Clinical impact of measurable residual disease monitoring by ultradeep next generation sequencing in *NPM1* mutated acute myeloid leukemia

SUPPLEMENTARY MATERIALS

SUPPLEMENTARY METHODS

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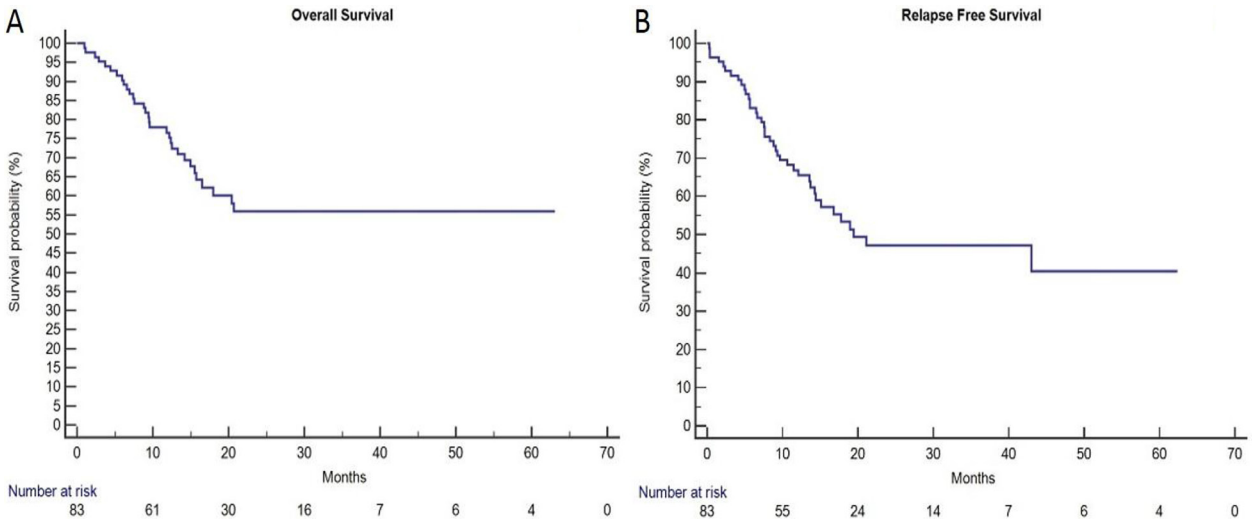
Clinical Outcome



Supplementary Figure 1: Kaplan Meier curves for OS (**A**) and RFS (**B**) for *NPM1*^{mut} AML.

Supplementary Table 1A: Patient characteristics

No.	Characteristics	Value
1	Sex	
	Male	46 (55.4%)
	Female	37 (44.6%)
2	Median age (range), years	36.7 (18 – 62)
3	Median WBC Count (range), × 10^9/L	36.35 (0.1 – 270.7)
4	*FLT3*-ITD	33 of 83 (39.8%)
5	PI NGS VAF median % (range) - 82 patients	2.15% (0 – 36.4%)
6	PC NGS VAF % (Range) - 55 patients	0.21% (0 – 5.8%)

Supplementary Table 1B: Cytogenetics data

NPM1_ID	*Age*	*Sex*	*FISH*[$]	*Conventional Karyotyping*[*]
NPM1-3	**23**	**M**	Negative	**NA**
NPM1-6	**36**	**F**	Negative	**46,XX**
NPM1-9	**46**	**F**	Negative	**46,XX,del(13)(q14q21)[3]/45,X,-X [3]/46,XX[13]**
NPM1-10	**32**	**M**	Negative	**NA**
NPM1-11	**52**	**F**	Negative	**NA**
NPM1-13	**55**	**F**	Negative	**46,XX**
NPM1-14	**41**	**M**	Negative	**NA**
NPM1-17	**50**	**M**	Negative	**NA**
NPM1-18	**41**	**M**	Negative	**NA**
NPM1-19	**33**	**M**	Negative	**NA**
NPM1-21	**22**	**F**	Negative	**NA**
NPM1-22	**38**	**M**	Negative	**46,XY**
NPM1-23	**62**	**M**	Negative	**45,X,-Y[10]/45,X,-Y,t(?;9)(?;q34)[3]/44-45,XY,-Y[3],t(9)(q34)[1] [cp3]**
NPM1-25	**42**	**M**	Negative	**NA**
NPM1-26	**47**	**F**	Negative	**NA**
NPM1-27	**28**	**M**	Negative	**NA**
NPM1-28	**52**	**F**	Negative	**NA**
NPM1-30	**20**	**M**	Negative	**NA**
NPM1-32	**20**	**M**	Negative	**46,XY**
NPM1-33	**49**	**M**	Negative	**NA**
NPM1-34	**28**	**M**	**t(8;21)**	**NA**
NPM1-35	**25**	**M**	Negative	**NA**
NPM1-36	**54**	**M**	Negative	**NA**
NPM1-37	**40**	**M**	Negative	**NA**
NPM1-38	**56**	**F**	Negative	**46,XX**
NPM1-39	**54**	**M**	Negative	**NA**
NPM1-40	**37**	**M**	Negative	**47,XY, +21[10]/46,XY[3]**
NPM1-41	**25**	**M**	Negative	**NA**
NPM1-42	**50**	**M**	Negative	**46,XY**
NPM1-44	**30**	**F**	Negative	**46,XX**
NPM1-45	**44**	**F**	Negative	**NA**
NPM1-46	**44**	**M**	Negative	**46,XY**
NPM1-47	**37**	**M**	Negative	**NA**
NPM1-48	**24**	**F**	Negative	**NA**
NPM1-49	**56**	**M**	Negative	**NA**
NPM1-50	**38**	**M**	Negative	**NA**
NPM1-51	**29**	**M**	Negative	**NA**
NPM1-52	**21**	**M**	Negative	**NA**
NPM1-53	**42**	**F**	Negative	**NA**
NPM1-54	**34**	**F**	Negative	**46,XX**
NPM1-55	**21**	**F**	Negative	**NA**
NPM1-56	**36**	**F**	Negative	**NA**
NPM1-57	**19**	**M**	Negative	**46,XY, +9[2][cp2]/46,XY[17]**
NPM1-58	**43**	**M**	Negative	**NA**
NPM1-59	**52**	**F**	Negative	**NA**
NPM1-60	**57**	**M**	Negative	**NA**
NPM1-61	**41**	**F**	**NA**	**NA**
NPM1-62	**45**	**F**	Negative	**NA**

