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Current Status of Flow Cytometric Immunophenotyping of Hematolymphoid Neoplasms in Korea

Mikyoung Park, M.D., Ph.D.[1], Jihyang Lim, M.D., Ph.D.[1], Ari Ahn, M.D., Ph.D.[2], Eun-Jee Oh, M.D., Ph.D.[3], Jaewoo Song, M.D., Ph.D.[4], Kyeong-Hee Kim, M.D., Ph.D.[5], Jin-Yeong Han, M.D., Ph.D.[5], Hyun-Woo Choi, M.D., Ph.D.[6], Joo-Heon Park, M.D.[6], Kyung-Hwa Shin, M.D., Ph.D.[7], Hyerim Kim, M.D., Ph.D.[7], Miyoung Kim, M.D., Ph.D.[8], Sang-Hyun Hwang, M.D., Ph.D.[8], Hyun-Young Kim, M.D., Ph.D.[9], Duck Cho, M.D., Ph.D.[9], and Eun-Suk Kang, M.D., Ph.D.[9]

[1]Department of Laboratory Medicine, Eunpyeong St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea; [2]Department of Laboratory Medicine, Incheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea; [3]Department of Laboratory Medicine, Seoul St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Seoul, Korea; [4]Department of Laboratory Medicine, Severance Hospital, Yonsei University College of Medicine, Seoul, Korea; [5]Department of Laboratory Medicine, Dong-A University Hospital, College of Medicine, Dong-A University, Busan, Korea; [6]Department of Laboratory Medicine, Chonnam National University Hwasun Hospital, Chonnam National University Medical School, Hwasun, Korea; [7]Department of Laboratory Medicine, Pusan National University Hospital, Pusan National University School of Medicine, Busan, Korea; [8]Department of Laboratory Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Korea; [9]Department of Laboratory Medicine and Genetics, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, Korea

Background: Flow cytometric immunophenotyping of hematolymphoid neoplasms (FCI-HLN) is essential for diagnosis, classification, and minimal residual disease (MRD) monitoring. FCI-HLN is typically performed using in-house protocols, raising the need for standardization. Therefore, we surveyed the current status of FCI-HLN in Korea to obtain fundamental data for quality improvement and standardization.

Methods: Eight university hospitals actively conducting FCI-HLN participated in our survey. We analyzed responses to a questionnaire that included inquiries regarding test items, reagent antibodies (RAs), fluorophores, sample amounts (SAs), reagent antibody amounts (RAAs), acquisition cell number (ACN), isotype control (IC) usage, positive/negative criteria, and reporting.

Results: Most hospitals used acute HLN, chronic HLN, plasma cell neoplasm (PCN), and MRD panels. The numbers of RAs were heterogeneous, with a maximum of 32, 26, 12, 14, and 10 antibodies used for acute HLN, chronic HLN, PCN, ALL-MRD, and multiple myeloma-MRD, respectively. The number of fluorophores ranged from 4 to 10. RAs, SAs, RAAs, and ACN were diverse. Most hospitals used a positive criterion of 20%, whereas one used 10% for acute and chronic HLN panels. Five hospitals used ICs for the negative criterion. Positive/negative assignments, percentages, and general opinions were commonly reported. In MRD reporting, the limit of detection and lower limit of quantification were included.

Conclusions: This is the first comprehensive study on the current status of FCI-HLN in Korea, confirming the high heterogeneity and complexity of FCI-HLN practices. Standardization of FCI-HLN is urgently needed. The findings provide a reference for establishing standard FCI-HLN guidelines.

Key Words: Flow cytometry, Hematolymphoid neoplasm, Immunophenotyping, Minimal residual disease, Plasma cell neoplasm

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Corresponding author:
Jihyang Lim, M.D., Ph.D.
Department of Laboratory Medicine, Eunpyeong St. Mary's Hospital, College of Medicine, The Catholic University of Korea, 1021 Tongil-ro, Eunpyeong-gu, Seoul 03312, Korea
E-mail: ljh117@catholic.ac.kr

INTRODUCTION

Flow cytometric immunophenotyping (FCI) plays a key role in the diagnosis, classification, and monitoring of hematolymphoid neoplasms (HLNs) [1-4]. According to the 2022 European Leukemia Net (ELN) guidelines, immunophenotyping using multiparameter flow cytometry (MFC) is required for identifying cell surface and intracellular markers during work-up for AML [5]. In addition, minimal residual disease (MRD) monitoring using MFC is recommended in AML, ALL, multiple myeloma (MM), and CLL [5-8]. According to the National Comprehensive Cancer Network guidelines for MM, next-generation flow cytometry (NGF) with a minimum sensitivity of 1 in 10^5 nucleated cells, corresponding to the sensitivity of molecular genetic tests such as PCR or next-generation sequencing (NGS) in MRD monitoring, is essential for assessing the therapeutic response in MM [9]. Therefore, accurate and precise flow cytometric measurements are required.

Although multicolor antibody (Ab) panels with new Abs and fluorophores have been introduced in clinical practice, numerous clinical laboratories still use laboratory-developed tests comprising in-house Ab panels and methodologies [1, 2]. FCI of HLNs (FCI-HLN) can be affected by various factors, including equipment, sample handling, reagents, instrument setup, procedures, and data analysis [1, 10, 11-13]. Staining patterns can markedly differ according to the types of Ab clones and conjugated fluorophores used, causing discrepancies between positive and negative cell populations [11]. As each laboratory has a different environment, standardization is strictly necessary to accurately analyze FCI-HLN results.

To the best of our knowledge, there have been no studies on the standardization of FCI-HLN in Korea. We aimed to gather basic data by surveying the current situation in Korea to establish a standardization plan for FCI-HLN, improve the quality of FCI-HLN, and provide highly objective and reliable results.

