Clin. Lab. Haem.
2000, **22,** 89–96

Standardization of lymphocyte antibody binding capacity – a multi-centre study

D. BARNETT*
I. STORIE*
V. GRANGER*
L. WHITBY*
J.T. REILLY*
S. BROUGH†
S. GARNER†
J. LAWRY‡
S. RICHARDS§
A.E. BELL¶
B.K. SHENTON¶

**UK NEQAS for Leucocyte Immunophenotyping, Royal Hallamshire Hospital (co-ordinating centre), Sheffield, UK;* †*National Blood Service (North London Centre), London, UK;* ‡*Institute for Cancer Studies, Sheffield University Medical School, UK;* §*Haematology Malignancy Diagnostic Service, Leeds, UK;* ¶*Department of Surgery, Newcastle University Medical School, UK*

Summary As quantitative flow cytometry is being increasingly used to characterize non-malignant and malignant disorders, interlaboratory standardization becomes an important issue. However, the lack of standardized methods and process controls with predefined antibody binding capacity values, limits direct interlaboratory comparison. The present study has addressed these issues using a stable whole blood product and a standardized antigen quantification protocol. It was demonstrated that: (i) a standard technical protocol can result in a high degree of interlaboratory concordance; (ii) interlaboratory variation of less than 12% can be achieved for CD4 antibody binding capacity values; and (iii) stable whole blood can be used as a process control with predefined antibody binding capacity values. Furthermore, using such an approach, a normal range was established for CD3, CD4 CD8 and CD19. These antigens appear to be expressed in a hierarchical manner, a factor that could be used as a procedural quality control measure.

Keywords Antigen density, flow cytometry, normal range, quality control, standardization

Introduction

The use of flow cytometric antigen detection has become an increasingly important technique for the identification and monitoring of cell populations (Givan 1992; Macey 1994). Recently, an extension of this technique has been the quantification of antigens on both normal and abnormal cells (Lavabre-Bertrand *et al.* 1994a; Lavabre-Bertrand *et al.* 1994b; Lavabre-Bertrand *et al.* 1994c; Peters *et al.* 1994; Farahat *et al.* 1995; Storie *et al.* 1995). Such techniques have been made possible by the introduction of latex particles for use as calibration standards (Poncelet & Carayon 1985; Poncelet, Lavabre-Bertrand & Carayon 1986; Schwartz & Fernandez-Repollet 1993).

Several methods for antigen quantification have been described; relative linear fluorescence intensity (RFI) (Muirhead, Schmitt & Muirhead 1983; Schmid, Schmid & Giorgi 1988), CD4 reference standard (Hultin, Matud & Giorgi 1998), molecules of equivalent soluble fluorochrome (Schwartz *et al.* 1998), quantitative indirect immunofluorescence (QIFI) (Poncelet & Carayon 1985), quantum simply cellular antibody binding capacity (QSC ABC) (Schwartz & Fernandez-Repollet 1993) and stabilized cell immunofluorescence assay (Gratama *et al.* 1998;

Accepted for publication 8 February 2000
Correspondence: Dr D. Barnett, UK NEQAS for Leucocyte Immunophenotyping, Department of Haematology, Royal Hallamshire Hospital, Glossop Road, Sheffield S10 2JF, UK. E-mail: d.barnett@sheffield.ac.uk

Janossy *et al.* 1998). The calibration of the flow cytometer with molecules of equivalent soluble fluorochrome (MESF) and the subsequent use of antibody conjugates with known MESF/antibody ratios (Davis *et al.* 1996) forms the basis for the recently introduced QuantiBrite™ system (Becton Dickinson, San Jose, CA, USA) which uses anti-CD4 with a fluorochrome:protein ratio of 1:1. The majority of published studies, however, have used either the QIFI or the QSC ABC method. The former technique uses beads, coated with known amounts of murine monoclonal antibody (IgG anti-CD5), that serve as a control for indirect immunofluorescence analysis. In contrast, the ABC method employs a cocktail of five highly uniform microbead populations, one blank and four coated with a defined, and different, quantity of goat anti-mouse. Using these methods it has been possible to define antigen density on both normal and leukaemic cells, and monitor antigen expression changes that occur during viral infections and with age (Lavabre-Bertrand *et al.* 1994a; Lavabre-Bertrand *et al.* 1994b; Lavabre-Bertrand *et al.* 1994c; Peters *et al.* 1994; Farahat *et al.* 1995; Lavabre-Bertrand *et al.* 1995; Lenkei & Andersson 1995a; Lenkei & Andersson 1995b; Rebuck, Gibson & Finn 1995; Storie *et al.* 1995).