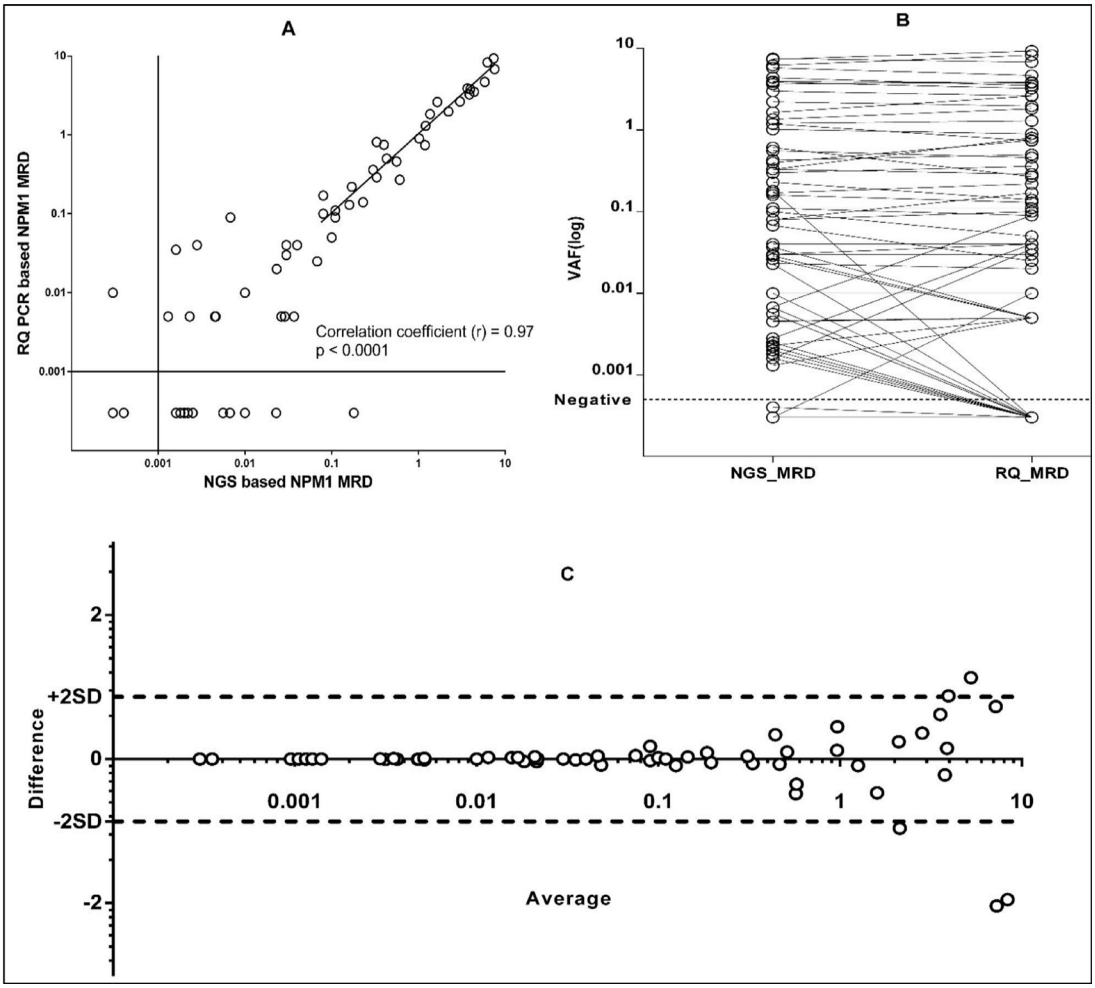
NPM1-63	**48**	**F**	Negative	**NA**
NPM1-64	**32**	**M**	Negative	**NA**
NPM1-65	**45**	**M**	Negative	**46,XY**
NPM1-67	**44**	**F**	Negative	**NA**
NPM1-68	**37**	**F**	**t(9;22)**	**NA**
NPM1-69	**21**	**M**	Negative	**46,XY**
NPM1-70	**32**	**F**	Negative	**NA**
NPM1-71	**24**	**M**	Negative	**NA**
NPM1-74	**45**	**F**	Negative	**46,XX, ?inv(10) (p13;q22)[10] / 46,XX [7]**
NPM1-75	**25**	**F**	Negative	**46,XX**
NPM1-77	**26**	**F**	Negative	**46,XX**
NPM1-78	**37**	**F**	Negative	**46-47,XX, i(Xq)[2],del(9q11)[2],del(11q23)[2],+21[3][cp7]/**
NPM1-79	**41**	**F**	Negative	**NA**
NPM1-80	**18**	**M**	Negative	**44-45,XY, random abnormalities[2]/ 46,XY[13]**
NPM1-81	**58**	**M**	Negative	**NA**
NPM1-82	**32**	**M**	Negative	**NA**
NPM1-83	**22**	**M**	Negative	**46,XY, del(11)(q13)[2]/ 43-46,XY,del(5q)[2], -8[3], +8[2], t(13;?)(p11;?)[2],+21[2][cp5]/ 46,XY[6]**
NPM1-84	**20**	**F**	Negative	**NA**
NPM1-85	**30**	**F**	Negative	**46,XX,del(5)(q13.1q13.1)[4]/ 46,XX,t(Xp)[2][cp2]/**
NPM1-86	**32**	**M**	**Trisomy 8**	**44-46, XY, del (5) (q? 15/q22)[1], random aberrations[4]/ 46, XY[11]**
NPM1-87	**22**	**F**	Negative	**44-46,XX,/X, -X[3], -21[3],+mar1[2][cp5]/ 46, XX[10]**
NPM1-89	**45**	**F**	Negative	**46,XX,t(11;?)(q23;?)[5]/45,XX,t(11;?)[3][cp3]/46,XY[11]**
NPM1-90	**19**	**M**	**t(8;21)**	**45,XY,t(8;21)(q22;q22),ins(13)(?;q12.2)[3]/ 45,XY,-Y[3], t(8;21)[13],+8[5],ins(13)[11],-13[2],-14[8],-17[3],+21[2][cp14]/ 46,XX,[1]**
NPM1-91	**24**	**M**	**trisomy 3**	**NA**
NPM1-92	**23**	**F**	**inv(16),del(7q)**	**NA**
NPM1-93	**49**	**F**	Negative	**NA**
NPM1-94	**30**	**M**	Negative	**46,XY**
NPM1-96	**41**	**M**	Negative	**47-49,XY, +4[2],-9[2],+12[2], +22[6][cp6]/ 46,XY[12]**
NPM1-97	**41**	**F**	Negative	**NA**
NPM1-98	**54**	**M**	**NA**	**NA**
NPM1-99	**38**	**F**	Negative	**NA**
NPM1-101	**25**	**F**	**del (5q)**	**46,XX,i(3q)[3]/46,XX, del(5)(q33)[3]/46,XX, i(3q),del(5)(q33)[2]/45-46,XX, i(3q)[3],del(5q)[1],-18[3][cp4]46,XX[8]**
NPM1-103	**24**	**M**	Negative	**46,XY**
NPM1-104	**49**	**M**	**Trisomy 8**	**NA**
NPM1-111	**43**	**F**	Negative	**NA**

(NA- not available due to metaphase failure, [*]- As per International System for Cytogenetic Nomenclature, [$]- FISH was done for t(8;21), inv(16)/t(16;16), t(v;11q23): MLL , deletion (5q), deletion (7q), Trisomy 8, TP53 deletion, t(9;22).