MATERIALS AND METHODS

Study population

The study protocol was approved by the Institutional Review Board (IRB) of Eunpyeong St. Mary's Hospital, The Catholic University of Korea, Seoul, Korea (IRB No. PC22QASI0213). The questionnaire survey was designed and critically reviewed by laboratory medicine specialists from the Korean Society of Laboratory Hematology and Korean Society of Diagnostic Immunology. We conducted the survey by e-mailing the specialists at eight university hospitals in Korea actively performing FCI-HLN procedures.

Questionnaire survey

The questionnaire (Supplemental Data Table S1) consisted of 12 questions in three main categories to assess the current laboratory practice status of FCI-HLN: (1) basic characteristics of each hospital: hospital grade, number of licensed beds, annual flow cytometric test numbers, and type of flow cytometer used (manufacturer); (2) analytic phase (test phase) variables for each test panel: reagent Abs (RAs), fluorophores, amount of sample per tube, amount of RA per tube, and acquisition cell count; and (3) post-analytic phase (report phase) variables of each test panel: positive criterion (positive cut-off), negative criterion (negative control), and report contents. The test panels included acute HLN, chronic HLN, plasma cell neoplasm (PCN), and MRD panels. All eight laboratories completed the survey. We analyzed the survey results in detail.

RESULTS

The basic characteristics of the clinical laboratories in the eight university hospitals performing FCI-HLN are presented in Table 1. The hospitals used flow cytometers from Beckman Coulter (Navios series) or Becton Dickinson (FACS series).

RAs and fluorophores used

Acute HLN panels

The eight hospitals used acute HLN panels comprising 16–32 RAs (Table 2). In seven hospitals, the acute HLN panel comprised 18–32 RAs, regardless of the acute HLN cell type. In one hospital, the number of RAs in the acute HLN panel varied according to the cell type: 18 Abs for AML, 16 Abs for ALL, 22 Abs for mixed-phenotype acute leukemia (MPAL), and 18 Abs for T-lymphoblastic leukemia (T-ALL). Four hospitals added optional 2–8 RAs for cases requiring further confirmation after the primary test. The common RAs in the acute HLN panels used in the eight hospitals were cluster of differentiation (CD)2, CD3, CD5, CD7, CD10, CD13, CD14, CD19, CD20, CD33, CD34, CD41, CD45, CD56, CD64, CD117, cytoplasmic CD (cCD)3, cCD22, human leucocyte antigen DR (HLA-DR), myeloperoxidase (MPO), and terminal deoxynucleotidyl transferase (TdT).

Chronic HLN panels

The eight hospitals used chronic HLN panels comprising 15–26 RAs (Table 2). Seven hospitals used common panels comprising

Table 1. Basic characteristics of eight Korean university hospitals performing FCI-HLN

Characteristics	Hospitals							
	A	B	C	D	E	F	G	H
Hospital grade	Tertiary	General	Tertiary	Tertiary	Tertiary	Tertiary	Tertiary	Tertiary
Number of licensed beds*	1,362	740	2,426	991	684	1,184	2,723	1,985
Number of flow cytometry tests in 2021†	45,681	743	19,420	1,206	1,871	2,152	17,687‡	5,976
Flow cytometer (manufacturer)	FACS Lyric (BD) DxFLEX (BC)	FACS Canto II (BD)	Navios EX (BC)	Navios (BC)	Navios (BC)	Navios (BC)	FACS Canto II (BD) FACS Lyric (BD)	FACS Lyric (BD)

*Data are from the Health Insurance Review and Assessment Service (April 2022).
†These were counted except for the numbers of flow cytometric cross-matching.
‡These were counted except for the numbers of flow cytometric cross-matching and lymphocyte subset analysis.
Abbreviations: FCI-HLN, flow cytometric immunophenotyping of hematolymphoid neoplasms; BD, Becton Dickinson; BC, Beckman Coulter.

16–23 RAs, regardless of the chronic HLN cell type. In one hospital, the number of RAs in the chronic HLN panel varied: 15 Abs for B cells and 26 for T/natural killer cells. The common RAs in the chronic HLN panels used in the eight hospitals were CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD20, CD23, CD45, CD56, Flinders Medical Centre (FMC)7, surface immunoglobulin kappa (sIgκ), and sIg lambda (sIgλ).

PCN panels

Only seven of the eight hospitals used PCN panels comprising 5–12 RAs (Table 2). The common RAs in the PCN panels used in the eight hospitals were CD19, CD38, CD45, CD56, CD138, cytoplasmic (c)Igκ, and cIgλ.

MRD panels

Seven hospitals used various MRD panels (Table 2). ALL-MRD and MM-MRD panels were used in six and four hospitals, respectively, and three hospitals used both ALL-MRD and MM-MRD panels. The ALL-MRD panels comprised 6–14 RAs, and the MM-MRD panels comprised 7–10 RAs. One hospital used CLL-MRD (eight RAs), mantle cell lymphoma (MCL)-MRD (10 RAs), and mature B cell neoplasm-MRD (five RAs) panels. In the B-lymphoblastic leukemia (B-ALL)-MRD panels, the commonly used RAs included CD10, CD19, CD20, CD34, CD38, CD45, and CD58. In the MM-MRD panels, the commonly used RAs included CD19, CD27, CD38, CD45, CD56, CD81, CD117, CD138, cIgκ, and cIgλ.

Fluorophores

The number of fluorophores in each test panel ranged from 4–10. The fluorophores used in each test panel are described in detail in Table 2.

