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Pre-lysis technique for MRD detection

Validation of a modified pre-lysis sample preparation technique for flow cytometric minimal residual disease assessment in multiple myeloma, chronic lymphocytic leukemia, and B-non Hodgkin lymphoma.

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Abstract

Background: Minimal residual disease (MRD) assessment of hematopoietic neoplasia below 10^{-4} requires more leukocytes than is usually attainable by post-lysis preparation. However, not all laboratories are resourced for consensus Euroflow pre-lysis methodology. Our study aim was to validate a modified pre-lysis protocol against our standard post-lysis method for MRD detection of multiple myeloma (MM), chronic lymphocytic leukemia (CLL), and B-non Hodgkin lymphoma (B-NHL), to meet demand for deeper MRD assessment by flow cytometry.

Method: Clinical samples for MRD assessment of MM, CLL and B-NHL (50, 30 and 30 cases respectively) were prepared in parallel by pre and post-lysis methods for the initial validation. Total leukocytes, MRD and median fluorescence intensity of antigen expression were compared as measures of sensitivity and antigen stability. Lymphocyte and granulocyte composition were compared, assessing relative sample processing stability. Sensitivity of the pre-lysis assay was monitored post validation for a further 18 months.

Results: Pre-lysis achieved at least 10^{-4} sensitivity in 85% MM, 81% CLL and 90% B-NHL samples, versus 24%, 48% and 26% by post-lysis respectively, with stable antigen expression and leukocyte composition. Post validation over 18 months with technical expertise improving, pre-lysis permitted 10^{-5} MRD assessment in 69%, 86%, and 82% of the respective patient groups.

Conclusion: This modified pre-lysis procedure provides a sensitive, robust, time efficient and relatively cost-effective alternative for MRD testing by MFC at 10^{-5} , facilitating clinically meaningful deeper response assessment for MM, CLL and B-NHL. This method adaptation may facilitate more widespread adoption of highly sensitive flow cytometry-based MRD assessment.

Introduction

Minimal residual disease (MRD) assessment is a powerful prognostic tool and an essential part of patient management for a number of hematologic malignancies¹⁻⁸. With novel therapies resulting in more frequent deeper responses, the number of patients achieving longer remissions and improved overall survival continues to increase^{1,4}. The need for highly sensitive laboratory assays for assessment of MRD has evolved as a central component of patient monitoring, prognosis and ongoing care. For example, the emergence of 'flow MRD-negative' as a response criteria by the International Myeloma Working Group (IMWG)⁹, and dramatic responses of chronic lymphocytic leukemia (CLL) and other B-non Hodgkin lymphomas (B-NHL)

to bcl-2 and other B-cell receptor pathway inhibitors^{10,11} drove us to progress our laboratory's multiple myeloma (MM) MRD and CLL MRD flow cytometry panels from 0.01% sensitivity, using post-lysis sample preparation^{12,13}, to 0.001% using pre-lysis to keep pace with the increasing demand for deeper response assessment.

Multi-parametric flow cytometry (MFC) is a timely, relatively cost effective and sensitive method for assessment of MRD^{14,15}. With continued improvements in diagnostic performance, highly sensitive MFC (10^{-5} - 10^{-6}) is rapidly transitioning from research to routine clinical care^{1-9,14}. However, implementing recent advances in MFC MRD assessment presents economic and technical challenges for diagnostic laboratories. Assay sensitivity can be hindered by insufficient acquisition of white blood cells (WBC). A number of pre-analytical factors affect WBC acquisition, such as marrow hypocellularity or poorly collected samples, but a major variable that lies within the laboratory's control, is sample preparation¹⁶.

While there are consensus guidelines towards a harmonized approach for MM and CLL MRD testing, these are not prescriptive about sample preparation^{4,17-19}. Stain-lyse-wash (SLW) methods are generally considered to provide better signal discrimination, however, analysis of large numbers of WBC for MRD purposes is less practical because of increased antibody reagent and

acquisition time required to achieve a sufficient level of sensitivity. Therefore, lyse-stain-wash (LSW) methods are preferred when a high number of WBC is required in a relatively concentrated sample, devoid of erythrocytes, before antibody staining²⁰. While washing steps in both methods result in incremental cell loss, 'bulk lysis' methods involve removal of erythrocytes from a large volume of specimen before washing to allow for a more concentrated sample with the goal of acquiring more leukocytes for analysis per aliquot of sample. The EuroFlow Consortium outlines a preferred bulk lysing approach for its recommended MRD panels and is considered a reference method by the IMWG for assessment of MRD by MFC⁹. While adopting this approach results in the acquisition of more than 10^7 cells per sample, the method may not be feasible for widespread adoption due to time, labor and cost requirements²¹⁻²³. Alternatively, while pooling the analysis of multiple aliquots of unconcentrated bone marrow processed by post-lysis achieves a similar high sensitivity, this results in extra reagent cost and acquisition time²⁴.

To date, few studies have directly compared assay performance of pre-lysis against post-lysis^{22,24-26}. In this paper, we report on the evaluation of our department's standard post-lysis preparation (SLW) against a modified EuroFlow pre-lysis (LSW) method to assess overall assay sensitivity in assessment of MRD using routine samples from patients with hematological malignancies for which MFC MRD plays an important clinical role.

