Evaluation of diagnostic sensitivity and specificity of 10-colour flow cytometry testing for disease monitoring/detection on the Navios/NaviosEx, Kaluza C platform, in comparison to RQ-PCR testing.

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A research report (in the format of a "submissible" paper) submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in Haematological Pathology.

Johannesburg, 2025.

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DECLARATION

I, Tsholofelo Lungile Malaka declare that this Research Report is my own, unaided work. It is being submitted for the Degree of Master of Medicine in Pathology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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Tsholofelo Lungile Malaka

_____31st__day of__October ___2025_____in Johannesburg

Contribution of the Candidate to the Paper

Declaration: Student's contribution to the article and agreement of co-authors

I, Tsholofelo Lungile Malaka, student number, 552798, declare that this Research Report is my own work and that I contributed adequately towards the research findings published in the article stated below which are included in my Research Report.

Signature of Student…….....**Date**…….....…

Supervisor 1…….....……

Signature of Supervisor 1 ……**Date**……...…….....…

Supervisor 2…….....……

Signature of Supervisor 2 ……**Date**……...…….....…

Agreement by co-authors:

By signing this declaration, the co-authors listed below agree to the use of the article by the student as part of her Research Report.

Article Title: Evaluation of diagnostic sensitivity and specificity of 10-colour flow cytometry testing for disease monitoring/detection on the Navios/NaviosEx, Kaluza C platform, in comparison to RQ-PCR testing.

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List of Tables

Nomenclature

ALL	Acute lymphoblastic leukaemia
BFM	Berlin–Frankfurt–Münster
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CI	Confidence interval
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
DNA	Deoxyribonucleic acid
EQA	External quality assurance
FISH	Fluorescence *in-situ* hybridisation
IgH	Immunoglobulin heavy chain
IQR	Interquartile range
LIS	Laboratory Information System
LOQ	Lower Limit of Quantification
LOD	Lower Limit of Detection
MRD	Measurable residual disease
NGS	Next generation sequencing
NHLS	National Health Laboratory Services
NPV	Negative predictive value
PCR	Polymerase chain reaction
PPV	Positive predictive value
RQ-PCR	Real-time quantitative polymerase chain reaction
RNA	Ribonucleic acid
SANAS	South African National Accreditation System
SCGU	Somatic Cell Genetics Unit
SE	Standard error
VDJ	Variability-diversity-joining

RESEARCH ARTICLE – "SUBMISSIBLE FORMAT"

Title

Evaluation of diagnostic sensitivity and specificity of 10-colour flow cytometry testing for disease monitoring/detection on the Navios/NaviosEx, Kaluza C platform, in comparison to RQ-PCR testing.

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Author contributions

TL Malaka collected, analysed the data and drafted the manuscript report. A Baiden and K Hodkinson conceived the study, supervised and edited the research report.

ABSTRACT

Background

Measurable residual disease (MRD) analysis is critical in acute lymphoblastic leukaemia (ALL) management protocols. Real-time quantitative polymerase chain reaction (RQ-PCR) is the accepted gold standard in MRD testing. Multiparameter flow cytometry has emerged as a comparative testing modality. We evaluated the concordance of our centre's 10-colour flow cytometry (Navios/NaviosEx) with RQ-PCR (ABI 7500 Fast Real Time PCR) for MRD detection.

Aim

To investigate the concordance of flow cytometry MRD results versus RQ-PCR; and immunoglobulin heavy chain (IgH) rearrangement PCR versus RQ-PCR.

Setting

The National Health Laboratory Services Flow Cytometry and Somatic Cell Genetics Units at the Charlotte Maxeke Johannesburg Academic Hospital.

Methods

Flow cytometry results between March 2020 and June 2023 were compared to RQ-PCR results in follow-up samples from patients with a diagnosis of B-cell ALL, bearing one of the following recurrent genetic lesions; t(1;19), t(12;21) or t(9;22).

Results

A total of 74 bone marrow aspirate samples were eligible for inclusion. The flow cytometry assay showed a low sensitivity (13%) but a high specificity (98%) when compared to RQ-PCR. The positive and negative predictive values were 75% and 71%, below the acceptable levels of 80% and 90% respectively. The IgH assay showed better sensitivity (43%) and a positive predictive value of 91%.

Conclusion

The flow cytometry assay at our center exhibited significantly lower sensitivity compared to PCR-based methods for the detection of MRD in follow-up cases of B-ALL. Among the PCR techniques, RQ-PCR demonstrated superior sensitivity for MRD detection.

Keywords

B-ALL, flow cytometry, MRD, RQ-PCR, IgH rearrangement

INTRODUCTION

Acute lymphoblastic leukaemia (ALL) is the most common cancer in childhood, constituting up to 25% of cancers in children under the age of 15 years.(1) A critical part of the management of ALL includes the assessment of measurable residual disease (MRD) at several time points during the treatment protocol. MRD is recognised as an independent predictor of survival in ALL (2,3) and is regarded as the most reliable predictor of relapse-free survival.(4) In ALL, early negativity for MRD is associated with very good outcomes in both children and adults.(5) In essence, MRD provides useful information for prognostication, guiding therapy and risk stratification of patients.(6)

Advancement in technology and understanding of the underlying disease pathogenesis has led to the development of different techniques for the detection of MRD. Examples include various polymerase chain reaction (PCR) based techniques such as immunoglobulin heavy chain gene (IgH) rearrangement studies, digital droplet PCR, real-time quantitative PCR (RQ-PCR) next generation sequencing (NGS), and flow cytometry-based methods. The sensitivity and specificity varies among the different MRD testing modalities, with RQ-PCR recognised as the gold standard for the detection of MRD in MRD-based ALL protocols.(7,8) However, multi-parameter flow cytometry has emerged as a comparative technique with studies showing good correlation of flow cytometry results with RQ-PCR MRD results.(9,10,11) Flow cytometry has several advantages over RQ-PCR namely, a shorter turn-around time, being less labour-intense and more widely available.(2) Thus, in resource limited areas, particularly in Africa and other developing countries, multi-parameter flow cytometry is an appealing modality for MRD monitoring where RQ-PCR is not readily available. Notably, RQ-PCR can only be performed in cases bearing specific recurrent genetic translocations, resulting in fusion gene proteins. Therefore, alternative methods for MRD testing such as flow cytometry or NGS may offer alternative means of testing in cases that do not bear recurrent genetic lesions.

In this study, we evaluated the sensitivity and specificity of the ClearLab 10-colour flow cytometry assay for MRD monitoring/detection in comparison with the gold standard RQ-PCR. Additionally, we evaluated the concordance between RQ-PCR and IgH PCR MRD testing.

METHODS

Study Setting

The National Health Laboratory Services (NHLS), Flow Cytometry laboratory, and Somatic Cell Genetics Unit (SCGU), at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). These laboratories are South African National Accreditation System (SANAS) accredited with associated subscription to both local and international external quality assurance (EQA) testing programs.

