

Evaluation of a Novel Stable Whole Blood Quality Control Material for Lymphocyte Subset Analysis: Results From the UK NEQAS Immune Monitoring Scheme

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The UK NEQAS Immune Monitoring Scheme (UK NEQAS) evaluates the performance of laboratories routinely performing T-lymphocyte subset analysis on HIV-infected individuals. The scheme originally issued fresh whole blood, but a significant problem was that of analyte stability, especially 36 h postphlebotomy. To circumvent this problem, we have developed a novel stabilisation procedure that ensures retention of leucocyte light scatter and immunological staining characteristics for up to 300 days. In addition, the stabilised whole blood preparation is fully compatible with flow cytometer technology, incorporating either whole blood lysis or "no wash, no lyse" techniques. The ranges of interlaboratory coefficient of variation for the stabilised material are now tighter than those previously obtained with fresh whole blood. Development of this novel material has enabled overseas laboratories to participate in the UK NEQAS Immune Monitoring Scheme and could, in the future, lead to the production of reference and/or calibration reagents for leucocyte immunophenotyping. © 1996 Wiley-Liss, Inc.

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The use of flow cytometry for immunophenotyping has had a significant impact on the diagnosis, monitoring, and therapeutic control of diseases, including leukaemia and HIV infection (14,19). The management of HIV-infected individuals, for example, requires the precise and accurate measurement of absolute CD4⁺ T-lymphocyte counts (7,22). It is vitally important, therefore, that laboratory data be supported by accurate daily and longitudinal quality control (QC) programmes (21). To date, however, such internal quality control facilities (IQA) have been difficult to provide for flow cytometric immunophenotyping, because fresh whole blood collected into EDTA becomes unsuitable for analysis 30 h postphlebotomy (24). This rapid deterioration necessitates the daily use of a fresh specimen for IQA, thus placing an additional burden upon the laboratory to find suitable material, usually from a member of staff. Most centres, therefore, employ either fluorescent latex beads or frozen cells to monitor both flow cytometer performance and sample processing. These preparations, although of value, fail to provide full-process quality control.

External quality assurance (EQA) programs have generally resorted to the use of either fresh whole blood or frozen cells (17,25,31). The instability of these materials

necessitates that samples be shipped by express courier, making the price of transportation one of the heaviest cost burdens in EQA. Furthermore, wide coefficients of variation (CV) can be observed with the use of fresh whole blood (>12%), potentially masking the detection of factors important in influencing laboratory performance. Ideally, therefore, preparations which are able to generate low CV's should be used in EQA.

Lymphoid and myeloid antigens have been shown to be sensitive to chemical fixation processes (6,16). Nevertheless, where antigen stabilisation has been achieved with either the use of lyophilised lymphocytes (e.g., Cytotrol, Coulter Electronics, Hialeah, FL) or leucocyte suspensions (e.g., CD-Chex, Becton Dickinson, San Jose, CA), only part of the immunophenotyping process can be assessed. Neither of these commercial products are, for example, recommended for use with lysing reagents, rou-

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tinely employed in immunophenotyping laboratories to obtain leucocyte suspensions.

We have previously reported the development of a stable whole blood preparation that retains light scatter and immunological staining characteristics similar to fresh whole blood, even following 300 days of storage (2,12). In this report, we demonstrate its suitability for use on a day-to-day basis with the whole blood lysis techniques, on the FACScan (Becton Dickinson) and Ortho Cyturon Absolute (Ortho Diagnostics Systems, Inc., Raritan, NJ), as well as with the "no wash, no lyse" technology of the FACSCount (Becton Dickinson). We also review our experience with this material in the UK NEQAS Immune Monitoring Scheme.

MATERIALS AND METHODS

Stabilisation Procedure

Five hundred milliliters of human peripheral blood were obtained by voluntary donation, with consent from the individual donor, into Citrate Phosphate Dextrose-Adenosine (CPD-A) anticoagulant, and screened for HIV-1, HIV-2, hepatitis B, hepatitis C, and syphilis. The blood was stabilised using a procedure which has been described in detail previously (4), and is now used under license to produce OrthoAbsolute Control (Ortho Diagnostic Systems). This batch of blood was designated BTS-1. For longitudinal studies, 100 × 5 ml aliquots were stored between 4 and 8°C until use, and allowed to equilibrate at room temperature prior to assay. A new aliquot of BTS-1 was opened weekly, or as and when required.