Lastly, multiple steps of sample preparation affect antigen expression, upon which the ability to detect abnormal cells relies. This is crucial for identification and enumeration of a target cell population at a particular level of sensitivity.^{22,25} Various preparation methods also result in differential loss of normal leukocyte subtypes as well as affecting discrimination between major cell populations to variable degrees.²² Performance of a MFC MRD assay is thus dependent on both level of sensitivity (denominator cell acquisition) and quality of antigen expression, which influence the lower limit of detection (LOD) and degree of certainty of a negative result.¹⁹

Therefore we also assessed for any significant relative changes in antigen expression and preferential loss of lymphocytes and granulocytes, comparing median fluorescence intensity (MFI) of all antigens in the MRD panels, leukocyte percentages, and light scatter properties of lymphocytes between paired samples as a measure of stability of antigen expression and sample composition between the two techniques.

Materials and methods

Patient characteristics and sample collection

All samples were from patients with a history of MM, CLL or B-NHL undergoing treatment at Peter MacCallum Cancer Centre between 2016 and

2018. Ethics approval was obtained according to institutional protocol. Data collection and analysis were anonymized. Peripheral blood (PB) (4mL) and bone marrow (BM) (2 – 4mL) samples were collected in lithium heparin as part of routine clinical care following therapy. A total of 50 samples from patients with MM, 30 from CLL patients and 30 from patients with B-NHL were collected and analyzed within 24 hours on a BD FACSCanto II 8 color instrument. Additionally, 30 of the MM samples were also analyzed for cytoplasmic immunoglobulin light chain expression by both methods. MM samples were all derived from first pull bone marrow aspiration, whereas CLL and B-NHL samples comprised a mixture of PB and BM aspirations. B-NHL subtypes comprised mantle cell (n=17), follicular (n=4) and CD5 negative B-non Hodgkin lymphoma (n=9).

Sample preparation and processing

Modified pre-lysis and standard post-lysis techniques were performed in parallel for the 110 patient samples to compare diagnostic performance. Pre-lysis sample preparation was modified from the EuroFlow method by reducing the number of washing steps during the erythrocyte lysis phase from three to two, and during antibody staining from two to one, in order to fit within departmental workflow and resources. Sample processing steps are provided in detail in the following two sections and summarized in figure 1. Antibody panel composition aligned with consensus guidelines for MM MRD and CLL

MRD^{27,28}. B-NHL panel design was an in-house cocktail containing markers from the first tube of our diagnostic B cell lymphoma panel based on earlier recommendations.²⁹

The antibody cocktails (table 1) consisted of CD45 (APC-H7), CD38 (V450), CD138 (APC), CD19 (PE-Cy7), CD27 (PE), CD81 (FITC), CD56 (PerCPCy5.5), CD200 (V500) for MM surface marker ('MM-S') panel; cytoplasmic kappa (FITC), cytoplasmic lambda (PE), CD117 (PerCP-Cy5.5) substituted in the relevant channels for MM intracellular ('MM-I') panel; CD43 (APC), CD19 (PE-Cy7), CD3 (V500), CD5 (BV421), CD20 (APC-H7), CD22 (PC5-5), CD79b (PE), CD81 (FITC) for CLL; and CD45 (V500), CD19 (BV421), CD5 (PerCP-Cy5.5), CD10 (PE-Cy7), CD20 (APC-H7), CD22 (APC), kappa (FITC), lambda (PE) for B-NHL. Immunoconjugates were sourced from Beckton Dickinson (BD), Franklin Lakes, New Jersey, USA, apart from Beckman Coulter (BC), Brea, California, USA for CD22-PE-Cy5.5 and Agilent Dako, Santa Clara, California, USA for kappa and lambda.

Cell concentration of neat PB or BM sample was first determined by a CELL-DYN Sapphire™ Hematology Analyzer (Abbott). Sample volume reduction was subsequently performed with the aim to stain a minimum of 10^7 cells per tube for an intended sensitivity of 10^{-5} by acquiring $3-5 \times 10^6$ WBC per tube depending on the patient's cellularity and sample quality.

Standard post-lysis technique

Samples of BM or PB (1-2mL) were washed in phosphate buffered saline (PBS) (Oxoid BR0014G) and up to 3×10^6 WBC were stained with the appropriate antibody cocktail in a dark space at room temperature (RT) for 15 minutes. One mL FACSlyse (BD 349202) for every 100uL of sample was added and vortexed prior to incubation in the dark for 10 minutes. The sample was centrifuged for two minutes at 2000 rpm. The supernatant was removed, and the sample was washed and re-suspended in 0.5mL of PBS.

For intracellular staining of MM samples; after the first incubation step with cell surface membrane antibodies, 200µL of Dako Intrastain (fixation) Reagent A was added, the sample was immediately vortex mixed, and incubated at RT in the dark for 15 minutes. PBS (2mL) was added and the sample centrifuged for 2 minutes at 2000 rpm. Sample was decanted and 200 µL of Dako Intrastain (permeabilization) Reagent B and kappa and lambda light chain immune-conjugates were added, immediately vortex mixed and incubated at RT for 15 minutes in the dark. PBS (2mL) was added and sample centrifuged for 2 minutes at 2000 rpm, then decanted and re-suspended in 0.5mL PBS.

Modified pre-lysis technique

Sample volume was calculated based on number of WBCs obtained. Diluted (1 in 10) ammonium chloride lysing solution, (Beckman Coulter Lysing Solution, IOTest 3, 10X Concentrate containing a 10X-concentrated mixture of ammonium chloride, potassium bicarbonate and ethylenediamine tetraacetic acid, item number A07799) was added to the patient's sample at 50 mL for every 2 mL of BM or PB. The sample was incubated at RT on a roller for 15 minutes, then centrifuged at 800g for 10 minutes.

