

## Absolute CD4<sup>+</sup> T-lymphocyte and CD34<sup>+</sup> stem cell counts by single-platform flow cytometry: the way forward

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**Summary.** To determine the potential advantage of single-platform technology in the enumeration of CD4<sup>+</sup> T lymphocyte and CD34<sup>+</sup> stem cells, data has been analysed from the UK NEQAS for Leucocyte Immunophenotyping schemes. The inter-laboratory CVs for CD4<sup>+</sup> T lymphocyte counts were consistently lower for single-platform (mean 13.7%, range 10–18.3%) compared to dual-platform methodology (mean 23.4%, range 14.5–43.7%). Subgroup analysis of single-platform users demonstrated mean overall inter-laboratory CVs of 17.2%, 13% and 7.1% for the FlowCount, TruCount and volumetric approach respectively. The lowest inter-laboratory CVs obtained for a single sample by each single platform approach were 4% (TruCount), 4.4%

(volumetric), 4.6% (FACSCount) and 12.7% (FlowCount). Similarly, the mean inter-laboratory CV for CD34<sup>+</sup> stem cell enumeration using non-standardized single-platform approaches was 18.6% (range 3.1–36.9%) compared to 28.6% (range 19–44.2%) for the dual-platform technology. Our results suggest absolute cell subset enumeration should be performed by single-platform technology and that such an approach should improve the quality control of multi-centre clinical trial data for CD4<sup>+</sup> T lymphocyte and CD34<sup>+</sup> stem cells.

**Keywords:** CD34, CD4, haemopoietic stem cells, quality control, absolute counting.

The enumeration of absolute CD4<sup>+</sup> T lymphocyte and CD34<sup>+</sup> haemopoietic stem cells is important for the clinical management of HIV-infected individuals and for cancer patients undergoing peripheral blood stem cell transplantation, respectively. The need for an accurate and reproducible method for absolute cell counting may take on even more importance following the National Blood Authority (NBA) strategy to issue blood products with an absolute white cell count (WBC) of  $<5 \times 10^6$  leucocytes per blood product unit (BCSH, 1998).

Currently, peripheral stem cell and lymphocyte subset analysis is routinely undertaken by flow cytometry, employing either a dual or, less frequently, a single platform approach. The dual-platform technique utilizes immunophenotypic data derived from the flow cytometer together with the total white blood cell count (WBC), or total absolute lymphocyte count (TLyC), obtained from a haematology analyser. It is recognized, however, that a major factor contributing to the high inter-laboratory coefficients of variation reported for absolute CD4<sup>+</sup> lymphocyte counts is the WBC generated by different haematology analysers (Robinson *et al*, 1992). In addition, the inter-machine variance

for WBC increases significantly for values  $<0.1 \times 10^9/l$ , precluding this approach for the quality control of leucocyte-depleted blood products. In contrast, single-platform technology derives the absolute cell count directly from the flow cytometer, using either precision fluidics, or microbead technology (Mercolino *et al*, 1995; Verwer & Ward, 1997; Keeney *et al*, 1998). Theoretically, single-platform techniques should overcome the above limitations and therefore be the preferred method of analysis.

In this report we present data from UK NEQAS for Leucocyte Immunophenotyping that demonstrates the advantages of the single-platform approach. We conclude that such technology is the preferred method for the determination of absolute cell counts, especially when the populations of interest are present in low numbers.

### MATERIALS AND METHODS

Fifty millilitre samples of peripheral blood were obtained, after informed consent, from patients undergoing G-CSF stem cell mobilization, but prior to peripheral blood stem cell harvesting, for use within the CD34<sup>+</sup> stem cell enumeration scheme (Barnett *et al*, 1998). In addition, 500 ml peripheral blood donations were obtained for CD4<sup>+</sup> T lymphocyte enumeration (Barnett *et al*, 1996). Both preparations were stabilized using a previously described procedure (Barnett

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*et al*, 1995). Aliquots of stabilized blood were issued every 2 months for a period of 1 year (1997–98) to a maximum of 280 laboratories worldwide (180 in the CD4<sup>+</sup> T-lymphocyte scheme and 100 in the CD34<sup>+</sup> stem cell scheme). Each laboratory was required to report the results for WBC together with absolute CD4<sup>+</sup> T lymphocyte or CD34<sup>+</sup> stem cell counts, as appropriate, using local laboratory protocols. The mean and SD, or median and inter-quartile ranges, together with the respective inter-laboratory CVs were calculated for absolute CD4<sup>+</sup> T lymphocyte and CD34<sup>+</sup> stem cell counts respectively. Data were excluded from CD4<sup>+</sup> T-lymphocyte analysis if the TLYC was used rather than the WBC. Although the haematology analyser derived WBCs can routinely be obtained from the stabilized samples, the use of such material may inadvertently affect the TLYC due to differences in haematology analyser technology. Furthermore, the TLYC is, at present, not routinely subjected to external quality assessment and the inter-laboratory variation is therefore unknown.