Supplementary Table 2: Types and Frequency of *NPM1* mutations detected in our cohort

Chromosome	Start	Insertion	*NPM1* Allele Type	Frequency (%)
chr5	170837543	TCTG	A	69.14
chr5	170837545	TGCA	B	7.41
chr5	170837545	TGCG	C	1.23
chr5	170837544	CTGC	D	7.41
chr5	170837546	GCAG	G	2.47
chr5	170837545	TGCT	I	1.23
chr5	170837546	GCCG	J	1.23
chr5	170837546	GCCA	K	1.23
chr5	170837545	TGTT	L	1.23
chr5	170837545	TGTA	R	3.7
chr5	170837546	GTAG	Z	2.47
chr5	170837557	GCCA	Novel	1.23

Comparison between NGS-VAF and RQ-PCR for Type-A NPM1*^{mut} AML *MRD levels



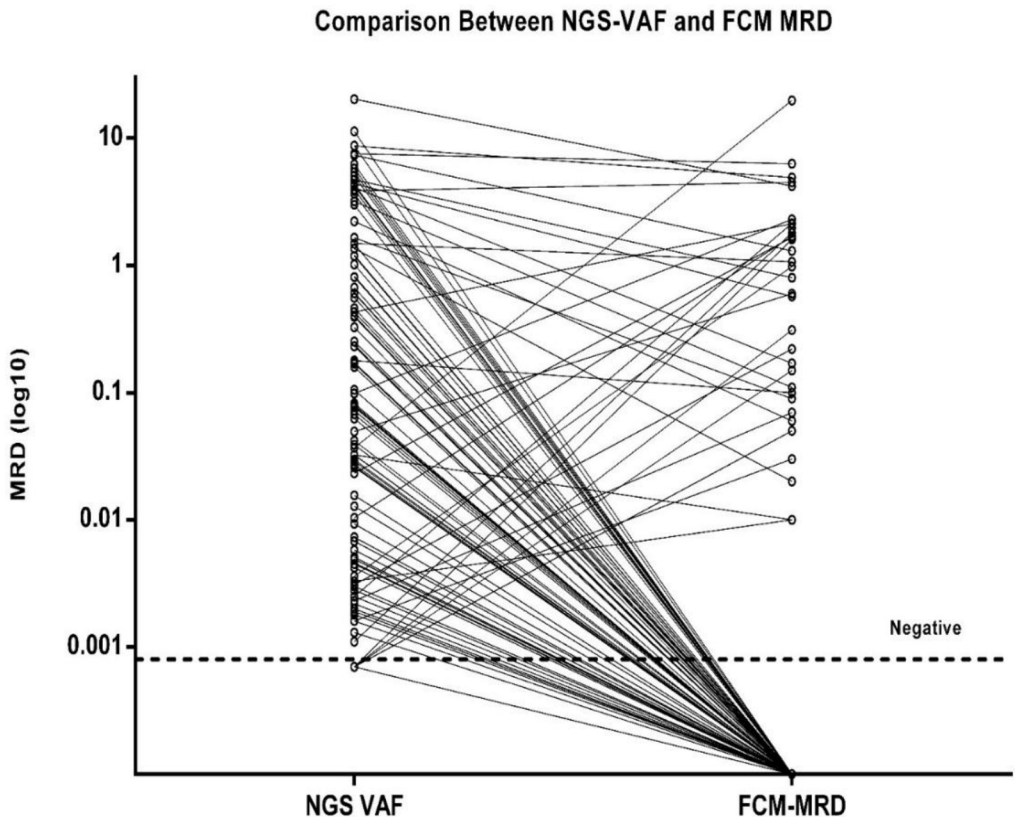
Supplementary Figure 2: (**A**) Correlation between real time PCR and NGS assays at MRD time points, (**B**) Comparison between NGS VAF and RQ-PCR MRD in *NPM1*^{mut}AML, (**C**) Difference vs. average: Bland-Altman analysis of *NPM1* NGS MRD as compared to RQ PCR. (Replacement approach was used for values below limit of detection for log calculation).

Supplementary Table 3: Types and Dynamics of *DNMT3A* mutations with respect to *NPM1*^{mut}AML at different treatment time points

Sample ID	HGVS genomic change	HGVS protein change	HGVS Coding DNA Change	Exon	DNMT3A VAF at Baseline	Corres ponding NPM1 VAF at Baseline	DNMT3A VAF at PI	Corres ponding NPM1 VAF at PI	DNMT3A VAF at PC	Corres ponding NPM1 VAF at PC
NPM1-3	2:g.25463266G>A	ENSP00000321117p.Pro743Ser	ENST00000321117.5c.2227C>T	exon19	42.34%	49.26%	Negative	Negative	NA	NA
NPM1-6	2:g.25457243G>A	ENSP00000321117p.Arg882Cys	ENST00000321117.5c.2644C>T	exon23	47.89%	42.55%	13.18%	1.3577%	NA	NA
NPM1-13	2:g.25463266G>A	ENSP00000321117p.Pro743Ser	ENST00000321117.5c.2227C>T	exon19	42.41%	38.94%	22.23%	0.5586%	NA	NA
NPM1-18	2:g.25457242C>T	ENSP00000321117p.Arg882His	ENST00000321117.5c.2644C>T	exon23	46.18%	41.45%	Negative	1.2116%	Negative	Negative
NPM1-32	2:g.25469170T>G	ENSP00000321117p.Asn430His	ENST00000321117.5c.1288A>C	exon11	4.82%	46.14%	Negative	0.6685%	NA	NA
NPM1-45	2:g.25457243G>A	ENSP00000321117p.Arg882Cys	ENST00000321117.5c.2644C>T	exon23	50.99%	24.84%	Negative	0.1693%	Negative	Negative
NPM1-46	2:g.25464460C>A	ENSP00000321117p.Gly685Trp	ENST00000321117.5c.2053G>T	exon17	1.87%	36.91%	4.35%	0.232%	5.08%	0.0037%
NPM1-47	2:g.25457242C>T	ENSP00000321117p.Arg882His	ENST00000321117.5c.2644C>T	exon23	47.11%	31.53%	Negative	0.0031%	Negative	0.1863%
NPM1-50	2:g.25469584_ 25469584insT	ENSP00000321117p.Thr395fs	ENST00000321117.5c.1184dupA	exon10	34.48%	35.83%	Negative	0.0798%	Negative	0.0152%
NPM1-54	2:g.25470577delT	ENSP00000321117p.Lys299fs	ENST00000321117.5c.897delA	exon8	47.50%	17.85%	Negative	0.0771%	NA	NA
NPM1-62	2:g.25463308G>A	ENSP00000321117p.Arg729Trp	ENST00000321117.5c.2185C>T	exon19	23.49%	23.52%	25.36%	0.0791%	NA	NA
NPM1-78	2:g.25457243G>A	ENSP00000321117p.Arg882Cys	ENST00000321117.5c.2644C>T	exon23	43.48%	28.94%	38.40%	0.0326%	46.49%	0.0025%
NPM1-82	2:g.25457242C>T	ENSP00000321117p.Arg882His	ENST00000321117.5c.2644C>T	exon23	43.40%	39.97%	Negative	0.1728%	Negative	Negative
NPM1-84	2:g.25469170T>G	ENSP00000321117p.Asn430His	ENST00000321117.5c.1288A>C	exon11	3.35%	25.85%	Negative	0.0724%	Negative	Negative
NPM1-97	2:g.25457242C>T	ENSP00000321117p.Arg882His	ENST00000321117.5c.2644C>T	exon23	47.70%	42.35%	6.39%	4.2246%	6.15%	3.9617%
NPM1-98	2:g.25457149_ 25457149insAC	ENSP00000321117p. X913delinsC	ENST00000321117.5c.2736_2 737dupGT	exon23	8.84%	13.51%	3.81%	0.3266%	NA	NA