Immunostaining and Analysis

Two- and three-colour immunophenotyping was performed using the Center for Disease Control Guidelines (7,22,23). Staining protocols were used in accordance with manufacturers' recommendations, all reagents being used at saturating concentrations (IMK Simulset or CD45 gating for the FACScan, Becton Dickinson; Trio Reagents for the Ortho Cyturon Absolute, Ortho Diagnostics; FACSpettes for the FACSCount, Becton Dickinson). To study longitudinal variation of BTS-1, calibration of the flow cytometers was performed in accordance with manufacturers' instructions, and compared with light scatter and immunostaining characteristics of a fresh sample of peripheral blood. The fresh sample was run initially to optimise the flow cytometer, followed immediately by BTS-1. The settings remained constant between the fresh specimen and the test sample. On the FACScan, absolute lymphocyte subset counts for BTS-1 were calculated using the total white cell count (WCC) generated by a Sysmex NE8000 haematology analyzer (Sysmex U.K., Ltd), and the percentage of total lymphocytes was determined by immunological gating procedures previously described (18,23). Simulset and FACScan Research software were used for analysis of two- and three-colour-stained specimens, respectively (Becton Dickinson). The Ortho Cyturon Absolute and the Becton Dickinson FACSCount

generated absolute lymphocyte subset values directly (8,30).

Lysing Properties

To evaluate the lysing properties of the stable whole blood preparation, parallel experiments were performed with fresh whole blood. Three dilutions of FACS lysing solution (Becton Dickinson) were tested (2×, 3.5×, and 5× concentrate) in addition to that recommended by the manufacturer (1×). The lysing properties of the stabilised and fresh whole blood were also tested at 4°C, 20°C, and 37°C with each dilution. Complete lysis was determined by visual inspection and confirmed by centrifugation (to visually detect unlysed RBC's), and also by flow cytometric analysis (to examine light scatter properties).

Pilot External Quality Assurance Study

The UK NEQAS Scheme for Leucocyte Immunophenotyping evaluates the performance of over 300 laboratories worldwide that routinely perform leukaemia immunophenotyping and HIV-lymphocyte subset analysis (3,13). As of November 1995, a total of 126 laboratories from the UK and overseas participated in the Immune Monitoring Scheme (T-lymphocyte subset scheme). To examine the performance of the stabilised whole blood prior to full implementation within the scheme, an external evaluation was undertaken. A second batch of 500 ml was prepared (BTS-2), and 2 × 5 ml aliquots issued to 21 participants, who were selected as suitable laboratories capable of rigorously testing the material based on experience, persistent good performance (using a defined scoring system), and the use of a relevant technology—FACScan (16 users), Coulter Profile II (2 users), Ortho Cyturon Absolute (2 users), and FACStar (1 user). Each laboratory was required to assay one specimen of BTS-2 on the day of receipt and to store the second at 4°C for 14 days, before analysing the percentage total CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ lymphocytes.

RESULTS

In-House Stability Testing

Seventy-one separate analyses were performed, over a period of 297 days, on BTS-1 using the FACScan. Cytometer PMT voltages and compensation levels remained constant throughout the duration of the study. Isotype controls consistently showed very low levels of both autofluorescence and nonspecific binding, similar to those recorded with fresh blood. The percentage values for each lymphocyte subset remained stable throughout, with CV's for the various T-lymphocytes ranging from 4.3% to 7.3% (Table 1). Importantly, the flow cytometric profiles were retained throughout the 297-day study period (see Fig. 1 for representative examples). Granulocyte, monocyte, and lymphocyte populations were easily identified, using both light scatter and immunological parameters, enabling cytometric WCC differentials to be

