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Multi-Tissue Controls and Multiplex Immunocytochemistry in Pulmonary Cytology

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Multi-tissue controls · Multiplex immunocytochemistry · Immunocytochemistry · Pulmonary cytology

Abstract
Introduction: The World Health Organization 2021 lung cancer classification highlights the central role of immunohistochemistry (IHC) in diagnostic pathology. Despite traditional IHC being essential, its limitation to one marker per tissue section brings challenges, particularly when facing cytological limitedly sized samples. To overcome these challenges, multiplex immunocytochemistry (mICC) techniques offer the simultaneous detection of multiple markers from a single section. These advances complement the highly complex imaging techniques that enable additional analyses of cellular interactions. ***Methods:*** The present study outlines a comprehensive mICC methodology of an automated multiplex immunoperoxidase staining method and multiple tissue hybrid controls for ICC/mICC. Protocols are presented in detail and demonstrate a careful approach to optimizing various markers for diagnostic workup including immunotherapy. ***Conclusion:*** Multiplex IHC/ICC emerges as a transformative force in biomedical diagnostics and research. Beyond simultaneous marker detection, it unravels complexities within tissues – unveiling co-localization nuances, deciphering expression patterns, and enhancing understanding of cellular populations. As personalized treatments gain prominence, the study emphasizes the heightened importance of diagnostic tools and sample adequacy. The present methodological study, encapsulating an automated multiplex immunoperoxidase staining method, symbolizes a stride towards precision in pulmonary carcinoma diagnosis. Multi-tissue controls represent a key element in quality assurance in pathology laboratories.

Introduction

Over the past decades, the field of immunohistochemistry (IHC) has made significant progress. The World Health Organization (WHO) 2021 classification of lung cancer incorporated IHC in the classification system [1, 2].

The traditional IHC is a valuable diagnostic tool in surgical pathology, and despite its limitations, such as the labelling of only one marker per tissue section, it remains an essential technique in a diagnostic workup. Examination of all requisite markers may not be possible if the samples are

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depleted, especially with samples acquired through minimally invasive methods like core needle biopsies or fine needle aspirations. This is a challenge, particularly in the evaluation of undifferentiated tumours, sarcomas, and lymphomas, which often demand an extensive panel exceeding 12 IHC stains for accurate diagnosis [3, 4]. However, in the era of targeted therapy and immunotherapy, an extensive panel of predictive markers is also required in the diagnostic workup of carcinomas [5].

The introduction of multiplex immunohistochemistry/immunofluorescence (mIHC/IF) technologies has addressed this limitation and is becoming more popular, enabling the simultaneous detection of multiple markers in a single section. Additionally, advancements in highly multiplexed imaging techniques offer comprehensive analyses of cell composition and interactions, increasing diagnostic potential. The ability to assess multiple markers in one section is especially beneficial for low cellular samples [6, 7].

The mIHC/IF enables the application of several diagnostic markers in a single tissue section. Furthermore, the mIHC/IF provides detailed, quantitative data on the quantity and spatial distribution of various cells including immune cells within tumours, serving as a powerful investigative tool for understanding the immune context of the tumour [6].

Since fixation and other pre-analytical steps can affect antigenicity and quality of the staining, using control samples with different processing methods can lead to unreliable results. Variability in staining patterns caused by incorrect controls can complicate result interpretation in cytological samples processed differently from surgical pathology specimens. When using an inappropriate control type or controls treated differently from the sample, the likelihood of obtaining a false-negative staining result is heightened [8–10].

According to the College of American Pathologists 2024 guideline update "Principles of Analytic Validation of Immunohistochemical Assays," laboratories should use validation tissues processed with the same fixative and methods as clinical cases whenever possible. The fixation and processing methods can affect certain epitopes in a manner that may alter the reliability of the assay, making consistent processing crucial. However, harvesting control tissues in-house has become increasingly challenging, leading many laboratories to rely on commercial suppliers, which may not replicate the laboratory's specific fixation and processing methods. Therefore, maintaining the flexibility of using in-house controls remains important. Additionally, laboratories should verify assay performance using at least one known positive and one known negative control tissue when introducing a new antibody lot for an existing validated assay [11].

In surveys conducted by the European Federation of Cytology Societies (EFCS) and the College of American Pathologists (CAP), the importance of positive and negative controls in ensuring accurate staining results was examined. Without proper controls, it is difficult to determine if the staining observed in the patient's sample is real or false due to an error in the staining process. Control samples with different fixation and procession methods compared to the patient's sample can lead to wrong interpretation and diagnostic mistakes [8–10].

In the CAP survey, more than half (59.2%) of laboratories used control samples that were processed differently from the patient's cytology specimens. Only 40.8% of laboratories used control samples that were processed in the same way as the patient's samples. Validation of non-FFPE (formalin-fixed, paraffin-embedded) fixation methods is expensive and time and labour consuming. Many cytology tests are low volume, meaning they are performed infrequently, which makes validation even less cost effective. The high cost and time investment required for validation create a challenge for many laboratories [8, 9]. Nevertheless, accreditation systems require those quality assurance steps, and the latest ISO 15189 is risk prevention oriented [12]. As treatments become more individualized and case specific, the importance of diagnostics and the adequacy of samples becomes even more crucial, particularly in immunotherapy, which is largely based on accurate patient profiling and biomarker analysis.

This methodological study describes an automated multiplex immunoperoxidase staining method using common diagnostic markers for the differential diagnosis and immunotherapy profiling of pulmonary carcinomas. The horseradish peroxidase (HRP) multimer was used for detection for its stability and reproducibility. The distinct feature of the multiplexes in this study is enhanced by the fact that these were made for brightfield microscopy with chromogenic visualization, thus preserving morphology and facilitating interpretation; translucent chromogens have been used for staining, allowing better visualization of the colocalization by highlighting it as a refractive colour; the automated method facilitates multiple protocol steps and does not require dark field microscopy and fluorochromes for visualization. In addition, cytology-specific multi-tissue controls were developed and applied in the present study.