Study design and study population

A retrospective study was conducted to review and analyse the degree of concordance between flow cytometry and RQ-PCR results, in B-ALL MRD follow-up cases performed on bone marrow aspirate samples from patients in morphological remission (<5% blasts). The study included paediatric and adult patients with a diagnosis of B-ALL, bearing one of the following recurrent genetic lesions; the translocation t(1;19)(q23;p13.3); TCF3-PBX1 (t(1;19)), translocation t(12;21)(p13.2;q22.1); ETV6-RUNX1 (t(12;21)), or translocation t(9;22)(q34.1;q11.2); BCR-ABL1 (t(9;22)) , for whom flow cytometry and RQ-PCR were performed for MRD testing at the same time point, at the CMJAH NHLS laboratories. The study period was from March 2020 to June 2023. Given that MRD monitoring occurs at several different time points during therapy (i.e. post-induction, post-consolidation, post-maintenance), each time point was viewed as separate analysis.

The following samples were excluded; morphologically haemodilute samples where the flow cytometry and RQ-PCR failed to detect MRD, samples for which RQ-PCR failed (low ribonucleic acid (RNA) yield defined as <1 ug of RNA (12), morphologic evidence of residual disease, and cases without a baseline positive RQ-PCR result. An exception was made for five cases where the blast percentage was more than 5% morphologically, ranging from 6% to 14%. In all cases there was no detectable disease on flow cytometry and thus comparison could be made with the corresponding RQ-PCR result in order to distinguish MRD from normal haematogones or recovering myeloid blasts.

The patient samples were analysed on the Navios and NaviosEx (Beckman Coulter, San Diego, United States of America) flow cytometer, using the Kaluza C™ software platform, at the CMJAH NHLS Flow Cytometry Laboratory. This assay employs a premixed ClearLLab 10-

color backbone B panel comprising of the antibodies: cluster of differentiation (CD) CD5, CD10, CD19, CD20, CD34, CD38, CD45, CD200, kappa and lambda. Other panels commonly used in addition to the backbone B panel, included the M2 tube which contained the following antibodies: CD13, CD15, CD19, CD33, CD34, CD45, CD38, CD117, CD123 and HLA-DR; and the Haematogone (H) panel containing the following markers: CD10, CD19, CD22, CD34, CD45, CD58 and CD81. The use of additional panels during flow cytometry was dependant on the tumour specific immunophenotype which was reported at presentation. The M2 tube was included for aberrant myeloid markers (if present), or to assess for CD123 and HLA-DR expression which are frequently expressed by leukaemic cells.

RQ-PCR was performed on the ABI 7500 Fast Real Time PCR system (Applied Biosystems ™, California, United States of America) at the SCGU. This assay uses fluorescent-based chemistry for qualitative and quantitative detection of fusion transcripts. RQ-PCR derives complementary deoxyribonucleic acid (DNA) products from RNA transcripts with quantitation made by use of end points and dissociation curves. The results were analysed with percentages and ratios of fusion gene to control gene. The control gene, *GUSB*, is used to gauge sample degradation (which is defined as <1000 copies of *GUSB*), validate any undetectable fusion transcript result and used in the calculation fusion transcript ratios)(12).

A list of RQ-PCR tests that were performed in the specified time frame was obtained from the SCGU database. Each item in the list was screened for eligibility alongside the data obtained from the NHLS laboratory information system (LIS). The eligible patient entries and data were transcribed onto an Excel (Microsoft, Washington, United States) spreadsheet. Additional flow cytometry data (number of events analysed for each case, and panel(s) used) was obtained from analysis files generated by the Navios and NaviosEx instruments via Kaluza C software interface.

The PCR IgH assay used was the IdentiClone® *IGH* Gene Clonality Assay (Invivoscribe, Inc., San Diego, United States of America) which contains multiple consensus DNA primers that target the variability-diversity-joining (VDJ) segments of the IGH gene to detect clonal rearrangements.(13)

Two separate analyses were performed, the first between flow cytometry and the gold-standard RQ-PCR, and the second between IgH rearrangement studies and RQ-PCR. The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for each

analysis were calculated and presented in two separate ‘two-way-tables’. Descriptive statistics (median, interquartile range (IQR), minimum and maximum values) were used to summarise results. Strength of agreement between MRD methods was assessed by means of the Cohens Kappa statistical measure. (14)

Ethical considerations

Ethics clearance for this study was obtained from the University of the Witwatersrand Human Research Ethics Committee under clearance certificate number M230840. The data was anonymised, with patient identifying information stored in a separate password protected Excel spreadsheet which was only accessible to the investigators.

RESULTS

Of the 303 bone marrow aspirate samples, a total of 74 were eligible for inclusion in the study. Two hundred and twenty-nine samples were excluded due to; absence of a corresponding flow cytometry analysis performed at CMJAH NHLS flow cytometry laboratory (185); sample degradation (9); insufficient RNA yield (19); lack of a baseline positive RQ-PCR result (13); or haemodilution (3). The median age (IQR) of the cohort was 5 years (2 to 9 years), all the samples were from children and adolescents except for one adult patient at age 28 years. There was an almost equivalent number of male and female patients. The most common genetic aberration was t(12;21) constituting 54% (n=40), followed by t(1;19) at 44.6% (n=33) and t(9;22) at 1.4% (n=1). Paediatric patients from the referring centres were most commonly treated with the ALL Berlin–Frankfurt–Münster (BFM) 95 protocol or the BFM 2000 protocol, without the use of immunotherapeutic targets, this precluded the need for modifications or special considerations of the flow cytometry analysis. In the majority of the samples a corresponding IgH gene rearrangement PCR had been performed (n=70), of these, two failed amplification, leaving 68 results (97%) available for comparative analysis with RQ-PCR and flow cytometry.

Flow cytometry versus RQ-PCR

A total of 74 cases were assessed for comparison of flow cytometry versus RQ-PCR. The majority of cases (~81%) tested for MRD by flow cytometry utilised two separate antibody panels including a premixed backbone B panel combined with M2 and/or H panels.

The median (IQR) of events analysed (excluding nucleated red blood cells) was 50 000 events. The flow cytometry assay showed a low sensitivity (13%) but a high specificity (98%) as compared with RQ-PCR (Table 1). The PPV and NPV values were 75% and 71%, below the acceptable levels of 80% and 90% respectively. The RQ-PCR results of the false negative cases (n=20) were below the limit of detection (LOD) of the flow cytometry assay (0.04%), with a median (IQR) *Fusion gene*:*GUSB* ratio of 0.55 $x10^{-3}$ (0.08 to 2.60 $x10^{-3}$)(Appendix I).
For the 3 true positive cases, the amount of disease detected on flow cytometry was between 0.2 and 1% of total events analysed, whilst the median (IQR) ratio of *Fusion gene*:*GUSB* was 0.014 (0.0095 to 0.027), which is higher than the *Fusion gene*:*GUSB* ratio in the false negative cases (Appendix II). Six samples were reported as haemodilute and all were negative for MRD on flow cytometry. Half of these samples (n=3) had detectable MRD on RQ-PCR and thus the latter were included in the overall analysis. The Cohen's kappa coefficient (K = 0.10 (95% confidence interval (CI), 0.08987 to 0.29079, standard error (SE) 0.09711) which was calculated to assess agreement between flow cytometry testing and RQ-PCR showed poor agreement. (14)

A small subset (n=5) of the samples had no immunophenotypic evidence of disease however, morphologically there were more than 5% blasts. For three out of the five cases, this finding was accounted for as normal reactive precursor B-cells on flow cytometric analysis. This correlated with the corresponding RQ-PCR and IgH results, which were also negative for MRD.

