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A multicentre study to evaluate an in-house multiparameter immunophenotypic panel to identify precursor B-cells in the determination of measurable residual disease in paediatric B-cell acute lymphoblastic leukaemia

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UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

Post-Graduate Research Report

A multicentre study to evaluate an in-house multiparameter immunophenotypic panel to identify precursor B-cells in the determination of measurable residual disease in paediatric B-cell acute lymphoblastic leukaemia

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ABBREVIATIONS

- ALB — Abnormal B-progenitor cells
- ALL — Acute lymphoblastic leukaemia
- APC-H7 — Allophycocyanin-cyanine dye analogue
- AUC — Area under the curve
- BFM — Berlin-Frankfurt-Münster (BFM)
- BMA — Bone marrow aspirate
- CD — Cluster of differentiation
- CHBAH — Chris Hani Baragwanath Hospital
- CMJAH — Charlotte Maxeke Johannesburg Academic Hospital
- DGMC — Donald Gordon Medical Centre
- DNA — Deoxyribonucleic acid
- ECD — Phycoerythrin-Texas Red conjugate
- EQA — External quality assurance
- FcR — Fragment region receptor
- FISH — Fluorescence in situ hybridisation
- FITC — Fluorescein isothiocyanate
- FS — Forward scatter
- HSCT — Haemopoietic stem cell transplant
- IgH — Immunoglobulin heavy chain
- IQC — Internal quality control
- LAIP — Leukaemia-associated immunophenotype
- LDT — Laboratory-developed test
- LLOQ — Lowest limit of quantification
- LMIC — Low and middle income countries
- LOD — Limit of detection
- mAb — Monoclonal antibody
- MFC — Multiparameter flow cytometry

- MFI Mean fluorescence intensity
- MRD Measurable residual disease
- M2 Myeloid-cell 2
- NSB Nonspecific binding
- NHLS National Health Laboratory Service
- PBS Phosphate-buffered saline
- PCR Polymerase chain reaction
    - cPCR (conventional PCR)
    - RQ-PCR (real-time quantitative PCR)
- ROC curve Receiver Operating Characteristic curve
- SOP Standard operating procedure
- SS Side scatter

DEFINITIONS AND TERMINOLOGY

- **Boolean gating** — Also logic gating
  Refers to individualised flow cytometric gating strategies allowing for the accurate identification of leucocyte populations based on an "AND", "OR" and "NOT" logic

- **Comparability** — Also reliability, agreement and accuracy
  Refers to the extent of agreement between results generated by a method and its true value

- **Carry-over assessment** — Refers to the analysis for any discrete amount of analyte carried by a measurement system from one sample reaction to the next
  Involves the calculation of the Broughton formula and T-test

- **Compensation** — Process by which intrinsic spectral overlap between fluorochromes is mathematically eliminated using computer software

- **EQA** — External quality assurance
  Refers to the planned and systematic set of quality activities focused on providing confidence that quality requirements will be fulfilled

- **Fluorochrome** — Fluorescent molecules attached to nucleotides that absorb energy from light and raise to an excited state where they emit energy as fluoresce when they return to ground state

- **FRET** — Fluorescence resonance energy transfer
  Refers to a visual signal technique detecting fluorochrome-emitted energy that is transferred from one fluorochrome to the next

- **Haemodilution** — Refers to the dilution of a bone marrow aspirate sample with sequential pulls on sample acquisition resulting in a sample resembling peripheral blood constituents

- **IQC** Internal quality control

  Refers to a set of activities or techniques whose purpose is to ensure that all quality requirements are being met

- **LDT** Laboratory-developed test

  Refers to an assay that has been developed in a single laboratory, or an approved test system which has been modified or used outside its intended scope

- **LOD** Limit of detection

  Refers to the lowest quantity of a component that can reliably be detected with a given analytical method with stated confidence or statistical significance

- **Logistic regression** A statistical method commonly used for the analysis of binary outcome variables when exposed to single or multiple exposure variables.

- **LLOQ** Lowest limit of quantification

  Refers to the lowest amount of an analyte detected by a test method with stated probability provided accuracy and precision have been demonstrated

- **MFI** Mean fluorescence intensity

  Measurement used to define the mean intensity and level of mAb expression, directly proportional to instrument optimisation (IQC)

- **NPV** Negative predictive value

  Refers to the proportion of test positives that are truly positive

- **PPV** Positive predictive value

  Refers to the proportion of test negatives that are truly negative

- **Precision** Refers to the closeness of agreement between repeated measurements

	in a sample
▪ **Sensitivity**	Refers to the proportion of true positives correctly identified as such
▪ **Specificity**	Refers to the proportion of true negatives correctly identified as such
▪ **SOPs**	Standard operating procedures Refers to a set of written instructions that describe in detail how a laboratory process is to be carried out so as to ensure standardisation within the laboratory
▪ **Spectral overlap**	Refers to the leakage of donor dye fluorescence and acceptor dye diminished fluorescence as a result of the introduction of multiple fluorochromes capable of being excited by a single light source
▪ **Target population**	Refers to leukaemic cells of interest that express a particular leukaemia-associated immunophenotype (LAIP)
▪ **Validation**	A defined process that provides objective evidence that a test system meets the requirements for intended use by manufacturer-specified claims. Minimum requirements include accuracy, precision and linearity assessment.
▪ **Verification**	An abbreviated process to demonstrate that a test system performs in substantial compliance to previously established manufacturer claims

ABSTRACT

Background: Periodic assessment of measurable residual disease (MRD) is an important prognostic factor in the management of paediatric B-cell acute lymphoblastic leukaemia (ALL). Conventional polymerase chain reaction (cPCR) and multiparameter flow cytometry (MFC) are well-established in MRD determination, the latter with no current optimal immunophenotypic panel by international consensus.

Objective: To determine whether an in-house immunophenotypic panel containing the discriminatory CD58-FITC (cluster of differentiation; fluorescein isothiocyanate) marker compares with cPCR in the detection of paediatric B-cell ALL MRD.

Methods: This prospective descriptive validation study was performed on diagnostic and follow-up bone marrow aspirate samples, comparing an in-house immunophenotypic panel against the standardised commercial ClearLLab 10C^{TM} B-cell/myeloid cell-2 (M2) panels in MRD assessment. These findings were then compared to cPCR to determine individual panel performance and predictive power.

Results: Both immunophenotypic panels demonstrated 100% concordance in the identification of the leukaemia-associated immunophenotype (LAIP) on all diagnostic samples. The in-house immunophenotypic panel showed a higher sensitivity and specificity, and greater association with cPCR in MRD assessment in follow-up samples. In combination with shared backbone markers of the ClearLLab 10C^{TM} B-cell/M2 panels, inclusion of CD58-FITC and CD81-APC-H7 (allophycocyanin-cyanine dye) proved most informative in accurate distinction between regenerating B-cell precursors and residual leukaemic cells.

Conclusion: This work confirms the findings of previous studies, where discriminatory marker CD58-FITC in combination with backbone informative markers demonstrates both superior diagnostic and monitoring utility in paediatric B-cell ALL. The in-house immunophenotypic panel offers an attractive, comparable alternative in MRD determination in this patient population whilst awaiting cPCR results,

raising the possibility of earlier clinical decision-making with potential improvement of morbidity and mortality outcomes.

Key words: B-cell acute lymphoblastic leukaemia; measurable residual disease; conventional polymerase chain reaction; multiparameter flow cytometry.

1. BACKGROUND AND INTRODUCTION

1.1. Overview of B-cell acute lymphoblastic leukaemia

B-cell acute lymphoblastic leukaemia (ALL) refers to the malignant transformation and proliferation of B-cell lymphoid progenitor cells arising within the bone marrow.[1] A classical bimodal age distribution has been described, with the majority of cases within the paediatric population, and a second peak later in adult life.[2,3] Accurate epidemiological data reflecting the true incidence of B-cell ALL within the sub-Saharan African paediatric population is limited by resource constraints, including accessibility to healthcare and cancer surveillance strategies employed.[3–5] High rates of loss-to-follow-up and delay in intervention compound the untimely loss of life in low and middle income countries (LMIC) in particular.[4]

Risk stratification and prognostication is central to paediatric B-cell ALL management, allowing for appropriate initial regimen choice, individualised treatment consideration and eligibility for haemopoietic stem cell transplantation (HSCT).[2,6] Traditional prognostic factors include patient age at diagnosis, baseline white cell count and early response to treatment by time to remission and measurable residual disease (MRD) determination, with recent molecular technologies having identified defined genetic abnormalities that contribute to this risk stratification strategy.[2,6–8] The majority of newly-diagnosed paediatric patients attain both morphological and molecular remission after completion of the induction phase of treatment with conventional cytotoxic chemotherapy regimens.[9]

1.2. Measurable residual disease in haematological malignancies

MRD refers to the subclinical presence of residual leukaemic cells that are below the level of morphological detection (5% cell threshold of detection) using molecular tools such as flow cytometry, cytogenetics and polymerase chain reaction (PCR)-based approaches.[9–12] The prognostic MRD threshold (also limit of detection, LOD) of $\leq 0.01\%$ suggests that, should a patient have cellular MRD levels above this demarcated value at a specified time point during therapy, it is associated with a significantly higher risk of future relapse.[13–15] The definition of true MRD negativity is debatable and depends on the MRD assay and corresponding sensitivity for detection of very small populations of residual leukaemic cells.[10]

A negative MRD status implies that no MRD is detected with a high level of certainty, using molecular techniques that have been validated to measure low quantitative levels of residual leukaemic cells.[10,16]

In paediatric B-cell ALL, MRD status informs the clinician about remaining disease burden after induction therapy using institution-specific, modified Berlin-Frankfurt-Münster (BFM) treatment protocols, reflecting chemosensitivity and treatment response.[15,17] Furthermore, MRD status forms a major predictor of overall and disease-free survival, with the potential for earlier clinical decision-making pertaining to regimen modification (i.e. therapy minimisation versus intensification, or review of HSCT eligibility), improving morbidity and mortality in this patient population.[2,6,9,11,18] The level of MRD in paediatric patients pre-HSCT conditioning has a significant impact on post-transplant outcomes and prediction for post-transplant disease relapse.[11] B-cell ALL MRD negativity has also been used as a surrogate therapeutic endpoint for drug approval in clinical trials over the last two decades.[16]

According to a number of studies, periodic B-cell ALL MRD measurements at days 15, 33 and 78 have proven to provide information for the identification of very early treatment responders as well as a small group of poor treatment responders. [9,10,19] MRD measurements at early and later treatment time points correlates with long-term relapse-free survival.[15] However, despite MRD exclusion patients may still relapse due to underlying clonal evolution and/or persistence of, and subsequent clonal evolution of subclonal populations with an “antigenic shift” as these cells acquire new immunophenotypes.[9,10,13,20]

MRD determination requires skilled interpretation to differentiate between normal haemopoietic cells (including B-cell lineage precursors or haematogones) and aberrant leukaemic target populations.[19,21,22] Expanded populations of haematogones are observed in children, comprising up to 50% of marrow cellularity and as a result, pose a diagnostic challenge in paediatric B-cell ALL MRD analysis.

1.3. Overview of existing B-cell ALL MRD strategies

Classical MRD strategies include conventional PCR (cPCR) for the identification of B-cell immunoglobulin heavy chain (IgH) gene re-arrangements, real time quantitative PCR (RQ-PCR) for defined genetic abnormality detection (if present), flow cytometry to identify the leukaemia-associated immunophenotype (LAIP), and the detection of specific genetic aberrations by cytogenetics [chromosomal analysis and fluorescence in situ hybridisation (FISH)].[7,10,18,23,24] For the purpose of this research report, further discussion is focused around flow cytometry and cPCR, both methods demonstrating good correlation in MRD detection in paediatric B-cell ALL.[7,9,25]

The cPCR method for B-cell ALL MRD determination is well-standardised, being the most studied molecular technique for MRD detection, large scale validations having been conducted worldwide using various manufacturer-specified kits.[8,25,26] The general principle involves rapid in vitro enzymatic amplification of specific deoxyribonucleic acid (DNA) segments using sequence-specific single-stranded primers that target desired DNA sequences within conserved genetic framework regions. Endpoint analysis of amplified products may then be interpreted using various assays, including fragment analysis by capillary electrophoresis.[8,25]

The National Health Laboratory Service (NHLS) at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) currently uses the IdentiClone IgH Gene Clonality PCR kit for B-cell IgH gene re-arrangement detection, with a sensitivity of 93% and specificity of 92% when compared to Southern Blot analysis.[8,26] According to various authors, the LOD of cPCR ranges between 0.01 – 0.001%, which may be increased using nested PCR or higher DNA input strategies, the latter not currently employed in our laboratory.[8,25] In comparison to its superior RQ-PCR counterpart where a specific defined genetic abnormality has been identified, cPCR offers a major advantage where it may be applied to the majority of B-cell ALL patients, with the exception of a few cases where IgH gene re-arrangement cannot be identified at diagnosis (i.e. polyclonal or failed initial analysis).[25]

However, despite the accurate and sensitive detection of low frequencies of B-cell ALL leukaemic cells by amplification of small DNA quantities, cPCR has its limitations.[8–11,14,25] In addition to the risk of contamination and initial start-up cost, the modality does not offer the opportunity for real-time, same-day clinical decision-making regarding patient management. Furthermore, owing to patient-specific unique variable, diversity and joining gene segment re-arrangements, each case requires individualised assessment.[25] These gene re-arrangements are not essential for leukaemic cell survival and in the advent of clonal evolution, patients may relapse with clones that do not harbour the initial diagnostic gene re-arrangement, leading to possible false negative interpretation of MRD status.[9–11,25]

Flow cytometry assigns an immunophenotypic profile to a target population of cells, identifying physical characteristics including cell size and complexity, as well as antigen expression. Antigen-specific fluorochrome-labelled monoclonal antibodies (mAbs) are added to the sample, excited by laser light source emission at varying wavelengths and detected by fluorescent detectors and mirrors which are in turn measured by flow cytometric software.[27,28] In comparison to cPCR, flow cytometry has a quicker turnaround time, offering timeous diagnostic reporting to facilitate appropriate patient management. Furthermore, the modality is able to detect subclonal populations and is less likely to pick up apoptotic blast cells which may yield false positive MRD results.[7] Limitations of flow cytometry include the need for expert analysis, particularly if a very small target population is present, as well the requirement for a minimum number of cellular events for accurate interpretation. Furthermore, when analysing a bone marrow aspirate (BMA) sample the quality of the sample directly implicates immunophenotypic assessment, particularly if a haemodilute specimen is obtained, as this may underestimate residual leukaemic burden and yield a false negative MRD status.[11,17,18,27–29]

Multiparameter flow cytometry (MFC) measures several cellular characteristics simultaneously for total cell analysis by incorporating a larger number of novel fluorochrome-labelled mAbs to better identify a target population.[30,31] Together with advanced instrumentation and software where “rare event” analysis may be employed to artificially amplify and enable easy visualistion of very small number of leukaemic

cells, MFC improves the overall precision of specimen immunophenotypic analysis.[11,28,29,32] MFC for LAIP detection has a LOD similar to cPCR ranging between 0.01 – 0.001%, which is increased when more than 50 000 events are analysed.[25] Determination of the assay's sensitivity and specificity is dependent on the number of fluorochrome-labelled mAbs detected by laser light application. In two recent multicentre observational studies, one conducted in Europe and North America and the other in South Africa, the diagnostic accuracy of the ClearLLab 10C™ panels using the Beckman Coulter's KaluzaC™ software system was determined when applied to patient specimens suspected of a haematological malignancy. Both studies yielded high sensitivities (96% and 100%) and specificities (95% and 100%) respectively within their cohorts.[33–36]

Participation in proficiency testing schemes and accreditation programmes with widespread incorporation of educational material may ensure transparency and reliability in MRD monitoring using MFC. [18,29] Compliance to manufacturer-described instrument setup additionally ensures reproducible and institution-specific standardised sample analysis.[25,33,34] Novel software tools may facilitate a more objective immunophenotypic interpretation, including specific automated gating strategies and maturation pathway analysis in an attempt to standardise the identification of various B-cell populations in MRD samples.[14,37]

A major limiting factor with MFC is the concept of "spectral overlap" with the introduction of tandem conjugate (multiple) mAbs into the system.[38,39] These tandem conjugates expand the pool of mAbs that may be detected, measuring fluorescent resonance energy transfer by excitation of multiple acceptor dyes using a single laser emitting light at a particular emission spectrum. Commonly used tandem conjugates vary in their transfer capabilities from donor to acceptor dyes across mAb lots over time, resulting in the leakage of donor dye fluorescence and diminished acceptor dye fluorescence (i.e. "spectral overlap").[39] This may be overcome by the application of "colour compensation" using electronic elimination of duplicated fluorescent emission.[38] Lastly, the antigenic shift that may occur with clonal evolution may reduce the assay's accuracy, leading to possible false negative MRD interpretation.[17]

1.4. Role of flow cytometry in B-cell ALL MRD

To correctly interpret MFC immunophenotypic information, a sound understanding of normal cell biology and antigenic expression (cluster of differentiation, CD) is necessary. Additionally, strict adherence to sample preparation and acquisition of as large a number of cellular events as possible is required.[40] Normal B-cell education and maturation occurs within primary and secondary lymphoid organs, with gain and/or loss of certain antigens at variable levels of expression occurring during B-cell development (see Table 1.4.1; Appendix A). [33,41,42] It is important to distinguish between haematogones and residual or subclonal leukaemic cells, as incorrect interpretation owing to immunophenotypic overlap may adversely affect patient care.[19,21,22,43] Table 1.4.2 (see Appendix B) summarises the usefulness of previously explored antigen markers in isolation and in combination with other maturity markers in the attempt to differentiate between the two populations by MFC.[12,19,20,22,33]

Various research groups (including Becton Dickinson and Beckman Coulter) have attempted to develop standardised B-cell ALL MRD MFC data analysis protocols, however no one optimal panel exists that minimises cost and spillover effect, whilst maximising relevant immunophenotypic information obtained.[14,38] Panel selection may be individualised as a laboratory-developed test (LDT), dependent on resource and expertise availability, whilst ensuring retention of the predictive power of larger panels.[13,21,44,45]

A wide range of surface mAbs have been validated in the determination of B-cell ALL MRD. Many of these mAbs are used regularly outside of the B-cell ALL diagnostic scope, as well as less familiar markers requiring more expert interpretation.[20] Consensus regarding the precise combination of aberrant markers which identify the presence of the LAIP and subclones, if present, has not yet been reached.[19,20] Novel markers may perform well in individual validations, but may not add value in combination with other markers, implying that their value is dependent on clinical context.[20,44] Shaver elegantly describes five important considerations when selecting an appropriate panel for B-cell ALL diagnostic and follow-up MRD determination.[20] An initial gating strategy may be employed to assign lineage to the leucocyte

population within the sample, followed by markers of immaturity to identify the target population. Aberrancies detected at presentation or acquired during induction therapy may include evidence of asynchronous and aberrant maturation patterns, as well as inappropriate myeloid antigens and certain markers associated with specific B-cell ALL genetic abnormalities.[11,20,33,46] Further panel design may include a targeted therapeutic marker, whereby a reduction and subsequent normal maturation pattern attained by the relevant informative marker may indicate the absence of MRD.[40]

Several studies have rationalised a limited number of mAbs with comparable sensitivity to cPCR for MRD determination in B-cell ALL by employing "design-test-evaluate-redesign" strategies.[18,39] General consensus amongst these studies includes a combination of at least two informative, LAIP-defining aberrant markers in addition to basic "backbone" markers confirming lineage and immaturity of the target population. Panel optimisation involves choosing informative markers that are both discriminatory enough to detect subsets of B-cell ALL cases that comprise less striking abnormal immunophenotypes, and detect subtle differences from background developing precursor B-cells.[16,44] The combinations of (CD38/CD58), (CD58/CD81) and (CD81/CD123) are most commonly proposed using this panel design strategy, with CD38 being the most informative across studies, with superior diagnostic utility of CD58 having been proposed.[14,16,22,40,44,47] Adoption of these mAb combinations assists in the development of an in-house immunophenotypic formulation, standardisation and informative MRD predictive values.[20]

At the time of submission of this research report, the predictive power of cPCR against the validated ClearLLab 10C™ panels had not yet been determined in the wider scope of laboratory practice.

2. AIMS AND OBJECTIVES

2.1. Study aim

To determine whether an in-house immunophenotypic panel containing the discriminatory CD58-FITC (fluorescein isothiocyanate) marker compares with cPCR in the detection of paediatric B-cell ALL MRD.

2.2. Study objectives

2.2.1. To verify the manufacturer-specified performances of reagents CD58-FITC and CD81-APC-H7 (allophycocyanin-cyanine dye) using remnant paediatric bone marrow samples submitted for investigation in which no active disease or infiltrate has been identified morphologically and/or by immunophenotypic analysis.

2.2.2. To compare the in-house immunophenotypic panel against the validated ClearLLab 10C™ B-cell/myeloid cell-2 (M2) in diagnostic and follow-up bone marrow investigations of paediatric patients with B-cell ALL.

2.2.3. To evaluate the predictive power of the in-house immunophenotypic panel as compared to the cPCR technique for paediatric B-cell ALL MRD determination.

3. MATERIALS AND METHODS

3.1. Ethical considerations

Ethics and research approval information and documents may be reviewed under "Ethics and research approval documents". Individual patient consent was not required as only remnant BMA samples were used for secondary analysis. All flow cytometric data analysed was anonymised with patient identifiers removed for the purpose of this study.

3.2. Study design

This was a prospective multicentre study conducted during the period of 01 June 2021 – 31 December 2022. BMA samples were received from specified paediatric facilities at the national referral flow cytometry laboratory at the CMJAH NHLS. Pilot work conducted prior to ethics clearance was covered under the principal supervisor's ethics clearance for the evaluation and validation of kits, reagents, data analysis protocols and methods. As part of research and development, Professor Deborah Glencross initiated investigation into the addition of drop-in CD58-FITC and CD81-APC-H7 as an alternative to the validated ClearLLab 10C™ B-cell/M2 panels in the identification of haematogones and leukaemic cells

in BMA samples of paediatric patients with B-cell ALL. The previously mentioned "design-test-evaluate-redesign" strategy was employed for this component of the study.

This qualitative validation study was conducted in two parts: Part 1 comprised verification of the manufacturer-specified performance of the reagents included in the in-house immunophenotypic panel (see Figure 3.2.1) against the validated ClearLLab 10C^{TM} panels in the identification of precursor B-cells in paediatric BMA samples with no demonstrable disease. During this process, an in-house haematogone data analysis protocol was developed using the validated Beckman Coulter KaluzaC^{TM} software system. The ClearLLab 10C^{TM} B-cell/M2 and in-house immunophenotypic panels with their respective data analysis protocols were then analysed in Part 2, using both diagnostic and follow-up paediatric B-cell ALL BMAs for final determination of predictive power as compared to cPCR in MRD assessment.

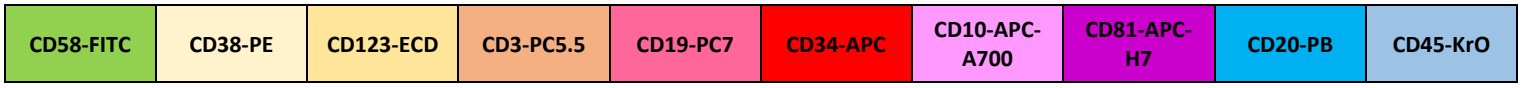


Figure 3.2.1. Components of the in-house immunophenotypic panel, including primary backbone (CD45-KrO), secondary backbone (CD19-PC7), tertiary (CD10-APC700; CD34-APC and other mAbs) and assessed markers of interest (CD58-FITC and CD81-APC-H7). CD, cluster of differentiation; FITC, fluorescein isothiocyanate; APC-H7, allophycocyanin-cyanine dye. Abbreviations of the non-assessed markers are not further described.

3.3. Sample size and eligibility

In their observational study to assess the genetic landscape of B-cell ALL in the South African Johannesburg state sector setting, Vaughn and colleagues identified all newly-diagnosed cases captured on the CMJAH NHLS flow cytometry database during a 36-month period between 2016 and 2019.[49] Their data showed that 108/461 cases (23.4%) comprised a B-cell immunophenotype, of which 82 cases (75.9%) fell within the paediatric age group (≤ 18 years old); approximately 36 – 40 new cases identified annually within the hospital complexes referring their molecular testing to CMJAH.[48] The sample size (n=45) for this study was determined using the statistical method of sensitivity and specificity (see Table 3.3.1).[50,51]

Table 3.3.1. Sample size estimation

Width of 95% CI	SN	SP	Disease prevalence	Sample size for SN	Sample size for SP	N
0.14	0.9	0.85	0.4	45	42	45

* CI, confidence interval; SN, sensitivity; SP, specificity; N, total number of samples.

The following inclusion and exclusion criteria were strictly adhered to in sample collection and eligibility determination for the duration of the study period:

Table 3.3.2. Research study patient inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
1. Paediatric age group (<18 years old or institution-dependent paediatric age range) 2. Referral paediatric centres included CMJAH, CHBAH and DGMC 3. Patients with newly-diagnosed or relapsed paediatric B-cell ALL cases with at least one follow up BMA sample submitted during the period of 01 June 2021 – 31 December 2022	1. Paediatric acute leukaemias with a mixed immunophenotype or lineage designation not possible at diagnosis 2. Inappropriate samples on which diagnostic immunophenotyping specific for this research cannot be performed (e.g. diagnostic trephine imprints and other fluids) 3. Diagnostic or relapsed paediatric B-cell ALL BMA samples in which no addition of the in-house immunophenotypic panel and/or cPCR was performed 4. Poor quality BMA samples (e.g. clotted sample or poor cell viability) 5. Incomplete sample collection (e.g. No follow-up sample received; insufficient remnant BMA sample; diagnostic sample analysed at a different facility)

* CMJAH, Charlotte Maxeke Johannesburg Academic Hospital; CHBAH, Chris Hani Baragwanath Academic Hospital; DGMC, Donald Gordon Medical Centre; ALL, acute lymphoblastic leukaemia; BMA, bone marrow aspirate; cPCR, conventional polymerase chain reaction.

