Immunocytochemistry Practices in European Cytopathology Laboratories—Review of European Federation of Cytology Societies (EFCS) Online Survey Results With Best Practice Recommendations

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BACKGROUND: Variability in preanalytical and analytical steps for immunocytochemistry (ICC) on cytology samples is poorly defined. The objective of this study was to evaluate current practices for ICC on cytology samples in European laboratories. **METHODS:** A link to an online survey with 19 questions about ICC practices was distributed to cytology laboratories through national representatives in the European Federation of Cytology Societies. **RESULTS:** In total, 245 laboratories responded to the survey by January 30, 2019. Cell blocks, cytospins, liquid-based cytology (LBC) preparations, and smears alone or in combination with other preparations were used for ICC in 38%, 22%, 21%, and 19% of laboratories, respectively. In general, various combinations of preparations were used for ICC in greater than one-half of laboratories (147 of 245; 60%), whereas only 1 specific type of cytology preparation was used in the remaining 98 of 245 laboratories (40%) laboratories. The majority of laboratories (217 of 226; 96%) performed ICC on automated platforms using protocols that were the same as those used for formalin-fixed, paraffin-embedded samples (238 of 527 laboratories; 45%), either optimized (138 of 527 laboratories; 26%) or optimized and validated (151 of 527 laboratories; 29%) for cytology preparations. Positive control slides, negative control slides, and external quality control were used in 174 of 223 (78%), 112 of 223 (50%), and 111 of 120 (50%) laboratories, respectively. Greater than 1000 ICC tests were performed yearly in 34% of laboratories (65 of 191; average, 1477 tests; median, 500 tests). **CONCLUSIONS:** ICC is extensively performed in European laboratories using variously prepared cytology preparations on automated platforms, mostly without quality-assurance measures. ***Cancer Cytopathol* 2020;0:1-10**.

KEY WORDS: cell blocks; cytology; cytospin preparations; external quality control; immunocytochemistry; liquid-based cytology; quality assurance; quality control; smears.

INTRODUCTION

Immunocytochemistry (ICC) has become an indispensable ancillary method for establishing a diagnosis and assessing prognostic and predictive markers because only cytology samples are available for diagnostic workup in a significant proportion of patients.[1-5] Several studies have confirmed that ICC can be used on variously prepared and fixed slides prepared from cytology samples.[3,6-13] However, in contrast to immunohistochemistry (IHC) on formalin-fixed, paraffin-embedded (FFPE) tissue sections, to date, quality assurance (QA) and quality control (QC) for ICC on cytology samples have been ignored.

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TABLE 1. Questions and Predefined Answers Included in the Web-Based Survey

Question	Predefined Answers
What kind of preparations are used for diagnostic ICC on cytology samples in your laboratory?	(Cytospins/direct smears/cell blocks/LBC/other)[a]
Main fixative(s) used for the fixation of cytospins/direct smears/cell blocks/LBC/other for ICC?	Methanol/ethanol/aceton/Delaunay/spray/formalin/commercial[b]/air drying/other
After fixation, the slides for ICC are:	Air-dried/Papanicolaou-stained/Romanowsky-stained/PEG/in fixative/other
When ICC is not performed immediately, how do you store the slides?	RT/between 4°C and 8°C/−20°C/−80°C/other
Which platform is used for ICC on cytology samples?	Agilent (DAKO)/BioGenex/Leica/Roche (Ventana)/other
The staining protocol/procedure for ICC on cytology samples used in your laboratory is:	Same as for FFPE/optimized/optimized and validated/other
Which steps of ICC staining procedure are different for cytology samples?	Pretreatment/retrieval/dilution/detection/platform/counterstaining/other
What kind of positive control slides do you use for ICC on cytology samples?	Cytospins/smears/cell blocks/FFPE/none/other
Do you use negative control slides for ICC on cytology samples?	Always/usually/occasionally/rarely/never
What kind of external quality control do you use for ICC on cytology samples?	National service/UK NEQAS ICC/comparison between laboratories/other/none
Do you have any troubles with ICC on cytology samples?	Usually/occasionally/rarely/never
Who is responsible for the ICC (taking care for the controls, testing, optimization, etc)?	Cytotechnologist/cytopathologist/IHC technologist/none/other
Immunocytochemistry is performed in:	Cytology laboratory/histology laboratory/IHC laboratory/other