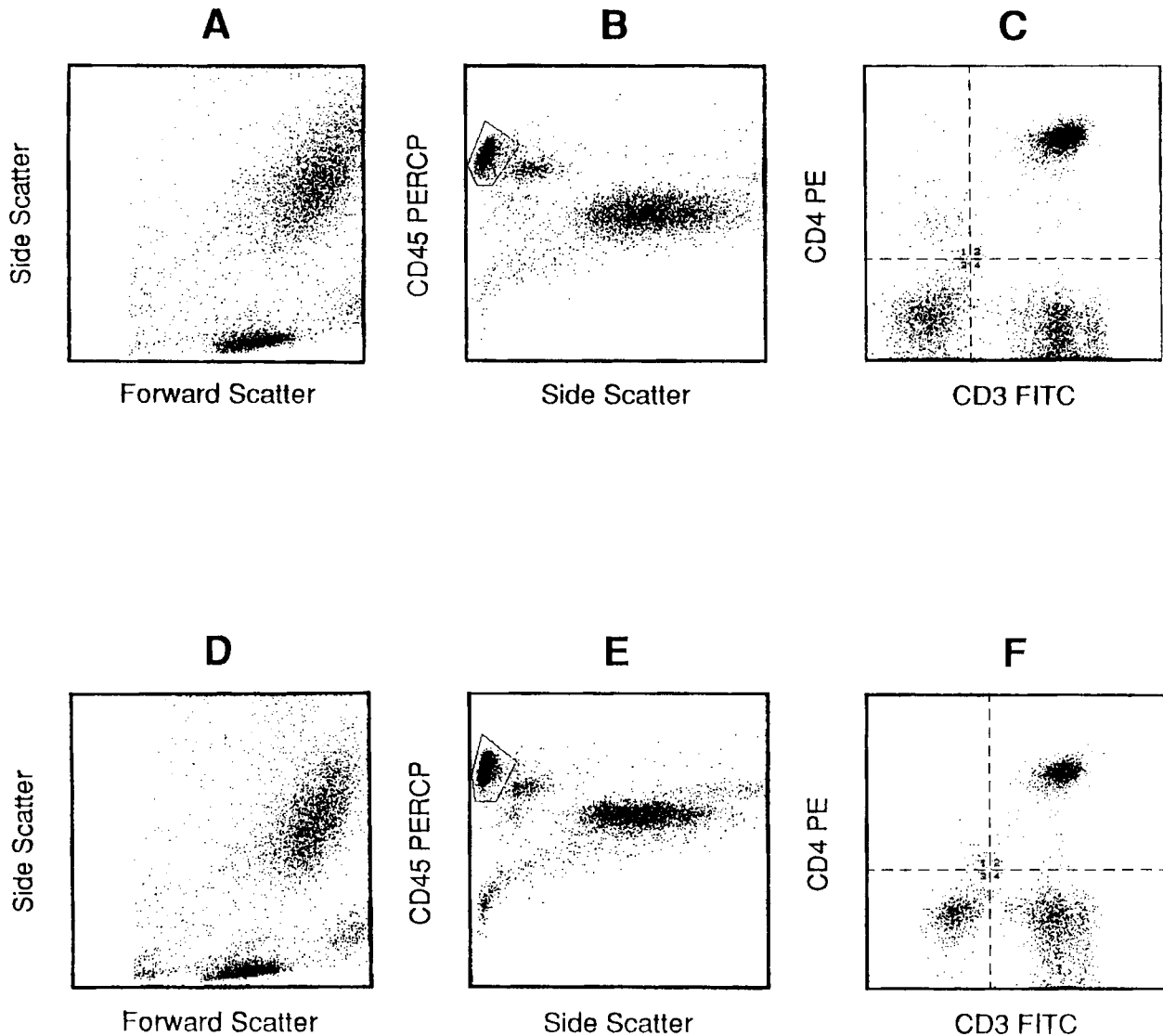


FIG. 1. Comparison of flow cytometric profiles of a stabilized whole blood specimen at day 5 (plots A-C) and day 220 (plots D-F). Plots A and D illustrate the FSC versus SSC profiles. The regions depicted in the SSC

versus CD45 PERCP plots (B and E) were used to generate the CD3 FITC versus CD4 PE plots (C and F). Data were acquired on a standard FACScan with the PMT values remaining constant throughout.

generated. Statistical analysis of absolute lymphocyte values derived for BTS-1 are summarised in Table 2. In addition, complete erythrocyte lysis was achieved, such that lymphocyte gate purity was >90% and nonlymphocyte contamination was low (<10%) (7).