3.4. Research laboratory procedures

3.4.1. KaluzaC™ in-house haematogone data analysis protocol development and technicalities

Under the guidance of the principle supervisor, an in-house haematogone data analysis protocol was developed using the Beckman Coulter KaluzaC™ software system, developed using the 20 verification samples collected in Part 1 of the study. The newly-devised data analysis protocol was applied to each verification sample (normal paediatric BMA samples containing precursor B-cells with no active disease

or infiltrate demonstrable) and tailored so that the final in-house haematogone data analysis protocol would correctly identify any precursor B-cells in the sample based on their expected immunophenotypic expression profile (see Figure 3.4.1.1; Appendix C). Several factors were taken into consideration in developing this in-house haematogone data analysis protocol, summarised below.[33,49,50]

Pre-analytical data analysis considerations

- Time versus CD45 dual plot: This ungated plot was included to show all events collected in sequence, evaluating fluidic perturbation during sample acquisition. Acceptable samples yield a uniform pattern of events.
- Forward scatter (FS) peak and FS INT (integral) dual plot: This plot was included to exclude all doublet events (i.e. non-singly occurring cells), debris and dead or dying cells. If included in analysis, these events would yield marker overexpression and false positive interpretation.
- FL3 (fluorescence; CD123) versus FL5 (CD19) dual plot: This plot allowed for the exclusion of any nonspecific binding (NSB) of mAbs to leucocyte fragment region receptors (FcRs) which may yield erroneous background fluorescence.
- CD45 versus side scatter (SS) dual plot: This plot allowed for the identification of normal background leucocyte populations in the context of CD45 expression and their respective cell complexity. Haematogones were identified based on their characteristic non-discrete, beaded-like appearance. Haematogones may express very dim CD45 and should not accidentally be excluded from analysis.
- Colour compensation: Intrinsic spectral overlap was eliminated in order to avoid artificial population identification and false positive interpretation.

SS versus CD dual plots

Using a CD45-gating strategy, separation and identification of the various leucocyte populations present was done using SS versus CD plots. In addition to the haematogone populations within normal paediatric

samples, other discernable populations identified in these plots included myeloblasts, monocytes, basophils, granulocytes, T-cells and background mature B-cells (see Figure 3.4.1.2).

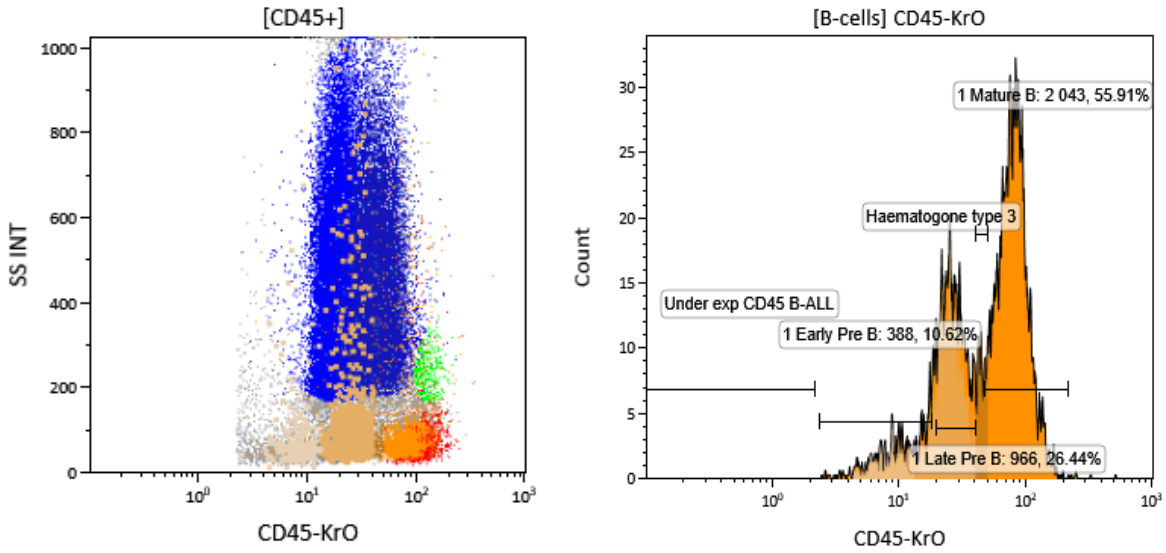


Figure 3.4.1.2. Normal paediatric bone marrow aspirate leucocyte populations, including maturing precursor B-cells (haematogones) represented by varying shades of orange. CD, cluster of differentiation; SS, side scatter; ALL, acute lymphoblastic leukaemia.

Boolean gating strategy employed

The following Boolean gating strategy was employed to correctly identify the precursor B-cell population within normal paediatric BMA samples, labelled "Precursor B-cells" in the in-house haematogone data analysis protocol:

(CD45+ AND B-cells) AND (NOT Gr) AND (NOT CD3+) AND (NOT Mo) AND (NOT NSB) AND (NOT Eosinophils)

Figure 3.4.1.3. Boolean gating strategy. Gr, granulocytes; Mo, monocytes; NSB, nonspecific binding.

In its formulation, the various leucocyte populations present were identified by applying stepwise, logical inferences and gating strategies based on the knowledge of normal cell biology and antigenic expression.[40,49]

- Granulocytes were first identified on the CD45 versus SS dual plot, showing a pattern of appropriate variability in size and complexity seen in its maturation series (labelled Gr; lighter

blue). This was followed by the identification of more mature, CD10-expressing forms on the CD10 versus SS dual plot (labelled A; dark blue), concurring with their brightest CD45 expression on the CD45 versus SS dual plot.

- Monocytes were then identified on the CD45 versus SS dual plot, showing bright CD45 expression with higher side scatter (labelled Mo; green) when compared to lymphocytes.
- T-cell lymphocytes were next identified by CD3 expression on the CD45 versus CD3 dual plot (labelled CD3+ cells; red). Colour compensation was required to correctly identify this population on the CD45 versus SS dual plot, where together with mature B-cells, they characteristically have the brightest CD45 expression and lowest complexity of all leucocytes. This involved using the "Pre-B_Plot Sheet" and slight manipulation of the CD3/CD81, CD3/CD34, CD3/CD10 and CD123/CD3 dual plots, with careful correlation on the "Analysis_haematogones panel" to ensure no erroneous background fluorescence was introduced.
- NSB was then applied using the ungated CD123 versus CD19 dual plot (labelled NSB; black). This specific dual plot was included following a pattern of NSB noted on verification analysis of these fluorochromes for leucocyte FcRs.
- Lastly, eosinophils were identified using the CD19 versus CD45 dual plot (labelled Eosinophils; teal) by their autofluorescence in violet light and distinct separation from the leucocyte populations highlighted in previous steps.

Dual plots were then analysed using the stand-alone discriminatory markers (CD19, CD58, CD81 and CD123) against the primary (CD45 and CD34) and secondary (CD10 and CD38) backbone markers and allowed for the identification of maturing precursor B-cells within a background of mature B-cells. "Rare event" analysis was incorporated to visually assist in identifying very small B-cell populations by effectively magnifying the cells without affecting its actual percentage within the sample, as described earlier in this research report.

An example of the immunophenotypic analysis of a normal paediatric sample with abundant haematogones may be found in Figure 3.4.1.4 (see Appendix D). The histogram plot included in this figure depicts the total B-cell population within the sample (using the CD45 versus CD19 gating strategy) and the variable antigenic expression and intensities of the precursor B-cells present (using the Boolean gating logic described above).

3.4.2. Study-specific bench procedure

At the CMJAH NHLS flow cytometry laboratory, trained technologist personnel performed daily bench internal quality control (IQC) and formally processed samples for immunophenotypic analysis according to the laboratory's standard operating procedures (SOPs), which may be provided on request. Beckman Coulter Navios flow cytometers were used, maintained and quality-controlled as per the manufacturer's instructions and laboratory SOPs.

Specialist haematopathologists on the flow cytometry bench reviewed all clinical history and laboratory parameters for samples received, determining whether samples would be processed for immunophenotypic analysis or not. This was done according to the Bethesda International Consensus guidelines on flow cytometric immunophenotypic analysis of haematolymphoid neoplasms, optimised and modified for local use.[51] Upon reception of suspected new or relapsed paediatric acute leukaemias, haematopathologists on the bench performed their routine immunophenotypic investigation using the established ClearLLab 10C™ and cytoplasmic panels for lineage assignation and further characterisation. Follow-up paediatric B-cell ALL samples generally required ClearLLab 10C™ B-cell/M2 panels for MRD analysis. The in-house immunophenotypic panel was requested as a secondary investigation in newly-diagnosed or relapsed paediatric B-cell ALL and their follow-up samples. This did not interfere with concurrent diagnostic practice, as only remnant patient BMA samples were utilised.

3.5. Data collection and patient records

All newly-diagnosed and relapsed paediatric B-cell ALL patients were followed for the duration of their treatment in the specified study period. Patient record of diagnostic and follow-up flow cytometry and cPCR findings were reviewed using the NHLS Webview and TrakCare laboratory information systems. This information was documented using a password-protected Excel spreadsheet with patient identifiers removed. An example of a single patient's diagnostic and follow-up information captured may be found in Table 3.5.1 in Appendix E. The completed spreadsheet may be provided on request. Further patient characteristics (e.g. age, gender and defined abnormality presence) were not evaluated and were beyond the scope of this research report.

4. DATA ANALYSIS

4.1. Part 1

In a prior validation study conducted in the CMJAH NHLS flow cytometry laboratory, CD58-FITC proved efficacious in differentiating haematogones from LAIPs and polyclonal B-cells in paediatric B-cell ALL using the Beckman Coulter DURAClone ALB (abnormal B-progenitor cells) panel.[52,53] Furthermore, although not formally included in a United Kingdom National External Quality Assurance (EQA) Service programme report in May 2021, our flow cytometry laboratory confirmed CD58-FITC's consistent and expected level of expression in the identification of the prepared sample's leukaemic cell population.[54] Specific to this study, the manufacturer-specified performance of discriminatory markers CD58-FITC and CD81-APC-H7 required verification by their inclusion in the in-house immunophenotypic panel, in which precursor B-cells at varying stages of development were identified in normal paediatric BMA samples (i.e. BMAs with an expected haematogone population and no identifiable target population). 20 remnant paediatric samples on which the ClearLLab 10C™ B-cell diagnostic panel was originally applied, were included in the verification component of this study (see Table 4.1.1; Appendix F).

Instrument and method precision was established by running 10 separate, consecutive acquisition runs (of at least 50 000 events each) on residual paediatric BMA samples from two paediatric patients, one with no disease (i.e. rich in haematogones) and one with disease (i.e. rich in B-cell ALL leukaemic cells). The in-house haematogone data analysis protocol was applied, tailored and superimposed (see description below) on consecutive samples for which the analysed data may be reviewed on request. A carry-over assessment was conducted for the diseased sample to confirm minimal carry-over between samples run on the Beckman Coulter Navios flow cytometer machine as per international standards (see Figure 4.1.1; Appendix G).

Individual lowest limit of quantification (LLOQ) assessment of the ClearLLab 10C™ B-cell/M2 and the in-house immunophenotypic panels was determined using a serial dilution approach with phosphate-buffered saline (PBS) in a paediatric BMA sample with a high B-cell leukaemic burden. Beads were added to each dilution to confirm the persistent number of white cell events in each sequential sample as the diluent (PBS) was added. Fixed quantities of the appropriate panels were then added to each labelled dilution, processed according to the flow cytometry laboratory SOPs and analysed by application of panel-specific data analysis protocols using the Beckman Coulter KaluzaC™ software system**.** Owing to limited sample quantity and time constraints, 20 000 events were analysed for each dilution. Individual panel precision was confirmed by conducting five consecutive analyses of the respective panel dilution in which no leukaemic cells were detectable. Table 4.1.2 in Appendix H may be reviewed for further information.

4.2. Part 2

The in-house immunophenotypic panel was added to each patient's diagnostic remnant BMA sample, following which individual case analysis involved tailoring the diagnostic gates of the in-house haematogone data analysis protocol to more accurately identify the leukaemic cell population, including "rare event" analysis where required. In the case of the addition of drop-in CD58-FITC and CD123-ECD (phycoerythrin-Texas Red conjugate) reagents to the established ClearLLab 10C™ B-cell tube in the

initial pilot stage of the study, the sample was analysed as per ClearLLab 10C™ B-cell data analysis protocol, with Kappa-FITC and CD10-ECD replaced by the discriminatory markers of interest.

Figure 4.2.1 shows the diagnostic gating strategy used to identify a patient's LAIP (CD19 versus CD10 dual plot gated with the "Precursor B-cell" Boolean logic described previously), that is small and non-complex (FS versus SS dual plot) showing aberrant dim CD45 expression (CD45 versus SS dual plot) and an early precursor B-cell immunophenotype. An example of the full immunophenotypic analysis of a patient's diagnostic remnant BMA sample is shown in Figure 4.2.2 (see Appendix I).

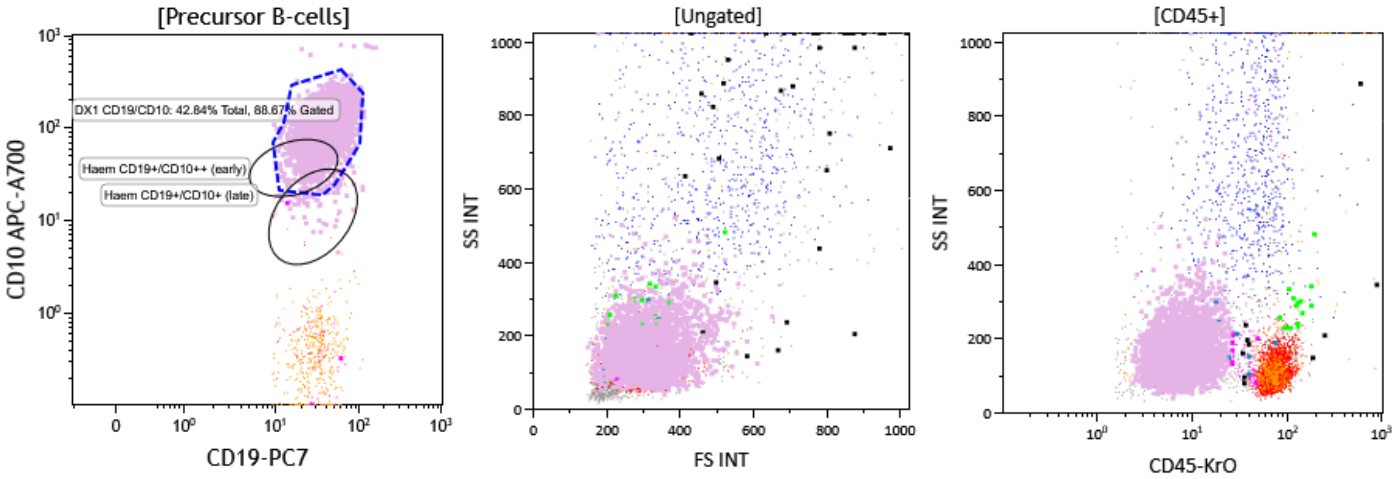


Figure 4.2.1. Example of a paediatric B-cell ALL with strategies employed to identify the LAIP. ALL, acute lymphoblastic leukaemia; LAIP, leukaemia-associated immunophenotype; CD, cluster of differentiation; SS, side scatter; FS, forward scatter; INT, integral; DX, diagnostic gate; Haem, haematogone.

Follow-up samples were analysed either with the drop-in of the reagents by means of logical inference using the in-house haematogone data analysis protocol, or by the unique superimposition analysis strategy introduced in the CMJAH NHLS flow cytometry unit. The latter involved initial analysis of patient-unique LAIPs [defined by mean fluorescence intensity (MFI)] on diagnostic samples using the in-house haematogone data analysis protocol. This was followed by the creation of a patient-specific, tailored data analysis protocol into which follow-up sample data could be selected and dragged into; effectively being superimposed on their diagnostic LAIP. This strategy is only able to be employed in current state of the art flow cytometers such as the Beckman Coulter Navios instrument used in this study.

Figure 4.2.3 below shows an example of distinct clustering within dual plots incorporating the in-house haematogone data analysis protocol discriminatory markers, indicative of residual disease. Distinct clustering was determined qualitatively by visual identification, with "rare event" analysis applied in cases with very small numbers of residual leukaemic cells. Absolute count determination was beyond the scope of this research report. An example of the full immunophenotypic analysis of a single patient's follow-up samples, both with (distinct clustering in the majority of diagnostic gates) and without residual disease (no distinct clustering present) may be found in Figures 4.2.4 and 4.2.5 respectively (see Appendix J).

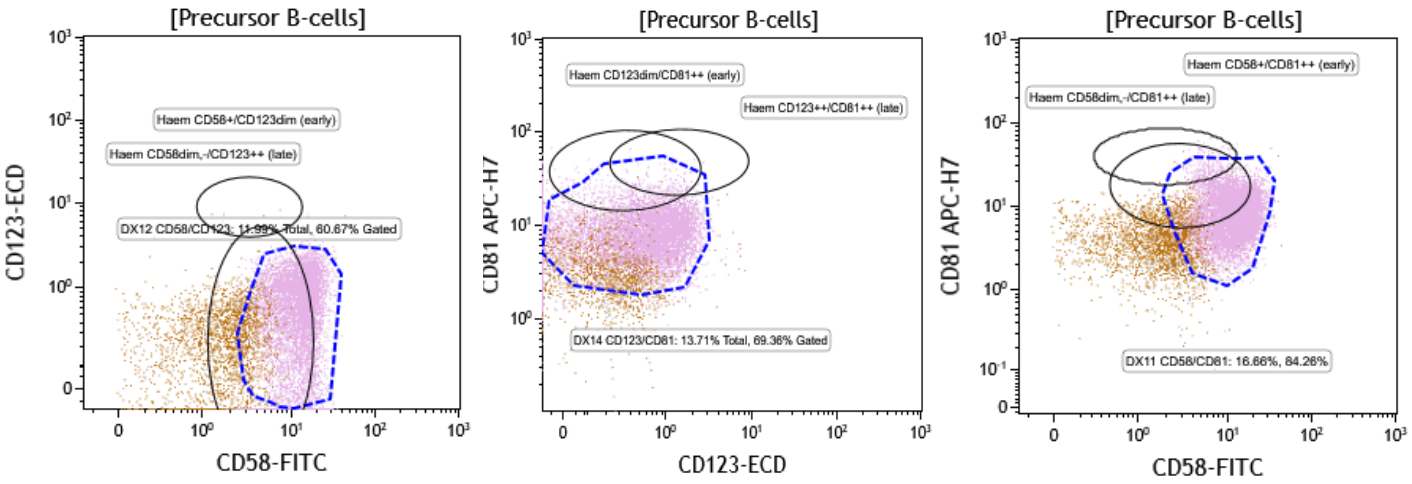


Figure 4.2.3. Example of a paediatric B-cell ALL residual disease with distinct clustering noted in the dual plots incorporating the discriminatory markers of interest in the in-house haematogone data analysis protocol. ALL, acute lymphoblastic leukaemia; CD, cluster of differentiation; DX, diagnostic gate; Haem, haematogones; FITC, fluorescein isothiocyanate; APC-H7, allophycocyanin-cyanine dye; ECD, phycoerythrin-Texas Red conjugate.

Performance and predictive powers of the ClearLLab 10C™ B-cell/M2 and in-house immunophenotypic panels as compared to cPCR were then determined, retaining binary outcome data for all eligible patients (see Tables 4.2.1 and 4.2.2; Appendix K). Due to quasi separation of points, qualitative multivariate analysis using a Firth bias-correction logistic regression model was performed to establish the association between the dependent binary outcome variable (cPCR) and independent exposure variables (individual immunophenotypic panels). Taking into consideration that some patients had more than one follow-up

sample, the first observation per patient was utilised for the purpose of estimating the area under the curve (AUC) measure, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the individual immunophenotypic panels.

5. RESULTS

5.1. Part 1

The in-house immunophenotypic precursor B-cell marker expression matched the expected variable expression in this leucocyte population. No abnormal B-cell populations (i.e. leukaemic B-cells) were identified in all samples tested. The additional markers included in the in-house immunophenotypic panel thus met the requirements for intended use and manufacturer performance specifications documented in applicable Beckman Coulter product specifications.[55] Of note, despite varying degrees of remnant sample haemodilution, NSB and requirement for colour compensation, the in-house haematogone data analysis protocol was still able to effectively visualise small populations of B-cells at various stages of maturation using "rare event" analysis allowing for visual magnification.

Instrument and method precision was confirmed, with minimal carry-over between samples run on the Beckman Coulter Navios flow cytometer machine as per international standards (i.e. less than 5% after injection of a blank sample following the running of a high concentration diseased sample), documented in Table 4.1.1 (see Appendix G). The ClearLLab 10C™ B-cell/M2 panels both had a LLOQ at a 1:1024 dilution (0.001%), whilst the in-house immunophenotypic panel had a LLOQ at 1: 512 (0.02%). Lastly, individual panel precision was confirmed by conducting five consecutive analyses at their respective identified LLOQ.

5.2. Part 2

5.2.1 Overview of eligible patients

The study fell short of the calculated sample size (n = 45) with a total of 40 eligible patients for final statistical analysis (see Figure 5.2.1.1). The sample size of 40 was sufficient enough to detect 90%

sensitivity and 85% specificity with a ± 15% precision. Reasons for ineligibility have been summarised in the figure below. Four patient samples were analysed for demonstration interest.

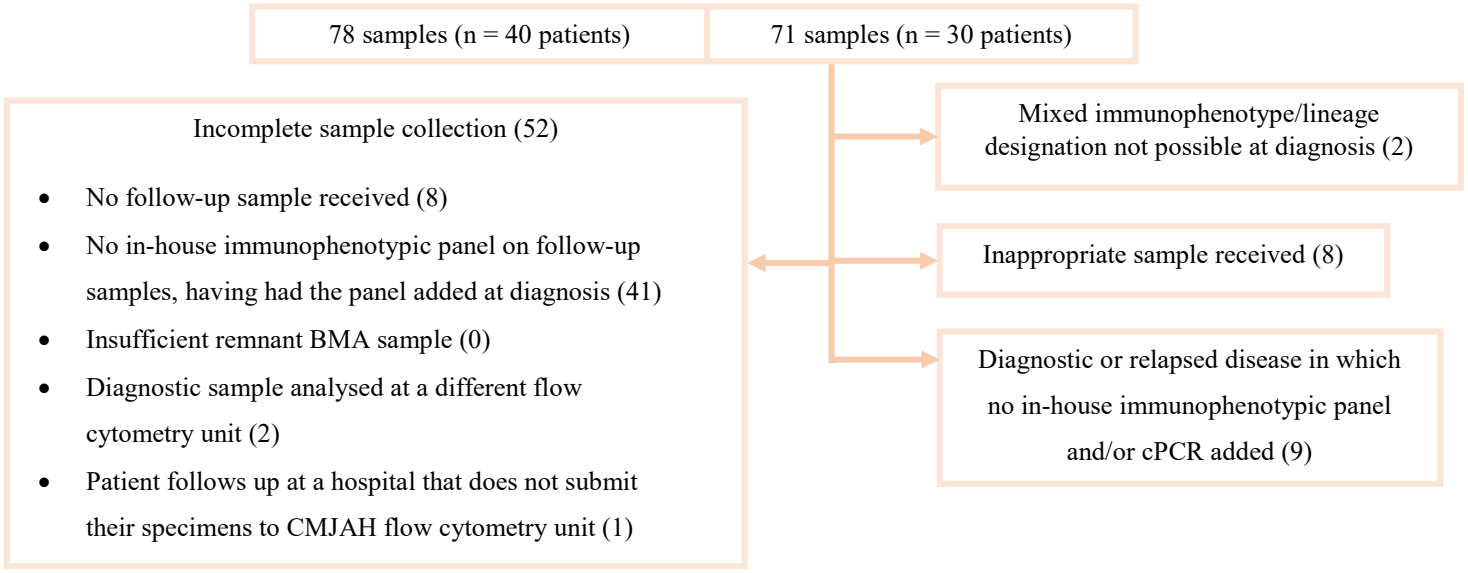


Figure 5.2.1.1. Research study patient and sample eligibility. n, number; cPCR conventional polymerase chain reaction; CMJAH, Charlotte Maxeke Johannesburg Academic Hospital.

5.2.2. Qualitative method analysis

Individual immunophenotypic panel sensitivity, specificity, PPV and NPV measures are presented in Table 5.2.2.1 below. When compared to cPCR, the ClearLLab 10C™ B-cell/M2 panels demonstrated low sensitivity (44%) and high specificity (90%), with a PPV and NPV of 0.7 and 0.8 respectively. In contrast, the in-house immunophenotypic panel demonstrated both a high sensitivity (81%) and specificity (100%), with a very high PPV (1.0) and NPV (0.9). Receiver Operating Characteristic (ROC) curve analysis further revealed an AUC of 0.7292 and 0.9583 for the ClearLLab 10C™ B-cell/M2 and in-house immunophenotypic panels respectively (see Figure 5.2.2.1). These findings provide evidence that the in-house immunophenotypic panel is overall superior in performance and predictive power as compared to the ClearLLab 10CTM B-cell/M2 panels in the determination of MRD in paediatric B-cell ALL whilst awaiting the results of cPCR.

Table 5.2.2.1. Sensitivity and specificity measures for ClearLLab 10C™ B-cell/M2 and in-house immunophenotypic panels.

	ClearLLab 10C™ B-cell/M2 panels				In-house immunophenotypic panel			
Statistic	**Estimate**	**Standard error**	**95% Confidence Interval**		**Estimate**	**Standard error**	**95% Confidence Interval**	
Sensitivity	0.4444	0.1656	0.1198	0.7691	0.8182	0.1163	0.5903	1.0000
Specificity	0.9048	0.0641	0.7792	1.0000	1.0000	0.0000	1.0000	1.0000
PPV	0.6667	0.1925	0.2895	1.0000	1.0000	0.0000	1.0000	1.0000
NPV	0.7917	0.0829	0.6292	0.9541	0.9231	0.0523	0.8207	1.0000

M2, myeloid-2; PPV, positive predictive value; NPV, negative predictive value.

