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 2024/2

Sciensano/CD34/35-E

Biological health risks
Quality of laboratories
J. Wytsmanstreet, 14
1050 Brussels | Belgium

www.sciensano.be

ISSN: 2294-3498

COMMITTEE OF EXPERTS

Sciensano					
Secretariat		PHONE:	02/642.55.22	FAX:	02/642.56.45
		e-mail:	QL_secretariat@sciensano.be		
Dr. Bouacida L.	Scheme coordinator	PHONE:	02/642.53.83		
		e-mail:	lobna.bouacida@sciensano.be		
Dr. Vernelen K.	Alternate coordinator	PHONE:	02/642.55.29		
		e-mail:	kris.vernelen@sciensano.be		

Experts	Institute
Dr Chatelain Bernard	UCL Louvain
Dr Defour Jean Philippe	CHC Mont Légia
Dr Gothot André	ULG Liège
Dr Hofmans Mattias	UZ Gent
Dr Keutgens Aurore	ULG Liège
Dr Kornreich Anne	Grand Hopital de Charleroi
Dr Mullier Francois	UCL Louvain
Dr Nguyen Vo Thanh Phuong	Centre Hospitalier EpiCURA
Dr Poutakidou Danai	LHUB-ULB

A draft version of this report was submitted to the experts EQA Flow Cytometry on: 26/06/2024
This report was discussed at the meeting of the committee of experts EQA Flow Cytometry on: 09/07/2024

Authorization of the report: by Lobna Bouacida, scheme coordinator

Date of publication: 23/07/2024

All the reports are also available on our webpage:

- NL: https://www.sciensano.be/nl/kwaliteit-van-laboratoria
- FR: https://www.sciensano.be/fr/qualite-des-laboratoires

TABLE OF CONTENTS

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 ± 2.7 SD)

  $O_{M/G}$: external limits (M ± 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

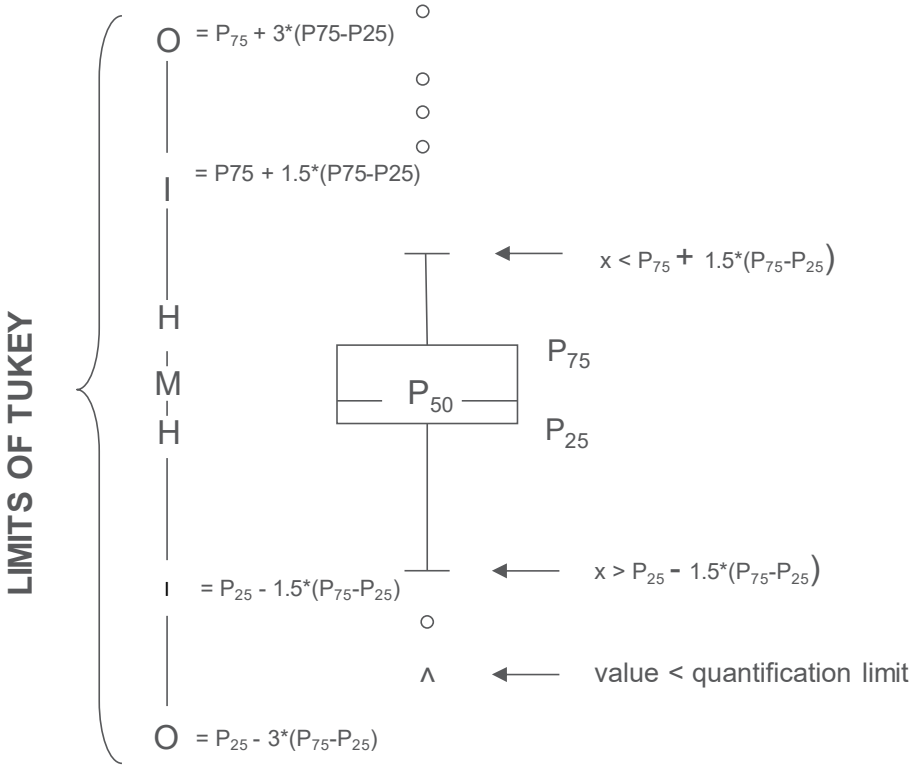
Santé clinique | EEQ biologie clinique | sciensano.be

