Minimal residual disease monitoring in acute myeloid leukemia with non-A/B/D *NPM1* mutations by digital polymerase chain reaction: feasibility and clinical use

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Supplemental appendix

Minimal residual disease monitoring in acute myeloid leukemia with non-A/B/D-NPM1 mutations by digital PCR: feasibility and clinical use

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Supplemental method: NPM1-mutated transcript quantification by droplet dPCR

Optimum hybridization temperature determination

For each studied mutation, patients' samples collected at diagnosis were used to test the protocol in a temperature gradient. Hybridization temperature decreases from 60 to 52°C from line A to line H of the 96 wells plate. Optimum temperature should allow the biggest difference of fluorescent amplitude between clusters of positive droplets and clusters of negative droplets and may not induce nonspecific amplifications. Results for the *NPM1*-type I mutation are shown in the **Supplemental Figure 2**. In this example the best discrimination between positive and negative signals was obtained in wells heated with a hybridization temperature of 55.1 and 53.5°C. The final temperature selected was 55°C for the 16 rare mutations. For *NPM1*-type A mutation, the optimum hybridization temperature was 60°C.

Fluorescence thresholds

Thresholds were fixed using fluorescence distribution curves. For each variant and for each probe we chose the amplitude for which positive and negative droplets frequency was the lowest. Fluorescence thresholds for *NPM1* are determined from positive samples results and the threshold for *ABL* was determined from negative control results. Results for the *NPM1*-type I mutation are shown in the **Supplemental Figure 3**.

Limit of blank

To define the limit of blank (LoB), i.e. the number of « false positive » droplets for *NPM1*, we analyzed each variant by droplet dPCR with previously described parameters on a negative control. The limit of blank was determined by the quantification of the negative control for each variant 8 times (**Supplemental Figure 4**).

Linearity limit

The linearity limit was determined with the quantification of 7 positive controls obtained by serial dilutions of a *NPM1*-type A commercial plasmid with known copy number (10^5 copies/µL) (Qiagen®). Each *NPM1*-type A plasmid dilution was mixed with an *ABL* plasmid dilution (Qiagen®) to have for each 32 000 copies of *ABL* by test sample. The following dilutions were obtained: 312%, 156%, 100%, 10%, 1%, 0.1% and 0.01% of *NPM1*-type A transcript. The dynamic range went from 10^5 to 3.2 copies of *NPM1*-type A. The straight line obtained by comparing the observed results against the expected results provided the following equation $y = 0.968x + 0.110$ which is approximately $y = x$. Considering the low quantity of available material for rare *NPM1* mutations, the linearity limit was determined for type A mutation only (**Supplemental Figure 5**) and results were extrapolated to other variants.

Limit of detection

The limit of detection (LoD) was determined by measuring a sample containing 5 copies of mutated-*NPM1*-transcript 20 times in order to overcome the sampling bias (i.e. a ratio of 0.015% for a sample containing 32 000 copies of *ABL*). This 0.015% control was performed from *NPM1*-type A, type B and type D commercial plasmids (Qiagen®). At least one copy was detected in 19 wells of NPM1 type A and in the 20 wells of NPM1 type B and NPM1 type D (**Supplemental Figure 6**). For *NPM1*-type A, B and D, the LoD was equal to 5 copies, thus for a sample containing 50 000 copies of *ABL*: LoD = 0.01%.

Detection of contaminations

A complete cDNA-free reaction mixture (No Template Control, NTC) showed the absence of contaminations of the reagents, the material, and the environment by mutated or wild type cDNA copies.

Accuracy

Accuracy reflects random errors distribution. It is assessed by repeatability and intermediate precision. Repeatability was determined testing 3 samples with different ratios (100%, 1% and 0.1%) of *NPM1*-type A mutation 10 times in a one-time series (**Supplemental Table 4A**). Intermediate precision was determined from 3 samples with different ratios (100%, 1% and 0.1%) of *NPM1*-type A mutation tested in duplicate wells in 7 different series (**Supplemental Table 4B**). The 3 positive samples were obtained from 3 dilutions of a *NPM1*-type A commercial plasmid (Qiagen®) mixed with an *ABL* plasmid dilution (Qiagen®) to have for each *NPM1*-A dilution 32 000 copies of *ABL* by test sample.