These techniques, however, are poorly standardized on an interlaboratory basis (Lavabre-Bertrand *et al.* 1994b; Farahat *et al.* 1995; Lenkei & Andersson 1995b; Gratama *et al.* 1998). Variables include the source and amount of antibody used, the type of fluorochrome and red cell lysing solution, the use of either mononuclear cells or whole blood, variations in incubation time and temperature, the use of single or multi-parameter analysis and the recording of either mean or median channel values (Lavabre-Bertrand *et al.* 1994b; Farahat *et al.* 1995; Lenkei & Andersson 1995b). These factors, in addition to differences in flow cytometer calibration (Vogt *et al.* 1991), have almost certainly contributed to the reported variations in antigen density when expressed as molecules per cell (Lavabre-Bertrand *et al.* 1994b; Farahat *et al.* 1995).

Recently, several technical factors have been highlighted that can influence the ABC values obtained with the quantum simply cellular approach and a 'benchmark' method to reduce interlaboratory variation has been suggested (Barnett *et al.* 1998b). However, to monitor this variation, a stable whole blood quality control material, with a predefined antibody binding capacity, is required. A prior study reported on the use of a novel stable whole blood preparation as an analyte by UK NEQAS for Leucocyte Immunophenotyping (Barnett *et al.* 1996; Barnett *et al.* 1998a). The present study uses a standardized protocol coupled with such a preparation and demonstrates that a high degree of concordance is possible.

Materials and methods

Antibody binding capacity determination

Five UK laboratories, randomly coded 1–5 to retain confidentiality, participated in this study. Flow cytometric analysis was undertaken on a FACScan (Becton-Dickinson) at three laboratories and a FACSort (Becton-Dickinson) by the other two. ABCs for CD3, CD4, CD8, CD19, as well as for the two isotype controls (IgG1 and IgG2a) were determined using single colour fluorescein isothiocyanate (FITC) conjugated antibodies (Sigma Immunochemicals, Poole, Dorset, U.K.). Each laboratory, on a predetermined day, collected 10 normal peripheral blood samples into either potassium or sodium EDTA (five males, five females; age range 18–65 years). All samples were stained within 6 h of collection and analysed within 24 h using the method described below.

In addition, all centres received two aliquots from the same batch of stabilized whole blood, prepared as previously described (Barnett & Granger 1998), for analysis 15 days apart. Each laboratory stained the samples using a standardized staining protocol (agreed prior to the study). Briefly, 100 μl whole blood (or 50 μl QSC beads to produce a calibration curve) was added to 10 ml antibody (manufacturer recommended volume) and incubated, at 18–22°C, for 1 h in the dark. The red cells where then lysed according to the appropriate protocol for the reagent used. FACS Lysing solution (Becton Dickinson) was employed by four centres, while one used Optilyse-B (Immunotech, Marseille, France). After lysis, samples were washed twice by centrifuging at 500 ***g*** for 7 min at 10°C with 2 ml Dulbecco's phosphate buffered saline (DPBS) pH 7.4, containing 1% bovine serum albumin (BSA) and 0.1% NaN_3 (Sigma Immunochemicals, Poole, Dorset, UK). Each tube was fixed with 0.6 ml (0.2 ml for beads) 1% paraformaldehyde in DPBS containing 1% BSA (to improve peak resolution of QSC beads). Prior to flow cytometry, a common window of analysis was established using QC Windows (Sigma Immunochemicals, Poole, Dorset, UK).

To facilitate the calculation of ABC for both normal and stabilized whole blood, all flow cytometric information (including bead data) was analysed centrally (UK NEQAS) using TallyCal software (Applied Cytometry Systems, Sheffield, UK). TallyCal software enables the standardization of the median channel values derived by different flow cytometer software, thus enabling direct interlaboratory comparison of ABC values. The ABC values calculated for CD3, CD4, CD8 and CD19 were expressed as molecules/cell after subtracting the appro-

Table 1. Statistical summary for antibody binding capacity normal range for isotype controls, CD3, CD4, CD8 and CD19 by centre. Each centre analysed 10 normal subjects (five males, five females).

