

**Comparison of Measurable Residual Disease in Pediatric B-Lymphoblastic Leukemia
Using Multiparametric Flow Cytometry and Next-Generation Sequencing**

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Supplemental Material

Validation of in-house multiparametric flow cytometric-measurable residual disease (MFC-MRD) assay

The analytical performance was assessed by evaluating accuracy, precision, linearity, limit of detection (LOD) and lower limit of quantitation (LLOQ) based on Clinical and Laboratory Standards Institute H62 guideline with modification [1]. Accuracy was validated by comparison against validated molecular results in 20 samples. The MRD results should reach $\geq 90\%$ concordance for qualitative results. For precision, low-level samples were tested at least twice and coefficient of variation (CV) should be within 35%. For linearity, leukemic samples and normal samples were mixed and created at least 3 levels and the correlation coefficient (R^2) > 0.95 . The limit of blank (LOB) and LOD were assessed using three normal samples. Calculation for LOB and LOD were based on the following formulas: $LOB = \text{mean} + 1.654SD$ (standard of deviation), $LOD = \text{mean} + 3SD$. For LLOQ, samples with low level of target population were assessed twice and the MRD events within 35% CV was selected for LLOQ.

1) Accuracy: Comparison with previously validated tests

The MFC-MRD results of 20 samples with *BCR::ABL1*, *ETV6::RUNX1* RT-PCR results were compared. Out of 20 samples, 1 discordant case was present, within the prespecified acceptance criteria ($\geq 90\%$ concordance).

ID	MFC-MRD	MFC-MRD (%)	Molecular test results
1	Negative		<i>BCR-ABL1/ABL1</i> minor % ratio = Not detected
2	Negative		<i>BCR-ABL1/ABL1</i> minor % ratio = 0.002
3	Negative		<i>BCR-ABL1/ABL1</i> minor % ratio = Not detected
4	Positive	0.0013	<i>BCR-ABL1/ABL1</i> minor% ratio = 0.1
5	Negative		<i>BCR-ABL1/ABL1</i> minor % ratio = Not detected
6	Negative		<i>BCR-ABL1/ABL1</i> minor % ratio = Not detected
7	Negative		<i>BCR-ABL1/ABL1</i> minor % ratio = Not detected