Exactitude

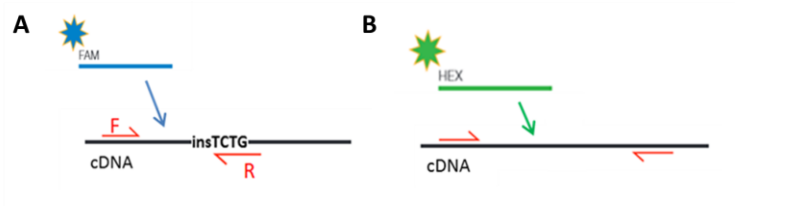
Exactitude was assessed using 3 quality controls from external quality assessments with different levels of *NPM1*-type A expression provided by the GBMHM (Groupe des Biologistes Moléculaires des Hémopathies Malignes)(**Supplemental Table 5**).

Method comparison

Quantification of *NPM1*-type A transcript levels was performed from 28 patients' samples both using RT-qPCR and a droplet dPCR (**Supplemental Figure 7**). Both methods were performed using nucleic acids from the same DNA extraction and the same reverse transcription, in a limited time lapse. Patients for whom the two methods gave an indetectable result were not considered for the comparison. The correlation between the two techniques was assessed using the Least Squares regression and the Bland Altman plots which assesses the conversion factor and the systematic bias.

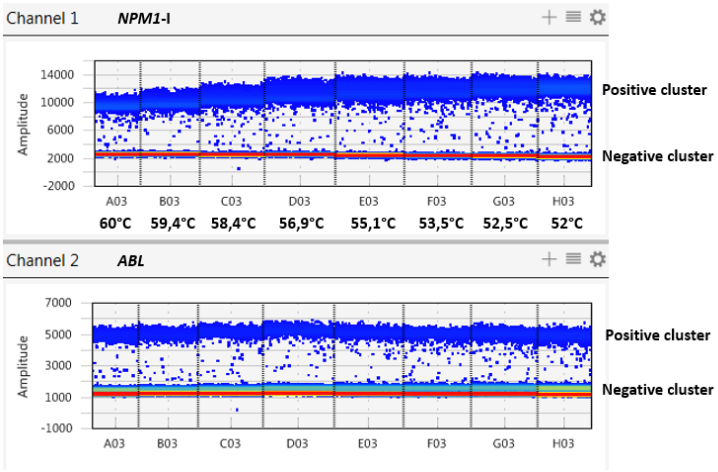
Supplementary Figure 1: Schematic representation of NPM1 and ABL systems.

NPM1-mutated and reference *ABL* transcripts quantifications were performed in multiplex. Two probes were used; one specific to *NPM1* transcript, the other specific to *ABL* and amplification was detected by a TaqMan system. **A**. *NPM1* system: with a reverse primer (R) specific to *NPM1*-mutated transcript (red arrow), a forward primer (F) common to all *NPM1* variants (red arrow) and a generic *NPM1* probe tagged with FAM (blue sequence). **B**. *ABL* system: with primers (red arrows) and a probe (EAC system) tagged with HEX (green sequence).

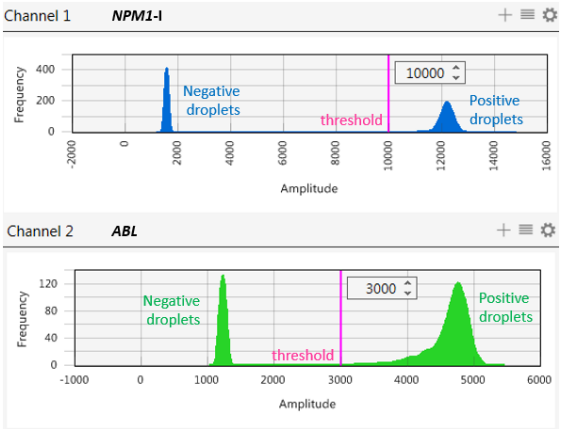


Supplemental Figure 2: 1D representation of temperature gradient experiment for *NPM1*-type I mutation.

A blue dot represents a droplet and each column a reaction well in two different channels (*NPM1* and *ABL* are quantified simultaneously). In this example the best discrimination between positive and negative signals is obtained in wells E and F.