Abbreviations: FFPE, formalin-fixed paraffin-embedded; ICC, immunocytochemistry; IHC, immunohistochemistry; LBC, liquid-based cytology; PEG, polyethylene glycol; RT, room temperature;UK NEQAS, United Kingdom National External Quality Assessment Service.
[a]Details of specific cytology preparation procedures were not included in the survey.
[b]No specification was required for commercial fixatives.

Fixation and other preanalytical steps in FFPE tissue processing can change antigenicity[14-17] and influence IHC detection of antigens, which has led to standardization and recommended practices for tissue processing.[18-20] Any change or modification of the standard FFPE procedure should be thoroughly validated to assure reliable IHC results.[18,21-23] Moreover, assay revalidation is also required when a validated IHC assay is performed on cytology specimens for which different processing techniques have been used.[18] In addition, the application of appropriate positive controls and participation in external QC (EQC) is already widely accepted practice in diagnostic IHC.[24]

In view of these recommendations, theoretically, the best and easiest way would be to process cytology samples as cell blocks using the standard FFPE procedure, in which standard and validated IHC procedures can be applied. Unfortunately, achieving adequate and optimal cell blocks from cytology samples can be challenging and time consuming.[25,26] Therefore, laboratories have developed and published various strategies for preparing slides for ICC and fulfilling demands for advanced diagnosis on scanty cytology samples.[7,9,11,13,27-29]

The actual variability in cytology sample preparation for ICC, and especially the application of QA/QC measures for assuring accurate and reliable ICC results are still largely unknown. The first inquiry conducted by the European Federation of Cytology Societies (EFCS) a decade ago revealed diversity in some aspects of ICC practices in 28 laboratories from 13 European countries.[30] To obtain an up-to-date and comprehensive overview of preanalytical, analytical, and QA/QC aspects of ICC on cytology samples, we performed a web-based survey, which was disseminated to European cytology laboratories.

MATERIALS AND METHODS

A survey with 19 questions was designed by 2 cytotechnologists who had extensive experience in all practical and QA/QC aspects of ICC on cytology samples using the open source web application 1KA (One Click Survey). The questions were revised and confirmed by a panel of 5 cytopathologists. An invitation letter with a link to the survey was distributed to cytology departments through the official national representatives of all cytology societies affiliated with the EFCS. The number of laboratories that received the invitation to the survey was not followed.

A selection of questions with predetermined, multiple-choice answers prepared from the original survey (One Click Survey; available at: https://www.1ka.si/a/155920%26preview=on%26pages=all%26mobile=0, accessed June 19, 2020) is shown in Table 1. All participants' answers were collated. Multiple responses were allowed in some questions.

A chi-square test of independence was performed to examine the relation between ICC quality issues and different slides as well as the protocols used for ICC on

TABLE 2. Cytology Preparations Used for Immunocytochemistry in European Laboratories, n = 245

	No. of Laboratories (%)
Combination	147 (60)
2 Types: CB/CY, CB/LBC, CB/S, CY/S, CY/LBC, LBC/S	75 (31)
3 Types: CB/CY/LBC, CB/CY/S, CY/LBC/S, CB/LBC/S	47 (19)
4 Types: CB/CY/LBC/S	25 (10)
One Type	98 (40)
CB	72 (29)
LBC slides	20 (8)
CY	4 (2)
S	2 (1)

Abbreviations: CB, cell block; CY, cytospin; LBC, liquid-based cytology; S, smear.

cytology preparations. The results were considered significant at $P < .05$.

RESULTS

In total, 245 laboratories responded to the survey in the period from November 27, 2018 to January 30, 2019, and were included in the analysis. In addition to the 210 laboratories from 23 European countries, replies also were received from 17 non-European laboratories located in Azerbaijan, Canada, Chad, Hong Kong, Japan, Russia, Serbia, and Turkey. The location of 23 respondent laboratories remained unknown because of a short temporary error that occurred on the platform.

