

Quality Control of CD4⁺ T-Lymphocyte Enumeration: Results From the Last 9 Years of the United Kingdom National External Quality Assessment Scheme for Immune Monitoring (1993–2001)

Liam Whitby, Viv Granger, Ian Storie, Karen Goodfellow, Alex Sawle,
John T. Reilly, and David Barnett*

UK NEQAS for Leucocyte Immunophenotyping, Department of Haematology, Royal Hallamshire Hospital, Sheffield, United Kingdom

The human immunodeficiency virus (HIV) global epidemic has necessitated the routine enumeration of T-lymphocyte subsets, which has created a need for external quality assurance (EQA). The United Kingdom National External Quality Assessment Scheme (UK NEQAS) for Immune Monitoring provides EQA for 296 laboratories in 40 countries. In 1993, UK NEQAS developed and incorporated into its program stabilized whole blood that enables the accurate monitoring of laboratory performance. Overall, the mean interlaboratory coefficient of variation (CV) for percentage CD4⁺ T-lymphocyte subset enumeration has fallen from 15% to less than 5%, as a direct result of the increased use of CD45/ side scatter (SSC) gating. Laboratories using alternative gating strategies (i.e., CD45/CD14 or forward scatter [FSC]/SSC) were about 7.4 times more likely to fail an EQA exercise. Furthermore, the adoption of single-platform technology resulted in a reduction of the overall mean interlaboratory CV for absolute CD4⁺ T lymphocytes from 56% (prior to the widespread use of single-platform technology) to 9.7%. Individual laboratory deficiencies were also identified using a performance monitoring system and, through re-education by collaboration with the coordinating center, satisfactorily resolved. In conclusion, during the last 9 years, the UK NEQAS for Immune Monitoring program has highlighted the significant technological advances made by laboratories worldwide that undertake lymphocyte subset enumeration. *Cytometry (Clin. Cytometry)* 50:102–110, 2002. © 2002 Wiley-Liss, Inc.

Key terms: flow cytometry; CD4⁺ T lymphocytes; quality control; quality assessment

The accurate determination of absolute and percentage values for CD4⁺ T lymphocytes is a crucial parameter in the monitoring and treatment of individuals infected with the human immunodeficiency virus (HIV). CD4⁺ T-lymphocyte counts are used to assess the degree of immune deterioration and speed of progression toward AIDS (1–4); to improve AIDS surveillance (5); to determine the optimum timing for prophylaxis of opportunistic infections (6); to monitor the efficacy of antiretroviral therapy (7–9); and to monitor the efficacy of therapeutic vaccines (10). It is vitally important, therefore, that laboratory data are both precise and accurate and supported by well-documented quality control (QC) procedures that include participation in an external quality assurance (EQA) program.

EQA programs have generally used either fresh whole blood or frozen cells (11–13). However, the instability of these materials requires that samples be shipped by express courier, making the price of transportation one of the heaviest cost burdens in EQA. Furthermore, the wide

coefficients of variation (CV) observed with the use of fresh whole blood potentially mask the detection of factors important in affecting laboratory performance (12,13). Ideally, therefore, sample preparations that are stable and able to generate low CVs are desirable for use in EQA programs.

The United Kingdom National External Quality Assessment Scheme (NEQAS) for Leucocyte Immunophenotyping evaluates the performance of more than 600 laboratories worldwide. These laboratories undertake flow cytometric diagnosis of hematological malignancies, as well as CD34⁺ stem cell enumeration, low-level leukocyte

*Correspondence to: Dr. D. Barnett, UK NEQAS for Leucocyte Immunophenotyping, First Floor, Rutledge Mews, 3 Southbourne Road, Sheffield S10 2QN, United Kingdom.

E-mail: d.barnett@sheffield.ac.uk

Received 14 January 2002; Accepted 15 February 2002

Published online in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/cyto.10094

counting, and lymphocyte subset analysis. To overcome the problem of sample stability, we developed a unique whole blood stabilization procedure that ensures retention of leukocyte light scatter and immunological staining characteristics for up to 300 days of the leukocytes (14,15). Because the matrix is similar to that of "fresh" blood, it acts as a full process control. As a result, since 1993, UK NEQAS for Leukocyte Immunophenotyping has incorporated stabilized samples in all its EQA programs.

In this report, we summarize and discuss the findings from the last 9 years of the UK NEQAS for Immune Monitoring, one of the largest CD4⁺ T-lymphocyte enumeration schemes reported to date. Currently, this program has 296 registered laboratories from more than 40 countries.

MATERIALS AND METHODS

The immune monitoring program (lymphocyte subset enumeration scheme) involves 296 laboratories from more than 40 countries (December 2001). During the last 9 years, the scheme has collected and analyzed more than 30,000 individual data sets derived from the issue of stabilized whole blood samples with CD4⁺ T-lymphocyte counts that ranged from 140 to 1,390 cells per microliter. For each bimonthly survey, two separate 500-ml voluntary donations (following informed consent) from apparently normal healthy individuals of human peripheral blood were obtained from the National Blood Service. The blood was collected into citrate phosphate dextrose-adenosine (CPD-A) anticoagulant, stabilized without further manipulation of lymphocyte subsets (14,15), and screened for HIV-1, HIV-2, hepatitis B, hepatitis C, and syphilis prior to issue. Two samples with CD3⁺CD4⁺ T-lymphocyte counts of <300 cells per microliter (samples 96 and 106) were obtained by dividing the 500-ml sample into two equal-sized volumes, depleting one half with paramagnetic microbeads specific for CD3⁺CD4⁺ T lymphocytes (Dynal A.S, Oslo, Norway) and then mixing the depleted with the nondepleted unit to obtain the desired CD3⁺CD4⁺ T-lymphocyte cell count. Stabilization then progressed as previously described (14,15) without further manipulation of the lymphocyte subsets.

All participants received a 1.5-ml sample from each stabilized donation and were requested to enumerate the percentage and absolute values for CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ T lymphocytes, using their normal protocol. Recently, the CD19⁺ B-lymphocyte population has been subjected to performance monitoring (data not shown). Each center was also asked to return methodological details, including the type of flow cytometer used, the commercial source of antibodies, whether dual or single-platform analysers were used to generate absolute counts, as well as the particular gating strategy employed. Laboratory results were returned to UK NEQAS within 1 month of the issue date and full data analysis was returned to the participants within a week following trial closure.