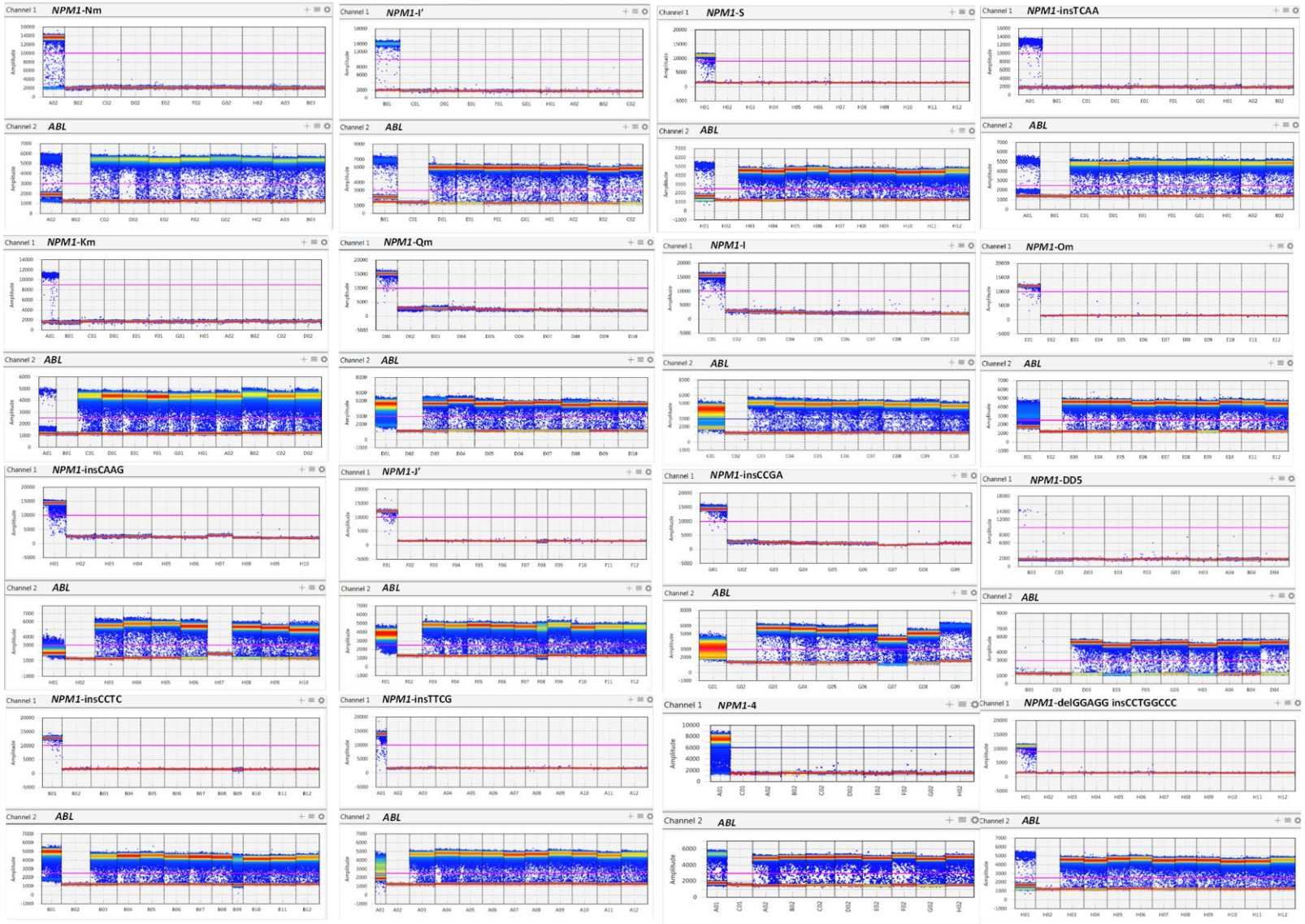


Supplemental Figure 3: Fluorescence thresholds setting for *NPM1* mutation and *ABL*.