BTS-1 was also analyzed using an Ortho Cyturon Absolute, between days 14 and 118. The PMT values were retained throughout the duration of the study. Percentage values obtained (Table 3) were comparable to the FACScan data, with absolute lymphocyte subset counts remaining constant throughout (Table 4). The mean of the absolute values, as well as the range of results obtained with the Cyturon Absolute, were lower than those obtained by the standard method of FACScan plus NE8000 (Table 2). As with the FACScan, flow cytometric profiles

obtained on the Cyturon Absolute remained unchanged over the analysis period. Assays of BTS-1 were made for ten consecutive days (days 30-40 of study), using the FACScan—an analyzer that generates absolute values for total CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ lymphocyte populations, using a “no wash, no lyse” technique. The CV values obtained with this technology were the lowest obtained (Table 5).

Lysing Properties

The stable whole blood preparation possesses lysing properties similar to those of fresh whole blood (Table 6). The lysing time was only marginally shorter at 20°C, when using a 1× concentration of FACS lysing solution, to that obtained with fresh blood. Interestingly, complete

Table 1
Percentage Values Obtained on BTS-1 Over a 297 Day Period Using a FACScan

	Total CD3 ⁺ lymphocytes	CD3 ⁺ CD4 ⁺ lymphocytes	CD3 ⁺ CD8 ⁺ lymphocytes	CD19 ⁺ lymphocytes	CD3 ⁻ CD16 ⁺ CD56 ⁺ lymphocytes
Observations	71	71	71	71	71
Minimum	60	41	17	2	17
Maximum	73	51	24	13	30
Mean	66.7	45.2	20.5	7.82	24.3
Median	67	45	21	8	24
Standard deviation	2.9	2.1	1.5	1.6	2.3
CV%	4.3	4.6	7.3	20.5	9.5

Table 2
Absolute Values Obtained on BTS-1 Using a FACScan Plus Haematology Analyzer (NE8000)
Generated White Cell Count Over a 297 Day Period

	Total CD3 ⁺ lymphocytes	CD3 ⁺ CD4 ⁺ lymphocytes	CD3 ⁺ CD8 ⁺ lymphocytes	CD19 ⁺ lymphocytes	CD3 ⁻ CD16 ⁺ CD56 ⁺ lymphocytes
Observations	66	66	66	66	66
Minimum	410	290	130	20	160
Maximum	720	540	230	120	290
Mean	560	381	172	65	205
Median	560	387	170	64	200
Standard deviation	72	52	24	14	29
CV%	12.9%	13.6%	14.13%	21.29%	14.15%

Table 3
Percentage Values Obtained on BTS-1 Over 122 Day Period When Analyzed Using an Ortho
Cytoron Absolute

	Total CD3 ⁺ lymphocytes	CD3 ⁺ CD4 ⁺ lymphocytes	CD3 ⁺ CD8 ⁺ lymphocytes	CD19 ⁺ lymphocytes	CD3 ⁻ CD16 ⁺ lymphocytes
Observations	45	45	45	45	45
Minimum	55	35	15.5	4	16
Maximum	79	61.5	24.5	10	26
Mean	67	44	19	7	24
Median	68	43	19	7	24
Standard deviation	5.3	5.7	1.9	1.1	5.8
CV%	7.8%	12.9%	10.0%	15.9%	22.3%

lysing times for fresh whole blood were shorter than the stable whole blood material at 4°C and 20°C when using FACS lysing solution at concentrations not recommended by the manufacturer.

Pilot External Quality Assurance Study

The results are summarised in Table 7. The between-laboratory CV values for the 21 centres were 3.4% (total percentage of CD3⁺ lymphocytes), 4.1% (percentage of CD3⁺CD4⁺ lymphocytes), and 5.1% (percentage of CD3⁺CD8⁺ lymphocytes). All laboratories stated that the flow cytometric and fluorescence characteristics of the preparation were comparable to those seen with fresh whole blood specimens. Five laboratories reported that minor adjustments to the forward/side scatter were required to achieve optimum resolution, but that these adjustments were not detrimental to specimen performance or analysis. No laboratory reported problems with lysis, autofluorescence, or nonspecific binding. In addition, the specimen was fully compatible with the automatic gating

software in use at the participating centres. Performance, following the second analysis 14 days later (the preparation was already 20 days postdonation), was unchanged (two laboratories actually stated that it looked better 14 days later than on day 0) (Table 7). The between-laboratory CV's were further reduced: 1.8% for total %CD3⁺ lymphocytes, 2.4% for %CD3⁺CD4⁺ lymphocytes, and 2.5% for the %CD3⁺CD8⁺ population.

