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An International Collaborative Study to Establish a WHO International Reference Reagent for CD4 T cell counting

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NOTE:
This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by **8 October 2018** and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Technologies, Standards and Norms (TSN). Comments may also be submitted electronically to the Responsible Officer: **Dr Ivana Knezevic** at email: knezevici@who.int.

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Summary

The World Health Organization (WHO) has recognised the lack of harmonisation in CD4 T cell counting as an issue for HIV patient care. This report describes the evaluation of a freeze-dried preparation of pooled human leukocytes, NIBSC code 15/270, for use as a reference reagent for CD4 T cell enumeration technologies. The material was evaluated by twelve laboratories from eight different countries. Participants used different CD4 T cell counting technologies all of which have shown suitable performance through independent peer-reviewed data. These include single-platform and dual-platform flow cytometry, dedicated CD4 systems and point of care (PoC) technologies. We found that the vast majority of users employ standard flow cytometers for their counting. The material worked well in flow cytometry platforms and with the point of care technology tested. The material could not be read by the dedicated CD4 systems BD FACS Count and BD FACS Presto and was incompatible with one of the commercial lysis reagents used. We conclude this material is fit-for-purpose for use with standard flow cytometry platforms and the point-of-care device Instacount. The issue with the two dedicated CD4 systems will need to be further investigated with the manufacturer in a follow-up study. Red blood cell lysis reagents will need to be independently evaluated in each centre to determine suitability. In addition, we were unable to recruit participants to cover all the technologies used for CD4 T cell counting. Specifically, it would be important to test the material in the PoC device Alere Pima CD4 test commonly used in Sub-Saharan Africa. However, we still feel the material has value. Therefore, we propose that the candidate be established as a WHO reference reagent for CD4 T cell counting. The IFU will state the technologies for which it has been qualified and the expected performance of the material, as obtained by participants in this study. A follow-up study will be organised with manufacturers to allow for testing of the material in a wider breadth of technologies.

Introduction

Infection with HIV leads to the development of Acquired Immune Deficiency Syndrome (AIDS) characterized by loss of CD4+ T cells required to mount an effective immune response against infections. In 2005 the World Health Organisation (WHO) issued an open letter to manufacturers of CD4+ T cell enumeration technologies emphasizing the need for laboratory monitoring of immunological parameters to support the clinical monitoring of human immunodeficiency virus-1 (HIV-1) infected patients. In particular, this letter states that 'All CD4+ cell enumeration technologies need to be compatible with a form of external quality assessment programme'.

Accurate CD4+ T cell count measurements ensure that patients receive the appropriate anti-retroviral therapy (ART) against HIV-1 and chemoprophylaxis for opportunistic infections.

The WHO Expert Committee on Biological Standardization (ECBS) endorsed the proposal to develop a WHO Reference Reagent to serve as a standard for CD4+ cell counting methods at their 61st annual meeting in 2010.

Over 59 international laboratories were invited to participate in the collaborative study in order to represent the range of methodologies for which the standard would be applicable. Fourteen competent laboratories representative of the six WHO regions agreed to participate. Twelve laboratories in four WHO regions returned data. The other two laboratories were unable to perform the testing due to either moving of their facilities or problems with customs. Any CD4 counting technology that has shown acceptable performance through independent peer-reviewed data was included in the study design.

Bulk materials, processing and characterization (15/270)

Material 15/270 was prepared from human blood leukocytes isolated from donations to the UK National Blood Service (NHSBT). The blood was tested by NHSBT by serology and found negative for antibodies against HIV, HCV, HBsAg and syphilis. The blood was also tested by NHSBT with NAT for Hepatitis B, Hepatitis C, HIV-1 and HIV-2 (Roche MPX v2.0) and for Hepatitis E (Roche HEV v1.0). All virology results were negative. Six leukocyte cones and four whole blood packs were used. Leukocytes were isolated from whole blood by treatment with ammonium chloride red blood cell lysis buffer and from leukocyte cones by density gradient centrifugation. The cells were resuspended in complete media containing fixative. After fixation, the cells were stored in 90%FCS 10%DMSO at -80°C until all donations had been processed. On the day of filling, the cells were thawed, washed and pooled. The CD4 T cell concentration was calculated by single-platform flow cytometry using BD Trucount tubes to determine the filling volume. The cells were suspended in freeze-drying formulation and distributed into ampoules. The ampoule contents were freeze-dried and sealed under nitrogen. The finished product characteristics are as follows:

Code number	15/270
Presentation	Sealed, 3 mL glass ampoules
Number of ampoules available	5672
Date filled	February 2016
Mean fill mass	0.5209 g
Precision of fill (CV of fill mass) (=192)	0.16%
Residual moisture (n=12)	0.4%
Mean dry weight	0.01338
Mean oxygen head space (n=12)	0.26%
Microbiological results	Negative
Storage conditions	-20°C
Address of processing facility	NIBSC, Potters Bar, EN6 3QG, UK
Address of custodian	NIBSC, Potters Bar, EN6 3QG, UK

Participants

Twelve participants from eight different countries returned data and are listed in Table 1, alphabetically, by country. The participants are competent laboratories in CD4 T cell counting as demonstrated by their participation in EQA schemes for CD4 T cell counting (UK NEQAS and QASI). Each participating laboratory is referred to in the study by a code number. The code numbers were randomly assigned and do not reflect the order of listing.

Table 1: List of participants in order of country

AUSTRALIA	Dr Joseph Manitta Victorian infectious disease reference laboratory, Melbourne
BELGIUM	Dr Luc Kestens Institute of Tropical Medicine Antwerp, Antwerpen
CANADA	Mr Michael Keeney London Health Sciences Centre, London, Ontario
FRANCE	Dr Guillaume Monneret Hopital E. Herriot, Lyon
INDIA	Dr Madhuri Thakar National AIDS Research Institute, Pune
INDIA	Dr P Balakrishnan YRG Centre for AIDS Research and Education (YRG Care)
NETHERLANDS	Dr Markus Beck University of Twente, Enschede
PORTUGAL	Dr Maria Arroz Hospital S. Francisco Xavier, Lisboa
PORTUGAL	Dr Marta Alvim Instituto Nacional de Saude Doutor Ricardo Jorge, Lisboa
UK	Mr Dan Payne Leicester Royal Infirmary UHL NHS Trust, Leicester
UK	Mr David Wilson Aberdeen Royal Infirmary, Aberdeen
UK	Mr Liam Whitby UK NEQAS for Leucocyte Immunophenotyping, Sheffield

Collaborative study for the value assignment of 15/270

The collaborative study was organised by NIBSC. Each laboratory was asked to perform their in-house method for CD4 T cell counting after reconstitution of each study sample in 1mL of sterile distilled water. The methodologies used by each laboratory are shown in Table 2. A study protocol, shown in Appendix 1, and instructions for use were provided with the samples.

Five ampoules were provided to participants. Participants tested one ampoule in eight replicate runs in order to assess the precision of the assay. The following four ampoules were tested in single runs. Participants were asked to return CD4+ T cell concentrations expressed as number of CD4+ T cells per microliter and also as %CD4 of total white blood cells, when applicable to their routine method.

Table 2: CD4 T cell counting methods used by participants
Laboratory 6 received the samples but was unable to return data due to moving facilities. Laboratory 7 was unable to receive the samples due to problems with customs.

Type of technology	Instrument	Lab codes
Single-platform flow cytometry	AQUIOS CL by Beckman Coulter	1 and 14
	NAVIOS by Beckman Coulter	2 and 9
	BD FACS Calibur	3
	BD FACS Canto II	8 and 11
	Beckman Coulter FC500	10
Dual-platform flow cytometry	BD FACS Calibur	5 and 12
Dedicated CD4 systems	BD FACS Count system	3
	BD FACS Presto system	13
Point of care technologies	InstantCount	4

Statistical analysis
First, precision was assessed. The expected performance for CD4 technologies is a %CV less than 10% for CD4 counts more than 200 cells/µL (source: guidelines on the WHO website for 'Multicentre Evaluation of CD4 technologies as part of the WHO Prequalification of Diagnostics Programme'). Any results showing CVs greater than 10% were deemed to have unacceptable precision and excluded from value assignment. The remaining CD4 count data was tested using a Grubbs' test (Minitab) to detect an outlier value. The %CD4 data was analysed separately with a Grubb's test. The data was extrapolated to generate a normal distribution to assign a reference range covering 99.7% of extrapolated data.

Results

Data returned for analysis

Data were contributed by 12 laboratories who performed CD4 T cell counting by their routine method on 5 ampoules of 15/270. The first ampoule was assessed in 8 replicates to estimate the precision of the assay (Table 3). The other 4 ampoules were assessed for the purpose of value assignment of the material (Table 4). Participants using the dedicated CD4 systems BD FACS Count and BD FACS Presto were unable to assess the material as the software returned an error code and was unable to analyse the samples. BD was unable to help troubleshoot this problem. One participant looked only at %CD4 and did not return a CD4 T cell concentration.