Sample amount, antibody amount, and number of analyzed cells

Sample amount

For acute HLN, chronic HLN, or PCN panels, five to six hospitals used 50 µL or 100 µL of sample per tube, and two hospitals adjusted the sample amount to the desired cell count (1×10^6 cells). For the MRD panel, five hospitals adjusted the sample amount to a desired cell count (5×10^5–1×10^7 cells), and two hospitals used 50 µL or 100 µL of the sample (Table 2).

Antibody amount

The amount of RA per tube in all panels ranged from 2–20 µL. Four hospitals used 5 µL or 10 µL of RA, and four hospitals used 2 µL to 20 µL, depending on the fluorophore, antigen, and type of Ab used (Table 2).

Acquisition cell count

The acquisition cell count varied among the hospitals according to the test panel: 5×10^3–2×10^5 cells for acute and chronic HLN panels, 5×10^3–1×10^6 cells for the PCN panels, and 5×10^3–1×10^7 cells for the MRD panels. In one hospital (E), the acquisition cell count varied according to the Ab type used (1×10^4 cells for surface Abs [sAbs] and 5×10^3 cells for cytoplasmic Abs [cAbs]) in acute and chronic HLN panels (Table 2).

Post-analytic phase variables

Positive criterion (positive cut-off)

All hospitals except hospital D used ≥20% of target cells as a positive criterion for the acute and chronic HLN panels. Notably, for cytoplasmic or nuclear markers, including MPO and TdT, 10% was used as a positive criterion in three hospitals (E, F, and G)

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Table 2. Analytic phase (test phase) variables of each test panel for FCI-HLN in eight university hospitals

Variables	Hospitals							
	A	B	C	D	E	F	G	H
Reagent antibodies								
Acute HLN	23 Abs CD2, CD3, CD5, CD7, CD10, CD11c, CD13, CD14, CD19, CD20, CD22, CD33, CD34, CD41a, CD45, CD56, CD64, CD117, cCD3, cCD79a, HLA-DR, MPO, TdT	19 Abs (+3) CD3, CD5, CD7, CD10, CD11c, CD13, CD14, CD19, CD20, CD33, CD34, CD45, CD56, CD64, CD117, cCD3, cCD79a, HLA-DR, MPO (+ if needed, TdT, cCD22, CD61)	32 Abs CD1a, CD2, CD4, CD5, CD7, CD8, CD10, CD11c, CD13, CD14, CD19, CD20, CD33, CD34, CD36, CD38, CD41b, CD42, CD45, CD56, CD61, CD64, CD71, CD117, CD123, CD235a, cCD3, cCD22, cCD79a, HLA-DR, MPO, TdT	23 Abs CD2, CD5, CD7, CD10, CD11c, CD13, CD14, CD19, CD20, CD33, CD34, CD41a, CD45, CD56, CD64, CD71, CD79a, CD117, cCD3, cCD22, HLA-DR, MPO, TdT	21 Abs CD2, CD3, CD5, CD7, CD10, CD13, CD14, CD19, CD20, CD22, CD33, CD34, CD41, CD45, CD56, CD61, CD64, CD117, HLA-DR, MPO, TdT	20 Abs (+2) CD3, CD5, CD7, CD10, CD13, CD14, CD19, CD20, CD33, CD34, CD41, CD45, CD56, CD117,cCD3, cCD22, cCD79a, HLA-DR, MPO, TdT (+ if needed, CD11c, CD64)	- AML: 18 Abs CD2, CD7, CD10, CD13, CD14, CD15, CD19, CD33, CD34, CD41, CD45, CD56, CD64, CD117, cCD3, cCD22, HLA-DR, MPO if MPAL: 22 Abs (4 Abs added CD20, cIgM, sIgM, TdT) - ALL: 16 Abs CD2, CD3, CD5, CD7, CD10, CD13, CD19, CD20, CD33, CD34, CD45, cCD3, cCD22, cIgM, sIgM, TdT if T-ALL: 18 Abs (2 Abs added CD4, CD8)	- 18 Abs (+4) CD3, CD10, CD13, CD14, CD19, CD33, CD34, CD45, CD64, CD66c, CD71, CD117, cCD3, cCD22, cCD79a, HLA-DR, MPO, TdT (+ if needed, CD56, CD123, CD61, CD41) if T-ALL: 26 Abs (8 Abs added CD1a, CD2, CD4, CD5, CD7, CD8, TCRαβ, TCRγδ)
Chronic HLN	18 Abs CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD14, CD19, CD20, CD23, CD43, CD45, CD56, CD103, FMC7, sIgκ, sIgλ	18 Abs CD3, CD4, CD5, CD8, CD10, CD11c, CD19, CD20, CD23, CD38, CD43, CD45, CD56, CD103, FMC7, sIgκ, sIgλ, TCRγδ	23 Abs CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD20, CD22, CD23, CD25, CD38, CD45, CD49d, CD79b, CD138, FMC7, ZAP70, sIgκ, sIgλ, TCRαβ, TCRγδ	16 Abs CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD14, CD19, CD20, CD23, CD45, CD56, FMC7, sIgκ, sIgλ	18 Abs CD2, CD3, CD5, CD7, CD10, CD19, CD20, CD22, CD23, CD34, CD45, CD56, CD138, FMC7, HLA-DR, sIgκ, sIgλ, TdT	16 Abs CD3, CD4, CD5, CD7, CD8, CD10, CD13, CD19, CD20, CD23, CD33, CD34, CD45, CD56, cCD79a, TdT	16 Abs CD2, CD3, CD5, CD7, CD10, CD19, CD20, CD22, CD23, CD45, CD56, cCD3, FMC7, sIgκ, sIgλ, TdT	- B cells: 15 Abs CD5, CD10, CD11c, CD19, CD20, CD22, CD23, CD25, CD38, CD45, CD79b, CD103, FMC7, sIgκ, sIgλ - T/NK cells: 26 Abs CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD13, CD14, CD16, CD25, CD26, CD28, CD30, CD33, CD45, CD45RA, CD56, CD57, CD94, CD103, CD279, cCD3, TCRγδ, TdT