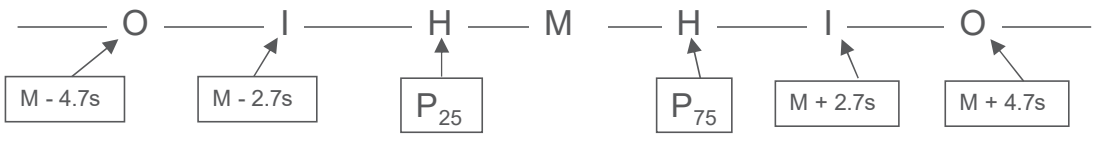
- Brochure d'information générale EEQ
- Méthodes statistiques appliquées à l'EEQ
- Traitement des valeurs censurées

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 ($\mathbf{P_{25}}$) to percentile 75 ($\mathbf{P_{75}}$)
- a central line representing the median of the results ($\mathbf{P_{50}}$)
- a lower limit showing the smallest value $\mathbf{x > P_{25} - 1.5 * (P_{75} - P_{25})}$
- an upper limit representing the largest value $\mathbf{x < P_{75} + 1.5 * (P_{75} - P_{25})}$
- 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 samples (FC/20466 and FC/20467, 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 (27/05/2024).

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 Belgian clinical laboratories participated in this survey.

METHODOLOGY OF THE BELGIAN CLINICAL LABORATORIES

Single or dual-platform

75% of the laboratories used a single-platform approach.

- Among these, 11 laboratories utilized Trucount technology (BD Biosciences).
- 3 laboratories employed Flow-Count or Stem-count beads (Beckman-Coulter).
- One participant used a volumetric single platform approach with the MACSQuant analyzer (Miltenyi Biotec).

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

Flow cytometer	Number of laboratories
BD Biosciences FACSLyric	9
Beckman-Coulter Navios	4
BD Biosciences FACSCanto II	4
Beckman Coulter AQUIOS CL	1
Beckman Coulter DxFLEX	1
Miltenyi Biotec MACSQuant analyzer	1

Sample preparation

Among the participants, twelve used a sample volume of 100 µL, five used 50 µL, one used 43 µL, another used 30 µL, and one more used 25 µL. All participants employed a lyse-no-wash method.

Below is a summary of the lysing reagents used:

Lysing reagent	Number of laboratories
BD Biosciences Ammonium chloride lysing solution	7
Ammonium chloride (NH_4Cl)	5
BD Biosciences Pharm Lyse	3
Beckman-Coulter VersaLyse Lysing Solution	1
Beckman-Coulter Ammonium chloride	1
BD Biosciences FACS Lysing Solution	1
Qiagen EL-buffer	1
Beckman-Coulter AQUIOS STEM Lysing Solution	1

Monoclonal antibodies

In all laboratories except one (PC5.5/PE-Cy5.5), a phycoerythrin (PE)-conjugated CD34 monoclonal antibody was used.

Except for three participants (Horizon V500, Krome Orange, VioBlue), all others utilized a fluorescein isothiocyanate (FITC)-conjugated CD45 monoclonal antibody.

Gating strategy

Out of the participants, 14 followed the ISHAGE (International Society of Hematotherapy and Graft Engineering) gating protocol, 4 utilized the BD Biosciences Stem Cell Enumeration kit, one participant employed the BD Biosciences ProCount Kit, and another used the Stem-Kit from Coulter/Immunotech.

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=20).

FC/20466

	Median	SD	CV,%	Range	N
% CD34+ cells within total WBC	0.160	0.020	12.7	0.132 – 0.223	20
Absolute CD34+ cell count (cells/μL)	9.9	1.3	13.5	8.0 – 15.0	20