Methods

Pulmonary samples were ethanol-fixed biopsies obtained from 486 patients who underwent endobronchial ultrasound bronchoscopy (EBUS) between January 2017 and December 2018 and

Table 1. Control block composition and fixations

N	Tissue fragment	Diagnosis	Fixation, duration
1	Small intestinal smooth muscle	Normal tissue	Formalin, 24–48 h
2	Intestinal epithelium	Normal tissue	Formalin, 24–48 h
3	Skin	Normal tissue	Formalin, 24–48 h
4	Pancreas	Normal tissue	Formalin, 24–48 h
5	Tonsil	Normal tissue	Formalin, 24–48 h
6	Placenta	Normal tissue	Formalin, 24–48 h
7	Skin	Normal tissue	Ethanol 50%, 2 weeks
8	Tonsil	Normal tissue	Ethanol 50%, 2 weeks
9	Intestinal epithelium	Normal tissue	Ethanol 50%, 2 weeks
10	Tonsil	Normal tissue	Ethanol 50%, 1 week
11	Small intestinal smooth muscle	Normal tissue	Ethanol 50%, 2 weeks
12	Placenta	Normal tissue	Ethanol 50%, 1 week
13	Small intestinal smooth muscle	Normal tissue	Ethanol 50%, 1 week
14	Intestinal epithelium	Normal tissue	Ethanol 50%, 1 week

were retrospectively selected from C5Lims – laboratory information system of the Fimlab Laboratories Pathology Department, Tampere, Finland [13]. Control samples were collected from fresh normal tissues from diagnostic specimens. The study was approved by the Ethical Committee of Pirkanmaa Hospital District and was performed according to the guidelines of the Declaration of Helsinki. This consent protocol was reviewed and the need for written and informed consent was waived by the Ethical Committee of Pirkanmaa Hospital District, decision reference number R17174.

Multi-Tissue Hybrid Controls for Immunocytochemistry

A multi-tissue control block consisting of both FFPE and ethanol-fixed paraffin-embedded tissue cores was introduced for immunocytochemistry (ICC). Control tissues from the same original specimen were separated for two different fixation processes, ethanol fixation, and formalin fixation (Table 1). The comparison between FFPE and ethanol-fixed paraffin-embedded control blocks was not fully in line, as the hybrid block was intended to contain pancreatic tissue, which could not be utilized in ethanol-fixed form. The block still allowed to control the normal function of the staining, considering insulinoma-associated protein 1 (INSM1) expression levels, and to verify the reliability of the staining.

During the grossing step, fresh tissue was dissected into multiple 2–3 mm pieces to ensure similarity to cytological material. Different fresh tissue types (tonsil, placenta, skin, and small intestine) as normal tissue remnants from the grossing laboratory were collected.

A 3 mm puncher for ethanol-fixed tissue and a 4 mm puncher for formalin-fixed tissue were used as internal quality assurance and control parameters. By this method, we were able to practically distinguish them from each other with the naked eye during processing and under microscopy, thus reducing misinterpretation.

Formalin fixation, for 24 h–48 h, was performed with neutral buffered 10% formalin at room temperature. Ethanol fixation was performed with two different time points: 1-week fixation in 50% ethanol at room temperature and 2-week fixation, respectively. Post-fixation for 4–6 h in formalin was performed for ethanol-fixed tissues before tissue processing.

The different fixation times are due to the fact that formalin fixes quickly, but ethanol fixes slowly. In our practice, we check the morphology of the cytospins first, and based on the findings, pathologists order the cell blocks (CBs) and ICC later, so the ethanol fixation is longer, and the time interval mimics the actual laboratory flow. In addition, the pre-analytical fixation period may be long as our central laboratory serves a large geographic area of several provinces, and the distances are long, so samples can reach the laboratory in a few days after collection.

Formalin- and ethanol-fixed tissues were processed using Pathos Delta hybrid processing technology (Milestone Medical, Kalamazoo, MI, USA). Ethanol-fixed material was processed with a short biopsy protocol, mimicking the cytological material process. Formalin-fixed tissues were processed with the routine protocol for histological specimens.

Embedded blocks were sectioned and stained with haematoxylin and eosin to ensure the quality and correct orientation of the tissue. Cores were punched using a Kai medical disposable biopsy puncher (Kai Industries Co., Ltd., Osaka, Japan). Ethanol-fixed tissue cores were punched from donor blocks with a 3 mm puncher and formalin-fixed material with a 4 mm puncher, respectively. Tissue cores were embedded with a similar orientation as in the donor block as shown in Figure 1.

Multiplex Immunocytochemistry Staining

The automated multiplex immunoperoxidase staining methodology was designed utilizing the common diagnostic markers for the differential diagnosis and immunotherapy profiling of pulmonary carcinomas, including adenocarcinoma, squamous cell carcinoma, and small cell carcinoma. Two chromogenic triple staining methods with five different chromogens were designed. The synaptophysin, chromogranin A, and INSM1 combination for the diagnosis of small cell carcinoma and the programmed death-ligand 1 (PD-L1), p40, and thyroid transcription factor 1 (TTF-1) combination for the diagnosis and immunotherapy profiling of pulmonary non-small cell carcinomas were introduced. All primary antibodies were first optimized with a single staining method before combining multiplex staining. The immunohistochemical multiplex staining consists of three sequential staining methods, respectively.

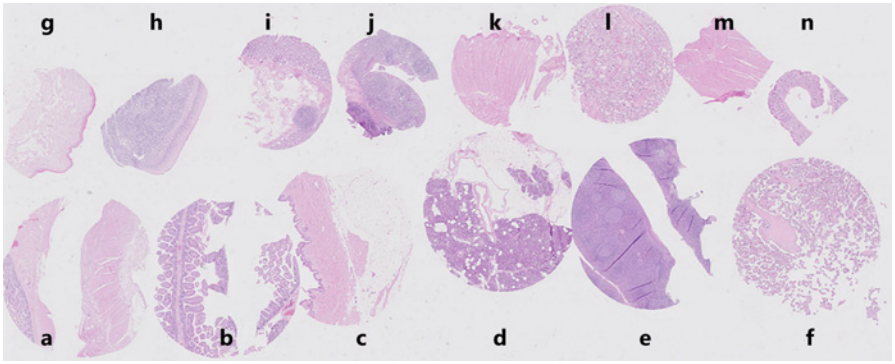

Fig. 1. Haematoxylin and eosin-stained slide section from a hybrid block containing both ethanol-formalin-fixed control tissues (3-mm punches, upper row) and formalin-fixed control tissues (4-mm punches, lower row). **a** Intestinal smooth muscle, formalin fixed. **b** Intestinal epithelia, formalin fixed. **c** Skin, formalin fixed. **d** Pancreas, formalin fixed. **e** Tonsil, formalin fixed. **f** Placenta, formalin fixed. **g** Skin, 50% ethanol fixed. **h** Tonsil, 50% ethanol fixed. **i** Intestinal epithelia, 50% ethanol fixed. **j** Tonsil, 50% ethanol fixed. **k** Intestinal smooth muscle, 50% ethanol fixed. **l** Placenta, 50% ethanol fixed. **m** Intestinal smooth muscle, 50% ethanol fixed. **n** Intestinal epithelia, 50% ethanol fixed.

