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

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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;
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; Re buck, 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 lysis 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 Immunochromicals, 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. FACSLysing 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% NaN₃ (Sigma Immunochromicals, 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 Immunochromicals, 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-
pripriate 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 924 (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 CD8dim population to have a mean ABC of 46,612 ± 8606 mol/cell, compared to 44,7816 ± 141,084 for the CD8bright 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 immunofluorochromes.

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).

<table>
<thead>
<tr>
<th>Centre</th>
<th>Mean IgG1</th>
<th>Mean IgG2a</th>
<th>Mean CD3</th>
<th>Mean CD4</th>
<th>Mean CD8</th>
<th>Mean CD19</th>
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<tbody>
<tr>
<td>1</td>
<td>1840 (273)</td>
<td>1127 (76)</td>
<td>178053 (39113)</td>
<td>80044 (14268)</td>
<td>447816 (141084)</td>
<td>40567 (12407)</td>
</tr>
<tr>
<td>2</td>
<td>1945 (142)</td>
<td>1221 (86)</td>
<td>148940 (9660)</td>
<td>76366 (2423)</td>
<td>347923 (90224)</td>
<td>23318 (3366)</td>
</tr>
<tr>
<td>3</td>
<td>1754 (113)</td>
<td>836 (60)</td>
<td>194127 (24659)</td>
<td>78500 (4507)</td>
<td>279347 (79480)</td>
<td>3127 (4891)</td>
</tr>
<tr>
<td>4</td>
<td>2170 (283)</td>
<td>1545 (139)</td>
<td>150887 (12706)</td>
<td>76175 (6345)</td>
<td>327803 (51627)</td>
<td>36515 (9599)</td>
</tr>
<tr>
<td>5</td>
<td>1933 (134)</td>
<td>1142 (130)</td>
<td>152854 (17533)</td>
<td>69423 (6802)</td>
<td>337159 (130120)</td>
<td>45478 (3641)</td>
</tr>
<tr>
<td>Overall mean</td>
<td>1928 (246)</td>
<td>1174 (249)</td>
<td>164972 (23906)</td>
<td>76102 (8743)</td>
<td>348010 (117610)</td>
<td>35441 (10832)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Centre</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD19</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>22.0%</td>
<td>17.8%</td>
<td>31.5%</td>
<td>30.6%</td>
</tr>
<tr>
<td>2</td>
<td>6.5%</td>
<td>3.2%</td>
<td>25.9%</td>
<td>14.4%</td>
</tr>
<tr>
<td>3</td>
<td>12.7%</td>
<td>5.7%</td>
<td>28.5%</td>
<td>15.6%</td>
</tr>
<tr>
<td>4</td>
<td>8.4%</td>
<td>8.3%</td>
<td>15.7%</td>
<td>26.3%</td>
</tr>
<tr>
<td>5</td>
<td>11.5%</td>
<td>9.8%</td>
<td>38.6%</td>
<td>8.0%</td>
</tr>
<tr>
<td>Mean CV</td>
<td>12.2%</td>
<td>9.0%</td>
<td>28.0%</td>
<td>19.0%</td>
</tr>
<tr>
<td>Overall interlaboratory CV</td>
<td>17.8%</td>
<td>11.5%</td>
<td>33.8%</td>
<td>30.6%</td>
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</table>
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 CD8dim 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.

**Discussion**

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

![Figure 1](image1.png)  
**Figure 1.** The relationship of antibody binding capacity for CD3, CD4, CD8 and CD19 defined from 50 normal individuals (error bars shown).

![Figure 2](image2.png)  
**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).

![Figure 3](image3.png)  
**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 3.** Statistical summary of antibody binding capacity by sex for isotype controls, CD3, CD4, CD8 and CD19

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

Data presented as mean (SD).
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), 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 setup 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 site 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

<table>
<thead>
<tr>
<th>Centre</th>
<th>Day</th>
<th>IgG1</th>
<th>IgG2a</th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD19</th>
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<tr>
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<td>3699</td>
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<td>122245</td>
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<td>174049</td>
<td>15333</td>
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<tr>
<td></td>
<td>15</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
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<td>ND</td>
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<tr>
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<td>1</td>
<td>3843</td>
<td>2604</td>
<td>96351</td>
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<td>39925</td>
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<td>4206</td>
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<td>105117</td>
<td>47668</td>
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<td>1084</td>
<td>1209</td>
<td>11265</td>
<td>3477</td>
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<td>3371</td>
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<td>52100</td>
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<tr>
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<td>726</td>
<td>18713</td>
<td>11970</td>
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<td>2968</td>
<td>103424</td>
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<td>151954</td>
<td>25379</td>
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<tr>
<td>Overall SD</td>
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<td>979</td>
<td>13324</td>
<td>7615</td>
<td>25579</td>
<td>8398</td>
<td></td>
</tr>
</tbody>
</table>

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

possibility of stearic hindrance from additional fluorochromes. Furthermore, all antibodies were obtained from the same source and batch (Sigma Immunocoummensions).

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<sup>dim</sup> cells in the analysis, while the lowest interlaboratory variance was observed for CD4 (11.4%). Three other studies have reported quantification values 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. Re-analysis 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<sup>dim</sup> 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 fluorescent–protein ratio. However, even in view of these considerations, this 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.

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