(HGVS- Human Genome Variation Society, PI- Post Induction, PC- Post First consolidation, VAF- Variant Allelic Frequency, NA- Not available).

Comparison between NGS VAF and FCM-MRD



Supplementary Figure 3: Comparison of NGS variant allele frequencies with corresponding FCM-MRD value. No significant correlation was detected between the two methods.

Difference in Overall Survival and Relapse Free Survival between MRD positive and negative groups

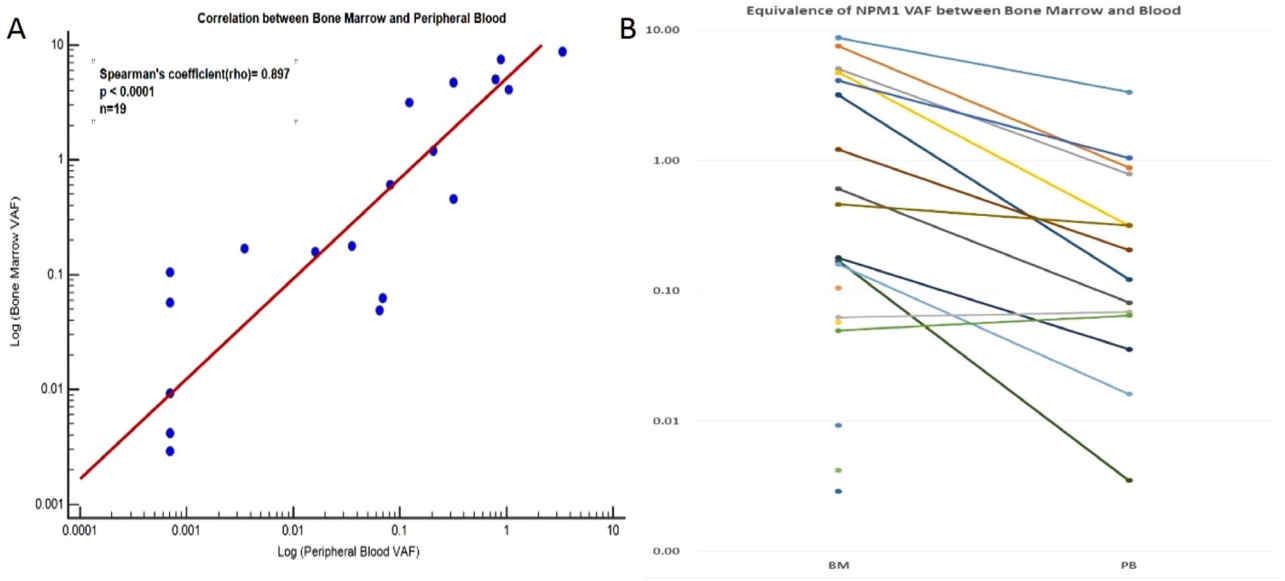
Supplementary Table 4: Survival statistics of patients stratified as per MRD results

	Overall Survival (OS)		Relapse Free Survival (RFS)	
PI FCM-MRD				
MRD Negative	Mean OS: 43.9 months; 95% CI (37.0–50.9 months), Median OS: not reached	***p = 0.006***	Mean RFS: 35.7 months; 95% CI (28.1–43.4 months), Median RFS: 43.0 months; 95% CI (17.7–43.03 months),	***p = 0.0097***
MRD Positive	Mean OS: 26.8 months; 95% CI (16.4–37.2 months) Median OS: 14.9 months; 95% CI (9.0 to 20.4 months)		Mean RFS: 23.1 months; 95% CI (13.2–33.0 months); Median RFS: 9.7 months; 95% CI (7.2 to 15.1 months)	
NGS-MRD				
MRD Negative	Mean OS: 42.2 months; 95% CI (33.1–51.2 months), Median OS: not reached	***p = 0.0009***	Mean RFS: 38.5 months; 95% CI (29.4–47.5 months), Median RFS: 43.0 months; 95% CI (16.8–43.0 months),	***p < 0.0001***
MRD Positive	Mean OS: 19.4 months; 95% CI (8.1–30.8 months) Median OS: 12.1 months; 95% CI (8.8 to 20.4 months)		Mean RFS: 11.3 months; 95% CI (3.9–18.7 months); Median RFS: 7.2 months; 95% CI (5.1 to 9.3 months)	

CI, confidence interval.

