRD cases (n=75; *NPM1* mutated AML) detected by *NPM1* NGS MRD assay. Since the axis is logarithmic, zero values (n=44) have been omitted.

Orthogonal MRD testing was performed in 75 MRD samples (75 out of 323 MRD samples; 23.2%). These were detected using a previously published ultradep sequencing based MRD assay for *NPM1* mutated AML. 7 smMIPS MRD positive *NPM1* mutated AML cases (45 out of 75; 60%) could be detected by the *NPM1* NGS MRD assay. For the rest of the cases, *NPM1* mutation was either negative or detected at a threshold below the smMIPS MRD assay limit. For smMIPS MRD positive cases the median MRD value was 0.86% as compared to 0.82% for *NPM1* NGS MRD (at a LOD cut-off of 0.03%).
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4. FCM based AML-MRD:

**Supplementary Figure 14:** Survival analysis of adult AML patients by flow cytometric MRD assessment (FCM-MRD) after induction and consolidation phases of chemotherapy.
Supplementary Figures

**Supplementary Figure 15**: Comparison of genetic profiles between FCM-MRD and NGS-MRD groups (FCM+NGS+ vs FCM-NGS-) at PI timepoint. Comparison is performed using Fischers exact test on all genes between two cohorts (*RUNX1* mutation, *p*=0.04).
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**Supplementary Table 5:** Clinical, cytogenetic risk, molecular risk stratification, NGS MRD coverage, NGS MRD and Flow MRD results and outcome of patients who are FCM MRD positive but NGS MRD negative. NA: MRD not performed
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### Post Induction MRD Assessment

|                | NGS+FCM+ vs NGS-FCM- | NGS+FCM- vs NGS-FCM- | NGS-FCM+ vs NGS-FCM- |
|----------------|----------------------|----------------------|----------------------|
| **Value**      | 95% CI               | Value                | 95% CI               | Value                | 95% CI               |
| Sensitivity    | 81.25%               | 69.54% to 89.92%     | 78.95%               | 66.11% to 88.62%     | 50.00%               | 29.12% to 70.88%     |
| Specificity    | 63.64%               | 47.77% to 77.59%     | 52.83%               | 38.64% to 66.70%     | 84.85%               | 68.10% to 94.89%     |
| Positive Predictive Value (*) | 76.47% | 68.36% to 83.02% | 64.29% | 56.78% to 71.15% | 70.59% | 49.36% to 85.53% |
| Negative Predictive Value (*) | 70.00% | 57.21% to 80.28% | 70.00% | 57.05% to 80.39% | 70.00% | 60.40% to 78.12% |
| Accuracy (*)   | 74.07%               | 64.75% to 82.03%     | 66.36%               | 56.73% to 75.09%     | 70.18%               | 56.60% to 81.57%     |

### Post Consolidation MRD Assessment

|                | NGS+FCM+ vs NGS-FCM- | NGS+FCM- vs NGS-FCM- | NGS-FCM+ vs NGS-FCM- |
|----------------|----------------------|----------------------|----------------------|
| **Value**      | 95% CI               | Value                | 95% CI               | Value                | 95% CI               |
| Sensitivity    | 28.57%               | 13.22% to 48.67%     | 48.72%               | 32.42% to 65.22%     | 25.93%               | 11.11% to 46.28%     |
| Specificity    | 95.65%               | 78.05% to 99.89%     | 70.97%               | 51.96% to 85.78%     | 95.65%               | 78.05% to 99.89%     |
| Positive Predictive Value (*) | 88.89% | 51.88% to 98.34% | 67.86% | 52.74% to 79.98% | 87.50% | 48.14% to 98.14% |
| Negative Predictive Value (*) | 52.38% | 46.14% to 58.55% | 52.38% | 42.93% to 61.66% | 52.38% | 46.40% to 58.29% |
| Accuracy (*)   | 58.82%               | 44.17% to 72.42%     | 58.57%               | 46.17% to 70.23%     | 58.00%               | 43.21% to 71.81%     |

**Supplementary Table 6:** Sensitivity, Specificity, PPV, NPV and Accuracy of both FCM-MRD and NGS-MRD assays.
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Supplementary Figure 16: Cytogenetic and genomic landscape of cases which on which NGS-MRD could not be performed. Out of 65 cases, 36 had detectable mutations (A) whereas, no mutations were detected in 29 cases (B). Genomic landscape of cases in which mutations were detected can be seen in a waterfall plot (C). Number of cases are represented in box with percentages in parenthesis. The solitary CEBPA mutation seen here was a case sequenced by Illumina Trusight Myeloid Sequencing Panel.
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**Supplementary Figure 17:** This figure highlights genes that were informative in making a NGS-MRD positive call.

**Supplementary Figure 18:** Survival analysis for end of induction and post consolidation using NGS-MRD (After excluding samples sourced from blood).
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**Supplementary Figure 19:** Impact of presence of FLT3 NGS MRD on overall and relapse free survival at PI and PC.

**Supplementary References**

1. Boyle EA, O’Roak BJ, Martin BK, Kumar A, Shendure J. MiPgen: optimized modeling and design of molecular inversion probes for targeted resequencing. *Bioinformatics*. 2014;30(18):2670-2672.
2. Waalkes A, Penewit K, Wood BL, Wu D, Salipante SJ. Ultrasensitive detection of acute myeloid leukemia minimal residual disease using single molecule molecular inversion probes. *Haematologica*. 2017;102(9):1549-1557.
3. Zhang J, Kobert K, Flouri T, Stamatakis A. PEAR: a fast and accurate Illumina Paired-End read merge. *Bioinformatics*. 2014;30(5):614-620.
4. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009;25(14):1754-1760.
5. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009;25(16):2078-2079.
6. Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res*. 2010;38(16):e164.
7. Patkar N, Kodgule R, Kakirde C, et al. Clinical impact of measurable residual disease monitoring by ultradeep next generation sequencing in NPM1 mutated acute myeloid leukemia. *Oncotarget*. 2018;9(93):36613-36624.
8. Huang Q, Chen W, Gaal KK, Slovak ML, Stein A, Weiss LM. A rapid, one step assay for simultaneous detection of FLT3/ITD and NPM1 mutations in AML with normal cytogenetics. *Br J Haematol*. 2008;142(3):489-492.
9. Blatte TJ, Schmalbrock LK, Skambraks S, et al. getITD for FLT3-ITD-based MRD monitoring in AML. *Leukemia*. 2019;33(10):2535-2539.