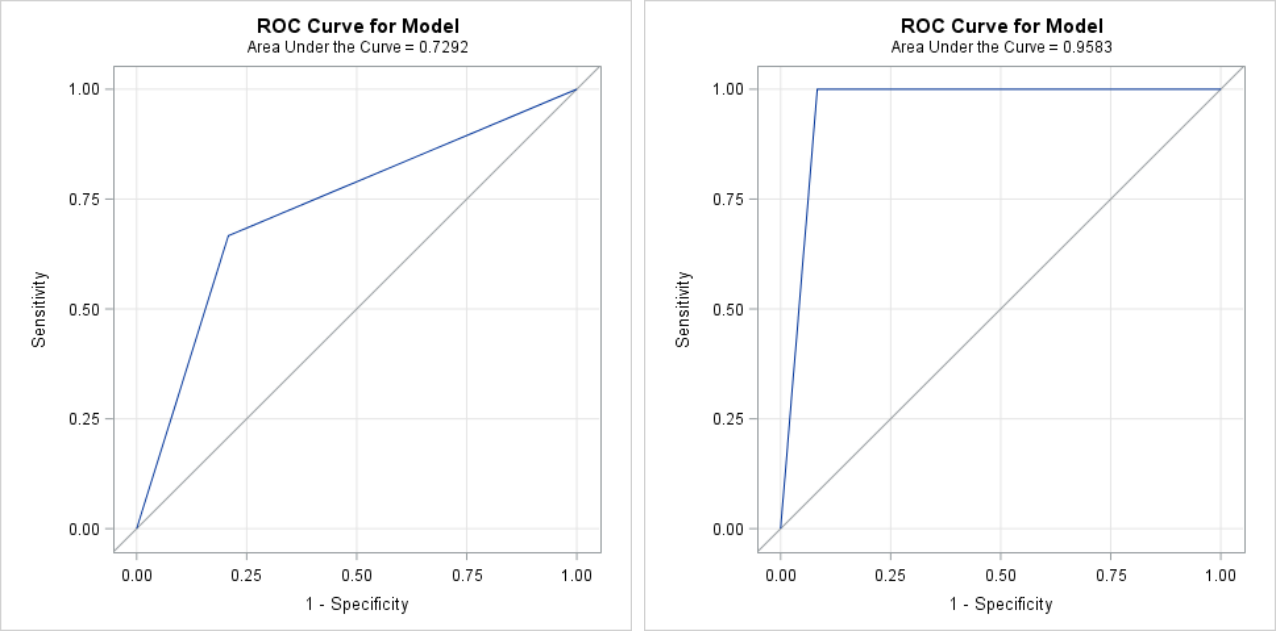


Figure 5.2.2.1. Receiver Operating Characteristic curve used to assess the association between the binary outcome (cPCR) and independent exposure variables (ClearLLab 10C™ B-cell/M2 and in-house immunophenotypic panels). cPCR, conventional polymerase chain reaction; M2, myeloid-2; ROC, Receiver Operating Characteristic; AUC, area under the curve.

5.2.3. Other noteworthy findings and observations

All samples analysed contained sufficient remnant sample upon which the in-house immunophenotypic panel could be applied. Despite variable degrees of haemodilution, immunophenotypic analysis was

possible even with a low leukaemic burden at diagnosis or follow-up, the latter visualised and amplified graphically using “rare event” analysis.

- Six samples (6/78; 8%) showed prominent NSB for which accurate analysis using the in-house immunophenotypic panel could not confirm immunophenotypic remission status. One sample (1/78; 1%) was too degenerate for any meaningful interpretation, with a similarly degenerate sample analysed using the ClearLLab $10C^{TM}$ B-cell/M2 panels.
- Overall, 16 samples (16/78; 19%) did not have specific constituents of the in-house immunophenotypic panel added at diagnosis or follow-up. This was due to intermittent unavailability (e.g. CD19-PC7, CD10-APC700 and CD3-PC5.5) with a short period when the primary backbone marker (CD45-Kro) was unavailable towards the end of the study. CD81-APC-H7 and CD123-ECD was not added in one follow-up sample owing to human error. Despite this technicality, the in-house immunophenotypic panel was able to reliably identify precursor B-cells and the LAIP in these samples by application of knowledge of normal B-cell maturation patterns, positive and negative internal controls and aberrant marker expression on leukaemic cells.

The ClearLLab $10C^{TM}$ B-cell/M2 and in-house immunophenotypic panels demonstrated 100% concordance in the identification of the LAIP on all diagnostic samples, as well as the identification of background leucocyte populations (characteristic expression profile and internal negative or positive controls). After having identified the leukaemic CD19/CD10 co-expressing population, discriminatory markers CD58-FITC and CD81-APC-H7 of the in-house immunophenotypic panel were of most value, especially when interpreted in conjunction with CD34-APC and CD38-PE, with “rare-event” analysis applied when applicable. CD123-ECD was less informative in terms of distinguishing regenerating B-cell precursors from residual disease.

- Five samples (5/78; 6%) concurred with cPCR negativity for MRD, whilst the ClearLLab $10C^{TM}$ B-cell/M2 panels suggested or could not exclude a small population of residual disease. The

main reason for the inability of exclusion of residual disease in the established panels were due to the absence of aberrant markers to better distinguish residual disease versus regenerating precursor B-cells. The in-house immunophenotypic panel is superior in this regard, capable of distinguishing the full maturation series based on appropriate discriminatory marker expression and level of brightness.

- Eight samples (8/78; 10%) concurred with cPCR positivity for MRD, whilst ClearLLab 10C™ B-cell/M2 panels were not suggestive of residual disease or could not exclude residual disease due to the absence of aberrant markers described above.
- Eight samples (8/78; 10%) showed out of range clonal products of uncertain clinical significance by cPCR, for which the concurrent in-house immunophenotypic panel confirmed no residual disease.
- Two samples (2/78; 3%) showed cPCR negativity for MRD whilst both the ClearLLab 10C™ B-cell/M2 and in-house immunophenotypic panels showed immunophenotypic evidence of residual disease.
- One sample (1/78; 1%) was extremely haemodilute, essentially resembling that of peripheral blood and both immunophenotypic panels were unable to exclude residual disease, whilst cPCR for MRD was positive for residual disease.
- One sample (1/78; 1%) showed immunophenotypic evidence of two distinct leukaemic subpopulations, identified on both immunophenotypic panels. This specific case showed evidence of monocytic disease evolution, best detected by the CD45 versus SS dual plots of the in-house immunophenotypic panel. Further immunophenotypic information was not possible with this in-house haematogone data analysis protocol alone.
- Four samples (4/78; 5%) expressed dim to negative leukaemic CD45 expression. By sound logic and inference, these populations were not excluded from analysis.

The following was noted on the addition of the in-house immunophenotypic panel to the samples of demonstration interest:

- One sample showed a polyclonal IgH gene re-arrangement at diagnosis, and thus cPCR was not required on subsequent follow-up samples unless relapsed disease was suspected. The in-house immunophenotypic panel was added to four of this patient's follow-up samples, one of which showed prominent NSB and could not reliably be interpreted. The remaining three samples, however confirmed no residual leukaemic cells, with variable precursor B-cells present in keeping with no MRD.
- Two samples were in consensus with the CMJAH NHLS flow cytometry laboratory EQA proficiency testing scheme and accreditation programme in the identification of a diagnostic sample with B-cell ALL. The first sample incorporated the drop-in of CD58-FITC, whilst the second sample incorporated the full in-house immunophenotypic panel. These findings show comparability to international standards for flow cytometric analysis of diagnostic B-cell ALL.

5.3 Conclusive remark

This work confirms the findings of previous studies, where discriminatory marker CD58-FITC in combination with backbone informative markers demonstrates both superior diagnostic and monitoring utility in paediatric B-cell ALL. Furthermore, the study suggests that the in-house immunophenotypic panel is both superior to the established ClearLLab 10C™ B-cell/M2 panels, and compares to cPCR for MRD determination, supporting its introduction as a LDT into routine flow cytometry laboratory practice in this patient population.

6. DISCUSSION

Risk stratification and prognostication is central to paediatric B-cell ALL management, allowing for appropriate initial regimen choice, individualised treatment consideration and potential future HSCT planning.[2,6] As an important prognostic factor in this patient population, MRD status informs the clinician about remaining disease burden after induction therapy using institution-specific, modified BFM protocols, reflecting chemosensitivity and treatment response [15,17]. MRD status also forms a major

predictor of overall and disease-free survival, with the potential for earlier clinical decision-making pertaining to regimen modification (i.e. therapy minimisation versus intensification, or HSCT eligibility evaluation), improving morbidity and mortality.[2,6,9,11,18,56]

cPCR for the identification of B-cell IgH gene re-arrangements as well as flow cytometry play well-established roles in MRD determination in paediatric B-cell ALL, the former being the most studied and superior molecular technique in the absence of defined genetic aberrations.[25] In comparison to cPCR, flow cytometry has a quicker turnaround time, offering timeous diagnostic reporting to facilitate appropriate patient management. However, international consensus offers no one optimal MFC panel that exists in terms of minimising cost and spectral overlap, whilst maximising relevant immunophenotypic information obtained.[14,38] Panel selection may thus be individualised as a LDT, dependent on resource and expertise availability whilst ensuring retention of the predictive power of larger panels.[13,21,44,45]

This study aimed to determine whether an in-house immunophenotypic panel containing the discriminatory CD58-FITC compared to cPCR in the detection of paediatric B-cell ALL MRD, with concurrent comparison to the validated ClearLLab 10C™ B-cell/M2 panels used at the CMJAH NHLS flow cytometry laboratory. The study found that the in-house immunophenotypic panel was overall superior in performance and predictive power as compared to its counterpart, the latter demonstrating a notable low sensitivity in correctly identifying residual disease when present, largely owing to the absence of aberrant markers on LAIPs at diagnosis. By application of its highly specific Boolean gating strategy in the identification of diagnostic patient-specific LAIPs, the in-house immunophenotypic panel offers an attractive, comparable alternative in MRD determination in this patient population whilst awaiting PCR findings. Furthermore, adoption of this LDT into routine flow cytometry laboratory practice raises the possibility of earlier clinical decision-making with potential improvement of morbidity and mortality outcomes. This is particularly an incentive within resource-constrained sub-Saharan Africa where feasibility of performing one modality over the other is a reality.[2,6,9,11,18]

Many additional observations of clinical importance were made during the construct of this research report. The samples of demonstration interest showed both comparability to international standards for flow cytometric analysis of diagnostic B-cell ALL, and clinical utility in cases with polyclonal IgH gene re-arrangements. This study may form the backbone for future MFC validation studies in our flow cytometry laboratory (e.g. T-cell ALL MRD determination), as well as its application to different sample types for evidence of disease infiltration (e.g. pleural fluid, lymph node fine-needle aspiration and cerebrospinal fluid). Furthermore, long-term follow-up and correlation with the current knowledge of risk of relapsed disease in this patient population may be considered using this data.[56] Lastly, in addition to current literature these findings may advocate for the development of a pre-titrated commercial lyophylised panel (much like the ClearLLab 10C™ panel), obviating the need for flow cytometric titration and technical effort in sample preparation.[35]

In addition to the smaller sample size recruited, this study encountered limitations predominantly related to the pre-analytical stage of analysis. These included limited remnant patient sample for secondary analysis, delayed sample processing (e.g. if a sample was received over non-business working days) and technical error (e.g. single panel constituents erroneously not added or due to reagent unavailability). These difficulties are not unique to this study and may occur in general flow cytometry laboratory work practice.[35] However, despite these technicalities, the in-house immunophenotypic panel was still able to overall reliably detect the presence or absence of the LAIP or precursor B-cells. The following solutions were instituted in an attempt to overcome these limitations experienced: the application of "rare event" analysis; inclusion of "NSB" into the Boolean gating strategy; real-time error correction and monitoring of reagent stock, and alternative marker assessment to identify the LAIP or precursor B-cell populations.

The future of B-cell ALL MRD analysis is promising, with various novel modalities in study including high throughput sequencing MRD technologies and artificial intelligence MFC machine learning. The former includes next generation flow cytometry and sequencing, PCR-based high throughput sequencing of IgH gene re-arrangements, and third generation digital droplet cPCR.[10,11,13,18,27,57] The latter has been

hypothesised to negate the need for calibration curve analysis, whilst still accurately quantifying DNA target sequences.[13,17,25,57]

The breakthrough phenomenon of artificial intelligence machine learning in the context of haematopathology is increasingly being recognised, with advancing algorithms identifying common immunophenotypic profiles, whilst uncovering populations and subpopulations undetectable by subjective human analysis and streamlining workflow.[58–61] These future modalities address the need for fast, standardised technologies with greater MRD diagnostic sensitivity within this patient population, however still require large scale validation studies, their feasibility in resource-constrained LMICs remaining reserved.[4,10,11,12,13,22,63]

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9. ETHICS AND RESEARCH APPROVAL DOCUMENTS

All pilot work conducted prior to formal MMed candidate ethics clearance was covered under supervisor Professor Deborah Glencross' blanket ethic clearance for the evaluation and validation of kits, reagents, data analysis protocols and methods (M1704129). Formal ethics approval for the current research study was obtained from the University of the Witwatersrand's Human Research Ethics Committee (M220153). Permission to use NHLS facilities and the CMJAH flow cytometry unit's Beckman Coulter KaluzaC™ software system was obtained from the NHLS Academic Affairs and Research Office (PR2222608) and head of department respectively. Registration with the National Health Research Database (GP_202203_014) and formal approval to conduct research from the CMJAH chief executive officer was further obtained.

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APPENDIX A

Table 1.4.1. Normal maturation of the B-cell. Major immunophenotypic markers are included for the purposes of this research report.

<table>
<tr><th colspan="3">Bone marrow</th><th colspan="4">Lymph node</th></tr>
<tr><th>Pro-B-cell</th><th>Pre-B-cell</th><th>Immature B-cell</th><th>Mature Naive B-cell</th><th>Germinal Centre B-cell</th><th>Marginal Zone B-cell</th><th>Plasma cell</th></tr>
<tr><td>CD34</td><td>+/- CD34</td><td colspan="5"></td></tr>
<tr><td colspan="7">HLA-DR</td></tr>
<tr><td colspan="7">CD45</td></tr>
<tr><td>nTdT</td><td>+/- nTdT</td><td colspan="5"></td></tr>
<tr><td colspan="2">CD10</td><td colspan="2"></td><td>CD10</td><td colspan="2"></td></tr>
<tr><td colspan="6">CD19</td><td>Dimmer CD19</td></tr>
<tr><td>+/- CD20</td><td colspan="5">Increasing CD20 and cCD22</td><td></td></tr>
<tr><td colspan="3"></td><td>CD23</td><td colspan="3"></td></tr>
<tr><td colspan="2">Bright CD38</td><td colspan="2"></td><td colspan="3">Increasing CD38</td></tr>
<tr><td>Weak CD58</td><td colspan="6"></td></tr>
<tr><td>+/- CD79a</td><td colspan="6">CD79a</td></tr>
<tr><td>CD81</td><td colspan="6"></td></tr>
<tr><td>+/- CD123</td><td colspan="6"></td></tr>
<tr><td>+/- PAX5/BSAP</td><td colspan="5">PAX5/BSAP</td><td></td></tr>
<tr><td colspan="6"></td><td>CD138</td></tr>
<tr><td></td><td>cμ chain Ig</td><td colspan="4">Surface Kappa/Lambda light chain (dependent on antigen exposure)</td><td></td></tr>
</table>

* c, cytoplasmic; CD, cluster of differentiation; nTdT, nuclear terminal deoxynucleotidyl transferase; HLA-DR, human leucocyte antigen-DR isotype; PAX5, paired-box containing protein 5; BSAP, B-cell lineage-specific activator protein; μ, mu; Ig, immunoglobulin.

** **Orange:** denotes generalised antigen expression

*** **Grey:** denotes non-antigen expression

**** **White:** denotes antigenic expression at specific stages of maturation

APPENDIX B

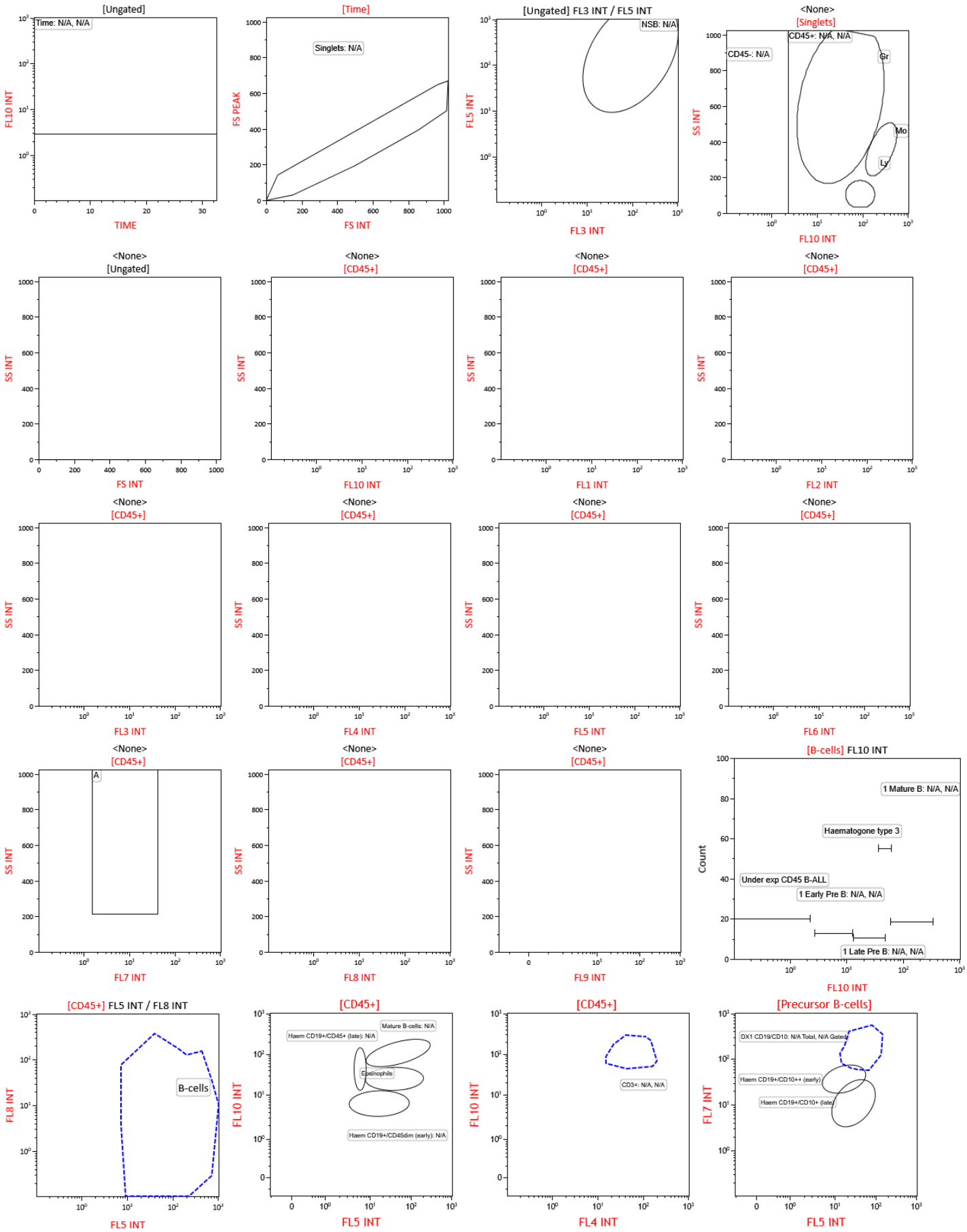
Table 1.4.2. Useful markers to differentiate between LAIP and haematogone populations within B-cell ALL MRD bone marrow samples.

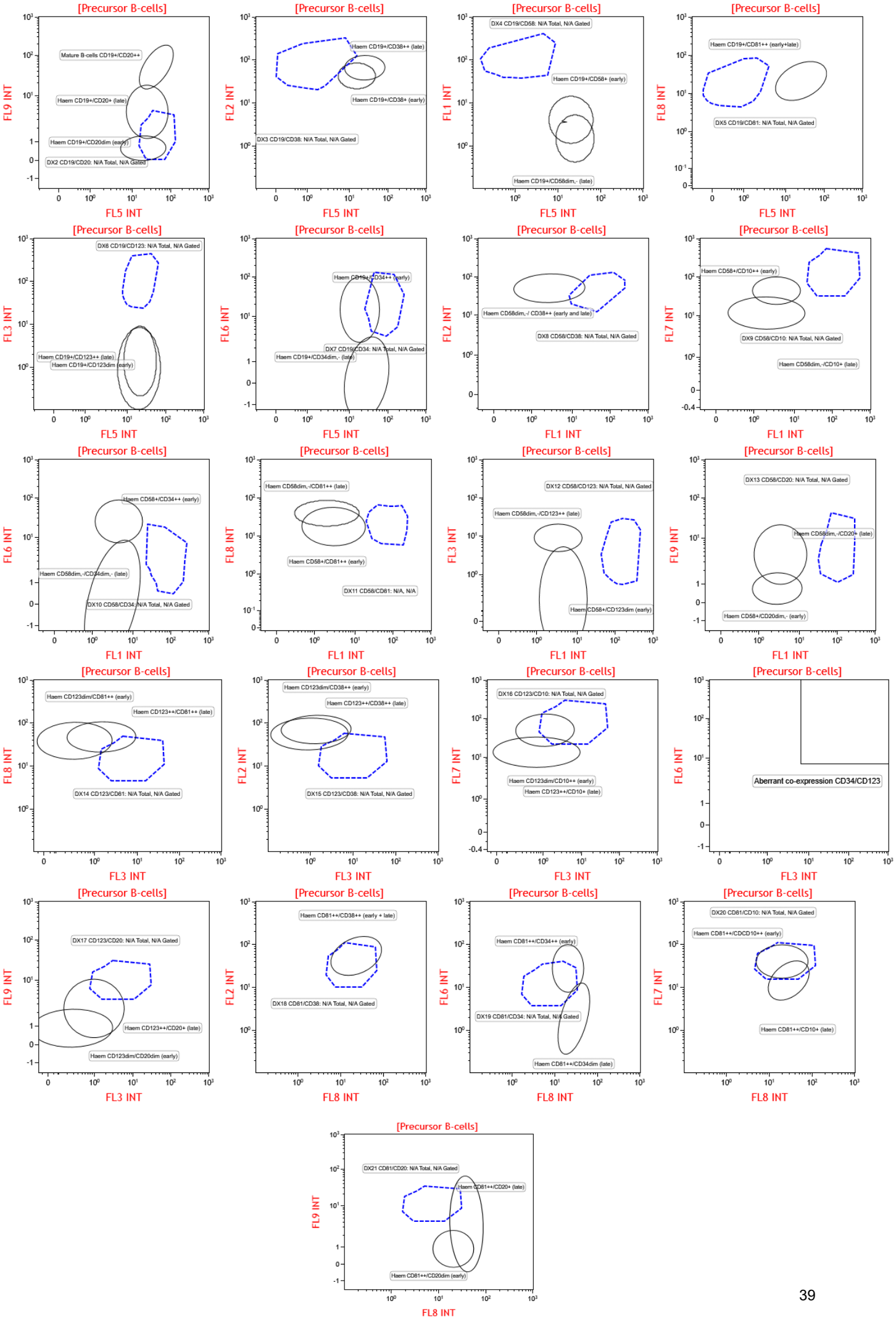
	Relevant marker considerations	LAIP (and subclones if present)	Maturing haematogone population
CD34	○ Marker of immaturity ○ Not lineage specific	▪ Persistent expression suggests asynchronous maturation ▪ May lack CD34 expression	▪ May or may not be expressed ▪ Lost relatively early in B-cell maturation
CD45	○ Marker for all leucocytes ○ Common initial gating strategy	▪ Presents as a discrete population within the negative-to-dim CD45 gate ▪ May diminish with clonal evolution or disease relapse	▪ Presents as a non-discrete population within the negative-to-dim CD45 gate, indicating a maturing population ▪ Dimmer than background normal polyclonal B-cells ▪ Brighter than LAIP expression
nTdT	○ Associated with early B- and T-cell development ○ Not specific for ALL	▪ Useful only to assist in immaturity determination at initial diagnosis	▪ May or may not be expressed
CD10	○ Expression is lost with B-cell maturation as cells acquire surface Ig expression ○ Re-expressed at the germinal centre stage of B-cell development	▪ Brighter than normal B-cell precursors ▪ Persistent expression suggests asynchronous maturation ▪ Certain subtypes may have absent expression and yield early prognostic value	▪ Decreased expression
CD20	○ Defines an early pre-B-cell ○ PAX5 is considered more sensitive and specific to detect pre-B-cells immunohistochemically	▪ May or may not be expressed ▪ Earlier expression may suggest asynchronous maturation	▪ Continued and brighter expression with maturation
CD22	○ Expression is restricted to B-cells	▪ Brighter than normal B-cell precursors ▪ Earlier expression may suggest asynchronous maturation	▪ Decreased expression
CD38	○ Widely expressed in most leucocytes with its intensity largely correlating with the degree of cellular activation	▪ Variable expression and decreased as compared to haematogones	▪ Uniform bright expression
CD58	○ CD38/CD58 co-expression is most frequently used to identify LAIP (insufficient power if used in isolation)	▪ Bright and overexpressed ▪ Correlates with CD10 identification of LAIP in terms of brightness and asynchronous maturation	▪ Expression is lost in early maturation
CD81	○ Widely expressed on immune cells	▪ Dim and underexpressed	▪ Uniform bright expression
CD123	○ Low or negative expression in primitive haemopoietic cells	▪ Bright and overexpressed	▪ Discordant expression favours haematogones at varying stages of

		▪ Concordant expression favours LAIP (CD34+/CD123+; CD34-/CD123-)	maturation (CD34+/CD123-) and (CD34-/CD123+)
Surface Igs	o Used in cPCR for clonal determination	▪ Very rarely express surface kappa and lambda light chains	▪ Absent surface expression

MRD, measurable residual disease; CD, cluster of differentiation; ALL, acute lymphoblastic leukaemia; nTdT, nuclear terminal deoxynucleotidyl transferase; LAIP, leukaemia-associated immunophenotypes; PAX5, paired-box containing protein 5; cPCR, conventional polymerase chain reaction; Ig, immunoglobulin.