The designed hybrid control sections and EBUS biopsies [13] were sectioned at a thickness of 3 μm on the positively charged TOMO® slides (Matsunami Glass Ind., Ltd., Osaka, Japan). IHC was performed using a Ventana DISCOVERY ULTRA automated slide stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA). Deparaffinization and rehydration were performed on the instrument. Cell Conditioning 1 (CC1) alkaline antigen retrieval solution (Ventana Medical Systems, Inc., Tucson, AZ, USA) was used as a pretreatment solution for heat-induced epitope retrieval (HIER). HIER was performed only before the first primary antibody incubation step. Endogenous peroxidases were quenched using the DISCOVERY inhibitor (Ventana Medical Systems, Inc., Tucson, AZ, USA) for 8 min. Between the sequential staining steps, the deactivation of previous primary antibodies and the detection systems was performed using the antibody denaturation step and applying Cell Conditioning 2 (CC2) acidic antigen retrieval solution for 8 min at 99°C (Ventana Medical Systems, Inc., Tucson, AZ, USA). After the final chromogenic visualization, the slides were counterstained with haematoxylin II, dehydrated, and coverslipped (Fig. 2, 3). The detailed protocols and reagent information are summarized in Tables 2 and 3.

In the present study, we tested the effect of antibody sequence in multiplex staining. Vendors do not select the antibodies based on feasibility for ethanol fixation. It was the reason behind we have both formalin- and ethanol-fixed material in a control panel. We found that staining is impaired in all clones of ethanol-fixed tissue material compared to formalin-fixed material (Fig. 4, 5). This has also been shown in previous studies that ethanol fixation impairs antigenicity [14–20]. All markers were optimized, and multiplex ICC results were compared to formalin-fixed IHC and ethanol-fixed single ICC. The comparison was made with DAB as chromogen, and based on this implementation testing protocols, antibodies, and its optimized protocols were implemented in the multiplex protocols. Antigen depletion was particularly observed in tonsillar follicular macrophages. Reduced staining was also observed in tonsillar epithelial crypt cells. The weakest staining result was obtained specifically using the SP142 clone, which is typically used in an IC algorithm that considers the staining of immune cells in relation to the sample surface area. The clearest staining result and most intense staining were obtained with staining using the SP263 clone. In placental syncytiotrophoblasts, staining was seen at both the apical membrane and the basal membrane.

In our study, we found that Ventana DISCOVERY's purple chromogen and yellow chromogen translucency allowed the co-localization resulting in orange stain colour. This technique allows more accurate analysis and diagnosis of antigens localized in the same cell and the same cell compartment. In the past, multiplex chromogenicity has been more obscuring and has not allowed for more accurate diagnostics.

It should be noted that the Ventana DISCOVERY ULTRA is an open immunostaining automation machine, which allows for protocol amplification and optimization for ethanol-fixed sample material. For triple staining, the clone SP263 was selected as the PD-L1 antibody due to its best staining results. The protocol without amplification allows sensitization of the protocol and thus compensation for antigen loss in the intensity of staining.

PD-L1-Stained Controls

The performance of PD-L1 staining with hybrid controls was tested using pathology laboratory diagnostic protocols (Table 4). Table 4 presents the final optimized PD-L1 protocols for four different clones. In summary, all HIER steps were performed on board with an alkaline antigen retrieval buffer at 99°C. All PD-L1 antibodies were optimized with hybrid block sections with Ventana BenchMark ULTRA instrument for multiplex protocol in Ventana DISCOVERY ULTRA. Clone SP263 was selected according to its robust and sensitive staining pattern during the validation of multiplex staining. Finally, fine-tuning of antibody incubation and detection method was performed.

PD-L1 staining with clones SP263 and SP142 were Ventana PD-L1 IHC assays, while 22C3 and 28-8 were laboratory-prepared proprietary tests. Ventana's protocols are closed and in line with the Pharmacotherapy Recommendation. The 22C3 and 28-8 are laboratory-validated protocols validated on the Ventana BenchMark ULTRA platform. Tyramide amplification was used for staining with SP142, 22C3, and 28-8 as clones.