Three single-platform technologies were used by participants to determine absolute CD4<sup>+</sup> T-lymphocyte counts: (i) FlowCount (Coulter, Florida, U.S.A.) in which a defined number of latex beads is added to a known volume of peripheral blood, (ii) TruCount (Becton Dickinson, San Jose, U.S.A.), where a known volume of peripheral blood is added to tubes containing a known number of manufacturer pre-dispensed latex beads and (iii) the Cyturon *Absolute* (Ortho Clinical Diagnostics, Raritan, U.S.A.), a volumetric flow cytometer that determines absolute counts using precision fluidics. Absolute CD34<sup>+</sup> stem cell counts were obtained using the same three single-platform approaches, with the exception that FlowCount and TruCount beads were incorporated into the CD34<sup>+</sup> stem cell detection kits, StemKit and ProCount respectively. The StemKit is based upon the ISHAGE gating strategy (Keeney *et al*, 1998), whereas the ProCount kit uses a proprietary nucleic acid dye (NAD), anti-CD45, anti-CD34 and the ProCount software (Verwer & Ward, 1997).

Statistical analysis was undertaken using MiniTab software (MiniTab Inc., State College, Pennsylvania, U.S.A.).

## RESULTS

### *Absolute CD4<sup>+</sup> T-lymphocyte determination*

Of the 180 laboratories, a maximum of 102 returned CD4<sup>+</sup> T-lymphocyte counts for each of the 10 samples issued during the study period, resulting in a total of 884 data sets. Initially, 66% of laboratories used the dual-platform approach although, during the 12-month period, this fell to 56% (Table I). The use of bead-based methods was the preferred technology for the single-platform approach (66%). The inter-laboratory CVs were consistently lower for laboratories using the single-platform approach (mean 13.7%, range 10–18.3%) when compared to dual-platform users (mean 23.4%, range 14.5–43.7%). Furthermore, subgroup analysis of the single-platform users demonstrated overall inter-laboratory CVs of 17.2%, 13% and 7.1% for the FlowCount ( $n=110$ ), TruCount ( $n=100$ ) and volumetric ( $n=110$ ) approaches respectively. However, no significant difference was found between the mean absolute counts for dual platform versus single platform (Table I) or between single-platform methods (Table II). The lowest inter-laboratory CVs that were obtained for each single-platform method were 4% using TruCount (sample 1), 4.4% using volumetric (sample 5) and 12.7% using FlowCount (samples 1 and 6) (Table III). In addition, four participants used the FACSCount (Becton Dickinson, San Jose, U.S.A.) for samples 5, 6 and 8. This technology, that employs pre-pipetted beads, antibodies and recommends 'reverse pipetting' (Strauss *et al*, 1996), gave inter-laboratory CVs of 4.6%, 6.3% and 5.2% for the three samples.

### *Absolute CD34<sup>+</sup> stem cell count determination*

A maximum of 99 laboratories returned CD34<sup>+</sup> stem cell counts for each of the nine samples issued, giving rise to 803 data sets (Table III) of which 16% were analysed using a single-platform approach. Only one issue gave an inter-laboratory CV of <20% for dual-platform users (overall mean 28.6%, range 19–44.2%) compared to 3/9 samples analysed by single-platform flow cytometry (overall mean 18.6%, range 3.1–36.9%) (Table III). Due to the small

**Table I.** Comparative analysis of CD4<sup>+</sup> T lymphocyte determination by all methods, dual-platform and single-platform flow cytometry.