The data analysis included graphical and statistical analysis of the methods used, together with presentation of individual performance in comparison to all participants

and groups using similar methodology. The computer analysis algorithm was written using Borland Visual dBase and the data analyzed as described previously (16). Briefly, the overall mean and SDs were calculated and the outliers removed, following which the trimmed data (the data were normally distributed following removal of outliers) were reanalyzed to obtain both a consensus trimmed mean and a SD for each lymphocyte subset parameter. Individual participant results were then compared directly to the trimmed SD value. Prior to implementation, the analytical approach was validated using 10,000 randomly generated computer data sets by Professor G. Raab (Department of Medical Statistics, Napier University, Edinburgh, Scotland; data not shown).

Individual laboratory performance monitoring was assessed for both absolute and percentage values for total CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ T lymphocytes. The overall performance score for each sample was derived from the sum of the scores for total CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ T lymphocytes, with the scores for percentage and absolute values being derived separately. A performance score for each parameter was determined as follows: A reported result that fell within 1 trimmed SD value of the trimmed mean was assigned two points; a result outside 1 trimmed SD, but within 2 trimmed SD values of the trimmed mean was assigned one point; any result outside 2 trimmed SD values was assigned zero points. Furthermore, any laboratory that failed to return a result was assigned zero points. Using these criteria, a maximum number of six points was attainable for each specimen for each of the percentage and absolute results (i.e., two points for each percentage and absolute value obtained for CD3⁺, CD3⁺CD4⁺, and CD3⁺CD8⁺ T lymphocytes).

A cumulative performance score was then obtained by summing the scores for the last six samples, with separate scores being generated for percentage and absolute values. This approach removed the possibility that a single error could result in a laboratory being classified as a persistent poor performer, defined as obtaining less than 15 points over a six-sample window. In the United Kingdom, if a laboratory performance score falls below 15 points, remedial action is undertaken, either by the program organizers, or if this course of action fails, by the National Quality Assurance Advisory Panel for Immunology (NQAAP). Evidence of adequate performance in an accredited EQA program is currently required for laboratory accreditation by Clinical Pathology Accreditation (UK) Ltd.

RESULTS Participation Numbers

The introduction of fixed whole blood in 1993 has enabled a steady increase in scheme participation from non-UK sites. Currently, there are 296 UK and non-UK-based participants (equivalent to an average of 180 laboratories per trial since 1993). During this period, an average of 94% of centers returned results for each issue,

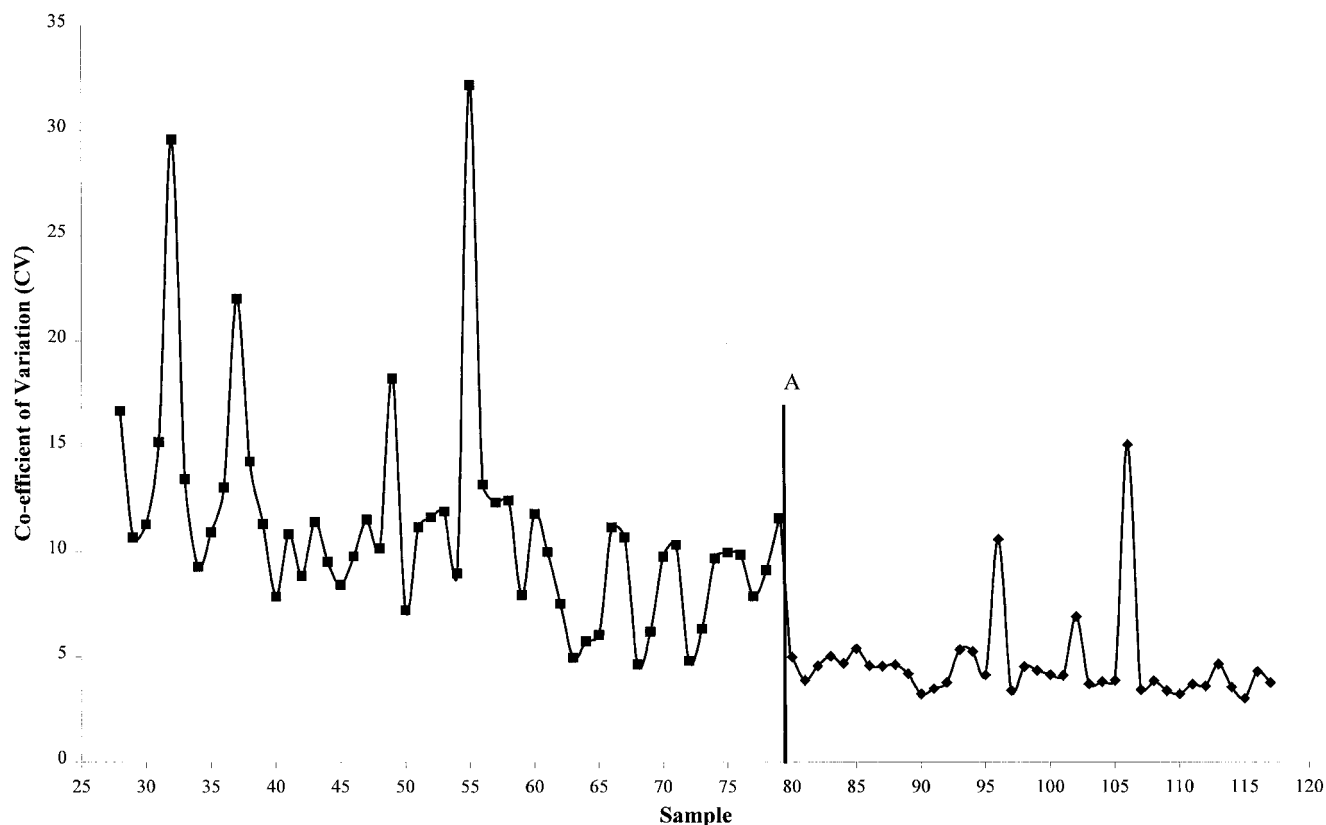


Fig. 1. CV for percentage of CD3⁺CD4⁺ T lymphocytes pre- and post-publication of the BCSH guidelines in 1997 (17). Publication date denoted by vertical line A.

generating 30,000 data sets (all parameters were found to be normally distributed).