Correlation of mutant NPM1 variant allelic frequency detected in bone marrow and blood

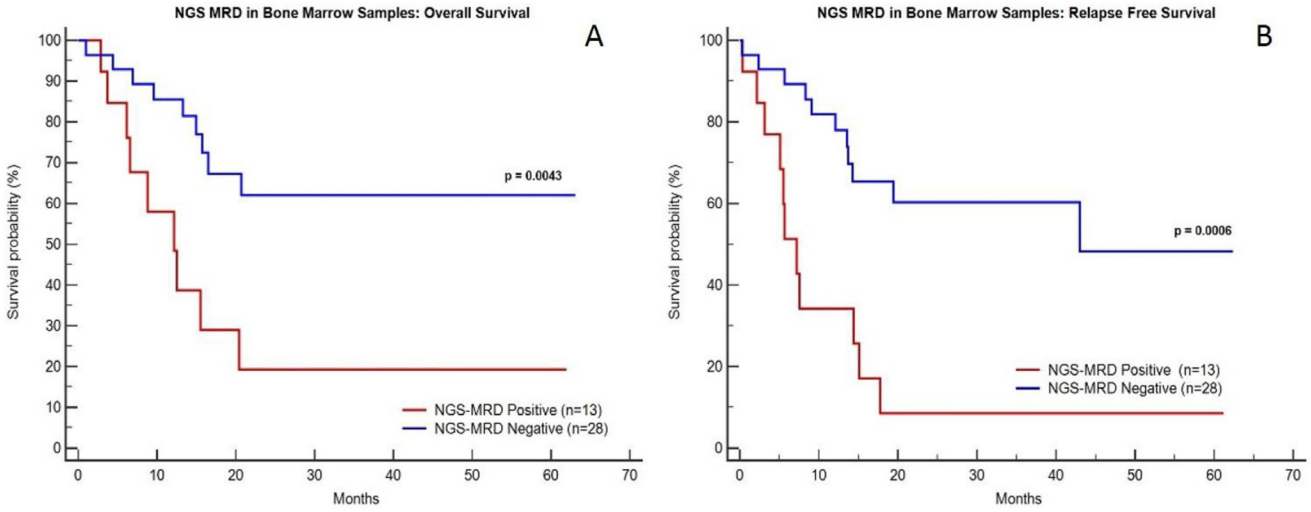
There were 19 patients in which bone marrow (BM) and peripheral blood (PB) samples were available at same time point. There was a reasonable correlation between mutant *NPM1* VAF in BM and PB. (Spearman's r = 0.897, $P < 0.0001$). (Supplementary Figure 4A) Based on these results we also calculated that there was a mean 0.7 Log (median 0.41Log) difference between *NPM1* MRD measurements in the BM and blood. (Supplementary Figure 4B).



Supplementary Figure 4: Matched samples of BM and PB (**A**) The plot showing correlation between the peripheral blood and bone marrow using Pearson's index. (**B**) Mean difference of 0.7 log was observed between matched samples.

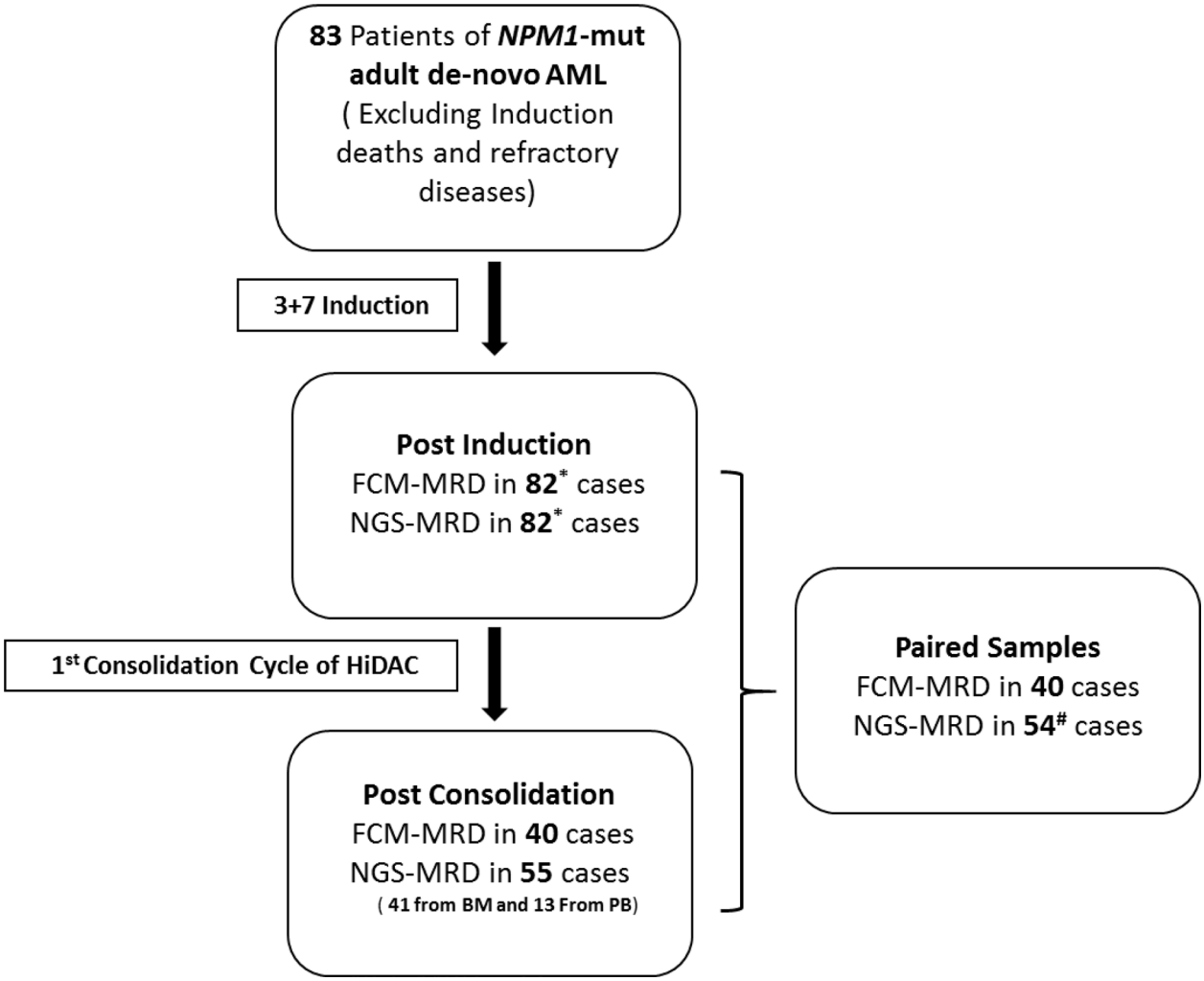
Prognostic impact of NGS MRD using only Bone Marrow samples

We excluded paired cases in which NGS was done on peripheral blood samples at post consolidation time point as bone marrow sample was not available to determine if there was a bias in the results due to a combination of BM and PB. As can be seen in Supplementary Figure 5 NGS MRD was still significantly predictive of outcome.