Two wash and centrifugation (540g/5min) steps were performed with phosphate buffered saline (PBS) (Oxoid BR0014G)/0.5% bovine serum albumin (BSA) (Sigma Aldrich A7030-100G)/0.1% sodium azide (NaN_3) (Sigma Aldrich S-8032) (PBS/BSA/NaN_3) after discarding the supernatant each time. Samples were then re-suspended in PBS/BSA/NaN_3 and aliquoted to a WBC count of 10×10^6 in 300 μL per tube, stained with the relevant panel of antibodies and incubated in the dark for 15 minutes.

After adding 2 mL of FACSlyse and incubating in the dark for a further 10 minutes, the sample was washed in PBS/BSA/NaN_3 , centrifuged at low spin (2000 rpm) for 2 minutes and finally re-suspended in 0.5mL of PBS/BSA/NaN_3 prior to data acquisition. The additional lysis step assisted in removing any remaining erythrocytes under a gentle hypotonic condition, while preserving leukocytes and acting as a fixative to stabilize membrane

epitopes. This enables antigenic fluorescence to remain stable over time during the acquisition phase. For intracellular staining of MM samples, the same method used for post-lysis was applied after the first incubation step with cell surface membrane antibodies.

MRD analysis – data acquisition

The goal of MRD analysis for both methods was to acquire as many events as possible, with anticipation that final leukocyte recovery might be as low as approximately 50% of the original number of cells stained by the completion of either sample preparation protocol due to inherent incremental cell loss at each stage of processing.³⁰ The instrument's acquisition template for the post-lysis method was set to 'stop acquiring' at the attainment of two million leukocytes or after four minutes, whichever occurred first. The pre-lysis acquisition template was adjusted to acquire on either low or medium flow rate and manually stopped when the sample was estimated as 'almost dry'.

Instrument set-up, calibration and quality checks were performed daily as per departmental protocols. Quality control assessment utilized cytometry set-up and tracking beads (BD - 642412), run according to the manufacturer's recommendations, to ensure consistent performance and accurate results.

Post-acquisition data analysis

The total number of WBC and MRD events was enumerated to compare sensitivity of both methods for each disease group. Target MRD populations (MM, CLL and B-NHL) were identified and enumerated by employing respective pre-defined gating strategies using Kaluza™ analysis software version 2.0 (Beckman-Coulter, IN, USA) and consensus gating methods. MFI for each antigen following pre-lysis and post-lysis was compared within each disease specific panel.

WBC enumeration

Figure 2 shows how lymphocytes and granulocytes were gated. To assess for any differential loss or alteration of light scatter properties, the percentage out of total WBC, forward and sideward light scatter area parameters (FSC-A, SSC-A) were compared between paired samples for each method, within each disease group. For MM and B-NHL cases, lymphocytes and granulocytes were first defined as CD45^{high}/SSC^{low}, and CD45^{intermediate}/SSC^{high} respectively using the CD45 vs. SSC plot (figure 2A). Then, each leukocyte subset was individually transposed back onto a SSC-A vs. FSC-A plot for light scatter evaluation. For CLL cases, lymphocytes were defined as SSC-A^{low}/FSC-A^{low} and granulocytes as CD43⁺/SSC^{high} on respective plots (figures 2B and 2C), as CD45 is not part of the consensus panel.

Total WBC were enumerated differently for the three disease groups (figures 3, 4 and 5). After always checking the time parameter for continuous data acquisition, and doublet and debris exclusion using light scatter (figure 3A), total WBC were enumerated for MM by subtracting the cluster of CD45-/CD38- events, which represent nucleated red blood cells (NRBC), from all 'live cells' to account for CD45+ WBC plus CD45- PC (figure 3B). For CLL, as CD45 is not included in the MRD panel, a combination of SSC-A vs. FSC-A and CD43 vs. SSC-A plots were used for WBC enumeration (figure 4A). As total 'event number' can include a highly variable number of non-lysed erythrocytes and debris, we based the WBC calculation on the European Research Initiative in CLL (ERIC) recommendation to use total leukocytes, defined as CD43+ events plus the CD43-CD19+ events that have light scatter properties consistent with leukocytes.³¹ WBC were defined as all CD45+ 'live cells' for B-NHL cases (figure 5A).

Identification and enumeration of MRD

The absolute number of abnormal, normal and total plasma cells was derived following plasma cell gating recommendations of the European Myeloma Network and International Clinical Cytometry Society/European Society for Clinical Cell Analysis (ICCS/ESCCA) guidelines^{27,32}. Briefly, following doublet and debris exclusion, a sequential gating algorithm was applied to all cells to firstly arrive at a relatively pure plasma cell gate, using CD38, CD138

and CD45 (figure 3A). Abnormal plasma cells were then differentiated from normal plasma cells based on well-described aberrant antigen expression patterns, with the assistance of local pooled normal reference data (figures 3B and 3C). MRD was defined as a cluster of 30 or more phenotypically abnormal PCs based on a minimum of two of the following aberrancies: under-expression of CD19, CD27, CD38, CD45, and/or CD81, overexpression of CD56 and/or CD200, and asynchronous expression of CD117. A kappa:lambda ratio of <0.5 or $>4:1$ in a cluster of >50 PCs was considered abnormal when using the intracellular tube. PC populations were vetted by back-gating on all light scatter and fluorescence plots. A cell population of interest that did not show a plausible cluster on FSC/SSC/CD45/singlet plots or in overall fluorescence pattern were excluded.²⁷ MM MRD was defined as the number of abnormal PC expressed as a percentage of total WBC.