Use of Interlaboratory CVs as a Measure of Improving Standards

The CVs, derived from the trimmed mean and SD for percentage and absolute CD3⁺CD4⁺ T lymphocytes, were used to monitor improvements in the cohort performance (any methodological change, or technological improvement, affecting this parameter will also affect the precision and accuracy of the CD3⁺ and CD3⁺CD8⁺ T-lymphocyte populations and thus similar trends will be mirrored; data not shown).

The program has demonstrated a reduction in CVs for both percentage and absolute values over the 9-year period (Figs. 1, 2). Interestingly, these two parameters are influenced by different analytical variables and, therefore, multiple factors are likely to be involved. Figure 1 demonstrates that over a 4.5-year period (up to and including sample number 79) the CVs for CD3⁺CD4⁺ T-lymphocyte percentage values fell from 15% to 8% and that further improvement coincided with the publication of the UK guidelines (denoted by the vertical line A, sample 80) for the determination of CD4⁺ T-lymphocyte subsets (17). The introduction of this guideline was associated with the CV falling to 4.8% (sample 82) although on three subse-

quent occasions, mean interlaboratory CVs of >5% were obtained. All these samples, however, had CD3⁺CD4⁺ T-lymphocyte counts of ≤500 cells per microliter. The interlaboratory CV for all parameters improved further (sample 80 onward) and appeared to be associated with the adoption of the recommended CD45/ side scatter (SSC) gating procedure. Currently, 71% of participants use CD45/SSC gating, 7% CD45/CD14 gating, 17% forward scatter (FSC)/SSC gating, 4% T gating, and 1% unknown gating strategies.

Similar results were obtained for absolute CD3⁺CD4⁺ T-lymphocyte enumeration (Fig. 2), with the mean interlaboratory CV falling from 40.5% to 28% up to and including sample 85. In 1998 (sample 86), UK NEQAS encouraged laboratories to use single-platform analysis for absolute counting, an approach supported by the publication of scheme data (18; denoted by vertical line B). This improvement in interlaboratory CV has been maintained (sample 94 onward) with 15 of 22 (68%) samples having an interlaboratory CV of ≤25% compared with 8 of 67 (12%) prior to sample 94 (three of eight were samples 86, 87, and 90), suggesting an association of the adoption of single-platform technology with proactive education and the publication (18).

Single-platform technology, employed by 42% of all participants, gave a lower interlaboratory CV for

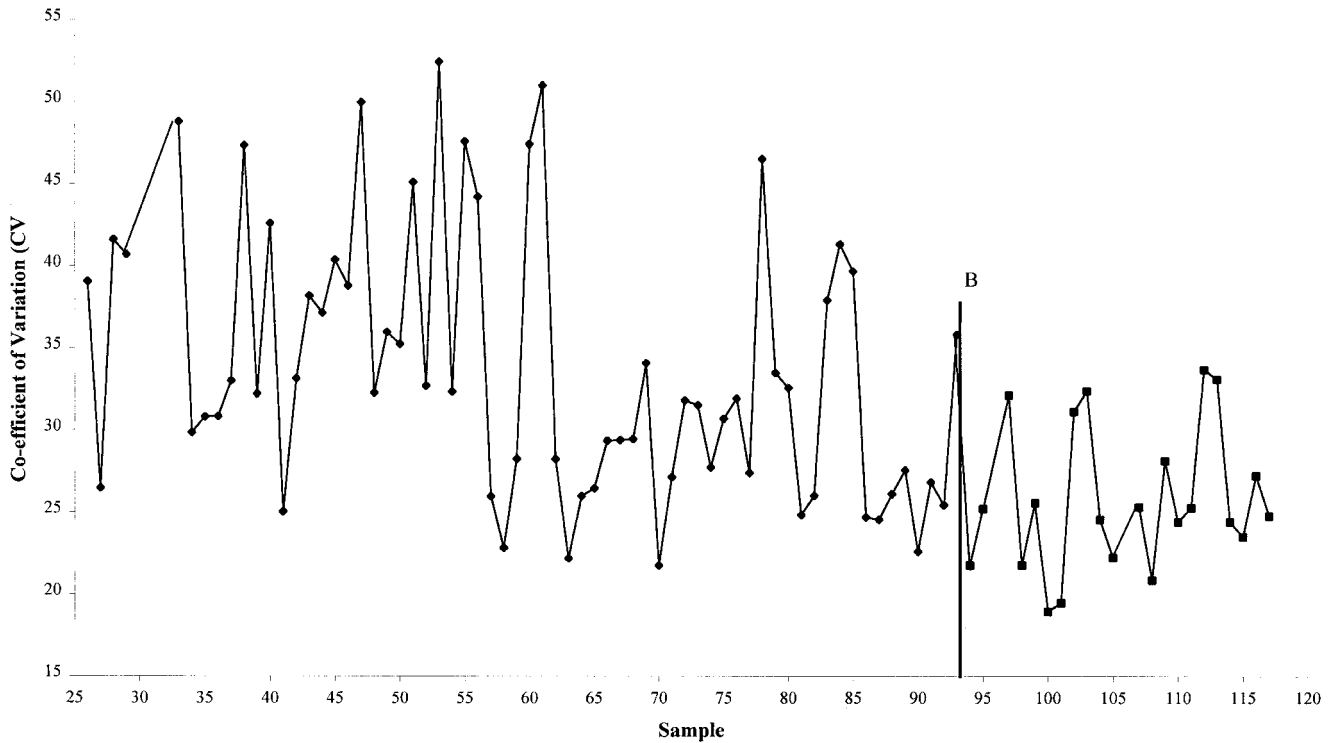


FIG. 2. CV trend lines for absolute CD3⁺CD4⁺ T lymphocytes pre- and post-publication of a study from UK NEQAS highlighting the major differences between single and dual-platform techniques (18). Publication date denoted by vertical line B.