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Table 2. Continued 1

Variables	Hospitals							
	A	B	C	D	E	F	G	H
PCN	NP	12 Abs β2-MG, CD19, CD27, CD28, CD38, CD45, CD56, CD81, CD117, CD138, cIgκ, cIgλ	7 Abs CD19, CD38, CD45, CD56, CD138, cIgκ, cIgλ	7 Abs CD19, CD38, CD45, CD56, CD138, cIgκ, cIgλ	6 Abs CD19, CD45, CD56, CD138, cIgκ, cIgλ	5 Abs CD19, CD38, CD45, CD56, CD138	5 Abs CD19, CD38, CD45, CD56, CD138	9 Abs CD19, CD28, CD38, CD45, CD56, CD117, CD138, cIgκ, cIgλ
MRD	- B-ALL:12 Abs CD10, CD19, CD20, CD22, CD34, CD38, CD45, CD58, CD66c, CD73, CD123, CD304 - CLL: 8 Abs CD5, CD19, CD20, CD43, CD45, CD79b, CD81, ROR1 - MM: 9 Abs CD19, CD27, CD38, CD45, CD56, CD81, CD117, CD138, CD200	MM: 10 Abs CD19, CD27, CD38, CD45, CD56, CD81, CD117, CD138, cIgκ, cIgλ	B & T-ALL: 14 Abs CD1a, CD2, CD5, CD7, CD10, CD13 (or CD33), CD19, CD20, CD22, CD34, CD45, CD79a, cCD3, TdT	- B-ALL: 6 Abs CD10, CD19, CD34, CD20, CD38, CD45 - T-ALL: 6 Abs CD3, CD4, CD7, CD8, CD34, CD45 - MM: 7 Abs CD19, CD38, CD45, CD56, CD138, cIgκ, cIgλ	NP	- B-ALL: 7 Abs CD10, CD19, CD20, CD34, CD38, CD45, CD58 - T-ALL: 6 Abs CD3, CD4, CD7, CD8, CD34, CD45 - MBCN: 5 Abs CD5, CD19, CD20, CD23, CD45	B-ALL: 7 Abs (+1 or 2) CD10, CD19, CD20, CD34, CD38, CD45, CD58 (+ if needed, CD13 and/or CD33)	- B-ALL: 12 Abs CD10, CD19, CD20, CD34, CD38, CD45, CD58, CD66c, CD73, CD81, CD123, CD304 - MCL: 10 Abs CD3, CD5, CD19, CD20, CD23, CD45, CD62L, CD200, sIgκ, sIgλ - MM: 10 Abs CD19, CD27, CD38, CD45, CD56, CD81, CD117, CD138, cIgκ, cIgλ
Fluorophores								
Acute HLN	4 colors APC, FITC, PE, PerCP	8 colors APC, APC-H7, FITC, PE, PE-Cy7, PerCP-Cy5.5, V450, V500	9 colors APC, APC-A750, FITC, KO, OC515, PB, PE, PE-Cy7, PerCP-Cy5.5	4 colors FITC, PC5, PC7, PE	4 colors FITC, PC5, PC7, PE	5 colors APC-A750, FITC, PC5, PC7, PE	AML/MPAL: 4 colors APC, FITC, PE, PerCP - ALL: 5 colors APC, APC-Cy7, FITC, PE, PerCP	8 colors APC, APC-H7, BV421 (BV450, PB), FITC, PE, PE-Cy7, PerCP-Cy5.5 (PerCP), V500 (BV510)
Chronic HLN	4 colors APC, FITC, PE, PerCP	8 colors APC, APC-H7, FITC, PE, PE-Cy7, PerCP-Cy5.5, V450, V500	8 colors APC, APC-A750, FITC, OC515, PB, PE, PE-Cy7, PerCP-Cy5.5	4 colors FITC, PC5, PC7, PE	4 colors FITC, PC5, PC7, PE	5 colors APC-A750, FITC, PC5, PC7, PE	4 colors APC, FITC, PE, PerCP	8 colors APC, APC-H7, BV421 (BV450, PB), FITC, PE, PE-Cy7, PerCP-Cy5.5 (PerCP), V500 (BV510)