Table 1. Two-way table for sensitivity, specificity, positive and negative predictive values for Navios flow cytometry versus RQ-PCR

		Reference standard (RQ-PCR)		
		Positive	Negative	**Total**:
Index test (Flow cytometry)	Positive	A: 3 (true positive)	B: 1 (false positive)	4
	Negative	C: 20 (false negative)	D: 50 (true negative)	70
	Total:	23	51	74

Quantitative reverse transcriptase polymerase chain reaction (RQ-PCR)

RQ-PCR versus IgH

Table 2. Two-way table for sensitivity, specificity, positive and negative predictive values for IgH rearrangement PCR versus RQ-PCR

		Reference standard (RQ-PCR)		
		Positive	Negative	**Total**:
Index test (IgH PCR)	Positive	A: 10 (true positive)	B: 1 (false positive)	11
	Negative	C: 13 (false negative)	D: 44 (true negative)	57
	Total:	23	45	68

Real-time quantitative polymerase chain reaction: RQ-PCR

Immunoglobulin heavy chain polymerase chain reaction: IgH PCR

The IgH assay showed a low sensitivity (43%) as compared with RQ-PCR, but higher than flow cytometry, and a 98% specificity (Table 2). The PPV was 91% but the NPV was suboptimal at 77%. The RQ-PCR results of the false negative cases were below the LOD of the IgH assay (0.01%) with a median (IQR) ratio of *Fusion gene*:*GUSB* of 0.3 $x10^{-3}$ (0.025 to 0.7 $x10^{-3}$). In contrast, in the true positive cases, the median (IQR) of the ratio of *Fusion gene*:*GUSB* was expectedly higher at 3.0 $x10^{-3}$ (0.45 to 13.5 $x10^{-3}$), with 0.004 the lowest value obtained where both RQ-PCR and IgH were positive.

The calculated Cohen's kappa coefficient was 0.47 (95% CI, 0.25617 to 0.68957, SE 0.11056) which denotes moderate agreement between IgH PCR and RQ-PCR.(14)

DISCUSSION

Monitoring for MRD is pivotal in the management of ALL and is the standard of care in ALL treatment protocols. Results of MRD testing alter risk, prognostication and treatment approaches. As this data becomes available, clinical decisions are made, including identifying patients at high risk of disease relapse and those who are eligible for allogeneic stem cell transplantation.

The recommended sensitivity levels for MRD testing range from 10^{-4} to 10^{-5}, corresponding to the detection of 1 leukaemic cell per 10 000 and 100 000 cells respectively.(15) Historically, RQ-PCR techniques have met the desired sensitivity levels, and with the advent of improved high-throughput multiparameter flow cytometry assays, several reports have shown correlation in the sensitivities between molecular modalities and flow cytometry.(2,4,16)

As our flow cytometry assay is not MRD tailored, the sensitivity results are not unexpected, with only around 13% of MRD positive cases detected. These cases had a higher fusion transcript burden and fusion transcript to GUSB ratio when compared to the false negative cases, reflecting the low sensitivity of the assay.

According to the Australasian Cytometry Society Guidelines, for lower LOD, the minimum number of total events needed for a sensitivity level of 0.01% (10^{-4}) is 2x 10^5 cells, while the minimum number of leukaemic targets or tumour events required for an MRD positive result is 20. For lower limit of quantification (LOQ), the minimum number of total events needed for sensitivity level of 0.01% (10^{-4}) is 2 x 10^5 cells, while the minimum number of target cells required for an MRD positive result is 50.(6) Most MRD protocols are based on a sensitivity of 0.01% (10^{-4}) to 0.001%(10^{-5}).(15,17) If the recommendations are applied to our flow cytometry assay, where a minimum of 50 000 events are analysed, the LOD and LOQ would be below the desirable sensitivity at 0.04% and 0.1% respectively. In order to meet the sensitivity requirements as an MRD assay, the number of CD45 positive events would need to increase to 500 000, 10 times more than the current target.

Our flow cytometry assay showed high specificity of close to 100% as compared to the gold standard, demonstrating the low probability of calling MRD positivity when not present, i.e. low false positive rates. Ultimately, molecular assays would need to be awaited in flow cytometry negative cases to guide clinical decision making in our setting. Given that flow cytometry has shorter turn-around times, is less labour-intense, is more widely available than RQ-PCR, and is valuable in cases lacking a recurrent genetic abnormality, a move toward MRD flow cytometry is anticipated.(17) The PPV and NPV for flow cytometry were suboptimal, with the clinical utility limited to cases where MRD is detected.

The single false positive case (flow cytometry versus RQ-PCR) showed a 5% population of cells on flow cytometry with a similar immunophenotype to that seen at presentation and was therefore concluded to represent residual disease. Both the IgH PCR and RQ-PCR techniques were negative for MRD. In this specific case, the different from normal immunophenotype was that of CD38 under-expression and CD200 over-expression but with no non-B-cell lineage aberrancies. This highlights the challenge in delineating disease from normal B-cell precursors morphologically in the bone marrow and immunophenotypically on flow cytometry, the need for caution in interpreting MRD on flow cytometry when no definite aberrancies are present,

and the requirement to confirm suspected MRD with an alternative method such as RQ-PCR in these cases. For this case, both PCR techniques were negative for MRD. This may indicate that while assessment of cell population scatter patterns and associated aberrancies is valuable in delineating disease from normal B-cell precursors in the bone marrow, it is not definitive or absolute. This is particularly true as a phenomenon of clonal evolution is observed post-chemotherapy with antigen expression changes which can affect MRD testing. (3,6) This highlights the important role PCR techniques play in further clarifying such cases.

