

**BIOLOGICAL HEALTH RISKS
QUALITY OF LABORATORIES**

**CLINICAL BIOLOGY COMMISSION
COMMITTEE OF EXPERTS**

**EXTERNAL QUALITY ASSESSMENT
IN CLINICAL BIOLOGY**

DEFINITIVE GLOBAL REPORT

**FLOW CYTOMETRY: CD34+ STEM CELL
ENUMERATION**

SURVEY 2022/3

Sciensano/CD34/31-E

Biological health risks
Quality of laboratories
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All the reports are also available on our webpage:

<https://www.sciensano.be/nl/kwaliteit-van-laboratoria/eke-flow-cytometry-lymphocyte-subset-analysis-and-cd34-stem-cell-enumeration>
 EEQ Cytométrie en flux : Analyse des sous-populations lymphocytaires et Numération des cellules souches CD34+ | [sciensano.be](https://www.sciensano.be)

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INTERPRETATION OF THE INDIVIDUAL REPORT

Besides this global report, an individual report is at your disposal via toolkit.

Below you can find information to help you interpreting this report.

The position of your quantitative results is presented on the one hand in comparison with the results from all the participants and on the other hand in comparison with the results of the laboratories using your method.

Following information is provided:

- Your result (R)
- Your method
- Global median (M_G):
central value of the results obtained by all laboratories (all methods together).
- Global standard deviation (SD_G):
measure of the spread of the results obtained by all the laboratories (all methods together).
- Global median of your method (M_M):
central value of the results obtained by the laboratories using your method.
- Standard deviation of your method (SD_M):
measure of the spread of the results obtained by the laboratories using your method.
- The coefficient of variation CV (expressed in %) for all laboratories and for the laboratories using your method:
 $CV_M = (SD_M / M_M) * 100 (\%)$ and $CV_g = (SD_G / M_G) * 100 (\%)$.
- Z score:
difference between your result and the median of your method (expressed as a number of SD):
 $Z_M = (R - M_M) / SD_M$ and $Z_G = (R - M_G) / SD_G$.
The result is flagged when $|Z_M| > 3$.
- U score:
relative deviation of your result from the median of your method (expressed in %):
 $U_m = ((R - M_M) / M_M) * 100 (\%)$ and $U_g = ((R - M_G) / M_G) * 100 (\%)$.
The result is flagged when $|U_M| > d$, where "d" is a parameter-dependent fixed limit, namely the percentage maximal deviation from the method median.
- A graphical interpretation of the position of your result (R), towards the results of all the participants as well as the results of the participants using your method, based on the method of Tukey, for each parameter and for each analyzed sample.

R : your result
 $M_{M/G}$: median
 $H_{M/G}$: percentiles 25 en 75
 $I_{M/G}$: internal limits ($M \pm 2.7$ SD)
 $O_{M/G}$: external limits ($M \pm 4.7$ SD)

The global graph and the one of your method are presented on the same scale, which allows you to compare them. These graphs give you a rough estimation of the position of your result (R) with respect to the medians ($M_{M/G}$).

More information can be found in the brochures available on our website (only in Dutch and French):

Klinische gezondheid | EKE klinische biologie | sciensano.be

- Algemene informatiebrochure EKE
- Statistische methoden gebruikt voor EKE
- Verwerking van gecensureerde waarden

