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Professional paper

External Quality Assessment in Clinical Cell Analysis by Flow Cytometry. Why is It so Important?

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A B S T R A C T

Participation in external quality assessment is an integral part of laboratory work and mandatory when the results have a clinical application, which is one of the requirements of standard 15189 for accreditation of medical laboratories. Institute of Clinical Chemistry, the first laboratory accredited for clinical cell analysis by flow cytometry in Croatia, participated in UKNEQAS for Leukocyte Immunophenotyping in 3 schemes: »Immune Monitoring«, »CD34 Stem Cell Enumeration« and »Leukaemia Immunophenotyping«. For sample processing on EPICS XL flow cytometer, lyse/no wash preparation technique with ammonium chloride (NH_4Cl) or ImmunoPrep lysing reagent was employed. In »Immune monitoring« programme CD45/sideward light scatter (SSC) proposed gating strategy was adopted for lymphocyte subsets, while modified ISHAGE protocol was used for CD34+ cell enumeration. Absolute count determination was performed on flow cytometer using FlowCount beads solution. In the period from the beginning of 2006 until the middle of 2009 a total number of 100 stabilized whole blood samples were processed. The relative and absolute enumeration results for lymphocyte subsets were within tolerable limits, in 97.1 and 97.1% of cases, and 95 and 90% of CD34+ cell enumeration, respectively. In immune monitoring CD45/SSC proposed gating strategy is the most frequent analysis used (>85% participants) and ISHAGE protocol for CD34+ cell determination with continuous rise from 76 to 83%. A number of participants who accept beads method for absolute count enumeration on flow cytometer get greater, 69 to 86%, while FlowCount was the second of bead-based techniques used (25 and 35%). Sample treatment in lyse/no wash technique using NH_4Cl lysing solution was dominant procedure used by more than 1/3 participants, although its home made solution has replaced slowly by commercial reagents. The unacceptable results, 6 of 244, were obtained for 20 most frequently determined cell antigens in »Leukaemia Immunophenotyping« samples screened for leukaemia/lymphoma. Processing results of all participants showed that the deviation from laboratory guidelines and the use of older methods for cell identification, quantification of cell counting on haematology analyser, or usage an antibody conjugated with fluorochrome lesser fluorescence quantum often lead to an unacceptable result, although is noticeable trend to accept new referrals and protocols to reduce the inter-laboratory differences.

Key words: *external quality assessment, UKNEQAS for Leukocyte Immunophenotyping, laboratory guidelines*

Introduction

Clinical cell analysis by flow cytometry undergoes the same process of development, instrument and methods validation, internal control, external quality assessment (EQA) as any other laboratory medicine activity. Laboratory normization through certification or accreditation, present in the whole world, has become a reality with growing trend in Croatia. The previous standard ISO/IEC 17025 from 1999 is replaced with the present one ISO 15189 in 2003 and the majority of medical laboratories have been accredited according to them. They have entered the European and have become a part of Croatian legal regulations (HRN EN ISO17025 General requirement for competence of testing and calibrated laboratories, HRN ISO EN15189 Medical laboratories – Particular requirements for quality and competence)[1,2]. The Institute of Clinical Chemistry is the first medical

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laboratory in Croatia accredited for the field of cell immunophenotyping, for absolute cell count tests: CD3+ T-lymphocytes, CD4+ T-lymphocytes, CD8+ T-lymphocytes, B-lymphocytes, NK cells, Leukocytes (CD45), Stem Cells (CD34), and relative cell count tests: CD2+, CD3+, CD4+, CD5+, CD7+,CD8+, CD13+, CD14+, CD19+, CD33+, CD34+, HLA D/DR+, KAPPA light chain+ B-lymphocytes, LAMBDA light chain+B-lymphocytes. EQA is objective and necessary factor that compares and evaluates laboratory test results with the goal of assuring credibility of all laboratory performance aspects: sampling, analyzing and reporting, when the test results are included in clinical decision making[3–7]. In the norm 15189, there is also a requirement included in Article 5.6.4 »Assuring quality of examination procedures« that conditions participation of laboratory in international comparisons. One of today's existing internationally organized EQA is United Kingdom National External Quality Assessment Service (UKNEQAS) for Leukocyte Immunophenotyping. It is generally accepted world standard due to its robust organization and control apparatus; various control schemes that comprise different areas of laboratory activity like leukemia/lymphoma immunophenotyping, CD34+ stem cells enumeration, identification and quantification of lymphocyte populations, detection of minimal residual disease cells, detection of residual leukocyte count and other activities, but mostly due to its accreditation by the Clinical Pathology Accreditation (CPA) what represents a necessity for laboratories which want to be recognized, certified or accredited. UKNEQAS uses stabilized blood samples collected from different donors, processed and modified (e.g. selective elimination of cell populations) providing an image of pathological sample. It is extremely important that long-term stabilized samples have enabled elimination of time-, transportation- and storage-dependent factors and therefore that inter-laboratory differences originate from the applied procedure/analysis. This paper aims to show results of participation of the Institute of Clinical Chemistry in UKNEQAS for Leukocyte Immunophenotyping international control in 3 schemes on the area of cell immunophenotyping: Immune Monitoring, CD34+ Stem Cell Enumeration and Leukaemia Immunophenotyping with an overview on applied procedures and laboratory performance guidelines.