Several studies have examined these shifts in detail in order to optimize their panels, a practice that ensures MRD with antigen shift is detected.(2) The use of novel immunotherapeutic agents such as anti-CD19, anti-CD22 therapy, and chimeric antigen receptor (CAR) T-cell therapy further pose challenges to MRD monitoring due to key antigen (particularly CD19) loss. This requires specially tailored panels and algorithms which will be able to carefully discriminate disease populations, as shown in one study which employed a special all-colour panel consisting of 10 antibodies (namely, CD10, CD19, CD20, CD22, CD24, CD34, CD38, CD45, CD58, cytoplasmic CD79a) and Syto41, a DNA dye to identify nucleated cells. The combination of CD79a and CD22 was used as a main gating substitute for CD19 while CD10 and CD24 were alternatives if the tumour population expressed none of these antigens.(6)
As expected, IgH testing, a PCR technique, showed better test performance in contrast to flow cytometry, but with comparable specificity. In contrast to flow cytometry, the PPV fared better than the NPV, with no false positive cases. Thus, a positive result on IgH is reliable, whilst a negative result does not exclude MRD, and alternative more sensitive molecular techniques are required in the latter.

The reliability of MRD testing results is heavily dependent on the quality of samples analysed. Haemodilute samples are typically contaminated with peripheral blood and may yield falsely negative results. From our results, it would also appear that more sensitive MRD testing modalities such as RQ-PCR are more likely to detect disease in the face of sample haemodilution. This is also relevant and useful for post-induction bone marrow samples which are usually pauci-cellular and have a limited number of events on flow cytometry. Haemodilution was determined on morphology alone, and it is important to note that the quality of each sample submitted for the different testing modalities may not be uniform. It is therefore recommended that MRD reports should contain a comment on the degree of haemodilution,

when this assessment is possible.(18) This also speaks to the pre-analytical aspects that should be considered when samples are collected, so that optimal and representative samples are obtained. The European LeukaemiaNet MRD working group recommends the first-pull bone marrow aspirate for MRD testing.(19) In our context, preference should be given to RQ-PCR for first-pull samples, whilst IgH or flow cytometry should be prioritised in cases lacking a recurrent genetic abnormality (detectable by RQ-PCR). Therefore, MRD testing should not only focus on the analytical process but should be seen as a comprehensive workflow that seeks to streamline all the pre-analytical, analytical and post-analytical processes with the goal of ensuring accurate and sensitive MRD results for clinical decision-making.

Limitations

Limitations of this study include:

- Patient files were not examined, thus, evaluation of clinical outcomes in relation to laboratory results was not assessed.
- The number of events analysed may vary between the cases. The maximum number of events analysed was set at 50 000 on the Navios/NaviosEx instruments

CONCLUSION

The flow cytometry assay employed at our center exhibited significantly lower sensitivity compared to PCR-based methods for the detection of MRD in follow-up cases of ALL. Among the PCR techniques, RQ-PCR demonstrated superior sensitivity for MRD detection. There is a need for modification of the current flow cytometry assay to be tailored specifically for MRD testing by adapting it to accepted standards/guidelines. This would be of value as it would offer alternative methods of MRD testing that are comparatively sensitive for cases not amenable to RQ-PCR testing. As modification of the assay is underway, future studies could conduct a similar comparison looking at sensitivity/specificity of the modified MRD-tailored flow cytometry assay versus RQ-PCR or other PCR techniques.

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APPENDICES

Appendix I: Details of cases that were false negative on flow cytometry

		Number of events				Morphology	RQ-PCR	Fusion Gene: GUSB	
Patient number	**Panel(s) used**	**B**[a]	**T**[b]	**M2**[c]	**H**[d]	**Blast %**	**Primer**	**Ratio**	**%**
9	B	72496	-	-	-	<1%	t(12;21)	0.000025	0.0004
10	B,M2	61674	62307	62307	-	4%	t(12;21)	0.0008	N/A[e]
12	B,M2	53808	-	51241	-	2%	t(12;21)	0.00007	0.007
13	B,M2	43876	-	47552	-	2%	t(12;21)	0.0007	N/A[e]
72	Not available	Not available	-	-	-	Haemodilute	t(12;21)	0.0009	0.09
48	B,M2	48797	-	49951	-	<1%	t(1;19)	0.0004	0.03
49	B,H	49984	-	-	50478	2%	t(1;19)	0.0003	0.02
50	B,M2	50015	-	49990	-	2%	t(1;19)	0.003	0.19
51	B,M2,H	48229	-	51184	54951	3%	t(1;19)	0.15	9.5
52	B,H	46824	-	-	56282	1%	t(1;19)	0.00004	0.01
53	B,T,H	52466	45482	-	55584	<1%	t(1;19)	0.003	0.51
62	B,H	50199	-	-	50406	3%	t(1;19)	0.00014	N/A[e]
63	B,H	39295	-	-	32935	<1%	t(1;19)	0.00002	N/A[e]
66	B,M2,H	50709	-	55238	55578	2%	t(1;19)	0.0001	0.004
67	B,H	38937	-	-	65109	<1%	t(1;19)	0.00141	0.043
68	B,H	49280	-	-	57726	<1%	t(1;19)	0.00002	0.001
69	B,H	49710	-	-	50215	3%	t(1;19)	0.00014	N/A[e]
70	B,H	41030	-	-	31220	<1%	t(1;19)	0.0007	N/A[e]
73	Not available	Not available	-	-	-	Haemodilute	t(1;19)	0.000014	N/A[e]
74	B,T,H	17489	18550		18296	Haemodilute	t(1;19)	0.011	1.86

a Antigens assessed on the B panel include: CD5, CD10, CD19, CD20, CD34, CD38, CD45, CD200, surface kappa and lambda.

b Antigens assessed on the T panel include CD2, CD3, CD4, CD5, CD7, CD8, CD34, CD45, CD56, and T-cell receptor gamma-delta.

c Antigens assessed on the M panel include CD13, CD15, CD19, CD33, CD34,CD38, CD45, CD117, CD123, HLA-DR, AGP (anti-glycophorin).

d Antigens assessed on the H panel include CD10, CD19, CD22, CD34, CD38, CD45, CD59 and CD81.

e These are cases with no presentation RQ-PCR, but had a subsequent baseline positive RQ-PCR result.

Appendix II: Details of cases that were true positive on flow cytometry

		Number of events				Morphology	Flow	RQ-PCR	Fusion Gene: GUSB	
Patient number	**Panel(s) used**	**B**[a]	**T**[b]	**M2**[c]	**H**[d]	**Blast %**	**% disease**	**Primer**	**Ratio**	**%**
25	B,T,H	51173	54815		60019	0%	1%	t(12;21)	0.04	5.26
36	B	49440	-	-	-	1%	0.20%	t(12;21)	0.005	6.34
56	B	51001	-	-	-	2%	1%	t(1;19)	0.014	0.4

a Antigens assessed on the B panel include: CD5, CD10, CD19, CD20, CD34, CD38, CD45, CD200, surface kappa and lambda.

b Antigens assessed on the T panel include CD2, CD3, CD4, CD5, CD7, CD8, CD34, CD45, CD56, and T-cell receptor gamma-delta.

c Antigens assessed on the M panel include CD13, CD15, CD19, CD33, CD34,CD38, CD45, CD117, CD123, HLA-DR, AGP (anti-glycophorin).

d Antigens assessed on the H panel include CD10, CD19, CD22, CD34, CD38, CD45, CD59 and CD81.