Full Introduction into the NEQAS Scheme

In April, 1993, following the extensive in-house and pilot external evaluation studies, a further preparation was produced (designated sample 30) and distributed to all participants (n = 75). Results were returned from 67 laboratories (89%), with only two stating that the sample was unsuitable. Analysis of the data revealed the following interlaboratory CV's: 3.2% for total %CD3⁺ lymphocytes, 5.1% for %CD3⁺CD4⁺ lymphocytes, and 5.2% for %CD3⁺CD8⁺ lymphocytes, values lower overall than those seen when using "fresh" blood. This trend of low

Table 4
Absolute Values $\times 10^6/ml$ Obtained on BTS-1 Over a 122 Day Period When Analyzed Using an Ortho Cytoron Absolute

	Total CD3 ⁺ lymphocytes	CD3 ⁺ CD4 ⁺ lymphocytes	CD3 ⁺ CD8 ⁺ lymphocytes	CD19 ⁺ lymphocytes	CD3 ⁻ CD16 ⁺ lymphocytes
Observations	45	45	45	45	45
Minimum	439	288	108	33	100
Maximum	590	434	173	65	418
Mean	500	331	142	51	200
Median	501	329	143	51	185
Standard deviation	26.5	23	15	7	67
CV%	5.3%	6.9%	10.4%	12.7%	15.9%

Table 5
Absolute Values $\times 10^6/ml$ Obtained on BTS-1 Over an 11 Day Period When Analyzed Using a FACScount

	Total CD3 ⁺ lymphocytes	CD3 ⁺ CD4 ⁺ lymphocytes	CD3 ⁺ CD8 ⁺ lymphocytes
Observations	10	10	10
Minimum	590	330	152
Maximum	683	360	195
Mean	623	346	170
Median	621	347	170
Standard deviation	26	8	13
CV%	4.2%	2.37%	7.46%

Table 6
Lysing Properties of Stabilised and Fresh Whole Blood at Varying Concentrations of FACS Lysing Solution and Temperatures^a

	4°C		20°C		37°C	
	Stabilised	Fresh	Stabilised	Fresh	Stabilised	Fresh
1x	2' 10"	4' 20"	1' 20"	1' 48"	0' 40"	0' 54"
2x	>22'	12' 48"	12'	3'	1' 20"	2' 42"
5x	>22'	>22'	>22'	>22'	3' 30"	2'
5x	20'	13'	5' 30"	2' 17"	1'	1'

^aTimes are in minutes and seconds.

Table 7
Statistics for Pilot Trial of BTS-1

Parameter	Day 0 (n = 19), Mean \pm SD	Day 14 (n = 21), Mean \pm SD
CD3	73.2 \pm 5.4%	71.8 \pm 2.8%
CD4	50.8 \pm 4.2%	50.3 \pm 2.6%
CD8	18.5 \pm 1.7%	18.1 \pm 1.1%

CV values and standard deviations has been maintained since introduction of the stable whole blood preparation (Fig. 2). The two specimens (samples 37, 38), with low T-lymphocyte percentages to mimic HIV⁺ material, possessed slightly increased CV values.