General suitability of the reference material

One participant reported incompatibility with Q-Prep, a red blood cell lysis reagent distributed by Beckman Coulter. There was poor discrimination of the CD4+ T cells from other T cells when using this reagent but not when using a PBS diluent (Figure 1) or other brands of red blood cell lysis reagents used in the collaborative study (BD FACS Lysing solution, AQUIOS Lysing Reagent kit).

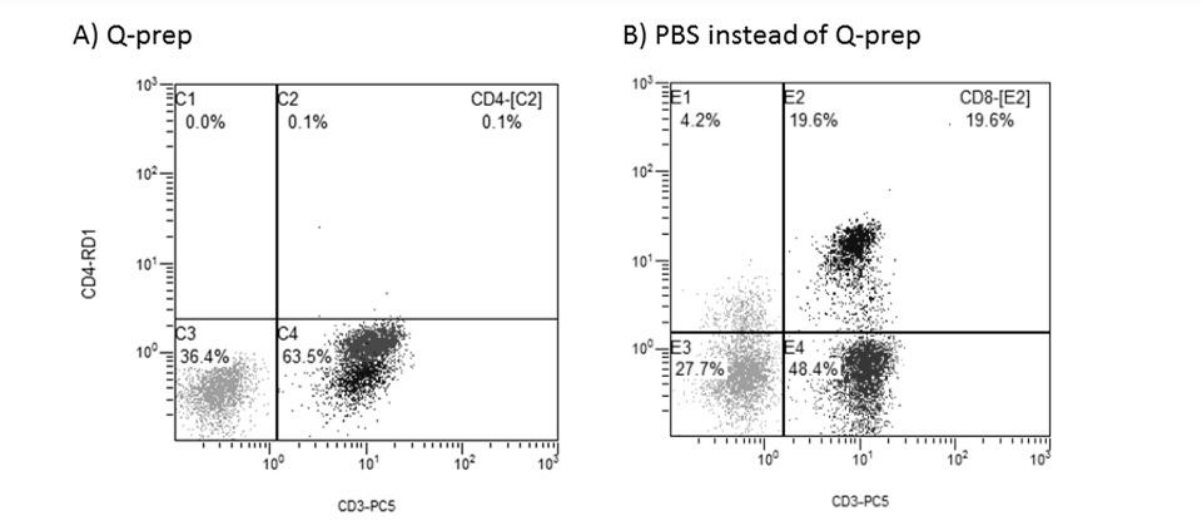

Figure 1 – The same sample analysed using either Q-Prep or PBS. One participant noted poor separation of CD4+ T cells when using their routine in-house method (4 colour flow cytometry CD45-FITC/CD4-PE/CD8-ECD/CD3-PC5 using Beckman Coulter Q-prep no wash, single-platform with Flow-count absolute counting beads). (A) Sample treated with Q-prep. (B) Same sample treated with PBS instead of Q-prep. The x axis depicts fluorescence in the CD3 channel and the y axis fluorescence in the CD4 channel. Note the separation of CD4+ cells from CD4- cells is poor when Q-prep is used (A) but not when PBS is used (B).

Assay validity
Precision assessment is shown in Table 3. Two participants returned %CVs greater than 10% (12.11% and 22.31%) when analysing one ampoule in eight replicates. The WHO guidelines state that the expected performance for CD4 technologies is a %CV of less than 10% for CD4 counts more than 200 cells/µL. Therefore, their data was excluded from the value assignment of 15/270. No outliers were found on the remaining data using the Grubb's test (Minitab).

Value assignment
Value assignment data is shown in Table 4. Laboratory means were used to calculate an overall mean and standard deviation and a normal distribution was extrapolated from that data. The overall mean was 336 CD4/µL with a standard deviation of 21.35 CD4/µL. Most laboratories achieved CVs between 4 and 6% with a maximum of 16%. A reference range spanning 99.7% of expected normally distributed values would be 272 to 400 CD4/µL which is equal to a distance of 3 standard deviations from the mean. Individual participant data segregated by assay type is shown in Figure 2.

Percentage CD4
CD4 percentage is sometimes used as an alternative to CD4 counts, particularly in children under 5 years old (source: 'Guidelines for the Use of Antiretroviral Agents in Pediatric HIV Infection', May 2018, U.S. Department of Health and Human Services webpage). The average percentage of CD4 T cells among white blood cells in the value assignment exercise was 46%. A reference range spanning 99.7% of expected normally distributed values would be 40 to 52%. All the values returned in the study were within this range.