Materials and Methods

A total of 100 blood samples has been processed in the period from the beginning of 2006 until the middle of 2009 in cycles 0601–0903: 42 in scope the scheme »Immune monitoring«, 40 in »CD34+ Stem Cell Enumeration«, and 18 in the scheme »Leukaemia Immunophenotyping«. The scheme »Immune monitoring« includes several lymphocyte parameters: T-lymphocytes (CD3+), CD4+ (CD3+CD4+)T and CD8+(CD3+CD8+) T-lymphocytes, B-lymphocytes (CD19+) and NK cells (CD3-CD16+CD56+), and the scheme »CD34+ Stem Cell Enumeration« only one, CD34+ cells. Positivity results are expressed as percentage (%) and absolute number ($\times 10^6$/L). Samples were stained with antibodies anti -CD3, -CD4, -CD16, -CD56, -CD19, -CD45, Dako, anti-CD19, -CD3, -CD8, IQP, or anti-CD3, -CD45, -CD4, -CD8, -CD16, -CD56, -CD19, Beckman-Coulter for lymphocyte markers, anti-CD45, Dako or Beckman-Coulter, isotopic control, Dako, anti -CD34, BD Biosciences were used for determination of CD34+ cells. Before acquisition on flow cytometer samples were treated with ammonium chloride (NH_4Cl) or ImmunoPrep Reagent System (Coulter) lysing solution in the »lyse/no-wash« procedure. All measures were made on EPICS XL flow cytometer, Coulter. For immune status testing CDC recommended multicolor analysis was adopted[8,9] and for CD34+ cells quantification ISHAGE protocol was used[10]. The absolute cell count on flow cytometer (single platform absolute count-

TABLE 1
PRESENTATION OF RESULTS RELATED TO ACCEPTABLE LIMITS AND CONCENSUS MEAN VARIATION FROM 42 SAMPLES IN SCHEME »IMMUNE MONITORING«

Cell's type	Institute results (N)			Consensus means (CV)	
	inside		outside	average	range
	$\overline{X}$±SD	$\overline{X}$±2SD	>$\overline{X}$±2SD	$\overline{X}$	(min–max)
Percentage of lymphocytes (%)	179/210 (85.2%)	25/210 (11.9)	6/210 (2.9%)		
T lymphocyte (CD3+)	33	5	4	3.5	2.2–8.7
CD4+ T lymphocyte	38	4	0	6.8	3.0–23.2
CD8+ T l lymphocyte	37	4	1	7.4	5.1–14.9
B lymphocyte (CD19+)	32	10	0	15.5	8.7–28.5
NK cell (CD3-CD16+CD56+)	39	2	1	16.7	9.3–40.4
Absolute number ($\times 10^6$/L)	155/210 (73.8%)	49/210 (23.3%)	6/210 (2.9%)		
T lymphocyte (CD3+)	27	13	2	10.7	8.5–15.0
CD4+ T lymphocyte	28	12	2	12.2	8.7–2.2
CD8+ T lymphocyte	35	6	1	13.5	10.8–20.6
B lymphocyte (CD19+)	27	14	1	21.0	12.5–37.5
NK cell (CD3-CD16+CD56+)	38	4	0	21.6	14.0–41.7

TABLE 2
PRESENTATION OF RESULTS RELATED TO ACCEPTABLE LIMITS AND CONCENSUS MEDIAN VARIATION FROM 40 SAMPLES IN SCHEME »CD34+ STEM CELL ENUMERATION«

CD34+ cell	Institute results (N)			Consensus medians (CV)	
	inside		outside	average	range
	median±25 centile	median±40 centile	>median±40 centile	$\overline{X}$	min-max
Percentage (% of CD45+)	20 (50%)	18 (45%)	2 (5%)	11.2	4.9–25.0
Absolute number ($\times 10^6$/L)	22 (55%)	14 (35%)	4 (10%)	9.4	5.6–16.3

ing) is determined by the method with FlowCount beads reagent, Beckman-Coulter, a suspension of artificial particles of defined number[11–13]. The »Leukaemia Immunophenotyping« programme is divided into two parts: analytical (»Leukaemia Immunophenotyping«) and diagnostic (»Leukaemia Diagnosis Interpretation«). In the first part a number of positive cells is required for: 6 standard, 8 recommended and maximal 6 optional cell antigens, expressed as percentage in relation to the total number of mononuclear cells. The certain marker is defined as positive or negative using defined criteria by the British Committee for Standards in Hematology (BCSH)[14]. Applied antibodies were: anti -CD45, -CD2, -CD3, -CD4, -CD5, -CD7,-CD10, -CD11c, -CD14, -CD19, -CD20, -CD56, -CD79a,-CD103, -MPO, -HLA DR, -KAPPA, -LAMBDA (DAKO), anti –CD3, -CD4, -CD7, -CD8, -CD10, -CD19, –CD23, -CD25, -CD33, -CD34, -CD38, -CD56, -CD79ß, -CD117, -CD138 (Beckman-Coulter), anti -CD13, -CD33, -CD34, -CD117, -HLA DR (BD Biosciences), or anti -CD23, -CD19 and -TdT (IQP). A total of 18 cycles each with one sample was performed: 8 with final diagnosis of acute leukemia (7 acute myeloid leukemia, AML and 1 plasma cell leukemia, PCL), and 10 of lymphoproliferative disease (6 B-chronic lymphocytic leukemia, B-CLL, 2 B-cell prolymphocytic leukemia, B-PLL, 1 T-prolymphocytic leukemia, T-PLL and 1 with hairy cell leukemia, HCL). After staining the samples were lysed by NH_4Cl solution and the analysis on flow cytometer was done according to CD45/sideward scatter (SSC) method for cells identification[14]. In the part »Leukaemia Diagnosis Interpretation« diagnosis is made on the basis of case history data, morphology images, consensus immunophenotype, cytogenetics and results of molecular diagnosis.