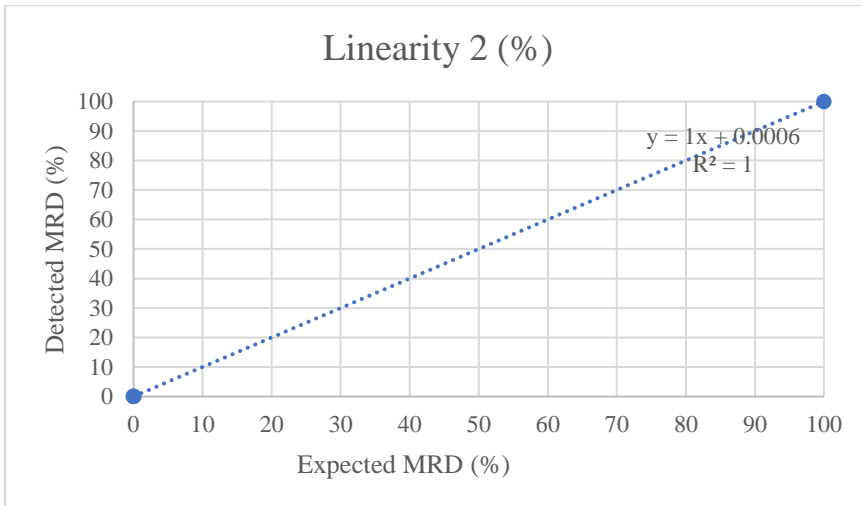
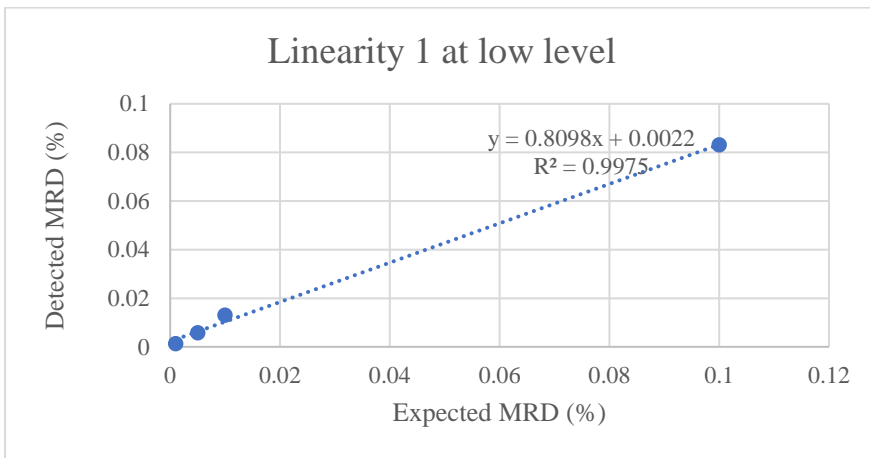
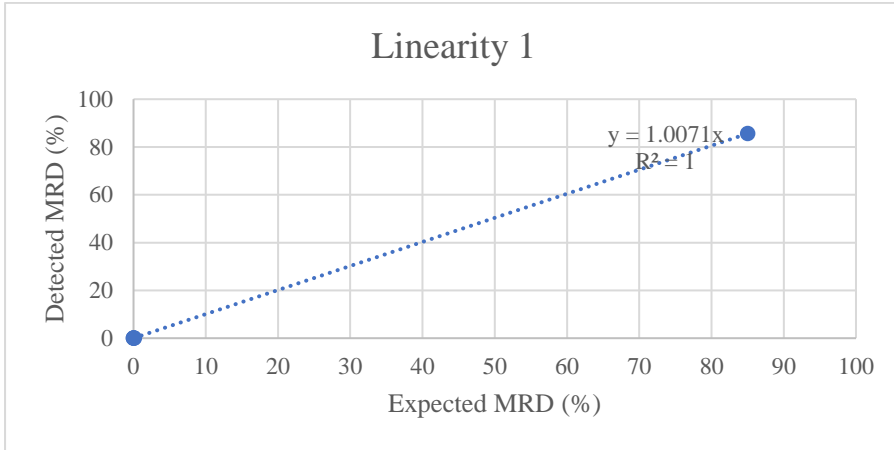
8	Negative		<i>BCR-ABL1/ABL1</i> minor % ratio = Not detected
9	Negative		<i>BCR-ABL1/ABL1</i> minor % ratio = Not detected
10	Negative		<i>BCR-ABL1/ABL1</i> minor% ratio = Not detected
11	Negative		<i>BCR-ABL1/ABL1</i> minor % ratio = Not detected
12	Positive	0.0021	<i>BCR-ABL1/ABL1</i> minor% = 0.02
13	Negative		<i>BCR-ABL1/ABL1</i> minor% = 0.004
14	Negative		<i>BCR-ABL1/ABL1</i> minor % ratio = Not detected
15	Negative		<i>BCR-ABL1/ABL1</i> minor % ratio = Not detected
16	Negative		<i>ETV6-RUNX1/ABL1</i> % ratio = Not detected
17	Negative		<i>ETV6-RUNX1/ABL1</i> % ratio = Not detected
18	Positive	0.3400	<i>ETV6-RUNX1/ABL1</i> % ratio = 5.8
19	Positive	0.0620	<i>ETV6-RUNX1/ABL1</i> % ratio = 3.0
20	Positive	0.0090	<i>ETV6-RUNX1/ABL1</i> % ratio = 0.7