Stability assessment

Accelerated degradation studies were performed at NIBSC with ampoules of 15/270 which had been stored at -70, -20, +4, +37, +45 and +56°C for 0.5 and 9 months. Four replicate vials were assessed by single-platform flow cytometry. A brownish colour and resistance to reconstitution were seen in the samples stored at +56°C for 9 months. This reflects degradation processes such as Maillard reactions which are irrelevant at the low temperatures that the material is stored but can be significant at higher temperatures.

Samples stored at higher temperatures showed a higher fluorescence background than samples stored at -70 to +4°C. The signal to noise ratio between CD4+ T cells and CD4 negative lymphocytes was used as a measure for degradation. The signal to noise values were normalised to the storage temperature (-20°C) results and the long-term stability of 15/270 was predicted using the Arrhenius equation. The estimated percentage loss at -20°C is 0.01% and the estimated percentage loss at 20°C is 1.59% per month. This equates to an estimate of 2187 years before a significant drop in CD4 T cell counts at continuous -20°C storage. The material will require a cold chain for transport which is not a significant issue as it is intended for use mainly by manufacturers.

Discussion

CD4 T cell counting is critical in the monitoring of HIV disease progression. However, there is a lack of harmonisation in CD4 T cell counting which has been highlighted by the WHO as an issue in patient care. To help improve international harmonisation of CD4 T cell counting, the WHO endorsed a project to develop an international reference reagent for CD4 T cell counting. The plan was to manufacture a stable preparation of leukocytes, to be used as a long-term reference sample for the comparison of different technologies or the investigation of instrument performance.

NIBSC manufactured reference material 15/270 from pooled donations from the UK National Blood Service. The material was sent out for evaluation to laboratories proficient in the counting of CD4 T cells. Fourteen international laboratories representative of the six WHO regions agreed to participate. Twelve laboratories returned data. The material worked well in all conventional flow cytometers and in the PoC device tested. However, participants were unable to analyse it in the dedicated CD4 systems BD FACS Count and BD FACS Presto which is unfortunate as BD is one of the largest manufacturers. Two participants used a plug-in offered by BD for automated counting of CD4 T cells in a standard flow cytometer with no issues which suggests that at least this automated application recognizes the candidate as 'like for like'.

In addition, it would be desirable to test the material in a wider range of technologies. The Alere Pima CD4 test in particular is popular in Sub-Saharan Africa, which suffers the greatest burden of HIV globally. Unfortunately, we were unable to recruit any participant using this test. The use of the red blood cell lysis reagent Q-Prep from Beckman Coulter resulted in poor separation of CD4 T cells from other lymphocytes, which the participant had previously seen with other stabilised cellular controls as well. However, there were no issues with any of the other red blood cell lysis reagents used in the study. Expected performance criteria for 15/270 were taken from the evaluation in single and dual-platform classical flow cytometers and the PoC device InstantCount and will be included in the Instructions for Use (IFU).

Conclusion and Recommendation

We propose that the candidate 15/270 be established as a WHO reference reagent for use in CD4 T cell counting. The IFU will state that the material has been qualified using single-platform and dual-platform classical flow cytometers, and the point of care device InstantCount. The expected performance will be stated in the IFU as:

In the hands of expert laboratories, material 15/270 returned an overall mean of 336 CD4 T cells/µL, with an intra-laboratory CV between 4 and 6% for most laboratories and a maximum intra-laboratory CV of 16%. The mean value obtained by an individual laboratory upon repeat testing is expected to fit within the range of 272-400 CD4 T cells/µL with a maximum CV of 16%.

Additionally, the IFU will state the need to validate red blood cell lysis reagents and the %CD4 expected range. A draft IFU can be found in Appendix 2. A follow-up study will be organised by NIBSC with manufacturers to allow for testing of the material in a wider breath of technologies including the Alere Pima CD4 test.

Responses from participants

Originally, it was proposed to participants that 15/270 be established as an international reference reagent for use in selected technologies with a reference range of 272 to 400 CD4 T cells/µL. A follow-up study would be organised with manufacturers of the technologies that were unable to be assessed. Eight of the nine laboratories who responded (8/12) agreed with this proposal. However, one laboratory disagreed. They felt the range was too wide and that this might be detrimental to the development of adequate CD4 instrumentation. After reflecting on this comment we revised the proposal to include the expected performance of the material, in line with that found by the expert laboratories in this study. The participant who had originally disagreed now supports the revised proposal. One participant felt the material needed more supporting data and the other (8/12) all agreed with the proposal.

The participant's comments and response from NIBSC are shown below.

