EXPERTISE AND SERVICE PROVISION
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 2020/2

Sciensano/CD34/28-E

Expertise and service provision
Quality of laboratories
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Authorization to release the report: By Lobna Bouacida, scheme coordinator, on 30/09/2020.

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All the reports are also available on our webpage:
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https://www.wiv-isp.be/QML/activities/external_quality/rapports/_fr/rapports_annee.htm

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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 ± 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 ($M_{M/G}$).

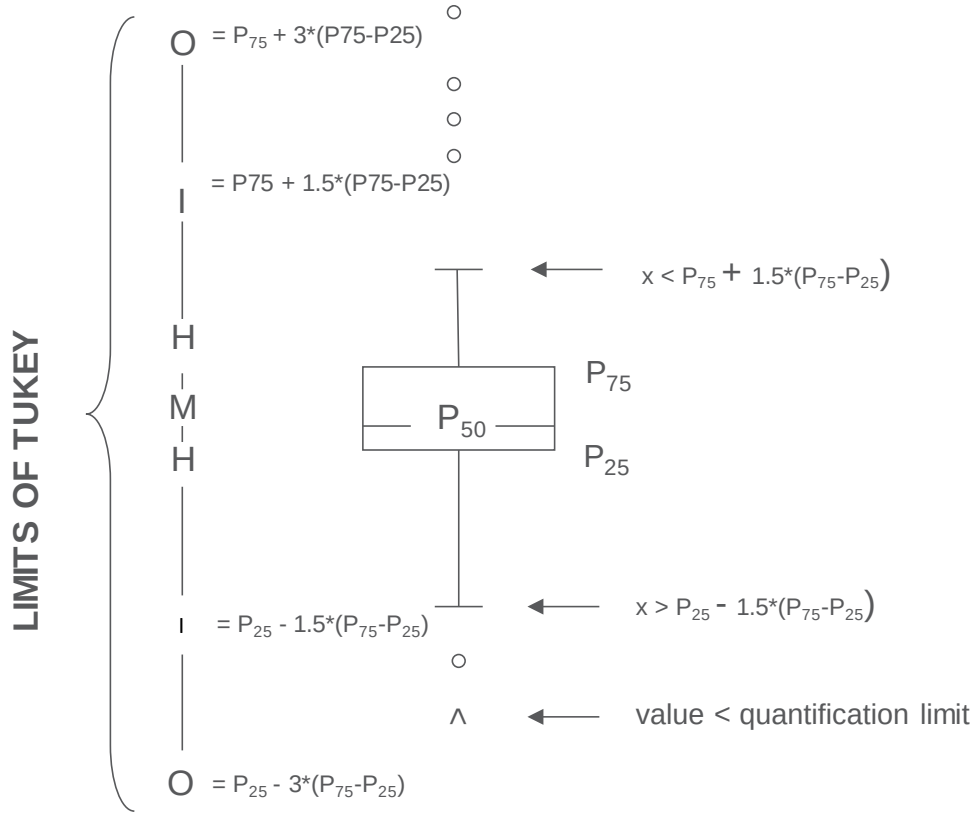
More information can be found in 3 brochures available on our website (only in Dutch and French):
https://www.wiv-isp.be/QML/index_nl.htm
https://www.wiv-isp.be/QML/index_fr.htm
(Choose “brochures” in the menu)
or directly on the following webpage (only in Dutch and French):
https://www.wiv-isp.be/QML/activities/external_quality/brochures/_nl/brochures.htm
https://www.wiv-isp.be/QML/activities/external_quality/brochures/_fr/brochures.htm