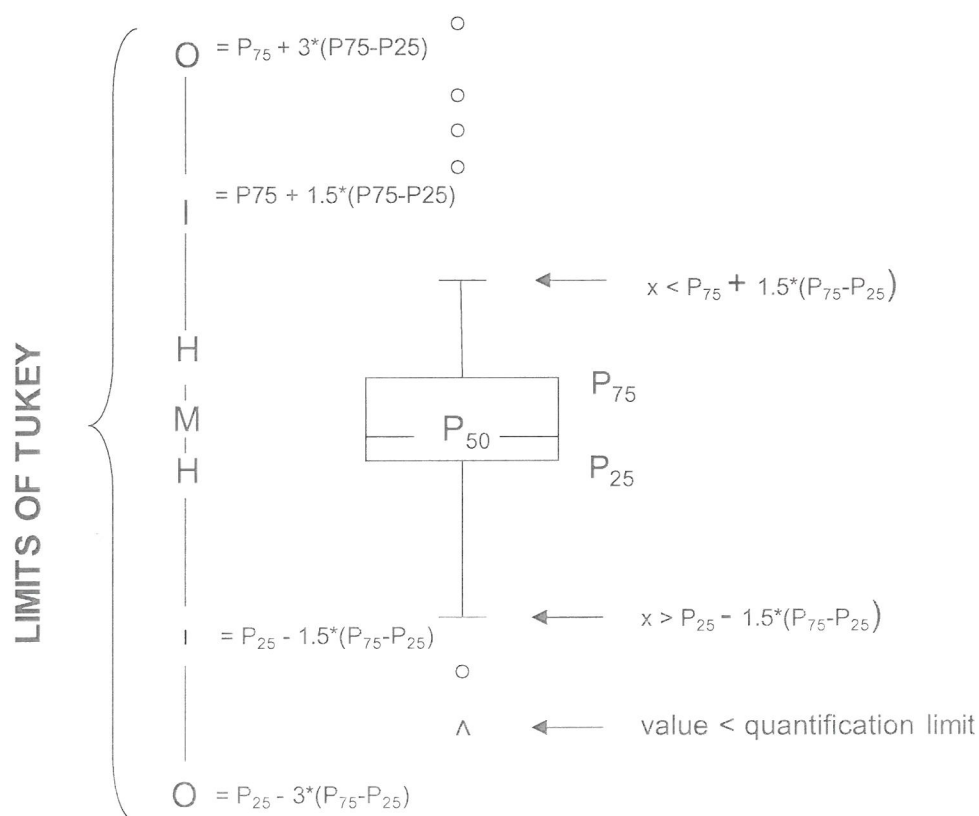
Santé clinique | EEQ biologie clinique | sciensano.be

- Brochure d'information générale EEQ
- Méthodes statistiques appliquées à l'EEQ
- Traitement des valeurs censurées

Graphical representation

Besides the tables with the results a "Box and whisker" plot is added. It contains the following elements for the methods with at least 6 participants:

- a rectangle ranging from percentile 25 (P_{25}) to percentile 75 (P_{75})
- a central line representing the median of the results (P_{50})
- a lower limit showing the smallest value $x > P_{25} - 1.5 * (P_{75} - P_{25})$
- an upper limit representing the largest value $x < P_{75} + 1.5 * (P_{75} - P_{25})$
- all points outside this interval are represented by a dot.



Corresponding limits in case of normal distribution

SAMPLE MATERIAL

Sent out specimens

The survey comprised two stabilized blood sample (FC/19307 et FC/19308, StatusFlow Pro, BIO-TECHNE).

The samples were sent by Taxipost 24h and the laboratories were informed by e-mail of the send-out of the control material (22/11/2022).

Requested analyses

The participants were asked to perform flow cytometric CD34+ stem cell enumeration and to indicate the date of receipt, the date of acquisition, and to provide details of the type of flow cytometer, the sample preparation technique, the source of antibodies, the gating strategy, and the data analysis software used.

PARTICIPATION

Twenty-one Belgian clinical laboratories participated in this survey.

METHODOLOGY OF THE BELGIAN CLINICAL LABORATORIES

Fourteen laboratories (67%) used a single platform approach for determining the absolute CD34+ cell count. Of these laboratories, 8 used Trucount technology (BD Biosciences), 4 Flow-Count or Stem-count beads (Beckman-Coulter) and one Perfect-Count Microspheres (Cytognos). One participant used a volumetric single platform approach (MACSQuant analyzer (Miltenyi Biotec)).

The next table gives an overview of the **flow cytometers** used:

Flow cytometer	Number of laboratories
BD Biosciences FACSCanto II	7
Beckman-Coulter Navios	6
BD Biosciences FACSLytic	6
Miltenyi Biotec MACSQuant analyzer	1
Beckman Coulter AQUIOS CL	1

Sample preparation

Nine participants used a sample volume of 100 µL, eight a sample volume of 50 µL, two a volume of 30 µL, one a volume of 25 µL and one a volume of 43 µL. All participants used a lyse no wash method.