Sample	All methods				Dual platform				Single platform			
	No. in group	Mean ( $\times 10^9/l$ )	SD	CV (%)	No. in group	Mean ( $\times 10^9/l$ )	SD	CV (%)	No. in group	Mean ( $\times 10^9/l$ )	SD	CV (%)
1	77	1.407	0.323	23	50	1.458	0.380	26.1	27	1.314	0.136	10.3
2	77	0.628	0.117	18.6	50	0.645	0.135	21.0	27	0.598	0.061	10.2
3	82	1.335	0.498	37.3	54	1.374	0.601	43.7	28	1.259	0.152	12.1
4	82	0.482	0.131	27.2	54	0.487	0.149	30.7	28	0.470	0.084	17.8
5	95	0.607	0.101	16.7	56	0.612	0.116	18.9	39	0.600	0.076	12.6
6	95	0.704	0.121	17.1	56	0.693	0.114	16.5	39	0.722	0.128	17.7
7	102	1.010	0.173	17.1	59	1.013	0.187	18.4	43	1.007	0.152	15.1
8	101	0.617	0.143	23.1	57	0.595	0.155	26.1	43	0.646	0.118	18.3
9	87	0.603	0.095	15.8	51	0.621	0.112	18.0	36	0.579	0.058	10.0
10	86	0.546	0.082	15	50	0.552	0.088	14.5	36	0.538	0.071	13.3

**Table II.** Comparative analysis of the different single-platform approaches for determination of absolute CD4<sup>+</sup> T lymphocytes.

Sample	FlowCount				TruCount				Volumetric			
	No. in group	Mean ( $\times 10^9/l$ )	SD	CV (%)	No. in group	Mean ( $\times 10^9/l$ )	SD	CV (%)	No. in group	Mean ( $\times 10^9/l$ )	SD	CV (%)
1	8	1.232	0.156	12.7	6	1.316	0.052	4.0	10	1.353	0.078	5.8
2	8	0.573	0.090	15.7	6	0.600	0.053	8.8	10	0.618	0.033	5.3
3	9	1.183	0.190	16.1	8	1.259	0.141	11.2	10	1.330	0.079	5.9
4	9	0.466	0.098	21.0	8	0.441	0.101	22.9	10	0.502	0.031	6.2
5	12	0.569	0.104	18.3	12	0.607	0.071	11.7	11	0.609	0.027	4.4
6	12	0.695	0.088	12.7	12	0.779	0.197	25.3	11	0.699	0.038	5.4
7	15	0.915	0.195	22.9	13	1.034	0.104	10.1	12	1.072	0.070	6.5
8	15	0.589	0.135	22.9	13	0.669	0.106	15.8	12	0.657	0.084	12.9
9	11	0.566	0.077	13.6	11	0.573	0.044	7.7	12	0.591	0.047	8.0
10	11	0.504	0.081	16.1	11	0.560	0.069	12.3	12	0.542	0.057	10.5
Overall	–	–	–	17.2	–	–	–	13.0	–	–	–	7.1

**Table III.** Comparative analysis of CD34<sup>+</sup> stem cell determination by all methods, dual-platform and single-platform flow cytometry.

Sample	All methods				Dual platform				Single platform			
	No. in group	Median (cells/ $\mu$ l)	Inter-quartile range	CV (%)	No. in group	Median (cells/ $\mu$ l)	Inter-quartile range	CV (%)	No. in group	Median (cells/ $\mu$ l)	Inter-quartile range	CV (%)
A	81	43	32–51	44.2	73	43	32–51	44.2	9	46	35–52	36.9
B	87	43	37–50	30.2	72	43	35–49	32.6	8	50	49–51	4.0
C	86	52	45–59	26.9	70	52	44–58	26.9	10	57	50–63	22.8
D	98	64	51–70	29.7	70	64	50–69	29.7	10	67	57–73	23.9
E	81	13	11–16	38.5	69	13	11–15	30.8	12	14	13–16	21.4
F	87	80	71–86	18.8	75	79	71–86	19.0	12	82	80–84	4.9
G	86	16	13–18	31.3	74	16	14–18	25.0	12	16	13–17	25.0
H	98	95	83–101	18.9	85	94	80–101	22.3	13	96	95–98	3.1
I	99	11	10–13	27.3	85	11	10–13	27.3	14	12	10–13	25.0

number of participants in each single-platform group for samples A–D, subgroup statistical analysis was limited to samples E–I. The overall mean inter-laboratory CV for each of the single-platform methods was 9.9% (range 0.6–26%), 10% (range 5.3–13.5%) and 12.4% (range 0.6–25.2%) for the volumetric ( $n=15$ ), ProCount ( $n=24$ ) and StemKit ( $n=24$ ) approaches respectively.