CLL MRD was identified and enumerated in accordance with the ERIC harmonised approach^{4,28,31,33} (figure 4). This single tube, 8 color panel was utilized once a case had been annotated as CLL at diagnosis by more detailed immunophenotypic and other clinicopathological data. Here, CD19+ events were partitioned from all other mononuclear cells, checked as true lymphoid cells by SSC-A vs. FSC-A properties (figure 4A), and assessed for differential expression of CD5, CD20, CD22, CD43, CD79b and CD81 compared to normal B-cells across three bivariate plots; CD43 vs. CD81, CD5 vs. CD79b,

and CD20 vs. CD22 (figure 4B). CLL MRD was quantified as the Boolean product of CLL-like events gated in the three plots in figure 4B, and expressed as a percentage of total WBC.

There are no consensus guidelines for a gating algorithm for B-NHL in general, except for the principle that “reagent combinations informative for MRD assays require a higher signal-to-noise ratio to allow complete separation of abnormal populations of low frequency from normal cells of similar immunophenotype”¹⁹. For identification of MRD in B-NHL samples, a sequential gating strategy was employed that first isolated CD19+ B-cells (figure 5A). Next, B-cells were ‘purified’ from extraneous events such as T-cells exhibiting high background expression into the CD19 region, which was particularly important when resolving small numbers of potential CD5+CD19+ MRD events (figure 5B). B-cells were then checked for the presence of hematogones (figure 5C) so that, finally, all Pure B-cells (excluding hematogones) were assessed for CD5, CD10, CD20, CD22 and surface light chain expression, paying particular attention to the tracking of any previously known abnormal immunophenotype. MRD was defined as the proportion of the number of events of the dominant light chain isotype when a kappa to lambda ratio was less than 0.5, more than 3.0, or a minimum of 20 events if cells of only one isotype were detectable, expressed as a percentage of total WBC.

Sensitivity

The level of sensitivity was dictated by the total number of leukocytes acquired and MRD was defined as a cluster of ≥ 30 cells in MM^{27,34} and ≥ 20 cells in CLL^{4,33} and B-NHL. Lower limit of detection (LOD) was then defined as MRD divided by the total number of leukocytes, expressed as a percentage (table 2). Following the 12 month validation period comparing post- and pre-lysis methods, the pre-lysis assay was adopted and performance at achieving a sensitivity of 10^{-5} monitored for a subsequent 18 months.

Antigen stability

Antigen expression (MFI) was compared between pre-lysis and post lysis samples for all antigens on target MRD populations, all antigens on normal plasma cells in the MM cohort, and CD5 on T-cells in CLL and B-NHL cases.

Stability of leukocyte composition

Lymphocytes and granulocytes were enumerated and compared between paired samples as a measure of stability of overall sample composition, and to ultimately ensure preservation of good discrimination of MRD from background haematopoietic cell populations. Parameters assessed were: Percentage of WBC represented by lymphocytes and granulocytes, ratio of granulocytes to lymphocytes per sample, and lymphocyte FSC-A.

Statistical analysis

Median and range were calculated for all continuous data. McNemar's test assessed for difference between pre and post-lysis MRD quantification, as MRD was considered a dichotomous dependent variable between two related groups. Paired t-test assessed for differences between MFI of antigen expression and other compared continuous data. A p value of <0.05 was considered statistically significant.

Results***Level of sensitivity***

Significantly greater numbers of WBC were acquired by pre-lysis, reflected by a median of 1,460,303 WBC in MM; 2,210,247 in CLL; and 1,918,381 in B-NHL samples versus 269,565; 546,454; and 210,690 WBC respectively (table 3). Compared to post-lysis, this represented a minimum of a four-fold increase in total WBC acquisition across all groups, resulting in a log increase in limit of detection for MRD (table 4).

Feasibility of MRD detection

For 85% of MM, 81% of CLL and 90% of B-NHL cases, a level of sensitivity of $\leq 0.01\%$ was achieved by pre-lysis (i.e. acquisition of $\geq 500,000$ total WBC) compared to 24%, 48% and 26% respectively by post-lysis ($p = 0.02$) (Table

4). Sensitivity of $<0.001\%$ was achieved in 18% of MM, 48% of CLL and 50% of B-NHL cases by pre-lysis compared with none by post-lysis.

Seventy-six (69%) samples processed by pre-lysis were MRD positive.

Nineteen (25%) of these samples (11 MM, 7 CLL and 1 B-NHL) would have otherwise been labelled MRD negative by post-lysis because of the lower number of total WBC acquired (table 3).

In the post validation phase, during the first 12 months (Jan 2018 – Dec 2018) 10^{-5} sensitivity was attained by pre-lysis in 53% of MM, 79% of CLL and 84% of B-NHL samples out of a total pool of cases analyzed that year by pre-lysis numbering 30, 129 and 135 for respective cohorts. During the next six months, to end of June 2019, 69% of MM, 86% of CLL and 82% of B-NHL samples out of a further 65, 115 and 84 cases respectively were analyzed at 10^{-5} .