CD3⁺CD4⁺ T lymphocytes than the dual platform in 33 of the last 34 samples (overall mean 14% [range 9.7-33] versus 21% [range 13-56], respectively; $P < 0.001$). However, no significant difference was detected in the mean absolute counts between these two groups. The samples were also analyzed according to absolute CD3⁺CD4⁺ T-lymphocyte counts, i.e., less than ($n = 6$) or greater than ($n = 28$) 500 CD4⁺ T-lymphocyte cells per microliter. Overall, the mean CV for ≤ 500 cells per microliter was 25% (range 19-46) compared with 17% (range 12-22) for samples with >500 cells per microliter ($P < 0.001$). Furthermore, lower and significantly different CVs ($P < 0.001$) were obtained for single-platform users, irrespective of cell count, when compared with the dual-platform group; CD4 count ≤ 500 cells per microliter: 31% (range 22-56; dual platform) versus 19% (range 15-33; single

platform) and CD4 count >500 cells per microliter 19% (range 13-27; dual platform) versus 14% (range 9.7-19; single platform).

Effect of Using Predicate Methodologies on the Scoring System

The UK NEQAS program does not employ a predicate method to determine the target values, the scoring system being derived from the results of all users. Data analysis from the last 10 samples revealed that 7% of laboratories using dual-platform technology scored 0 points. However, if the single-platform group was used as the predicate method, 12% of dual-platform users would have been identified as poor performers (Table 1).

In order to measure the performance effect of different gating strategies, we compared the trimmed means and

Table 1
Comparison of All Methods Versus the Single-Platform Technique as the Predicate Scoring Method on Performance for Absolute CD3⁺CD4⁺ T-Lymphocyte Enumeration

Scoring method	Participant method	Percentage in group scoring 0 points	Percentage in group scoring 1 point	Percentage in group scoring 2 points
All users	All	5	19	76
All users	Dual	7	20	73
All users	Single	2	18	80
Single platform	All	9	23	68
Single platform	Dual	12	25	63
Single platform	Single	5	21	74

Table 2
Comparison of All Methods Versus CD45/SSC as the Predicate Method on Performance for Percentage CD3⁺CD4⁺ T-Lymphocyte Determination

Scoring method	Participant method	Percentage in group scoring 0 points	Percentage in group scoring 1 point	Percentage in group scoring 2 points
All users	All	2.5	7.3	90.1
All users	CD45/CD14	3.1	7.8	89.1
All users	FSC/SSC	1.5	7.3	91.2
All users	CD45/SSC	0	2.7	97.3
CD45/SSC	All	25.1	30.0	44.9
CD45/SSC	CD45/CD14	15.6	37.5	46.9
CD45/SSC	FSC/SSC	30.4	31.9	37.7
CD45/SSC	CD45/SSC	4.1	26.7	69.2

SDs derived from all users with those obtained from users of a predicate method (Table 2). The first approach failed to identify a laboratory using CD45/SSC gating as a poor performer, compared with 4.6% of laboratories that used CD45/CD14 or FSC/SSC gating. If, however, trimmed mean and SDs were derived from laboratories using CD45/SSC only (herein defined as the predicate gating method), there was a significant increase in laboratories using either CD45/CD14 or FSC/SSC gating strategies identified to be poor performers (15.6% and 30.4%, respectively), i.e., consistently having results outside ± 2 SD values (equivalent to scoring 0 points). Therefore, a laboratory employing FSC/SSC or CD45/CD14 gating was 7.4 and 3.8 times, respectively, more likely to fail the EQA program compared with those using the predicate method (CD45/SSC).

Comparison of UK and Non-UK Centers

In 1999, 25 overseas laboratories joined the program simultaneously. There was no significant difference between the overall mean absolute values and interlaboratory CVs between this group and an identically sized UK group that had been in the program since 1993, indicating the absence of a learning curve. In addition, no significant difference was noted for percentage and absolute CD3⁺CD4⁺ T lymphocytes between the UK (n = 88) and overseas laboratories (n = 208).

Identification of Persistent Poor Performers

The educational nature of the UK NEQAS for Immune Monitoring program is an essential component of the scheme. To facilitate this, we developed a scoring system that allowed the monitoring of individual laboratory performance over a rolling 24-sample window (equivalent to a 2-year rolling period). This has enabled the coordinating center and individual laboratories to identify correctable causes of persistent poor performance. These include changes in a laboratory's working practice, inadequate machine maintenance, and the use of a nonrecommended technology. For example, in laboratory A (Fig. 3), the introduction of a shift system involved staff less experienced in the flow cytometric enumeration of lymphocyte subsets. Retraining of relevant staff resolved the problem. Another laboratory (laboratory B; Fig. 4) was identified

that failed to undertake routine instrument quality performance checks between regular maintenance. Once the laboratory was advised and adopted instrument performance verifiers, its performance improved. Finally, a laboratory was identified (laboratory C; Fig. 5) as a persistent poor performer as a result of using an instrument capable of only two-color analysis coupled with an FSC/SSC gating strategy. Performance improvement resulted from the purchase of an instrument with multiparametric capability (three-color) that enabled the laboratory to adopt a CD45/SSC gating strategy (Fig. 5).

DISCUSSION

The HIV global epidemic has resulted in routine enumeration of T-lymphocyte subsets which, in turn has created a need for EQA. In 1989, a T-lymphocyte subset program was initiated in the United Kingdom for an exclusive cohort of 23 UK laboratories participating in the MRC/Concorde trial (9). In 1991, the program was officially recognized as a UK NEQAS and has subsequently expanded to involve 296 laboratories in 40 countries. This has been made financially and logistically possible by the use of stabilized whole blood (14,15,18).