Not all laboratories provided a response to every question. The number of laboratories responding to a specific question is indicated appropriately.

Greater than one-half of the laboratories participating in this survey (147 of 245 laboratories; 60%) used a combination of 2, 3, or all 4 different types of cytology preparations for ICC (ie, cell blocks, cytospins, LBC slides, and smears), whereas 1 specific type of cytology preparation was used for ICC in 98 of 245 laboratories (40%) (Table 2).

The variability in cytology preparations and their combinations used for ICC in laboratories from participating countries is shown in Figure 1. In total, cell blocks were used for ICC in 186 of 489 laboratories (38%), and the frequency of using other cytology preparations was fairly equally distributed among cytospins (106 of 489 laboratories; 22%), LBC slides (105 of 489 laboratories; 21%), and smears (92 of 489 laboratories; 19%).

Fixatives and Slide Storage

The main fixative used was formalin for cell blocks (141 of 180 laboratories; 78%) and commercial fixatives for LBC slides (34 of 77 laboratories; 44%). Multistep fixation with a combination of different fixatives were most frequently used for smears (25 of 63 laboratories; 40%) and cytospins (30 of 93 laboratories; 32%) (Fig. 2). Cytospins, smears, and LBC slides also were fixed with ethanol (25%), methanol (15%), air drying (13%), commercial fixatives (12%), acetone (9%), formalin (9%), spray fixatives (9%), and, rarely, with Delaunay fixative (3%) or other fixatives (5%).

Immediately after fixation, the cytology preparations for ICC mainly were air dried (38%) or stained by Papanicolaou (31%) (Fig. 3) and stored at room temperature (60%) or in a refrigerator (27%). Only a few laboratories stored ICC slides in a freezer at −20°C (8%) or at −80°C (1%) (Fig. 4).

ICC Platforms and Protocols

Most laboratories (217 of 226; 96%) performed ICC using 1 or more automated platforms. The most frequently used platforms were Roche (Ventana) (54%) and Agilent (DakoCytomation) (21%), followed by Leica (BOND; 18%), and others (3%). Manual ICC still was performed in 9 of 226 laboratories (4%).

Almost one-half (238 of 527; 45%) of all ICC protocols used for cytology samples were the same as those used for FFPE tissue sections, and most were applied on cell blocks (165 of 238; 69%), whereas only 138 of 527 (26%) and 151 of 527 (29%) ICC protocols were optimized or validated for cytology preparations (Table 3).

Quality Assurance and Quality Control

Almost one-half of the laboratories (105 of 223; 47%) used FFPE tissue sections as a positive control for ICC on cytology samples, whereas others used cell blocks (20 of 223 laboratories; 9%) and various cytology preparations either alone (26 of 223 laboratories; 12%) or in combination with FFPE or cell blocks (23 of 223 laboratories; 10%). In 49 of 223 laboratories (22%), ICC was performed on cytology preparations without any positive control slides. Greater than one-half of the laboratories (149 of 223; 67%) rarely or never used negative controls for ICC on cytology preparations, and only one-half participated in external QC schemes (EQC) (Table 4).