Stability of antigen expression

There was no significant difference in the MFI of antigen expression resulting from pre-lysis except for the following: Dimmer CD138 (with or without an intracellular processing step), and brighter CD200 on plasma cells; and dimmer CD43 and CD45 on B-cells using the CLL and B-NHL panels respectively (supplemental table). Only the data demonstrating a significant difference in expression between the two methods is shown in the table,

alongside the difference in median MFI expressed as a logarithm. Of all the antigens showing a statistical difference in MFI, only CD138 expression on plasma cells that underwent intracellular processing showed a difference of almost 0.5 log (figure 6). This caused no issues in identifying PC's. Only seven (14%) of the MM samples analysed for surface marker expression and four earmarked for intracellular staining of light chains were processed by pre-lysis the day after post-lysis, and this did not correlate with diminished CD138 expression.

Stability of leukocyte composition

There was no significant difference between light scatter parameters of lymphocytes, or between percentages of granulocytes or lymphocytes across the two methods (data not shown) demonstrating relative stability of composition of these major leukocyte subsets between sample preparation methods.

Discussion

Various methods currently available for MFC sample preparation are selected based on providing the best signal discrimination between populations, improving signal to noise ratio, minimization of non-specific binding, auto-fluorescence and antibody specificity^{19,20}. SLW protocols are widely adopted

in the diagnostic setting, providing better signal discrimination^{25,35}. With novel sample preparation procedures, attaining a high number of events is a priority, however, clear distinctions between positive and negative populations remains essential. Our study showed that it is possible to acquire sufficiently higher numbers of leukocytes for more sensitive MRD assessment using a modified pre-lysis sample preparation method with fewer steps than what may be regarded as a reference standard at this juncture; and without hampering data analysis by affecting antigen expression or background noise.

Highly sensitive assays are required to improve the detection of MRD in bone marrow and peripheral blood samples of patients with haematological neoplasms undergoing treatment at multiple time points. Sample preparation and assay methodology play a key role in overall performance of flow cytometric MRD testing^{7,25}, including sample stability and ability to discriminate between major cell populations²¹. Our study shows that adopting a pre-lysis method results in the acquisition of significantly more leukocytes in samples from patients with MM, CLL and B-NHL, enabling a substantial improvement in achieving at least 10^{-4} sensitivity compared to post-lysis method, and in the majority of cases, 10^{-5} sensitivity. The increased level of sensitivity facilitated by pre-lysis translates to both higher MRD detection rates as shown in table 3 as well as improved degree of certainty of a negative result³⁶. Moreover, taking a pragmatic approach to the pre-lysis method over

the EuroFlow procedure enabled the laboratory to accommodate more samples in order to meet the clinical need for deeper response assessment, more often. Assay performance was otherwise equivalent, with no significant difference in the cellular composition between samples prepared by either method, or in the signal intensity of antigen expression upon which target cell identification depends. I.e. we show that pre-lysis by this modified method does not negatively impact the ability to discriminate all cells of the lineage of interest from remaining WBC in the PB or BM or to differentiate normal from abnormal (MRD) cells within the lineage of interest compared to post-lysis. While consistent antigen expression is paramount between the two methods, so is to a lesser extent the ability to provide some confidence of stability of sample composition of lymphocytes and granulocytes, therefore allowing comparison between samples prepared by either method over time, or between patients.

For those antigens in our panels that did show a mild reduction (CD43, CD45, and CD138) or gain (CD200) in expression intensity, it is important to note that none of these 'statistically significant' differences in MFI represented deviations of more than 0.3 log aside from CD138 with intracellular processing differing by 0.45 log. This made no impact on the discrimination of plasma cells from remaining WBC (figure 6). Deviations of MFI less than 0.5 log are not considered sufficiently robust for interpreting real biological

differences in antigen expression over and above other pre-analytical influences such as fluorochrome selection or lysis reagents³⁷.

Additionally, expression of CD138, the antigen that showed the most diminution following pre-lysis, is notoriously sensitive to variations in sample preparation and delay in analysis¹⁶. However, the latter did not apply in this cohort within a 24-hour time frame. Lastly, higher numbers of WBC acquired using pre-lysis may also contribute to diminished antigen expression resulting from an overall reduction in antibody availability, although this was factored into the design of our antibody cocktails as best as possible by adjusting individual antibody concentrations according to the target WBC count for an optimal staining index.

There was no significant deviation of CD45 or CD43 expression, or of light scatter parameters of lymphocytes or granulocytes to affect the operator's ability to visually gate populations between samples. This was reflected more objectively by the absence of significant differences in the proportion of lymphocytes, granulocytes or the ratio of granulocytes to lymphocytes between paired samples.

Similarly, discrepancies in antigen expression did not impact on the initial gating of plasma cells or B-cells, differentiation between normal or abnormal

cell populations, or the final MRD result for any disease group. Therefore, these results highlight acceptable phenotypic and sample stability across both methods.

Although the initial phase of the validation study fulfilled the aim of demonstrating stability of antigen expression and sample composition at 10^{-5} , this level of sensitivity was only achieved in relatively few cases presenting for MRD assessment during the validation phase (18% of MM, 48% of CLL and 50% of B-NHL samples). Importantly however, since the routine implementation of this method in early 2018, there was an incremental improvement in attainment of target sensitivity to 69% of MM, 86% of CLL and 82% of B-NHL samples (total 558 samples) that were referred for MRD assessment over a period of 18 months, with the shortfall due to sample hypocellularity. The more consistent attainment of 10^{-5} sensitivity post validation related to improvements in technical proficiency across the department over time, including increased confidence of the operator to extend an acquisition time nearer to 'running the sample dry'. Additional education and experience with sample procurement was also contributory.