Throughout the last 9 years, the mean interlaboratory CVs for both percentage and absolute T-lymphocyte subsets have declined. This has been due, in part, to the adoption of new technological methods (18–21), coupled with the establishment of good clinical laboratory practice. However, the variation in interlaboratory CVs (Fig. 2) is a result of the variety of techniques being employed to calculate absolute CD4⁺ T-lymphocyte counts, particularly up to sample 60. It has been noted that three approaches are essentially employed: (1) total white count and flow cytometric differential, (2) use of lymphocyte count derived from the hematology analyzer, and (3) single-platform technology. The high CVs observed prior to the influence of single-platform technology (up to 56%) are partially due to the adoption and use of the second approach and to the fact that although our stabilized whole blood is capable of providing a total white cell count with low interlaboratory CVs (18), it is not always compatible with the differential component on some hematology instruments.

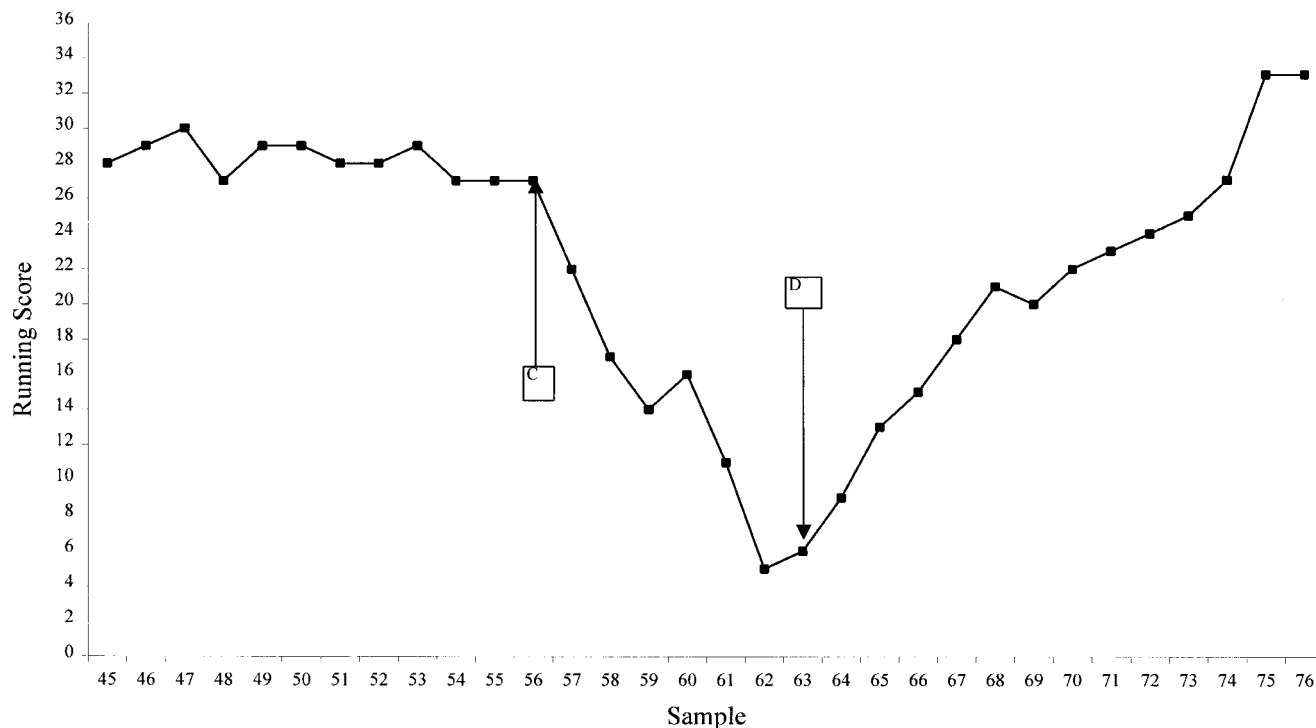


FIG. 3. Effect of changes in work pattern (shift system introduced denoted by arrow C) and introduction of new gating strategy, CD45/SSC (denoted by arrow D) on an individual laboratory's performance (laboratory A).

In the early 1990s, the value of incorporating leukocyte immunological characteristics into gating strategies to identify lymphocyte populations was recognized (22). As a result, many laboratories initially utilized a two-color tube combination of CD14/CD45 as a gating aid (2,22), a fact that led to the development of automated software programs to assist the operator in positioning the lymphocyte gate and to enable the calculation of the lymphosum (a quality control measure that sums the total T, B, and natural killer [NK] cell populations as a measure of lymphocyte purity; 2). This approach, however, does not allow lymphocyte gate positioning to be verified on a tube-to-tube basis. Fortunately, the availability of new fluorochromes has allowed the development of three and four-color assays based on the use of CD45/SSC gating strategy or on T-lymphocyte gating (19,23). This advance enables a common, single, immunological marker to be placed in each tube to act as a gate verifier and thus enable the operator to check tube-to-tube consistency. Our data have shown that although the majority of laboratories have now adopted the use of a CD45/SSC gating strategy, a significant number still use FSC/SSC, CD14/CD45 gating strategies. The interlaboratory CVs for percentage value enumeration were significantly lower for those laboratories using CD45/SSC compared with alternative strategies. Thus, if CD45/SSC gating was adopted as the predicate gating strategy with the trimmed mean and SD values calculated from this cohort, centres using FSC/SSC and CD14/CD45 gating strategies would be 7.4 and 3.8 times,

respectively, more likely to fail an EQA exercise, defined as falling outside 2 SD values.

The impact of two publications, the British Committee for Standardisation of Haematology (BCSH) guidelines for the enumeration of CD4⁺ T lymphocytes (17) and a study from UK NEQAS highlighting the major differences between single and dual-platform techniques (18), was evaluated. It is interesting to observe that, within 12 months of being sent a copy of the BCSH guidelines (17), the interlaboratory CV for percentage CD4⁺ T lymphocytes for all participants fell below the CV for the nonrecommended techniques of CD14/CD45 and FSC/SSC gating (data not shown). Furthermore, in the 12-month period following issue of the absolute counting study (18), there was a 50% increase in the use of single-platform methods. Although the fall in CV for percentage CD4⁺ T lymphocytes and the rise in single-platform use cannot be proved to be a direct result of these publications, it is interesting to observe that these changes were only apparent post publication and that they took place over a relatively short time (12 months equivalent to 12 samples) after document distribution.