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Table 2. Continued 2

Variables	Hospitals							
	A	B	C	D	E	F	G	H
PCN	NP	8 colors APC, APC-H7, FITC, PE, PE-Cy7, PerCP-Cy5.5, V450, V500	6 colors APC, APC-A750, FITC, PE, PE-Cy7, PerCP-Cy55	4 colors FITC, PC5, PC7, PE	4 colors FITC, PC5, PC7, PE	5 colors APC-A750, FITC, PC5, PC7, PE	5 colors APC, FITC, PE, PE-Cy7, PerCP-Cy5.5	7 colors APC, BV421 (BV450, PB), FITC, PE, PE-Cy7, PerCP-Cy5.5 (PerCP), V500 (BV510)
MRD	- B-ALL: 9 colors APC, APC-A700, APC-A750, ECD, FITC, KO, PC5.5, PC7, PE - B-CLL: 8 colors APC, APC-A750, FITC, KO, PB, PC5.5, PC7, PE - MM: 9 colors APC, APC-A750, ECD, FITC, KO, PB, PC5.5, PC7, PE	MM: 8 colors APC, APC-C750, BV421, BV510, FITC, PE, PE-CY7, PerCP-Cy5.5	ALL: 8 colors APC, APC-A750, FITC, OC515, PB, PE, PE-Cy7, PerCP-Cy5.5	ALL & MM: 4 colors FITC, PC5, PC7, PE	NP	- B-ALL: 7 colors APC-A700, APC-A750, ECD, FITC, KO, PC5.5, PC7 - T-ALL, MBCN: 5 colors APC-A750, FITC, PC5, PC7, PE	B-ALL: 7 colors APC, APC-Cy7, BV510, FITC, PE, PerCP, V450	- B-ALL: 10 colors APC, BV605, FITC, PE, PE-Cy7, PerCP-Cy5.5, V450, V500, R718, APC-H7 - MCL: 10 colors APC, APC-H7, APC-R700, BV421, BV605, FITC, PE, PE-Cy7, PerCP-Cy5.5, V500 - MM: 8 colors APC, APC-C750, BV421, BV510, FITC, PE, PE-CY7, PerCP-Cy5.5
Sample amount/tube								
Acute HLN	100 µL	100 µL	100 µL	100 µL	50 µL	50 µL	volume adjusted to 1×10^6 cells	volume adjusted (10–500 µL) to 1×10^6 cells
Chronic HLN	100 µL	100 µL	100 µL	100 µL	50 µL	50 µL	volume adjusted to 1×10^6 cells	volume adjusted (10–500 µL) to 1×10^6 cells
PCN	NP	100 µL	100 µL	100 µL	sAb: 100 µL cAb: 50 µL	50 µL	volume adjusted to 1×10^6 cells	volume adjusted (10–500 µL) to 1×10^6 cells
MRD	1×10^7 cells	volume adjusted to $\geq1\times10^7$ cells	volume adjusted to 5×10^5 cells	100 µL	NP	50 µL	volume adjusted to 1×10^6 cells	volume adjusted (10–500 µL) to 1×10^7 cells

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Table 2. Continued 3

Variables	Hospitals							
	A	B	C	D	E	F	G	H
Amount of reagent antibody/tube	5 μL	Depending on fluorophores or Ags - APC, APC-H7, PE-Cy7,PerCP-Cy5.5, V450, V500: 5 μL - FITC, PE: 20 μL - CD34-PerCP-Cy5.5: 20 μL - CD56-V450: 2 μL	5 μL	10 μL	10 μL (Isotype IgG1/G2a: 5 μL)	Depending on fluorophores - APC-A750: 3 μL - PC5, PC7: 5 μL - FITC, PE: 10 μL	8 μL - ALL CD10, CD19, CD20: 5 μL - ALL-MRD CD10, CD19, CD20: 5 μL CD58: 2 μL	Depending on Abs 2.5–20 μL
Acquisition cell count								
Acute HLN	2×10^4 cells	1×10^5 cells	$\geq5\times10^3$ cells	5×10^3 to 1×10^4 cells	- sAb: 1×10^4 cells - cAb: 5×10^3 cells	1×10^4 cells	5×10^4 cells	2×10^5 cells
Chronic HLN	2×10^4 cells	1×10^5 cells	$\geq5\times10^3$ cells	5×10^3 to 1×10^4 cells	- sAb: 1×10^4 cells - cAb: 5×10^3 cells	1×10^4 cells	5×10^4 cells	2×10^5 cells
PCN	NP	1×10^5 cells	$\geq5\times10^3$ cells	5×10^3 to 1×10^4 cells	1×10^5 cells	1×10^6 cells	5×10^5 cells	2×10^5 cells
MRD	5×10^6 cells	1×10^7 cells	$\geq5\times10^5$ cells	5×10^3 to 1×10^4 cells	NP	1×10^6 cells	1×10^6 cells	- B-ALL: 6×10^6 cells - MM: 1×10^7 cells

Abbreviations: FCI-HLN, flow cytometric immunophenotyping of hematolymphoid neoplasms; HLN, hematolymphoid neoplasms; Abs, antibodies; CD, cluster of differentiation; cCD, cytoplasmic cluster of differentiation; HLA-DR, human leucocyte antigen DR; MPO, myeloperoxidase; TdT, terminal deoxynucleotidyl transferase; MPAL, mixed-phenotype acute leukemia; cIgM, cytoplasmic immunoglobulin M; sIgM, surface immunoglobulin M; T-ALL, T-lymphoblastic leukemia; TCR, T cell receptor; FMC7; Flinders Medical Centre 7; sIgκ, surface immunoglobulin kappa; sIgλ, surface immunoglobulin lambda; ZAP70, zeta chain-associated protein kinase 70; NK, natural killer; PCN, plasma cell neoplasm; NP, not performed; β2-MG, β2-microglobulin; cIgκ, cytoplasmic immunoglobulin kappa; cIgλ, cytoplasmic immunoglobulin lambda; MRD, minimal residual disease; B-ALL, B-lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; ROR1, receptor tyrosine kinase-like orphan receptor 1; MM, multiple myeloma; MBCN, mature B cell neoplasm; MCL, Mantle cell lymphoma; APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin-chlorophyll protein; APC-H7, allophycocyanin-Hilite 7; PE-Cy7, phycoerythrin-cyanine 7; PerCP-Cy5.5, peridinin-chlorophyll protein-cyanine 5.5; APC-A750, APC-Alexa Fluor 750; KO, Krome Orange; OC515, Orange Cytognos 515; PB, Pacific Blue; PC, phycoerythrin-cyanine; BV, Brilliant Violet; ECD, phycoerythrin-Texas Red-X; APC-C750, allophycocyanin-tandem 750; sAb, surface antibody; cAb; cytoplasmic antibody; Ags, antigens.