Results

During the period from the beginning of 2006 until the middle of 2009 in 21 cycles of the scheme »Immune monitoring« 42 venous blood samples for assessing numbers of lymphocyte subsets expressed as absolute value and percentage were processed. Organizer's acceptance criteria are $\overline{X}$±1 SD as optimal value, and $\overline{X}$±2 SD as acceptable. Coefficient of variation (CV) is variable, dependent on consensus results in certain sample. Table 1 shows the results. For markers there were 179 results, out of 210 possible results (85.2%), expressed as percentage which were within optimal values and 6 results were outside the allowed limits (2.9%). 155 results for absolute cell count (73.8%) were within target values and 6 were outside the allowed limits In the scheme »CD34+ Stem Cell Enumeration« there were 40 determinations of absolute cell count and their portion expressed as percentage to all CD45+ cells. Results are presented in Table 2. The acceptance criterion was median ±25 centile for target values and the acceptable result had to be within range median ±40 centile and it varied with every sample. There were 20 (50%) results for percentage and 22

TABLE 3
PRESENTATION OF 20 MOST FREQUENTLY DETERMINED CD ANTIGENS RESULTS RELATED TO ACCEPTABLE LIMITS FROM 18 SAMPLES IN SCHEME »LEUKAEMIA IMMUNOPHENOTYPING«

CD antigen(s)	Inside median ±40 centile (N)	Wrongly assigned (N)	Consensus assignment
Percentage (% of mononuclear cells)	238/244 (97.5%)	6/244 (2.5%)	
CD2	16/16	0	15 neg/1 pos
CD3	18/18	0	17 neg/1 pos
CD5	18/18	0	11 neg/7 pos
CD7	15/16	1 false neg	12 neg/4 pos
CD10	16/16	0	15 neg/1 pos
CD13	18/18	0	11 neg/7 pos
CD14	9/9	0	8 neg/1 pos
CD19	17/18	1 false pos	8 neg/10 pos
CD20	15/16	1 false neg	7 neg/9 pos
CD23	8/8	0	3 neg/5 pos
CD33	8/8	0	1 neg/7 pos
CD34	8/8	0	1 neg/7 pos
CD38	8/8	0	5 neg/3 pos
CD45	16/16	0	0 neg/16 pos
CD117	7/7	0	3 neg/4 pos
HLA D/DR	16/16	0	3 neg/13 pos
MPO	9/9	0	3 neg/6 pos
TdT	6/6	0	5 neg/1 pos
KAPPA	5/7	2 false neg	0 neg/7 pos
LAMBDA	5/6	1 false pos	2 neg/4 pos

Neg – negative, pos – positive, according to positive/negative cut off point of 20% in acute leukemias, and 30% in chronic lymphoproliferative disorders

(55%) for absolute count of CD34+ cells within optimal values and outside the limits there were 2 (5%) and 4 (10%) results for relative and absolute count, respectively. Table 3 shows results of 20 most frequently determined antigens in our laboratory in 18 samples in the scope of the scheme »Leukaemia Immunophenotyping«. The result acceptance criterion was median ±25 centile, that is within limits of ±40 centile, under condition that they are in the consensus positive or negative area, whereas criteria for positivity/negativity were from ≥20% for acute leukemia and ≥30% for chronic lymphoproliferative diseases. That was all conditioned by the organizer and BCSH[13]. Out of 244 results for 20 most common markers, 6 (2.5%) of them were false positive or negative while the rest was within target and allowed limits.

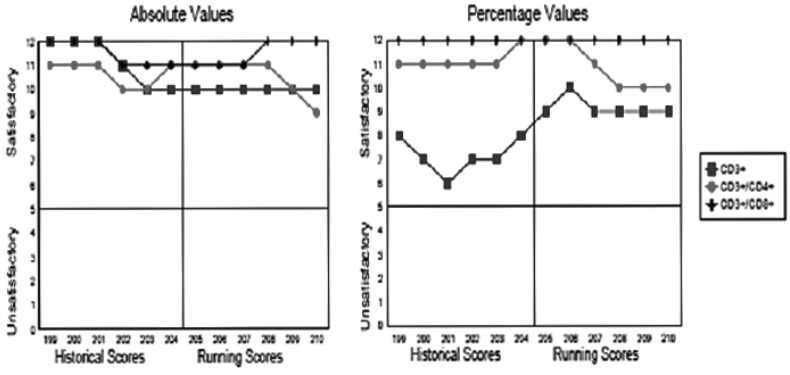
Discussion

Requirements from the Chapter 5 »Technical requirements« of the norm 15189 Medical laboratories – Particular requirements for quality and competence placed before accredited laboratory relate to instruments used in examination, methods and procedures for analysis, handling the samples – before, during and after the analysis, internal controls, reporting on obtained test results, evidences of performed actions and EQA[5–7,11–14,16–17]. EQA

	Absolute Values			
	Result (cells/µl)	Score	Running Score	Performance
CD3+ Lymphocytes	822	2	10	Satisfactory
CD3+/CD4+ Lymphocytes	223	1	9	Satisfactory
CD3+/CD8+ Lymphocytes	548	2	12	Satisfactory
CD19+ Lymphocytes	288	2	9	Satisfactory
CD16+/CD56+ Lymphocytes	246	1	11	Satisfactory

	Percentage Values			
	Result (%)	Score	Running Score	Performance
CD3+ Lymphocytes	60.00	2	9	Satisfactory
CD3+/CD4+ Lymphocytes	16.30	2	10	Satisfactory
CD3+/CD8+ Lymphocytes	40.00	2	12	Satisfactory
CD19+ Lymphocytes	21.70	2	9	Satisfactory
CD16+/CD56+ Lymphocytes	17.90	1	10	Satisfactory

Performance Graphs



Overall Statistics Tables

	Absolute Values cells/µl				Percentage Values			
	Returns	Mean	Trimmed Mean	Trimmed SD	Returns	Mean	Trimmed Mean	Trimmed SD
CD3+ Lymphocytes	479	776	766	74	426	61.69	61.53	1.82
CD3+/CD4+ Lymphocytes	510	202	195	23	452	16.85	15.74	1.02
CD3+/CD8+ Lymphocytes	462	513	506	57	425	40.89	40.78	2.29
CD19+ Lymphocytes	318	256	256	36	334	20.11	20.14	1.79
CD16+/CD56+ Lymphocytes	297	198	194	30	312	15.32	15.49	1.62

Fig. 1. Performance scoring system with positive point's award for each individual values in current and last 6 determinations coupled with overall statistics from »Immune Monitoring« scheme report. (With kindly permission of UKNEQAS for Leukocyte Immunophenotyping.)