Centre	Mean IgG1	Mean IgG2a	MeanCD3	Mean CD4	Mean CD8	Mean CD19
1	1840 (273)	1127 (76)	178053 (39113)	80044 (14268)	447816 (141084)	40567 (12407)
2	1945 (142)	1221 (86)	148940 (9660)	76366 (2423)	347923 (90224)	23318 (3366)
3	1754 (113)	836 (60)	194127 (24659)	78500 (4507)	279347 (79480)	31327 (4891)
4	2170 (283)	1545 (139)	150887 (12706)	76175 (6345)	327803 (51627)	36515 (9599)
5	1933 (134)	1142 (130)	152854 (17533)	69423 (6802)	337159 (130120)	45478 (3641)
Overall mean	1928 (246)	1174 (249)	164972 (29406)	76102 (8743)	348010 (117610)	35441 (10832)
Normal range (derived from all data)	1436–2420	676–1672	106160–223784	58616–93587	112790–583230	13779–57103

Data presented as mean (SD).

priate isotype control. Student's unpaired *t*-test was used in the statistical analyses.

Results

The mean ABC values for CD3, CD4, CD8, CD19 and isotype controls for 10 normal samples (Table 1) showed good agreement for the mean CD4 ABC values, with all centres obtaining values within approximately 10 000. Indeed, three centres obtained mean CD4 ABC values within 264. Similarly, four of the five centres produced CD19 ABC values within 9240 (centres 1, 3, 4 and 5). The interlaboratory coefficients of variation (CV) were lowest for CD4 (Table 2). Centre 1 had the highest intralaboratory CV for three of the four antigens tested (CD3, CD4 and CD19) (Table 2). However, two of the individual cases chosen by this centre exhibited ABC values significantly higher than their normal range for CD4 and CD19, probably skewing the data. If these values were excluded, the intralaboratory variation was reduced to 8% and 10.3% for CD4 and CD19, respectively, in line with results from the other centres.

The greatest variation, both intra-and interlaboratory, was observed for CD8 and CD19 (Table 2). The former probably reflects the inclusion of CD8dim cells in the analysis (Perussia, Fanning & Trinchieri 1983). For example, centre 1 calculated the $CD8^{dim}$ population to have a mean ABC of 46 612 ± 8606 mol/cell, compared to 447 816 ± 141 084 for the $CD8^{bright}$ cells (data not shown). The increased variation for CD19 probably reflects the fact that any slight variation in the median peak channel value will have a greater influence at low compared to high ABC values.

Analysis of the ABC values by sex showed that CD4 and CD19 antigen density is remarkably similar between males and females (Table 3). However, no statistical difference was demonstrated in mean ABC values for male and female individuals for any antigen. The antigen density of CD8, CD3, CD4 and CD19 demonstrated a 'linear' relationship when plotted on a log-linear scale (Figure 1). The hierarchical expression can be defined as follows: CD8 > CD3 > CD4 > CD19. A review of the literature reveals that such a relationship could also be demonstrated in two other studies that used a single-colour immunofluor-

Table 2. Comparison of intra and interlaboratory coefficients of variation (CV) for CD3, CD4, CD8 and CD19

Centre	CD3	CD4	CD8	CD19
1	22.0%	17.8%	31.5%	30.6%
2	6.5%	3.2%	25.9%	14.4%
3	12.7%	5.7%	28.5%	15.6%
4	8.4%	8.3%	15.7%	26.3%
5	11.5%	9.8%	38.6%	8.0%
Mean CV	12.2%	9.0%	28.0%	19.0%
Overall interlaboratory CV	17.8%	11.5%	33.8%	30.6%

Table 3. Statistical summary of antibody binding capacity by sex for isotype controls, CD3, CD4, CD8 and CD19

Sex	Mean IgG1a	Mean IgG2a	Mean CD3	Mean CD4	Mean CD8	Mean CD19
Male	1966 (281)	1197 (275)	17,2057 (35848)	77,561 (7846)	32,4999 (96034)	32,711 (11765)
Female	1888 (259)	1167 (279)	16,3946 (26409)	77,981 (9265)	37,6445 (129600)	33,778 (9749)

Data presented as mean (SD).

escence approach (Denny *et al.* 1996; Gratama *et al.* 1998)(Figure 2), in contrast to a third study (Lenkei & Andersson 1995a) that used a triple-colour immunofluorescence approach (Figure 2).