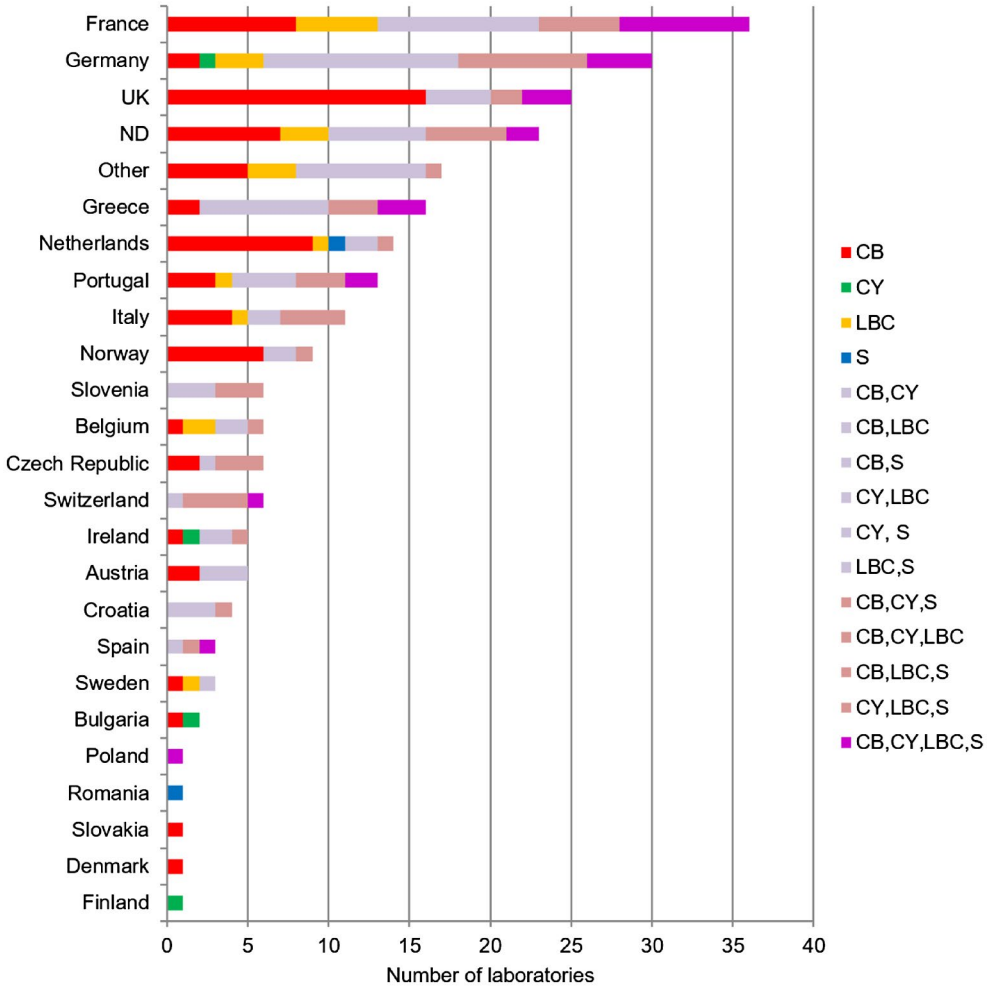

FIGURE 1. This chart illustrates cytology preparations and their combinations used for immunocytochemistry in participating laboratories (n = 245). CB indicates cell block; CY, cytospin; LBC, liquid-based cytology, ND, no data about location; S, smear.

Troubles With ICC on Cytology Samples

Laboratories participating in this survey usually or at least occasionally had problems with low cellular slides or an insufficient number of slides for all required biomarkers (121 of 195 laboratories [62%] and 105 of 181 laboratories [58%], respectively), followed by background staining (90 of 185 laboratories; 49%), difficult interpretation (82 of 183 laboratories; 45%), nonspecific staining (72 of 176 laboratories; 41%), weak staining (65 of 181 laboratories; 36%), poor cell morphology (66 of 185 laboratories; 36%), and inconsistent staining (59 of 171 laboratories; 35%).

The frequency of reported troubles with ICC did not differ among cytology preparations used for ICC ($P > .05$) (Table 5), whereas significantly higher frequencies of low cellular slides ($P = .027$), inconsistent staining ($P = .029$), and difficult interpretation ($P = .009$) were observed when FFPE protocols without optimization and validation were used on cytology preparations (Table 5).

Organization

In greater than one-half of laboratories (143 of 220; 65%), ICC was performed by noncytology staff in a noncytology department, such as a histology or IHC laboratory; ICC was performed by cytology staff in a histology or immunohistochemistry laboratory in 48 of 220 laboratories (22%), whereas ICC was performed in a cytology laboratory by cytology staff in only 29 of 220 laboratories (13%).