A further batch of stabilised whole blood was issued on two different occasions (6 weeks apart) to two groups of laboratories. (Group 1 comprised 77 laboratories within the UK, Eire, Portugal, and Sweden; Group 2 comprised 49 laboratories from Austria, Australia, Belgium, Canada, Denmark, France, Germany, Italy, Spain, Switzerland, and

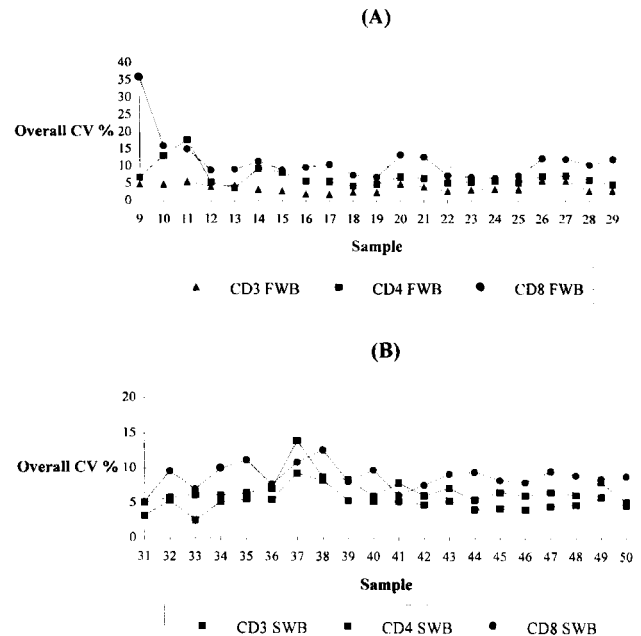


Fig. 2. Comparison of the overall CV's obtained from the UK NEQAS Immune Monitoring Scheme using fresh (graph A) and stabilised (graph B) whole blood.

Table 8
Stabilised Whole Blood UK NEQAS Distribution Involving Two Separate Groups Six Weeks Apart

Parameter	Group 1 (Issue day 0)			Group 2 (6 weeks later)		
	Returns	Mean %	SD %	Returns	Means %	SD %
Total	77	69.37	3.64	49	70.36	2.8
CD3 ⁺						
CD3 ⁺ 4 ⁺	77	45.87	2.85	49	46.44	2.8
CD3 ⁺ 8 ⁺	77	22.84	2.29	49	23.31	2.13

^aGroup 1 comprised laboratories from the UK, Eire, Portugal, and Sweden. Group 2 comprised laboratories from Austria, Australia, Belgium, Canada, Denmark, France, Germany, Italy, Spain, Switzerland, and the USA.

the USA). Table 8 shows the statistical analysis of data from each issue. It can be seen that the mean and standard deviations for percentage values are similar, with the later specimen (Group 2) demonstrating a slight improvement

in these values. No laboratory reported significant problems with the specimen on either occasion.

DISCUSSION

A previous study demonstrated the possibility of chemically preserving both the antigenic and the relative flow cytometric light scatter characteristics of B-lineage-associated cell lines, and advocated their use in the monitoring of internal and external laboratory performance (6). Although these are of value, such cellular preparations, as well as the currently available commercial products (e.g., Cytotrol & CD-Chex), are not whole blood and therefore do not provide full process control. It is important that sample preparation is thoroughly quality controlled, because the majority of HIV immunophenotyping is performed using whole blood techniques.

Historically, EQA has always used fresh whole blood, necessitating the rapid distribution of such material (11,17,25,27). However, there is no guarantee that the blood will be received at the testing laboratory in perfect condition, presenting problems in identifying poor or inadequately performing laboratories. Furthermore, because of the rapid deterioration of fresh whole blood, repeat samples cannot be issued to a laboratory that has experienced a problem. The use of fresh material results in a wide range of results (high CV and SD values) (27) attributable, in part, to sample condition. Ideally therefore, the use of a stable whole blood preparation will not only obviate the need for express delivery, but also reduce the deviation around the consensus mean caused by sample deterioration. Furthermore, it facilitates the reissue of material to laboratories experiencing problems, and allows a repeat trial weeks or even months later.

This study has demonstrated that such a stable whole blood preparation can be successfully introduced into an EQA program. Overall performance of the material has been excellent and has allowed a performance score to be determined for each participating laboratory. The preparation procedure allows bulk production and, when aliquoted into lots, can be issued at varying intervals (Table 8). This facilitates the monitoring of laboratory drift, or bias, over time, previously only possible by issuing at least duplicate specimens within one trial run.