#### WBC determination

To confirm that the stabilization process did not adversely affect the results generated by haematology analysers, the mean inter-laboratory CVs for the WBC on the issued specimens was compared to those obtained by the UK NEQAS for General Haematology. The overall mean CV for WBC for those laboratories performing CD4<sup>+</sup> T lymphocytes and CD34<sup>+</sup> stem cells was 10.5% (range 8–12.9%) and 8% (range 6.1–9.9%) respectively. These values compare favourably with those reported by UK NEQAS for General Haematology (J. Wardle, personal communication).

#### DISCUSSION

The absolute CD4<sup>+</sup> T lymphocyte count is now an accepted parameter for monitoring HIV disease progression (Centers for Disease Control, 1993; De Wolf *et al*, 1997). In addition, the enumeration of absolute CD34<sup>+</sup> stem cell counts in peripheral blood has been recognized as an important parameter for predicting the optimum timing for harvesting peripheral blood stem cells from patients mobilized with recombinant growth factors (Gratama *et al*, 1998; Chapple *et al*, 1998). The requirement for accuracy and precision is paramount, particularly if meaningful data is to be generated from multi-centre HIV drug trials and peripheral blood stem cell transplantation programmes. The total WBC generated by haematology analysers has been recognized as a significant factor in the high CVs for absolute CD4<sup>+</sup> T-lymphocyte enumeration (Robinson *et al*, 1992), a fact that has encouraged the development of single-platform approaches that use either bead or volumetric technology to generate absolute cell counts (Mercolino *et al*, 1995; Strauss *et al*, 1996; Verwer & Ward, 1997). However, such

technology has only recently been employed in the routine laboratory and its impact on inter-laboratory coefficients of variation has not been reported previously.

This study demonstrated that the single-platform approach produced consistently lower inter-laboratory CVs for both CD4<sup>+</sup> T lymphocyte and CD34<sup>+</sup> stem cell enumeration. Subgroup analysis indicated that the volumetric approach produced the lowest overall mean inter-laboratory CV for CD4<sup>+</sup> T-lymphocyte counting, a fact probably related to the use of a single pipetting stage. Similarly, the higher CVs obtained for the FlowCount approach, when compared to the TruCount, probably resulted from the need for two pipetting stages, one for bead and one for sample. Consequently, it has been proposed that 'reverse-pipetting' should be used to reduce such inaccuracies (Gratama *et al*, 1998; Barnett *et al*, 1999). Interestingly, the FACSCount instrument, that employs pre-pipetted beads, antibodies and recommends 'reverse pipetting', gave inter-laboratory CVs comparable to the volumetric approach. Strauss *et al* (1996) also reported that this instrument produced low CVs during a single site evaluation. Nevertheless, it is possible that CVs for the bead-based methods (e.g. TruCount and FlowCount) could be improved further by the standardization of the mixing and vortexing procedures. Furthermore, variation in the number of single beads included in the analysis gate, due to formation of doublet, triplet or quadruplet bead populations, or incorrect analysis region settings, will have an impact on absolute cell subset enumeration. This will be particularly evident when rare cell event analysis is being undertaken, as for CD34<sup>+</sup> stem cell counting, when intra- and inter-laboratory variation will be amplified as a result of low numbers of events being collected.

Although a detailed comparative analysis of the single-platform approaches for CD34<sup>+</sup> stem cell counting was limited, a number of conclusions can be drawn. Firstly, the individual single-platform approaches gave similar CVs, with all values being lower than the dual-platform approach. Secondly, the data for ProCount users supports the findings of McNiece *et al* (1998), who observed minimal variation between operators at a single site and suggested that the ProCount kit may be useful for standardization between laboratories. However, it should be noted that ProCount, although producing the lowest inter-laboratory CVs, still has software limitations with suboptimal samples that require operator intervention (Peel *et al*, 1998).

In conclusion, our data indicates that absolute cell subset enumeration should be performed by single-platform technology. Such an approach will improve the quality control of multi-centre clinical trial data for CD4<sup>+</sup> T lymphocytes and CD34<sup>+</sup> stem cells. Furthermore, such technology is likely to prove the method of choice for the quality control of leucodepleted blood products.