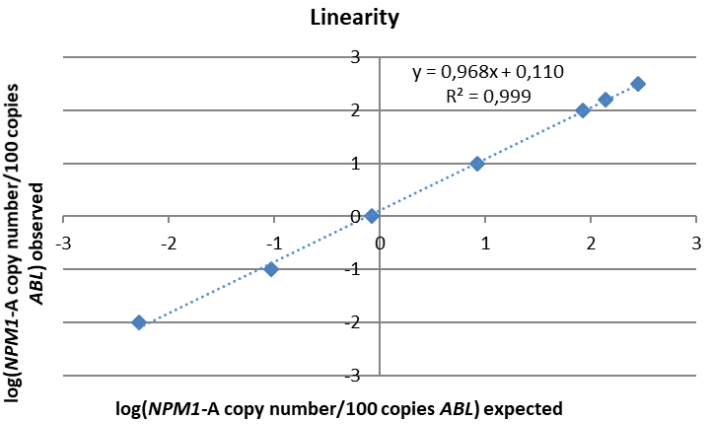


Supplemental Figure 4: Limit of blank (LoB) determination for *NPM1* mutation.

To define the LoB we analyzed each variant by ddPCR, on a negative control (8 wells) with a positive sample (first well) and H_2O (second well) as controls.

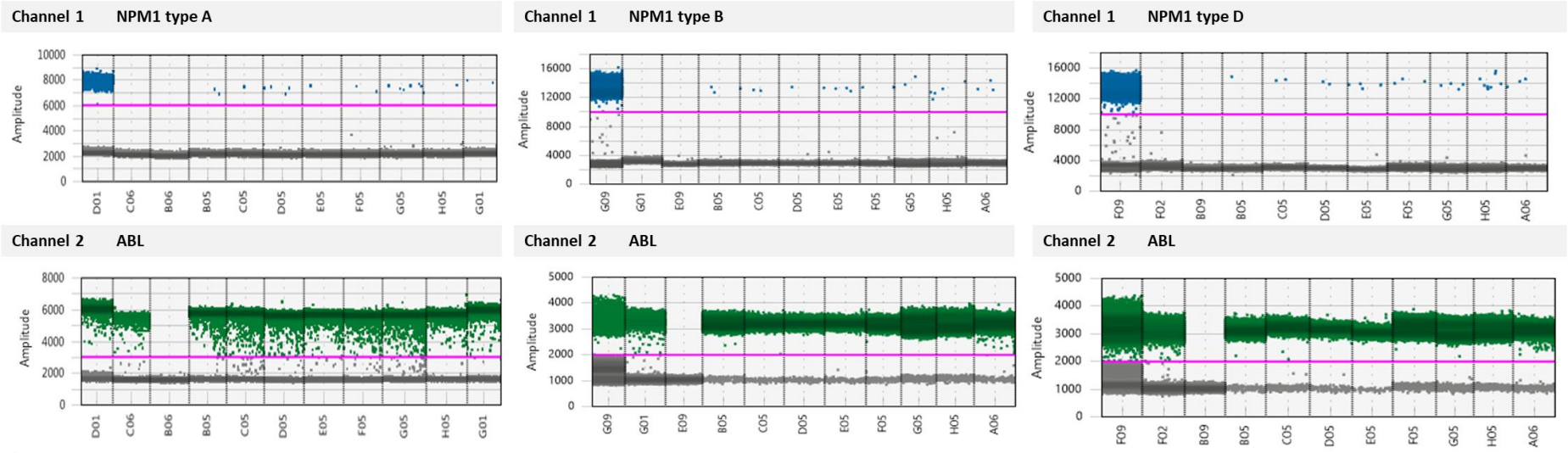


Supplemental Figure 5: Dilution range measurement of NPM1-type A mutation after logarithmic transformation.



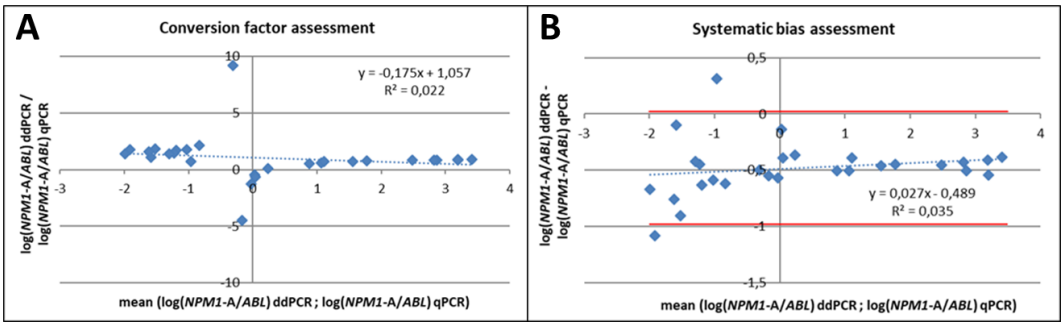
Supplemental Figure 6: Limit of detection (LoD) determination for *NPM1*-type A mutation.