Appendix III: Ethics Clearance Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R49 Dr T Malaka

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M230840

NAME: Dr T Malaka **DEGREE:** M Med
(Principal and Co-Investigators)

DEPARTMENT: School of Pathology
Department of Molecular Medicine & Haematology
Medical School
University

PROJECT TITLE: *Evaluation of diagnostic sensitivity and specificity of 10-colour flow cytometry testing for disease monitoring/ /detection on the Navios/NaviosEx, Kaluza C platform, in comparison to RQ-PCR testing*

DATE CONSIDERED: 25 August 2023

DECISION: Appproved unconditionally

CONDITIONS:

NOTE: If contact information regarding student study participants is required, please contact the Registrar's office <Nicoleen.Potgieter@wits.ac.za>

SUPERVISOR: Drs A Baiden and K Hodkinson

APPROVED BY:
Ms M van Niekerk, Co-Chairperson, HREC (Medical)

DATE OF APPROVAL: 20 December 2023 **EXPIRY DATE:** 19 December 2028

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report** in the format available at https://wits.ac.za/research/researcher-support/research-ethics/ethics-committees/. This report is due annually, for the duration of the Clearance Certificate, beginning on the first anniversary of Date of Approval above. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

27/12/2023
Date

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG	HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research and Innovation)

TO: Dr T Malaka
School of Pathology
Department of Molecular Medicine & Haematology
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E-mail: Tsholofelo.Malaka@nhls.ac.za

CC: Supervisor: Drs A Baiden and K Hodkinson
Anima.Baiden@nhls.ac.za
and <HREC-Medical Research Office@wits.ac.za>

FROM: Mr Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 20 December 2023

REF: R14/49

PROTOCOL NO: **M230840** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)

PROJECT TITLE: *Evaluation of diagnostic sensitivity and specificity of 10-colour flow cytometry testing for disease monitoring/ /detection on the Navios/NaviosEx, Kaluza C platform, in comparison to RQ-PCR testing*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to Government funding of the University.

MSWorks2000/Iain0007/Clearscan.wps

Appendix IV: Research Protocol

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

CANDIDATE'S SURNAME: [Please print] MALAKA		FIRST NAME/S: TSHOLOFELO	STUDENT NUMBER: 552798
CURRENT QUALIFICATIONS: MBChB			
TEL: -	CELL: 082 305 8086	E-MAIL: tsholofelo.malaka@nhls.ac.za	FAX: -
DEGREE FOR WHICH PROTOCOL IS BEING SUBMITTED: Master of Medicine in Haematological Pathology			
PART-TIME OR FULL-TIME: FULL-TIME			
FIRST REGISTERED FOR THIS DEGREE:	TERM : 1		YEAR: 2022
DEPARTMENT: Department of Molecular Medicine and Haematology			
TITLE OF PROPOSED RESEARCH: Evaluation of diagnostic sensitivity and specificity of disease monitoring/detection by 10-colour flow cytometry (set at capture of 50 000 events), on the Kaluza C platform, in comparison to the gold standard, RQ-PCR testing.			
CANDIDATE'S SIGNATURE:			DATE: 25/06/2023
SUPERVISOR 1 (NAME & SURNAME): Dr Anima Baiden			% Supervision 50%
SUPERVISOR'S QUALIFICATIONS			
SUPERVISOR'S DEPARTMENT: Department of Molecular Medicine and Haematology, University Of Witwatersrand and National Health Laboratory Services			
SUPERVISOR'S ADDRESS / TEL / E-MAIL: Anima.baiden@nhls.ac.za			
SUPERVISOR 2 (NAME & SURNAME): Dr Katherine Hodkinson			% Supervision 50%
SUPERVISOR'S QUALIFICATIONS			
SUPERVISOR'S ADDRESS / TEL / E-MAIL: Katherine.hodkinson@nhls.ac.za			
SUPERVISOR 3 (NAME & SURNAME): N/A			% Supervision
SUPERVISOR'S QUALIFICATIONS			
SUPERVISOR'S ADDRESS / TEL / E-MAIL:			
SYNOPSIS OF RESEARCH:(Brief summary of proposed research project; between 200-300 words only; with sub-headings: an introduction and justification for study, aim/s, proposed methodology and expected outcome/s) [Use reverse side of this page if more space is required] Please see reverse side for complete synopsis.			
WITS ETHICS NOT REQUIRED: Yes No WITS ETHICS PENDING: (Yes) No WITS ETHICS APPROVED: Yes No (circle appropriate symbol)* ***Please note the final human ethics clearance certificate or animal ethics certificate must be available prior to starting research**			IF Y SUPPLY ETHICS CLEARANCE CERTIFICATE AS ATTACHMENT AND INCLUDE ETHICS NUMBER HERE:
As supervisor/s, I/we confirm that I have read the protocol which has been submitted for assessment.			
SIGNATURE OF SUPERVISOR/S:	……	……	
SIGNATURE PG OFFICE STAFF ……	REGISTERED YES NO	STAMP	

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

FACULTY OF HEALTH SCIENCES

SYNOPSIS OF RESEARCH CONTINUED

Introduction

MRD analysis is a critical part in the management protocols of most ALL protocols. MRD assessment plays a pivotal role in the risk-stratification of patients, prognostication as well as guiding clinical decision making. Different testing modalities for MRD are available, with RQ-PCR being the accepted gold standard. Multiparameter flow cytometry has emerged as a comparative technique with several studies showing good correlation with RQ-PCR MRD. In this study we will evaluate the concordance of our centre's flow cytometry results performed on the 10-colour multi-parametric flow cytometer, Navios/NaviosEx by Beckman Coulter supported on the Kaluza C software platform with RQ-PCR MRD.

Aims

Primary

To evaluate the concordance between flow cytometry and RQ-PCR MRD testing in B-ALL cases with recurrent genetic abnormalities, that are in morphologic remission.

Secondary

If there are sufficient number of cases from the above analysis, which have matching IgH and RQ-PCR results, the concordance between IgH PCR and RQ-PCR MRD testing will be evaluated.

Method

Flow cytometry results will be compared to RQ-PCR MRD results for paediatric and adult patients with a diagnosis of B-ALL, bearing one of the following recurrent genetic lesions; t(1;19), t(12;,21) or t(9;22). The study period is from March 2020 – June 2023. The target sample size is a minimum of 42 samples. The list of RQ-PCR samples will be identified from a database at the NHLS CMJAH Somatic Cell Genetics Unit with further corresponding laboratory data obtained from the NHLS Laboratory Information System.

Expected outcome:

To determine the diagnostic sensitivity and specificity of MRD testing by 10-colour flow cytometry (set at capture of 50 000 events), on the Kaluza C platform, in comparison to the gold standard, RQ-PCR testing

11 March 2019/MP

Evaluation of diagnostic sensitivity and specificity of 10-colour flow cytometry testing for disease monitoring/detection on the Navios/NaviosEx, Kaluza C platform, in comparison to RQ-PCR testing.