In most participating laboratories (132 of 191; 69%) >100 and <5000 ICC tests are performed per year (chi-square statistic, 1477 ± 2159) (Table 6).

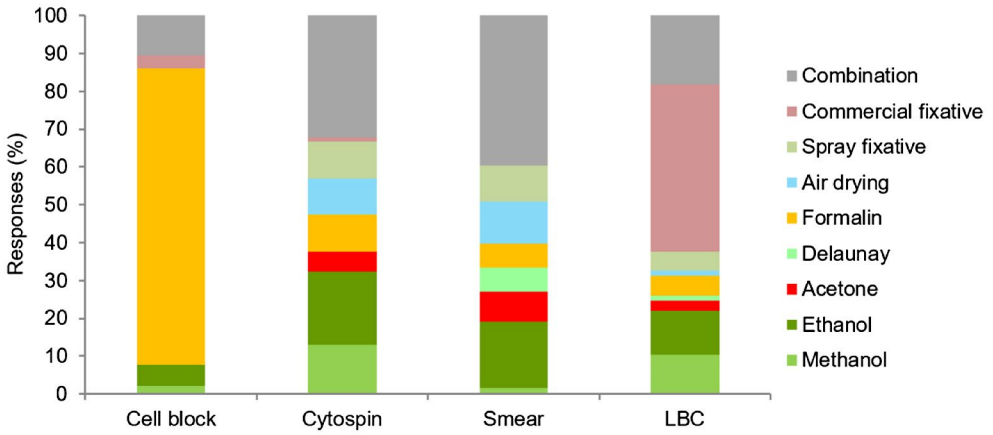

FIGURE 2. The fixatives used for the fixation of immunocytochemistry preparations are illustrated LBC indicates liquid-based cytology.

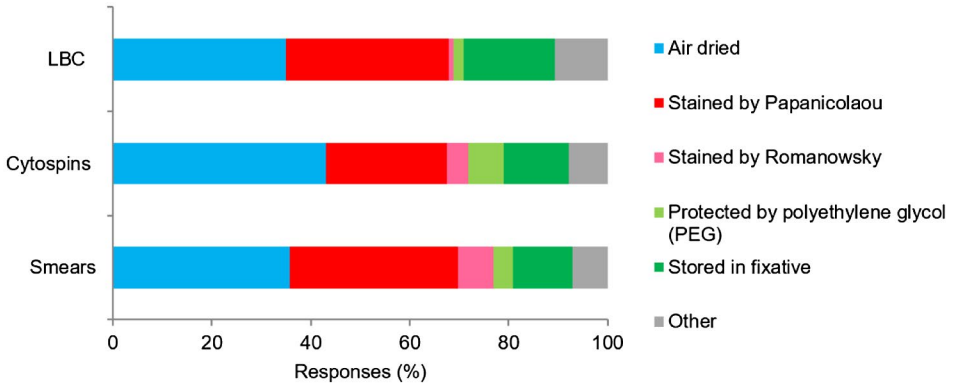

FIGURE 3. Postfixation steps for immunocytochemistry slides are illustrated. LBC indicates liquid-based cytology.

DISCUSSION

Our survey demonstrated that ICC is extensively used in European laboratories with great variation in practices and mostly without quality assurance measures. The high average and median numbers of ICC tests performed per year (1477 and 500, respectively) underlines the extensive role of ICC in European cytology laboratories, which is in contrast to a College of American Pathologists survey indicating that ICC on cytology samples is a low-volume test with a median of only 180 tests per year per laboratory.[31]

As expected and in line with other studies,[30,31] our survey confirmed great variation in ICC practices in European laboratories. This survey enabled us to demonstrate the range of variability in individual preanalytical and analytical steps for ICC on cytology preparations.

A previous EFCS survey published in 2011 indicated that various kinds of cytology preparations are used for ICC.[30] However, replies from only 28 laboratories were included and analyzed in that study; therefore, we decided to perform a new investigation to establish the comprehensive, up-to-date situation in European laboratories.