The stabilized whole blood samples were analysed on two occasions, 15 days apart (centre 1 failed to analyse on day 15 as a result of instrument failure). All scatter plots were compared to those obtained from fresh samples and were comparable. Over the 15-day period, the mean ABC for CD3 and CD8 decreased while those for CD4 and CD19 exhibited a slight increase (Table 4). The greatest decrease was for CD8 (6821 mol/cell), representing 4.4% of the original ABC. However, this may be as a result of variability in defining the $CD8^{dim}$ population. Although the stabilized whole blood had lower ABC values for each antigen when compared to fresh samples, the linear relationship between the antigens studied was preserved (Figure 3). There was no significant difference between the day 1 and day 15 values.

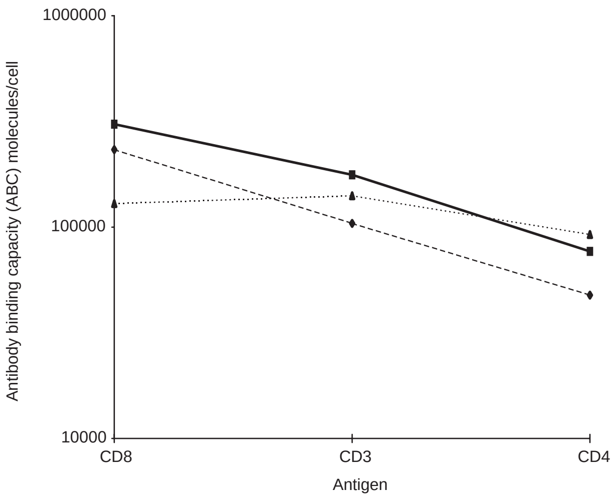

Figure 2. The relationship of antibody binding capacity for CD3, CD4 and CD8 as determined in previously published studies (Lenkei & Andersson 1995a solid diamonds, dashed line; Denny *et al.* 1996 filled squares, unbroken line; Gratama *et al.* 1998 filled triangles, dotted line).

Discussion

Several studies have demonstrated that it is possible to relate antigen density, determined by flow cytometry, to

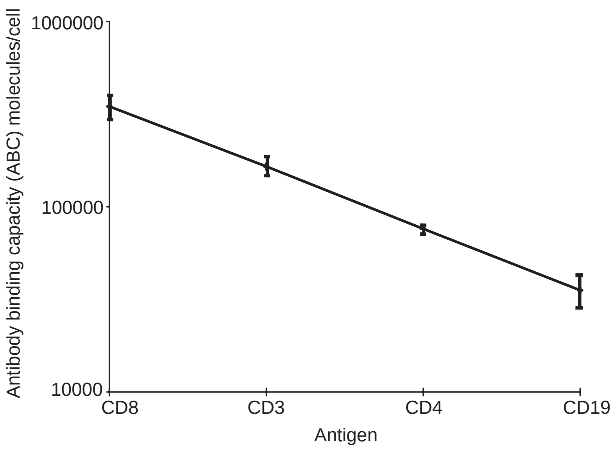

Figure 1. The relationship of antibody binding capacity for CD3, CD4, CD8 and CD19 defined from 50 normal individuals (error bars shown)

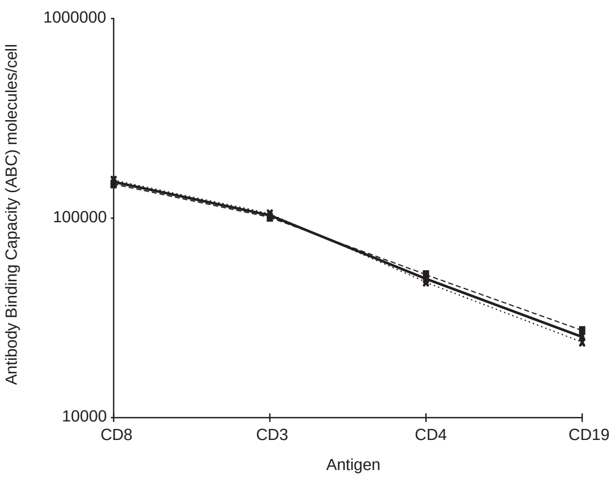

Figure 3. The relationship of antibody binding capacity for CD3, CD4, CD8 and CD19 on the stabilized whole blood samples (mean values of all five centres). Mean day 1, crosses, dotted line; Mean day 15, filled squares, dashed line; Overall mean, filled triangles, unbroken line).