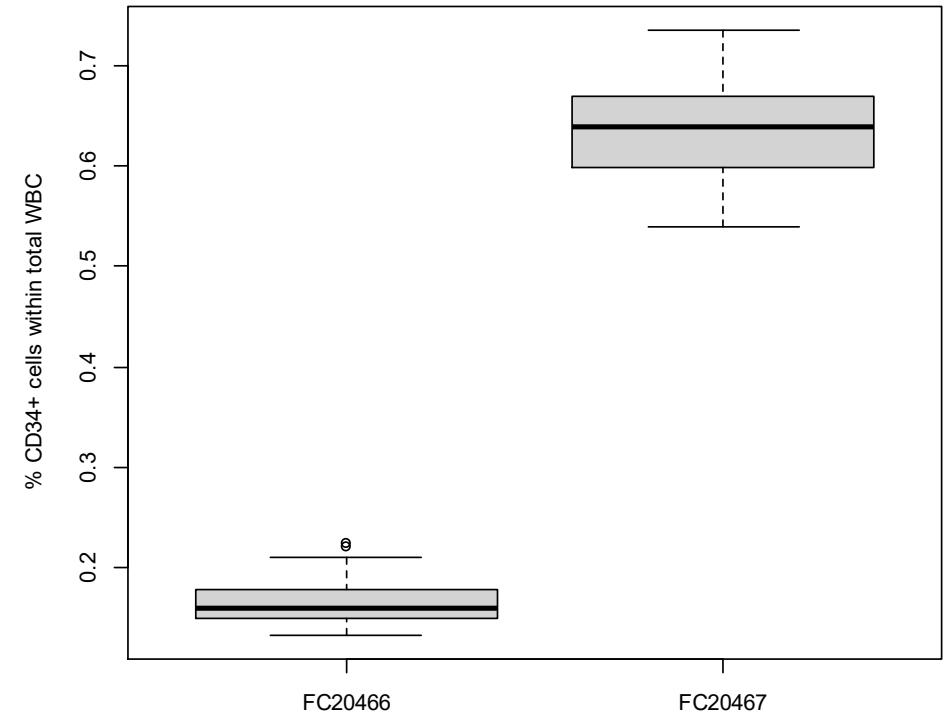
FC/20467

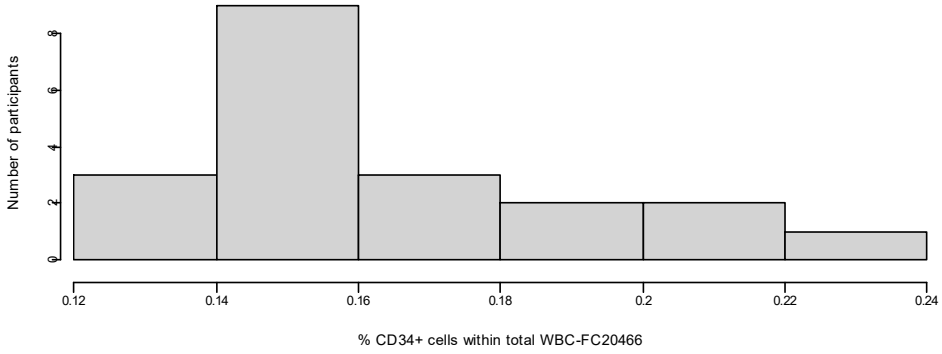
	Median	SD	CV,%	Range	N
% CD34+ cells within total WBC	0.640	0.052	8.1	0.540 – 0.736	20
Absolute CD34+ cell count (cells/μL)	39.7	4.6	11.5	31.2 – 45.0	20

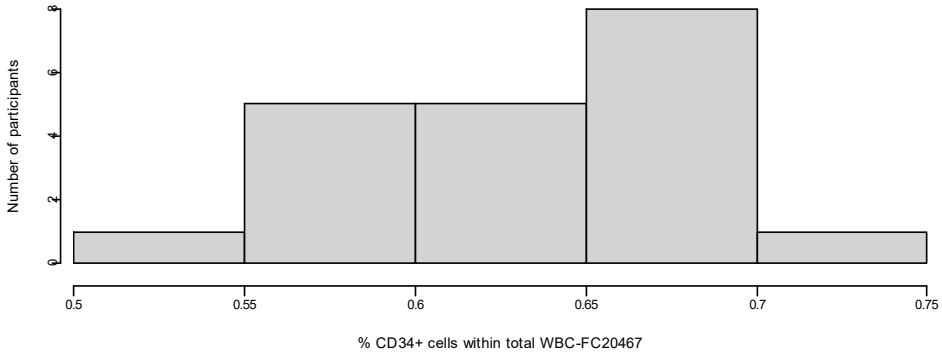
The median WBC count obtained by the laboratories using a double platform approach was 6.06 10^9/L (n=5) for sample FC/20466 and 6.20 10^9/L (n=5) for sample FC/20467. The overall CV's were 0.73% for sample FC/20466 and 0.71% for sample FC/20467.