While MRD assessment by MFC has advantages over molecular methods, next generation flow cytometry is labor intensive and may be impractical for universal adoption by many diagnostic laboratories³⁴ including ours. We have

demonstrated a modified pre-lysis method with a reduced number of steps compared to a reference approach^{21,23}. This represents an achievable alternative for laboratories seeking to adopt a cost-effective flow cytometric method of MRD assessment within feasible workflow constraints.

Our study findings are comparable with other recent reports, demonstrating improved sensitivity of flow cytometric MRD assays with implementation of a pre-lysis technique^{7,21,22,25}. Despite concerns for the potential of additional wash steps to increase cell loss, and the limited data available regarding the impact of pre-lysis on antigen-antibody stability, our study demonstrated no compromise in antigen stability across three different disease panels, and more reliably achieved a higher sensitivity. These findings are further supported by a recent publication comparing sample preparation methods, where no difference in the identification and quantification of target populations was demonstrated when utilizing a LSW method compared to SLW²⁵. Adopting a more pragmatic approach to pre-lysis technique consisting of fewer steps still resulted in the acquisition of more denominator events, meeting clinical demand for deeper response assessment, providing improved sensitivity and specificity of a positive result and specificity of a negative sample, whilst reducing the time, labor and cost demands of the Euroflow bulk lysis procedure.

MRD analysis to the standard of 10^{-5} or more adds significantly to our laboratory workflow as a national referral cancer centre given the high number of tests required. Incremental improvements in efficiency, particularly during the sample preparation phase, have a substantial additive impact on staff utilization, result turn-around, as well as reagent and other consumable costs. The pre-lysis technique takes approximately twice as long as the post-lysis technique, despite the acquisition phase of both assays being relatively short. Adoption of our modified technique represented a more pragmatic approach to the whole laboratory test complement and workflow in preference to adopting the EuroFlow reference bulk lysis procedure for MM MRD given the competing demands in other disease domains.

Conclusion

Implementation of a relatively time and resource efficient modified pre-lysis sample preparation method for MFC is feasible, resulting in the acquisition of more denominator events and identification of MRD at a higher level of sensitivity without compromising antigen stability.

The procedure represents a modification of a reference method with a reduced number of washing steps to accommodate departmental resources and workflow. We provide a validated and reasonable alternative for a diagnostic

laboratory to perform flow cytometric MRD assessment of three common haematological disorders for which the demand for higher sensitivity has become paramount for depth of response assessment and prognosis, namely multiple myeloma, chronic lymphocytic leukaemia and B-non Hodgkin lymphoma.

Many diagnostic laboratories have greater access to flow cytometry than next generation sequencing, but may be less well-resourced compared to a large reference centre. This cost-effective alternative pre-lysis method may facilitate more widespread adoption of flow cytometry-based MRD assessment. However, adherence to a robust validation process for sample preparation is critical if a modified pre-lysis technique is chosen.

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Tables

Table 1: Antibody cocktails

	FITC	PE	PerCP-Cy5.5	PC7	APC	APC-H7	BV421	V500
MM-S	CD81	CD27	CD56	CD19	CD138	CD45	CD38 (V450)	CD200 (BV510)
MM-I	Kappa*	Lambda*	CD117	CD19	CD138	CD45	CD38 (V450)	CD3
CLL	CD81	CD79b	CD22 (PE-Cy5.5)	CD19	CD43	CD20	CD5	CD3
BNHL	Kappa*	Lambda*	CD5	CD10	CD22	CD20	CD19	CD45

MM-S, multiple myeloma surface marker tube; MM-I, multiple myeloma intracellular marker tube; FITC, fluorescein isothiocyanate (BD); PE, phycoerythrin (BD); PerCP-Cy5.5, peridinin-chlorophyll-protein complex Cy5.5 conjugate (BD); PE-Cy5.5, phycoerythrin-cyanine 5.5 tandem conjugate (BD); PC7, phycoerythrin cyanin 7 (BD); APC, allophycocyanin (BD); APC-H7, allophycocyanin-H7 tandem conjugate (BD); BV421/510, Brilliant Violet 421/510 (BD); V450/V500, Violet 450/500 (BD); BD, Becton Dickinson, Franklin Lakes, NJ, USA; BC, Beckman Coulter, Brea, CA, USA; *Agilent Dako, Santa Clara, CA, USA.

Table 2: Estimated lower limit of detection (LOD) according to the total number of WBC acquired. $LOD = (30/\text{Total WBC}) \times 100\%$ for MM^{27,34}, and $(20/\text{Total WBC}) \times 100\%$ for CLL or B-NHL^{4,33}

Total WBC	LOD%	
	MM	CLL / B-NHL
100000	0.03	0.02
200000	0.015	0.01
300000	0.01	0.006
500000	0.006	0.004
1000000	0.003	0.002
2000000	0.0015	0.001
3000000	0.001	0.0006
5000000	0.0006	0.0004

Table 3: Comparable WBC acquisition and MRD detection for post-lysis and pre-lysis