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and 5% in one hospital (H). For the PCN panel, all hospitals used an abnormal phenotype as a positive criterion. For the MRD panel, four hospitals used the limit of detection (LoD) and lower limit of quantification (LLoQ), and the other three hospitals used an abnormal phenotype as a positive criterion (Table 3).

Negative criterion (negative control)

Five hospitals used an isotype control, and three used an internal control as a negative control (Table 3).

Report contents

Most hospitals reported positive/negative assignments, percentages, or general opinions for all panels. For the acute HLN, chronic HLN, and PCN panels, four hospitals reported the fluorescence intensity. One hospital (H) reported the acquisition cell count. For the MRD panel, four hospitals reported LoD, LLoQ, debris percentage, or previous results (Table 3).

DISCUSSION

This study demonstrated the variable pre-analytical, analytical, and post-analytical status of FCI-HLN in Korea. Several studies on the standardization of FCI-HLN have been conducted [1, 2, 4, 11, 14-25]. The EuroFlow Consortium developed a fully standardized approach for FCI, encompassing instrument setup, software tools, and data analysis [1, 16].

The WHO classification defines lineage assignment criteria for MPAL, and all acute leukemia panels include MPO for the myeloid lineage, CD10 and CD19 for the B lineage, and CD3 (cytoplasmic or surface) for the T lineage. Other Abs for monocytic differentiation (CD11c, CD14, and CD64) and the B lineage (CD22 and CD79a) are selectively included [26]. The EuroFlow Consortium suggests the use of a screening panel prior to comprehensive FCI using extended Ab panels for the diagnosis and classification of hematological malignancies, and two types of markers are combined in each 8-color tube: (i) backbone markers to identify distinct cell populations in a sample, and (ii) markers for the characterization of specific cell populations [22]. The CLSI H43-A2 guidelines recommend that sufficient Abs should be used to distinguish between abnormal and normal cell populations in FCI-HLN [3]. Extensive Ab panels with 20–24 Abs are required for both acute and chronic HLN [3]. Our data showed that each hospital used 16–32 Abs for acute HLN and 15–26 Abs for chronic HLN. For panel design for FCI-HLN, the following approaches are generally used [3]. The first approach is to use various Abs in a single step for the complete characterization of both abnormal and normal cell populations, regardless of the HLN subtype [3]. The second approach is to use a small number of Abs for an initial screening, followed by additional Abs selected based on the initial results [3]. Our data showed that most hospitals used the first approach for both acute and chronic HLN panels. Only a few hospitals used the second approach; two hospitals used this approach for acute HLN and one hospital for chronic HLN.

Typically, the larger the number of Abs used, the higher the sensitivity and specificity for the detection and characterization of abnormal cell populations in FCI-HLN [3]. Acute and chronic HLN panels proposed by the French Groupe d'Étude Immunologique des Leucémies (GEIL) include approximately 30 and 20–30 Abs, respectively [2]. However, most hospitals in Korea use panels with approximately 18 Abs for acute and chronic HLN, in compliance with the number of Abs permitted by the national health insurance standards. The current standards for cell marker analysis for national health insurance coverage in Korea are <18 Abs at initial diagnosis and <5 Abs (eight Abs for ALL since 2020) at MRD monitoring. At the time of initial HLN diagnosis, accurate and precise analysis of target cells is essential, and >18 Abs are required for FCI-HLN. Therefore, hospitals cannot claim health insurance fees from the Health Insurance Review and Assessment Service for Abs beyond the national health insurance coverage. As suggested by the EuroFlow Consortium, backbone markers must be used for accurate and objective FCI-HLN analysis [22]. However, national health insurance in Korea does not cover overlapping use of the same Ab. Therefore, multiple Abs (≥ 18) and overlapping use of Abs required in clinical practice must be considered when determining the national health insurance fee for FCI-HLN in Korea. In addition, diagnostic test kits and software for standardized FCI-HLN analysis have been introduced, and precise analytic methods are used in clinical practice [27-28]. Therefore, the national health insurance fee schedule in Korea must be urgently improved based on the number of Abs used in FCI-HLN.

In chronic HLN panels, 15 Abs are commonly used in Korea, including CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD19, CD20, CD23, CD45, CD56, FMC7, sIgκ, and sIgλ. CLL has a characteristic immunophenotype, expressing CD5, CD19, dim CD20, dim CD22, CD23, bright CD43, dim CD45, dim to negative CD79b, dim CD81, CD200, and dim monoclonal sIg [8, 29, 30]. The prevalence of CLL has recently been increasing in Korea [31]. As FCI is beneficial for diagnosing CLL, the number of Abs must be expanded to facilitate additional confirmatory tests following screening [8, 22, 29]. The GEIL proposed 8-color and 10-color

Table 3. Post-analytic phase (report phase) variables of each test panel for FCI-HLN in eight university hospitals