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#### REFERENCES

- Barnett, D., Granger, V., Mayr, P., Fay, S.P. & Reilly, J.T. (1995) Preparation and stabilization of Cells. WO 95/01796 (International Publication Number), pp. 1–31.
- Barnett, D., Granger, V., Mayr, P., Storie, I., Wilson, G.A. & Reilly, J.T. (1996) Evaluation of a novel stable whole blood quality control material for lymphocyte subset analysis: results from the UK NEQAS Immune Monitoring Scheme. *Cytometry*, **26**, 216–222.
- Barnett, D., Granger, V., Storie, I., Peel, J., Pollitt, R., Smart, T. & Reilly, J.T. (1998) Quality assessment of CD34<sup>+</sup> stem cell enumeration: experience of the United Kingdom National External Quality Assessment Scheme (UK NEQAS) using a unique stable whole blood preparation. *British Journal of Haematology*, **102**, 553–565.
- Barnett, D., Janossy, G., Lubenko, A., Matutes, E., Newland, A. & Reilly, J.T., for and on behalf of the General Haematology Task Force of BCSH, (1999) Guideline for the flow cytometric enumeration of CD34<sup>+</sup> haematopoietic stem cells. *Clinical and Laboratory Haematology*, in press.
- British Committee for Standards in Haematology (BCSH), Blood Transfusion Task Force (1998) Guidelines on the clinical use of leucocyte-depleted blood components. *Transfusion Medicine*, **8**, 59–71.
- Centers for Disease Control (1993) Revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *Centers for Disease Control: Morbidity and Mortality Weekly Report*, **41**, 1–35.
- Chapple, P., Prince, H.M., Quinn, M., Bertoncello, I., Juneja, S., Wolf, M., Januszewicz, H., Brettell, M., Gardyn, J., Seymour, C. & Venter, D. (1998) Peripheral blood CD34<sup>+</sup> cell count reliably predicts autograft yield. *Bone Marrow Transplantation*, **22**, 125–130.
- De Wolf, F., Spijkerman, I., Schellekens, P.T., Langendam, M., Kuiken, C., Bakker, M., Roos, M., Coutinho, R., Miedema, F. & Goudsmit, J. (1997) AIDS prognosis based on HIV-1 RNA, CD4<sup>+</sup> T-cell count and function: markers with reciprocal predictive value over time after seroconversion. *AIDS*, **11**, 1799–1806.
- Gratama, J.W., *et al* (1998) Flow cytometric enumeration of CD34<sup>+</sup> hematopoietic stem and progenitor cells. *Cytometry*, **34**, 128–142.
- Keeney, M., Chin-Yee, I., Weir, K., Popma, J., Nayar, R. & Sutherland, D.R. (1998) Single platform flow cytometric absolute CD34<sup>+</sup> cell counts based on the ISHAGE guidelines. *Cytometry*, **34**, 61–70.
- McNiece, I., Kern, B., Zilm, K., Brunaud, C., Dziem, G. & Briddel, R. (1998) Minimization of CD34<sup>+</sup> cell enumeration variability using the ProCount standardized methodology. *Journal of Hematotherapy*, **7**, 499–504.
- Mercolino, T.J., Connelly, M.C., Meyer, E.J., Knight, M.D., Parker, J.W., Stelzer, G.T. & DeChirico, G. (1995) Immunologic differentiation of absolute count with an integrated flow cytometric system: a new concept for absolute T cell subset determinations. *Cytometry*, **22**, 48–59.
- Peel, J., Storie, I., Smart, T., Pollitt, R., Granger, V., Reilly, J.T. & Barnett, D. (1998) Comparison of a lyse–wash and lyse–no-wash method using the ISHAGE strategy and the 'ProCount' reagent and software system. *Journal of Hematotherapy*, **7**, 280.
- Robinson, G., Morgan, L., Evans, M., McDermott, S., Pereira, S., Wansbrough-Jones, M. & Griffin, G. (1992) Effect of type of haematology analyser on CD4 count. *Lancet*, **340** (ii), 485.
- Strauss, K., Hannet, I., Engel, S., Shiba, A., Ward, D., Ullery, S., Jinguji, M.G., Valinsky, J., Barnett, D., Orfao, A. & Kestens, L. (1996) Performance evaluation of FACSCount: a dedicated system for clinical cellular analysis. *Cytometry*, **26**, 52–59.
- Verwer, B.J.H. & Ward, D.M. (1997) An automated classification algorithm for ProCount flow cytometric acquisition and analysis. *Journal of Hematotherapy*, **6**, 169.