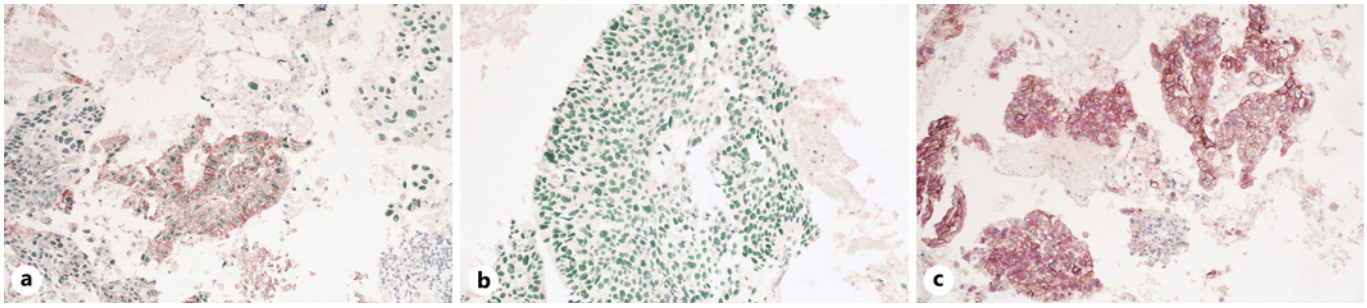

Fig. 2. mICC in pulmonary non-small cell carcinomas diagnostic workup and immunotherapy: p40 antibody nuclear positivity distinguishes squamous cell carcinoma from TTF-1 antibody nuclear positivity characteristic for adenocarcinoma. PD-L1 is applied as a marker for immunotherapy. **a** Case of squamous cell carcinoma in an EBUS-targeted lymph node in a 73-year-old male. PD-L1 membranous positivity in brown in 30% of carcinoma cells. Nuclear green positivity of p40 in most carcinoma cells in variably disperse fragments. Background lymphocytes are negative for all three markers. Multiplex immunostaining for PD-L1, p40, and TTF-1 (×200 magnification). **b** PD-L1 negative squamous cell carcinoma in an EBUS-targeted lymph node in a 70-year-old female. PD-L1 is negative in carcinoma cells. Nuclear green positivity of p40 in most carcinoma cells in a tight sheet. Multiplex immunostaining for PD-L1, p40, and TTF-1 (×200 magnification). **c** Adenocarcinoma in an EBUS-targeted lymph node in a 57-year-old male. PD-L1 membranous positivity in brown in the majority of carcinoma cells (95%). Nuclear red positivity of TTF-1 in most carcinoma cells in tubular and trabecular groups. Background lymphocytes are negative for all three markers. Multiplex immunostaining for PD-L1, p40, and TTF-1 (×200 magnification).

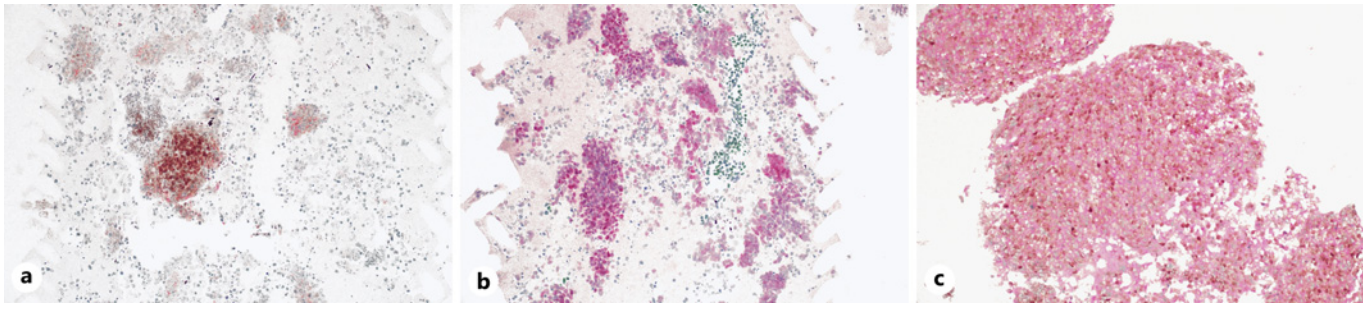

Fig. 3. mICC in pulmonary small cell carcinomas diagnostic workup. Three neuroendocrine markers (INSM1, synaptophysin, chromogranin) were applied as neuroendocrine markers sensitivity and specificity vary. Pulmonary small cell carcinomas are TTF-1 positive. **a** Case of small cell carcinoma in an EBUS-targeted lymph node in a 76-year-old female. INSM1 nuclear positivity in brown, synaptophysin cytoplasmatic granular positivity in purple, and chromogranin A cytoplasmatic granular positivity in yellow. Note that despite all markers are positive in this case, not all cells express all three markers; so with less sufficient samples, only one to two markers can be present. Multiplex immunostaining for INSM1, synaptophysin, and chromogranin A (×200 magnification). **b** TTF-1 nuclear positivity in red in a small cell carcinoma in an EBUS-targeted lymph node shown in **a**. Note a squamous cell metaplastic fragment with p40 nuclear positivity in green. PD-L1 is negative. Multiplex immunostaining for PD-L1, p40, and TTF-1 (×200 magnification). **c** Case of small cell carcinoma in an EBUS-targeted lymph node in a 68-year-old male. INSM1 nuclear positivity in brown in part of carcinoma cells, synaptophysin cytoplasmatic granular positivity in purple in almost all cells. Chromogranin A is negative. Expression of neuroendocrine markers is variable in various cases but also within the tissue of a single case as case **a**. showed expression of all three markers but not diffusely. In this case, only two markers were expressed with only one of them to be expressed diffusely. Multiplex immunostaining for INSM1, synaptophysin, and chromogranin A (×200 magnification).

To improve staining results, the antibody sequence was considered. We first applied those antigens that need to be counted and quantified (as PD-L1) as tissue is changing with the following subsequent processing sequences, morphology is less visible, and antigenicity weakens. In addition, chromogen characteristics should be considered: first, we use opaque chromogens and after translucent chromogens.

Discussion

Analysis of several immunohistochemical markers within a single tissue section is a complex task that requires multiplex chromogen or fluorescence detection. The

