Current Laboratory Practices in Flow Cytometry for the Enumeration of $CD4^+$ T-Lymphocyte Subsets

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Abstract

Background

$CD4^+$ T-lymphocyte subset enumeration is routinely used for monitoring HIV disease progression, with approximately 300,000 tests performed annually in the UK alone. Technical variables can impact upon any laboratory test and therefore the final result obtained. Here, we report the findings of a survey questionnaire issued to 1587 clinical flow cytometry laboratories to: a) determine if the UK NEQAS for Leucocyte Immunophenotyping (UK NEQAS LI) Lymphocyte Subset External Quality Assessment (EQA) programme was suitable for current laboratory needs and practices; and b) assess the impact of these responses on clinical practice where $CD4^+$ T-lymphocyte subsets analysis is undertaken. The survey covered areas not traditionally examined by EQA such as: staffing numbers, flow cytometer age and service intervals, plus six test specific sections covering: leukaemia immunophenotyping, $CD4^+$ T-lymphocyte subsets analysis (reported here), $CD34^+$ stem cell testing, low level leucocyte enumeration, minimal residual disease testing and PNH testing.

Results

The responses revealed major methodological variations between centres undertaking $CD4^+$ T-lymphocyte subset analysis. Significant differences existed in basic laboratory practices such as: normal range derivation; pipetting techniques; instrument maintenance and units of reporting, all of which results in non-adherence to international guidelines.

Discussion

Despite the availability of international guidelines our survey highlighted a lack of concordance amongst laboratory techniques. Such variation could adversely impact on patient care and clinical trial data. Therefore, it is recommended centres undertaking flow cytometric $CD4^+$ T-lymphocyte

subsets analysis urgently review their methodologies and normal ranges to ensure they are fit for purpose and meet current international guidelines.

Introduction

The use of the CD4+ T-lymphocyte count in HIV positive individuals is now a well established routine method for the monitoring of disease progression and initiate therapeutic intervention at clinically relevant levels (1–6). We have previously shown however, that variations in technique for $CD4^+$ T-lymphocyte enumeration, such as dual platform flow cytometry, can have a direct impact on the precision of the results obtained that in turn affects the confidence limits on any given result and thus potentially affect patient care (7,8).

UK NEQAS for Leucocyte Immunophenotyping (UK NEQAS LI)(www.ukneqasli.co.uk) is an accredited international External Quality Assessment (EQA) programme with over 3000 laboratories participating worldwide in 19 Haemato-oncology molecular and flow cytometry programmes. The primary role of UK NEQAS LI is educational, and as such, findings from our EQA programmes have been previously reported in order to drive best practice (9–11). However, to determine which clinical flow cytometric practices were in use within the UK NEQAS LI international cohort we issued a survey to all participating centres (n=1587). The survey attempted to elucidate how participating laboratories used staff resources, their flow cytometer and which areas of clinical flow cytometry were being practiced. Although the survey covered all of the UK NEQAS LI EQA programmes, it was designed in such a way as to survey only those areas relevant to the respondents' current clinical practices. It was also designed so UK NEQAS LI could identify future trends in clinical flow cytometry that would facilitate the planning of EQA development. We report here on the findings from those centres that responded to the technical section and the specific clinical section related to $CD4^+$ T-lymphocyte subset analysis.

Method

In May 2013, UK NEQAS LI issued an online survey to all laboratories currently registered in its flow cytometry EQA programmes (n=1587). The survey consisted of 141 different questions over 2 sections. Section A focussed upon general areas of flow cytometric practices, such as staffing, equipment and EQA requirements. All responding centres were required to complete section A. Section B, was test specific with laboratories requested to answer only questions regarding the flow cytometric tests they routinely offered. This section covered $CD4^+$ T-lymphocyte subset testing, leukaemia analysis, Paroxysmal Nocturnal Haemoglobinuria (PNH) testing, CD34+ stem cell enumeration, leukaemia minimal residual disease testing and low-level leucocyte enumeration. The survey was produced using web survey software provided by SurveyMonkey (www.surveymonkey.com). This allowed the use of conditional branching, such that if a laboratory did not offer a certain test then the survey would automatically move to the next section. Furthermore, it also enabled the inclusion of several questions following split progression depending on the initial answer(s) provided.