APPENDIX C


[Ungated]
Time: N/A, N/A
FL10 INT
TIME
[Time]
Singlets: N/A
FS PEAK
FS INT
[Ungated] FL3 INT / FL5 INT
NSB: N/A
FL5 INT
FL3 INT
<None>
[Singlets]
CD45+: N/A, N/A
CD45-: N/A
Gr
Mo
Ly
SS INT
FL10 INT
<None>
[Ungated]
SS INT
FS INT
<None>
[CD45+]
SS INT
FL10 INT
<None>
[CD45+]
SS INT
FL1 INT
<None>
[CD45+]
SS INT
FL2 INT
<None>
[CD45+]
SS INT
FL3 INT
<None>
[CD45+]
SS INT
FL4 INT
<None>
[CD45+]
SS INT
FL5 INT
<None>
[CD45+]
SS INT
FL6 INT
<None>
[CD45+]
A
SS INT
FL7 INT
<None>
[CD45+]
SS INT
FL8 INT
<None>
[CD45+]
SS INT
FL9 INT
[B-cells] FL10 INT
1 Mature B: N/A, N/A
Haematogone type 3
Under exp CD45 B-ALL
1 Early Pre B: N/A, N/A
1 Late Pre B: N/A, N/A
Count
FL10 INT
[CD45+] FL5 INT / FL8 INT
B-cells
FL8 INT
FL5 INT
[CD45+]
Mature B-cells: N/A
Haem CD19+/CD45+ (late): N/A
Eosinophils
Haem CD19+/CD45dim (early): N/A
FL10 INT
FL5 INT
[CD45+]
CD3+: N/A, N/A
FL10 INT
FL4 INT
[Precursor B-cells]
DX1 CD19/CD10: N/A Total, N/A Gated
Haem CD19+/CD10++ (early)
Haem CD19+/CD10+ (late)
FL7 INT
FL5 INT


[Precursor B-cells]
Mature B-cells CD19+/CD20++
Haem CD19+/CD20+ (late)
Haem CD19+/CD20dim (early)
DX2 CD19/CD20: N/A Total, N/A Gated
FL9 INT
FL5 INT
[Precursor B-cells]
Haem CD19+/CD38++ (late)
Haem CD19+/CD38+ (early)
DX3 CD19/CD38: N/A Total, N/A Gated
FL2 INT
FL5 INT
[Precursor B-cells]
DX4 CD19/CD58: N/A Total, N/A Gated
Haem CD19+/CD58+ (early)
Haem CD19+/CD58dim,- (late)
FL1 INT
FL5 INT
[Precursor B-cells]
Haem CD19+/CD81++ (early+late)
DX5 CD19/CD81: N/A Total, N/A Gated
FL8 INT
FL5 INT
[Precursor B-cells]
DX6 CD19/CD123: N/A Total, N/A Gated
Haem CD19+/CD123++ (late)
Haem CD19+/CD123dim (early)
FL3 INT
FL5 INT
[Precursor B-cells]
Haem CD19+/CD34++ (early)
DX7 CD19/CD34: N/A Total, N/A Gated
Haem CD19+/CD34dim,- (late)
FL6 INT
FL5 INT
[Precursor B-cells]
Haem CD58dim,-/ CD38++ (early and late)
DX8 CD58/CD38: N/A Total, N/A Gated
FL2 INT
FL1 INT
[Precursor B-cells]
Haem CD58+/CD10++ (early)
DX9 CD58/CD10: N/A Total, N/A Gated
Haem CD58dim,-/CD10+ (late)
FL7 INT
FL1 INT
[Precursor B-cells]
Haem CD58+/CD34++ (early)
Haem CD58dim,-/CD34dim,- (late)
DX10 CD58/CD34: N/A Total, N/A Gated
FL6 INT
FL1 INT
[Precursor B-cells]
Haem CD58dim,-/CD81++ (late)
Haem CD58+/CD81++ (early)
DX11 CD58/CD81: N/A, N/A
FL8 INT
FL1 INT
[Precursor B-cells]
DX12 CD58/CD123: N/A Total, N/A Gated
Haem CD58dim,-/CD123++ (late)
Haem CD58+/CD123dim (early)
FL3 INT
FL1 INT
[Precursor B-cells]
DX13 CD58/CD20: N/A Total, N/A Gated
Haem CD58dim,-/CD20+ (late)
Haem CD58+/CD20dim,- (early)
FL9 INT
FL1 INT
[Precursor B-cells]
Haem CD123dim/CD81++ (early)
Haem CD123++/CD81++ (late)
DX14 CD123/CD81: N/A Total, N/A Gated
FL8 INT
FL3 INT
[Precursor B-cells]
Haem CD123dim/CD38++ (early)
Haem CD123++/CD38++ (late)
DX15 CD123/CD38: N/A Total, N/A Gated
FL2 INT
FL3 INT
[Precursor B-cells]
DX16 CD123/CD10: N/A Total, N/A Gated
Haem CD123dim/CD10++ (early)
Haem CD123++/CD10+ (late)
FL7 INT
FL3 INT
[Precursor B-cells]
Aberrant co-expression CD34/CD123
FL6 INT
FL3 INT
[Precursor B-cells]
DX17 CD123/CD20: N/A Total, N/A Gated
Haem CD123++/CD20+ (late)
Haem CD123dim/CD20dim (early)
FL9 INT
FL3 INT
[Precursor B-cells]
Haem CD81++/CD38++ (early + late)
DX18 CD81/CD38: N/A Total, N/A Gated
FL2 INT
FL8 INT
[Precursor B-cells]
Haem CD81++/CD34++ (early)
DX19 CD81/CD34: N/A Total, N/A Gated
Haem CD81++/CD34dim (late)
FL6 INT
FL8 INT
[Precursor B-cells]
DX20 CD81/CD10: N/A Total, N/A Gated
Haem CD81++/CDCD10++ (early)
Haem CD81++/CD10+ (late)
FL7 INT
FL8 INT
[Precursor B-cells]
DX21 CD81/CD20: N/A Total, N/A Gated
Haem CD81++/CD20+ (late)
Haem CD81++/CD20dim (early)
FL9 INT
FL8 INT

KEY

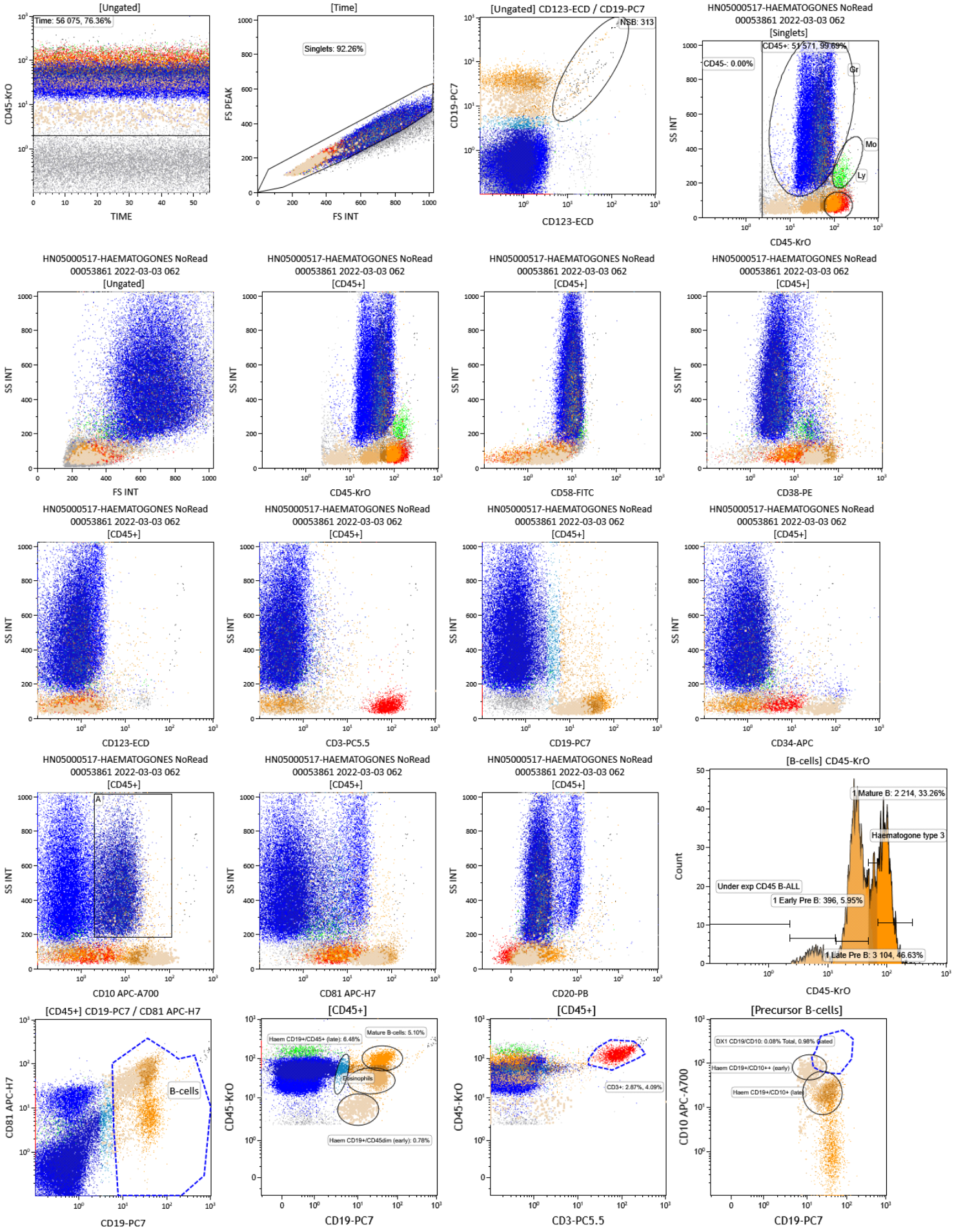
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FL2 INT: CD38-PE
FL3 INT: CD123-ECD
FL4 INT: CD3-PC5.5
FL5 INT: CD19-PC7
FL6 INT: CD34-APC
FL7 INT: CD10 APC-A700
FL8 INT: CD81 APC-H7
FL9 INT: CD20-PB
FL10 INT: CD45-KrO

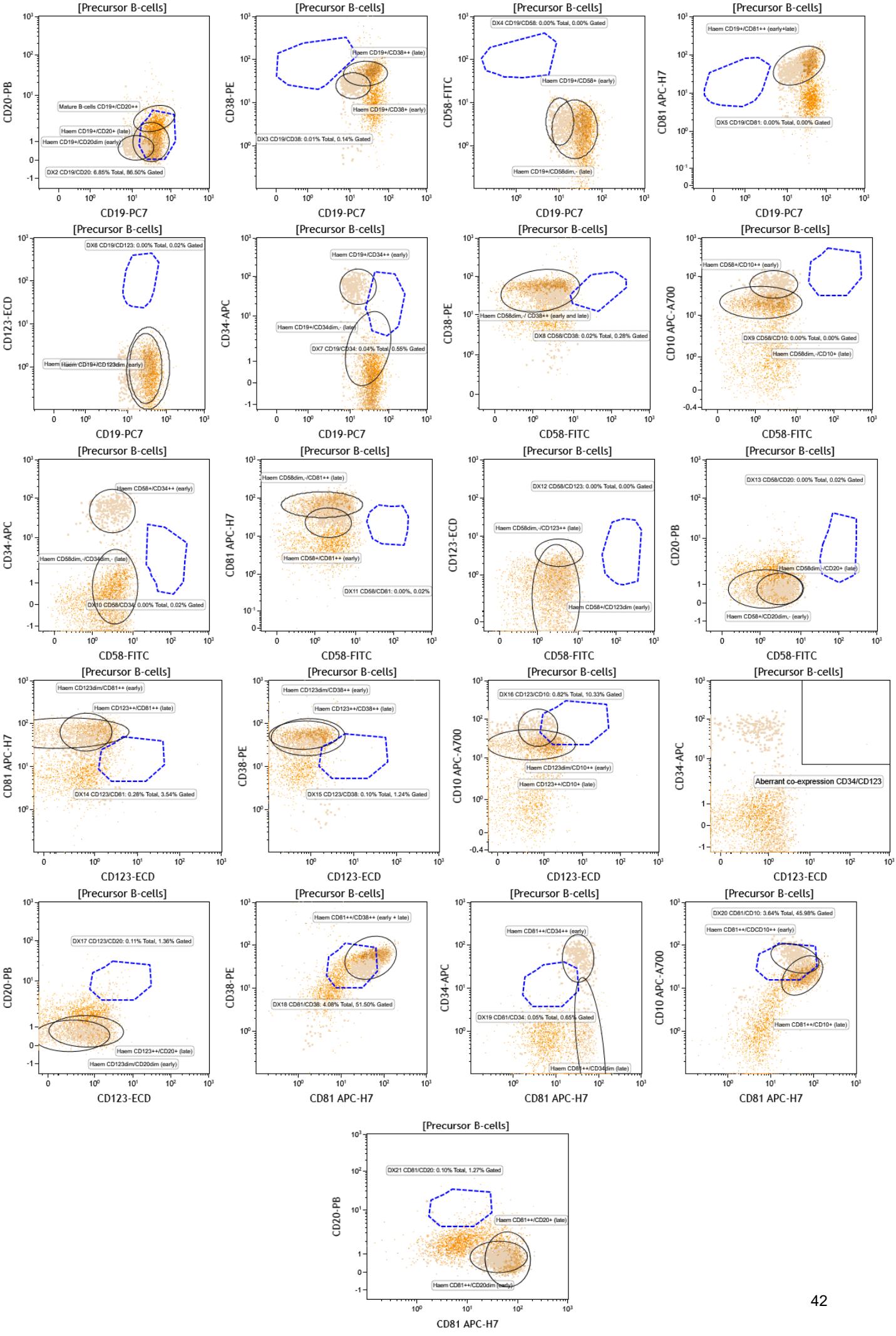
Figure 3.4.1.1. In-house haematogone data analysis protocol using the Beckman Coulter KaluzaC^{TM} software system. CD, cluster of differentiation; SS, side scatter; FS, forward scatter; NSB, nonspecific binding; Mo, monocytes; Gr, granulocytes; Ly, lymphocytes; Haem, haematogones; DX, diagnostic gate; FITC, fluorescein isothiocyanate; APC-H7, allophycocyanin-cyanine dye.

∗ Abbreviations of the non-assessed markers are not further described

∗ The blue diagnostic gates are discussed in the research report under "Data analysis" - Part 2 (pages 16 - 18)

APPENDIX D


[Ungated]
Time: 56 075, 76.36%
CD45-KrO
TIME
[Time]
Singlets: 92.26%
FS PEAK
FS INT
[Ungated] CD123-ECD / CD19-PC7
NSB: 313
CD19-PC7
CD123-ECD
HN05000517-HAEMATOGONES NoRead
00053861 2022-03-03 062
[Singlets]
CD45+: 51 571, 99.69%
CD45-: 0.00%
Gr
Mo
Ly
SS INT
CD45-KrO
HN05000517-HAEMATOGONES NoRead
00053861 2022-03-03 062
[Ungated]
SS INT
FS INT
HN05000517-HAEMATOGONES NoRead
00053861 2022-03-03 062
[CD45+]
SS INT
CD45-KrO
HN05000517-HAEMATOGONES NoRead
00053861 2022-03-03 062
[CD45+]
SS INT
CD58-FITC
HN05000517-HAEMATOGONES NoRead
00053861 2022-03-03 062
[CD45+]
SS INT
CD38-PE
HN05000517-HAEMATOGONES NoRead
00053861 2022-03-03 062
[CD45+]
SS INT
CD123-ECD
HN05000517-HAEMATOGONES NoRead
00053861 2022-03-03 062
[CD45+]
SS INT
CD3-PC5.5
HN05000517-HAEMATOGONES NoRead
00053861 2022-03-03 062
[CD45+]
SS INT
CD19-PC7
HN05000517-HAEMATOGONES NoRead
00053861 2022-03-03 062
[CD45+]
SS INT
CD34-APC
HN05000517-HAEMATOGONES NoRead
00053861 2022-03-03 062
[CD45+]
A
SS INT
CD10 APC-A700
HN05000517-HAEMATOGONES NoRead
00053861 2022-03-03 062
[CD45+]
SS INT
CD81 APC-H7
HN05000517-HAEMATOGONES NoRead
00053861 2022-03-03 062
[CD45+]
SS INT
CD20-PB
[B-cells] CD45-KrO
1 Mature B: 2 214, 33.26%
Haematogone type 3
Under exp CD45 B-ALL
1 Early Pre B: 396, 5.95%
1 Late Pre B: 3 104, 46.63%
Count
CD45-KrO
[CD45+] CD19-PC7 / CD81 APC-H7
B-cells
CD81 APC-H7
CD19-PC7
[CD45+]
Haem CD19+/CD45+ (late): 6.48%
Mature B-cells: 5.10%
Eosinophils
Haem CD19+/CD45dim (early): 0.78%
CD45-KrO
CD19-PC7
[CD45+]
CD3+: 2.87%, 4.09%
CD45-KrO
CD3-PC5.5
[Precursor B-cells]
DX1 CD19/CD10: 0.08% Total, 0.98% Gated
Haem CD19+/CD10++ (early)
Haem CD19+/CD10+ (late)
CD10 APC-A700
CD19-PC7


[Precursor B-cells]
CD20-PB
CD19-PC7
Mature B-cells CD19+/CD20++
Haem CD19+/CD20+ (late)
Haem CD19+/CD20dim (early)
DX2 CD19/CD20: 6.85% Total, 86.50% Gated
[Precursor B-cells]
CD38-PE
CD19-PC7
Haem CD19+/CD38++ (late)
Haem CD19+/CD38+ (early)
DX3 CD19/CD38: 0.01% Total, 0.14% Gated
[Precursor B-cells]
CD58-FITC
CD19-PC7
DX4 CD19/CD58: 0.00% Total, 0.00% Gated
Haem CD19+/CD58+ (early)
Haem CD19+/CD58dim,- (late)
[Precursor B-cells]
CD81 APC-H7
CD19-PC7
Haem CD19+/CD81++ (early+late)
DX5 CD19/CD81: 0.00% Total, 0.00% Gated
[Precursor B-cells]
CD123-ECD
CD19-PC7
DX6 CD19/CD123: 0.00% Total, 0.02% Gated
Haem CD19+/CD123dim (early)
[Precursor B-cells]
CD34-APC
CD19-PC7
Haem CD19+/CD34++ (early)
Haem CD19+/CD34dim,- (late)
DX7 CD19/CD34: 0.04% Total, 0.55% Gated
[Precursor B-cells]
CD38-PE
CD58-FITC
Haem CD58dim,-/ CD38++ (early and late)
DX8 CD58/CD38: 0.02% Total, 0.28% Gated
[Precursor B-cells]
CD10 APC-A700
CD58-FITC
Haem CD58+/CD10++ (early)
DX9 CD58/CD10: 0.00% Total, 0.00% Gated
Haem CD58dim,-/CD10+ (late)
[Precursor B-cells]
CD34-APC
CD58-FITC
Haem CD58+/CD34++ (early)
Haem CD58dim,-/CD34dim,- (late)
DX10 CD58/CD34: 0.00% Total, 0.02% Gated
[Precursor B-cells]
CD81 APC-H7
CD58-FITC
Haem CD58dim,-/CD81++ (late)
Haem CD58+/CD81++ (early)
DX11 CD58/CD81: 0.00%, 0.02%
[Precursor B-cells]
CD123-ECD
CD58-FITC
DX12 CD58/CD123: 0.00% Total, 0.00% Gated
Haem CD58dim,-/CD123++ (late)
Haem CD58+/CD123dim (early)
[Precursor B-cells]
CD20-PB
CD58-FITC
DX13 CD58/CD20: 0.00% Total, 0.02% Gated
Haem CD58dim,-/CD20+ (late)
Haem CD58+/CD20dim,- (early)
[Precursor B-cells]
CD81 APC-H7
CD123-ECD
Haem CD123dim/CD81++ (early)
Haem CD123++/CD81++ (late)
DX14 CD123/CD81: 0.28% Total, 3.54% Gated
[Precursor B-cells]
CD38-PE
CD123-ECD
Haem CD123dim/CD38++ (early)
Haem CD123++/CD38++ (late)
DX15 CD123/CD38: 0.10% Total, 1.24% Gated
[Precursor B-cells]
CD10 APC-A700
CD123-ECD
DX16 CD123/CD10: 0.82% Total, 10.33% Gated
Haem CD123dim/CD10++ (early)
Haem CD123++/CD10+ (late)
[Precursor B-cells]
CD34-APC
CD123-ECD
Aberrant co-expression CD34/CD123
[Precursor B-cells]
CD20-PB
CD123-ECD
DX17 CD123/CD20: 0.11% Total, 1.36% Gated
Haem CD123++/CD20+ (late)
Haem CD123dim/CD20dim (early)
[Precursor B-cells]
CD38-PE
CD81 APC-H7
Haem CD81++/CD38++ (early + late)
DX18 CD81/CD38: 4.08% Total, 51.50% Gated
[Precursor B-cells]
CD34-APC
CD81 APC-H7
Haem CD81++/CD34++ (early)
DX19 CD81/CD34: 0.05% Total, 0.65% Gated
Haem CD81++/CD34dim (late)
[Precursor B-cells]
CD10 APC-A700
CD81 APC-H7
DX20 CD81/CD10: 3.64% Total, 45.98% Gated
Haem CD81++/CDCD10++ (early)
Haem CD81++/CD10+ (late)
[Precursor B-cells]
CD20-PB
CD81 APC-H7
DX21 CD81/CD20: 0.10% Total, 1.27% Gated
Haem CD81++/CD20+ (late)
Haem CD81++/CD20dim (early)

Gate	Number	%Gated	%GP Gated	"FL5 INT"-GMean-Log	"FL6 INT"-GMean-Log	"FL8 INT"-GMean-Log	"FL1 INT"-GMean-Log	Logic
All	60 538	100.00	N/A	0.43	0.73	0.79	6.47	Ungated
NSB	90	0.15	N/A	19.43	1.79	72.26	16.78	NSB
B-cells	3 654	7.00	7.00	17.19	2.27	14.95	2.13	B-cells AND CD45+ AND Singlets AND Time
Haematogone type 3	192	5.25	0.37	12.73	2.45	18.26	2.80	"Haematogone type 3" AND B-cells AND CD45+ AND Singlets AND Time
CD3+	1 720	3.29	3.29	0.11	0.66	6.23	1.50	CD3+ AND CD45+ AND Singlets AND Time
Eosinophils	1 200	2.30	2.30	2.87	4.14	33.19	3.00	Eosinophils AND CD45+ AND Singlets AND Time
Gr	39 823	76.27	69.95	0.33	0.55	0.44	8.46	Gr AND Singlets AND Time
Haem CD19+/CD45+ (late)	883	1.69	1.69	6.73	4.90	41.52	2.98	"Haem CD19+/CD45+ (late)" AND CD45+ AND Singlets AND Time
Haem CD19+/CD45dim (early)	178	0.34	0.34	6.59	9.92	39.16	2.33	"Haem CD19+/CD45dim (early)" AND CD45+ AND Singlets AND Time
Mature Gr	17 169	32.89	32.88	0.38	0.77	0.47	9.07	"Mature Gr" AND CD45+ AND Singlets AND Time
Mo	456	0.87	0.80	0.45	1.34	3.32	11.08	Mo AND Singlets AND Time

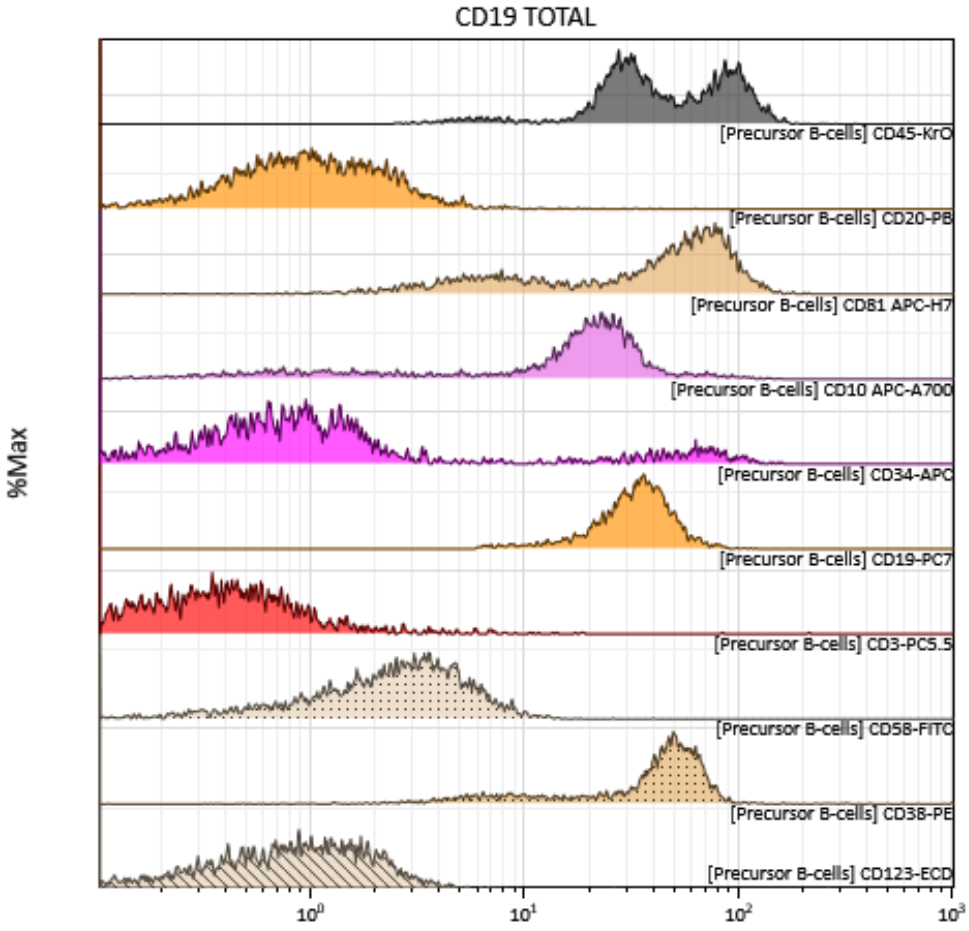


Figure 3.4.1.4. Example of the immunophenotypic analysis of a normal paediatric bone marrow aspirate sample using the in-house haematogone data analysis protocol. Included here, is the report sheet of the hierarchical gating strategies employed to identify the various populations, as well as the histogram plot depicting the CD19 B-cell population and haematogone composition within the sample. CD, cluster of differentiation; SS, side scatter; FS, forward scatter; NSB, non-specific binding; Mo, monocytes; Gr, granulocytes; Ly, lymphocytes; Haem, haematogones; DX, diagnostic gate; FITC, fluorescein isothiocyanate; APC-H7, allophycocyanin-cyanine dye. Abbreviations of the non-assessed markers are not further described.