The following boxplots and histograms show these data graphically:

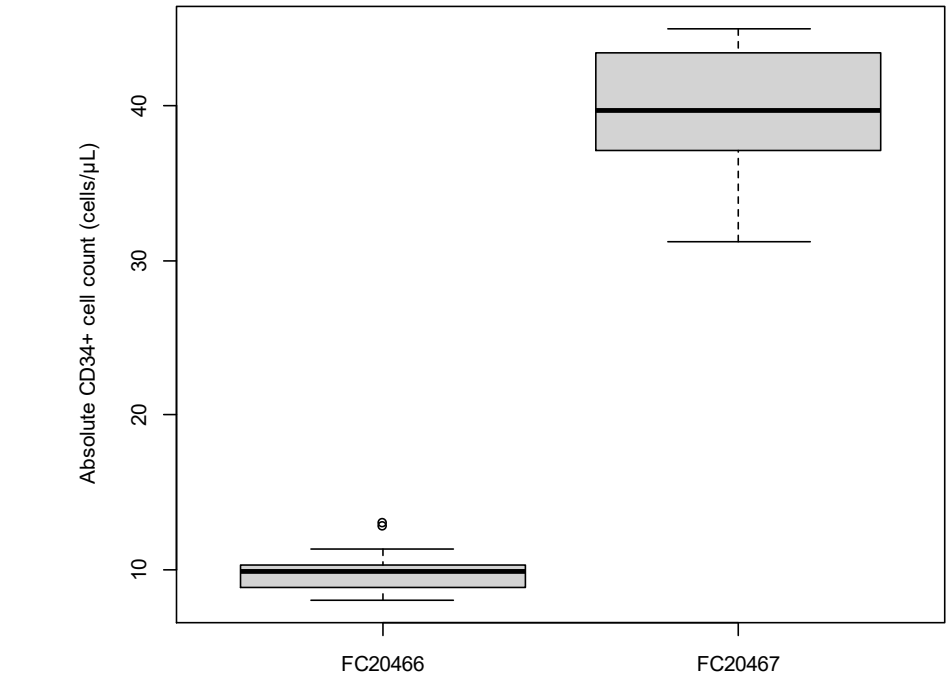
% CD34+ cells within total WBC

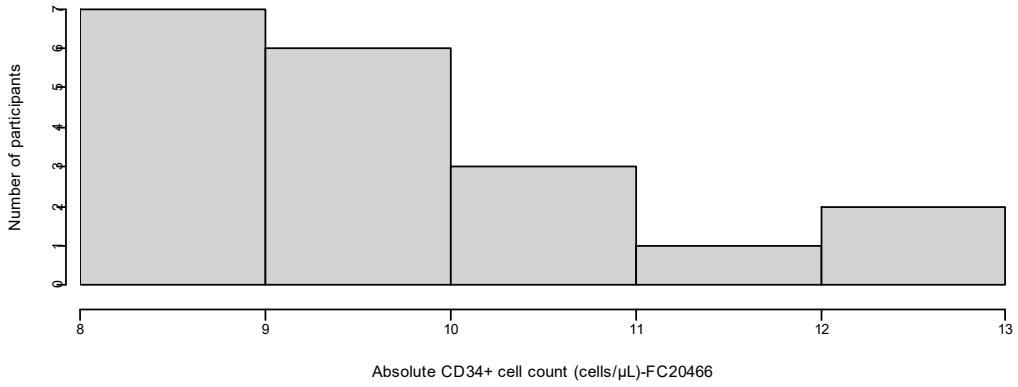


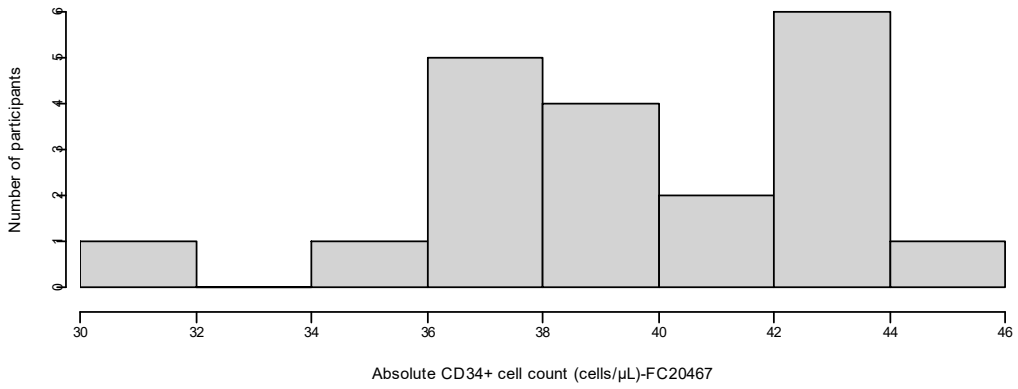




Absolute CD34+ cell count (cells/µL)