We have shown that, by using stabilised whole blood, individual laboratory results for each issue are tightly distributed about the mean, as defined by a low SD value. The majority of the CV values are less than 10% for each of the three parameters ($CD3^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$) (Fig. 2). Only three batches from the 19 issued to date have had CV values greater than 10%, the highest being 14% for the $CD3^+CD4^+$ population in batch 37. This sample had an increased $CD3^+$ NK population, resulting in a low % $CD3^+CD4^+$. In contrast, using fresh whole blood within the UK NEQAS programme, 11 of the 20 batches resulted in CV values greater than 10%, the highest CV being 36% (% $CD3^+CD8^+$, specimen 9). Furthermore, using fresh whole blood, higher SD values

have been observed ($\pm 7.39\%$ sample 27, total $CD3^+$ lymphocytes), compared with the stable preparation ($\pm 4.5\%$ sample 49, total $CD3^+$ cells).

Absolute T-lymphocyte counts are calculated from the absolute lymphocyte count and %T-lymphocyte subset value (20). Recent reports have highlighted the variability in absolute T-lymphocyte counts as a result of using different haematology analyzers (15,26). To overcome this problem, flow cytometers capable of generating absolute T-lymphocyte values have been developed (8,30). An additional benefit of our preparation is its ability to evaluate the performance of absolute T-lymphocyte subset counting on such instruments. A recent issue (sample 36), for example, highlighted a centre with flow cytometer calibration problems, in that the returned data was out of consensus with the relative user group (data not shown). Once the problem had been rectified, reissue of the same sample 1 month later produced results within ± 1 SD of the target values.

In-house studies have confirmed stability of both the light scatter and staining characteristics of the preparation. These parameters are similar to those observed with fresh whole blood, allowing identification of lymphocyte subpopulations, even when antigen expression is weak, e.g., $CD8^{\text{dim}}$ cells. The excellent light scatter patterns enable easy identification of lymphocytes, monocytes, and granulocytes using either CD14/CD45 or CD45/SSC gating, with both autogating software and manual gating methods. Complete compatibility was demonstrated with both FACScan and Ortho Cytoron Absolute cytometers. In addition, the preparation was compatible with the two commercial lysing reagents tested, namely, FACS lysing solution (Becton Dickinson) and Ortho-mune lysing reagent (Ortho Diagnostic Systems), thus meeting the requirements for total process control. When using FACS lysing solution at the manufacturer's recommended concentration, the lysis of our preparation compared well with those of fresh whole blood. Furthermore, the use of incorrect concentrations of FACS lysing solution was detectable with our preparation. Clearly, the lysing properties of alternative reagents need to be evaluated, but use of this preparation within the UK NEQAS Immune Monitoring Scheme has not highlighted any technical difficulties with other systems. One batch of the preparation performed well on the FACSCount system, which uses a "no wash, no lyse" method.

The stability of the white cell and lymphocyte counts will enable the preparation to be used as a reference material for absolute lymphocyte subset enumeration. Methods to evaluate the precision, accuracy, and sensitivity of measurements by flow cytometry are of paramount importance, particularly if quantitative analysis of antigen density or antibody binding capacity are to be of clinical value (1,28). The use of antibody-coated beads, such as Quantum Simply Cellular (Flow Cytometry Standards, Puerto Rico) or QIFI (Biocytex, Marseille, France), in conjunction with this material may facilitate the production of a reference/control with a predefined antibody binding capacity or antigen density for individ-

ual cell populations. A multicentre UK study using this material and the QSC beads is currently in progress.

The use of flow cytometry to measure DNA content in neoplastic conditions is well described (29). The need for adequate quality control measures to reduce the intra- and interlaboratory variation is important (5,9,10,32). Thus there is a need for a DNA-cell-cycle quality control product that will stain in a manner comparable to fresh whole blood but have the capacity to be stored for prolonged periods. Independent studies using this material have indicated that it may be suitable for such a purpose (manuscript in preparation).

In conclusion, we have demonstrated the suitability of a novel stable whole blood preparation for use as a reagent for both daily and longitudinal quality control. The material has been commercially available in Europe since June, 1995 (Ortho*Absolute* Control, Ortho Diagnostics Systems, Inc., Raritan, NJ). In addition, the stabilisation process may enable the production of abnormal specimens to reflect CD34⁺ PBSCs and leukaemic samples, as well as HIV cases with low T-lymphocyte subset counts.

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