	Post-lysis	Pre-lysis	P value
Multiple myeloma			
Total no. WBC events	269,565 (16,858 – 1,020,319)	1,460,303 (183,339 – 3,992,717)	<0.001
Total no. MRD events	43 (0 – 12743)	415 (0 – 76,226)	0.07
MRD+	N=28	N=39 (11 more than post-lysis)	<0.05*
Chronic lymphocytic leukemia			
Total no. WBC events	546,454 (10,791 – 1,446,321)	2,210,247 (66,745 – 8,891,400)	<0.001
Total no. MRD events	44 (0 – 197,313)	1190 (0 – 2,002,661)	0.03
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Total no. WBC events	210,690 (36,103 – 1,588,122)	1,918,381 (112,643 – 7,430,948)	<0.001
Total no. MRD events	2,2024 (0 – 28,184)	38,733 (0 – 1,004,031)	0.27
MRD+	N=13	N=14 (1 more than post-lysis)	NS*
Total MRD+	N=57	N=76 (19 more than post-lysis)#	

*McNemar's test; NS, not significant. #19 (25%) cases considered MRD- by post-lysis were shown to be MRD+ by pre-lysis

Table 4: Comparable rates of achieving incremental limits of detection

	MM			CLL			B-NHL	
WBC acquired	LOD%	Post-lysis N (%)	Pre-lysis N (%)	LOD%	Post-lysis N (%)	Pre-lysis N (%)	Post-lysis N (%)	Pre-lysis N (%)
>100000	0.03	30 (88%)	34 (100%)	0.02	25 (81%)	28 (90%)	20 (65%)	30 (100%)
>200000	0.015	23 (68%)	33 (97%)	0.01	25(81%)	28(90%)	17 (55%)	29 (97%)
>300000	0.01	15 (44%)	32 (94%)	0.006	24(77%)	27(87%)	13 (42%)	29 (97%)
>500000	0.006	8 (24%)	29 (85%)	0.004	15(48%)	25(81%)	8 (26%)	27 (90%)
>1000000	0.003	1 (3%)	25 (74%)	0.002	3 (10%)	23 (77%)	1 (3%)	23 (77%)
>2000000	0.0015	0	10 (29%)	0.001	0	15(48%)	0	15 (50%)
>3000000	0.001	0	6 (18%)	0.0006	0	12(39%)	0	9 (30%)
>5000000	0.0006	0	0	0.0004	0	4(13%)	0	1 (3%)

Limit of detection (LOD) and proportion of patients achieving the corresponding level of sensitivity according to the total number of WBC acquired (MRD defined as 30 cells for MM and 20 cells for CLL & B-NHL). Bold figures represent proportion of cases achieving $\leq 0.001\%$ sensitivity.

Supplementary Table: Median fluorescence intensity of antigen expression for those antigens that showed a statistically significant change following pre-lysis.

	Post-lysis	Pre-lysis	P value	Δ MFI (log)
	MFI	MFI		
Plasma cells				
CD138	94.2 (4.4 – 254)	47.59 (0.68 – 253.5)	0.001	0.29
CD138 (MM-I)	129.4 (16.2 – 304.7)	45.5 (3.3 – 222.7)	0.003	0.45

CD200	14.0 (1.25 – 89.2)	24.2 (0.74 – 167.1)	0.03	0.23
CLL B-cells				
CD43	4.43 (0.13 – 10.6)	2.95 (0.45 – 5.9)	0.001	0.18
B-NHL B-cells				
CD45	111.0 (18.56 – 149.27)	94.31 (23.49 – 141.4)	0.002	0.07

Δ MFI (log) = difference between the log value for median fluorescence intensity of antigen expression following post- and pre-lysis; MM-I, multiple myeloma intracellular marker tube

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure legends

Figure 1: Overview of main sample analysis steps for in-house post-lysis, pre-lysis and reference method, and approximate overall relative time required for each entire process. *Personal communication from a specialist flow cytometry laboratory in Melbourne, Australia that participates in MM MRD research studies with the Black Swan Research Initiative of the International Myeloma Foundation.

Figure 2: Identification of lymphocytes (blue) and granulocytes (orange) for enumeration and comparison of light scatter parameters between post- and pre-lysis methods. For MM and B-NHL cases, lymphocytes ($CD45^{hi}$, $SSC-A^{lo}$) and granulocytes ($CD45^{med}$, $SSC-A^{hi}$) were first derived from the CD45 vs. SSC-A plot (A). SSC-A and FSC-A were then measured individually on a

separate SSC-A vs. FSC-A plot, like **(B)**. For CLL cases, lymphocytes (SSC-A^{lo}, FSC-A^{lo}) were derived directly from the 'Live cell' gate **(B)**, while granulocytes were first derived from a CD43 vs. SSC-A plot **(C)** (CD43⁺, SSC-A^{hi}), then measured individually for SSC-A and FSC-A on a separate plot, like **(B)**.

Figure 3. Gating algorithm for MM MRD.

Figure 3A illustrates how plasma cells (PC) were first identified, by sequential gating of regions [R1] through [R6].²⁷ [R3] = 'Live cells' as in figure 2. For all live cells in [R3], a region was drawn generously around CD38⁺, CD138⁺ dual bright events [R4]. Within [R4], projected as CD38 vs. CD45, implausibly bright CD45⁺ or other non-specific events were excluded by [R5]. Within [R5], projected as SSC-A vs. FSC-A, events with implausible light scatter properties for real PC were excluded by a region [R6], labelled 'TOTAL PC'. 'TOTAL PC' were back gated on [R2] to ensure none were excluded.²⁷

Figure 3B illustrates how abnormal PC were identified. Gating on 'TOTAL PC', regions [A], [B] and [C] were drawn in the first three plots, taking advantage of common PC aberrancies. Any events outside of merged normal PC reference regions [R7], [R8], and [R9] were included. MRD was derived from [R10] which represented only CD19⁻ cells from the Boolean product of

[A], B] and [C] depicted on the fourth plot (colored red). CD19- PC were selected last to avoid including normal CD19- PC in the final MRD result. No MM cases were CD19+. Blue dots signified normal PC, derived from [R6] MINUS [R10]. WBC = [R3] minus 'NRBC [R11]' (colored green), which accounted for CD45+ 'Live cells' and CD45- PC. 'TOTAL PC' were back gated on reference plot [R12] and cross checked on plots in figure 3C.