Overall Results

	Returns	Median	Lower Quartile	Upper Quartile
CD34+ Percentage	245	0.12	0.11	0.14
CD34+ Absolute (cells/ul)	254	10.00	9.00	11.00
White Cell Count	204	8.00	7.61	8.66
Total Events	253	100000	77282	146018

Performance

	Score for sample	Running Score (Last 3 Samples)	Overall Performance
CD34+ Absolute	20	75	Satisfactory
Non Returns	0	0	Satisfactory

Centile Ranges CD34+ Absolute

5%	10%	25%	Median	75%	90%	95%
7.200	7.800	9.000	10.000	11.000	12.500	13.658

Fig. 2. Performance score with negative point penalty for CD34+ absolute number in current and last 3 samples with consensus results for each testing parameters given in „CD34+ Stem Cell Enumeration« scheme report. (With kindly permission of UKNEQAS for Leukocyte Immunophenotyping.)

assures measurements to be reliable in all phases of laboratory process that includes sampling, testing and reporting in case when results of analysis are involved in clinical decision making. UKNEQAS for Leukocyte Immunophenotyping, CPA accredited organizer of EQA on the field of cell immunophenotyping represents nowadays generally accepted standard with diverse schemes which evaluate independently and objectively different tests with application for clinical purposes like leukemia/lymphoma immunophenotyping, CD34+ stem cells

Overall Results					Your Results				
CD No.	Returns	Median	Lower Quartile	Upper Quartile	Result	Positive or Negative *	Score	Running Scores	
								Performance	Non-Returns
CD2	213	1	0	4	4.00	-	0	20	0
CD3	213	2	0	3	4.00	-	10	20	0
CD5	213	2	0	3	4.00	-	10	20	0
CD13	213	50	40	72	92.00	+	20	20	0
CD19	213	1	0	2	2.00	-	10	20	0
CD20	213	1	0	2	2.00	-	10	10	0
CD117	198	2	1	2	2.00	-	0	0	0
Myeloperox	191	30	14	51	31.00	+	0	0	0
CD4	199	73	38	84	77.00	+	0	10	0
CD33	206	93	89	97	93.00	+	0	0	0
CD34	207	0	0	1	1.00	-	10	10	0
HLADR	205	58	40	80	97.00	+	40	50	0
CD14	203	79	73	86	83.00	+	0	0	0
CD7	38	1	0	3	4.00	-	n/a	n/a	n/a
CD11c	25	93	85	95			n/a	n/a	n/a
CD45	39	100	99	100	100.00	+	n/a	n/a	n/a
CD15	46	76	15	92			n/a	n/a	n/a
CD11b	37	92	86	96			n/a	n/a	n/a
CD56	36	13	2	34	2.00	-	n/a	n/a	n/a

* Assigned by your value being equated to the positive/negative cut off point of 20%. See "Revised Guideline on Immunophenotyping in acute leukaemias and Chronic Lymphoproliferative Disorders".
General Haematology Task Force of the BCSH. Clinical and Laboratory Haematology 2002, 24, 1-13.

Fig. 3. Performance scoring system with negative point penalty for each individual antigens in current and 3 last samples compared to overall results from »Leukaemia Immunophenotyping« scheme report. (With kindly permission of UKNEQAS for Leukocyte Immunophenotyping.)

analysis, CD4+ T-cell count and others. Beside result evaluation, this assessment includes numerical and graphic diagrams, charts and performance scoring with results from the last 3 cycles. Figures 1, 2 and 3 show examples from 3 different schemes. There is also a consultative aspect of this EQA that recommends acceptance of adequate methods and helps in control of factors that influence the accuracy and reproducibility of cell identification and quantification. Today it is possible to assure satisfying lymphocyte purity and recovery, >90%, by implementing the CD45/SSC gating strategy for cells identification proposed by CDC[9–10]. The method is also recommended by the Working Group for Laboratory Immunology that is active in scope of Croatian Chamber of Medical Biochemists[15] and it has been used in laboratory practice. In the scheme »Immune Monitoring« more than 85% participants apply this method, while at the same time the number of laboratories using older methods, forward-scatter (FSC)/sideward-scatter (SSC) and CD45/CD14 leuko gate, for lymphocyte gating is decreasing from 11.3 to 0.8%, regardless the increase in participant number from 350 to 500 in the period from the beginning of 2006 until the middle of 2009. The second important information from this scheme is that the number of participants which use flow cytometer to determine absolute cell count by beads[11–12] has been continuously increasing from 69 to 86% (Figure 4). FlowCount beads used in our measurements for determination of absolute cell count on flow cytometer is the second used method in line according to frequency of use, about 25%. Analysis performed using older methods and generating absolute cell count from lymphocyte or leukocyte count on hematological counter are factors related to frequent result deviation from allowed limits. There was no influence of lysator type observed to obtained results, ammonium chloride *vs*. ImmunoPrep reagents, nor the influence of diversity of antibody and conjugated fluorochrome manufacturers what indicates that the quality of the reagents for analyses of well known cell antigens on the market is uniform, and it depends rather on individual choice which reagent will be used. Out of total count 85.2% of our results for relative and 73.8% for absolute cell count were within optimal values, and outside allowed limits were 6% and 6% respectively, due to momentary analyzer technical condition or antibody combination, however not due to system error. Average CV for relative cell