The following table summarises the lysing reagents used:

Lysing reagent	Number of laboratories
Ammonium chloride (NH ₄ Cl)	6
BD Biosciences Ammonium chloride lysing solution	4
BD Biosciences Pharm Lyse	3
Beckman-Coulter VersaLyse Lysing Solution	2
Beckman-Coulter Ammonium chloride	2
BD Biosciences FACS Lysing Solution	1
Cytognos Quicklysis	1
Qiagen EL-buffer	1
Beckman-Coulter AQUIOS STEM Lysing Solution	1

Monoclonal antibodies

All but 2 laboratories (PC5.5/PE-Cy5.5, APC) used a phycoerythrin (PE)-conjugated CD34 monoclonal antibody. All but 4 participants (Horizon V500 (n=2), Krome Orange, VioBlue) used a fluorescein isothiocyanate (FITC)-conjugated CD45 monoclonal antibody.

Gating strategy

With 3 exceptions (BD Biosciences ProCount Kit (n=1) and BD Biosciences Stem Cell Enumeration Kit (n=2)), all participants applied the ISHAGE (International Society of Hematotherapy and Graft Engineering) gating protocol.

RESULTS

Since the samples were stabilized, the laboratories were able to carry out the analysis throughout the full duration of survey. Statistics for the evaluation are therefore based on all results from the Belgian clinical laboratories regardless of the date of analysis (n=21).

FC/19307

	Median	SD	CV, %	Range	N
% CD34+ cells within total WBC	0.140	0.019	13.2	0.100-0.166	21
Absolute CD34+ cell count (cells/ μ L)	8.1	1.0	11.9	6.5-9.0	21