Table 2. Multiplex protocol of non-small cell carcinoma and small cell carcinoma

Step	Reagent	Time, min	Temperature, °C
Multiplex: PD-L1 + p40 + TTF-1			
Deparaffinization	DISCOVERY Wash	12	70
Pretreatment, HIER in CC1	CC1	64	99
Inhibitor CM for quenching endogenous peroxidases	Inhibitor CM	8	37
Primary antibody 1: PD-L1 (SP263)	Primary antibody 1	32	36
Detection: Anti-Rabbit-HQ	DISCOVERY HQ HRP	16	Ambient
Detection: Anti-HQ-HRP	DISCOVERY HQ HRP	16	Ambient
Chromogen 1: DAB	DISCOVERY ChromoMap DAB	8	Ambient
Denaturation step for deactivation antibodies and HRP	CC2	8	99
Primary antibody 2: p40 (BC28)	Primary antibody 2	48	36
Detection: OptiView Linker	OptiView DAB IHC Detection	20	Ambient
Detection: OptiView HRP Multimer	OptiView DAB IHC Detection	20	Ambient
Chromogen 2: Green HRP Kit	DISCOVERY Green HRP Kit	64	Ambient
Denaturation step for deactivation antibodies and HRP	CC2	8	99
Primary antibody 3: TTF-1 (SP141)	Primary antibody 3	48	36
Detection: OptiView Linker	OptiView DAB IHC Detection	20	Ambient
Detection: OptiView HRP Multimer	OptiView DAB IHC Detection	20	Ambient
Chromogen 3: RED HRP Kit	DISCOVERY RED HRP Kit	64	Ambient
Counterstaining with Haematoxylin II	Haematoxylin II	8	Ambient
Bluing of haematoxylin	Bluing reagent	4	Ambient
Multiplex: INSM1 + synaptophysin + chromogranin A			
Deparaffinization	DISCOVERY Wash	24	70
Pretreatment, HIER in CC1	CC1	64	99
Inhibitor CM for quenching endogenous peroxidases	Inhibitor CM	8	37
Primary antibody 1: INSM1 (A-8), 1:50	Primary antibody 1	60	36
Detection: Anti-Rabbit-HQ	DISCOVERY HQ HRP	24	Ambient
Detection: Anti-HQ-HRP	DISCOVERY HQ HRP	24	Ambient
Chromogen 1: DAB	DISCOVERY ChromoMap DAB	8	Ambient
Denaturation step for deactivation antibodies and HRP	CC2	8	99
Primary antibody 2: Synaptophysin (SP11), RTU	Primary antibody 2	52	36
Detection: OptiView Linker	OptiView DAB IHC Detection	20	Ambient
Detection: OptiView HRP Multimer	OptiView DAB IHC Detection	20	Ambient
Chromogen 2: Purple HRP Kit	DISCOVERY Purple HRP Kit	32	Ambient
Denaturation step for deactivation antibodies and HRP	CC2	8	99
Primary antibody 3: Chromogranin A (LK2H10), RTU	Primary antibody 3	60	36
Detection: OptiView Linker	OptiView DAB IHC Detection	20	Ambient
Detection: OptiView HRP Multimer	OptiView DAB IHC Detection	20	Ambient
Chromogen 3: Yellow HRP Kit	DISCOVERY Yellow HRP Kit	96	Ambient
Counterstaining with Haematoxylin II	Haematoxylin II	8	Ambient
Bluing of haematoxylin	Bluing reagent	4	Ambient
Dehydration with rising ethanol series, xylene clearing, and coverslipping.			

growing popularity of both chromogenic and fluorescent multiplex staining is due to the development of reliable multiplex staining techniques and sophisticated multispectral imaging. The benefits of multiplex staining include the ability to preserve valuable tissue samples or even the use of limited specimens and the ability to co-localize antigens and improve the accuracy of interpretation. This is especially important for the emerging field of immunotherapy as it allows the identification and detection of immune cells, as well as the characterization of response biomarkers [21–27].

The mIHC/IF approaches have become significantly more powerful, providing enhanced insights into disease heterogeneity and the underlying systems biology mechanisms. These approaches also contribute to the preservation of limited tissue material [6]. Tumour heterogeneity and the limited representativeness of small biopsy or cytology samples

Table 3. Multiplex reagents for non-small cell carcinoma and small cell carcinoma

(a) Multiplex: PD-L1 + p40 + TTF-1				
Primary antibody	Clone	Dilution	Antibody incubation, min	Vendor
PD-L1	SP263	Prediluted	32	Roche Ventana
p40	BC28	Prediluted	48	Roche Ventana
TTF-1	SP141	Prediluted	48	Roche Ventana
Detections	Steps	Dilution	Incubation(s), min	Vendor
DISCOVERY HQ HRP hapten-linked multimer detection	2	Prediluted	16 + 16	Roche Ventana
OptiView DAB IHC Detection Kit (using only detection part)[a]	2	Prediluted	20 + 20	Roche Ventana
Chromogens	Colour	Dilution	Incubation(s), min	Vendor
DISCOVERY ChromoMap DAB Kit (HRP)	Brown	Prediluted	8	Roche Ventana
DISCOVERY Green HRP Kit	Green	Prediluted	32 + 32	Roche Ventana
DISCOVERY RED HRP Kit	Red	Prediluted	32 + 32	Roche Ventana
(b) Multiplex: INSM1 + synaptophysin + chromogranin A				
Primary antibody	Clone	Dilution	Antibody incubation, min	Vendor
INSM1	A-8	1:50	60	Santa Cruz Bioscience
Synaptophysin	SP11	Prediluted	52	Roche Ventana
Chromogranin A	LK2H10	Prediluted	60	Roche Ventana
Detections	Steps	Dilution	Incubation(s), min	Vendor
DISCOVERY HQ HRP hapten-linked multimer detection	2	Prediluted	24 + 24	Roche Ventana
OptiView DAB IHC Detection Kit (using only detection part)[a]	2	Prediluted	20 + 20	Roche Ventana
Chromogens	Colour	Dilution	Incubation(s), min	Vendor
DISCOVERY ChromoMap DAB Kit (HRP)	Brown	Prediluted	8	Roche Ventana
DISCOVERY Purple HRP Kit	Purple	Prediluted	32	Roche Ventana
DISCOVERY Yellow HRP Kit	Yellow	Prediluted	48 + 48	Roche Ventana

[a]OptiView detection was performed using Ventana user fillable dispensers.

may lead to discrepancies between cytological specimens and/or core biopsy and the final histologic diagnosis from resection specimens [28, 29]. The mIHC/IF holds significant utility in various aspects of diagnostics and biomedical research. First, it enables the simultaneous detection of numerous markers in a single tissue section, extracting optimal information from limited or precious samples. Second, it allows the exploration of markers co-localization and interaction. Thirdly, studies benefit from mIHC/IF by the identification of expression patterns, providing insights into tumour organization and enhancing our understanding of the presence of various populations within the tumour. Lastly, by revealing spatial relationships among cells and tissues and uncovering disease heterogeneity, mIHC/IF contributes to increasing diagnostic and predictive accuracy as well as advances biomedical understanding of the diseases [7, 26, 30].