Participant A:
thank you for your email and the update on the reference material. Whilst I appreciate that there is currently no reference material for CD4 T cell counting I regret to say that I cannot endorse the use of this material as an international reference.
My decision to not endorse is based on the publication 'Daneau G, Buyze J, Wade D, Diaw PA, Dieye TN, Sopheak T, Florence E, Lynen L, and Kestens L. CD4 Results with a Bias Larger than Hundred Cells Per Microliter Can Have a Significant Impact on the Clinical Decision During Treatment Initiation of HIV Patients. Cytometry Part B 2017 Nov;92(6):476-484'
The manuscript by Daneau et al clearly states that CD4 results with a bias of larger than 100 cells/uL can affect clinical decisions and that new technologies should not have a bias that exceeds +/- 50 cells/uL. The range for the proposed reference material is 272-400 cells/uL, so with a mean of 336 cells/uL for the material this gives a bias of +/- 64 cells/uL that would still be within the reference range and so classified as acceptable. As such, given that the range for the reference material is greater than the target range proposed for new instrumentation by Daneau et al I cannot see how the introduction of this material at this time would be beneficial. In fact the wide range of the material could actually lead to the development of instrumentation that is not of the required standard required by the laboratory community and this is why I cannot endorse its use.
I would however suggest an alternative approach. It has been seen that different technologies give different results with different levels of precision (e.g single platform vs dual platform). So if it were possible to reassess the material (using single platform technologies only as the gold standard) then this may give a more favourable outcome. If this were the case I would be happy to reconsider.

NIBSC response:
The participant raises the issue that if the reference reagent were used to validate a new instrument then in theory a bias greater than 50 CD4 cells/µL between two instruments could be missed as the data would still be within range (272 to 400 CD4 T cells/µL).

This proposed range reflects a normal distribution extrapolated from laboratory means. However, the expected variability within one individual laboratory would be much lower so a manufacturer of a new technology would expect to achieve a much narrower range in their own testing. If you are taking into account data from various participants, then you have additional sources of uncertainty than those that apply within one laboratory. Sources of uncertainty might include differences in cell reconstitution due to pipetting and vial-to-vial variation within a batch, differences in antibody reagents, data analysis, the use of single or dual platform methods, the value assigned and variability within a batch of internal reference counting beads, different operators and instruments, all contributing to data spread. We can describe the expected variability within one individual laboratory in terms of the %CVs obtained by the participants. In the value assignment exercise, material 15/270 returned an overall mean of 336 CD4/µL, with an intra-laboratory CV of 5-6% for most laboratories and not more than 16%. We can state that the range of values for an individual laboratory should fit within the overall range of 272-400 CD4/µL but respect the %CV conditions described above. This should help prevent the scenario described above where the reference material is used to justify inadequate instrumentation.

It has been reported in the past that single-platform flow cytometry for CD4 T cell counting shows better inter-laboratory coefficients of variation than double-platform methods. However, I would argue two points. Firstly, to evaluate a reference reagent for international use you would want to include as many relevant technologies as possible, as there are various variables that might determine what technology end users might use including availability, cost, logistics and resources. Any CD4 counting technology that has shown acceptable performance through independent peer-reviewed data was invited to participate in the study. Importantly, the data in this particular study do not support the exclusion of double-platform methods for value assignment. The average precision for all methods was 6.52% whereas for single-platform methods only it was 6.98%. Therefore, whilst I appreciate that the collaborative study has weaknesses, it does not support the exclusion of non single-platform methods for value assignment.

Participant B:
I do think that the candidate 15/270 has the potential to be used as a reference material and should therefore be tested more in depth. But the final conclusion on whether or not it should indeed be used as a WHO standard requires better statistical significance.

NIBSC response:
The proposal is to establish 15/270 as a reference reagent which is established by WHO in a situation where the full criteria for an international standard cannot be met. We agree that the evidence is not strong enough to support its establishment as an international standard. The status of WHO reference reagent has been assigned to interim standards before, so it is possible to change the expected performance criteria for 15/270 as we learn more from further testing after establishment.

Acknowledgments

We are deeply thankful to the participants in this collaborative study who have dedicated their time to this project.

Table 3: Precision assessment. The table shows the precision of the assay as assessed by repeat testing of one ampoule in eight replicates. Laboratory 6 received the samples but was unable to return data due to moving facilities. Laboratory 7 was unable to receive the samples due to problems with customs.