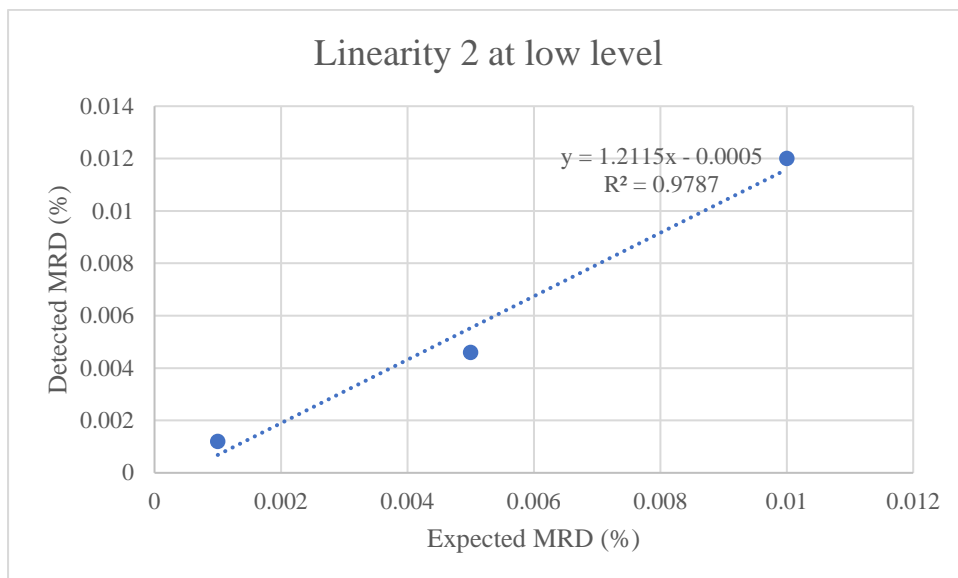
2) Precision

Low-level leukemic samples were tested at least twice and the mean event of leukemic cell was 34, SD 5.66 with CV 16.6% and another level sample with mean leukemic events 75 with SD 19.80 and CV of 26.4% within 35% CV.

3) Linearity or reportable range

R² of the simulated samples (leukemic samples mixed with normal sample) at least three levels were >0.95. The linearity was validated from 0.001 to 85.0%.





4) Limit of detection and lower limit of quantitation

Using bone marrow samples from three non-leukemic samples, LOB and LOD were assessed and resulted in LOB of 8 events, 0.000132%, and LOD of 10 events, 0.000169%. LLOQ was assigned as a level at <35% CV. Since the level of MRD events with 25 did not pass CV criteria, MRD events with mean 75, and 80.5 passed the criteria. The MRD events with highest value was 100, thus 100 events were set as the LLOQ value.

sample 1	Total nucleated cell events	3984652	3906414	Mean	SD	CV	CV <35%
	MRD event	12	38	25	18.38478	73.53911	Not passed
	MRD %	0.000301	0.000973				
sample 2	Total nucleated cell events	4301160	4287261				
	MRD event	61	89	75	19.79899	26.39865	OK
	MRD%	0.001418	0.002076				
sample 3	Total nucleated cell events	4314412	4309081				
	MRD event	61	100	80.5	27.57716	34.25735	OK
	MRD%	0.001414	0.002321				

References

1. Clinical and Laboratory Standards Institute (CLSI). Validation of Assays performed by flow cytometry. 1st ed. CLSI guideline H62. Clinical and Laboratory Standards Institute, USA, 2021.

Supplementary Table 1. Details of the discordant cases.

Case	Patient	MFC-MRD	MFC-MRD (%)	NGS-MRD	NGS-MRD (%)	Major MRD clone	Other molecular studies
1	1	Negative		Positive	0.0050	V3-J4	
2	2	Negative		Positive	0.0034	V4-J3	
3	3	Negative		Positive	0.0022	V1-J6	
4	4	Negative		Positive	0.0023	V3-J4	
5	5	Negative		Positive	0.0037	V3-J4	
6	6	Negative		Positive	0.0263	V4-J5	
7	7	Negative		Positive	0.0229	V3-J2	
8	8	Negative		Positive	0.0012	V1-J2	
9	9	Negative		Positive	0.5250	V3-none	<i>BCR::ABL1</i> detected
10	10	Negative		Positive	0.0005	V1-J4	
11	11	Negative		Positive	0.0054	V3-J6	<i>BCR::ABL1</i> not detected
12	12	Negative		Positive	0.0029	V4-J4	
13	13	Negative		Positive	0.0052	V3-J6	
14	13	Negative		Positive	0.0074	V3-J6	
15	13	Negative		Positive	0.0080	V3-J6	
16	14	Negative		Positive	0.0041	V2D-DEL	<i>ETV6::RUNX1</i> Not detected
17	14	Negative		Positive	0.0018	V2D-DEL	<i>ETV6::RUNX1</i> Not detected
18	14	Negative		Positive	0.0026	V2D-DEL	<i>ETV6::RUNX1</i> Not detected
19	15	Negative		Positive	0.0027	V1-J4	
20	16	Negative		Positive	0.0122	V3-J4	
21	17	Negative		Positive	0.0009	V2-IGKDEL	<i>BCR::ABL1</i> detected
22	17	Negative		Positive	0.0001	V2-IGKDEL	<i>BCR::ABL1</i> not detected
23	18	Negative		Positive	0.0057	V6-J4	<i>BCR::ABL1</i> detected
24	19	Negative		Positive	0.0035	V1-J4	<i>ETV6::RUNX1</i> Not detected
25	20	Negative		Positive	0.0001	V4_39-J6	<i>BCR::ABL1</i> detected

26	21	Positive	0.0025	Negative	V1-J5	
27	22	Positive	0.0026	Negative	V1-J4	
28	23	Positive	0.0089	Negative	V3-J6	<i>BCR::ABL1</i> detected