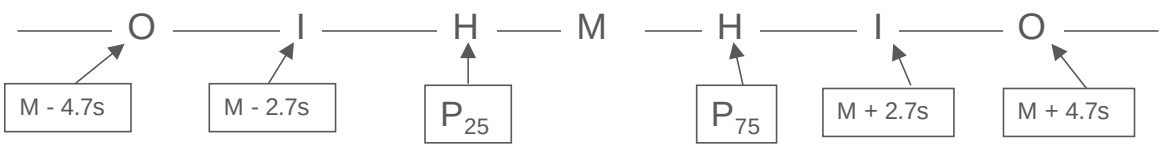
1. Informatiebrochure over de externe kwaliteitsevaluatieprogramma's voor klinische laboratoria (Algemene informatiebrochure over de externe evaluatie)/
   https://www.wiv-isp.be/QML/Informatiebrochure_EKE.pdf
   Brochure d’information sur les programmes d’évaluation externe de la qualité pour les laboratoires cliniques (Brochure d’information générale sur l’évaluation externe).
   https://www.wiv-isp.be/QML/Brochure_information_EEQ.pdf
2. Statistische brochure (Algemene statistische berekeningsprocedure opgesteld door Professor Albert)/ Brochure statistique (Procédure générale de calcul statistique mis au point par le professeur Albert).
3. Verwerking van gecensureerde waarden (Statistische berekeningsprocedure toegepast op de gecensureerde waarden opgesteld door Professor Albert)/
   Traitement des valeurs censurées (Procédure de calcul statistique appliquée aux valeurs censurées rédigée par le Professeur Albert).

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 one fresh umbilical cord blood sample (FC/17264).

The sample material was kindly provided by the Cliniques universitaires Saint-Luc, Brussels and distributed into aliquots at Sciensano. The sample material was collected into citrate-phosphate-dextrose. The samples were sent by Taxipost 24h and the laboratories were informed by e-mail of the send-out of the control material (day 0: 11/08/2020).

The sample tested negative for HIV 1 and 2, hepatitis B surface antigen and hepatitis C. Homogeneity was confirmed on the basis of WBC count.

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.

Since the samples were not stabilised, the laboratories were asked to perform sample testing as soon as possible upon receipt.

PARTICIPATION

Nineteen Belgian clinical laboratories participated in this survey.

METHODOLOGY OF THE BELGIAN CLINICAL LABORATORIES

Thirteen laboratories (68%) used a single platform approach for determining the absolute CD34+ cell count. Of these laboratories, 9 used Trucount technology (BD Biosciences), 2 Flow-Count or Stem-count beads (Beckman-Coulter) and 1 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	9
Beckman-Coulter Navios	5
BD Biosciences FACSLyric	3
Beckman-Coulter Cytomics FC 500	1
Miltenyi Biotec MACSQuant analyzer	1

Sample preparation

Nine participants used a sample volume of 50 µL and eight a sample volume of 100 µL. Two participants used other volumes: 25 µL for one and 30 µL for the other. All participants used a lyse no wash method.

The following table summarises the lysing reagents used:

Lysing reagent	Number of laboratories
Ammonium chloride (NH_4Cl)	6
Beckman-Coulter VersaLyse	4
BD Biosciences Pharm Lyse	3
BD Biosciences Ammonium chloride lysing solution	3
Beckman-Coulter Ammonium chloride	1
Qiagen EL-buffer	1
Cytognos Quicklysis	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.

Viability

89% of the laboratories evaluated CD34+ cell viability using 7-AAD (7-Aminoactinomycin D, n=16) or TO-PRO-3 (n=1).

Gating strategy

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

RESULTS

All laboratories received the samples on day 1 or 2. Fifteen laboratories (79%) received the samples on day 1 and four (21%) received them on day 2.

63% of the laboratories (n=12) performed the analyses on day 1, 26% on day 2 (n=5) and 11% on day 3 (n=2).

Since the samples are fresh and not stabilised, it is extremely important to perform sample testing as soon as possible upon receipt.