Duration of the ethanol fixation varies in the laboratory based on the decision for the preparation of the CB. Increasing the duration of the ethanol fixation, the staining intensity gradually fainted for some antibody clones. However, we noticed the same effect using the hybrid block-controlling method for the optimization of PD-L1

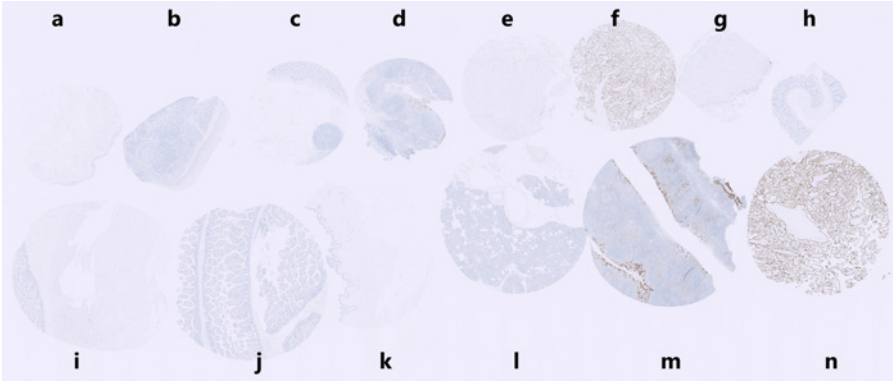

Fig. 4. Multi-tissue section stained with PD-L1 (SP263) clone. Ethanol-fixed tissues are punched with a 3 mm puncher and formalin-fixed tissues with 4 mm, respectively. The staining intensity of the PD-L1 is optimal using formalin-fixed tissues and protocol recommendation by the vendor. Placenta and tonsil are good control tissues for quality controlling of PD-L1 staining. Other tissues of the hybrid control are negative control of PD-L1 antigen. Note: staining intensity decreased in ethanol-fixed tissues compared to the formalin-fixed tissues. Staining intensity decreased especially in tonsil tissue. Top row, ethanol-fixed tissue; bottom row, formalin-fixed tissue. Controls from top left. **a** Skin, 1 week. **b** Tonsil, 2 weeks. **c** Intestinal epithelium, 2 weeks. **d** Tonsil, 1 week. **e** Small intestinal smooth muscle, 1 week. **f** Placenta, 1 week. **g** Small intestinal smooth muscle, 2 weeks. **h** Intestinal epithelium, 1 week. Controls from bottom left. **i** Small intestinal smooth muscle. **j** Intestinal epithelium. **k** Skin. **l** Pancreas. **m** Tonsil. **n** Placenta (×5 magnification).

clones. Tissue cores fixed 1 week in 50% ethanol stained more intensively (tonsil, placenta), then 2 weeks fixed tissues, respectively. For standardization of the ethanol fixation, the fixation time should be kept as short as possible, similar to the formalin fixation time. Post-fixation with the formalin could decrease the adverse effect of ethanol fixation, especially if the antibody is designed against the formalin-fixed and linearized epitope.

Therefore, one notable advantage of mIHC/IF is the improved accuracy facilitated by image analysis, which utilizes landmark markers to indicate tissue architecture [6, 27]. Nevertheless, interpreting multiplexed stained samples, particularly those using fluorescence, can be challenging. The use of fluorescence may cause multiple targets to blend, complicating resolution and potentially muddling visual assessment. Additionally, in FFPE tissues, there is the potential for tissue autofluorescence, further complicating visual interpretation [6]. Previous studies examined whether staining with a specific antibody in a multiplex protocol is qualitatively comparable to a single staining and concluded that multiplex staining can be qualitatively comparable to single staining. However, achieving this requires standardization, validation, and careful consideration of detection methods and antibody characteristics [31, 32]. In addition, previous research has concluded that multiplex IHC/ICC can quantitatively replicate single staining when thoroughly validated and optimized. It should also be noted that the location of antibodies in the multiplex panel affects all antibodies in the panel, requiring careful optimization to avoid problems of antibody shedding and interference [33–35].

Prior to the possible integration of mIHC/IF technology into clinical applications, it is crucial to establish a standardized and validated workflow that encompasses the entire process. This comprehensive workflow should be capable of supporting multisite trials and aligning with the requirements of clinical laboratory procedures and accreditation requirements [24].

In optimizing tissue availability for molecular testing, it is advisable to undertake a restricted diagnostic workup. Pulmonary cancer diagnosis necessitates a multidisciplinary approach. The landscape of lung cancer therapy is increasingly personalized, considering individual patient factors such as histologic cell type, subtypes, and molecular status. The role and approach of pathologists in diagnosing lung cancer in small biopsies and cytology specimens have undergone significant transformation [29].

Quality Assurance

The European Federation of Cytology Societies (EFCS) survey from 245 laboratories highlights the need for standardized protocols and robust quality assurance/quality control (QA/QC) practices in ICC [9]. Similar findings were identified in a CAP survey with 345 respondent laboratories [8], as well as in the UK NEQAS survey [36] and the meta-analysis already performed in the year 2011 [37]. Further research efforts are crucial to develop optimal fixation and processing methods for diverse cytology samples, establish validated ICC protocols applicable to various cytology preparations, and implement standardized QA/QC measures, including positive controls and internal and external quality