Each participating centre was notified by email when the survey was opened and it remained open for a period of 2 months. When the survey period closed, response data was downloaded into Microsoft Access and Microsoft Excel for analysis. The results presented here cover the Section A (General flow cytometric practice) and flow cytometric $CD4^+$ T-lymphocyte subset analysis covered in Section B.

Results

Section A-Flow Cytometric General Practices

The survey was issued to all participants in UK NEQAS flow cytometry programmes, n=1587 (May 2013). There were 496 respondents (31%) with 335 (21%) of these fully completing the survey with regards to all questions that applied to them and 161 (10%) providing partial answers.

There were 353 of the 496 responses that answered the question 'How old is your flow cytometer?'. Analysis showed that 265/353 (75%) used a flow cytometer less than 6 years old. However, 29/353 (8%) of respondents were using flow cytometers that were at least 10 years old with 15 of these having no planned replacement programme. Of the laboratories using flow cytometers that were >10 years old there was no specific clustering related to economic profile or geographic region. Additionally, the survey asked laboratories how many staff in their centre were responsible for performing flow cytometry. A total of 354 responded with 143/354 (40%) having 3 or 4 staff routinely undertaking clinical flow cytometry, this being the most common response. However 71/354 (20%) of centres responded that they only had 1 or 2 staff responsible for performing flow cytometry.

The survey then progressed by asking specific questions regarding servicing and calibration schedules [Table 1]. The majority of centres 342/349 (98%) followed their instrument manufacturers recommended service intervals of 6 to 12 months (depending on flow cytometer model). However, our survey revealed that 7/349 (2%) failed to have their flow cytometers serviced at least once per year. The survey also asked questions regarding calibration practices, of which there were 336 respondents. Of these, 278 (83%) undertook calibration at least every 6 months, with 172 of these

calibrating every 3 months or less. There were 3/336 respondents that failed to calibrate their machines at least annually with 1 of these that neither calibrated or serviced their flow cytometer in any 12-month period.

As technology has evolved this has facilitated the development of flow cytometers with 8+ fluorochrome detection capability. However, older flow cytometers have restricted fluorochrome detection capability (≤ 4 colours). The use of such technology can restrict the implementation of newer sophisticated multi-colour techniques. This survey identified there were 90/345 centres using instruments with ≤ 4-colour capability and 26 centres using instruments that had only a maximum of 3-colour capability. The data was examined by cross checking the number of fluorescent channels available for use on each centres flow cytometer with the actual number each centre actually used in clinical practice. This highlighted that 179/345 (52%) use the maximum number of fluorescent channels available on their instruments [Table 2]. However, of those laboratories that do not use the maximum fluorochrome capability of their flow cytometer (166/345 (48%)) there were 26/166 (7.5%) that used only single- (5/26) or dual-colour flow cytometry (21/26) despite having instruments with a flow cytometer that ≥3-colour capability. The most common technique in use is 4 colours used by 24% (83/345).

The survey then covered the use of pipettes. It is well documented that pipetting is a key part of any flow cytometric assay, especially when single platform enumeration methods are used (12). There were 351 responses to this section [Table 3]. The majority, 289/351 (82%), use manual pipettes with 155 of these using reverse pipetting. Only 62/351 employ an electronic pipette with 42 of these using reverse pipetting. Overall, reverse pipetting is used by 197/351 (56%). Furthermore, we identified that 33/351 (9%) failed to have their pipettes serviced/calibrated at least once within any

12 month period with 11/351 (3%) stating they never having the pipettes serviced/calibrated. Only 38/351 (11%) employed best practice of using electronically adjustable pipettes employing reverse pipetting technique and undertook pipette service/calibration at least once in a given 12-month service interval. Worryingly, there were 2 laboratories that never serviced/calibrated their flow cytometer and their pipettes.