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Contents

ABBREVIATIONS

ALL	Acute lymphoblastic leukaemia
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
DNA	Deoxyribonucleic acid
EQA	External Quality Assurance
FISH	Fluorescence *in-situ* hybridisation
IgH	Immunoglobulin heavy chain
LAIP	Leukaemia-associated immunophenotypes
LIS	Laboratory Information System
MRD	Measurable/minimal residual disease
NGS	Next Generation Sequencing
NHLS	National Health Laboratory Services
NPV	Negative predictive value
PCR	Polymerase chain reaction
PPV	Positive predictive value
RQ-PCR	Real-time quantitative polymerase chain reaction
RNA	Ribonucleic acid
SANAS	South African National Accreditation System
VDJ	Variability-diversity-joining

1. INTRODUCTION

1.1. Background

Acute lymphoblastic leukaemia (ALL) is the most common cancer in childhood, constituting up to 25% of cancers in children who are under the age of 15 years. (1) A critical part of the management of ALL includes the assessment of minimal/ measurable residual disease (MRD) at several time points during the treatment protocol. The presence of early MRD during treatment is regarded as the most reliable predictor of relapse-free survival (2), and it is also considered the most independent outcome predictor in ALL (3,4). In ALL, early negativity for MRD is associated with very good outcomes in both children and adults. (5) In essence, MRD provides useful information for prognostication, guiding therapy and risk stratification of patients. (6)

Advancement in technology and understanding of the underlying disease pathogenesis has led to the development of different techniques for the detection of MRD. Examples include various polymerase chain reaction (PCR) based techniques such as immunoglobulin heavy chain gene (IgH) rearrangement studies, digital droplet PCR, real-time quantitative polymerase chain reaction (RQ-PCR) next generation sequencing (NGS), and flow cytometry-based methods. The sensitivity and specificity varies among the different MRD testing modalities. RQ-PCR is the recognised gold standard for detection of MRD in MRD-based ALL protocols. (7,8) However, multi-parameter flow cytometry has emerged as a comparative technique with studies showing good correlation with RQ-PCR MRD results. (9,10,11) Flow cytometry has several advantages over RQ-PCR namely, a shorter turn-around time, being less labour-intense and more widely available. (2)

1.2. Principles of flow cytometry

Flow cytometric analysis is based on the characterisation of cells suspended in a fluid medium. These cells undergo hydrodynamic focusing to allow individual cells to pass through a laser beam, with resultant emission of light at different wavelengths and in different directions. This information is processed by the flow cytometer and provides information as to the size and complexity of each cell (forward light scatter and side light scatter respectively). The addition of fluorochrome conjugated antibodies to the suspension allows for the detection of specific surface and/or cytoplasmic antigen expression by the cells, which provides information on the immunophenotype of the cells.

Flow cytometry is used for MRD monitoring through the identification of leukaemia-associated immunophenotypes (LAIP), (3) and tracking of tumour populations using markers of aberrancy which may include abnormal antigen expression, antigen over or under expression and/or asynchronous maturation. In addition, this technique requires sound knowledge of immunophenotypic markers and expression patterns of cells to delineate disease/leukemic populations.

1.2.1 Multi-parametric/multi-colour flow cytometry

Multi-colour flow cytometry has refined the technical capability of immunophenotypic profiling by enabling the simultaneous analysis of multiple immunophenotypic markers/ parameters in a single cell thereby improving the sensitivity and specificity of MRD assessment. Most panels include 6-8 monoclonal antibodies with some ranging up to 18. (5) This reduces sample preparation time eliminating the need to pipette individual antibodies while mitigating human processing errors. This advancement in analytic capability has equated to advancements in hardware and software technology to meet this complex analytic capability. It is recommended that ALL MRD assays should run at a sensitivity of at least 1 leukaemic cell per 10 000 normal cells [i.e. 0.01%] (1,4), as this is the current threshold for MRD clinical decision making. (3,2) The sensitivity of 3-4 colour flow cytometry panels is reported to be between 0.1% - 0.01%, with 6-10 colour panels reaching sensitivities of 0.01% - 0.001%. (3) Furthermore, the more cellular events analysed, the lower the MRD false negative rate, with 4.4 million events required to obtain a sensitivity of 0.001%. (13) Thus, the sensitivity of flow cytometry assays is highly dependent on the panel used and the total number of events analysed.

1.2.2 Special considerations in flow cytometry

Flow cytometry requires viable cells for accurate analysis, with sample degradation a risk when there are delays in sample processing (5). Haemodilute samples may lead to an erroneously low yield of MRD which may result in a false negative MRD result. (10) In the absence of aberrant surface antigens on a tumour population at presentation, with which to aid disease identification, monitoring and in particular distinguishing regenerating marrow elements from a small percentage of disease in post-induction samples may not be possible. (9)

During the course of treatment, subclones with a different immunophenotypic profile may emerge. There may also be loss of surface antigen expression following treatment, such as in

the setting of anti-CD19 targeted therapy (loss of CD19). (3,6) All the above factors affect MRD assessment and require an astute pathologist/ scientist to interpret the results in the context of all the laboratory and clinical information.

1.2.3 Flow cytometry instruments and analysis platform

The flow cytometry unit of the National Health Laboratory Service (NHLS) at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) has both Beckman Coulter Navios and NaviosEx flow cytometers. These instruments are supported by the Beckman Coulter (BC) Kaluza C™ software platform which is used for data analysis and reporting and was introduced to the unit in March 2020. Multi-parameter immunophenotypic analysis is employed via four premixed, dry ClearLLab 10-color panels: Lymphoid (B-cells, T-cells), Myeloid (M1, M2). These panels use the FITC, PE, ECD, APC PB and KrO flurochromes, and the tandem dyes PC5.5, PC7, APC- A700 and APC-A50 dyes. (14) A multi-centre study evaluated the reliability of ClearLLab 10C panels and showed that these panels achieved a sensitivity of 96% and specificity of 95% with respect to the patients' final clinical diagnosis after an analysis of 453 spent samples. (15)

In addition to the above, there are cytoplasmic panels, which are compiled in-house and are mainly utilised for lineage assignment for first presentation/ diagnostic samples. The cytoplasmic panels are not routinely used for follow-up.

Based on the immunophenotype of the tumour population at presentation, only a subset of the above standard premixed panels are used for follow-up MRD assessment. The maximum number of events currently analysed in our centre is 50 000 events.