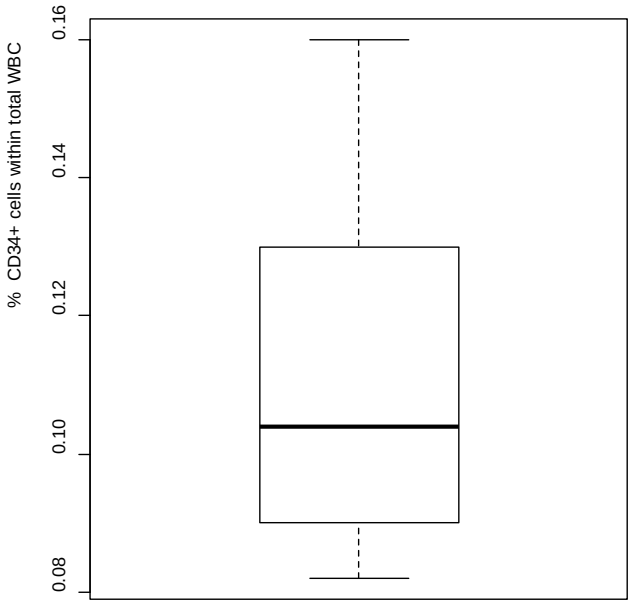
Statistics for the evaluation are solely based on the results of the Belgian clinical laboratories that performed the analyses on day 1 or 2 (n=17).

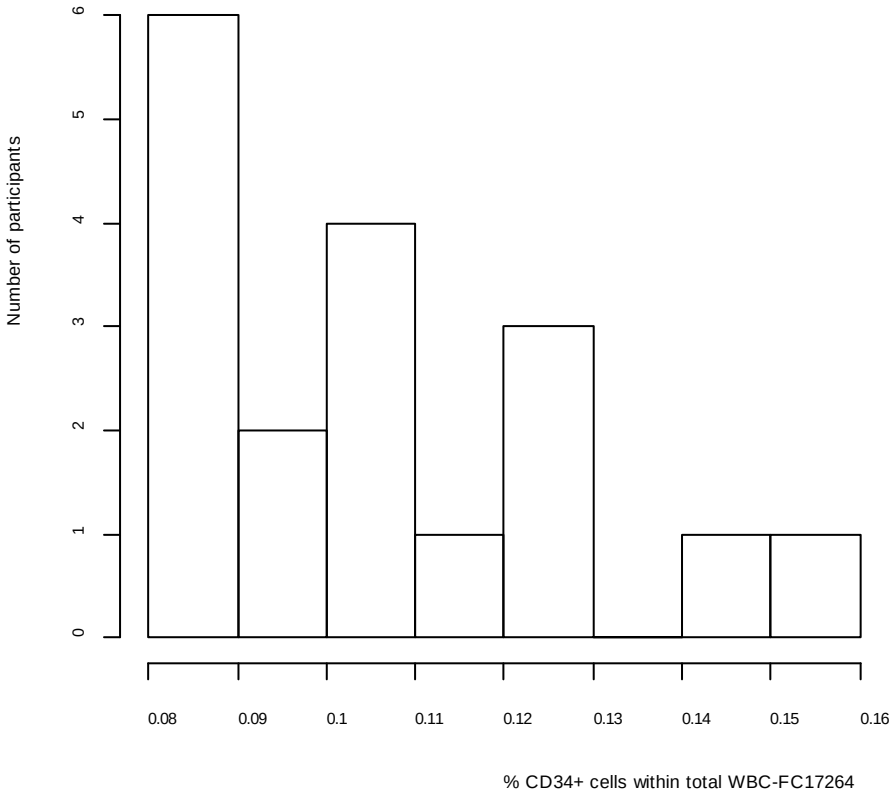
The median % viable CD34+ cells within total WBC was 0.104% with a CV of 28.5%. The median absolute viable CD34+ cell count was 10.1 cells/µL with a CV of 12.1%.