Supplementary Figure 5: NGS MRD in only bone marrow samples. MRD positivity is shown to be significantly predictive of inferior OS (**A**) and RFS (**B**) even when only BM samples were considered.

Enrolment summary



Supplementary Figure 6: Characteristics of the patient cohort. (*Out of 83 cases at Post Induction, in one case FCM data was not available and, in another case, DNA was not available for NGS MRD. #Of 55 cases sequenced at Post Consolidation, in one case Post Induction Sample was not available for NGS MRD, hence there were 54 Paired NGS -MRD cases).

Details of primers used for next generation sequencing

Supplementary Table 5: Adapter tagged locus specific and sequencing primers

NPM1 Primers	Sequences
Forward Primer*	5′AATGATACGGCGACCACCGAGATCTACAC**XXXXXXXXXX**TATGGTGCCTGTAAACACGGTAGGGAAAGTTCTC-3′
Reverse primer*	5′CAAGCAGAAGACGGCATACGAGAT**XXXXXXXXXX**AGTCAGTCAGTCTGTCTATGAAGTGTTGTGGTTCC-3′
Read 1 Primer	5′-TATGGTGCCTGTAAACACGGTAGGGAAAGTTCTC-3′
Read 2 primer	5′AGTCAGTCAGTCTGTCTATGAAGTGTTGTGGTTCC-3′
Index Primer	5′-GGAACCACAACACTTCATAGACAGACTGACTGACT-3′

*XXXXXXXXXX represents 10 bp sample specific index sequence.

DETECTION OF MRD USING MULTICOLOUR FLOW CYTOMETRY

Panel for Flow Cytometry MRD (FCM-MRD) detection in AML

Supplementary Table 6: Panel of antibodies and their clones used in three tubes 8 colour MRD assay

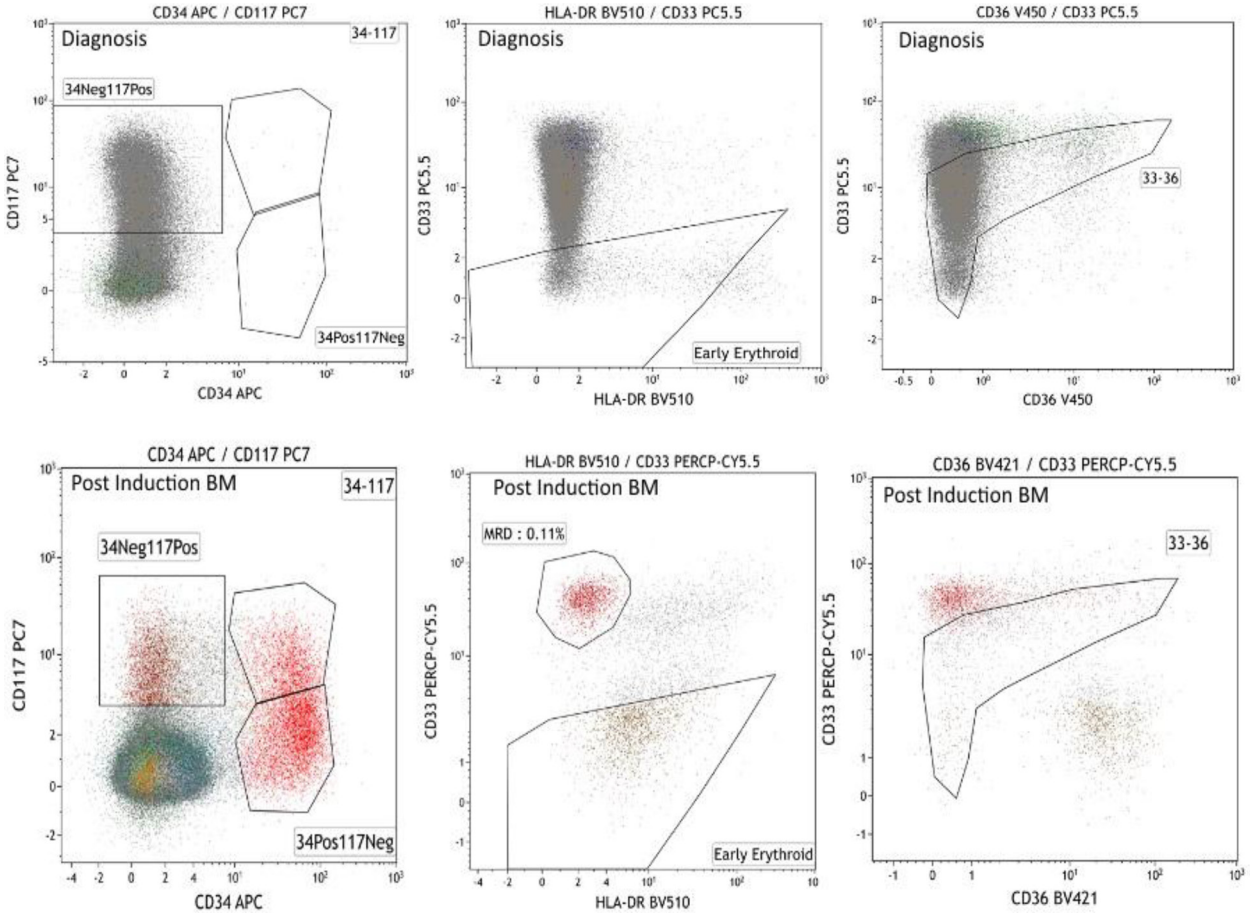
	FITC	PE	PerCP Cy5.5	PE Cy7	APC	APC H7	BV450/ BV421	BV510
TUBE1	CD38	CD123	CD33	CD117	CD34	CD45	CD36	HLA-DR
Clone	T16	9F5	P67.6	104D2D1	581	J.33	CB38	L243
TUBE2	CD56	CD13	CD33	CD19	CD34	CD45	CD7	HLA-DR
Clone	NCAM16.2	SJ1D1	P67.6	SJ25C1	581	J.33	M-T701	L243
TUBE3	CD16	CD11b	CD15	CD13	CD34	HLA-DR	CD117 (BV421)	CD45
Clone	DJ130C	ICRF44	HI98	Immu103.44	581	G46-6	YB5.B8	HI30

Supplementary Table 7: Panel of antibodies and their clones used in two tubes 10 colour MRD assay

	FITC	PE	ECD	PerCP Cy 5.5 /PC5.5	PECy7/ PC7	APC	APC AF700	APC AF750	BV421	BV510
TUBE1	CD14	CD123	CD64	CD33	CD117	CD34	CD45	CD38	CD36	HLA-DR
Clone	RM052	9F5	CLONE 22	P67.6	104D2D1	581	J.33	LS198.4.3	CB38	L243
TUBE2	CD15	CD13	CD19	CD34	CD56	CD7	CD45	CD11b	CD117	HLA-DR
Clone	80H5	SJ1D1	J3-119	581	N901 (HLDA6)	8H8.1	J.33	BEAR 1	YB5.B8	L243