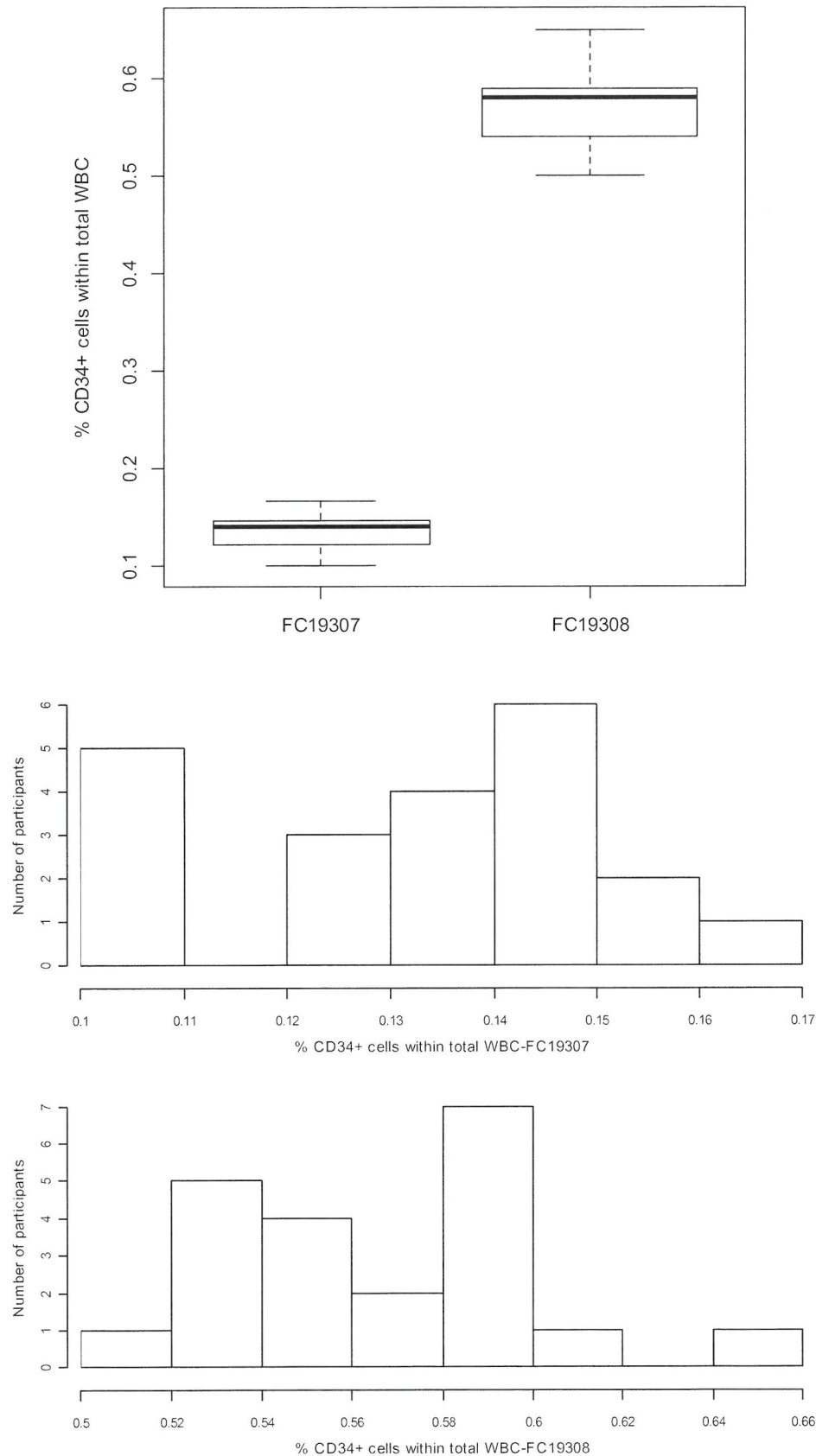
FC/19308

	Median	SD	CV, %	Range	N
% CD34+ cells within total WBC	0.580	0.037	6.4	0.500-0.650	21
Absolute CD34+ cell count (cells/ μ L)	33.8	2.5	7.4	28.7-38.5	21

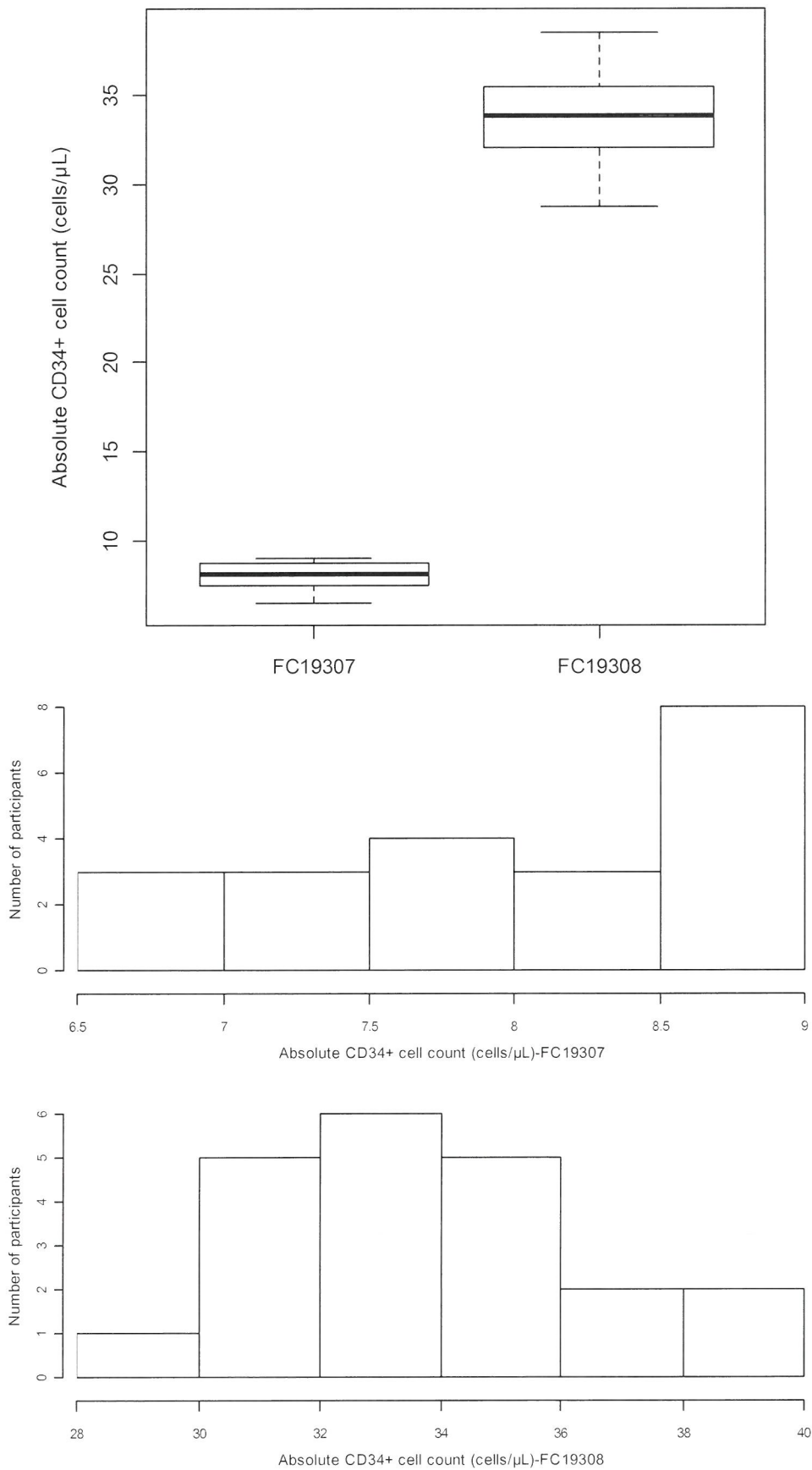
The median WBC count obtained by the laboratories using a double platform approach was $6.0 \times 10^9/L$ (n=7) for both samples. The overall CV's were 1.5% for sample FC19307 and 1.6% for sample FC19308.