FC 17264

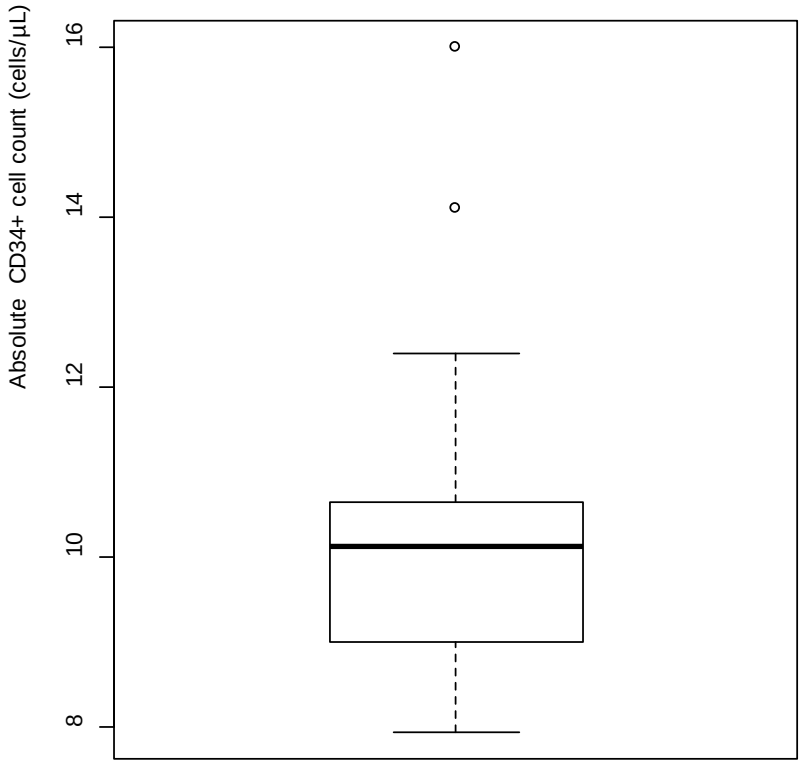
	Median	CV %	P25	P75	Range	N
% CD34+ cells within total WBC	0.104	28.5	0.090	0.130	0.080-0.770	17
Absolute CD34+ cell count (cells/µL)	10.1	12.1	9.0	10.7	7.9-16.0	17

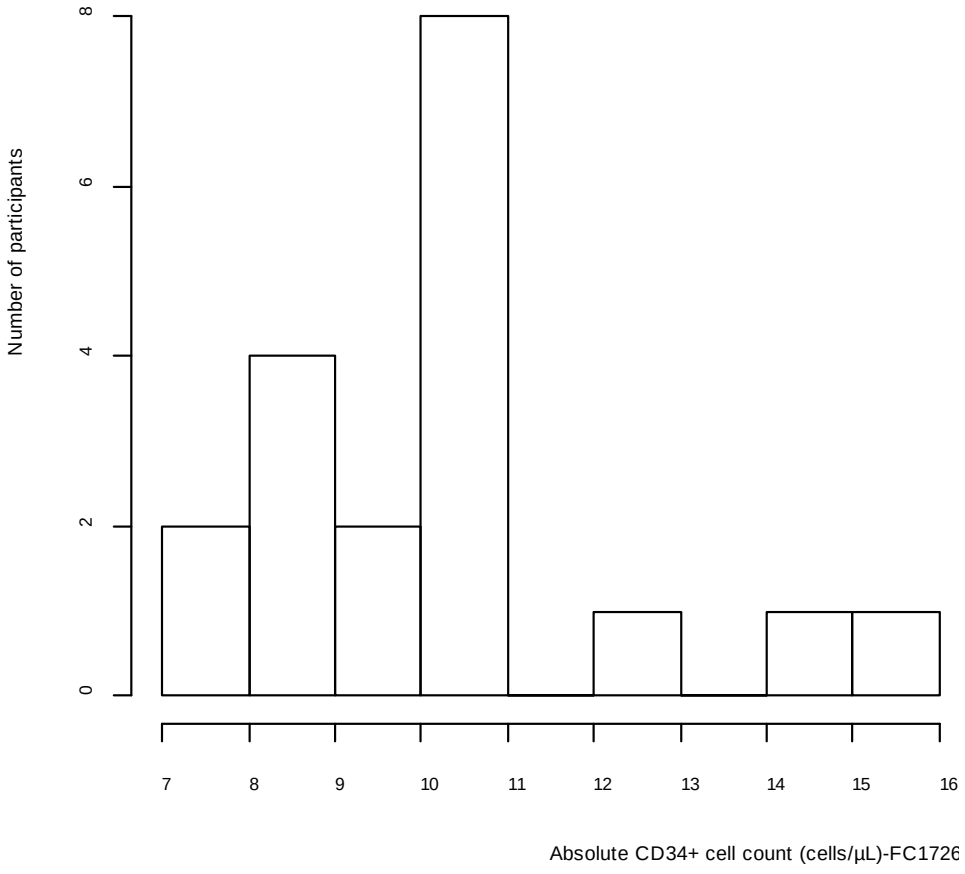
The following boxplots and histograms show these data graphically:





Data out of graph : 0.77

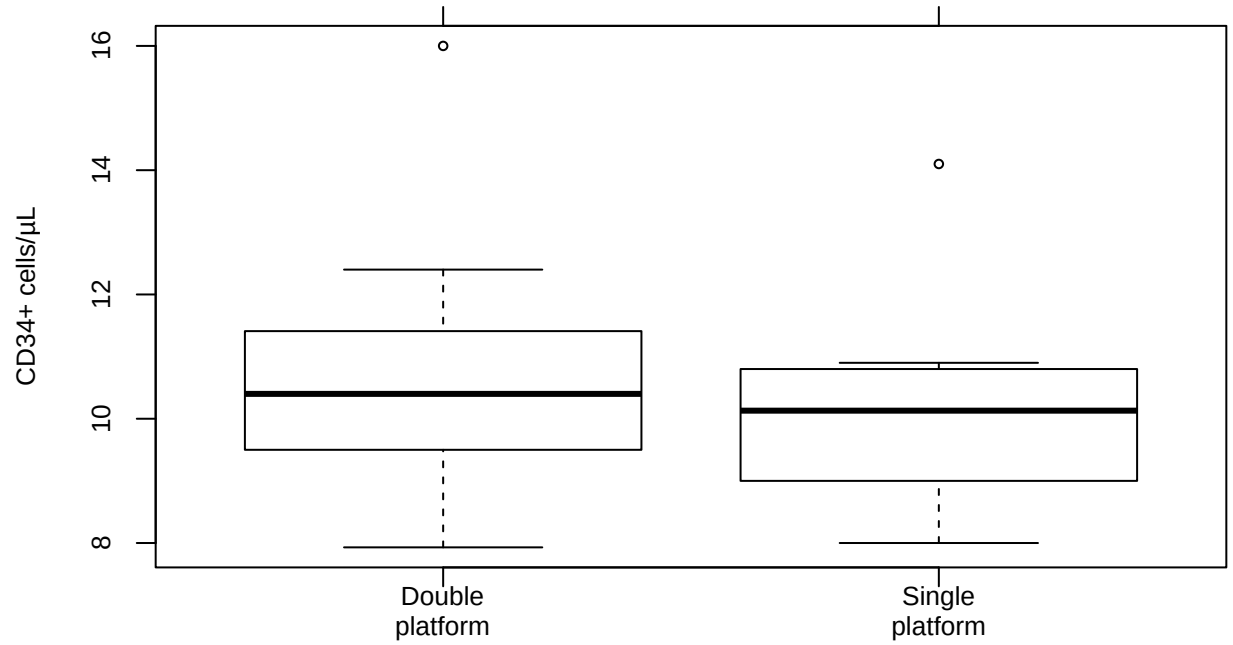

Absolute CD34+ cell count (cells/µL)
8
10
12
14
16


Number of participants
0
2
4
6
8
7
8
9
10
11
12
13
14
15
16
Absolute CD34+ cell count (cells/µL)-FC1726

The next table compares the results from the double (n=7) and single (n=12) platform users:

FC 17264

	Median cells/µL	CV %	P25 cells/µL	P75 cells/µL	Range cells/µL	N
Double platform	10.4	13.6	9.5	11.4	7.9-16.0	7
Single platform	10.1	11.5	9.0	10.6	8.0-14.1	12



The median WBC count obtained by the laboratories using a double platform approach was 9.9 10^9/L (n=7). The overall CV was 3.1%.