Appendix E

Table 3.5.1. Example of a single patient's diagnostic and follow-up captured information

Patient number	Hosipital number and institution	Date of diagnosis	Diagnostic BMA information			Follow-up BMA information			
			Episode number	Immunophenotypic findings using ClearLLab 10C panels	IgH cPCR result	Follow-up BMA episode numbers	IgH cPCR result	ClearLLab B/M2 panel immunophenotypic findings	Immunophenotypic findings using in-house immunophenotypic panel
21	This work is protected and covered under Wits HREC Ethics Clearance Certificate M220253	23-Mar-22	This work is protected and covered under Wits HREC Ethics Clearance Certificate M220253	~25% blast cells **Positive surface markers:** (under-expressed) CD45, CD19, CD10, CD38, CD200 and HLA-DR. CD34 expressed in 20% blasts. Aberrant CD33. **Negative surface markers:** Kappa, Lambda, CD20, CD123, T-cell or other myeloid markers. **Unusual, is presence of eosinophils (~4-5%)**	FR1 (+); FR2 (+) and FR3 (+) Out of range clones present **Clonal cell population detected**	HN05067738	FR1 (+), FR2 (-) and FR3 (+) Out of range clonal product detected **Suggestive of residual disease**	Revealed ~7-8% population of cells with a similar immunophenotype to that seen at presentation. In addition, the blasts express CD13+. **Suggestive of residual disease**	Revealed 0.27% discrete population of cells with a similar immunophenotype to that seen at presentation **Suggestive of residual disease**
						HN05094507	FR1 (-), FR2 (-) and FR3 (-) **Negative for clonal cell population**	No discrete population of cells with a similar immunophenotype to that seen at presentation. Haematogones virtually absent.	No discrete population of cells with a similar immunophenotype to that seen at presentation **Mature B-cells: 4.18%** CD19/CD10 co-expressing cells: <1% (Early haematogones: 0.01%) **(Late haematogones: 0.16%)**
						HN05166559	FR1 (+), FR2 (+) and FR3 (+) Out of range clonal product detected **Suggestive of residual disease**	No discrete population of cells with a similar immunophenotype to that seen at presentation. <1% CD19/CD10 co-expressing cells are favoured to be reactive precursor B-cells	Revealed 0.16% population of cells with a similar immunophenotype to that seen at presentation. **Suggestive of residual disease**
						HN05278099	FR1 (-), FR2 (-) and FR3 (-) **Out of range monoclonal product detected - significance unknown (not documented at presentation)**	No discrete population of cells with a similar immunophenotype to that seen at presentation. ~1% CD19/CD10 co-expressing cells are favoured to be reactive precursor B-cells	No discrete population of cells with a similar immunophenotype to that seen at presentation **Mature B-cells: 0.06%** CD19/CD10 co-expressing cells: <1% (Early haematogones: 0.14%) **(Late haematogones: 0.12%)**
						HN05333096	FR1 (-), FR2 (-) and FR3 (-) **Negative for clonal cell population**	No discrete population of cells with a similar immunophenotype to that seen at presentation.	No discrete population of cells with a similar immunophenotype to that seen at presentation **Mature B-cells: 0.47%** CD19/CD10 co-expressing cells: <1% (Early haematogones: 0.18%) **(Late haematogones: 0.21%)**

CMJAH, Charlotte Maxeke Johannesburg Academic hospital; BMA, bone marrow aspirate; CD, cluster of differentiation; IgH, immunoglobuline heavy chain; cPCR, conventional polymerase chain reaction; M2, myeloid 2.

APPENDIX F

Table 4.1.1. Documentation of verification findings of the manufacturer-specified performances of reagents CD58-FITC and CD81-APC-H7 by their addition to remnant normal paediatric BMA samples to identify precursor B-cells.

Case	Episode number	BMA morphological assessment	ClearLLab 10C™ B-cell panel (control)	CD58-FITC and CD81-APC-H7 (in-house immunophenotypic panel)	Verification status
			Identification of haematogones in normal paediatric BMA sample (%)		
1	This work is protected and covered under WITS HREC Ethics Clearance Certificate M220253	1-year-11-month old male presenting with bleeding diathesis and an associated hepatosplenomegaly • Suboptimal quality owing to aparticulate and haemodilute nature, thickly spread • Peripheral cause for thrombocytopaenia with iron deficiency	~ 15% CD19/CD10 co-expressing cells (which are favoured to represent reactive precursor B-cells by virtue of their light scatter pattern)	<1% early precursors ~ 1 – 2% late precursors	Yes
2		2-year-old female known with retinoblastoma of the right eye – BMA submitted for staging purposes • Good quality • No overt evidence of a non-haemopoietic infiltrate	~ 6 – 7% reactive precursor B-cells (based on their light scatter pattern)	<1% early precursors (near absent) <1 % late precursors	Yes
3		5-year-old male presenting with recurrent fevers and generalised lymphadenopathy, with an associated pancytopaenia • Aparticulate with paucicellular trails • Reactive inflammatory process	Not stated	<1% early precursors (near absent) <1% very late precursors (near mature B-cells) Prominent dead/degenerate cells and NSB	Yes
4		1-year-3-month old male presenting with an abdominal mass and hypertension, with imaging showing a mass above the left kidney – BMA submitted for staging purposes • Adequate quality • No overt evidence of a non-haemopoietic infiltrate	~ 1% haematogones	<1% early precursors (near absent) <1% late precursors Majority B-cells likely late mature B-cells or plasma cells (loss of CD20, bright CD38 and lack of immaturity markers)	Yes

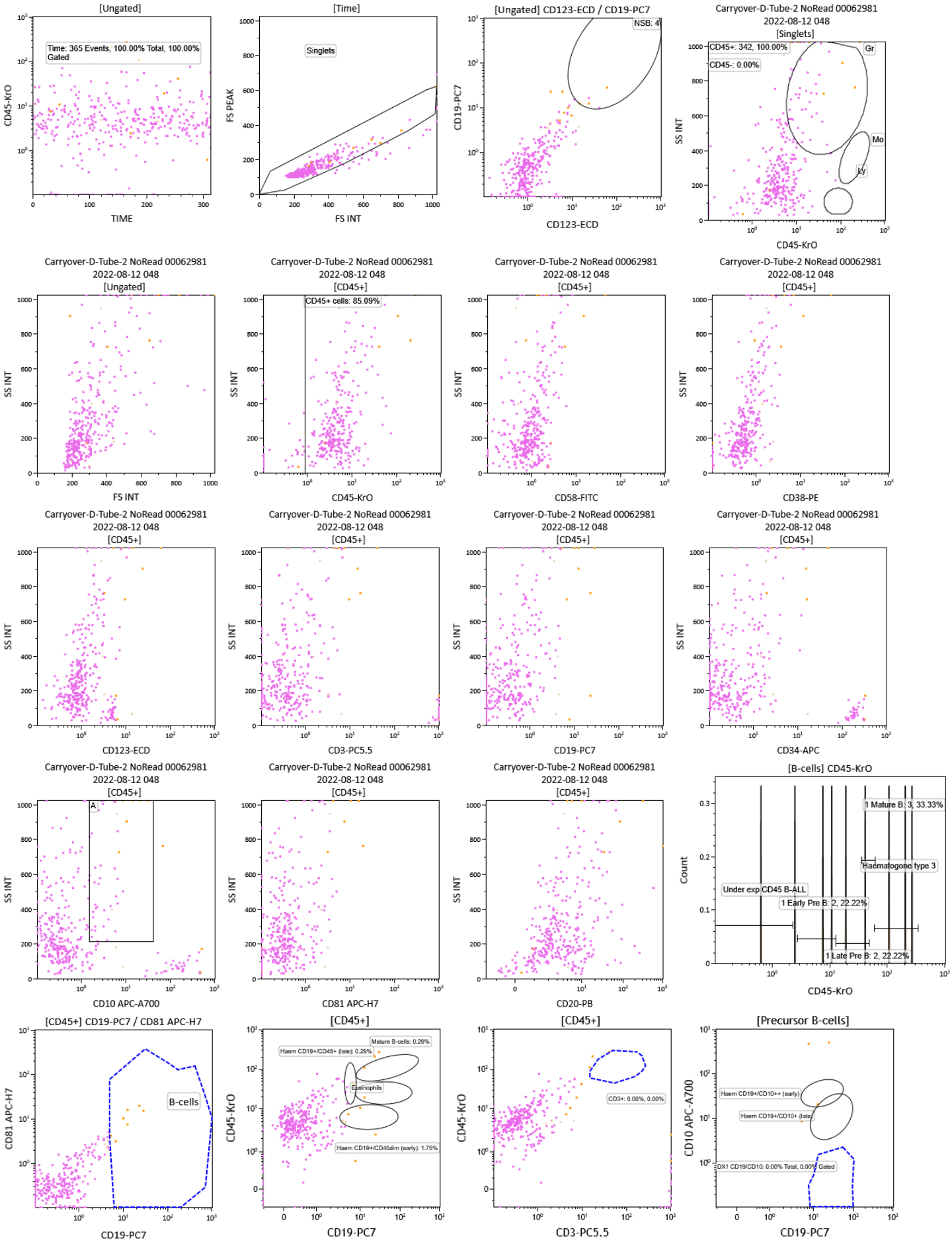
5	**This work is protected and covered under** **WITS HREC** **Ethics Clearance Certificate** **M220253**	6-year-old female known with B-cell ALL with t (12;21) diagnosed in April 2021 – BMA submitted to assess for response to treatment • Adequate quality • No overt increase in blasts • RQ-PCR for t (12;21) negative	<1% late precursor B-cells	<1% early precursors (predominate) <1% late precursors * interruption in run towards end – did not affect analysis overall	Yes
6		2-year-old female known with B-cell ALL NOS, diagnosed May 2021 – BMA submitted to assess for response to treatment • Aparticulate and haemodilute • No overt increase in blasts • PCR for IgH gene re-arrangement negative	~ 3 – 4% CD19/CD10 co-expressing cells which are favoured to be reactive precursor B-cells (by virtue of light scatter pattern)	~ 2 – 3% early precursors <1% late precursors	Yes
7		11-month old male with long-standing history of a bicytopaenia with a massive splenomegaly • Good quality • No overt evidence of an infiltrate or increase in blasts • Peripheral causes for cytopaenias favoured	~ 13 – 14% precursor B-cells	<1% early precursors ~ 6 – 7% late precursors Haemodilute sample with prominent dead and dying cells	Yes
8		13-year-old female known with B-cell ALL with t (1;19), diagnosed in March 2019 – BMA submitted for end-of-treatment assessment • Good quality • No overt increase in blasts • PCR for IgH gene re-arrangement negative	~ 0.3% immature B-cells (haematogones)	<1% early precursors (near absent) <1% late precursors (near absent) Majority of B-cells present are mature forms	Yes
9		8-year-old female known with T-cell ALL common thymocyte phenotype, diagnosed in September 2021 – BMA submitted to assess for response to treatment • Good quality • No overt increase in blasts, with ~4% larger more primitive lymphocytes noted • PCR for TCR gene re-arrangement polyclonal	~ 8 – 9% reactive precursor B-cells	~ 1 – 2% early precursors ~ 6 – 7% late precursors Haemodilute sample	Yes

10	**This work is protected and covered under** **WITS HREC** **Ethics Clearance Certificate** **M220253**	4-year-old female presenting for workup of a pancytopaenia • Adequate quality • Peripheral cause for cytopaenias	~ 18 – 20% CD19/CD10 co-expressing cells are present favoured to represent precursor B-cells (haematogones)	~ 3 – 4% early precursors ~ 13 – 14% late precursors	Yes
11		3-year-old male presenting for workup of an anterior mediastinal mass and massive pleural effusion with pleural fluid immunophenotypic analysis revealing a T-cell ALL best fitting the common thymocyte stage (See HN05250528) – BMA submitted to assess for response to treatment • Good quality • No overt evidence of an infiltrate or increase in blasts	Not stated	<1% early precursors (near absent) <1% late precursors (near absent) Majority of B-cells present are mature forms	Yes
12		3-year-old female recently diagnosed with left-sided retinoblastoma with cranial nerve involvement – BMA submitted for staging purposes • Excellent quality • No overt features of a non-haemopoietic infiltrate	~ 6 – 7% haematogones (by virtue of their light scatter and maturation patterns)	~ 1 – 2% early precursors ~ 3 – 4% late precursors Prominent late precursors with late maturing early precursors	Yes
13		9-year-old female known with AML with t (8;21), diagnosed in June 2022 – BMA submitted to assess for response to treatment • Aparticulate and haemodilute • No overt increase in blasts • RQ-PCR for t (8;21) negative	Not stated	<1% early precursors (near absent) <1% late precursors (near absent) Prominent NSB	
14		15-year-old male known with T-cell ALL, diagnosed in March 2022 – BMA submitted to assess for response to treatment • Excellent quality • No overt increase in blasts	Not stated	<1% early precursors (best represented) <1% late precursors (near absent)	Yes
15		3-year-old male known with B-cell ALL, NOS, diagnosed in July 2022 – no diagnostic haematogone panel added*. Day 15 BMA submitted to assess for response to treatment • Adequate quality • No overt increase in blasts • PCR for IgH gene re-arrangement **pending**	Not stated	<1% early precursors (near absent) <1% late precursors (near absent) Majority of B-cells present are mature forms	Yes

16	**This work is protected and covered under** **WITS HREC** **Ethics Clearance Certificate** **M220253**	7-year-old female presenting with a bleeding diathesis, cervical lymphadenopathy and enlarged tonsils, with an associated severe thrombocytopaenia • Aparticulate and haemodilute with paucicellular trails • No overt increase in blasts or evidence of an infiltrate	~ 1 – 2% reactive precursor B-cells	<1% early precursors (very late early forms noted – losing CD10) ~ 1 – 2% late precursors (near-mature) Majority of B-cells present are mature forms	Yes
17		1-year-old male being worked up for an acute leukaemia • Aparticulate and haemodilute with paucicellular trails • Mentzer's index <13 – consider workup for thalassaemia trait	~ 1 – 2% reactive precursor B-cells (haematogones)	~ 1 – 2% early precursors <1% late precursors (near absent) – majority are very late, late precursors (i.e. near-mature) ** No CD10 available - however, this does not affect immunophenotypic analysis	Yes
18		3-year-old female, severely malnourished and presenting with diarrhoea and vomiting, with an associated severe anaemia • Adequate quality • No overt increase in blasts or evidence of an infiltrate	Not stated	<1% early precursors (near absent) <1% late precursors majority are very late, late precursors (i.e. near-mature)	Yes
19		4-year-old female with retroviral disease, now presenting for workup of a persistent bicytopaenia • Good quality • No overt increase in blasts or evidence of an infiltrate	~3% haematogones	<1% early precursors ~ 2 – 3% late precursors ** No CD10 available - however, this does not affect immunophenotypic analysis	Yes
20		13-year-old male admitted to ICU with status asthmaticus with a large mediastinal mass and localised lymphadenopathy – BMAT submitted to exclude an acute lymphoblastic leukaemia • Excellent quality • No overt increase in blasts or evidence of an infiltrate	Not stated	<1% early precursors (near absent) <1% late precursors (near absent) ** No CD10 available - however, this does not affect immunophenotypic analysis	Yes

* CD, cluster of differentiation; FITC, fluorescein isothiocyanate; APC-H7, allophycocyanin-cyanine dye; BMA, bone marrow aspirate; PCR, polymerase chain reaction; t; translocation; IgH, immunoglobulin heavy chain; RQ-PCR, real-time quantitative PCR; ALL, acute lymphoblastic leukaemia; NSB, nonspecific binding; LIS, laboratory information system; HREC, Human Research Ethics Committee.

APPENDIX G


[Ungated]
Time: 365 Events, 100.00% Total, 100.00% Gated
CD45-KrO
TIME
[Time]
Singlets
FS PEAK
FS INT
[Ungated] CD123-ECD / CD19-PC7
NSB: 4
CD19-PC7
CD123-ECD
Carryover-D-Tube-2 NoRead 00062981
2022-08-12 048
[Singlets]
CD45+: 342, 100.00%
CD45-: 0.00%
Gr
Mo
Ly
SS INT
CD45-KrO
[Ungated]
FS INT
[CD45+]
CD45+ cells: 85.09%
CD58-FITC
CD38-PE
CD123-ECD
CD3-PC5.5
CD19-PC7
CD34-APC
A
CD10 APC-A700
CD81 APC-H7
CD20-PB
[B-cells] CD45-KrO
1 Mature B: 3, 33.33%
Haematogone type 3
Under exp CD45 B-ALL
1 Early Pre B: 2, 22.22%
1 Late Pre B: 2, 22.22%
Count
[CD45+] CD19-PC7 / CD81 APC-H7
B-cells
Mature B-cells: 0.29%
Haem CD19+/CD45+ (late): 0.29%
Eosinophils
Haem CD19+/CD45dim (early): 1.75%
CD3+: 0.00%, 0.00%
[Precursor B-cells]
Haem CD19+/CD10++ (early)
Haem CD19+/CD10+ (late)
DX1 CD19/CD10: 0.00% Total, 0.00% Gated

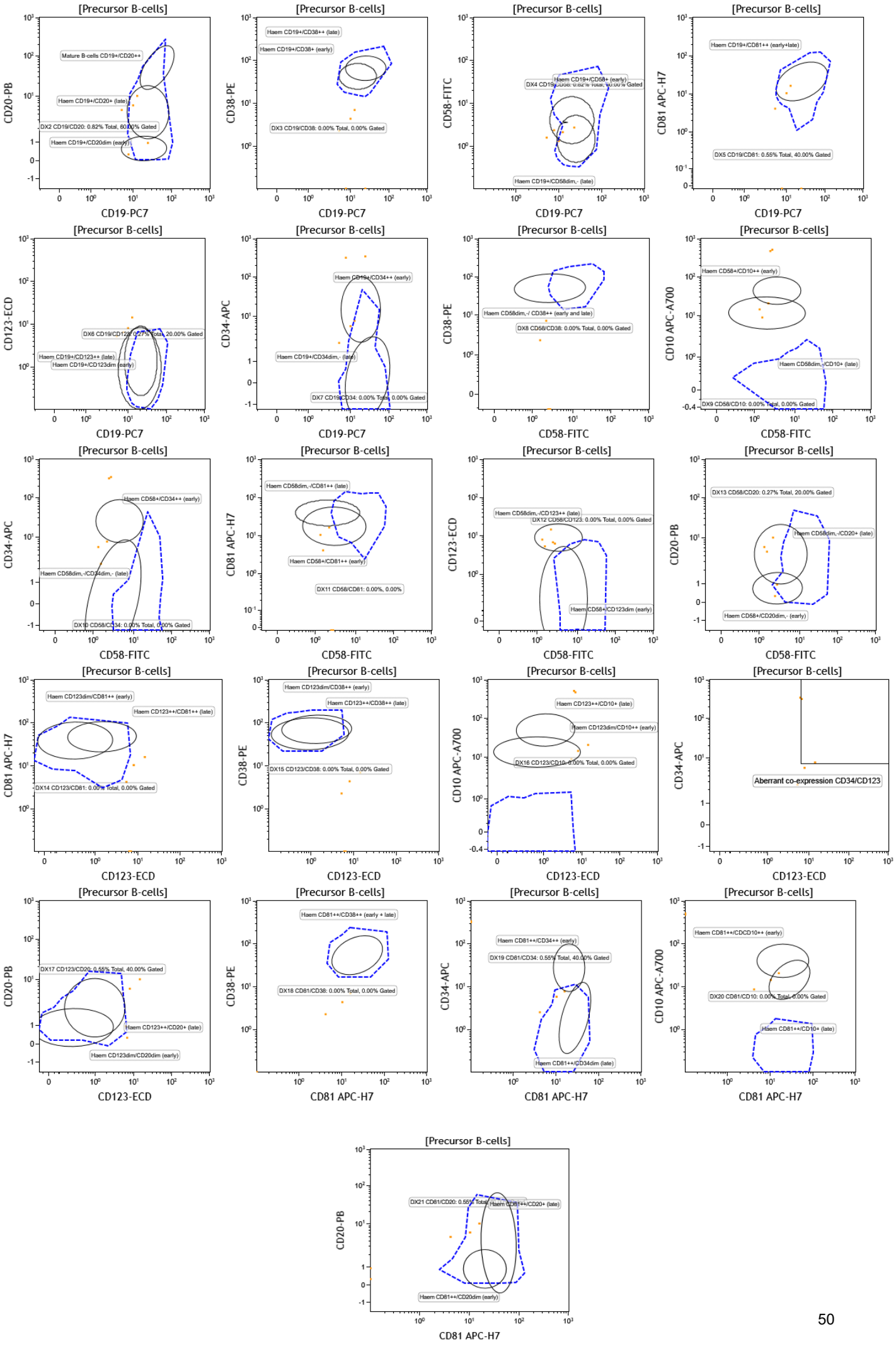

[Precursor B-cells]
CD20-PB
CD19-PC7
Mature B-cells CD19+/CD20++
Haem CD19+/CD20+ (late)
DX2 CD19/CD20: 0.82% Total, 60.00% Gated
Haem CD19+/CD20dim (early)
[Precursor B-cells]
CD38-PE
CD19-PC7
Haem CD19+/CD38++ (late)
Haem CD19+/CD38+ (early)
DX3 CD19/CD38: 0.00% Total, 0.00% Gated
[Precursor B-cells]
CD58-FITC
CD19-PC7
DX4 CD19/CD58: 0.82% Total, 60.00% Gated
Haem CD19+/CD58dim,- (late)
[Precursor B-cells]
CD81 APC-H7
CD19-PC7
Haem CD19+/CD81++ (early+late)
DX5 CD19/CD81: 0.55% Total, 40.00% Gated
[Precursor B-cells]
CD123-ECD
CD19-PC7
DX6 CD19/CD123: 0.27% Total, 20.00% Gated
Haem CD19+/CD123++ (late)
Haem CD19+/CD123dim (early)
[Precursor B-cells]
CD34-APC
CD19-PC7
Haem CD19+/CD34++ (early)
Haem CD19+/CD34dim,- (late)
DX7 CD19/CD34: 0.00% Total, 0.00% Gated
[Precursor B-cells]
CD38-PE
CD58-FITC
Haem CD58dim,-/ CD38++ (early and late)
DX8 CD58/CD38: 0.00% Total, 0.00% Gated
[Precursor B-cells]
CD10 APC-A700
CD58-FITC
Haem CD58+/CD10++ (early)
Haem CD58dim,-/CD10+ (late)
DX9 CD58/CD10: 0.00% Total, 0.00% Gated
[Precursor B-cells]
CD34-APC
CD58-FITC
Haem CD58+/CD34++ (early)
Haem CD58dim,-/CD34dim,- (late)
DX10 CD58/CD34: 0.00% Total, 0.00% Gated
[Precursor B-cells]
CD81 APC-H7
CD58-FITC
Haem CD58dim,-/CD81++ (late)
Haem CD58+/CD81++ (early)
DX11 CD58/CD81: 0.00%, 0.00%
[Precursor B-cells]
CD123-ECD
CD58-FITC
Haem CD58dim,-/CD123++ (late)
DX12 CD58/CD123: 0.00% Total, 0.00% Gated
Haem CD58+/CD123dim (early)
[Precursor B-cells]
CD20-PB
CD58-FITC
DX13 CD58/CD20: 0.27% Total, 20.00% Gated
Haem CD58dim,-/CD20+ (late)
Haem CD58+/CD20dim,- (early)
[Precursor B-cells]
CD81 APC-H7
CD123-ECD
Haem CD123dim/CD81++ (early)
Haem CD123++/CD81++ (late)
DX14 CD123/CD81: 0.00% Total, 0.00% Gated
[Precursor B-cells]
CD38-PE
CD123-ECD
Haem CD123dim/CD38++ (early)
Haem CD123++/CD38++ (late)
DX15 CD123/CD38: 0.00% Total, 0.00% Gated
[Precursor B-cells]
CD10 APC-A700
CD123-ECD
Haem CD123++/CD10+ (late)
Haem CD123dim/CD10++ (early)
DX16 CD123/CD10: 0.00% Total, 0.00% Gated
[Precursor B-cells]
CD34-APC
CD123-ECD
Aberrant co-expression CD34/CD123
[Precursor B-cells]
CD20-PB
CD123-ECD
DX17 CD123/CD20: 0.55% Total, 40.00% Gated
Haem CD123++/CD20+ (late)
Haem CD123dim/CD20dim (early)
[Precursor B-cells]
CD38-PE
CD81 APC-H7
Haem CD81++/CD38++ (early + late)
DX18 CD81/CD38: 0.00% Total, 0.00% Gated
[Precursor B-cells]
CD34-APC
CD81 APC-H7
Haem CD81++/CD34++ (early)
DX19 CD81/CD34: 0.55% Total, 40.00% Gated
Haem CD81++/CD34dim (late)
[Precursor B-cells]
CD10 APC-A700
CD81 APC-H7
Haem CD81++/CDCD10++ (early)
DX20 CD81/CD10: 0.00% Total, 0.00% Gated
Haem CD81++/CD10+ (late)
[Precursor B-cells]
CD20-PB
CD81 APC-H7
DX21 CD81/CD20: 0.55% Total, 40.00% Gated
Haem CD81++/CD20+ (late)
Haem CD81++/CD20dim (early)

Figure 4.1.1. Carry-over assessment of the Beckman Coulter Navios flow cytometer. CD, cluster of differentiation; SS, side scatter; FS, forward scatter; NSB, nonspecific binding; Mo, monocytes; Gr, granulocytes; Ly, lymphocytes; Haem, haematogones; DX, diagnostic gate; FITC, fluorescein isothiocyanate; APC-H7, allophycocyanin-cyanine dye.