Table 4. The antigen binding capacity results obtained in each centre for the stabilized whole blood when analysed 15 days' apart

Centre	Day	IgG1	IgG2a	CD3	CD4	CD8	CD19
1	1	3699	1741	122245	48699	174049	15333
	15	ND	ND	ND	ND	ND	ND
2	1	3843	2604	96351	47152	162352	26049
	15	4080	2753	89811	43794	107728	21264
3	1	3471	1797	105812	44776	158897	15569
	15	3541	ND	91910	63553	145746	19766
4	1	3895	2768	93665	44625	155794	21895
	15	4137	3189	128896	61127	199776	33743
5	1	6122	4724	107511	53090	123839	40376
	15	4170	4170	92967	39925	139409	34420
Mean	1	4206	2727	105117	47668	154986	23844
SD	1	1084	1209	11265	3477	18733	10282
Mean	15	3982	3371	100896	52100	148165	27298
SD	15	296	726	18713	11970	38216	7861
Overall mean		4106	2968	103241	49638	151954	25379
Overall SD		751	979	13324	7615	25579	8398

health and disease and thus assist in clinical diagnosis (Lavabre-Bertrand *et al.* 1994a; Lavabre-Bertrand *et al.* 1994b; Lavabre-Bertrand *et al.* 1994c; Peters *et al.* 1994; Farahat *et al.* 1995; Lavabre-Bertrand *et al.* 1995; Lenkei & Andersson 1995b; Rebuck *et al.* 1995; Storie *et al.* 1995). One study has suggested that antigen quantification could be used as a routine laboratory technique (Lavabre-Bertrand *et al.* 1994b). However, the methodology is still poorly standardized with no interlaboratory studies having been published that used standardized reagents and a pre-agreed protocol. Different protocols have been used in previously published reports, particularly with regard to incubation temperature (Lavabre-Bertrand *et al.* 1994b; Farahat *et al.* 1995), incubation time (Farahat *et al.* 1995; Lavabre-Bertrand *et al.* 1994a; Lavabre-Bertrand *et al.* 1994b; Lenkei & Andersson 1995b) and the use of the median or mean channel in the calculation (Lavabre-Bertrand *et al.* 1994a; Lavabre-Bertrand *et al.* 1994b; Lenkei & Andersson 1995b).

A prior study reported on the factors affecting antigen density and concluded that it is important to have a standardized method to enable the generation of comparative and meaningful data and proposed a 'benchmark' method that includes: (i) single staining using FITC conjugated antibodies; (ii) all reagents at pH 7.4 ± 0.1; and (iii) incubation and lysing should be undertaken at 20 ± 1°C (Barnett *et al.* 1998b). However, other factors will also affect the results obtained. For example, the antigen density will be influenced by the use of mean, or median, channel values in the calculation, particularly for antigens that are not normally distributed, including activation antigens. Additionally, the choice of fluorochrome and factors affecting stearic hindrance, e.g. multicolour assays, will affect the end result and the lack of a suitable reference material hinders interlaboratory standardization. These factors, coupled with the variability in flow cytometer set-up and calibration, account for the conflicting published data (Lavabre-Bertrand *et al.* 1994b; Farahat *et al.* 1995).

Limited data is available with regard to interlaboratory studies of flow cytometric antigen density determination, although interlaboratory proficiency testing has demonstrated a high degree of consensus when determining percentage values of a target population (Paxton *et al.* 1989; Homburger *et al.* 1993; Kagan *et al.* 1993; Barnett, Granger & Reilly 1994; Barnett *et al.* 1996; Barnett *et al.* 1998a). Current flow cytometers however, are not calibrated for quantitative fluorescence measurement and results are highly variable (Vogt *et al.* 1991). In an attempt to overcome this limitation we have attempted to standardize the methodology for antigen quantification. Although the number of participating laboratories was small, each sited tested 10 normal specimens, in addition to the stabilized whole blood preparation. Instruments were calibrated using QC windows and a 'common window of analysis' identified, following which, using an agreed protocol, ABC for isotype controls, CD3, CD4, CD8 and CD19 were calculated. Single colour staining, using FITC-conjugated antibodies was used throughout to remove the possibility of stearic hindrance from additional fluorochromes. Furthermore, all antibodies were obtained from the same source and batch (Sigma Immunochemicals).