We found that cell blocks were exclusively used for ICC in 29% of laboratories, whereas cell blocks in combination with other preparations were used in 38% of laboratories. Although the frequency of using cell blocks in European laboratories had increased from the 20% reported in the first EFCS survey,[30] it is still lower than the 49% reported in the College of American Pathologists survey.[31]

The frequency of using smears alone or in combination with other preparations for ICC had decreased

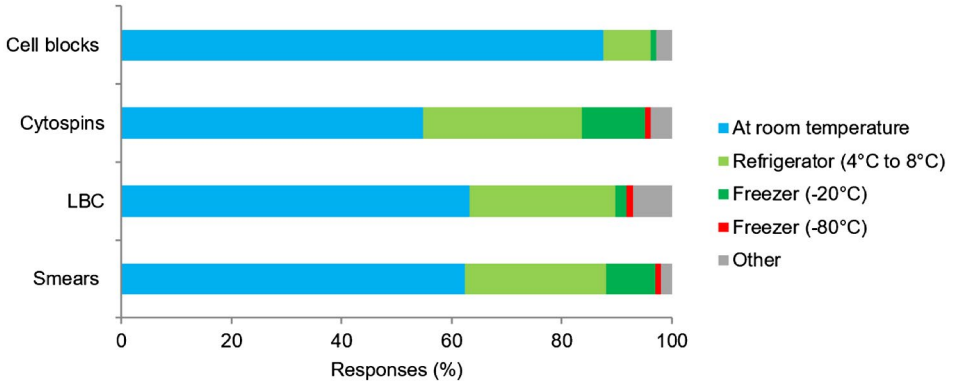

FIGURE 4. Storage of immunocytochemistry slides is illustrated. LBC indicates liquid-based cytology.

TABLE 3. Immunocytochemistry Protocols Used for Different Cytology Preparations

Protocols: Multiple Responses Allowed, n = 527	No. of ICC protocols (%)				
	Cell Blocks	Cytospins	LBC Slides	Smears	Total
Same as for FFPE sections	165 (69)	22 (9)	28 (12)	23 (10)	238 (45)
Optimized	10 (7)	43 (31)	44 (32)	41 (30)	138 (26)
Validated	14 (9)	49 (32)	39 (26)	49 (32)	151 (29)

Abbreviations: FFPE, formalin-fixed, paraffin-embedded; ICC, immunocytochemistry; LBC, liquid-based cytology.

compared with the first EFCS survey, from 35% to 19%, whereas there was only a slight difference in the frequency of using cytospins (from 29% to 22%) and LBC preparations (from 16% to 21%). In general, our survey showed that various combinations of cytology preparations are used for ICC in greater than one-half of laboratories (60%), whereas the remaining 40% of laboratories use only 1 specific type of slide. Differences in procedures of preparing cell blocks, cytospins, smears, and LBC slides were not included in the survey or analysis.

In addition to the variability in preparations used for ICC, our survey also revealed a range of variability in fixation and postfixation steps. As expected, we found the least variability in fixation and postfixation steps for cell blocks and marked variability for all other cytology preparations.

Another important aspect of variability in ICC is the staining procedure. The manual ICC staining procedure used in a significant proportion of laboratories (36%) in 2011[30] has now been replaced with various automated platforms in most of laboratories (96%).

Crucial steps in the IHC staining procedure (antigen retrieval, blocking nonspecific activities, antibody dilution, detection system, counterstaining) can significantly affect the final results.[14,32] This led to the recommendation

TABLE 4. Participation in External Quality Control

Provider of EQA, n = 220	No. of Laboratories (%)
None	109 (50)
UK NEQAS	44 (20)
Comparison between laboratories	43 (19)
National EQA	16 (7)
NordiQC	8 (4)

Abbreviations: NordiQC, Nordic Immunohistochemical Quality Control; EQA, external quality assessment; UK NEQAS, United Kingdom National External Quality Assessment Service.

that each step in the IHC staining procedure should be carefully tailored and that the whole procedure should be standardized and validated in each laboratory to assure the reliable demonstration of antigens in FFPE tissue sections.[18,20,22,33] The whole process for optimization and validation of IHC tests on FFPE tissue sections is well defined, although with only brief reference to cytology specimens.[18-20] Laboratories are advised to test only a sufficient number of cytology specimens to ensure that assays consistently achieve the expected results,[18] whereas there are no guidelines to date about the adjustment of standard IHC staining procedures for differently prepared cytology preparations in a process of optimization.