Section B- CD4+ T-lymphocyte Subset Practices

The second part of the survey focussed on clinically specific flow cytometry practices. This section details the findings from 224 laboratories that responded stating they undertake CD4+ T-lymphocyte subset analysis. However, not every lab in this group responded to every question.

The section covered workload, reporting units, normal ranges in use and adherence to best practice (following guidelines). There were 212/224 respondents that provided details of their workload with 53/212 (25%) reporting that they undertook between 1 and 25 $CD4^+$ T-lymphocyte subsets per month. Conversely, 17/212 (8%) reported that they had a workload of >1000 samples per month. We also asked specific questions regarding whether their workload was increasing, decreasing or remaining static. There were 208 responses to the question 'Has this number increased over the last 12 months?', 40% (n=83) stated that workload was increasing, whilst 33% (n=69) and 27% (n=56) stated that their workload in this area was remaining static or decreasing respectively. Whilst there were 208 responses to this question there were not sufficient responses from individual countries to allow analysis of changes in workload based on the geographic location of the laboratories.

We also asked specific questions with regard to what units are used when reporting $CD4^+$ T lymphocyte/lymphocyte subset values (n=214 respondents) and derivation/implementation of normal ranges (n=221 respondents). The findings showed that 66/214 (31%) of participants still

report $CD4^+$ T-lymphocyte subsets results in units of $x10^9$/L (n=62; 29%) or x 10^6/L n=4; 2%). The remainder (n=148; 69%) report results in the internationally accepted units of cells/µL. A total of 221 laboratories responded to questions on normal range derivation and application and we found that only 89/221 (40%) used an in-house derived normal range. Of the remainder, 132/221 (60%) had never determined their own normal range but instead adopted normal ranges from publications. Of the 132 that stated they used a publication to define their normal range(s), 66 provided further details by stating the publication used. We also found that 29 centres used a paper published before the year 2000; 15 quoted a paper that only contained paediatric normal ranges despite them testing adult samples only; 7 centres used normal ranges automatically programmed into analysis software by the manufacturer; 2 centres quoted papers that did not contain any normal ranges and 2 centres stated they did not know the publication on which their normal ranges are based. Centres that developed their own normal ranges were asked how many samples were analysed in order to establish this. A total of 72 centres provided further details and the results are shown in Table 4.

Laboratories were asked which $CD4^+$ T-lymphocyte subset values (percentages and/or absolute values) were provided to clinicians in order to assist in HIV patient treatment/monitoring and whether this was case dependent. Thus, we asked what results centres would be reported for an adult case and what results centres would report when dealing with a paediatric case. The results are summarised in Table 5.

Finally, the survey was designed in such a way such that we could determine the exact procedures being used in each centre and which laboratories applied best practice for $CD4^+$ T-lymphocyte subset analysis. We have defined best practice for this test as: Having the flow cytometer serviced at least every 12 months (as per manufacturer recommendations); regularly calibrating pipette(s) (as per ISO 15189 recommendations) (13); using a single platform technique (7); using reverse pipetting (7,14) with either an electronic pipette or automated preparation station (to avoid potential repetitive

strain injury by use of automation (15)); and finally; reporting absolute values as cells/µL (4–6)[Table 6]. In this analysis a total of 199 laboratories provided the relevant information to allow analysis. When the data was stratified according to the above algorithm, only 23/199 (12%) where found to fulfil the criteria of following best practice. We also examined the relevant EQA performance and accreditation status of these 199 laboratories. There was no link between the use of incorrect methodologies and performance in the programme (data not shown). However, a total of 71 (36%) laboratories were identified as being accredited to either their prevailing National standards or ISO 15189 (data was not available for the rest of the cohort) with 9/71 (13%) complying with all of the criteria for $CD4^+$ T-lymphocyte subset analysis best practice as defined above.