The next table shows the individual results (10^9/L) per type of haematology analyser:

Haematology analyser	FC 17264
Abbott Cell-Dyn Sapphire	9.5
	9.5
Sysmex XN 1000/XN 3000/XN 9000	10.2
	10.0
	10.0
	9.7
	10.0

The next table compares the absolute CD34+ cell counts obtained by the laboratories evaluating CD34+ cell viability (n=15) or not (n=2):

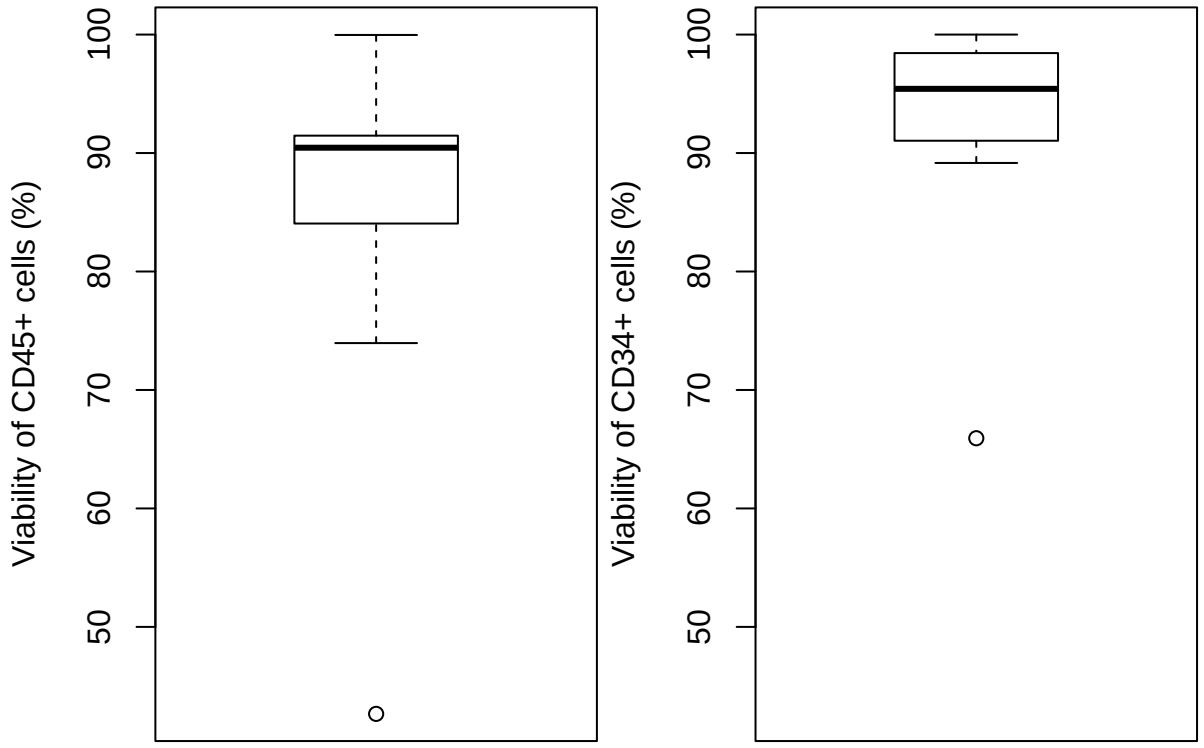
FC 17264

	Median/ *Results* cells/µL	**CV %**	**P25 cells/µL**	**P75 cells/µL**	**Range cells/µL**	**N**
Determination of viability	10.1	9.9	9.3	10.7	8.0-16.0	15
No determination of viability	*12.4* *7.9*					2

The median viability of CD45+ cells was found to be 90% and the median viability of CD34+ cells was found to be 96%.

FC 17264

	Median %	**CV %**	**P25 %**	**P75 %**	**Range %**	**N**
Viability of CD45+ cells	90	9.0	80	91	15-100	16
Viability of CD34+ cells	96	5.0	92	99	66-100	15



CONCLUSION

% CD34+ cells within total WBC (n=17)

One Belgian clinical laboratory (6%) obtained an unacceptable result (z-score >3 or <-3).

Absolute CD34+ cell count (n=17)

One Belgian clinical laboratory (6%) obtained an unacceptable result (z-score >3 or <-3).

Participant	FC 17264 % CD34+ cells	z-score	FC 17264 CD34+ cells/μL	z-score
1	0.77*	22.5	10.1	-0.023
2	0.16	1.892	16.0	4.407

*encoding error

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).

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[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.

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