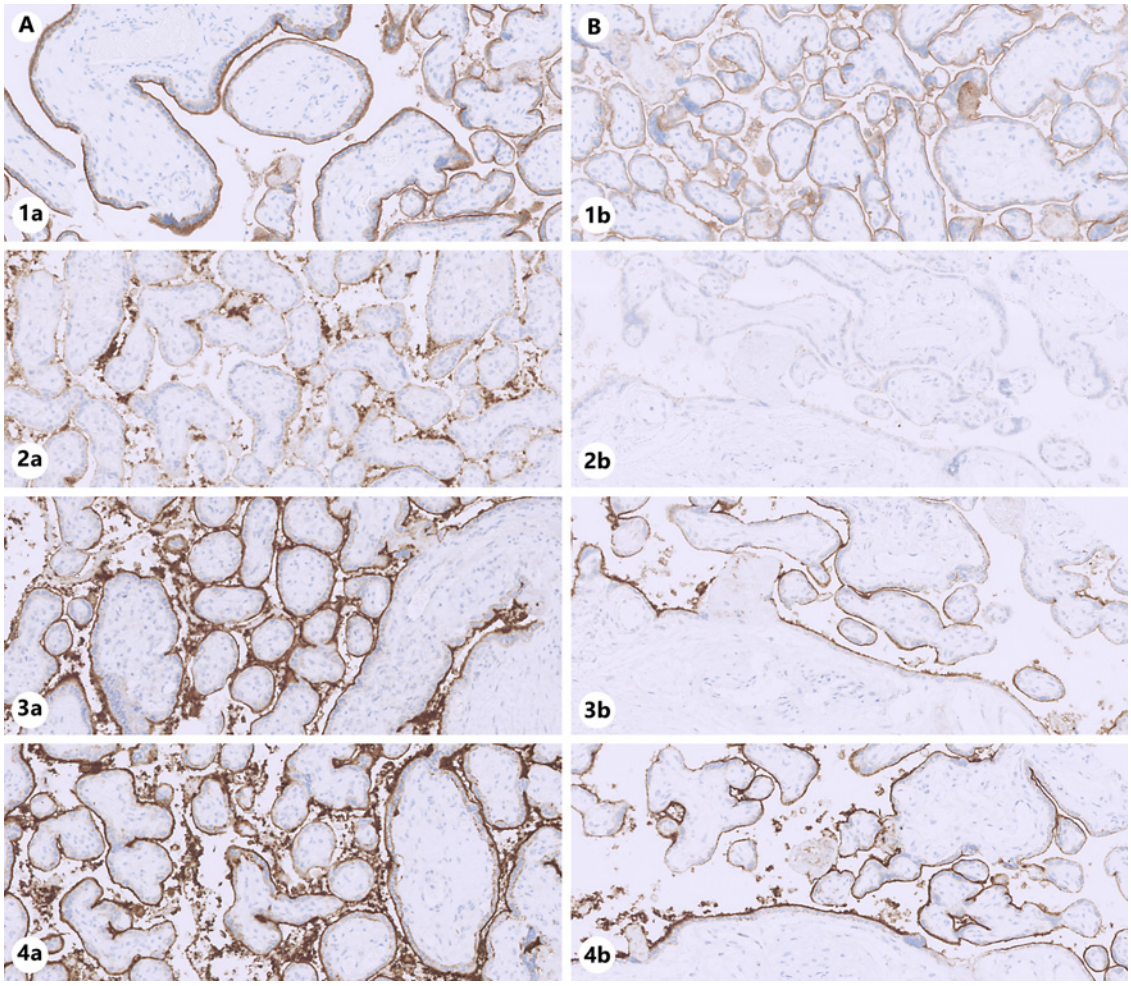

Fig. 5. Placenta tissues from hybrid control stained with different PD-L1 clones. **A** represent formalin-fixed placenta tissue and **B** represent ethanol-fixed placenta tissue, respectively. Syncytiotrophoplasts of formalin-fixed placenta stained intensively. The basal part of formalin-fixed syncytiotrophoblasts stained moderately or weakly with PD-L1 clones SP263, 28-8, and 22C3. Decreased sensitivity was observed with ethanol-fixed placenta. The most intensive straining pattern was observed with 22C3 and SP263 clones. The weakest technical sensitivity was observed with clone SP142, using formalin- and ethanol-fixed tissues. **1a**, **1b** PD-L1, SP263. **2a**, **2b** PD-L1, SP142. **3a**, **3b** PD-L1, 28-8. **4a**, **4b** PD-L1, 22C3 (×200 magnification).

Table 4. Optimized PD-L1 protocols

PD-L1 Clone	Dilution	Antigen retrieval (HIER)	Antibody incubation	Detection	Amplification	Vendor
SP142	Ready to use	CC1, 64 min	40 min	OptiView Detection Reagent by Ventana, 12 + 12 min	8 + 8 min	Roche Ventana
SP263	Ready to use	CC1, 64 min	16 min	OptiView Detection Reagent by Ventana, 8+8 min	No amplification	Roche Ventana
22C3	1:50	CC1, 56 min	32 min	OptiView Detection Reagent by Ventana, 8+8 min	4 + 4 min	Agilent, Dako
28-8	Ready to use	CC1, 64 min	24 min	OptiView Detection Reagent by Ventana, 8 + 8 min	4 + 4 min	BioSB

control programs, specifically for ICC on cytology samples. Traditional FFPE CBs are the most common cytology preparations used for ICC, but non-CB preparations like smears and liquid-based cytology are also feasible after proper validation [1].

IHC and ICC serve distinct purposes, but both face limitations in pre-analytical and QA/QC practices. While IHC benefits from standardized FFPE processing, ICC encounters diverse sample preparation techniques and lacks well-established protocols, especially primary antibodies dedicated to ethanol-fixed material. A concerted effort towards standardization and supportive QA/QC is essential for ICC to reach its full potential in clinical practice, particularly when dealing with limited cytology samples [9].

Interestingly, the EFCS survey identified that utilizing non-optimized and unvalidated FFPE protocols on cytology samples significantly increased the frequencies of low cellularity, inconsistent staining, and difficult interpretation. This suggests that blindly applying protocols designed for tissue specimens to cytology samples might lead to diagnostic challenges [9].

Addressing these troubles requires a multi-faceted approach, including optimizing sample collection and processing techniques to ensure sufficient material and cellularity for analysis. Furthermore, developing and validating ICC protocols specifically tailored to diverse cytology sample preparations is essential to achieve consistent and reliable staining patterns. It requires implementing rigorous QA/QC measures, including positive and negative controls, internal and external proficiency testing, and standardized interpretation criteria. Altogether, it is vital for ensuring result accuracy and reproducibility. Exploring novel technologies, such as automation and digital image analysis, could potentially aid in standardizing staining protocols and facilitating result interpretation.

The main limitation of traditional IHC compared to mIHC/IF is its inability to label more than one marker in a tissue section. Although it is a practical and cost-effective diagnostic and prognostic method, this single-marker approach fails to capture the complex tumour microenvironment (TME) [7].

In dualplex IHC, which is performed utilizing simultaneous primary antibody, the same tissue section undergoes staining with two contrasting markers, usually employing DAB (brown) chromogen and red or blue chromogens. To prevent cross-reactivity with the secondary marker, each primary marker must come from a different species. The assessment of these markers is then conducted simultaneously [23, 38].