Absolute Count Determination by Platform cells/µl

	CD3+ Lymphs				CD3+/CD4+ Lymphs				CD3+/CD8+ Lymphs				CD19+ Lymphs				CD16+/CD56+ Lymphs			
Method	Returns	Mean	SD	CV	Returns	Mean	SD	CV	Returns	Mean	SD	CV	Returns	Mean	SD	CV	Returns	Mean	SD	CV
All	466	766	143	19	494	199	57	28	449	506	94	19	307	255	63	25	287	193	45	23
Single	398	765	110	14	424	199	54	27	381	506	71	14	252	257	51	20	236	193	34	18
TRUCOUNT	185	790	100	13	187	204	43	21	182	530	64	12	137	268	28	11	136	205	24	12
FACSCOUNT	61	730	98	13	65	181	18	10	48	481	46	9	2	294	23	8	1	188	0	0
FLOW COUNT	93	746	127	17	105	202	66	33	95	482	77	16	80	240	74	31	69	169	39	23
PERFECT COUNT	1	826	0	0	1	207	0	0	1	619	0	0	1	337	0	0	1	194	0	0
CYTORON	0	0	0	0	2	334	187	56	1	406	0	0	0	0	0	0	0	0	0	0
OTHER	4	766	46	6	5	148	73	49	3	467	25	5	2	249	36	15	2	178	6	4
CYTOCOUNT	3	772	109	14	3	191	25	13	3	495	52	11	3	280	34	12	2	234	44	19
Dual	68	768	265	34	70	197	72	37	68	506	174	34	55	246	102	42	51	192	79	41
HAEM. WBC	50	756	293	39	50	193	78	40	49	491	187	38	39	243	107	44	36	192	86	45
HAEM. LYMPH	16	805	177	22	18	210	58	28	17	548	138	25	14	255	99	39	13	186	65	35

Percentage Count by Gate

	CD3+ Lymphs				CD3+/CD4+ Lymphs				CD3+/CD8+ Lymphs				CD19+ Lymphs				CD16+/CD56+ Lymphs			
Method	Returns	Mean	SD	CV	Returns	Mean	SD	CV	Returns	Mean	SD	CV	Returns	Mean	SD	CV	Returns	Mean	SD	CV
CD3	2	61.75	54.09	87.60	10	23.35	22.96	98.34	5	56.10	24.32	43.35	0	0.00	0.00	0.00	0	0.00	0.00	0.00
CD4	0	0.00	0.00	0.00	3	27.01	18.43	68.24	0	0.00	0.00	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
CD4/FSc	0	0.00	0.00	0.00	1	19.00	0.00	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00
CD4/SSC	2	63.94	1.50	2.34	4	18.44	7.19	39.02	2	34.58	10.71	30.99	1	21.21	0.00	0.00	1	15.08	0.00	0.00
CD45/14	2	62.39	0.54	0.87	2	16.03	0.04	0.22	2	40.82	1.68	4.11	2	19.70	1.85	9.37	1	15.00	0.00	0.00
CD45/SSC	382	61.69	2.50	4.06	381	15.85	1.31	8.25	377	40.64	2.49	6.12	303	20.19	3.29	16.27	284	15.33	2.01	13.10
Facscount Gating	5	61.30	2.35	3.83	5	15.79	1.40	8.85	5	43.34	1.61	3.72	4	21.58	1.22	5.66	3	16.35	1.17	7.14
FSC/SSC	29	61.43	4.35	7.08	35	20.94	30.66	146.42	29	42.29	6.83	16.16	22	18.59	2.96	15.90	21	14.87	3.23	21.73
Panleucogate	3	61.30	0.89	1.45	7	14.63	1.87	12.75	4	35.88	9.59	26.74	2	21.15	1.20	5.68	2	16.35	0.21	1.30
Partec Gating	0	0.00	0.00	0.00	1	186.00	0.00	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00	0	0.00	0.00	0.00

Fig. 4. Methods comparisons for absolute number determination by platform and percentage count by gate used in »Immune Monitoring« scheme. (With kindly permission of UKNEQAS for Leukocyte Immunophenotyping.)

count is lower than CV for absolute cell count, what is detailed described in Table 1. The lowest CV was calculated for CD3+ cells (%) and amounted in average 3.5% with the lowest value of all parameters that was 2.2%. The highest average CV values for CD3+(%), CD4+(%) and NK cells(%) were obtained expectedly in samples with the lowest determined value of 48.21, 2.89 and 4.21 respectively, different than for CD8+(%) and B-lymphocytes(%) where the results were within normal area, 25.04 and 10.24, respectively. For absolute cell count the average CV for all parameters was higher than 10% and the lowest was for CD3+(abs) 10.7%, followed by CD4(abs) 12.2%, CD8(abs) 13.5%, B-lymphocytes(abs) 21.0% and for NK cells(abs) 21.6%. The highest CV for CD4+(abs) and NK cells(abs) was 26.2 and 41.7% respectively and was calculated from the samples with the lowest cell count (60 for CD4+ and 36×10^6/L for NK cells). Introduction of CD34 marker for identification and quantification of stem cells brought the possibility of performing analysis on flow cytometer, however only ISHAGE protocol provided procedure standardization and result reproducibility[10]. Normally, there is very small number of CD34+ cells and therefore, the basis of ISHAGE protocol is to include multiple logical gates to get the purest possible population by excluding autofluorescence of cells, non-specific binding of antibodies, platelets aggregates and optical noise of instrument. Modification of this protocol has enabled getting absolute CD34+ cells count on flow cytometer what is also recommendation of the scheme organizer and the Working Group for Laboratory Immunology of the Croatian Chamber of Medical Biochemists[13,15,18–19] and it has been used in laboratory practice. Since the beginning of 2006 the number of participants in the scheme »CD34+Stem Cell Enumeration« that used ISHAGE protocol has been increased from 76 to 80% and at the same time the number of participants that apply single platform for absolute cell count has also significantly increased from 73% to 83%. FlowCount is in this scheme also the second used method in line according to frequency what makes 35%. Analysis not performed under ISHAGE protocol and generating absolute cell count from leukocyte count on hematological counter are factors related to frequent result deviation from allowed limits. There was no influence of applied antibodies for CD34 observed, although in our case the antibody anti CD45, Dako, showed poorer quality in discrimina-