* Abbreviations of the non-assessed markers are not further described.

APPENDIX H

Table 4.1.2. Individual lower limit of quantification and precision of the ClearLLab 10C™ B-cell/M2 and in-house immunophenotypic panels.

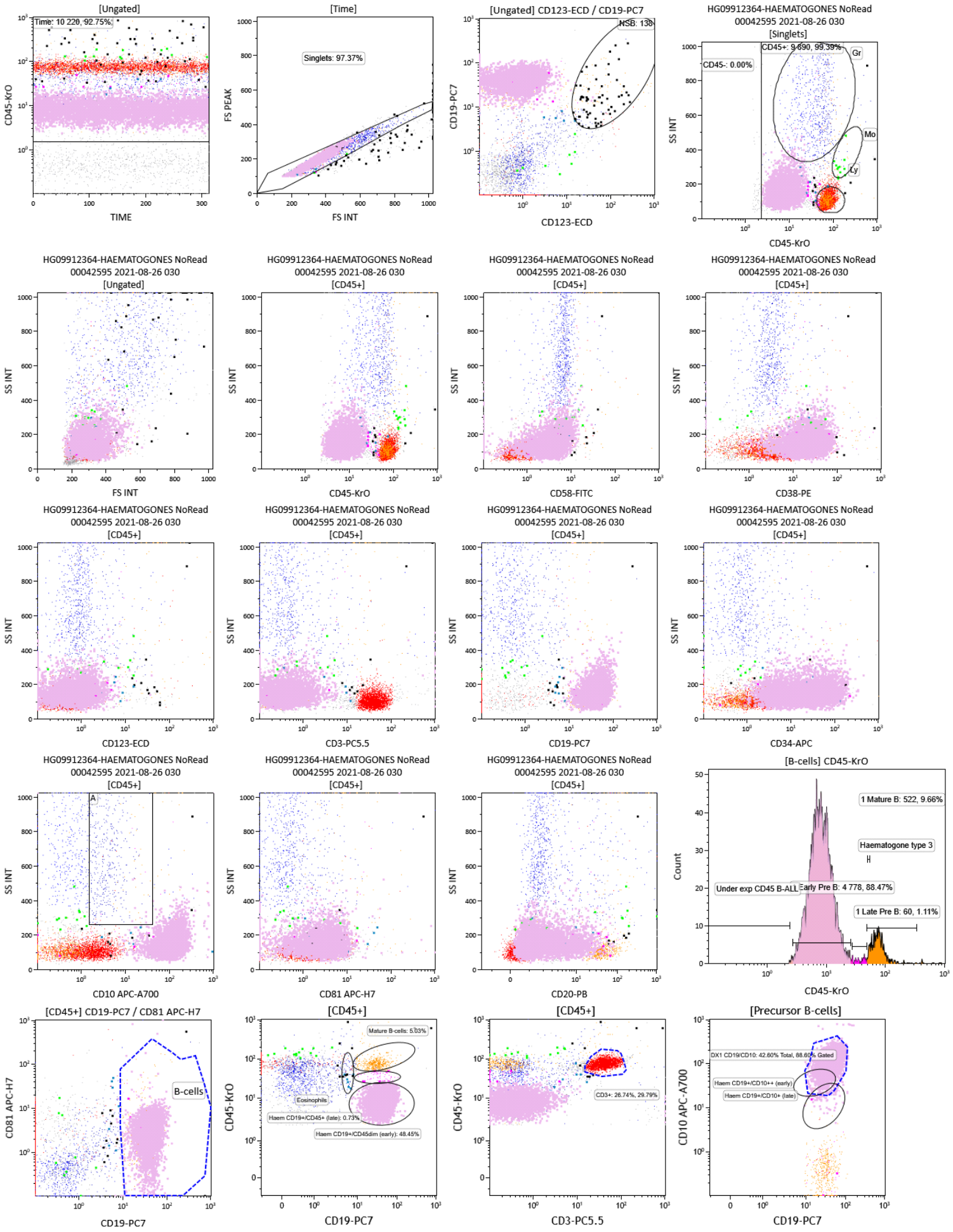
Dilution description	**ClearLLab 10C™ B-cell panel**			**ClearLLab 10C™ M2 panel**			**In-house immunophenotypic panel**		
Neat (% B-cell leukaemic cells)	~ 57 – 58			~59 – 60% (NSB noted)			~ 63 – 65		
Dual plot analysed	CD19/CD10 (cells/uL)	CD19/CD34 (cells/uL)	CD19/CD38 (cells/uL)	CD19/CD10 (cells/uL)	CD19/CD34 (cells/uL)	CD19/CD123 (cells/uL)	CD19/CD58 (cells/uL)	CD19/CD81 (cells/uL)	CD19/CD123 (cells/uL)
1: 2 dilution (dilution 1)	921	898	914	1070	1073	1066	1534	1519	1521
1: 4 dilution (dilution 2)	445	432	440	537	537	534	741	734	737
1: 8 dilution (dilution 3)	177	170	173	188	189	188	341	337	338
1: 16 dilution (dilution 4)	151	146	148	111	111	110	182	180	181
1: 32 dilution (dilution 5)	56	53	56	47	48	47	81	79	79

1: 64 dilution (dilution 6)	33	31	32	33	34	33	53	52	52
1: 128 dilution (dilution 7)	16	14	13	16	17	16	30	29	29
1: 256 dilution (dilution 8)	10	9	10	8	9	8	13	12	12
1: 512 dilution (dilution 9)	2	1	3	1	2	1	**0**	**0**	**0**
1: 1024 dilution (dilution 10)	**1**	**0**	**1**	**0**	**1**	**1**	0	0	0
1: 2048 dilution (dilution 11)	2	1	2	0	3	1	0	0	0
Precision dilution level identified and confirmed with consecutive runs	1: 1024 dilution (dilution 10)			1: 1024 dilution (dilution 10)			1: 512 dilution (dilution 9)		

* M2, myeloid-cell 2; CD, cluster of differentiation.

** CD34-APC out of stock for during this component of the study for the haematogone component**

APPENDIX I


[Ungated]
Time: 10 220, 92.75%
CD45-KrO
TIME
[Time]
Singlets: 97.37%
FS PEAK
FS INT
[Ungated] CD123-ECD / CD19-PC7
NSB: 138
CD19-PC7
CD123-ECD
HG09912364-HAEMATOGONES NoRead
00042595 2021-08-26 030
[Singlets]
CD45+: 9.690, 99.39%
CD45-: 0.00%
Gr
Mo
Ly
SS INT
CD45-KrO
HG09912364-HAEMATOGONES NoRead
00042595 2021-08-26 030
[Ungated]
SS INT
FS INT
HG09912364-HAEMATOGONES NoRead
00042595 2021-08-26 030
[CD45+]
SS INT
CD45-KrO
HG09912364-HAEMATOGONES NoRead
00042595 2021-08-26 030
[CD45+]
SS INT
CD58-FITC
HG09912364-HAEMATOGONES NoRead
00042595 2021-08-26 030
[CD45+]
SS INT
CD38-PE
HG09912364-HAEMATOGONES NoRead
00042595 2021-08-26 030
[CD45+]
SS INT
CD123-ECD
HG09912364-HAEMATOGONES NoRead
00042595 2021-08-26 030
[CD45+]
SS INT
CD3-PC5.5
HG09912364-HAEMATOGONES NoRead
00042595 2021-08-26 030
[CD45+]
SS INT
CD19-PC7
HG09912364-HAEMATOGONES NoRead
00042595 2021-08-26 030
[CD45+]
SS INT
CD34-APC
HG09912364-HAEMATOGONES NoRead
00042595 2021-08-26 030
[CD45+]
A
SS INT
CD10 APC-A700
HG09912364-HAEMATOGONES NoRead
00042595 2021-08-26 030
[CD45+]
SS INT
CD81 APC-H7
HG09912364-HAEMATOGONES NoRead
00042595 2021-08-26 030
[CD45+]
SS INT
CD20-PB
[B-cells] CD45-KrO
1 Mature B: 522, 9.66%
Haematogone type 3
Under exp CD45 B-ALL
Early Pre B: 4 778, 88.47%
1 Late Pre B: 60, 1.11%
Count
CD45-KrO
[CD45+] CD19-PC7 / CD81 APC-H7
B-cells
CD81 APC-H7
CD19-PC7
[CD45+]
Mature B-cells: 5.03%
Eosinophils
Haem CD19+/CD45+ (late): 0.73%
Haem CD19+/CD45dim (early): 48.45%
CD45-KrO
CD19-PC7
[CD45+]
CD3+: 26.74%, 29.79%
CD45-KrO
CD3-PC5.5
[Precursor B-cells]
DX1 CD19/CD10: 42.60% Total, 88.60% Gated
Haem CD19+/CD10++ (early)
Haem CD19+/CD10+ (late)
CD10 APC-A700
CD19-PC7

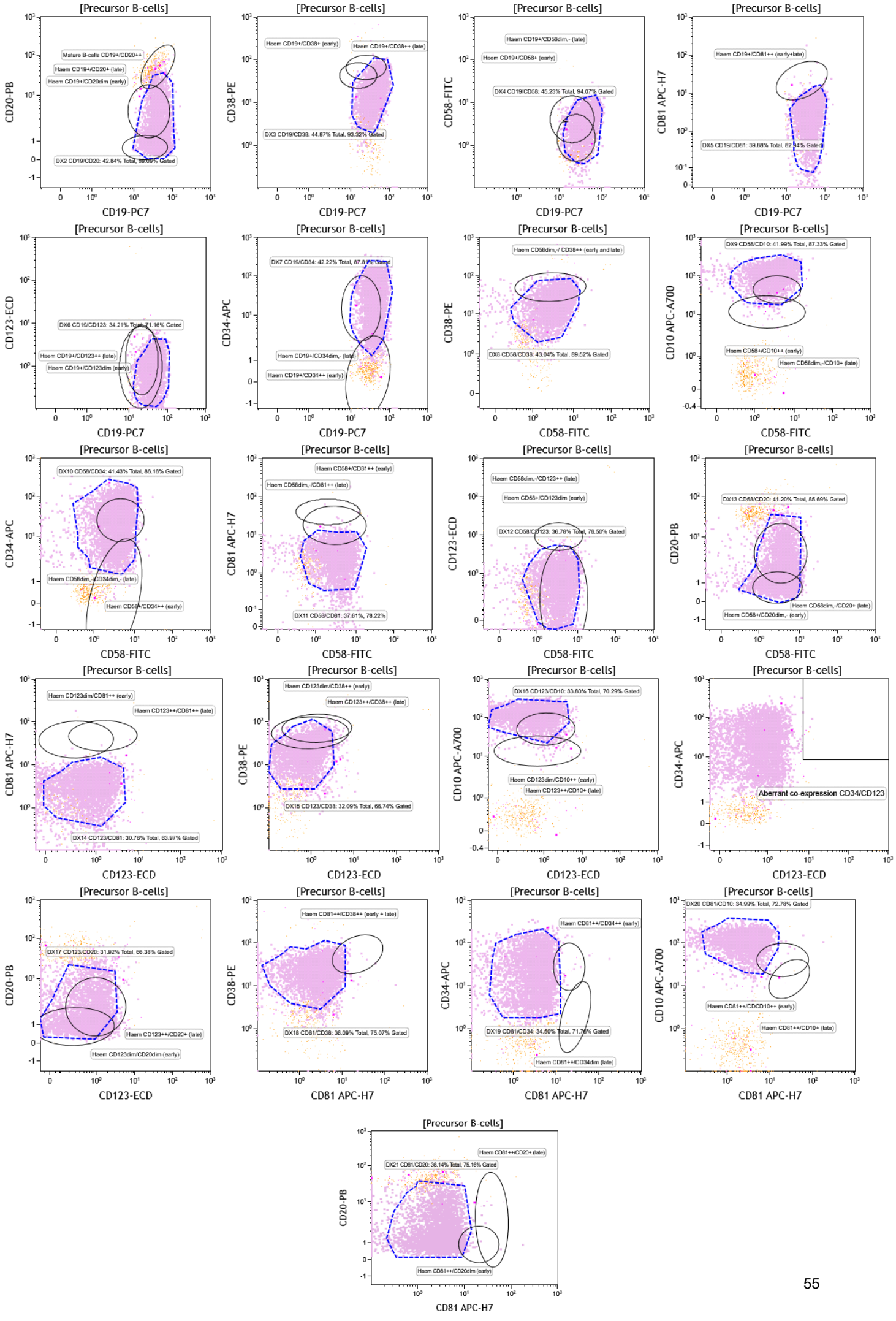
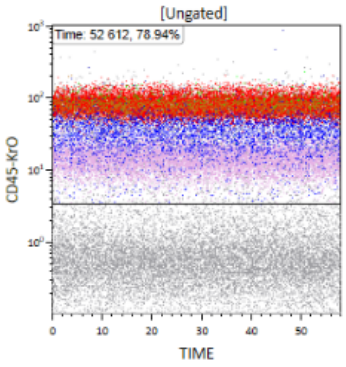
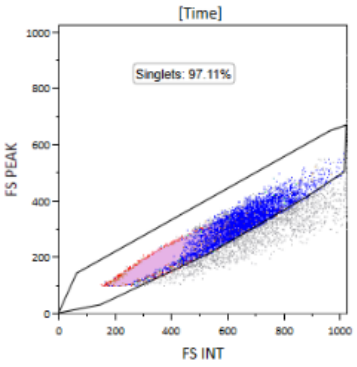

[Precursor B-cells]
Mature B-cells CD19+/CD20++
Haem CD19+/CD20+ (late)
Haem CD19+/CD20dim (early)
DX2 CD19/CD20: 42.84% Total, 88.09% Gated
CD20-PB
CD19-PC7
Haem CD19+/CD38+ (early)
Haem CD19+/CD38++ (late)
DX3 CD19/CD38: 44.87% Total, 93.32% Gated
CD38-PE
Haem CD19+/CD58dim,- (late)
Haem CD19+/CD58+ (early)
DX4 CD19/CD58: 45.23% Total, 94.07% Gated
CD58-FITC
Haem CD19+/CD81++ (early+late)
DX5 CD19/CD81: 39.88% Total, 82.94% Gated
CD81 APC-H7
DX6 CD19/CD123: 34.21% Total, 71.16% Gated
Haem CD19+/CD123++ (late)
Haem CD19+/CD123dim (early)
CD123-ECD
DX7 CD19/CD34: 42.22% Total, 87.81% Gated
Haem CD19+/CD34dim,- (late)
Haem CD19+/CD34++ (early)
CD34-APC
Haem CD58dim,-/ CD38++ (early and late)
DX8 CD58/CD38: 43.04% Total, 89.52% Gated
DX9 CD58/CD10: 41.99% Total, 87.33% Gated
Haem CD58+/CD10++ (early)
Haem CD58dim,-/CD10+ (late)
CD10 APC-A700
CD58-FITC
DX10 CD58/CD34: 41.43% Total, 86.16% Gated
Haem CD58dim,-/CD34dim,- (late)
Haem CD58+/CD34++ (early)
Haem CD58+/CD81++ (early)
Haem CD58dim,-/CD81++ (late)
DX11 CD58/CD81: 37.61%, 78.22%
Haem CD58dim,-/CD123++ (late)
Haem CD58+/CD123dim (early)
DX12 CD58/CD123: 36.78% Total, 76.50% Gated
DX13 CD58/CD20: 41.20% Total, 85.69% Gated
Haem CD58dim,-/CD20+ (late)
Haem CD58+/CD20dim,- (early)
Haem CD123dim/CD81++ (early)
Haem CD123++/CD81++ (late)
DX14 CD123/CD81: 30.76% Total, 63.97% Gated
Haem CD123dim/CD38++ (early)
Haem CD123++/CD38++ (late)
DX15 CD123/CD38: 32.09% Total, 66.74% Gated
DX16 CD123/CD10: 33.80% Total, 70.29% Gated
Haem CD123dim/CD10++ (early)
Haem CD123++/CD10+ (late)
Aberrant co-expression CD34/CD123
DX17 CD123/CD20: 31.92% Total, 66.38% Gated
Haem CD123++/CD20+ (late)
Haem CD123dim/CD20dim (early)
Haem CD81++/CD38++ (early + late)
DX18 CD81/CD38: 36.09% Total, 75.07% Gated
Haem CD81++/CD34++ (early)
DX19 CD81/CD34: 34.50% Total, 71.76% Gated
Haem CD81++/CD34dim (late)
DX20 CD81/CD10: 34.99% Total, 72.78% Gated
Haem CD81++/CDCD10++ (early)
Haem CD81++/CD10+ (late)
Haem CD81++/CD20+ (late)
DX21 CD81/CD20: 36.14% Total, 75.16% Gated
Haem CD81++/CD20dim (early)

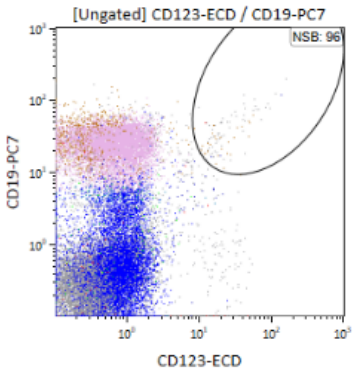
Figure 4.2.2. Example of the full immunophenotypic analysis of a patient's diagnostic remnant BMA sample using the in-house haematogone data analysis protocol. CD, cluster of differentiation; SS, side scatter; FS, forward scatter; NSB, non-specific binding; Mo, monocytes; Gr, granulocytes; Ly, lymphocytes; Haem, haematogones; DX, diagnostic gate; FITC, fluorescein isothiocyanate; APC-H7, allophycocyanin-cyanine dye.

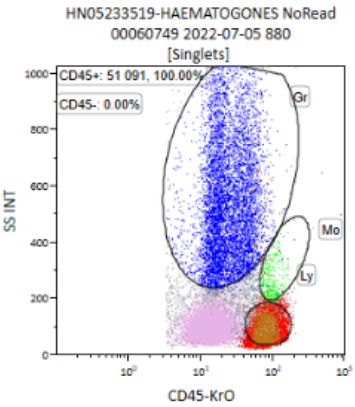
* Abbreviations of the non-assessed markers are not further described.

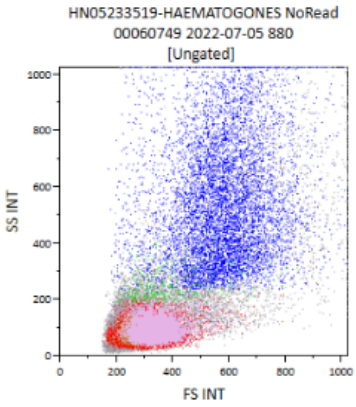
APPENDIX J

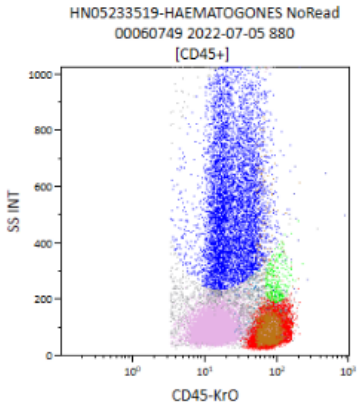

[Ungated]
Time: 52 612, 78.94%
CD45-KrO
TIME

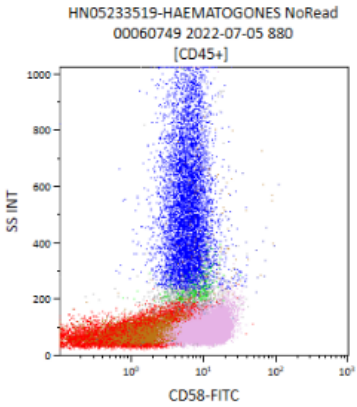

[Time]
Singlets: 97.11%
FS PEAK
FS INT

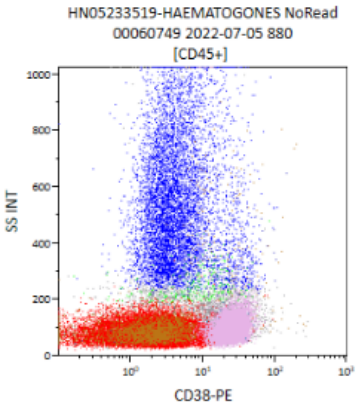

[Ungated] CD123-ECD / CD19-PC7
NSB: 96
CD19-PC7
CD123-ECD

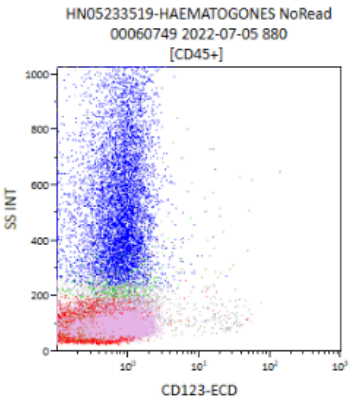

HN05233519-HAEMATOGONES NoRead
00060749 2022-07-05 880
[Singlets]
CD45+: 51 091, 100.00%
CD45-: 0.00%
Gr
Mo
Ly
SS INT
CD45-KrO

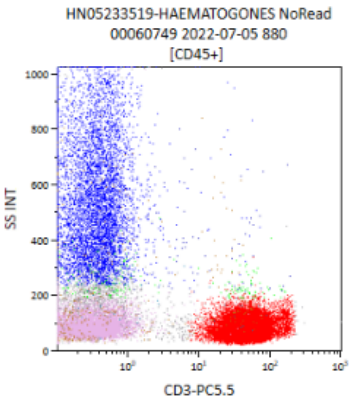

HN05233519-HAEMATOGONES NoRead
00060749 2022-07-05 880
[Ungated]
SS INT
FS INT

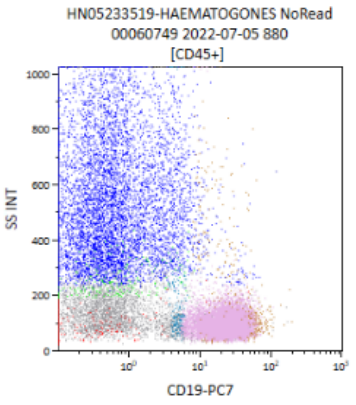

HN05233519-HAEMATOGONES NoRead
00060749 2022-07-05 880
[CD45+]
SS INT
CD45-KrO

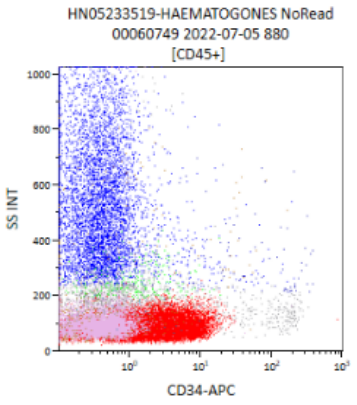

HN05233519-HAEMATOGONES NoRead
00060749 2022-07-05 880
[CD45+]
SS INT
CD58-FITC

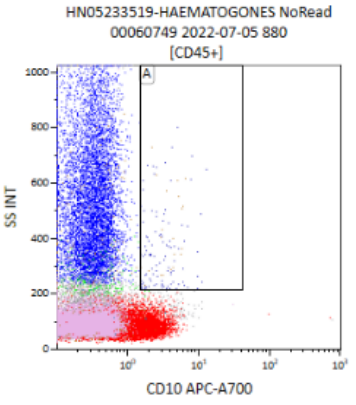

HN05233519-HAEMATOGONES NoRead
00060749 2022-07-05 880
[CD45+]
SS INT
CD38-PE

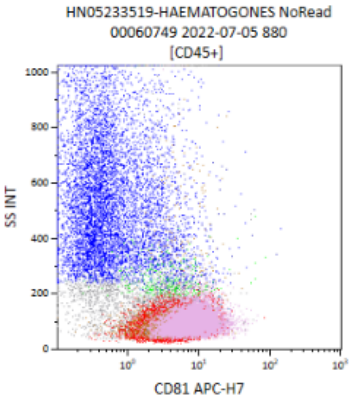

HN05233519-HAEMATOGONES NoRead
00060749 2022-07-05 880
[CD45+]
SS INT
CD123-ECD

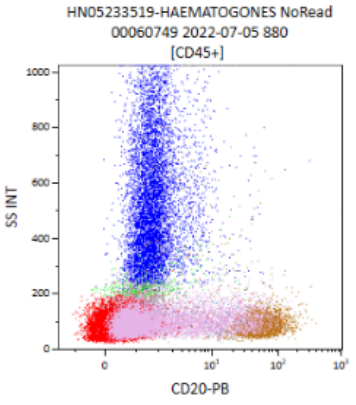

HN05233519-HAEMATOGONES NoRead
00060749 2022-07-05 880
[CD45+]
SS INT
CD3-PC5.5

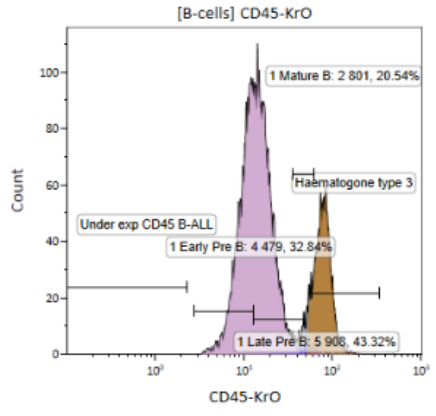

HN05233519-HAEMATOGONES NoRead
00060749 2022-07-05 880
[CD45+]
SS INT
CD19-PC7

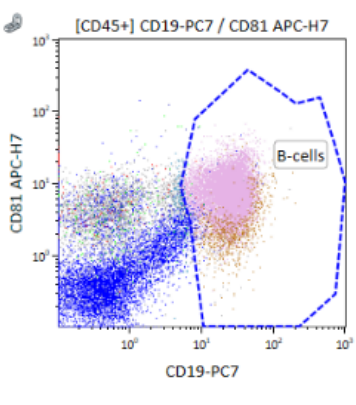

HN05233519-HAEMATOGONES NoRead
00060749 2022-07-05 880
[CD45+]
SS INT
CD34-APC

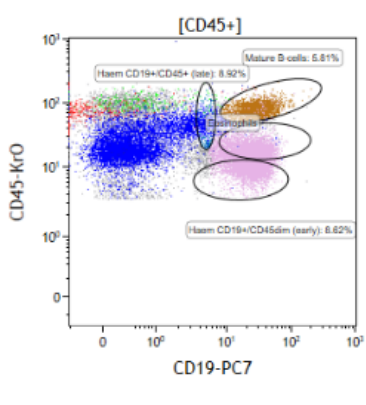

HN05233519-HAEMATOGONES NoRead
00060749 2022-07-05 880
[CD45+]
A
SS INT
CD10 APC-A700

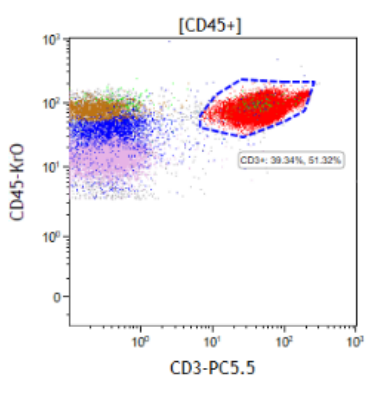

HN05233519-HAEMATOGONES NoRead
00060749 2022-07-05 880
[CD45+]
SS INT
CD81 APC-H7

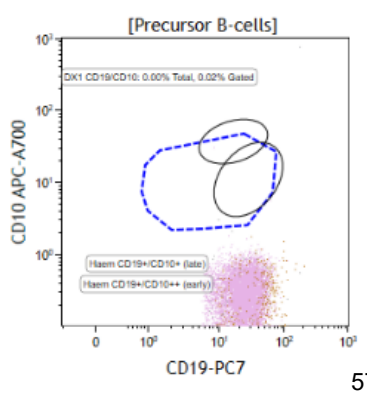

HN05233519-HAEMATOGONES NoRead
00060749 2022-07-05 880
[CD45+]
SS INT
CD20-PB


[B-cells] CD45-KrO
1 Mature B: 2 801, 20.54%
Haematogone type 3
Under exp CD45 B-ALL
1 Early Pre B: 4 479, 32.84%
1 Late Pre B: 5 908, 43.32%
Count
CD45-KrO