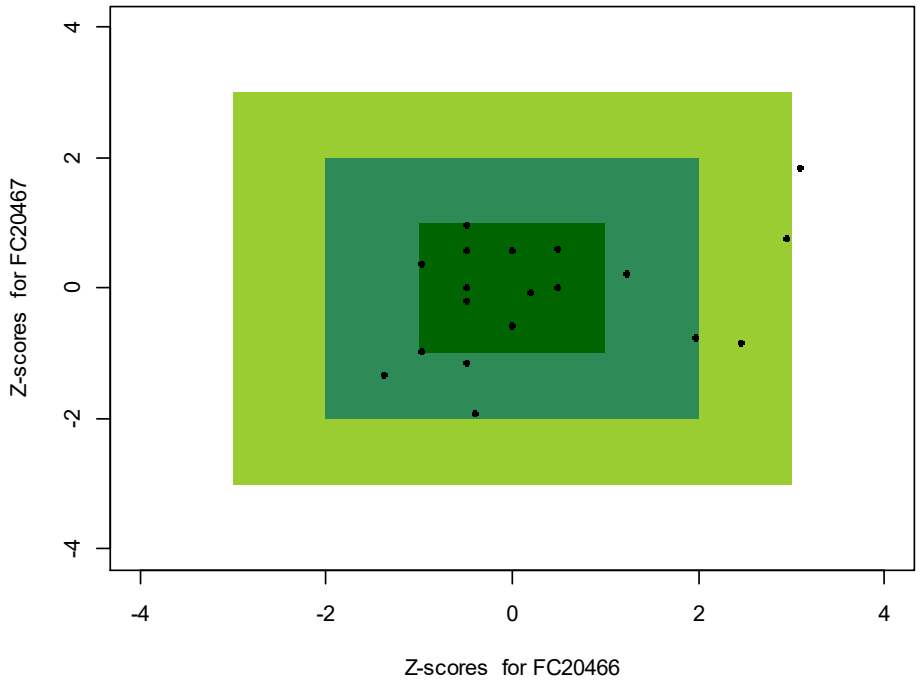




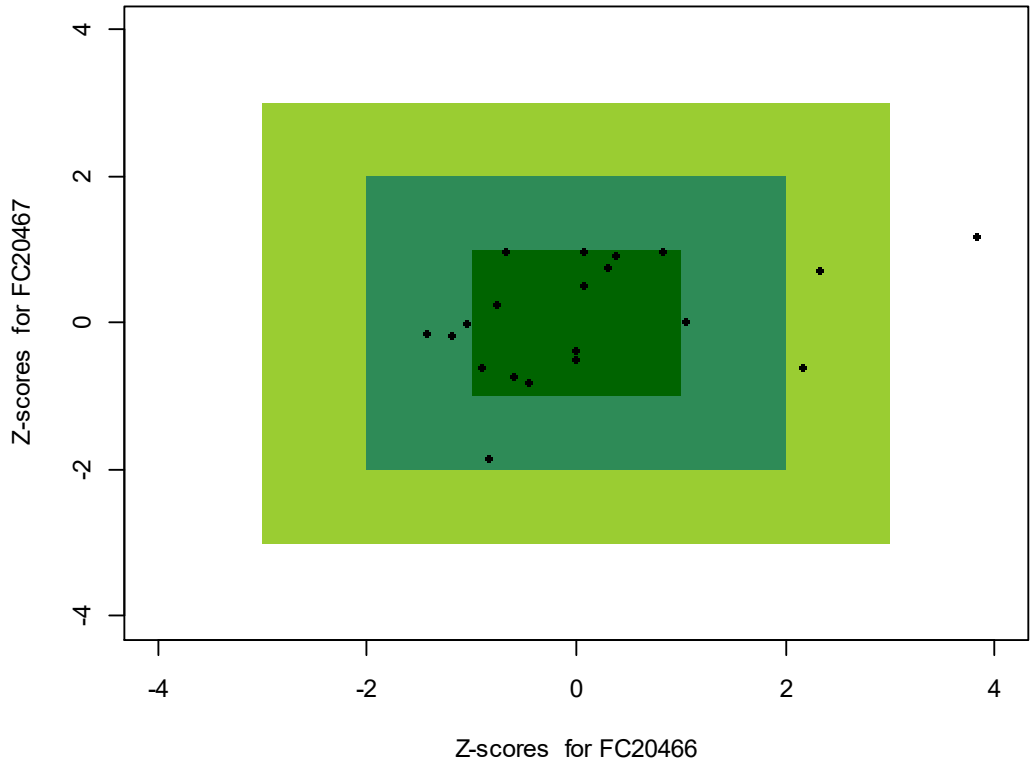
In the next graphs, the z-scores obtained for the two samples are plotted against each other for each laboratory.