1.3 Gene fusion transcript assessment by RQ-PCR

Up to 40% of ALL cases harbour recurrent chromosomal translocations which result in chimeric/fusion gene transcripts. (3) These fusion transcripts are thought to be expressed during active disease, thus serve as good targets for MRD monitoring. (5) The B-ALL recurrent translocations for which our lab offers RQ-PCR testing, include: t(9;22)(q34;q11) *BCR/ABL1*, t(12;21)(p12,q22) *ETV6/AML1*, and t(1;19)(q23;p13) *TCF3/PBX1*. This is performed on the ABI 7500 Fast Real Time PCR system and uses fluorescent-based chemistry for qualitative and quantitative detection of deoxyribonucleic acid (DNA) sequences. (16)

RQ-PCR derives DNA products from ribonucleic acid (RNA) transcripts with quantitation made by using standard curves. (9) The sensitivity is estimated to be 1 leukemic cell per $10^{\wedge 4}$ to $10^{\wedge 5}$ (higher than 4-colour panel flow cytometry) or 0.01% - 0.001%. (5) RQ-PCR is highly sensitive but there are certain limitations. These include RNA instability and the lack of standardization of RNA quantification, which is due to variability in the number of RNA transcripts per cell and amongst patients. (9) Furthermore, in the absence of recurrent genetic abnormalities which are tested by RQ-PCR, this test cannot be employed for MRD monitoring.

1.4 PCR for IgH gene rearrangement

Part of the development of B cells includes antigen receptor gene rearrangement which is unique to each cell. This involves VDJ (variability-diversity-joining) segment recombination (17), which produces DNA sequences of different sequences and lengths. PCR can then be used to amplify targeted DNA segments (amplicons) in the immunoglobulin gene by using specific primers. (17) Subsequently, these DNA products can be analysed to determine clonality. If a clonal population is present, the DNA products reflect one or two prominent amplicons whereas in normal cell populations, a polyclonal background of products is seen.

Our laboratory uses the IdentiClone® *IGH* Gene Clonality Assay which contains multiple consensus DNA primers that target the regions within the variable and the joining regions; and the diversity and joining regions. (18) The end products are analysed by differential fluorescence detection using capillary electrophoresis.

This molecular method can be applied to almost all B-cell ALL cases, however, it is particularly useful in cases that do not bear recurrent genetic lesions (thus cannot be monitored on RQ-PCR) as well as cases lacking aberrant surface antigens making it challenging to identify disease on flow cytometry. (19)

The sensitivity of this method is estimated to be between 0.01% - 0.001%. (3) The conventional PCR method can be further refined to techniques such as nested PCR, digital droplet PCR or high throughput NGS to further improve sensitivity. (3,5) However, these techniques are may not be standardised and are not widely available. (3)

Limitations for IgH PCR include the potential for false negative results as VDJ rearrangements that were initially present may be lost during clonal evolution and secondly the platform cannot distinguish viable and non-viable cells (20). Studies have shown good correlation between multiparametric flow cytometry and PCR for IGH rearrangement (13,21).

2. STUDY OBJECTIVES

2.1 Hypothesis

MRD monitoring by 10 colour flow cytometry, on the Kaluza C™ platform, has similar sensitivity and specificity to RQ-PCR, which is the gold standard for MRD testing.

2.2 Aim

- To determine the diagnostic sensitivity and specificity of MRD testing by 10 colour flow cytometry (set at capture of 50 000 events), on the Kaluza C™ platform, in comparison to the gold standard, RQ-PCR testing.

2.3 Primary Objectives

- To evaluate the concordance between flow cytometry and RQ-PCR MRD testing in B-ALL cases with recurrent genetic abnormalities, that are in morphologic remission.

2.4 Secondary Objectives

- If there are sufficient number of cases (i.e. minimum number of cases required for statistical significance, please refer to the sample size calculation below) from the above analysis, which have matching IgH and RQ-PCR results, the concordance between IgH PCR and RQ-PCR MRD testing will be evaluated.

3. STUDY METHODS

In this retrospective study, we will review and analyse the degree of concordance of our flow cytometry results with RQ-PCR MRD in B-ALL follow-up cases. Notwithstanding our laboratory's flow cytometry assay is not MRD tailored, we aim to take into consideration any insights this analysis may reveal for the adaptation of the assay for MRD in future.

3.1. Study Setting

The NHLS Flow cytometry, Molecular Haematology Laboratory and Somatic Cell Genetics unit at the CMJAH. These laboratories are SANAS accredited with associated subscription to both local and international EQA (external quality assurance) testing programs.

3.2. Study design

A retrospective cohort study.

3.3. Study population

Paediatric and adult patients with a diagnosis of B-ALL, bearing one of the following recurrent genetic lesions; t(1;19), t(12;21) or t(9;22), for whom flow cytometry and RQ-PCR were performed at the CMJAH NHLS, at the same time point, for MRD monitoring. Given that MRD monitoring occurs at several different time points during therapy (i.e., post-induction, post-consolidation, post-maintenance), each of these can be used as a separate analysis point, as long as the patient is in morphological remission at the time, and both flow cytometry and RQ-PCR were performed.

3.3.1. Inclusion criteria

- Bone marrow aspirate samples submitted for MRD testing on flow cytometry and RQ-PCR, at the same time point, which were analysed between March 2020 and June 2023.
- The corresponding bone marrow aspirate findings must confirm morphologic remission (defined as less than 5% blasts)
- Bone marrow aspirate samples analysed on the Navios and NaviosEx flow cytometer, on the Kaluza C™ software platform, at the CMJAH NHLS flow cytometry laboratory.
- Bone marrow aspirate samples analysed with RQ-PCR for t(9;22)(q34;q11) *BCR/ABL1*, t(12;21)(p12,q22) *ETV6/AML1*, and t(1;19)(q23;p13) *TCF3/PBX1* fusion transcripts.

3.3.2. Exclusion criteria

- Bone marrow aspirate samples, in morphologic remission that were analysed with RQ-PCR without corresponding flow cytometry analysis.
- Bone marrow aspirate samples, in morphologic remission, that were analysed with flow cytometry without corresponding RQ-PCR.

- Cases in which a presentation RQ-PCR was not performed. This as the subsequent RQ-PCR results are calculated as a ratio of the presentation percentage.
- Bone marrow aspirate samples for which RQ-PCR has failed (low RNA yield, or technical issues)
- Bone marrow aspirate samples for which flow cytometry and RQ-PCR failed to detect MRD and the corresponding aspirate reported that the sample was haemodilute and non-representative of the marrow status.
- Bone marrow aspirate samples which are not in morphological remission.
- Cases that fall outside of the stipulated study period.

A total of , 83 samples were included in the data analysis

- Sample degradation
- Insufficient RNA yield
- No baseline RQ-PCR
- Haemodilute samples
-

3.4. Data collection

3.4.1 Laboratory parameters to be recorded

Results available on the laboratory information service (LIS) to be recorded include the patient age, sex, recurrent translocation at presentation, presentation cytogenetic analysis and ploidy results (flow cytometry, cytogenetic), sample collection date, time point in treatment, bone marrow sample quality, morphology blast percentage, percentage disease on flow cytometry, percentage disease on RQ-PCR, results for PCR for IgH rearrangement studies and follow-up fluorescence *in-situ* hybridisation (FISH) results.