[CD45+] CD19-PC7 / CD81 APC-H7
B-cells
CD81 APC-H7
CD19-PC7


[CD45+]
Mature B-cells: 5.81%
Haem CD19+/CD45+ (late): 8.92%
Eosinophils
Haem CD19+/CD45dim (early): 6.62%
CD45-KrO
CD19-PC7


[CD45+]
CD3+: 39.34%, 51.32%
CD45-KrO
CD3-PC5.5


[Precursor B-cells]
DX1 CD19/CD10: 0.00% Total, 0.02% Gated
Haem CD19+/CD10+ (late)
Haem CD19+/CD10++ (early)
CD10 APC-A700
CD19-PC7

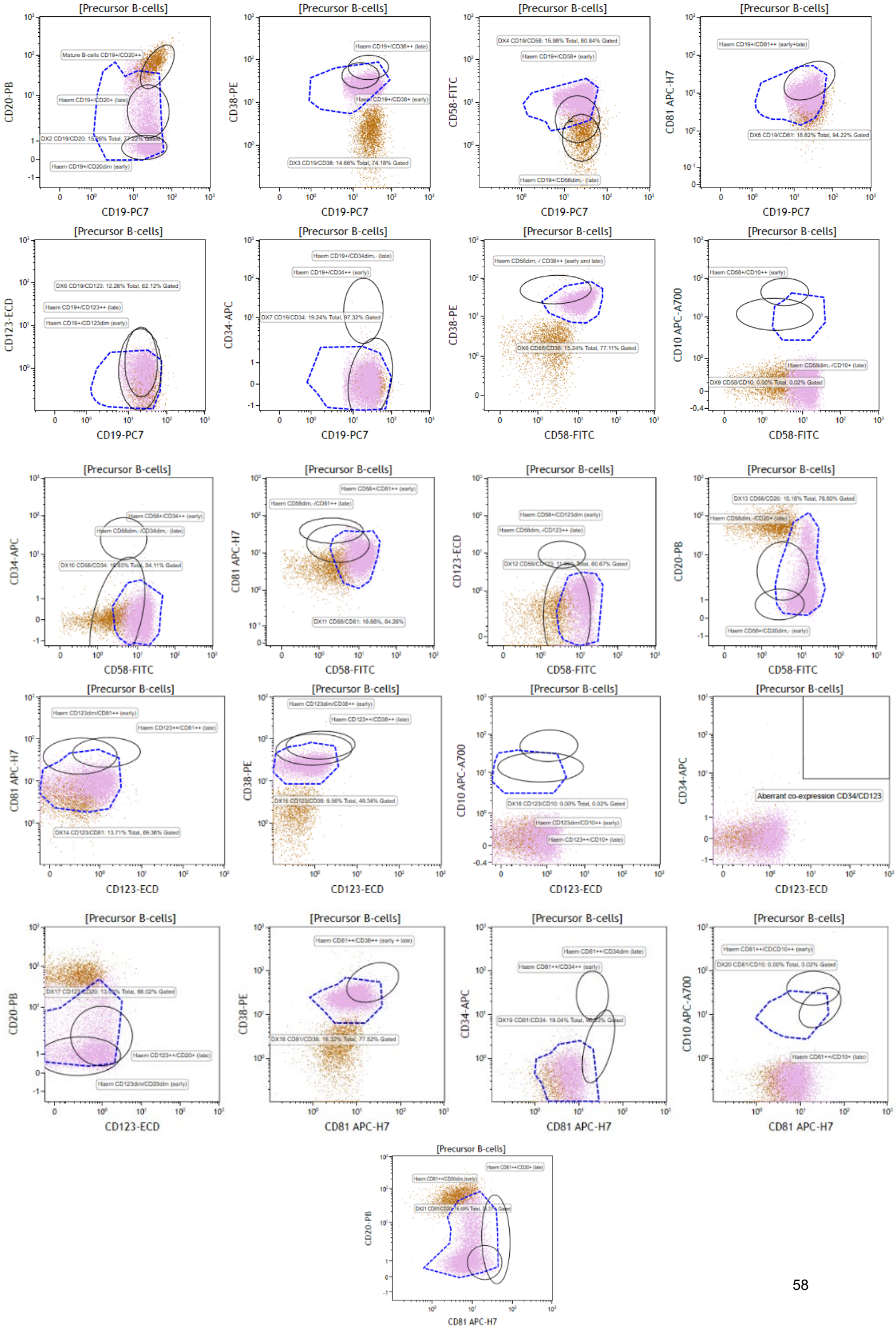
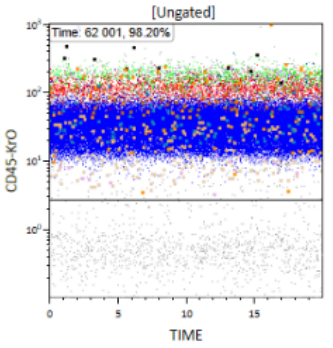
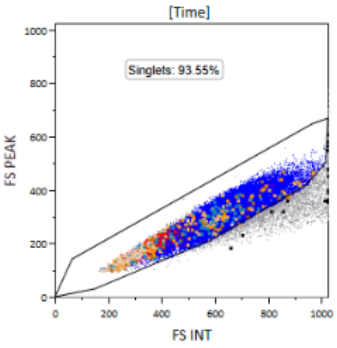
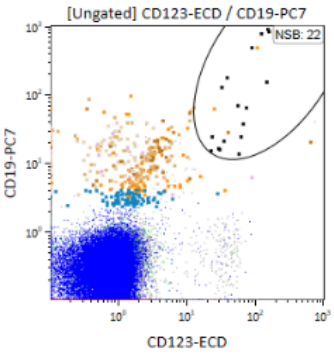

[Precursor B-cells]
CD20-PB
CD19-PC7
Mature B-cells CD19+/CD20++
Haem CD19+/CD20+ (late)
Haem CD19+/CD20dim (early)
[Precursor B-cells]
CD38-PE
CD19-PC7
Haem CD19+/CD38++ (late)
Haem CD19+/CD38+ (early)
DX3 CD19/CD38: 14.66% Total, 74.18% Gated
[Precursor B-cells]
CD58-FITC
CD19-PC7
DX4 CD19/CD58: 15.98% Total, 80.84% Gated
Haem CD19+/CD58+ (early)
Haem CD19+/CD58dim,- (late)
[Precursor B-cells]
CD81 APC-H7
CD19-PC7
Haem CD19+/CD81++ (early+late)
DX5 CD19/CD81: 18.62% Total, 94.22% Gated
[Precursor B-cells]
CD123-ECD
CD19-PC7
DX6 CD19/CD123: 12.28% Total, 62.12% Gated
Haem CD19+/CD123++ (late)
Haem CD19+/CD123dim (early)
[Precursor B-cells]
CD34-APC
CD19-PC7
Haem CD19+/CD34dim,- (late)
Haem CD19+/CD34++ (early)
DX7 CD19/CD34: 19.24% Total, 97.32% Gated
[Precursor B-cells]
CD38-PE
CD58-FITC
Haem CD58dim,-/ CD38++ (early and late)
DX8 CD58/CD38: 15.24% Total, 77.11% Gated
[Precursor B-cells]
CD10 APC-A700
CD58-FITC
Haem CD58+/CD10++ (early)
Haem CD58dim,-/CD10+ (late)
DX9 CD58/CD10: 0.00% Total, 0.02% Gated
[Precursor B-cells]
CD34-APC
CD58-FITC
Haem CD58+/CD34++ (early)
Haem CD58dim,-/CD34dim,- (late)
DX10 CD58/CD34: 16.33% Total, 84.11% Gated
[Precursor B-cells]
CD81 APC-H7
CD58-FITC
Haem CD58+/CD81++ (early)
Haem CD58dim,-/CD81++ (late)
DX11 CD58/CD81: 16.66%, 84.26%
[Precursor B-cells]
CD123-ECD
CD58-FITC
Haem CD58+/CD123dim (early)
Haem CD58dim,-/CD123++ (late)
DX12 CD58/CD123: 11.49% Total, 60.67% Gated
[Precursor B-cells]
CD20-PB
CD58-FITC
DX13 CD58/CD20: 15.16% Total, 78.80% Gated
Haem CD58dim,-/CD20+ (late)
Haem CD58+/CD20dim,- (early)
[Precursor B-cells]
CD81 APC-H7
CD123-ECD
Haem CD123dim/CD81++ (early)
Haem CD123++/CD81++ (late)
DX14 CD123/CD81: 13.71% Total, 69.36% Gated
[Precursor B-cells]
CD38-PE
CD123-ECD
Haem CD123dim/CD38++ (early)
Haem CD123++/CD38++ (late)
DX15 CD123/CD38: 9.56% Total, 48.34% Gated
[Precursor B-cells]
CD10 APC-A700
CD123-ECD
DX16 CD123/CD10: 0.00% Total, 0.02% Gated
Haem CD123dim/CD10++ (early)
Haem CD123++/CD10+ (late)
[Precursor B-cells]
CD34-APC
CD123-ECD
Aberrant co-expression CD34/CD123
[Precursor B-cells]
CD20-PB
CD123-ECD
Haem CD123++/CD20+ (late)
Haem CD123dim/CD20dim (early)
[Precursor B-cells]
CD38-PE
CD81 APC-H7
Haem CD81++/CD38++ (early + late)
DX18 CD81/CD38: 15.32% Total, 77.52% Gated
[Precursor B-cells]
CD34-APC
CD81 APC-H7
Haem CD81++/CD34dim (late)
Haem CD81++/CD34++ (early)
[Precursor B-cells]
CD10 APC-A700
CD81 APC-H7
Haem CD81++/CD10++ (early)
DX20 CD81/CD10: 0.00% Total, 0.02% Gated
Haem CD81++/CD10+ (late)
[Precursor B-cells]
CD20-PB
CD81 APC-H7
Haem CD81++/CD20+ (late)

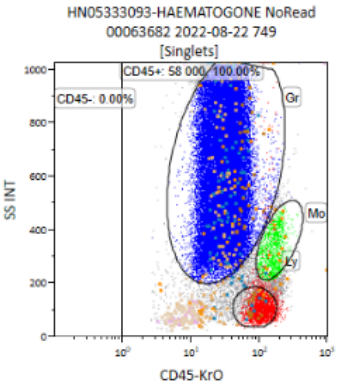
Figure 4.2.4. Example of the full immunophenotypic analysis of a patient's follow-up remnant BMA sample using the in-house haematogone data analysis protocol, showing significant residual disease. CD, cluster of differentiation; SS, side scatter; FS, forward scatter; NSB, non-specific binding; Mo, monocytes; Gr, granulocytes; Ly, lymphocytes; Haem, haematogones; DX, diagnostic gate; FITC, fluorescein isothiocyanate; APC-H7, allophycocyanin-cyanine dye.

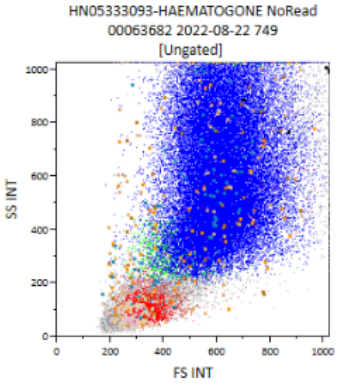
* Abbreviations of the non-assessed markers are not further described.

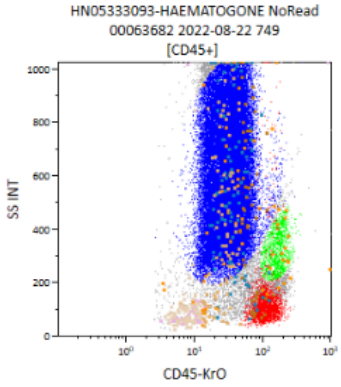

[Ungated]
Time: 62 001, 98.20%
CD45-KrO
TIME

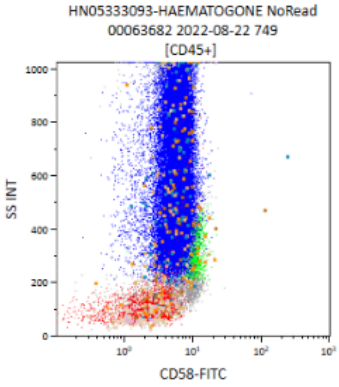

[Time]
Singlets: 93.55%
FS PEAK
FS INT

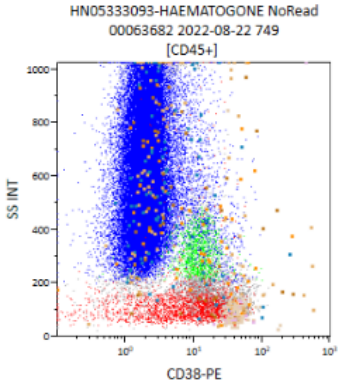

[Ungated] CD123-ECD / CD19-PC7
NSB: 22
CD19-PC7
CD123-ECD

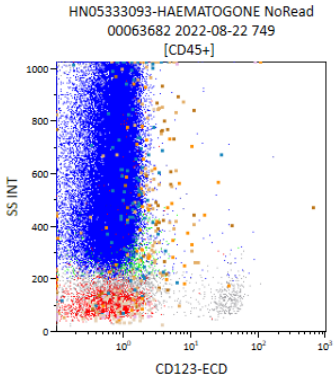

HN05333093-HAEMATOGONE NoRead
00063682 2022-08-22 749
[Singlets]
CD45+: 58 000, 100.00%
CD45-: 0.00%
Gr
Mo
Ly
SS INT
CD45-KrO

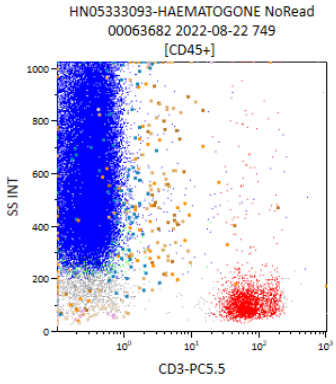

HN05333093-HAEMATOGONE NoRead
00063682 2022-08-22 749
[Ungated]
SS INT
FS INT

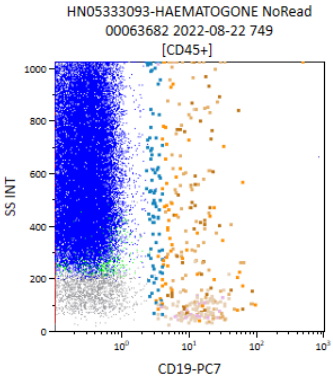

HN05333093-HAEMATOGONE NoRead
00063682 2022-08-22 749
[CD45+]
SS INT
CD45-KrO

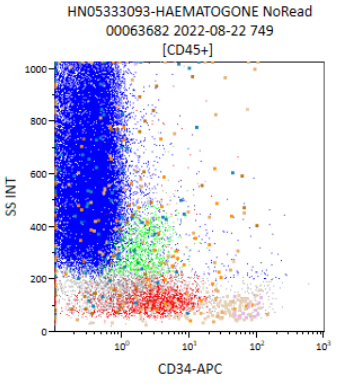

HN05333093-HAEMATOGONE NoRead
00063682 2022-08-22 749
[CD45+]
SS INT
CD58-FITC

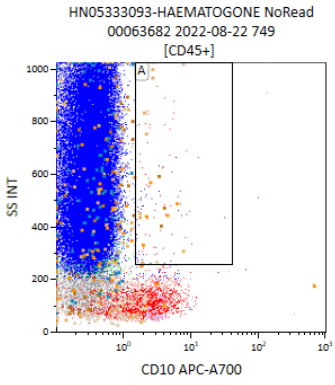

HN05333093-HAEMATOGONE NoRead
00063682 2022-08-22 749
[CD45+]
SS INT
CD38-PE

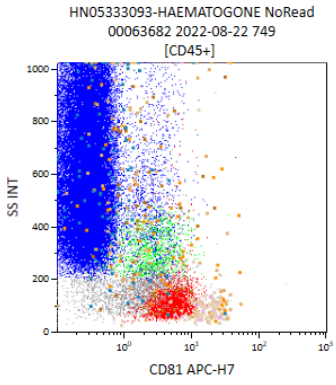

HN05333093-HAEMATOGONE NoRead
00063682 2022-08-22 749
[CD45+]
SS INT
CD123-ECD

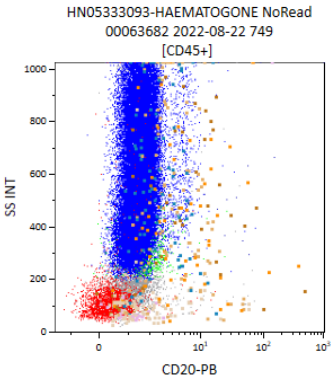

HN05333093-HAEMATOGONE NoRead
00063682 2022-08-22 749
[CD45+]
SS INT
CD3-PC5.5

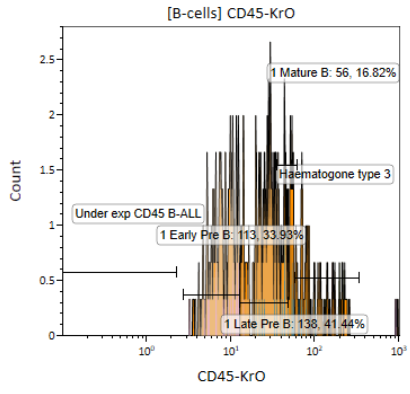

HN05333093-HAEMATOGONE NoRead
00063682 2022-08-22 749
[CD45+]
SS INT
CD19-PC7

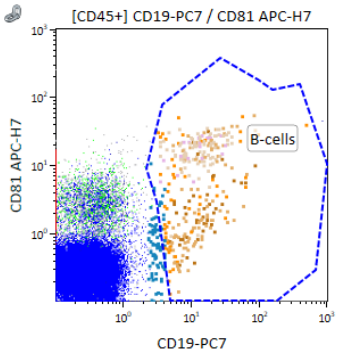

HN05333093-HAEMATOGONE NoRead
00063682 2022-08-22 749
[CD45+]
SS INT
CD34-APC

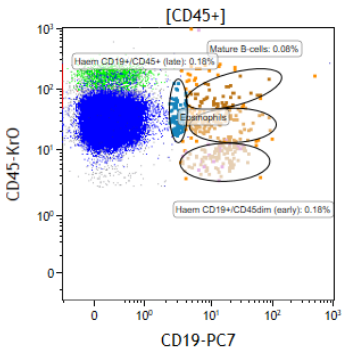

HN05333093-HAEMATOGONE NoRead
00063682 2022-08-22 749
[CD45+]
A
SS INT
CD10 APC-A700

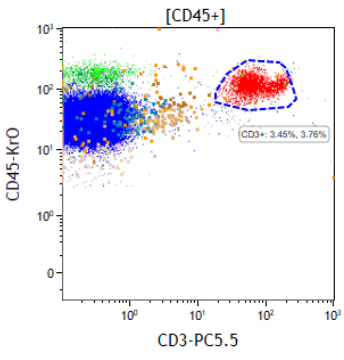

HN05333093-HAEMATOGONE NoRead
00063682 2022-08-22 749
[CD45+]
SS INT
CD81 APC-H7

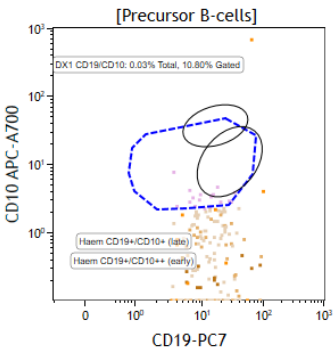

HN05333093-HAEMATOGONE NoRead
00063682 2022-08-22 749
[CD45+]
SS INT
CD20-PB

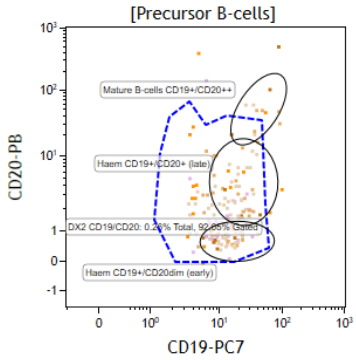

[B-cells] CD45-KrO
1 Mature B: 56, 16.82%
Haematogone type 3
Under exp CD45 B-ALL
1 Early Pre B: 113, 33.93%
1 Late Pre B: 138, 41.44%
Count
CD45-KrO

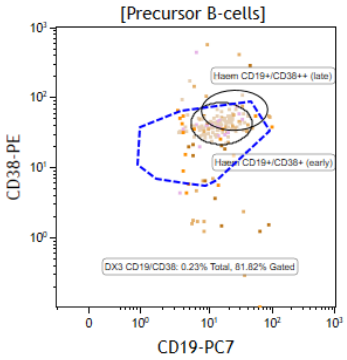

[CD45+] CD19-PC7 / CD81 APC-H7
B-cells
CD81 APC-H7
CD19-PC7

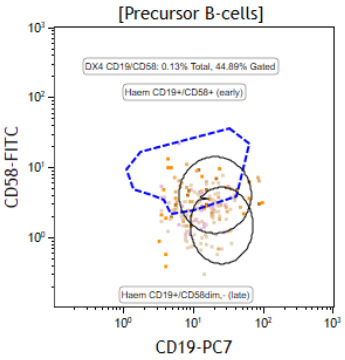

[CD45+]
Mature B-cells: 0.08%
Haem CD19+/CD45+ (late): 0.18%
Eosinophils
Haem CD19+/CD45dim (early): 0.18%
CD45-KrO
CD19-PC7

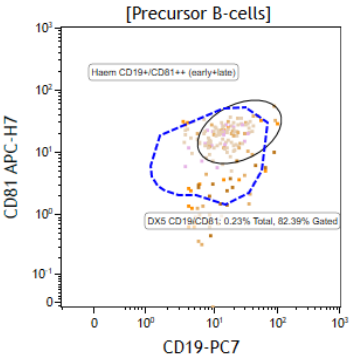

[CD45+]
CD3+: 3.45%, 3.76%
CD45-KrO
CD3-PC5.5

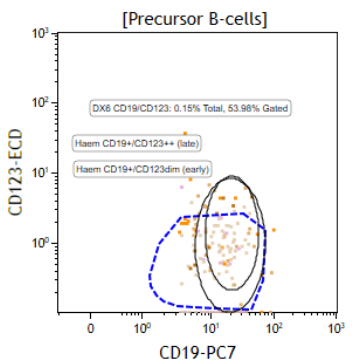

[Precursor B-cells]
DX1 CD19/CD10: 0.03% Total, 10.80% Gated
Haem CD19+/CD10+ (late)
Haem CD19+/CD10++ (early)
CD10 APC-A700
CD19-PC7

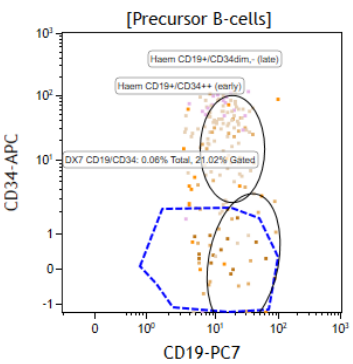

[Precursor B-cells]
Mature B-cells CD19+/CD20++
Haem CD19+/CD20+ (late)
Haem CD19+/CD20dim (early)
CD20-PB
CD19-PC7

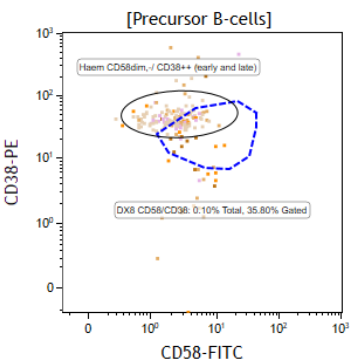

[Precursor B-cells]
Haem CD19+/CD38++ (late)
Haem CD19+/CD38+ (early)
DX3 CD19/CD38: 0.23% Total, 81.82% Gated
CD38-PE
CD19-PC7

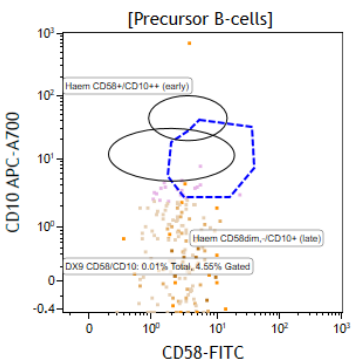

[Precursor B-cells]
DX4 CD19/CD58: 0.13% Total, 44.89% Gated
Haem CD19+/CD58+ (early)
Haem CD19+/CD58dim,- (late)
CD58-FITC
CD19-PC7