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).

% CD34+ cells within total WBC



Absolute CD34+ cell count (cells/µL)



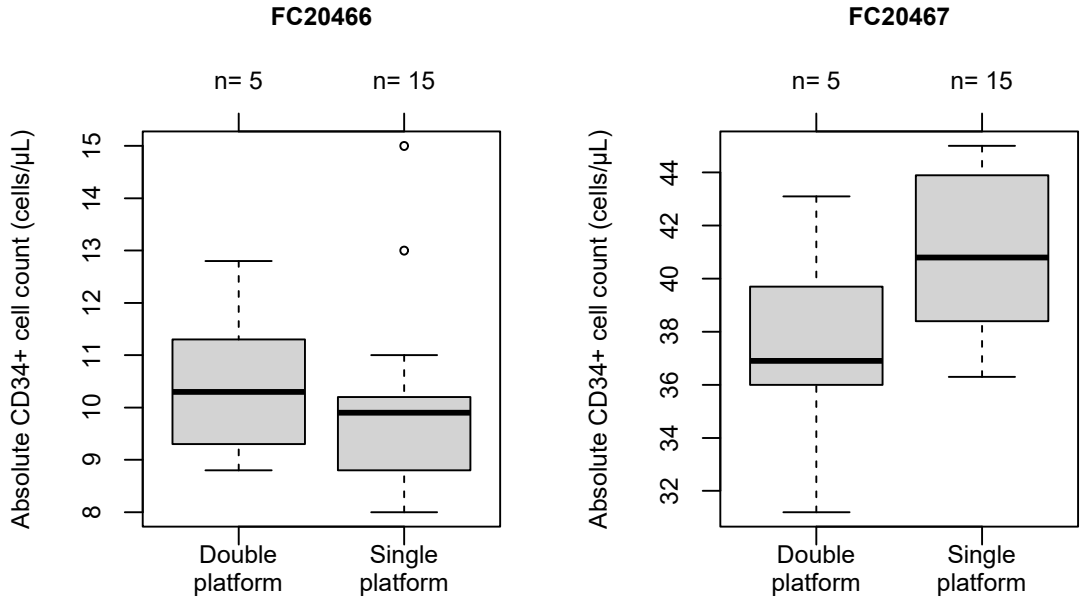
The next tables compare the results from the double (n=5) and single (n=15) platform users:

FC/20466

	Median cells/µL	CV %	P25 cells/µL	P75 cells/µL	Range cells/µL	N
Double platform	10.3	14.4	9.3	11.3	8.8 – 12.8	**5**
Single platform	9.9	10.5	8.8	10.2	8.0 – 15.0	**15**

FC/20467

	Median cells/µL	CV %	P25 cells/µL	P75 cells/µL	Range cells/µL	N
Double platform	36.9	7.4	36.0	39.7	31.2 – 43.1	**5**
Single platform	40.8	10.0	38.4	43.9	36.3 – 45.0	**15**



CONCLUSION

% CD34+ cells within total WBC (n=20)

One laboratory obtained an unacceptable result (z-score >3 or <-3).

Participant	FC/20466 % CD34+ cells	z-score	FC/20467 % CD34+ cells	z-score
1	0.223	3.096	0.736	1.853

Absolute CD34+ cell count (cells/µL) (n=20)

One laboratory obtained an unacceptable result (z-score >3 or <-3).

Participant	FC/20466 (cells/µL)	z-score	FC/20467 (cells/µL)	z-score
2	15.0	3.829	45.0	1.176

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

[1]Sutherland 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.

[2]Whitby 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.

[3]Keeney 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.

[4]Gratama 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

[5]Sutherland 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.

[6]Levering 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.

Pharm. Hugues Jacqmin, Prof. F. Mullier and Prof. B. Chatelain
CHU UCL Namur

END