This assay was performed with a positive sample (first well), a negative sample (second well) and H2O (third well) as controls. At least one copy was detected in 19 wells of NPM1 type A and in the 20 wells of NPM1 type B and NPM1 type D (only 8 wells are shown here for each transcript).Each reaction was performed in duplicate.



Supplemental Figure 7: Quantification of *NPM1*-type A mutation transcript levels in samples from 28 AML patients using both RT-qPCR by TaqMan chemistry assay and a droplet dPCR assay.

(A) Conversion factor assessment by the Bland Altman plots after logarithmic transformation. (B) Systematic bias assessment by the Bland Altman plots after logarithmic transformation.



Supplemental Table 1: Reaction mixture composition.

ddPCR™ Supermix for Probes (No dUTP) is a mix including dNTP and $MgCl_2$. The total reaction volume is 24 µL. For type A and type 4 mutations, forward and reverse *NPM1* primers volumes were divided by 4.

Amplification reaction mixture	
Reagents	**Volume (µL)**
ddPCR Supermix for Probes (No dUTP) (2X)	12
H_2O	5,016 / 0,016
ABL forward primer ENF1003 (100 pmol/µL)	0,216
ABL reverse primer ENR1063 (100 pmol/µL)	0,216
NPM1 forward primer (100 pmol/µL)	0,216
NPM1 reverse primer (100 pmol/µL)	0,216
ABL probe ENP1043_ddPCR (100 pmol/µL)	0,06
NPM1 probe (100 pmol/µL)	0,06
cDNA diagnosis (2 ng/µL) / follw-up (20 ng/µL)	6 / 11
Mix volume	18 / 13
Total reactionnal volume	24

Supplemental Table 2: Quantification of *NPM1*-type A mutation transcript levels performed by RT-qPCR and droplet dPCR

	***NPM1* copy number / *ABL* copy number**					
	ddPCR		**qPCR**		**log(ddPCR)/log(qPCR)**	**log(ddPCR)-log(qPCR)**
	ratio*100 (%)	**log(ratio*100)**	**ratio*100 (%)**	**log(ratio*100)**		
Patient 1	0,91	-0,04	1,24	0,09	-0,46	-0,14
Patient 2	1640	3,21	3986	3,60	0,89	-0,39
Patient 3	0,03	-1,51	0,13	-0,88	1,72	-0,63
Patient 4	0,02	-1,63	0,03	-1,54	1,06	-0,10
Patient 5	0,04	-1,45	0,10	-1,01	1,44	-0,45
Patient 6	0,16	-0,81	0,08	-1,12	0,72	0,32
Patient 7	0,07	-1,15	0,30	-0,53	2,17	-0,62
Patient 8	0,03	-1,51	0,08	-1,09	1,39	-0,42
Patient 9	0,05	-1,32	0,19	-0,73	1,80	-0,59
Patient 10	0,28	-0,56	0,87	-0,06	9,22	-0,50
Patient 11	972	2,99	2507	3,40	0,88	-0,41
Patient 12	838	2,92	2937	3,47	0,84	-0,54
Patient 13	408	2,61	1300	3,11	0,84	-0,50
Patient 14	404	2,61	1087	3,04	0,86	-0,43
Patient 15	35,4	1,55	99,5	2,00	0,78	-0,45
Patient 16	6,42	0,81	20,7	1,32	0,61	-0,51
Patient 17	0,01	-1,97	0,09	-1,07	1,85	-0,91
Patient 18	0,00	-2,46	0,04	-1,38	1,79	-1,08
Patient 19	0,36	-0,45	1,26	0,10	-4,46	-0,55
Patient 20	0,49	-0,31	1,79	0,25	-1,24	-0,57
Patient 21	0,01	-2,00	0,06	-1,24	1,61	-0,76
Patient 22	4,27	0,63	13,6	1,13	0,56	-0,50
Patient 23	180	2,26	515	2,71	0,83	-0,46
Patient 24	21,1	1,32	60,8	1,78	0,74	-0,46
Patient 25	1,13	0,05	2,63	0,42	0,13	-0,37
Patient 26	8,10	0,91	20,0	1,30	0,70	-0,39
Patient 27	0,71	-0,15	1,73	0,24	-0,63	-0,39
Patient 28	0,005	-2,32	0,02	-1,65	1,40	-0,67

Supplemental Table 3: Sequences of rare *NPM1* mutations studied.