Discussion

Flow cytometry is a technology with many variables: antibody choice, antibody clone, fluorochrome selection, gating technique used and how results are finally reported (16,17). Thus, every aspect of a flow cytometric assay is open to interpretation – and, unfortunately to misinterpretation. This survey was the largest international survey ever undertaken into the flow cytometric clinical practices in the areas of general flow cytometry. As such, this is the first time that numerous non-standardised approaches in many areas of clinical flow cytometry have been identified. The survey was issued to a total of 1587 laboratories worldwide (840 of which performed $CD4^+$ T-lymphocyte subset enumeration) with response rates of 335 (21%) for the overall survey and 225 (27%) for the $CD4^+$ section giving 95% and 90% confidence levels respectively with a 5% margin of error (sample size calculator www.surveymonkey.com). This was sufficient to conclude the practices in use worldwide had no significant bias. Locations of non-responding centres were analysed and no geographical pattern was observed, although any future surveys will be issued in a multi-lingual format to remove any language barrier that may have been in effect.

The survey asked specific questions with respect to routine aspects of flow cytometry that have not been investigated on such a large scale. The results showed that the majority of machines currently in use are relatively new (less than 6 years old). Whilst multi-colour flow cytometry is essential in leukaemia/lymphoma testing (18) there are many other flow cytometric techniques that can easily be performed using as little as 2 or 3 colours (e.g. CD34+ stem cell enumeration and $CD4^+$ T-lymphocyte subset enumeration). Thus, age of a flow cytometer is not a restrictive factor in many routine clinical practices. However, regardless of the age of the flow cytometer in use, our study identified a small number of sites (2%) failing to have their instrument serviced appropriately (i.e. at least every 12 months), despite previous evidence that not undertaking essential machine maintenance impacts upon laboratory performance in PT/EQA studies (10).

When the data for pipetting technique was analysed we found that the majority of centres used manual pipettes (82%) rather than electronic pipettes (18%) even though evidence shows electronic pipettes provide greater precision and pipetting reliability, particularly when used in conjunction with a reverse pipetting technique (12). We identified that 58% of laboratories in this survey that employed a single platform technique for $CD4^+$ T-lymphocyte subset enumeration did not use such an approach. It is also concerning that 9.4% of laboratories never service or calibrate their pipettes. One factor that laboratory managers should consider, especially when a large amount of pipetting is performed is that of staff health and safety. It has previously been shown that overuse of manual pipettes increases the risk of repetitive strain injury (RSI), particularly when used for >300 hours per year (19). This survey found 24 centres having less than 5 staff performing flow cytometry stated that they processed more than 100 $CD4^+$ T-lymphocyte subset counts per week using manual pipettes suggesting that these staff have an increased risked of RSI.

Further investigation of staff numbers per centre showed that 71/354 (20%) of the respondents had only 1 or 2 staff performing flow cytometry. Whilst this may be adequate for the needs of the service it highlights that a centres lack any spare capacity in case of staff absence or departure. Therefore to ensure long-term continuation of flow cytometry services it is recommended centres examine their knowledge transfer procedures.

The survey also examined workload, and found that all centres processing more than 1000 $CD4^+$ T-lymphocyte subset samples per month were in major urban areas and supports previously published findings (20). Furthermore, the easier access to medical facilities for patients in urban areas, as opposed to rural settings, could also be a contributing factor (21).

The HIV pandemic is a worldwide concern and as such requires co-ordinated efforts in order to find a suitable treatment. With such an international issue it is essential to use consistent terminologies , particularly where results are used in clinical trials. Thus, for $CD4^+$ T-lymphocyte subset counting, the internationally accepted reporting unit is cells/µL (4–6). However, despite such publications our

survey has highlighted that 28% of responding centres continue to report values either as cellsx10^9/L or x10^6/L. This means that clinicians would have to convert these values to cells/µL and should be avoided as any unit conversion introduces potential errors (22,23). As such it would be best practice for laboratories to adopt a harmonised reporting method to minimise the risk of confusion and error and report as cells/µL.

Our study also investigated how normal range(s) were derived. It should be stressed that normal ranges would not typically be used when HIV patients are receiving highly active anti-retroviral therapy (HAART) because the laboratory/clinician should be undertaking trend analysis of $CD4^+$ T-lymphocyte subsets rather than applying a normal range. We surveyed laboratories asking how many samples were used to establish their normal range (when used) and identified that 71% of centres are using incorrectly established normal ranges due to using insufficient sample numbers (24). In addition this survey also found variability in the parameters reported for $CD4^+$ T-lymphocytes (percentages/absolute values) based on patient age (Table 5).