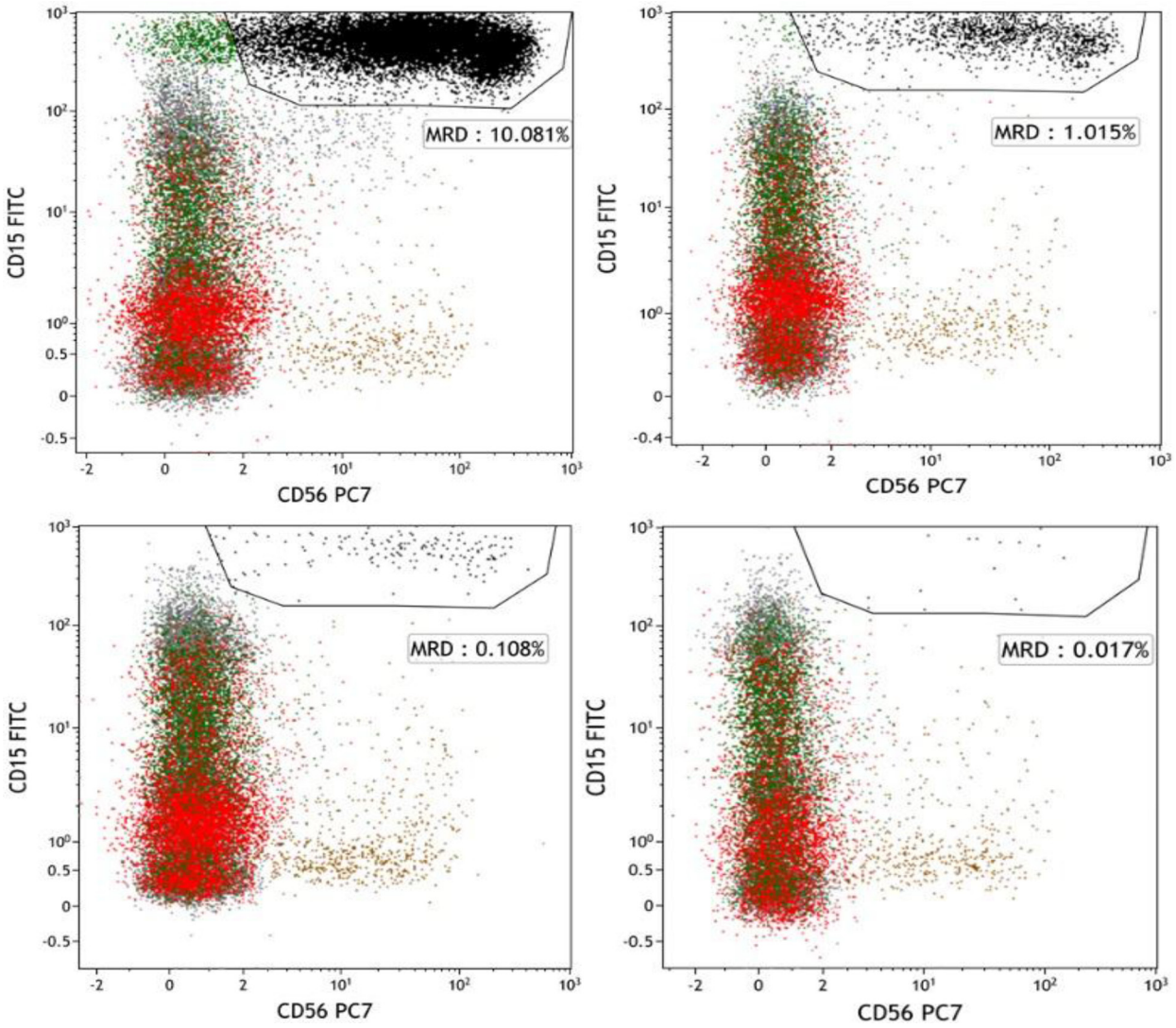
Analysis of FCM-MRD Data

Familiarity with normal myeloid maturation and standardization of normal templates was achieved on stressed regenerative bone marrows (for e.g. ALL post induction that were MRD negative) using the antibody panels as seen above. A representative case of *NPM1*-mut AML which is positive at the post induction time point is shown in Supplementary Figure 7.



Supplementary Figure 7: An example of FCM-MRD detection in a typical *NPM1*-mut AML. The panel on top shows that the blasts (grey) are CD34 negative, heterogeneous for CD117 and negative for HLA-DR and CD36. The panel below shows that these blasts are present (brown) at a frequency of 0.11% (of all nucleated cells) in the post induction BM. Normal myeloid progenitors are coloured red.