Because different fixatives (and, in fact, any step in the preanalytical sample processing procedure)

TABLE 5. Troubles With Immunocytochemistry Quality on Different Cytology Preparations and for Different Immunocytochemistry Protocols

	Percentage of ICC Protocols		Percentage of Preparations			
Quality Issue	Same as FFPE	Optimized and Validated	Cell Block	Cytospin	LBC	Smear
Low cellular slides	61[a]	47	62	58	66	55
Not enough slides	70	61	59	69	66	69
Inconsistent staining	50[a]	31	37	32	40	37
Background staining	44	54	48	51	52	62
Nonspecific staining	42	38	43	45	45	48
Weak staining	40	31	37	35	36	40
Poor cell morphology	32	38	34	38	30	41
Difficult interpretation	57[a]	34	50	45	40	48

Abbreviations: FFPE, formalin-fixed, paraffin-embedded; ICC, immunocytochemistry; LBC, liquid-based cytology.
[a]$P < .05$.

TABLE 6. Number of Immunocytochemistry Tests Performed in a Laboratory per Year

ICC Tests per Year	No. of Laboratories (%)
≤100	46 (24)
101-1000	80 (42)
1001-5000	52 (27)
>5000	13 (7)

Abbreviation: ICC, immunocytochemistry.

can affect the spatial structure of antigens and, consequently, the final IHC reactions, it can be expected that staining procedures optimized and validated for standard FFPE tissue sections might not be suitable for differently prepared and fixed cytology preparations.[14,15,18,23] This was previously confirmed in 1 of the rare such studies by Sauter et al, who found that standard IHC staining procedures were not always suitable for the demonstration of antigens on cell blocks fixed with alcohol fixatives and that nearly one-half of the tested antibodies required procedure adjustment.[34] Similarly, 1 of our own studies demonstrated that cytospins fixed in methanol required adjustment of IHC staining protocols for all tested antigens.[35] So, theoretically, staining protocols validated for FFPE tissues should be adjusted for cytology preparations in the process of optimization, in which the dilution, pretreatment, incubation time, and detection system yielding the best results on cytology preparations are set in each laboratory. Moreover, the accuracy and reliability of results obtained with such optimized ICC protocols should be validated on suitable cases by correlation of the results with other methodologies, eg, IHC on corresponding tissue samples.

However, our survey showed that optimization and validation of ICC protocols for cytology preparations are not yet common practice. Staining procedures validated for FFPE tissue sections in participating laboratories are used not only for cell blocks but also for variously fixed and prepared cytology preparations (31%), but only a few laboratories optimized or validated their ICC protocols (26% and 29%, respectively). In laboratories that optimized ICC staining procedures, all crucial steps in the ICC staining procedure (slide pretreatment, antigen retrieval, antibody dilution, detection system, platform, counterstaining) were more or less equally included. Details about validation and optimization of ICC protocols were not included in our survey.

Another inadequately addressed area to date in QC for ICC on cytology preparations is control slides. Basic QC and good IHC laboratory practice require that control samples used for optimization, standardization, validation, and daily controls are prepared and fixed in the same manner as patient samples.[18,20,24] However, our survey demonstrated that 22% of laboratories performed ICC on cytology preparations without control slides, and almost one-half used FFPE tissue sections as control slides. Similar results were also found in a review of the UK National External Quality Assessment Service ICC (NEQAS, cytology module), in which 66% of participants sent FFPE tissue sections as in-house control slides for ICC on cytology samples,[6] and by Colasacco and colleagues in a meta-analysis in which only 11 of 87 ICC articles (13%) mentioned positive and negative controls run on identically prepared samples.[36] Moreover, we observed that 50% of laboratories did not use any kind of

EQA, which is also considered to be a basic QA tool.[24] Among several EQC programs for IHC,[24] UK NEQAS ICC is the only 1 offering a specialized cytology module. Although the program is part of the UK NEQAS, it also accepts participants from other countries. However, we found that only 20% of European laboratories made use of this specialized EQC program.