The following boxplots and histograms show these data graphically:

% CD34+ cells within total WBC

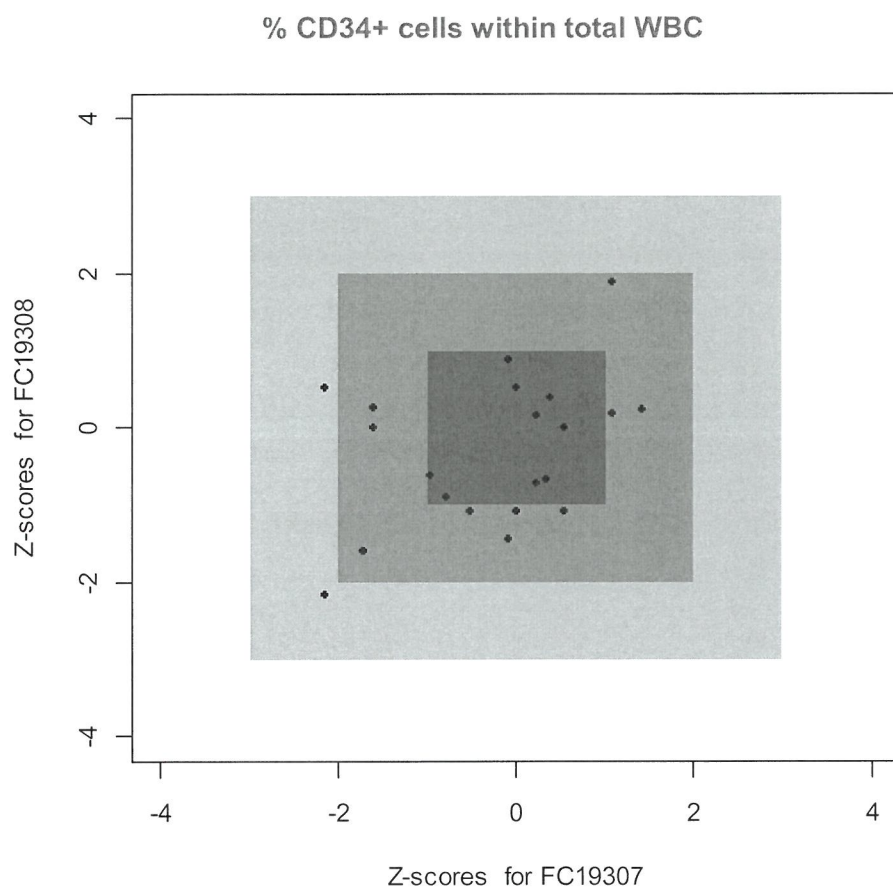


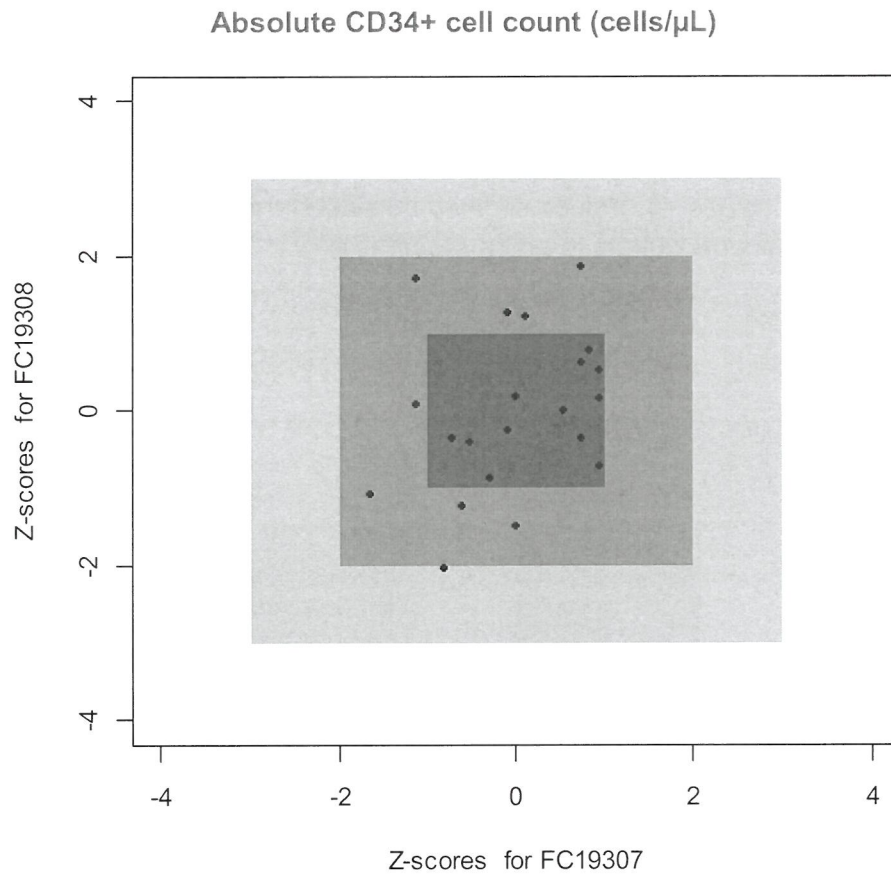
Absolute CD34+ cell count (cells/ μ L)



In the next graphs, the z-scores obtained for the two samples are plotted against each other for each laboratory.

The inner square of the plot represents the z-scores with absolute values <1 , the next larger square represents the z-scores with absolute values <2 , and the outer square represents z-scores with absolute values <3 . Values situated outside of the outer square are considered unacceptable for at least one sample (z-score <-3 or >3).





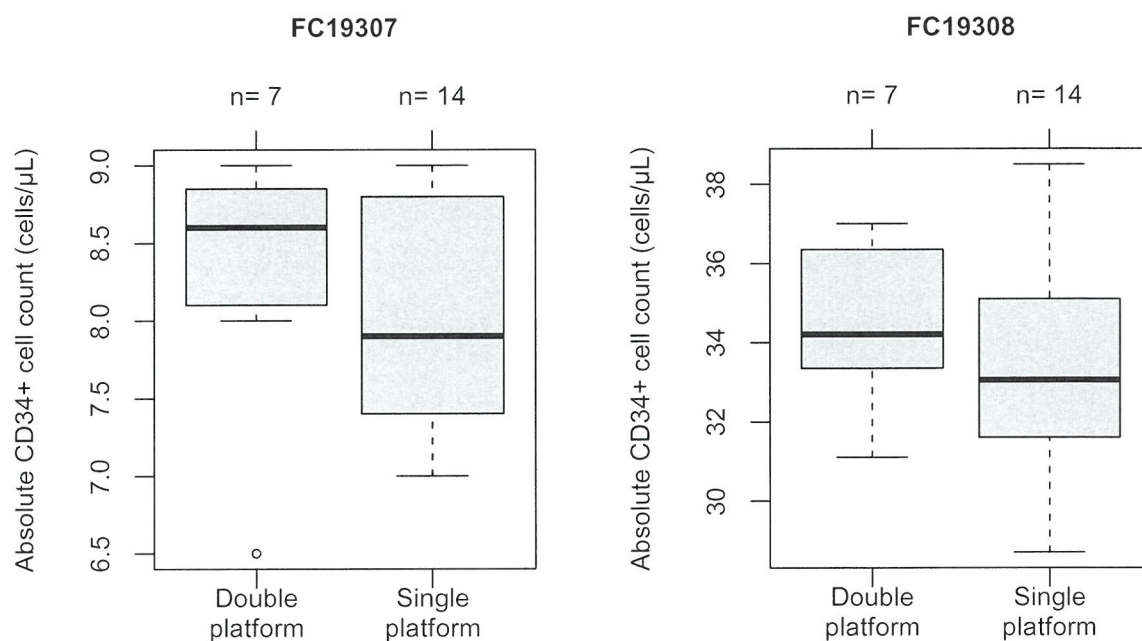
The next tables compare the results from the double (n=7) and single (n=14) platform users:

FC/19307

	Median cells/ μ L	CV %	P25 cells/ μ L	P75 cells/ μ L	Range cells/ μ L	N
Double platform	8.6	6.5	8.1	8.9	6.5-9.0	7
Single platform	7.9	11.3	7.4	8.6	7.0-9.0	14

FC/19308

	Median cells/ μ L	CV %	P25 cells/ μ L	P75 cells/ μ L	Range cells/ μ L	N
Double platform	34.2	6.5	33.4	36.4	31.1-37.0	7
Single platform	33.1	7.2	31.7	34.9	28.7-38.5	14



CONCLUSION

None of the 21 participating laboratories obtained an unacceptable result (z-score >3 or <-3).

RECOMMENDATIONS

The ISHAGE guidelines have been established to overcome a lack of standardization for the enumeration of CD34+ stem cells in routine clinical laboratories. Using these guidelines seems to be the best way to guarantee an acceptable inter-laboratory reproducibility for a better clinical application. Here, we will remind some features of these guidelines with referral to literature.

Sutherland et al. established the ISHAGE guidelines in 1996 (1). These are freely available on the Internet and describe in detail how to enumerate CD34+ cells with a double platform technology. The simple gating strategy that had then been validated is the basis of the one that should be used today. However, a gap that must be highlighted in this first description is how to identify the lymph/blast region. Indeed, an EQA survey published by Whitby et al. in 2012, showed that the most common error made for the correct application of the ISHAGE protocol was the omission of the lymphocyte gating P5/R5 region in region P1/R1 to place optimally the lymph-blast region P4/R4 (2). This part of the ISHAGE protocol was first described by Keeney et al. in 1998 (3). In this paper, two other crucial points were added (fluorescent counting beads and 7-AAD viability dye) to convert the ISHAGE protocol into a single-platform (SP) assay capable of determining the absolute viable CD34+ cell content of a sample using only a flow cytometer.

While the integration of viability dye (like 7-AAD) and the use of a lyse no wash procedure is strongly recommended to date, the replacement of conventional dual-platform by single-platform assay formats is still a matter of debate (4). The reason therefore is that the balance between advantage and disadvantage of this feature depend on many factors like the type of reagent used, the type of cytometer/hematology analyzer used and even the type of analysis software. However, some combinations of reagent kits/instrument platform have been tested for single platform use and the results of it are published in literature (5). Although these results confirm and extend the utility of 'single-platform ISHAGE protocols', no official guidelines have yet been published.

Anyway, the best way to evaluate the efficacy of your lab's enumeration protocol of CD34+ cells (whether it is a simple or double platform assay) is to participate to external quality controls and to revisit your protocol if you do not reach the quality expectations (6).

References

- ¹Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. *International Society of Hematotherapy and Graft Engineering. J Hematother* 1996;3:213-26.
- ²Whitby A, Whitby L, Fletcher M, Reilly, JT, Sutherland DR, Keeney M, Barnett D. ISHAGE protocol: Are we doing it correctly? *Cytometry Part B* 2012; 82B: 9-17.
- ³Keeney M, Chin-Yee I, Weir K, Popma J, Nayar R, Sutherland DR. Single platform flow cytometric absolute CD34+ cell counts based on the ISHAGE guidelines. *International Society of Hematotherapy and Graft Engineering. Cytometry* 1998; 34: 61-70.
- ⁴Gratama J, Orfao A, Barnett D, Brando B, Huber A, Janossy G, *et al.* Flow cytometric enumeration of CD34+ hematopoietic stem and progenitor cells. *Cytometry (Commun Clin Cytom)* 1998;34:128-42
- ⁵Sutherland DR, Nayyar R, Acton E, Giftakis A, Dean S, Mosiman VL. Comparison of two single-platform ISHAGE-based CD34 enumeration protocols on BD FACSCalibur and FACSCanto cytometers. *Cytotherapy* 2009;11:595-605.
- ⁶Levering W, Preijers F, van Wieringen W, Kraan J, van Beers W, Sintnicolaas K, van Rhenen D, Gratama J. Flow cytometric CD34+ stem cell enumeration: lessons from nine years' external quality assessment within the Benelux. *Cytometry Part B* 2007; 72B:178-88.

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