CD34+ Absolute Count Determination By Platform

(All values given as Cells/µl)

Method	Total CD34+ Absolutes				
	Returns	Median	Lower Quartile	Upper Quartile	CV
All	244	27.39	25.22	30.00	17.45
Single	186	27.64	25.23	30.00	17.25
TRUCOUNT	89	27.40	25.24	30.61	19.60
FLOW COUNT	74	27.97	25.70	30.00	15.37
Dual	58	27.12	25.15	29.15	14.77
HAEMATOLOGY ANALYSER	57	27.10	25.10	29.20	15.13

Gating Strategies	CD34+ Percentage		CD34+ Absolute (cells/ul)	
	Returns	Median	Returns	Median
ISHAGE	205	0.220	212	27.170
STEM KIT	27	0.230	28	28.300
PROCOUNT	7	0.228	7	31.800
MILAN PROTOCOL	10	0.225	10	27.750

Preparation Method	CD34+ Percentage				CD34+ Absolute (cells/ul)			
	Lysed & Washed		Lysed No Wash		Lysed & Washed		Lysed No Wash	
	Returns	Median	Returns	Median	Returns	Median	Returns	Median
ALL	50	0.22	198	0.22	50	26.85	206	27.73
Facslyse	20	0.22	12	0.23	20	26.09	13	27.43
Ammonium Chloride (NH4CL)	20	0.23	75	0.23	20	27.20	77	28.36
Stem Kit Lyse	0	0.00	39	0.23	0	0.00	41	27.99
Quicklysis	0	0.00	7	0.21	0	0.00	7	26.00
Pharmlyse	6	0.22	41	0.21	6	26.56	44	27.55
Dako Easylyse	2	0.22	11	0.22	2	26.25	11	26.50
Versalyse	0	0.00	7	0.21	0	0.00	7	26.20

Fig. 5. Methods comparisons for CD34+ absolute number and/or percentage count determination by platform, gating strategy or preparation method for current sample in »CD34+ Stem Cell Enumeration« scheme. (With kindly permission of UKNEQAS for Leukocyte Immunophenotyping.)

tion between leukocytes *vs*. no-leukocytes and it was replaced by Coulter's. According to the organizer's opinion, lysator carries also significant influence on results variability (Figure 5). Vast majority of participants applies ammonium chloride or its commercial preparations due to its minimal influence on leukocytes and fluorescence intensity, although sometimes homemade solutions give higher results, what is also observed in our case, and have been gradually replaced with commercial reagents with decreasing tendency from 43 to 38%. This is a control with a very low CVs, average CV for relative was 11.2 and for absolute count was 9.4%, regarding the higher complicity of the method in comparison with immune profile cell analysis and enumeration of cells that are normally present in very small number. The lowest value was 2.58×10^6/L (for absolute count) and 0.04% (for relative count) and the highest values were 110.00×10^6/L and 2.24%, the average was within range of $10–30\times10^6$/L and 0.20–0.50%, respectively (Figure 6). Cell immunophenotyping on flow cytometer has become a routine practice in management and monitoring of patients with hematological malignancy. Technical progress in the last years regarding fluidity, laser technology, optics, analog and digital data transmission, computers, software, fluorochromes and new antibodies has introduced mass application of complex multicolor analysis by flow cytometry in diagnosis of leukemia and lymphoma[19]. Following there events, certain groups have developed recommendations about sample processing methods, relevant antigens, choice of adequate antibodies and panel generation, analysis and reporting on obtained results[4–7,14,20,21]. In 2006 Subdivision for Laboratory Hematology and Cytology of the National Group for Hematology and Transfusion (today: Croatian Co-operative Group for Hematologic Diseases KROHEM) developed a diagnostic algorithm for Non-Hodgkin lymphoma (NHL) and acute leukemia (AL) with recommendation of diagnostically valuable marker, and in 2007 the procedure »Standard