Data from a cohort of non-UK laboratories that simultaneously joined the UK NEQAS program were analyzed for evidence of a learning process, as previously reported by a Canadian group (24). In contrast to the Canadian findings, we did not observe a significant difference between the two cohorts for either interlaboratory CVs or mean absolute CD3⁺CD4⁺ T-lymphocyte counts even when the

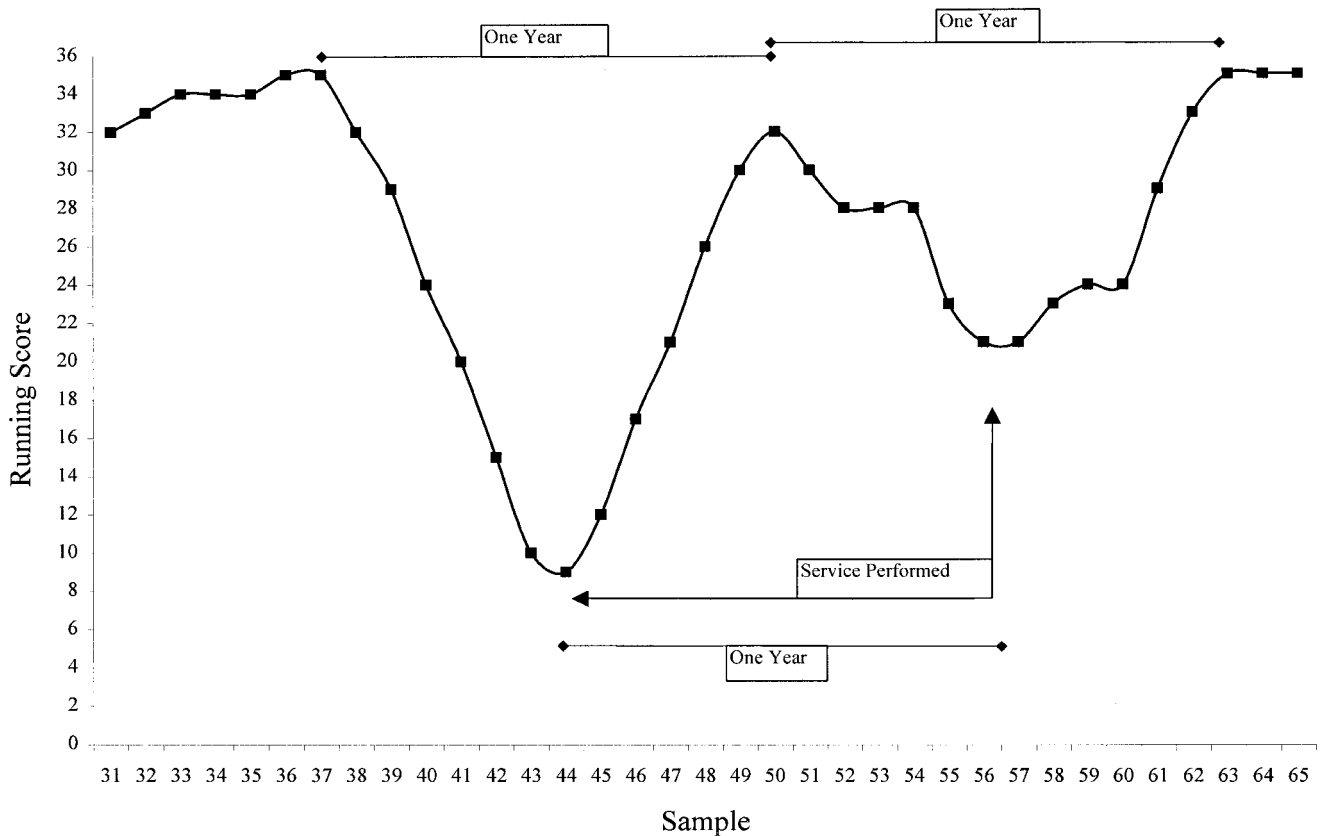


Fig. 4. Effect of instrument performance and instrument servicing on an individual laboratory's performance (laboratory B).

data were analyzed in the same manner as the Canadian study (data not shown). In the study reported by Bergeron et al. (24), active education was undertaken to promote synchronization of protocols. Interestingly, within the UK NEQAS program, we have identified several laboratories (see the two examples shown in Figs. 3 and 5), where proactive education and the adoption of a new improved protocol do result in a significant improvement in performance. Furthermore, it has been demonstrated that targeted training, standard protocols, and the use of stabilized whole blood material produce significant improvements in both interlaboratory and intralaboratory CVs (25,26). The UK NEQAS program achieves education via distribution of relevant publications (2,17,18,) and direct interaction with laboratories through national meetings and one-to-one teaching.

The incorporation of a scoring system enabled the identification of poor-performing laboratories. In addition, we were able to highlight a number of factors that contributed to poor performance, including changes in working practice, inadequate machine maintenance, and use of less accurate/less precise techniques. Reeducation and assistance by UK NEQAS staff helped resolve the problems with a return to satisfactory performance. The use of such a performance monitoring procedure, coupled with educational support, helped to raise the awareness of individ-

ual staff members and improve the quality of laboratory results.

In conclusion, over the last 9 years, the UK NEQAS for Immune Monitoring program has made significant advances in the quality control and monitoring of national and international laboratories undertaking the measurement of lymphocyte subsets. The timely production and distribution of scientific and educational material by the program, coupled with a robust performance monitoring system, has ensured that participants can use EQA (complementary to good internal QC procedures) as a tool to rapidly respond to technological advances. As a result, the quality of results generated by participating centers has improved significantly. Future studies in collaboration with organizations, such as the Communicable Disease Surveillance Centre (CDSC, Colindale, UK), are required to assess how the improvement in laboratory results affects the long-term monitoring of patients (27).