Whilst all of these individual technical issues highlighted herein cause concern, if an overall analysis is undertaken on the data to establish usage of best practice and adherence to published $CD4^+$ T lymphocyte guidelines we calculated that only 12% of laboratories comply (Table 6). This highlights that although $CD4^+$ T-lymphocyte subset enumeration is an established laboratory technique with millions of tests performed globally per annum there is still a lack of basic standardisation, adherence to good practices and application of guidance documents. Of interest is the potential effect that methodological variation has had on PT/EQA performance. The $CD4^+$ T-lymphocyte subset enumeration operated by UK NEQAS LI monitors performance based on consensus values obtained from all participants. Examination of trial data has not identified a link between incorrect methodologies and performance in the programme (data not shown). However, because the majority of methodologies are incorrect it is likely that this is affecting the derivation of overall

consensus values such that it is not possible at this time to monitor the relationship between technique and PT/EQA performance.

Interestingly, when accreditation status of laboratories was examined, 71 laboratories were identified as being accredited to either their prevailing National standards or ISO 15189 (data was not available for the rest of the cohort) but only 9 of these were identified as using correct techniques as we have defined. The best practice flow cytometric techniques we have defined within this document could be potentially used by accreditation authorities at inspection to improve $CD4^+$ T-lymphocyte subset analysis by flow cytometry.

Recent publications recommend reducing the frequency of $CD4^+$ T-lymphocyte subset monitoring in some HIV patients (25,26). However, this survey has identified highly variable practices with respect to deriving such counts which could impact upon the quality of results obtained. Because of a reduction in frequency of counting and the highly variable practices we have highlighted, it is extremely important that laboratories undertake a comprehensive review of their techniques to ensure that all protocols, practices and legacy documents are fit for purpose.

References

1. Phillips A, Sabin CA, Elford J, Bofill M, Janossy G, Lee CA. Use of CD4 lymphocyte count to predict long-term survival free of AIDS after HIV infection. BMJ 1994;309:309–313. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2540903&tool=pmcentrez&rendertype=abstract. Accessed December 2, 2013.

2. Post FA, Wood R, Maartens G. CD4 and total lymphocyte counts as predictors of HIV disease progression. QJM 1996;89:505–508. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8759490.

3. Phillips AN, Lundgren JD. The CD4 lymphocyte count and risk of clinical progression. Curr. Opin. HIV AIDS 2006;1:43–49. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19372783.

4. Anon. European AIDS Clinical Society Guidelines Version 7.0. Ann. Neurol. 2013;74:A28–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24320883.

5. Anon. British HIV Association guidelines for the treatment of HIV-1-positive adults with antiretroviral therapy 2012 British HIV Association guidelines for the treatment of HIV-1-positive adults with antiretroviral therapy 2012. 2012;13:1–85.

6. World Health Organization. Consolidated Guidelines on the Use of Antiretroviral Drugs for Treating and Preventing HIV Infection. 2013. Available at: http://www.who.int/hiv/pub/guidelines/arv2013/en/.

7. 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. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10520014.

8. Whitby L, Whitby A, Fletcher M, Helbert M, Reilly JT, Barnett D. Comparison of methodological data measurement limits in $CD4^+$ T lymphocyte flow cytometric enumeration and their clinical impact on HIV management. Cytometry B. Clin. Cytom. 2013;84:248–254. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23788473. Accessed November 7, 2013.

9. Reilly JT, Barnett D. UK NEQAS for leucocyte immunophenotyping: the first 10 years. J. Clin. Pathol. 2001;54:508–511.

10. Whitby L, Granger V, Storie I, Goodfellow K, Sawle A, Reilly JT, Barnett D. 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). Cytometry 2002;50:102–110. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12116352. Accessed November 7, 2013.