Theoretically, with conventional IHC, it is possible to conduct six individual IHC staining procedures on six consecutive sections, creating a "multiplex effect" comparable to mIHC/IF. This approach eliminates the necessity for investing in a multiplex microscopy machine or incurring additional costs associated with staining. However, the conventional consecutive section method typically yields only three successive sections of the same cell, which limits cross-referencing capabilities to just three markers for a single cell. Achieving this for six consecutive sections proves challenging. Additionally, tissue depletion poses a significant issue in clinical settings, particularly in clinical trials and limited (mainly biopsy and cytology) sample analyses. After routine diagnostic panels for TTF-1, napsin A, tumour protein p63 (p63), and cytokeratin, there may be minimal tissue remaining for molecular testing such as epidermal growth factor receptor, anaplastic lymphoma kinase, and reactive oxygen species proto-oncogene (ROS1). Furthermore, the evaluation of PD-L1 may be compromised. In this scenario, sectioning more than three consecutive sections may prove impossible, depriving the patient of the potential advantages offered by an advance in precision medicine. For tumour diagnosis requiring multiple IHC stains, small samples may be depleted before completing the full marker panel, posing challenges in diagnosing undifferentiated tumours, sarcomas, and lymphomas, especially when procured by minimally invasive techniques like core needle biopsies or fine needle aspiration. Moreover, even experienced pathologists may face challenges when attempting to cross-reference two or more consecutive slides to observe the co-localization of more than two markers. These routine challenges, which are often encountered in the practice of pathology, can be solved by mIHC/IF [3, 7, 39].

PD-L1 Challenges in Cytological Specimens

PD-L1 IHC has become a standard practice for selecting patients with NSCLC for targeted immunotherapy. However, several challenges remain in its validation and application, particularly when using cytological specimens instead of tissue samples. PD-L1 expression can vary within a tumour, leading to sampling bias when using small biopsies or cytology samples. Studies suggested that paired samples obtained concurrently show better correlation and cytology-histology discordance increases with case-related higher heterogeneity. While alcohol-based fixatives are common in cytology, they may require adjustments in ICC protocols. Non-formalin preservatives often lack specific data on their impact on PD-L1 staining. Non-CB preparations lack distinct membranous staining due to intact cell membranes, making it difficult to differentiate true positivity from cytoplasmic staining of other cells. This is particularly challenging with effusion samples containing mixed cell populations. Although some studies report successful scoring with less than 100 cells, testing samples with limited

cellularity is generally discouraged due to inherent interpretation difficulties [40].

In addition, there are pre-analytical challenges with PD-L1 in cytological samples. Specimen collection, handling, and processing require precise protocols and validation studies. The evaluation criteria for PD-L1 IHC tests are heterogeneous, encompassing staining patterns, scoring systems, and cut-off levels, which contribute to interpretational complexities and variability in treatment recommendations. Standardized training and assessment protocols are crucial in addressing the challenges posed by interobserver variability. Accurate PD-L1 assessment requires attention to the technical challenges of distinguishing inflammatory cells from tumour cells in cytological samples and optimizing staining protocols [41, 42]. In our study, this challenge was minimized by highlighting tumour positivity with either TTF-1-positive adenocarcinoma samples or p40-positive squamous cell carcinoma samples. This simplified identification and potentially together with the PD-L1 positivity reduced interobserver variability and interpretation uncertainty, which is typically high in PD-L1 diagnostics.

PD-L1 expression in the TME involves complex adaptive and constitutive mechanisms. Therapies targeting programmed death 1 (PD-1)/PD-L1 are most effective when PD-L1 is adaptively expressed alongside PD-1. Biomarkers measuring combined PD-1/PD-L1 expression predict treatment response reliably. Emerging mIHC/IF techniques offer comprehensive TME characterization, potentially enhancing understanding of PD-1/PD-L1 patterns and their link to treatment response in personalized cancer therapies [24, 43].

A systematic review of 27 articles assessing interobserver variability for PD-L1 IHC assays, including SP263, SP142, 22C3, and 28-8, reveals that agreement between pathologists is generally moderate to high for all assays [44]. In addition, one study reported interobserver agreement of 98.1% and concordance of 90.5% with SP263 assay [45]. In our study, the introduction of multiplex ICC did not differ significantly from the introduction of a single antibody. The sample interpretations were consistent.

Limitations of the Current Study

The study has a few limitations. Different fixation times, even if they mimic the laboratory processing flow, increase the risk of antigen depletion and may lead to misinterpretation of the sample in practice. The study had only a limited number of antibodies and tissue was limited to EBUS material. Another limitation of the study was that it did not assess interobserver variability and potential interpretation bias. It should also be noted that ethanol fixation can cause antigen depletion leading to weaker staining compared to formalin-fixed samples. This variability can affect the reliability of results across different tissue types and fixation methods.

Future Directions

Chromogenic multiplexing will be a promising tool together with artificial intelligence (AI)-powered image analysis. Multiplexing will save important cytological and histological tissue material, but implemented together with the image analysis, accurate and reproductive interpretations using bright field digitized whole slide images can be achieved. Morphology is still important in the interpretations of cytopathological and histopathological slides.

However, there are a few challenges with AI-powered analysis in cytopathology. Samples have too many variables including staining intensity, stain type, the thickness of smears, cellularity, architectural pattern, and the three-dimensionality of the sample, and its interpretation. It should also be considered what is the goal of AI in cytopathology. The remaining questions are as follows:

- Is AI to replace the pathologist?
- To improve diagnostic accuracy?
- To reduce turn-around time?
- To cut costs?

These are some of the issues to consider in future endeavours.

Conclusions

Overall, the application of mIHC/IF techniques enhances the medical value of analyses by providing comprehensive information about cellular interactions, tissue organization, and disease heterogeneity, thereby fostering more accurate diagnostics and deeper insights into various biomedical phenomena. The ICC in cytology has enormous potential, but solving its problems is crucial for its successful clinical use. By acknowledging the challenges identified, adopting standardized protocols, implementing thorough QA/QC measures, and exploring innovative technologies, the way can be paved for a more reliable and accurate use of ICC in cytological diagnostics. Addressing these challenges will ensure the accuracy and reliability of ICC, allowing the full potential of ICC in disease diagnosis and treatment to be realized even when tissue samples are limited.

Statement of Ethics

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Pirkanmaa Hospital District (R17174). This consent protocol was

reviewed and the need for written and informed consent was waived by the Ethics Committee of Pirkanmaa Hospital District (data of decision/decision reference number R17174).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Conceptualization, formal analysis, investigation, project administration, and funding acquisition: A.V. and I.K.; methodology, data curation, writing – original draft preparation, writing – review and editing, and visualization: A.V., I.K., and T.H.; validation: A.V. and T.H.; resources: T.H. and I.K.; and supervision: I.K. All authors have read and agreed to the submitted version of the manuscript.

Data Availability Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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