In view of the inadequately addressed QA/QC measures identified in our survey, it is actually not surprising that up to one-half of the laboratories usually or at least occasionally had issues with the quality of ICC on cytology samples, such as weak background and nonspecific or inconsistent ICC staining. Furthermore, more than one-half of the laboratories had problems with low cellular slides and an insufficient number of slides for all required biomarkers, which probably could be prevented with improved sample processing procedures as well sample triaging and the implementation of rapid on-site evaluation of sample adequacy.[37,38] Moreover, our analysis showed that low cellular slides, inconsistent staining, and difficult interpretation were related to the use of IHC protocols that were not optimized and validated for cytology preparations ($P = .027$, $P = .029$, and $P = .009$, respectively). Longer and harsher protocols used for FFPE tissue sections can probably cause damage and loss of cells, also affecting staining and interpretation.

In general, comprehensive optimization, standardization, and validation procedures recommended for FFPE tissue samples are obviously not feasible to the same extent for small and specific cytology samples; however, laboratories should test a sufficient number of cases to ensure that assays consistently achieve the expected results.[20,39]

Furthermore, it is crucial to optimize and standardize the preparation of cytology preparations for ICC and to reduce as much as possible preanalytical variables that could affect the final results. Only high-quality cytology preparations with sufficient, well preserved, and well distributed diagnostic cells ensure high-quality ICC reactions and reliable interpretation.

Control preparations prepared in the same manner as patient preparations should be used for optimization, standardization, validation, and daily controls to ensure correct and reliable ICC results. Absolute compliance with this requirement obviously will not be feasible for laboratories that use 3 different types of cytology preparations for ICC, in addition to cell blocks. However, laboratories should reduce as far as possible the variability in procedures for preparation of cytology samples for ICC and at least prepare adequate cytology controls according to the procedure most often used. An excellent source of cells for preparation of controls for ICC are human cell lines with defined expression of biomarkers, although they are not easy to access and are not available for all antigens. Suitable cytology control preparations can also be prepared from leftover effusion fluids or brushings from cut surfaces of unfixed fresh biopsy samples,[3] which is especially important for new predictive markers such as ALK, ROS1, PD-L1.

EQC is of utmost importance for achieving and improving the quality of any laboratory test, and laboratories should actually participate in EQC for all provided tests.[20,24] Therefore, cytology laboratories also should participate in EQC for ICC. Currently, the single EQC service for ICC on cytology samples—UK NEQAS ICC—offers only a limited number of markers. We believe that a cytology-tailored EQC service should be expanded in the future and further developed to enable widely available EQC suitable for the majority of cytology laboratories.

In conclusion, it seems that modern clinical practice requires ICC as an ancillary diagnostic method on a significant number of cytology samples, and an even greater increase in demand probably can be expected. To be able to ensure accurate and reproducible ICC results on cytology samples, laboratories will certainly have to implement at least the following QA/QC measures in their practice:

- Optimize and standardize the preparation of cytology preparations for ICC,
- Optimize and validate ICC staining protocols,
- Use appropriate cytology control slides for QA/QC procedures (positive controls, negative controls, optimization, and validation), and
- Make use of external QC programs.

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CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

AUTHOR CONTRIBUTIONS

Irena Srebotnik Kirbiš: Conceptualization, methodology, formal analysis, and writing–original draft. **Rúben Rodrigues Roque:** Data curation, formal analysis, and writing–review and editing. **Massimo Bongiovanni:** Supervision and writing–review and editing. **Margareta Strojan Fležar:** Supervision and writing–review and editing. **Béatrix Cochand-Priollet:** Project administration, supervision, and writing–review and editing.

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