ACKNOWLEDGMENTS

We thank Mrs. Angela Ford, Mrs. June Pidd, and Mrs. Joanne Antcliffe for their clerical and laboratory support of the UK NEQAS programs and Mr. David J. Ainscough for the computer programming support. We also thank the members of our Steering Committee for their continued support: Dr. K. Hyde, The Royal Infirmary, Manches-

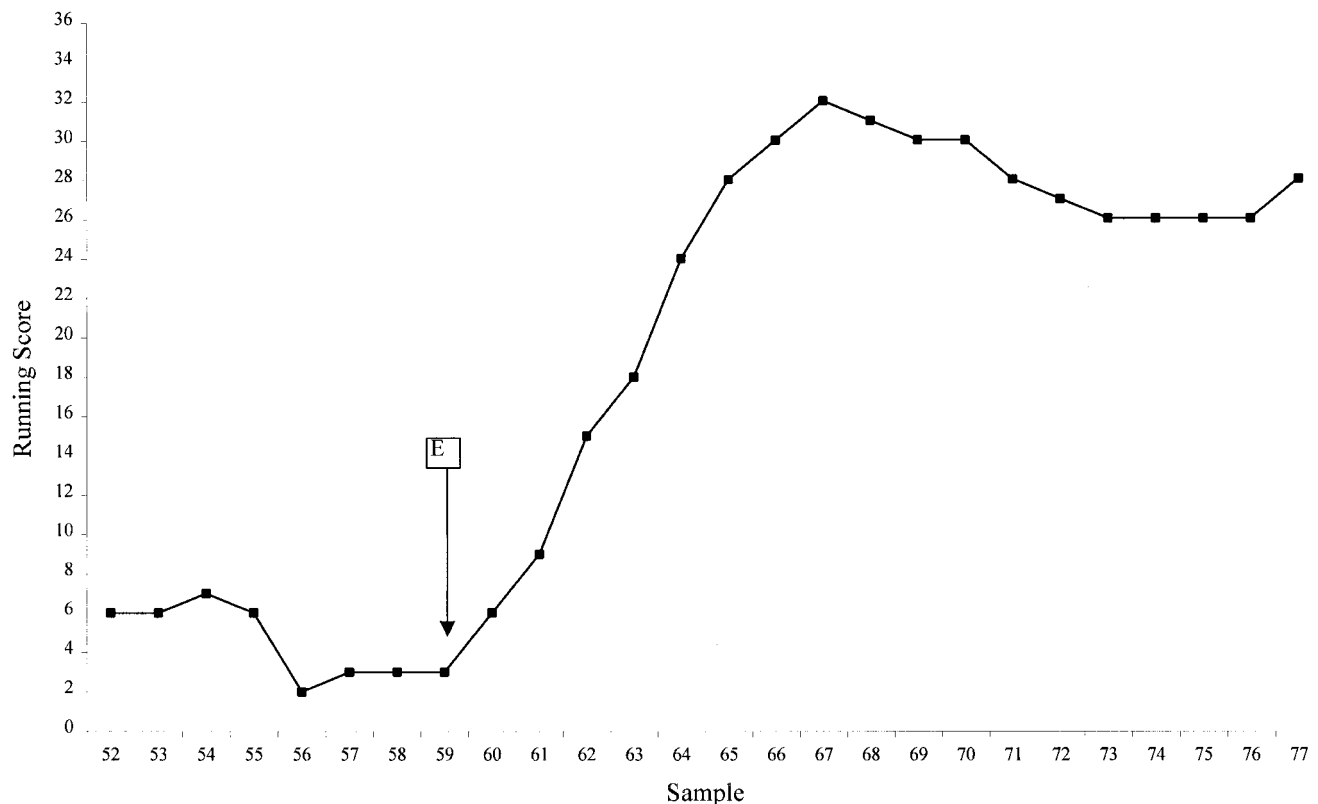


Fig. 5. Effect of changing from a flow cytometer with two-color capability to an instrument with multiparameter (three-color) capability to enable CD45/SSC gating (change denoted by arrow E) on an individual laboratory's performance (laboratory C).

ter (Chairman); Mr. N.R. Porter, Royal Hallamshire Hospital, Sheffield (NQAAP haematology observer); Dr. D.R.E. Jones, University Hospital, Nottingham (NQAAP immunology observer); Dr. M. Bofill, Royal Free Hospital, London; Dr. S. Richards, Leeds General Infirmary, Leeds; Dr. M. Macey, The Royal London Hospital, London; Dr. S. MacLennan, Leeds Blood Centre, Leeds; Mr. N. Beckman, National Blood Service, Birmingham; Dr. D. Swirsky, Leeds General Infirmary, Leeds; and Dr. E. Matutes, Royal Marsden Hospital, London. We thank Dr. J.W. Gratama for critical review of this manuscript, as well as all the participants in the UK NEQAS programs without whom none of these data could have been collected. A. Sawle is supported by grant QLRI-2000-00436 awarded by Directorate General XII of the European Commission (Brussels, Belgium) in the setting of the Fifth Framework Programme "Quality of Life and Management of Living Resources."