Variables	Hospitals							
	A	B	C	D	E	F	G	H
Positive criterion (positive cut-off)	- Acute and chronic HLN: 20% - PCN: NP - MRD: LoD, LLoQ	- Acute and chronic HLN: 20% - PCN: abnormal phenotype - MRD: LoD, LLoQ	- Acute and chronic HLN: 20% - PCN: abnormal phenotype - MRD: LoD, LLoQ	- Acute and chronic HLN: 10% - PCN: abnormal phenotype (consider κ/λ ratio) - MRD: LoD, LLoQ	- Acute and chronic HLN: 20% (except MPO 10%) - PCN: abnormal phenotype - MRD: NP	- Acute and chronic HLN: 20% (except TdT, MPO 10%) - PCN: abnormal phenotype - MRD: abnormal phenotype	- Acute and chronic HLN: 20% (except TdT, MPO 10%) - PCN: abnormal phenotype - MRD: abnormal phenotype	- Acute and chronic HLN: 20% (except MPO 5%) - PCN: abnormal phenotype - MRD: abnormal phenotype
Negative criterion (negative control)	Isotype control	Internal control	Internal control	Isotype control	Isotype control	Isotype control	Isotype control	Internal control
Report contents								
Acute HLN	- Positive/negative - Percentage - General opinion	- Positive/negative - Percentage - General opinion	- Positive/negative - Percentage - General opinion - Fluorescence intensity	- Positive/negative - Percentage - General opinion - Fluorescence intensity	- Percentage - General opinion	- Positive/negative - Percentage - General opinion - Fluorescence intensity	- Positive/negative - Percentage - General opinion - Fluorescence intensity	- Positive/negative - Percentage - General opinion - Acquisition cell count
Chronic HLN	- Positive/negative - General opinion	- Positive/negative - Percentage - General opinion	- Positive/negative - Percentage - General opinion - Fluorescence intensity	- Positive/negative - Percentage - General opinion - Fluorescence intensity	- Percentage - General opinion	- Positive/negative - Percentage - General opinion - Fluorescence intensity	- Positive/negative - Percentage - General opinion - Fluorescence intensity	- Positive/negative - Percentage - General opinion - Acquisition cell count
PCN	NP	Positive/negative	- Positive/negative (if needed, fluorescence intensity description) - Percentage - General opinion	- Positive/negative (if positive, the type of monoclonal light chain) - Percentage	- Percentage - General opinion	- Positive/negative - Percentage - General opinion - Fluorescence intensity	- Positive/negative - Percentage - General opinion - Fluorescence intensity	- Percentage - General opinion - Acquisition cell count
MRD	- Positive/negative - Percentage - General opinion - Acquisition cell count - LoD, LLoQ	- Positive/negative - Percentage - LoD, LLoQ - Debris percentage	- Positive/negative - Percentage - General opinion - LoD, LLoQ	- Positive/negative - Percentage - General opinion	NP	- Positive/negative - Percentage - General opinion - Fluorescence intensity	- Positive/negative - Percentage - General opinion - Fluorescence intensity	- Positive/negative - Percentage - Acquisition cell count - LoD, LLoQ - Previous results

Abbreviations: FCI-HLN; flow cytometric immunophenotyping of hematolymphoid neoplasms; HLN, hematolymphoid neoplasms; PCN, plasma cell neoplasms; NP, not performed; MRD, minimal residual disease; LoD, limit of detection; LLoQ, lower limit of quantification.; κ/λ, kappa/lambda; MPO, myeloperoxidase; TdT, terminal deoxynucleotidyl transferase.

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acute and chronic HLN panels [2]. In our study, 8-color Ab panels were the most common, followed by 4-color panels. Only one clinical laboratory (H) used 10-color Ab panels for B-ALL-MRD and MCL-MRD.

The International Clinical Cytometry Society and European Society for Clinical Cell Analysis suggested that the use of eight Abs (CD38, CD138, CD45, CD19, CD56, CD27, CD81, and CD117) is the best practice for MM-MRD monitoring, and routine evaluation of intracellular light chains is not recommended [23]. In our study, three hospitals used nine or 10 Abs for MM-MRD divided into two tubes following EuroFlow standard operating procedures (SOPs), including eight recommended Abs and two intracellular Ig light chains (cIgκ and cIgλ) [23]. In addition, CD19, CD27, CD38, CD45, CD56, and CD138 were used as backbone markers. In MFC-MM-MRD monitoring, the assessment of 5×10^6 cells and a minimum sensitivity of 10^{-5} are required [7]. NGF with improved sensitivity, applicability, and reproducibility than that of MFC has been developed and is used in clinical practice [7, 9, 10, 31]. Over 10×10^6 cells can be evaluated using NGF, with a sensitivity of 10^{-6}, which is comparable to the performance of molecular methods (e.g., NGS) [10, 31]. In our study, two hospitals (B and H) achieved this sensitivity in MM-MRD.

In FCI-HLN, the minimum acquisition cell count is 1×10^4–5×10^4 cells per tube, and at least 200–1,000 target cells should be assessed [3]. Our data showed that 5×10^3–2×10^5 cells were used for FCI of acute and chronic HLN. In MFC-MRD monitoring, obtaining a high number of events is recommended to detect rare cell populations [10, 12, 17, 23, 25, 32]. The ELN recommends $>5\times10^5$ CD45+ cells and at least 100 viable cells in the blast region for MFC-AML-MRD monitoring [33]. Studies have indicated that millions of cells (for instance, 3×10^6 cells for AML and CLL, 4×10^6 cells for B- and T-ALL, and 5–20×10^6 cells for MM) are required for MRD panels [10, 17]. A white blood cell count of 5×10^9/L suggests that 10 mL of whole blood likely contains approximately 5×10^7 cells [32]. To obtain a desired CV of ≤5%, a minimum of 4×10^3 cells at a frequency of 10% and a maximum of 10^{11} cells at a frequency of 0.00001% are required [32].