This study has demonstrated that interlaboratory consensus can be achieved using a standardized method. The highest interlaboratory variance was observed for CD8 (33.8%), and is almost certainly due to inclusion of $CD8^{dim}$ cells in the analysis, while the lowest interlaboratory variance was observed for CD4 (11.4%). Three other studies have reported quantification vales for CD3, CD4 and CD8 antigens (Lenkei & Andersson 1995a; Denny *et al.* 1996; Gratama *et al.* 1998). The present study obtained values for CD3, CD4 and CD8 comparable to those reported by Gratama *et al.* (1998), with both studies using the same antibody clones and fluorochrome conjugates. Conversely, the study reported by Denny *et al.* (1996) obtained lower ABC values for CD3, CD4 and CD8, probably as a result of the use of different antibody clones, while data from Lenkei & Andersson (1995a) differs as a result of the incorporation of three colour immunofluorescence (anti-CD3 FITC, anti-CD4 PE and anti-CD8 PerCP). A prior report demonstrated that significant differences occur depending on the use of single, two and three-colour staining (Barnett *et al.* 1998b). For example, CD3 ABC is 130 000 molecules/cell higher in a single colour assay than a triple colour assay.

There appears to be a 'log-linear' relationship between CD3, CD4, CD8 & CD19 (Figure 1). Janossy *et al.* (1998) have previously described a new concept for quantitative flow cytometry, termed stabilized cellular immunofluorescence assay (SCIFA), in which the hierarchical expression of CD45 and CD38 was used to construct an internal biological calibration curve. The present study has extended these observations and illustrates that a similar curve can be plotted using CD3, CD4, CD8 and CD19. Reanalysis of data published in two previous studies (Denny *et al.* 1996; Gratama *et al.* 1998) confirms this relationship between CD3, CD4 and CD8. Estimation of the ABC values in these two studies was performed using a single colour approach, whilst data from a third study (Lenkei & Andersson 1995a), which used three-colour analysis, did not exhibit this relationship. Therefore, when using a single colour approach, this relationship between CD3, CD4, CD8 and CD19 ABC could be routinely examined to provide internal quality control and help identify 'aberrant' or 'rogue' results.

To determine if biological controls could be used to provide additional quality control, a 'stabilised' normal whole blood specimen as previously described (Barnett & Granger 1998) was issued. This material has been of benefit in external and internal quality control for determination of percentage and absolute values (Peloquin *et al.* 1994; Barnett *et al.* 1996; Barnett *et al.* 1998a). The use of the stabilized whole blood, together with a standardized method, demonstrated that consensus can be achieved between centres on material that is at least 15-days-old. Although the ABC of those antigens studied was lower to those obtained using fresh samples, the values remain constant and demonstrate the linear relationship between CD3, CD4, CD8 and CD19 observed with the 50 normal specimens. The apparent decline in CD8 antigen is probably as a result of the variability in detection of $CD8^{dim}$ cells. However, it should also be noted that antigen quantification systems that 'capture' the monoclonal antibody, such as the QSC system (i.e. antibody–antibody) are not necessarily measuring the functional binding of an antibody to its antigen and therefore care should be taken in the interpretation of the data obtained. Furthermore, the antigen density value obtained can also be influenced by the use of monovalent or divalent antibodies and absolute antigen quantification is not possible without the use of monovalent antibodies having a known 1:1 fluorochrome–protein ratio. However, even in view of these considerations, the present study demonstrates that stabilized peripheral blood has the potential of being a candidate 'antigen density reference material' because it can be used as a full process control (Peloquin *et al.* 1994; Barnett *et al.* 1996) whilst retaining the linear relationship between antigens (Janossy *et al.* 1998).

To reduce the variation in antigen density determination, a standardized protocol should be used that defines flow cytometer set-up, antigen staining and data analysis. This study has demonstrated that using such a standardized approach, coupled with a stabilized whole blood preparation, interlaboratory antigen density determination is reproducible. As a result, antigen quantification will become a more reliable laboratory investigation enabling the discrimination of normal from abnormal states such as leukaemia and activation antigen changes during viral infections. Furthermore, it has been established that a linear relationship exists between CD3, CD4, CD8 and CD19 and that by generating a 'standard curve', outlying results can be easily identified. Finally, the use of a stable reference preparation with preassigned antigen density values that can be used as a full process control will enable the early identification of technical problems.

Acknowledgements

The authors wish to thank Mr R. Fergie of Sigma–Aldrich Company Ltd, Poole, Dorset, UK for kindly supplying the antibody and bead reagents used in this study.

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