Please see APPENDIX I for the data collection sheet.

3.4.2. Sample size

The Kaluza C™ software analysis platform was instituted in March 2020. All samples that meet the inclusion criteria from March 2020 to June 2023 will be included. The estimated number of processed RQ-PCR samples in this time period is estimated to be at least 327.

However, a sample size calculation using expected sensitivity and specificity method was performed to confirm the minimum number of samples required.

The formulas and calculation will be based on statistical methods described by Buderer. (22)

The base formulas: $TP + FN = Z^2_{\alpha/2} SN(1-SN)/W2$ where TP is the proportion of truly positive cases, FN false negative cases, SN the specified sensitivity of the test and Z is the value from a standard normal table with α being the type 1 error rate, $Z^2_{\alpha/2} = 1.96$ for 95% CI (confidence interval).

The sample size required for Sensitivity is N1 = TP + FN/P

[where P is the local prevalence i.e. 23.4% in our case (23)]

Assuming an expected sensitivity of 96%:

$$TP + FN = Z^2_{\alpha/2} SN(1-SN)/W2$$
$$= 1.96^2\ 0.96\ (1-0.96)/0.10^2$$
$$= 14.75$$
$$N1 = TP + FN/P$$
$$= 14.75/0.23$$
$$= 64.1$$

The sample size required for Specificity is N2 = FP+ TN/ (1-P)

Assuming an expected specificity of 95%:

$$FP + TN = Z^2_{\alpha/2} SP(1-SP)/W2$$
$$= 1.96^2\ 0.95\ (1-0.95)/0.10^2$$
$$= 18.24$$
$$N2 = FP + TN/ (1-P)$$
$$= 18.24/0.23$$
$$= 79.3$$

The larger sample size N2 (79) will be used as our minimum number.

4. DATA ANALYSIS

A list of all samples processed for RQ-PCR in the specified period will be obtained from the NHLS Somatic Cell Genetics Unit database at CMJAH. Each item in the list will be screened according to the inclusion and exclusion criteria described above by reference to the corresponding data on LIS.

The data will be transcribed onto an Excel spreadsheet (variables listed on Appendix 1). Two analyses will be performed, the first between flow cytometry and the gold-standard RQ-PCR, and the second, between IgH rearrangement studies and RQ-PCR. The sensitivity, specificity,

positive predictive value (PPV) and negative predictive value (NPV) for each analysis will be calculated and presented in two separate 'two-way-tables' (see Table 1).

True positive cases are defined as the number of patients with a positive test who have the disease; false positives -the number of patients with a positive test but do not have disease; false negatives - the number of patients with a negative test but have disease and true negative the number of patients with a negative test who do not have disease.

The formulas to calculate the four parameters are as follows:

1) Sensitivity= A/(A+C) × 100
2) Specificity= D/(D+B) × 100
3) Positive predictive value (PPV)= A/(A+B) x 100
4) Negative predictive value (NPV): D/(D+C) x 100

Table 2. Two-way table used to calculate sensitivity, specificity, positive and negative predictive values (24)

	Positive	Negative	Total
Positive	A (True Positive)	B (False positive)	T Test Positive
Negative	C (False negative)	D (True Negative)	T Test Negative
	T Disease	T Non-Disease	Total

5. ETHICAL CONSIDERATIONS

An application will be submitted to the Human Research Ethics Committee of the University of the Witwatersrand for approval to conduct the study. The data will be anonymised, with patient identifying information to be stored in a password protected Excel spreadsheet, with unique study numbers providing the link to patient results.

6. TIMELINE

	May	June	July	Aug	Sept	Oct	Nov	Dec	Jan	Feb	March	April
Literature review												
Protocol preparation												
Ethics application												
Protocol assessment												
Data collection												
Data analysis												
Write- up: thesis												

7. FUNDING

This study will make use of data that is already available on the LIS. There will be no new collection or processing of any samples from patients. A funding application will be submitted to the Faculty Research Committee of the University of the Witwatersrand to cover publication costs.

8. LIMITATIONS

Limitations of this study include:

- Patient files will not be examined, thus, evaluation of clinical outcomes in relation to laboratory results will not be examined.
- The number of events analysed may vary between the cases. The maximum number of events analysed is set at 50 000 on the Navios/NaviosEx instruments. This is notably lower than the recommended number of events used internationally for MRD testing. (2,5,6, 25)

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APPENDICES

APPENDIX I

DATA SHEET:

Sample number: ……

Patient age: …….. Patient sex: ……..

Patient location (hospital): ……..

Time point: Post-induction Post-consolidation End of maintenance Other

PRESENTATION INFORMATION:

FISH:…….

RQ-PCR:……

Cytogenetics:…….....

Ploidy results:…….

BONE MARROW ASPIRATE MORPHOLOGY:

% blasts:

FLOW CYTOMETRY:

Number of events:……

ClearLLab Panel(s)

	B		T		M1		M2

% disease:……..

RQ-PCR:

RQ-PCR		t(9;22)(q34;q11)		t(12;21)(p12,q22)		t(1;19)(q23;p13)

MRD RQ-PCR %: ……

PCR IgH: Monoclonal Polyclonal Failed

Other……

FOLLOW-UP CYTOGENETICS(if available): …….

FOLLOW-UP FISH …….

APPENDIX II

The pre-mixed ClearLLab 10C Panels used: (15)

	Fluorochromes/dyes									
Panels:	FITC	PE	ECD	PC5.5	PC7	APC	APC-A700	APC-A750	PC	KrO
ClearLab 10C B Cell Tube	Kappa	Lambda	CD10	CD5	CD200	CD34	CD38	CD20	CD19	CD45
ClearLab 10C T Cell Tube	TCRγδ	CD4	CD2	CD56	CD5	CD34	CD7	CD8	CD3	CD45
ClearLab 10C M1 Tube	CD16	CD7	CD10	CD13	CD64	CD34	CD14	HLA-DR	CD11b	CD45
ClearLab 10C M Tube	CD15	CD123	CD117	CD13	CD33	CD34	CD38	HLA-DR	CD19	CD45

In-house prepared cytoplasmic panels:

	FITC	PE	ECD	APC
Panel 1	cCD3	cMPO	cCD45	cCD34
Panel 2	nTDT	cCD79a	cCD45	-
Panel 3	cCD34	cCD22	cCD45	-

Appendix V: Turn-it-in Report

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CONTENT TYPES

An original research article

The article should be no longer than 4000 words and the layout should follow the following order: title page, abstract and keywords, main text, discussion and conclusion, acknowledgements and references. Ethics committee approval must be included in the methods section of the article.

The title page should include a short title, authors' names including initials, qualifications, address, telephone and the email address of the corresponding author to whom the proofs and requests would be directed. The title should not contain any abbreviations. The title page should also include, the ORCID ID of each author, and a declaration of conflict of interest and sources of funding.

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