We studied 16 rare mutations of the *NPM1* gene. Sequences are listed in accordance with the HGVS nomenclature (from the reference sequence NM_002520).

Variants of *NPM1* mutation	HGVS nomenclature
Mutation 4	NPM1 exon 11 c.863_864insCTTG : p.W288Cfs*12
Mutation Km	NPM1 exon 11 c.863_864insCCGG : p.W288Cfs*12
Mutation Nm	NPM1 exon 11 c.863_864insCCAG : p.W288Cfs*12
Mutation Om	NPM1 exon 11 c.863_864insTTTG : p.W288Cfs*12
Mutation Qm	NPM1 exon 11 c.863_864insTCGG : p.W288Cfs*12
Mutation DD5	NPM1 exon 11 c.863_864insTCAG : p.W288Cfs*12
Mutation I	NPM1 exon 11 c.863_864insCAGA : p.W288Cfs*12
Mutation S	NPM1 exon 11 c.863_864insCAAA : p.W288Cfs*12
Mutation J'	NPM1 exon 11 c.863_864insTATG : p.W288Cfs*12
Mutation I'	NPM1 exon 11 c.863_864insTAAG : p.W288Cfs*12
4 bp insertion: CAAG	NPM1 exon 11 c.863_864insCAAG : p.W288Cfs*12
4 bp insertion: CCTC	NPM1 exon 11 c.863_864insCCTC : p.W288Cfs*12
4 bp insertion: TTCG	NPM1 exon 11 c.863_864insTTCG : p.W288Cfs*12
4 bp insertion: CCGA	NPM1 exon 11 c.863_864insCCGA : p.W288Cfs*12
4 bp insertion: TCAA	NPM1 exon 11 c.865_866insTCAA : p.Q289Lfs*11
delGGAGG insCCTTGGCCC	NPM1 exon 11 c.869_873delinsCCTTGGCCC : p.W290Sfs*10

Supplemental Table 4: Accuracy assay results

(A) Repeatability (B) Intermediate precision. *NPM1*-mutated transcript levels were reported as the normalized values of *NPM1*m copy number/*ABL* copy number.

A-Repeatability

	***NPM1*-A copy number / *ABL* copy number**					
	Mean	**Confidence interval 95%**		**Standard deviation**	**Mean (log)**	**Standard deviation (log)**
		Min	**Max**			
Level 1	85%	78%	92%	4,1%	-0,072	0,018
Level 2	0,82%	0,72%	0,95%	6,7%	-2,09	0,030
Level 3	0,10%	0,05%	0,19%	34%	-3,03	0,14

B-Intermediate precision

	***NPM1*-A copy number / *ABL* copy number**					
	Mean	**Confidence interval 95%**		**Standard deviation**	**Mean (log)**	**Standard deviation (log)**
		Min	**Max**			
Level 1	91%	89%	92%	0,76%	-0,043	0,004
Level 2	0,80%	0,73%	0,89%	4,7%	-2,10	0,022
Level 3	0,09%	0,052%	0,17%	29%	-3,04	0,13

Supplemental Table 5: Exactitude assay results.

NPM1-mutated transcript levels were reported as the normalized values of *NPM1*m copy number/*ABL* copy number. Uncertainty calculation was made as follow:

Component from intermediate precision (IP): $U_1 = SD_{IP}$ (log) with SD: standard deviation

Component from bias: U_2 = bias / √3 with bias = log(expected value) - log(observed value)

Combined uncertainty: $Uc = \sqrt{(U_1^2+U_2^2)}$

	***NPM1*-A copy number / *ABL* copy number**					
	Expected value	**Observed value**	**Confidence interval 95%**		**Bias (log)**	**Combined uncertainty (log)**
			Min	**Max**		
Level 1	166%	180%	164%	198%	0,035	0,021
Level 2	18,6%	21,1%	17,6%	25,3%	0,056	0,039
Level 3	3,31%	4,27%	3,13%	5,83%	0,11	0,068