Lab	Technology	Precision (n=8) %CV
1	Single-platform AQUIOS	4.21
2	Single-platform NAVIOS	1.88
3	Single-platform BD FACS Calibur	3.07
3	Dedicated CD4 system BD FACS Count	ERROR
4	POC InstantCount	5.00
5	Dual-platform BD FACS Calibur	9.27
8	Single-platform BD FACS Canto II	1.92
9	Single-platform NAVIOS	3.36
10	Single-platform FC500	Participant only provided %CD4 data
11	Single-platform BD FACS Canto II	12.11
12	Dual-platform BD FACS Calibur	2.10
13	Dedicated CD4 system BD FACS Presto	ERROR
14	Single-platform AQUIOS	22.31

Table 4: Value assignment. The table shows the laboratory means used in the value assignment of 15/270, specifically, the calculation of expected means for CD4 counts and %CD4. Some laboratories were unable to return data or returned incomplete data as described in Table 3. Laboratories 11 and 14 were excluded from value assignment due to low assay precision.

Lab	CD4 T cells/µL		%CD4		No.vials
	Mean	%CV	Mean	%CV	
1	359.0	3.89	46.74	2.16	4
2	368.5	3.85	43.68	0.22	4
3	320.5	5.24	47.25	4.69	4
4	345.5	5.83	N/A	N/A	4
5	320.5	4.67	50.5	1.14	4
8	330.8	15.79	44.61	2.17	4
9	335.75	10.67	45.9	0.74	4
12	304.25	5.28	44.93	1.13	4

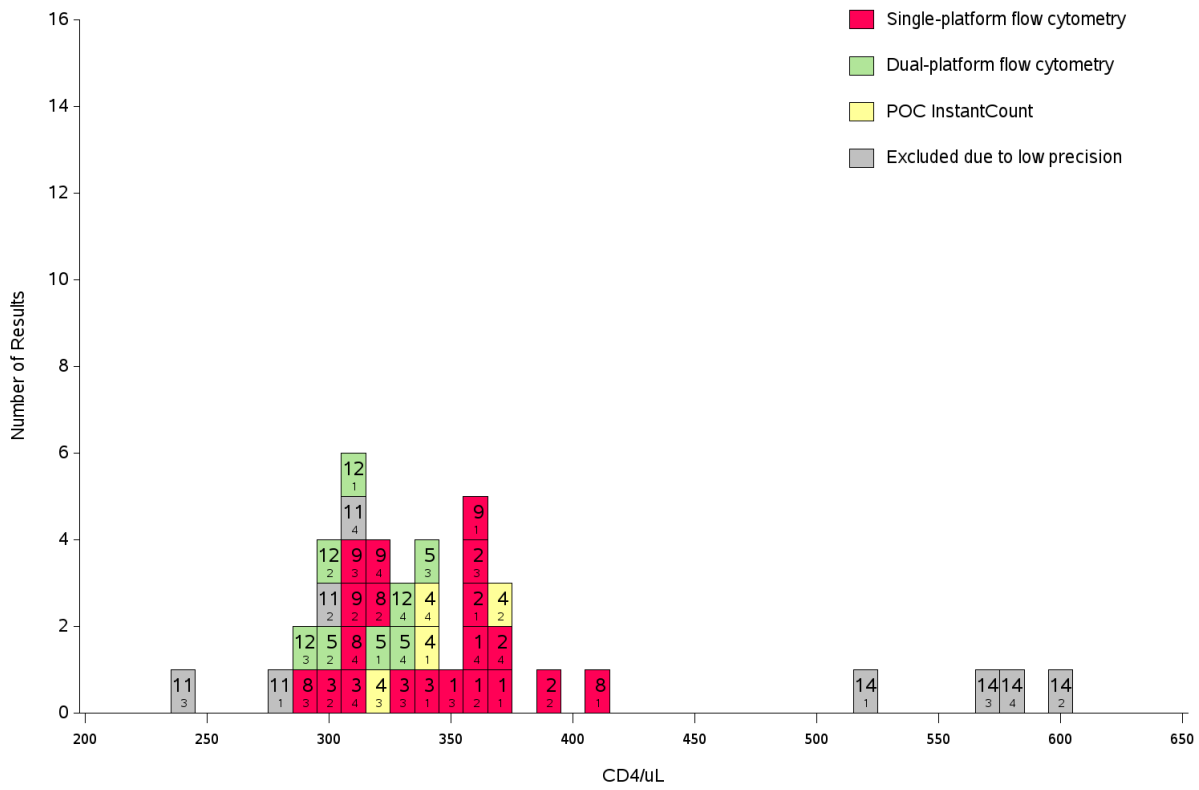

Figure 2 – Participant data in the value assignment exercise: data is shown segregated by value range on the x axis and number of values in the data in the y axis. For each box, the colour denotes the method used and the top number is the laboratory code. Each laboratory tested four ampoules and these are denoted by the small number 1, 2, 3, 4 on the bottom of the box. Laboratories 11 and 14 (grey) were excluded from value assignment due to low assay precision in the repeat testing exercise.