LITERATURE CITED

- Phillips A, Lee C, Elford J, Janossy G, Bofill M, Timms A, Kernoff P. Prediction of progression to AIDS by analysis of CD4 lymphocyte counts in a haemophilic cohort. *AIDS* 1989;3:737-741.
- Centers for Disease Control. 1993 Revised classification system for HIV infection and expanded surveillance case definition for AIDS among adolescents and adults. *MMWR* 1992;41:1-19.
- Phillips A, Sabin C, Elford J, Bofill M, Janossy G, Lee C. Use of CD4 lymphocyte count to predict long-term survival free of AIDS after HIV infection. *BMJ* 1994;309:309-313.
- Boutitie F, Pocock S. Predictive value of repeated measurements of CD4 lymphocyte counts on progression to AIDS. *AIDS* 1994;8:35-41.
- McAnulty JM, Modesitt SK, Yusem SH, Hyer CJ, Fleming DW. Improved AIDS surveillance through laboratory-initiated CD4 cell count reporting. *J Acquir Immune Defic Syndr Hum Retrovirol* 1997;16:362-366.
- Masur H, Ognibene F, Yarchoan R, Shelhamer J, Baird B, Travis W, Suffredini A, Deyton L, Kovacs J, Falloon J, Davey R, Polis M, Metcalf J, Baseler M, Wesley R, Gill V, Fauci A, Lane H. CD4 counts as predictors of opportunistic pneumonias in human immunodeficiency virus (HIV) infection. *Ann Intern Med* 1989;111:223-231.
- Selwyn P, Alcabes P, Hartel D, Buono D, Schoenbaum E, Klein R, Davenny K, Friedland G. Clinical manifestations and predictors of disease progression in drug users with human immunodeficiency virus infection. *N Engl J Med* 1992;327:1697-1703.
- Kravcik S, Magill A, Sanghvi B, Ogden R, Cameron W, Lewis R, Yu G, Badley A. Comparative CD4 T-cell responses of reverse transcriptase inhibitor therapy with or without nelfinavir matched for viral exposure. *HIV Clin Trials* 2001;2:160-170.
- Seligmann M, Warrell DA, for and on behalf of the Concorde Coordinating Committee. Concorde: MRC/ANRS randomised double-blind controlled trial of immediate and deferred zidovudine in symptom-free HIV infection. *Lancet* 1994;343:871-881.
- Kundu-Raychaudhuri S, Sevin A, Kilgo P, Nokta M, Pollard RB, Merigan TC. Effect of therapeutic immunization with HIV type 1 recombinant glycoprotein 160 ImmunoAG vaccine in HIV-infected individuals with CD4+ T cell counts of ≥ 500 and 200-400/mm³ (*AIDS Clinical Trials Group Study 246/946*). *AIDS Res Hum Retroviruses* 2001;17:1371-1378.
- Homburger HA, Rosenstock W, Paxton H, Paton ML, Landay AL. Assessment of interlaboratory variability of immunophenotyping. Results of the College of American Pathologists Flow Cytometry Survey. *Ann N Y Acad Sci* 1993;20:43-49.
- Paxton H, Kidd P, Landay A, Giorgi J, Flomenberg N, Walker E, Valentine F, Fahey J, Gelman R. Results of the flow cytometry ACTG

- quality control program: analysis and findings. *Clin Immunol Immunopathol* 1989;52:68-84.
13. Gratama JW, Kraan J, Van den Beemd R, Hooibrink B, Van Bockstaele DR, Hooijkaas H. Analysis of variation in results of flow cytometric lymphocyte immunophenotyping in a multicenter study. *Cytometry* 1997;30:166-177.
 14. Barnett D, Granger V, Mayr P, Storie I, Wilson GA, Reilly JT. Evaluation of a novel stable whole blood quality control material for lymphocyte subset analysis: results from the UK NEQAS immune monitoring scheme. *Cytometry* 1996;26:216-222.
 15. Barnett D, Granger V, Mayr P, Reilly JT. Preparation and stabilisation of leucocytes. UK patent number 2279653, pp 1-40, 1998.
 16. Healy MJR. Outliers in clinical chemistry quality-control schemes. *Clin Chem* 1979;25:675-677.
 17. Barnett D, Bird G, Newland AC, Linch DC, Matutes EM, Hodges E, Reilly JT. Guidelines for the determination of CD4⁺ T lymphocytes in immunosuppressed individuals. *Clin Lab Haematol* 1997;19:231-241.
 18. Barnett D, Granger V, Whitby L, Storie I, Reilly JT. Absolute CD4⁺ T-lymphocyte and CD34⁺ stem cell counts by single platform flow cytometry: the way forward. *Br J Haematol* 1999;106:1059-1062.
 19. Nicholson J, Hubbard M, Jones B. Use of CD45 fluorescence and side-scatter characteristics for gating lymphocytes when using the whole blood lysis procedure and flow cytometry. *Cytometry* 1996; 26:16-21.
 20. Reimann KA, O'Gorman MR, Spritzler J, Wilkening CL, Sabath DE, Helm K, Campbell DE. Multisite comparison of CD4 and CD8 T-lymphocyte counting by single- versus multiple-platform methodologies: evaluation of Beckman Coulter flow-count fluorospheres and the tetraONE system. *Clin Diagn Lab Immunol* 2000;7:344-351.
 21. Schnizlein-Bick CT, Spritzler J, Wilkening CL, Nicholson JK, O'Gorman MR. Evaluation of TruCount absolute-count tubes for determining CD4 and CD8 cell numbers in human immunodeficiency virus-positive adults. *Clin Diagn Lab Immunol* 2000;7:336-343.
 22. Loken MR, Brosnan JM, Bach BA, Ault KA. Establishing optimal lymphocyte gates for immunophenotyping by flow cytometry. *Cytometry* 1990;11:453-459.
 23. Mandy FF, Bergeron M, Recktenwald D, Izaguirre CA. A simultaneous three-color T cell subset analysis with single laser flow cytometers using T cell gating protocol. Comparison with conventional two-color immunophenotyping method. *J Immunol Methods* 1992;156: 151-162.
 24. Bergeron M, Faucher S, Minkus T, Lacroix F, Ding T, Phaneuf S, Somorjai R, Summers R, Mandy F, and the participating flow cytometry laboratories of the Canadian Clinical Trials Network for HIV/AIDS Therapies. Impact of unified procedures as implemented in the Canadian quality assurance program for T lymphocyte subset enumeration. *Cytometry* 1998;33:146-155.
 25. Barnett D, Granger V, Kraan J, Whitby L, Reilly JT, Papa S, Gratama JW. Reduction of intra- and interlaboratory variation in CD34⁺ stem cell enumeration by the use of stable test material, standard protocols and targeted training. *Br J Haematol* 2000;108:784-792.
 26. Gratama JW, Kraan J, Keeney M, Granger V, Barnett D. Reduction of variation in T-cell subset enumeration between 55 laboratories using single-platform, three or four-color flow cytometry based on CD45 and SSC based gating of lymphocytes. *Cytometry* 2002;50:92-101.
 27. Gupta SB, Gilbert RL, Brady AR, Livingstone SJ, Evans BG. CD4 cell counts in adults with newly diagnosed HIV infection: results of surveillance in England and Wales, 1990-1998. CD4 Surveillance Scheme Advisory Group. *AIDS* 2000;14:853-861.