Figure 3C depicts abnormal (red) and normal PC (blue) displayed together in multiple bivariate plots. $MRD\% = [R10] / ([R3] - NRBC [R11]) \times 100$

Figure 4. Gating algorithm for CLL MRD.

Figure 4A illustrates how B-cells were first identified by sequential gating. From left to right, following doublet (not shown) and debris exclusion, mononuclear cells of interest were defined by quadrant regions Q1+Q3 in the second plot (CD43+ and CD43-/SSC^{lo} events). By displaying mononuclear cells on a CD19 vs. CD3 bivariate plot, B-cells were confined to the upper left quadrant (third plot), isolated from CD19+CD3+ coincident events. In the last plot, events labelled as 'True B-cells' represented CD19+ cells displaying appropriate light scatter properties on a SSC-A vs. FSC-A projection.

$$WBC = Q1 + Q2 + \text{True B-cells in } Q3.^{31}$$

Figure 4B illustrates how abnormal B-cells were differentiated from normal 'True B-cells'. Gating on 'True B-cells', regions [R1], [R2] and [R3] were drawn in the first three plots, taking advantage of typical aberrant expression of each antigen for CLL as outlined by the ERIC consensus gating method^{28, 33}. [R1], [R2] and [R3] were drawn as generously as required to encompass all possible abnormal B-cells depending on separation from normal, colored green. The Boolean product of [R1], [R2] and [R3] was depicted on the fourth plot and MRD was enumerated from the region labelled 'CLL Final', colored blue. $MRD\% = \text{CLL Final} / \text{WBC}$ as defined in figure 4A

Figure 5. Gating algorithm for B-NHL MRD.

Figure 5A illustrates how B-cells were first identified by sequential gating. Following doublet (not shown) and debris exclusion (far left), WBC were defined as CD45+ 'Live cells' (second plot) and lymphocytes gated by a CD45^{hi}/SSC-A^{lo} region in the third plot. The lymphocyte region was drawn generously enough to encompass all CD19+ cells (far right), coloured and back-gated onto the first two plots, so as to not miss any potential larger B-cells with increased FSC-A or SSC-A, or hematogones (CD45^{lo}/SSC-A^{lo}).

Figure 5B illustrates how CD19+ cells were 'purified' from contamination by events showing non-specific CD19+ and/or surface immunoglobulin

expression. First, using the CD19 vs. CD5 projection (far left), a generous region [R1] was created in the CD19+CD5+ space. Gating on [R1] (second plot), a CD20-/dim, CD22-/dim region was created [R2], representing possible contaminating T-cells. Gating on [R2], CD20-/dim, CD22-/dim cells were checked for surface kappa and lambda light chain expression (third plot), with non-specific events labelled as [R3], colored red. These red events can be seen in the first plot as likely representing T-cells. A final pure B-cell population (far right) was derived from the Boolean formula, Pure B-cells = “CD19+ cells” AND (NOT [R3])).

Figure 5C illustrates how CD19+ cells were checked for the presence of hematogones (HG). Using the CD19 vs. CD10 projection (far left), a region [R4] was created in the CD19+CD10+ space. Gating on [R4] (second plot), a CD20-/dim, CD22-/dim region was created [R5]. Hematogones were defined as [R6], gated on [R5] representing B-cells without surface light chain expression, colored dark blue in the third plot, and better illustrated on the CD45 vs. CD10 and CD45 vs. SSC-A projections. Type I – Type III HG regions (far right) were derived from pooled normal bone marrow reference data.

Figure 6. Stability of CD138 expression.

Example of the case that demonstrated maximal diminished CD138 expression on plasma cells from the tube that underwent intracellular processing by pre-lysis. Median fluorescence intensity (MFI) of CD138 (horizontal axes) was calculated for the region labelled as CD38+br/CD138+br and displayed in Plot A following post-lysis (MFI = 216.77) and Plot B following pre-lysis (MFI = 74.87). Note that despite the reduction in MFI following post-lysis, there remains a clear separation of the plasma cell population in plot B from all other bone marrow cells.

CYTOB_21893_19-107R1_Figure-2.tif

3A

Gate	Number
All	3,871,319
R2	3,774,122

Gate	Number
All	3,774,122
R3	3,382,355

Gate	Number
All	3,382,355
R4	1,308

Gate	Number
All	1,308
R5	1,245

Gate	Number
All	1,245
TOTAL PC [R6]	764

CYTOB_21893_19-107R1_Figure-3A.tif

3B

CYTOB_21893_19-107R1_Figure-3B.tif

3C

CYTOB_21893_19-107R1_Figure-3C.tif

CYTOB_21893_19-107R1_Figure-4.tif

5A

CYTOB_21893_19-107R1_Figure-5A.tif

5B

CYTOB_21893_19-107R1_Figure-5B.tif

5C

CYTOB_21893_19-107R1_Figure-5C.tif

CYTOB_21893_19-107R1_Figure-6.tif

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