Appendix 1: Study protocol

An International Reference Reagent for the enumeration of CD4 T cells
COLLABORATIVE STUDY
Study Protocol
April 2016

Study coordinators: Luisa Saraiva, National Institute for Biological Standards and Control, UK Tel: +44 (0) 1707 641277, Luisa.Saraiva@nibsc.org; Sandrine Vessillier, National Institute for Biological Standards and Control, UK, Tel:+44 (0) 1707 641146, Sandrine.Vessillier@nibsc.org

Introduction

This study aims to assess an internationally recognised reference preparation for use as a comparator sample for the evaluation of CD4 technologies. Participants are competent laboratories representative of the six WHO regions. Any CD4 counting technology that has shown acceptable performance through independent peer-reviewed data has been included in the study.

The results of the study will be combined in a report to the WHO Expert Committee on Biological Standardization (ECBS) with a recommendation for establishment as a reference reagent together with any limitations on its use (e.g. suitability only for certain assay methods) and a consensus unit.

1. AIMS OF THE STUDY

This study sets out to assess specifically:

1. the suitability of the candidate international biological reference reagent for the enumeration of CD4 T cells in a variety of CD4 T cell counting technologies
2. reference values including a mean and a range expressed as CD4 T cells/μL and % CD4 to the reference reagent

2. REFERENCE MATERIAL

Participants will be sent four vials of reference material for calculation of CD4 T cell reference values. Also included is one vial for each method quoted in the participants' information sheet to be used to assess the precision of the method. Two spare additional vials are included.

The material is shipped at ambient temperature but please store at cold temperatures (+4°C or below) upon receipt. Reconstitute material just before use. If testing using more than one method use the same 4 ampoules for each method. If testing multiple methods is not feasible on the same day then it is possible to transfer material to new tubes so that it can be capped and stored at +4°C until use for a maximum of two days.

To reconstitute a sample:

1. take ampoule out of storage, place in a tube holder and allow to adjust to room temperature;
2. break ampoule seal;
3. pipette 1mL of sterile distilled water and allow 5-30 min for rehydration;
4. mix cell suspension well with a 1mL pipette;
5. sample adequate volume for your assay (treat the material as equivalent to a blood sample at about $1.5x10^6$ white blood cells/mL).

3. OUTLINE OF THE STUDY

Repeat 1) and 2) for each method used.

1) Assay precision comparison

Reconstitute 1 ampoule. Perform 8 replicate runs on this specimen using your own assay. Report CD4 T cells/µL and also %CD4, if applicable to the method used (Table 1).

2) Assessment of reference values

Reconstitute 4 ampoules. Report CD4 T cells/µL and also %CD4, if applicable to the method used (Table 3). Use the same 4 ampoules for each method.

4. DATA SUBMISSION

A report will be prepared and circulated to all participants. In the data analysis, participating laboratories will be identified by a laboratory number only. For submission of results and any further information please email:

Dr Luisa Saraiva
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Dr Sandrine Vessillier
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Sandrine.Vessillier@nibsc.org

Deadline for data submission – 31st August 2016

5. RESULTS

Repeat 1) and 2) for each method used.

Section 1 – Assay precision

Please fill in results in the table below.

Table 1 – Assay precision assessment

Sample	Replicate	CD4 T cells/µL	% CD4
1	**1**		
1	**2**		
1	**3**		
1	**4**		
1	**5**		
1	**6**		
1	**7**		
1	**8**		

Please select the assay method used in the table below:

Table 2 - Method

Method	Select below:
BD FACS Count system	
BD FACS Presto system	
Alere Pima CD4 test	
CyFlow Counter	
CyFlow miniPOC	
Single-platform flow cytometry with BD FACS Calibur	
Single-platform flow cytometry with BD FACS Canto II	
Single-platform flow cytometry with AQUIOS CL by Beckman Coulter	
Single-platform flow cytometry with NAVIOS by Beckman Coulter	
Single-platform flow cytometry with Beckman Coulter FC500 flow cytometer	
Dual-platform flow cytometry with Beckman Coulter FC500 flow cytometer	
Dual-platform flow cytometry with BD FACS Calibur flow cytometer	
Fluorescence imaging on a prototype point-of-care instrument using cell counting chambers with on-chip sample preparation for immunostaining	
Other method, please describe:	

Please describe or provide an example of the gating strategy:

Section 2 – Assay values

The data in this section will be used to assign target values to the reference reagent. Please fill in results in the table below. Report CD4 T cells/µL and also %CD4 if applicable to your method.