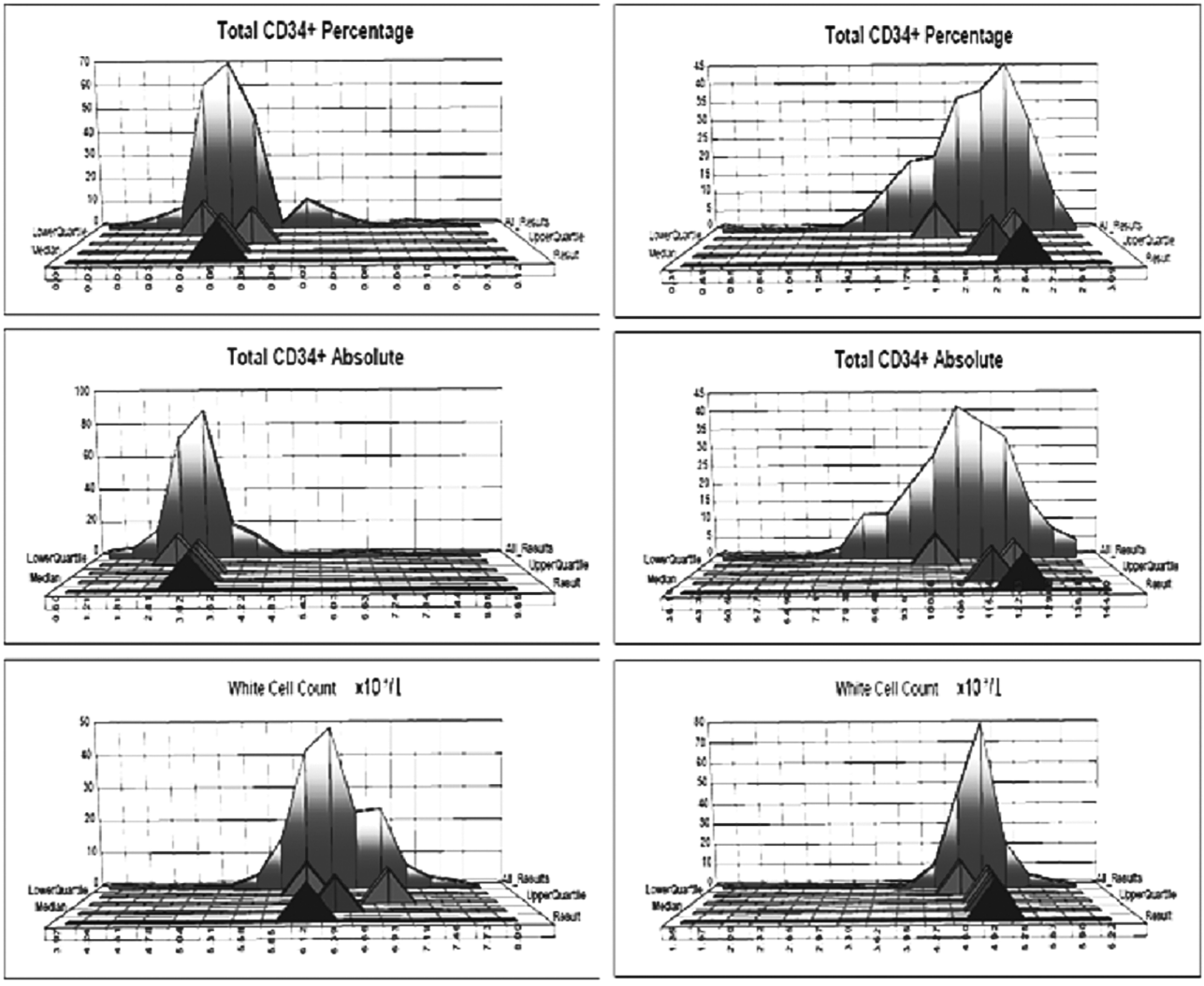


Fig. 6. Graphical interpretation of cumulative performance score for CD34 percentage and absolute number, and leukocytes determined on hematology analyzer from »CD34+ Stem Cell Enumeration« scheme. (With kindly permission of UKNEQAS for Leukocyte Immunophenotyping.)

Approach to Diagnosis and Treatment of Acute Leukemia« was accepted on the national level, where routine diagnostic methods were defined, with cell immunophenotyping among others. In the scheme »Leukaemia Immunophenotyping« where it is required to identify 20 different cellular markers in every sample, regardless of the fact that in samples there is dominant blast cell population, the main requirement to fulfil is to conduct CD45/side-scatter (SSC) analysis. The number of antigens to determine in each sample is higher than 30 between each cycle what implies that participants use different recommendations or wide panels, regardless of the assumed diagnosis. There were no differences in results that would depend on instrument manufacturers: BD

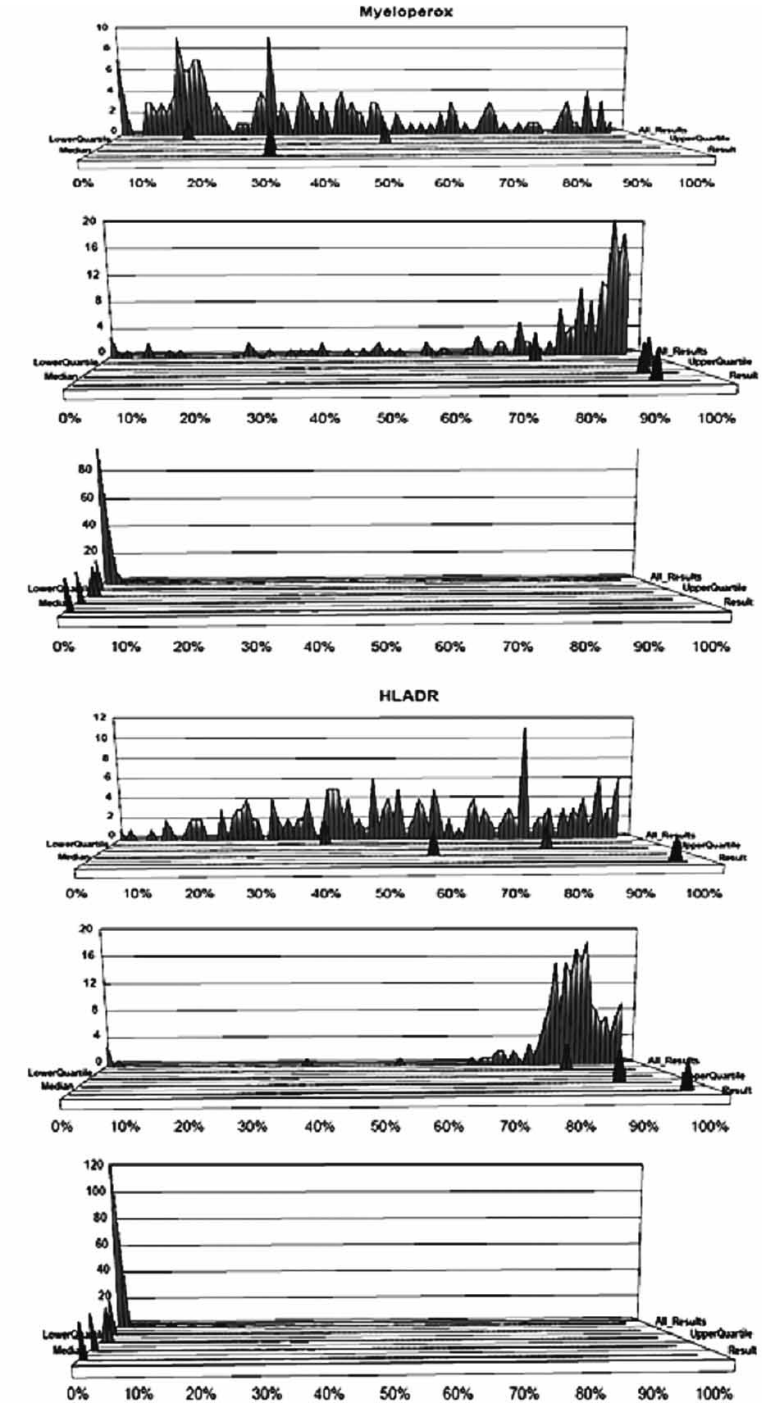


Fig. 7. Graphical interpretation of cumulative performance score for 2 cell antigens in 3 different cases: 2 positive and 1 negative. Myeloperox – myeloperoxidase, HLADR – HLA D/DR. (With kindly permission of UKNEQAS for Leukocyte Immunophenotyping.)