11. Clark JR, Scott SD, Jack AL, Lee H, Mason J, Carter GI, Pearce L, Jackson T, Clouston H, Sproul A, Keen L, Molloy K, Folarin N, Whitby L, Snowden J a, Reilly JT, Barnett D. Monitoring of chimerism following allogeneic haematopoietic stem cell transplantation (HSCT): Technical recommendations for the use of Short Tandem Repeat (STR) based techniques, on behalf of the United Kingdom National External Quality Assessment Service. Br. J. Haematol. 2014:n/a–n/a. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25145701. Accessed August 26, 2014.

12. Brando B, Barnett D, Janossy G, Mandy F, Autran B, Rothe G, Scarpati B, Avanzo GD, Hautcourt JD, Lenkei R, Schmitz G, Kunkl A, Chianese R, Papa S, Gratama JW. Cytofluorometric Methods for Assessing Absolute Numbers of Cell Subsets in Blood. 2000;346:327–346.

13. British Standards Instituion. BS EN ISO 15189:2012.; 2012.

14. Janossy G, Jani I, Göhde W. Affordable CD4(+) T-cell counts on "single-platform" flow cytometers I. Primary CD4 gating. Br. J. Haematol. 2000;111:1198–208. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11167762.

15. Fredriksson K. Laboratory work with automatic pipettes: a study on how pipetting affects the thumb. Ergonomics 1995;38:1067–73. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7737101. Accessed February 9, 2015.

16. Barnett D, Storie I, Wilson GA, Granger V, Reilly JT. Determination of leucocyte antibody binding capacity (ABC): the need for standardization. Clin. Lab. Haematol. 1998;20:155–164. Available at: http://doi.wiley.com/10.1046/j.1365-2257.1998.00116.x. Accessed November 25, 2014.

17. Reilly JT, Granger V, Temperton PF, Barnett D. Leukaemia immunophenotyping: effect of antibody source and fluorochrome on antigen detection. J. Clin. Pathol. 1995;48:186. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=502419&tool=pmcentrez&rendertype=abstract. Accessed November 25, 2014.

18. Johansson U, Bloxham D, Couzens S, Jesson J, Morilla R, Erber W, Macey M. Guidelines on the use of multicolour flow cytometry in the diagnosis of haematological neoplasms. Br. J. Haematol. 2014;165:455–488. Available at: http://www.ncbi.nlm.nih.gov/pubmed/24620735. Accessed March 26, 2014.

19. Björksten MG, Almby B, Jansson ES. Hand and shoulder ailments among laboratory technicians using modern plunger-operated pipettes. Appl. Ergon. 1994;25:88–94. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15676954.

20. Centers for Disease Control and Prevention. Cases of HIV infection and AIDS in urban and rural areas of the United States, 2006. HIV/AIDS Surveill. Supl. Rep. 2008;13(No. 2).

21. Reif S, Golin CE, Smith SR. Barriers to accessing HIV/AIDS care in North Carolina: rural and urban differences. AIDS Care 2005;17:558–565. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16036242. Accessed December 12, 2013.

22. Witkin R. JET'S FUEL RAN OUT AFTER METRIC CONVERSION ERRORS - NYTimes.com. new york times 1983. Available at: http://www.nytimes.com/1983/07/30/us/jet-s-fuel-ran-out-after-metric-conversion-errors.html. Accessed November 26, 2014.

23. Berg J, Lane V. Pathology Harmony; a pragmatic and scientific approach to unfounded variation in the clinical laboratory. Ann. Clin. Biochem. 2011;48:195–197. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21555538. Accessed December 12, 2013.

24. Lott JA, Mitchell LC, Moeschberger ML, Sutherland DE. Estimation of reference ranges: how many subjects are needed? Clin. Chem. 1992;38:648–650. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1582014.

25. Anon. Panel on Antiretroviral Guidelines for Adults and Adolescents. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services. 2015. Available at: http://aidsinfo.nih.gov/ContentFiles/Adultand AdolescentGL.pdf.