Table 3 – Assay values

Sample	CD4 T cells/µL	% CD4
1		
2		
3		
4		

6. GENERAL SUITABILITY

Please provide any comments on how you feel the material performed in your assay (e.g. did you find the CD4 level suitable, did you find it easy to use?)

Thank you for helping evaluate our standard!

Appendix 2 –Draft Instructions for Use

Medicines & Healthcare products Regulatory Agency

NIBSC Confidence in Biological Medicines

WHO Reference Reagent
15/270 - Reference Reagent for CD4 T cell counting
NIBSC code:
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE
This material is intended for use as a cellular control for CD4 T cell enumeration by flow cytometry. It has been qualified using single-platform and dual-platform classical flow cytometers, and the point of care device InstantCount. The material can be used to compare different technologies for CD4 T cell counting, to validate changes in equipment, operator or protocol, for inter- and intra-laboratory performance monitoring, and for training and qualifying new users or new assays. Each reconstituted vial contains unlabelled stabilised human leukocytes, evaluated for CD4 T cells per microliter and percentage CD4 T cells by expert laboratories.

2. CAUTION
This preparation is not for administration to humans or animals in the human food chain.

Human source material
As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE
In the hands of expert laboratories, material 15/270 returned an overall mean of 336 CD4 T cells/µL, with an intra-laboratory CV between 4 and 6% for most laboratories and a maximum intra-laboratory CV of 16%. The mean value obtained by an individual laboratory upon repeat testing is expected to fit within the range of 272-400 CD4 T cells/µL with a maximum CV of 16%.

Expert laboratories returned a mean %CD4 among leukocytes of 46%. Percentage CD4 values are expected to be between 40 and 52%.

4. CONTENTS
Country of origin of biological material: United Kingdom.
The material is stabilised lyophilised human blood leukocytes pooled from donations to the UK National Blood Service.

5. STORAGE
Store unopened ampoules at -20°C or below.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING
Din Ampoule
Care should be taken on opening to prevent loss of contents.

7. USE OF MATERIAL
No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
Take ampoule out of storage, place in a tube holder and allow to adjust to room temperature. Break ampoule seal.

RECONSTITUTE THE CONTENTS OF THE AMPOULE WITH 1mL OF STERILE DISTILLED WATER.

Allow 5-30 min for rehydration. Mix cell suspension well and transfer to a capped tube. Process the material as a normal patient sample. Red blood cell lysis reagents are not required. However, users can validate the use of a red blood cell lysis reagent with 15/270 in their laboratory.

The material has been qualified in single and dual-platform classical flow cytometers and the point of care device InstantCount. There is the possibility of lack of interpretable results using other assay systems.

8. STABILITY (Add or amend as necessary)
Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

For short term storage up to 2 days transfer the reconstituted material in a capped tube to 4°C. For longer storage, users should determine the stability of the reconstituted material according to their own storage facilities.

NIBSC follows the policy of WHO with respect to its reference materials.

9. REFERENCES
Please complete this section manually by typing over this text

10. ACKNOWLEDGEMENTS
Please complete this section manually by typing over this text

11. FURTHER INFORMATION
Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/standardisation/international_standards.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK
Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION
In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET (Add or amend as necessary)
Classification in accordance with Directive 2000/54/EC, Regulation (EC) No 1272/2008: Not applicable or not classified

Physical and Chemical properties			
Physical appearance: Freeze-dried powder		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	Yes	Irritant:	No
Flammable:	No	Handling:	See caution, Section 2
Other (specify):	Contains material of human origin		

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WHO International Laboratory for Biological Standards,
UK Official Medicines Control Laboratory

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Medicines & Healthcare products Regulatory Agency

NIBSC
Confidence in Biological Medicines

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin
Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.
Action on Spillage and Method of Disposal	
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.	

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 1.0g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable. **Attached:** No

17. CERTIFICATE OF ANALYSIS

NIBSC does not provide a Certificate of Analysis for WHO Biological Reference Materials because they are internationally recognised primary reference materials fully described in the instructions for use. The reference materials are established according to the WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards http://www.who.int/bloodproducts/publications/TRS932Annex2_Inter_biolefstandardsrev2004.pdf (revised 2004). They are officially endorsed by the WHO Expert Committee on Biological Standardization (ECBS) based on the report of the international collaborative study which established their suitability for the intended use.

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