According to the EuroFlow SOPs for 8-color MFC, 300 μL of sample and an appropriate volume of Abs are needed for using the EuroFlow Ab panel with sAbs [16]. In our study, most hospitals used 50–100 μL of sample and <20 μL of Abs, which is lower than the quantities recommended by EuroFlow. This variance is attributed to the common practice of using laboratory-developed tests for FCL-HLN in Korea. Each clinical laboratory should establish the optimal sample amount for FCI-HLN to ensure the minimum number of target cells [34]. In addition, the minimal Ab amount should be determined for optimal resolution and positive-staining intensity [3]. Using Ab titration, the Ab amount and concentration can be determined to achieve the highest signal and lowest noise levels [34]. Two hospitals adjusted their sample amounts to the cell count per tube (1×10^6 cells). This is in line with the CLSI H43-A2 guidelines. As most manufacturers recommend Ab amounts to be set in the normal range of target cells (0.2–2×10^6 cells), each clinical laboratory should adjust the target cell range either to 0.2–2×10^6 cells or according to the manufacturer's recommendations [3].

Clinical laboratories traditionally use 20% as the positive cut-off value for sAbs and 10% for cAbs (MPO, cCD3, cCD79a, and TdT) [35]. Internal (negative) or isotype controls, which are used to measure background fluorescence, can be used as negative controls to distinguish between positive and negative populations [3, 18, 34, 36]. For sample QC, both negative and positive controls are required [3]. Several materials can be used as positive controls to confirm the methods for target cells, RAs, and the optimal staining procedures, including human white blood cells, normal bone marrow cells, or cryopreserved human HLN cells (or cell lines) [3]. Our data showed that a conventional cut-off value (20%, except for hospital D, which used 10%) for internal or isotype controls was used to determine positive results in all panels. Previous studies have suggested cut-off values ranging from 5.4%–28% for MPO positivity in FCI-HLN [37-39]. As a negative criterion, isotype controls were used more than internal controls in this study. As the positive cut-off value is crucial for determining the lineage in HLN diagnosis, the positive cut-off value for FCI-HLN requires standardization. It can be defined by using an internal control or by comparing the degree of antigen expression in the patient sample with that in a normal cell sample under identical instrument settings [3]. For example, the normal T cell population can be defined based on the expression of CD3, CD4, and CD8 [3]. The cut-off values for MFC-MRD positivity were 0.01% (10^{-4}) for ALL, 0.01% (10^{-4}) for CLL, and 0.001% (10^{-5}) for MM [6-10]. When 5×10^6 cells are analyzed for MFC-MM-MRD, the LoD and LLoQ are 0.0006% and 0.001%, respectively [25].

In reporting quantitative or qualitative data, the following variables should be included: numeric results (such as percentage and cell counts), reference range (if applicable), abnormal phenotype, percentage of the population of interest and gated populations (such as gated CD45+ cells), summarized findings, the level of antigen expression (negative, positive, partially ex-

pressed, normal-positive, dim-positive, bright-positive, and heterogeneous or variably intense staining), list of Abs, and interpretational comments or recommendations [34]. When reporting MM-MRD panel results, including the LoD and LLoQ is recommended [25]. However, determining the LoD and LLoQ is challenging in clinical practice. As MRD panel design and evaluation are complicated, standardized guidelines reflecting the real hospital setting are required. In our study, most clinical laboratories reported positive/negative assignments, percentages, or general opinions. Some reported fluorescence intensity and acquisition cell count, and a few reported the LoD and LLoQ when reporting MRD panel results.

Regarding HLN diagnosis and MRD monitoring, variability in FCI may directly affect medical decisions on treatment and prognosis. Therefore, standardization of FCI is critical to ensure reliable results [14]. A standardized protocol including pre-analytical, analytical, and post-analytical steps would lead to harmonized and reproducible FCI results in clinical practice [15]. Multicolor flow cytometry has become a routine method of FCI-HLN, and clinical cytometers can measure up to 10 fluorescent parameters. To leverage a large number of channels in multicolor experiments and well-designed Ab panels, proper compensation for spectral spillovers of the dyes is essential [40].

We observed remarkable differences among the hospitals in terms of the RA combinations used and the acquisition cell count. This variance can be attributed to the lack of guidelines on standardized Ab use and the fact that the criteria are established solely for the number of Abs permitted by national health insurance in Korea. Hospitals should consider these differences as they may affect FCI-HLN results. Concordantly, these variations are reflected in the analysis of external quality assessment results in Korea.

We surveyed eight clinical laboratories actively conducting FCI-HLN, focusing on the most common challenges in panel design and data analysis and interpretation when implementing FCI-HLN panels in clinical practice. Detailed steps for QC were not within the study scope. There are various control materials or reagents for QC: setup controls, unstained controls, compensation controls, viability dyes for excluding dead cells, isotype controls, Fc receptor blockers (including commercial blocking solutions, unlabeled Ig, and animal serum), and fluorescence-minus-one controls [3, 16, 18, 21, 34, 36]. We surveyed only the use of isotype controls commonly used as negative controls in clinical practice. Further investigation is required to obtain practically useful information on QC of flow cytometry.

In conclusion, this was the first extensive evaluation of the current status of FCI-HLN in Korea. Our findings revealed a high heterogeneity and complexity of FCI-HLN practice. Standardization of FCI-HLN is urgently required, and the study data provide a reference for establishing standard FCI-HLN guidelines that are clinically useful. In addition, the national health insurance fee for FCI-HLN should be increased to facilitate the use of an appropriate number of Abs in accordance with international diagnostic standards.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.3343/alm.2023.0298

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AUTHOR CONTRIBUTIONS

Park M conducted the study, analyzed the data, and wrote the draft; Lim J conceived the study, analyzed the data, and finalized the draft; Ahn A, Oh EJ, Song J, Kim KH, Han JY, Choi HW, Park JH, Shin KH, K H, Kim M, Hwang SH, Kim HY, Cho D, and Kang ES discussed the data and reviewed the manuscript; all authors critically reviewed the manuscript and approved the final version.

CONFLICTS OF INTEREST

None declared.

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