Limit of dilution studies for FCM-MRD

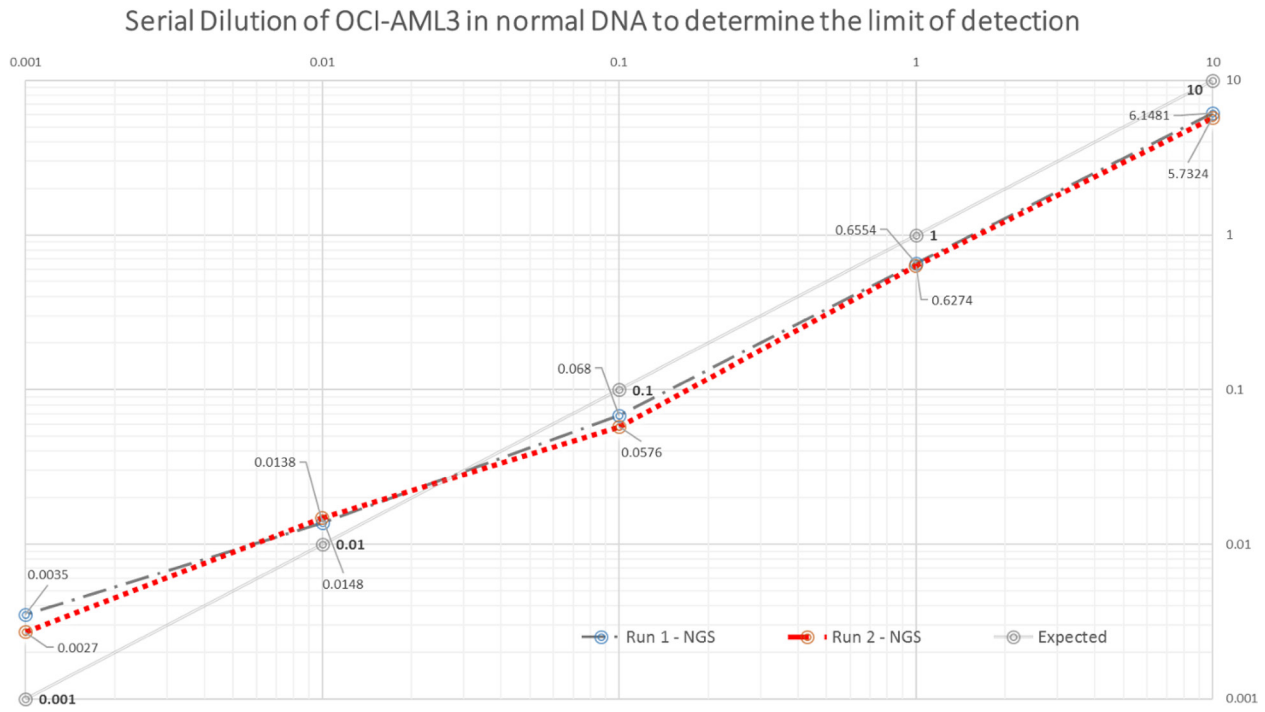


Supplementary Figure 8: Limit of detection testing for the AML-MRD assay. An OCI-AML3 cell line was serially diluted in a normal bone marrow. The analysis shows that these cells can be detected based on an abnormal immunophenotype (CD56 and CD16 positive, in black) at a frequency of 0.01%.

NGS MRD ASSAY VALIDATION USING ULTRA-DEEP SEQUENCING

Limit of detection testing

For establishment of limit of detection, we serially diluted an OCI-AML3 cell line (known to harbour a type-A *NPM1* mutation) in normal bone marrow ranging from 10% to 0.001%. As the Supplementary Figure 9 demonstrates we could successfully detect *NPM1* mutation at 1:100,000 dilution using NGS. Thus, for NGS 0.001% was the limit of detection of the assay (LOD) as already established. [2] Based on the results of these two experiments, we obtained inter run CV as can be seen in Supplementary Table 8.



Supplementary Figure 9: Results of serially diluted OCI-AML3 in normal BM sample. Observed variant allele frequencies (in percentages) of two dilution experiments are plotted against expected frequencies.

Inter Run Coefficient of Variation at different dilutions

Supplementary Table 8: Inter run coefficient of variation at different dilutions

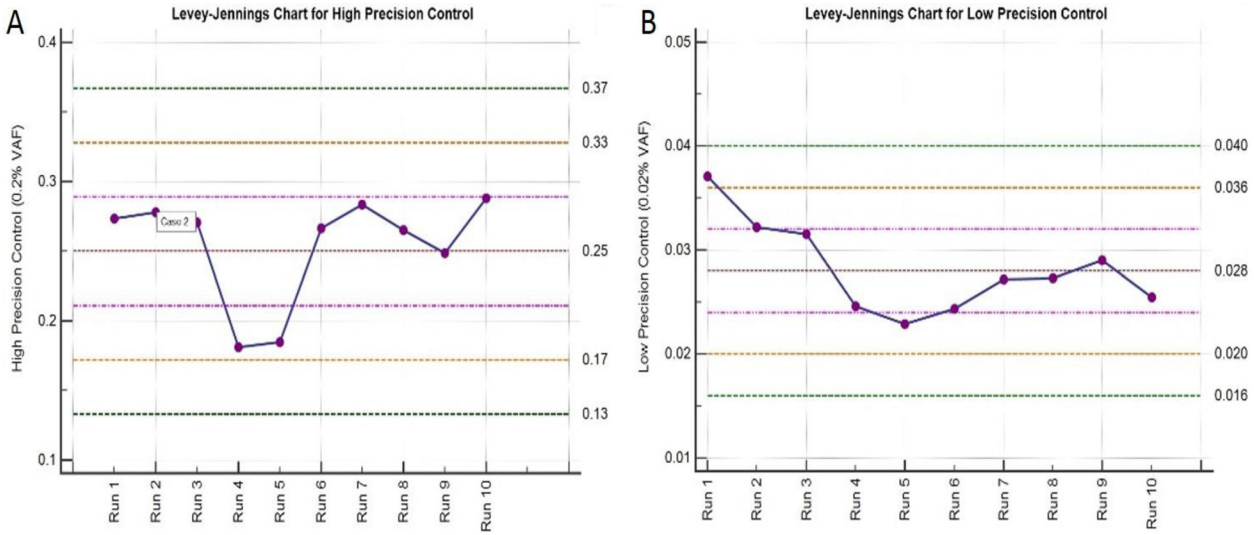
Percentage of *NPM1* mutant cells	Inter Run CV
10%	0.05
1%	0.03
0.1%	0.12
0.01%	0.05
0.001%	0.18

Determination of Limit of Blank

To determine the specificity of the assay we performed a limit of blank (LOB) study. For LOB, DNA was extracted from 30 normal peripheral blood samples and were subjected to ultradeep NGS to check that false calls of *NPM1* mutations were not made. LOB analysis did not show any four (or more) bp insertions. The average sequencing coverage for this experiment was 410,342X (average for 30 samples). This proves the specificity of the assay for determination of MRD in *NPM1*-mut AML.

CV and Standard Deviation of the assay

With each run, two *NPM1* mutation positive precision controls (OCI-AML3 in NA12878 DNA) were assayed (0.2% and 0.02%). Data were plotted in control chart for high precision (0.2%) and low precision (0.02%) controls as seen in Supplementary Figure 10. The CV for these low precision controls were 15.36% and 15.49% respectively.



Supplementary Figure 10: Levey-Jennings plots of precision controls. (**A**) High Precision Control (0.2% VAF) and (**B**) Low Precision Control (0.02% VAF).

Coverage Metrics and QC

The average coverage for MRD samples (PI and PC, n = 137) was 565,808x and median coverage was 551,227x. It was made sure that negative calls were made only if the sequencing coverage was more than 2,00,000x. We also added an NA12878 control (Coriell Bio Repository, Camden, NJ, USA) with every run to ensure that false positives variant calls were not made.

Calculation for Log Reduction between Post Induction and Post Consolidation Variant allelic frequency

While calculating log values of *NPM1* mutant allelic frequencies, replacement approach was used for values below limit of detection (i.e. less than 0.001%). As per Croghan *et.al*. [3] LOD/√2 is the best choice of replacement for left censored data. Hence in our cohort while calculating log difference between 'post induction' and 'post consolidation' time points, the cases in which original VAF values were less than 0.001% were substituted by a constant value of LOD/√2 i.e. (0.0007).

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