Biosciences, Beckman Coulter and Dako. Those differences originated from applied combinations/panels and choice of antibody/fluorochrome (Figures 8) and organizer's recommendation is (»...This programme has for many years been highlighting these issues and we urge participants to carefully select the antibody/fluorochrome. It should be noted that whilst say CD22 FITC conjugate may work well with normal B-lymphocytes and provide a good signal to noise separation, the expression of this antigen, as well as many others, can be highly variable in cellular development and the leukaemic process and thus may not be as highly conserved or as densely expressed...«) to use antibodies with fluorochrome of higher intensity for poorly expressed markers (PE or PC5 instead FITC). Our test results for markers which showed deviations outside of consensus acceptable values: negative CD20 in B-CLL, negative CD7 in AML, positive CD19 in AML, negative KAPPA and positive LAMBDA in B-CLL, are also a consequence of insufficiently clear positive/negative stratification in cases of antigen's weak expression in stabilized leukemic control samples and therefore, they are a consequence of false interpretation of raw measurement data. The problem was solved by using antibodies from another manufacturer/fluorochrome what was also apparent on results in following cycles. In the second part »Leukaemia Diagnosis Interpretation« which is in pilot phase establishment of diagnosis is conducted, based on consensus immunophenotype, case history data, digital blood/bone marrow smears for morphological analysis, cytogenetics and molecular genetics data, in case it is performed, by choosing one of offered diagnosis according to the Classification of Hematological Malignancies issued by the World Health Organization (WHO)[22]. In our case, physicians were involved in establishing diagnosis: hematologist, cytologist, and medical biochemist. Up to now there have not been any discrepancies in diagnoses. Educative character of this part is priceless because the explanation of definitive, consensus diagnosis includes comments and conclusions of one or more experts that are made on basis of relevant data and compared to the definition of WHO.

Conclusion

EQA is an objective and necessary factor that compares and evaluates laboratory test results with the goal of credibility improvement of test results and their comparability throughout the world. By application of recommended procedures and protocols in clinical cell analysis by flow cytometry what includes sample preparation for measuring and analysis on instrument, it is possible to assure a high level of inter-laboratory compliance with complete reliability of obtained test results what is also apparent in our example.

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VANJSKA PROCJENA KVALITETE U PODRUČJU IMUNOFENOTIPIZACIJE STANICA. ZAŠTO JE TOLIKO VAŽNA?

S A Ž E T A K

Sudjelovanje u vanjskoj procjeni kvalitete je sastavni dio rada laboratorija za imunofenotipizaciju stanica i neophodan u slučaju kada rezultati imaju kliničku primjenu, što je jedan od zahtjeva važeće norme 15189 za akreditaciju medicinskih laboratorija. Zavod za kliničku kemiju, prvi akreditirani laboratorij za područje imunofenotipizacije stanica u RH, sudjeluje u UKNEQAS for Leukocyte Immunophenotyping kontroli u 3 sheme: »Immune Monitoring«, »CD34+ stem Cell Enumeration« i »Leukaemia Immunophenotyping«. Za analizu na protočnom citometru EPICS XL uzorci su prethodno obrađeni amonij kloridom (NH_4Cl) ili ImmunoPrep lizatorom »liziraj/ne ispiri« metodom. U programu »Immune Monitoring« CD45/postranično raspršenje (SSC) je korišteno za analizu limfocita, a modificiran ISHAGE protokol za brojanje CD34+ stanica. Apsolutni broj je određen na citometru s FlowCount beads reagensom. U periodu od početka 2006 do sredine 2009. godine obrađeno je ukupno 100 uzoraka. Od ukupno 210 rezultata relativnog i apsolutnog broja limfocitnih stanica upotrebom preporučene CDC višebojne analize s CD45/SSC ograđivanjem stanica i FlowCount reagensom za apsolutni broj dobiveni su rezultati bili unutar dozvoljena raspona u 97,1 i 97,1% slučajeva, odnosno u 95 i 90% kod brojanja CD34+ stanica prema ISHAGE protokolu. U »Immune Monitoring« shemi najčešće je korištena CD45/SSC metoda ograđivanja (>85% učesnika), a ISHAGE protokol za određivanje CD34+ stanica sa stalnim porastom, 76 na 83%. Istovremeno je porastao i broj učesnika koji su prihvatili beads-metodu za apsolutni broj stanica s citometra, 69 na 86%, pri čemu je FlowCount druga tehnika po učestalosti (25 i 35%). Priprema uzorka NH_4Cl lizatorom u »liziraj/ne ispiri« tehnici je dominantan postupak i koristi ga više od 1/3 učesnika, iako se vlastoručni pripravci polako zamjenjuju komercijalnim. U shemi »Leukaemia Immunophenotyping« u 18 uzoraka neprihvatljivi rezultati su dobiveni u 6 od 244 rezultata za 20 najčešće određivanih staničnih antigena. Obrada rezultata svih učesnika pokazuje da je odstupanje od laboratorijskih smjernica i korištenje starijih metoda identifikacije stanica, kvantifikacija stanica brojanjem na hematološkom analizatoru uz korištenje protutijela vezanih fluorokromom slabije fluorescencije često uzrok neprihvatljivih rezultata.