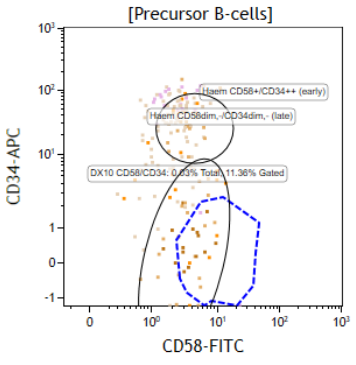

[Precursor B-cells]
Haem CD19+/CD81++ (early+late)
DX5 CD19/CD81: 0.23% Total, 82.39% Gated
CD81 APC-H7
CD19-PC7

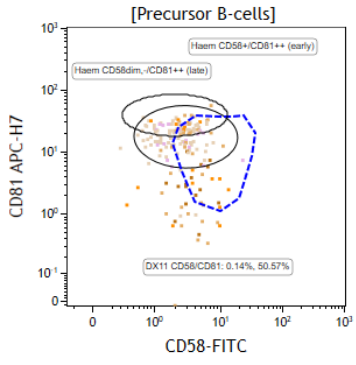

[Precursor B-cells]
DX6 CD19/CD123: 0.15% Total, 53.98% Gated
Haem CD19+/CD123++ (late)
Haem CD19+/CD123dim (early)
CD123-ECD
CD19-PC7

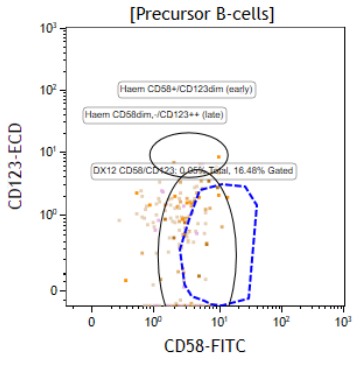

[Precursor B-cells]
Haem CD19+/CD34dim,- (late)
Haem CD19+/CD34++ (early)
DX7 CD19/CD34: 0.06% Total, 21.02% Gated
CD34-APC
CD19-PC7

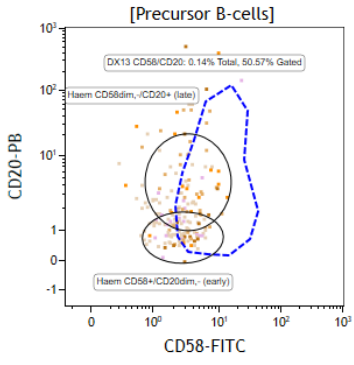

[Precursor B-cells]
Haem CD58dim,-/ CD38++ (early and late)
DX8 CD58/CD38: 0.10% Total, 35.80% Gated
CD38-PE
CD58-FITC

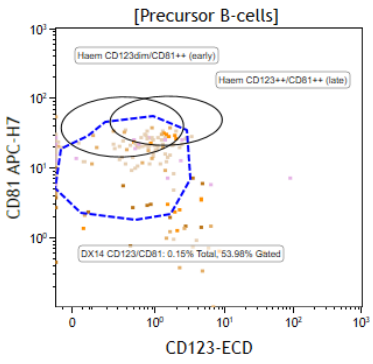

[Precursor B-cells]
Haem CD58+/CD10++ (early)
Haem CD58dim,-/CD10+ (late)
DX9 CD58/CD10: 0.01% Total, 4.55% Gated
CD10 APC-A700
CD58-FITC

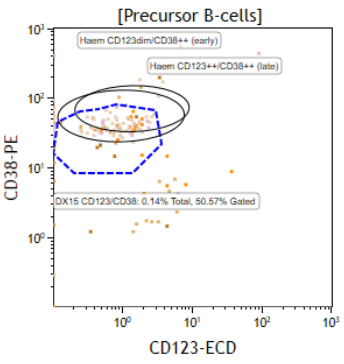

[Precursor B-cells]
Haem CD58+/CD34++ (early)
Haem CD58dim,-/CD34dim,- (late)
DX10 CD58/CD34: 0.03% Total, 11.36% Gated
CD34-APC
CD58-FITC

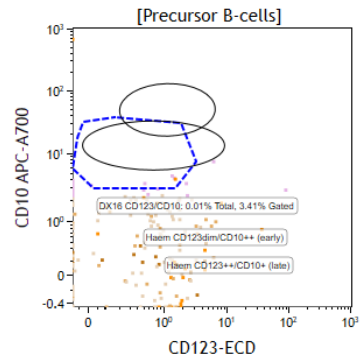

[Precursor B-cells]
Haem CD58+/CD81++ (early)
Haem CD58dim,-/CD81++ (late)
DX11 CD58/CD81: 0.14%, 50.57%
CD81 APC-H7
CD58-FITC

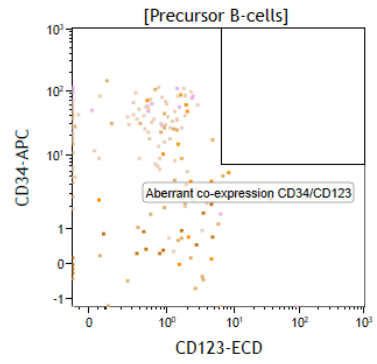

[Precursor B-cells]
Haem CD58+/CD123dim (early)
Haem CD58dim,-/CD123++ (late)
DX12 CD58/CD123: 0.06% Total, 16.48% Gated
CD123-ECD
CD58-FITC

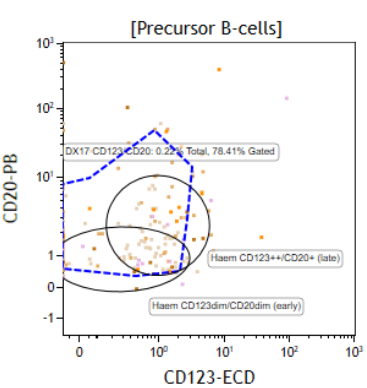

[Precursor B-cells]
DX13 CD58/CD20: 0.14% Total, 50.57% Gated
Haem CD58dim,-/CD20+ (late)
Haem CD58+/CD20dim,- (early)
CD20-PB
CD58-FITC

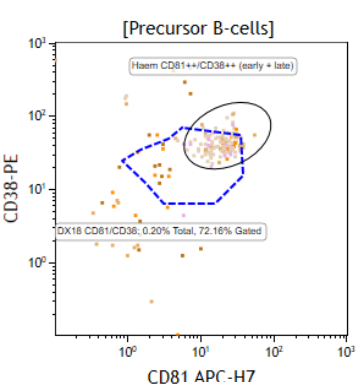

[Precursor B-cells]
Haem CD123dim/CD81++ (early)
Haem CD123++/CD81++ (late)
DX14 CD123/CD81: 0.15% Total, 53.98% Gated
CD81 APC-H7
CD123-ECD

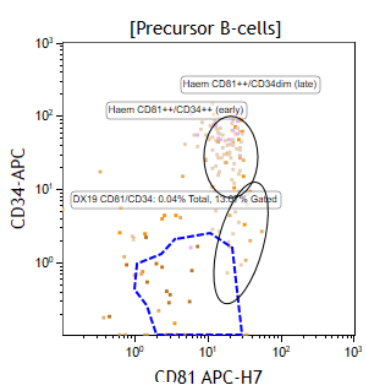

[Precursor B-cells]
Haem CD123dim/CD38++ (early)
Haem CD123++/CD38++ (late)
DX15 CD123/CD38: 0.14% Total, 50.57% Gated
CD38-PE
CD123-ECD

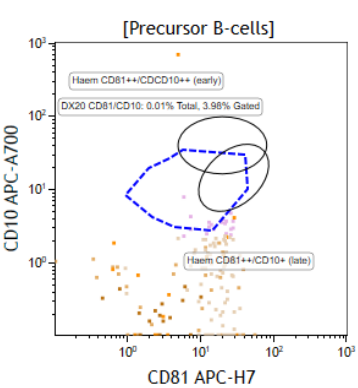

[Precursor B-cells]
DX16 CD123/CD10: 0.01% Total, 3.41% Gated
Haem CD123dim/CD10++ (early)
Haem CD123++/CD10+ (late)
CD10 APC-A700
CD123-ECD


[Precursor B-cells]
Aberrant co-expression CD34/CD123
CD34-APC
CD123-ECD


[Precursor B-cells]
DX17 CD123/CD20: 0.22% Total, 78.41% Gated
Haem CD123++/CD20+ (late)
Haem CD123dim/CD20dim (early)
CD20-PB
CD123-ECD


[Precursor B-cells]
Haem CD81++/CD38++ (early + late)
DX18 CD81/CD38: 0.20% Total, 72.16% Gated
CD38-PE
CD81 APC-H7


[Precursor B-cells]
Haem CD81++/CD34dim (late)
Haem CD81++/CD34++ (early)
DX19 CD81/CD34: 0.04% Total, 13.66% Gated
CD34-APC
CD81 APC-H7


[Precursor B-cells]
Haem CD81++/CDCD10++ (early)
DX20 CD81/CD10: 0.01% Total, 3.98% Gated
Haem CD81++/CD10+ (late)
CD10 APC-A700
CD81 APC-H7

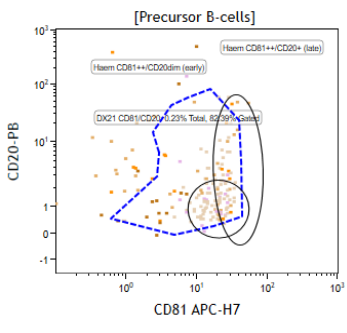

[Precursor B-cells]
Haem CD81++/CD20+ (late)
Haem CD81++/CD20dim (early)
DX21 CD81/CD20: 0.23% Total, 82.39% Gated
CD20-PB
CD81 APC-H7

Figure 4.2.5. Example of the full immunophenotypic analysis of a patient's follow-up remnant BMA sample using the in-house haematogone data analysis protocol, showing no overt immunophenotypic evidence of residual disease (i.e. no distinct clustering of LAIP). CD, cluster of differentiation; SS, side scatter; FS, forward scatter; NSB, non-specific binding; Mo, monocytes; Gr, granulocytes; Ly, lymphocytes; Haem, haematogones; DX, diagnostic gate; FITC, fluorescein isothiocyanate; APC-H7, allophycocyanin-cyanine dye.

* Abbreviations of the non-assessed markers are not further described.

APPENDIX K

Table 4.2.1. Raw data assessing the association between dependent binary outcome and independent exposure variables from which logistic regression analysis was performed.

Patient	Follow-up sample number	Binary outcome variable (cPCR)	Independent binary exposure variable	
			ClearLLab 10C™ B-cell/M2	In-house immunophenotypic panel
1	1	0	0	0
2	2	0	N	0
	3	0	0	0
3	4	0	N	0
	5	0	0	0
	6	0	0	N
4	7	1	1	1
	8	0	N	0
	9	0	0	0
	10	0	0	0
5	11	0	N	0
	12	0	0	0
6	13	0	0	0
	14	0	0	0
	15	1	0	N
	16	0	0	N
7	17	0	0	0
	18	0	0	0
	19	1	0	1
8	20	0	0	0
9	21	0	0	0
	22	0	N	0
10	23	0	N	0
11	24	0	0	0
	25	0	0	0
	26	0	N	N
12	27	0	0	0
13	28	0	N	0
14	29	0	0	0
	30	0	0	0
15	31	0	0	0
16	32	0	0	0
17	33	0	0	0
18	34	0	0	0
19	35	0	0	0
20	36	0	0	0
21	37	1	1	1
	38	0	0	0
	39	1	0	1
	40	0	0	0
	41	0	0	0
22	42	0	0	0

	43	1	0	0
	44	0	0	N
	45	0	0	0
23	46	1	N	1
	47	1	0	1
	48	1	0	N
	49	1	0	N
24	50	0	0	0
25	51	0	0	0
	52	0	0	0
26	53	0	N	N
	54	0	N	0
27	55	1	N	1
28	56	0	N	N
	57	0	0	0
29	58	N	0	N
30	59	0	0	0
	60	0	0	0
	61	1	N	N
31	62	1	N	1
32	63	0	0	0
	64	0	0	0
33	65	1	1	1
	66	1	N	N
34	67	1	N	1
	68	0	0	0
35	69	1	N	1
	70	0	0	0
36	71	0	0	0
	72	0	N	N
37	73	N	0	0
	74	N	0	0
38	75	1	1	1
39	76	0	0	0
40	77	0	0	0
	78	0	0	0

cPCR, conventional polymerase chain reaction; M2, myeloid-2.

Explanation of coding strategy using convention for coding

The binary outcome variable demarcated the presence or absence of disease as determined by the gold standard and superior cPCR. Independent binary exposure variables were the two mutually exclusive immunophenotypic panels in question.

Binary outcome variable (cPCR)

- **1 = presence of disease**
- **0 = absence of disease**

Includes the presence of out of range clones for which their clinical significance is unknown, as they were not detected at diagnosis. cPCR probes only detect clones with specific nucleotide lengths within a reportable range. Clonal markers outside of this range may be mono-, pseudo- or oligo-clonal.

- These out of range clones may be owing to evolution of an underlying subclone which has obtained a survival advantage (even with chemotherapy).
- For ease of interpretation, these specimens were considered 0 = absence of disease.
- This interpretation was carried forward in the contingency table, described under "Other noteworthy findings and observations" in the Part 2 results interpretation.

- **N = neutral**

Unable to exclude residual disease owing to no result or amplification failure of cPCR.

Independent binary exposure outcome variable (immunophenotypic panels)

- **0 = absence of disease by the binary outcome variable AND binary exposure outcome variable**
  e.g. cPCR (0) and immunophenotypic panels (0)

- **1 = presence of disease by the binary outcome variable AND binary the exposure outcome variable**
  e.g. cPCR (1) and immunophenotypic panels (1)

- **N = neutral**
  - Disease cannot be excluded using the binary outcome variable e.g. cPCR (N) and immunophenotypic panels (0/1).
  - Disease cannot be excluded using the binary exposure outcome variable e.g. cPCR (0/1) and immunophenotypic panels (N).
  - Binary exposure outcome variable detected disease when there was none detected by the binary outcome variable. e.g. cPCR (0) and immunophenotypic panels (N).
  - Binary exposure outcome variable did not detect disease when there was disease detected by the binary outcome variable e.g. cPCR (1) and immunophenotypic panels (N).

There are two main reasons for inability to exclude disease using the individual panels, described under "Other noteworthy findings and observations" in the Part 2 results interpretation.

1) Absence of aberrant markers for definitive exclusion of disease, particularly seen in the ClearLLab $10C^{TM}$ B-cell/M2 panels.
2) Prominent non-specific binding (NSB) which prevented meaningful immunophenotypic analysis, seen particularly in the in-house immunophenotypic panel owing to delay in processing.

Table 4.2.2. Tables of test by response: dependent binary outcome variable versus independent exposure variables.

Binary outcome variable versus ClearLLab 10C™ B-cell/M2 panels			
Test	**1**	**0**	**Total**
1	4 13.33 66.67 44.44	2 6.67 33.33 9.52	6 20.00
0	5 16.67 20.83 55.56	19 63.33 79.17 90.48	24 80.00
Total	9 30.00	21 70.00	30 100.0

Binary outcome variable versus in-house immunophenotypic panel			
Test	**1**	**0**	**Total**
1	9 25.71 100.00 81.82	0 0.00 0.00 0.00	9 25.71
0	2 5.71 7.69 18.18	24 68.57 92.31 100.00	26 74.29
Total	11 31.43	24 68.57	35 100.00

M2, myeloid-2.

APPENDIX L

R14/49 Prof Deborah K. Glencross et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M1704129

NAME: (Principal Investigator)	Prof Deborah K. Glencross et al
DEPARTMENT:	Molecular Medicine and Haematology University of the Witwatersrand Molecular Medicine and Haematology
PROJECT TITLE:	Class Ethics Clearance for Kit, Equipment, Method and Reagent Evaluation
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	Renewal 5 Years Valid for the Period 15 April 2017 - 30 April 2022 Previously M121020
SUPERVISOR:	
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	17/05/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the the conditions under which I am/we are authorised 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 resubmit to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. in this case, the study was initially review in April and will therefore be due in the month of April each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 14·07·2017

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

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

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

TO: Dr Z Nell
School of Pathology
Department of Molecular Medicine and Haematology
Medical School
University

E-mail: Zanre.Nell@nhls.ac.za

CC: Supervisor: Professors D Glencross and J Geel
<Deborah.Glencross@wits.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: 2022/04/28

REF: R14/49

PROTOCOL NO: **M220153** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)

PROJECT TITLE: *A multi-centre study to evaluate an in-house multi-parameter immunophenotypic panel to identify precursor B-cells in the determination of measurable residual disease in paediatric B-cell acute lymphoblastic leukaemia*

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

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R49 Dr Z Nell

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M220153

NAME: **(Principal Investigator)**	Dr Z Nell
DEPARTMENT:	School of Pathology Department of Molecular Medicine and Haematology Medical School University
PROJECT TITLE:	*A multi-centre study to evaluate an in-house multi-parameter immunophenotypic panel to identify precursor B-cells in the determination of measurable residual disease in paediatric B-cell acute lymphoblastic leukaemia*
DATE CONSIDERED:	2022/01/28
DECISION:	Approved unconditionally
CONDITIONS:	
NOTE:	If contact information regarding student study participants is required, please contact the Registrar's office - <Nicoleen.Potgieter@wits.ac.za>
SUPERVISOR:	Professors D Glencross and J Gee
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2022/04/28

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.** When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **January** and therefore reports and re-certification will be due in the month of **January** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator Date

Academic Affairs and Research
Modderfontein Road, Sandringham, 2031
Tel: +27 (0)11 386 6142
Fax: +27 (0)11 386 6296
Email: babatyi.kgokong@nhls.ac.za
Web: www.nhls.ac.za

23 February 2022

Applicant: Zanre Nell
Institution: National Health Laboratory Service
E-mail Address: zanre.nell@nhls.ac.za
Cell: 072 407 0619

CC: Deborah Glencross, Jennifer Geel

Project Title: A multi-centre study to evaluate an in-house multi-parameter immunophenotypic panel to identify precursor B-cells in the determination of measurable residual disease in paediatric B-cell acute lymphoblastic leukaemia
Reference Number: PR2222608

Application Type(s):
1. Permission to use NHLS Facilities

RE: APPROVAL LETTER: REQUEST TO CONDUCT RESEARCH

This letter serves to advise that an application requesting permission to conduct research has been reviewed and was **"Approved"**. Please note that approval is granted on your compliance with the NHLS conditions of service and that the study can only be undertaken provided that the following conditions have been met.

1. All material and data requested should be in accordance with the research protocol submitted and as approved by the relevant Health Research Ethics Committee (HREC) in South Africa.
2. Access to NHLS material and/or data will be limited to the minimum required for successful completion of the approved study and will be made available to you ***without patient names***.
3. Material and/or data obtained from the NHLS shall be used anonymously and shall not for any reason be used to track or recruit patients, as no pre-approval/consent is obtained from patients.
4. Processes are discussed with the relevant NHLS departments (i.e. Corporate Data Warehouse (CDW), NHLS Laboratory Management, and Operations Office, etc.) and are agreed upon.
5. Any changes and extensions to the study requirements should be submitted for approval by an approved HREC an application submitted on the AARMS system – https://aarms.nhls.ac.za.
6. Confidentiality is maintained at participant and institutional level and there is no disclosure of personal information or confidential information as described by NHLS policies.
7. The NHLS is indicted as a source of material and/or data requested in the original protocol that was approved by the (HREC) as NHLS does not have an institutional HREC.
8. A final report of the research study and any published paper resulting from this study are submitted to the NHLS AARMS or by email to academic.research@nhls.ac.za and that the NHLS has been acknowledged appropriately.

Please note that this letter constitutes approval by the NHLS Academic Affairs and Research Office. Data related queries may be directed to NHLS CDW, email: zarina.sabat@nhls.ac.za; contact number: 011 386 6074 and sample related queries (if applicable) shall be directed to the relevant business manager.

24Feb2022
Dr Babatyi Malope-Kgokong
National Manager: Academic Affairs and Research

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

Charlotte Maxeke Johannesburg Academic Hospital

7 York Road, Parktown, 2193

January 2022

To whom it may concern

I, Professor Deborah Glencross hereby provide consent for MMed candidate, Dr Zanré Nell to use the Charlotte Maxeke Johannesburg Academic Hospital flow cytometry unit's KaluzaC™ software system for secondary data analysis for the research protocol entitled *"A multi-centre study to evaluate an in-house multi-parameter immunophenotypic panel to identify precursor B-cells in the determination of measurable residual disease in paediatric B-cell acute lymphoblastic leukaemia"*.

Paediatric diagnostic and follow-up bone marrow aspirate samples submitted according to institution-specific treatment protocols will be analysed in real-time using the KaluzaC™ software system, with the in-house immunophenotypic panel requested as a secondary investigation and later analysed by the MMed candidate under my expert guidance.

Signature: _____ Date: 05 January 2022

Professor Deborah Glencross

MBBCh(Wits), MMed(Haem)(Wits)

HOD: CD4, Leukaemia and HIV Immunology Laboratory, CMJAH

Director: CD4 National Priority Programme, NHLS

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

CHARLOTTE MAXEKE JOHANNESBURG ACADEMIC HOSPITAL (CMJAH)
OFFICE OF THE SENIOR CLINICAL MANAGER

Enquiries: *Ms. TT Mahlangu*
Email: Thandi.Mahlangu4@gauteng.gov.za
Tel: *011 488 3365*
Ref: *1/7/2*
14 *March 2022*

To: Dr Zanre Nell

RE: PROVISIONAL APPROVAL OF STUDY

TITLE: A MULTI-CENTRE STUDY TO EVALUATE AN IN-HOUSE MULTI-PARAMETER IMMUNOPHENOTYPIC PANEL TO IDENTIFY PRECURSOR B-CELLS IN THE DETERMINATION OF MEASURABLE RESIDUAL DISEASE IN PAEDIATRIC B-CELL ACUTE LYMPHOBLASTIC LEUKAEMIA

Permission to conduct the above-mentioned study is provisionally granted subject to:

1. Submitting an Ethics Clearance Certificate from the University of Witwatersrand Human Research and Ethics Committee (HREC).
2. Proof of NHRD registration.

Please submit the above documents at your earliest convenient within 3 months from the date of this letter. This will prompt the Chief Executive Officer (CEO) of Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) to grant you a final approval. It is important to note that your research can only commence once a final approval is granted. Failure to comply with the above-stated requirements may result in the withdrawal of your application.

Supported / ~~Not Supported~~

Dr J. Punwasi
Clinical Director

Approved / ~~Not Approved~~

Ms. G Bogoshi
Chief Executive Officer

APPENDIX M

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I ____ Zanre Nell _____ (Student number: ____ 449 647 _____) am a student registered

for the degree of __ MMed(Haem)(Wits) _____ in the academic year ___ 2024 ____.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

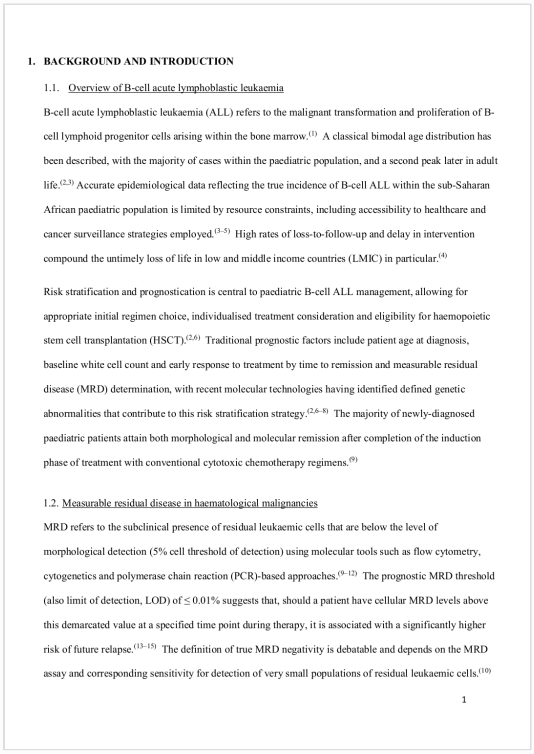
Signature: _____ Date: ___ 7 February 2024 _____

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1. **BACKGROUND AND INTRODUCTION**

1.1. Overview of B-cell acute lymphoblastic leukaemia

B-cell acute lymphoblastic leukaemia (ALL) refers to the malignant transformation and proliferation of B-cell lymphoid progenitor cells arising within the bone marrow.[1] A classical bimodal age distribution has been described, with the majority of cases within the paediatric population, and a second peak later in adult life.[2,3] Accurate epidemiological data reflecting the true incidence of B-cell ALL within the sub-Saharan African paediatric population is limited by resource constraints, including accessibility to healthcare and cancer surveillance strategies employed.[3–5] High rates of loss-to-follow-up and delay in intervention compound the untimely loss of life in low and middle income countries (LMIC) in particular.[4]

Risk stratification and prognostication is central to paediatric B-cell ALL management, allowing for appropriate initial regimen choice, individualised treatment consideration and eligibility for haemopoietic stem cell transplantation (HSCT).[2,6] Traditional prognostic factors include patient age at diagnosis, baseline white cell count and early response to treatment by time to remission and measurable residual disease (MRD) determination, with recent molecular technologies having identified defined genetic abnormalities that contribute to this risk stratification strategy.[2,6–8] The majority of newly-diagnosed paediatric patients attain both morphological and molecular remission after completion of the induction phase of treatment with conventional cytotoxic chemotherapy regimens.[9]

1.2. Measurable residual disease in haematological malignancies

MRD refers to the subclinical presence of residual leukaemic cells that are below the level of morphological detection (5% cell threshold of detection) using molecular tools such as flow cytometry, cytogenetics and polymerase chain reaction (PCR)-based approaches.[9–12] The prognostic MRD threshold (also limit of detection, LOD) of ≤ 0.01% suggests that, should a patient have cellular MRD levels above this demarcated value at a specified time point during therapy, it is associated with a significantly higher risk of future relapse.[13–15] The definition of true MRD negativity is debatable and depends on the MRD assay and corresponding sensitivity for detection of very small populations of residual leukaemic cells.[10]

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