26. Whitlock GG, Ahmed N, Benn P, Edwards S, Waters L. Stop routine CD4 monitoring in HIV-infected patients with fully suppressed virus and CD4 >=350 cells/ml. Clin. Infect. Dis. 2013;57:327–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/23537910. Accessed February 9, 2015.

Table 1. Service and calibration intervals of flow cytometers in routine clinical use for all tests

Time	Number of laboratories by Flow Cytometer Service Interval (percentage in brackets)	Number of laboratories by Flow Cytometer Calibration Interval (percentage in brackets)
0-3 months	28 (8%)	172(51%)
4-6 months	194(55%)	106(31%)
7-9 months	17(5%)	9(3%)
10-12 months	103(30%)	46(14%)
13 months +	7(2%)	3(1%)

Table 2. Comparison of the Flow Cytometric Colour Capability and Implementation

Number of Colours Implemented by laboratory (Maximum Fluorescent Colours Used)	Flow Cytometric Colour Capability by laboratory (Maximum Fluorescent Channels Available)*							
	3	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	2	2	0	0	0	1	0	0
2	11	4	1	1	2	2	0	0
3	13	17	7	4	0	2	1	0
4	0	41	14	13	0	10	1	4
5	0	0	34	3	1	4	2	6
6	0	0	0	33	0	26	0	4
7	0	0	0	0	0	3	0	1
8	0	0	0	0	0	38	0	10
9	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	19

*Please note only data up to 10 colours shown. 8/345 (2%) stated they routinely used >10 colours. 1 laboratory used 16 colour flow cytometry (this laboratory is not shown in table).

Table 3. Current Laboratory Practices For All Laboratories With Regards to Pipette Usage and Maintenance

Laboratories responding to Pipetting section	Do you use a manual or electronic pipette	Do you utilise reverse pipetting	How often are the pipettes calibrated
n=351 (100%)	Manual n=289 (82%)	Yes n=155 (44%)	>13 months/never n=19 (5%)
			≤12 months n=135 (38%)
		No n=134 (38%)	>13 months/never n=9 (3%)
			≤12 months n=124 (35%)
	Electronic n=62 (18%)	Yes n=42 (12%)	>13 months/never n=4 (1%)
			≤12 months n=38 (11%)
		No n=20 (6%)	>13 months/never n=1 (0.3%)
			≤12 months n=17 (5%)

Table 4.

Number of Specimens Tested to Develop a Normal Range for $CD4^+$ T-Lymphocyte Subsets

Number of samples tested to develop normal range	Number of Laboratories (n=72)
1-5	3 (4%)
6-10	0 (0%)
11-20	8 (11%)
21-30	5 (7%)
31-40	6 (8%)
41-50	11 (15%)
51-100	18 (25%)
101-150	6 (8%)
151-200	1 (1%)
>200	8 (11%)
Unknown Number (Lab statement)	5 (7%)
Done by another centre	1 (1%)

Table 5.

Breakdown of how $CD4^+$ T-Lymphocyte results are issued to the clinician based on patient age

Test Parameter Provided	Paediatric Cases (n=89)	Adult Cases (n=116)
Percentage Values	3(3%)	2(2%)
Absolute Values	10(11%)	22(19%)
Percentage and Absolute Values	76(85%)	92(79%)

Table 6.

Algorithm for identification of laboratories undertaking all best practices for $CD4^+$ T-Lymphocyte Subset Enumeration

Recommendations*/Good Laboratory Practice**	Initial Number of Laboratories Meeting All Previous Best Practices	Number of Laboratories Performing Correctly
Flow Cytometer Regularly Serviced (≤12 months)**	199(100%)	197(99%)
Single Platform Technique*	197(99%)	142(71%)
Electronic Pipette/Automated Preparation Station*	142(71%)	60(30%)
Regularly Calibrated Pipette (≤12 months)*	60(30%)	57(29%)
Reverse Pipetting**	57(29%)	38(19%)
Reporting in Clinically Relevant Units (Cells/μL)*	38(19%)	23(12%)

*Recommendations are classed as information from internationally published guidelines

**Good Laboratory Practice is classed as established practices that ensure the best performance of equipment