Acta Cytologica

Nongynecologic Cytopathology

Acta Cytologica
DOI: 10.1159/000518452

Received: April 15, 2021
Accepted: July 11, 2021
Published online: September 14, 2021

# Efficacy of the Antigenicity-Retaining Ability of Fixative Solutions for Liquid-Based Cytology: Immunocytochemistry of Long-Term Storage

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**Keywords**
Antigenicity-retaining ability · Fixative solution · Liquid-based cytology · Immunocytochemical staining · Long-term storage

**Abstract**
***Introduction/Objective:*** Liquid-based cytology (LBC) is advantageous as multiple stained specimens can be prepared and used for additional assays such as immunocytochemical and molecular-pathological investigations. Two types of preservative-fixative solutions (fixatives) are used for nongynecologic specimens used in the BD SurePath-LBC (SP-LBC) method, and their components vary. However, few studies have evaluated the differences in antigen-retaining ability between these fixatives. Therefore, we investigated and compared the antigen-retaining ability of the fixatives in immunocytochemical staining (ICC) under long-term storage conditions. ***Materials and Methods:*** Sediments of cultured RAJI cells (derived from Burkitt's lymphoma) were added to each fixative (red and blue) and stored at room temperature for a specified period (1 h; 1 week; and 1, 3, and 6 months). The specimens were then prepared using the SP-LBC method and subjected to ICC. Positivity rate was calculated using the specimens fixed at room temperature for 1 h as a control. Antibodies against Ki67 expressed in the nucleus and against CD20 and leukocyte common antigen (LCA) expressed on the cell membrane were used. ***Results:*** For CD20 and LCA, the positivity rate increased with time in the red fixative compared with that in the control. In the blue fixative, the positivity rate was highest at 1 h and was maintained at a high level throughout the storage period. In contrast, the Ki67 positivity rate was highest at 1 h in both red and blue fixatives and markedly decreased with time. Therefore, although refrigerated (8°C) storage was used, no improvement was noted. ***Conclusions:*** Long-term storage is possible for cell membrane antigens at room temperature; however, it is unsuitable for intranuclear antigens. Therefore, we conclude that suitable fixative type and storage temperature differ based on antigen location. Further investigation is warranted.


## Introduction

GLOBOCAN 2017 (International Agency for Research on Cancer) has reported a sustained decline in the worldwide age-specific incidence rate of cervical cancer based, in part, on Papanicolaou screening [1]. Liquid-based cytology (LBC), a system used for cervical cancer screening, was officially approved in the USA by the Food

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and Drug Administration in 1996. For LBC, specimens are suspended in a cell fixative and then smeared from a homogenous state onto glass slides, so that the collected cells can be effectively analyzed without loss. The resulting specimens are of excellent quality compared with the previous method of directly smearing specimens onto glass slides. Therefore, the results are superior to that of the direct smear method (rate of unsatisfactory specimens and diagnostic accuracy) [2–4].

In Japan, cervical cytology has been widely used for the primary screening of cervical cancer since 1983. LBC has been implemented in screening programs since 2008 with the adoption of the Bethesda System for reporting cervical cytology. Recently, LBC has been reported to potentially decrease the unsatisfactory smear rate that occurs in cervical cytology compared with conventional cytology in Japan [5]. Furthermore, cytology using LBC has been used in not only gynecologic specimens such as the uterine cervix and endometrium [6, 7] specimens but also other specimens such as the mammary [8] and thyroid [9] gland specimens.

One advantage of LBC is that multiple specimens can be prepared and utilized for immunocytochemical staining (ICC) [10–13] and gene analysis [14, 15]. Norimatsu et al. [16–18] combined ICC with cell morphology findings using the BD SurePath™ (BD Diagnostics, Burlington, NC, USA)-LBC (SP-LBC) for the accurate diagnosis of endometrial cytology. However, there are 3 types of cell fixative solutions used in SurePath-LBC: BD SurePath™ vial (gyne) for gynecologic analysis and BD Cytorich™ red (red) and BD Cytorich™ blue (blue) for nongynecologic analysis, and their components vary [19]. Accordingly, the antigen-retaining ability for ICC is predicted to differ depending on the type of fixative used. At present, limited studies have been conducted on the antigen-retaining properties of the fixatives used for ICC [20–22].

Norimatsu et al. [21] performed ICC in pleural effusions from 4 patients with pulmonary adenocarcinoma; they used 4 antibodies against intranuclear antigens (i.e., Ki67, P53, cyclin A, and MCM7) and cell sediments and various fixation periods (1 h, 1 week, 1 month, and 3 months). They calculated the positivity rate and relative ratio, setting the baseline to those of 1 h. There was no notable difference in the antigen-retaining ability among the 3 fixatives, but the positivity rate decreased by 30% after 1 week of storage depending on the antibody, suggesting a possibility of inaccurate judgment of the positivity rate. On the basis of the results of this study, we evaluated the antigenicity-retaining ability of the fixatives used in ICC over long-term storage conditions. Using a malignant lymphoma-derived cell line in conjunction with BD SurePath-LBC, the antigen-retaining ability of the fixatives was determined.

## Materials and Methods

*Cell Culture*

RAJI cells from human Burkitt's lymphoma were obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Kanagawa, Japan). The cell lines were cultured in RPMI 1640 medium (Gibco GlutaMAX™; Thermo Fisher Scientific Inc., Merelbeke, Belgium) with 10% fetal bovine serum in a humidified incubator at 37°C containing 5% $CO_2$. The cultured cells were harvested 6 days after seeding and pelleted by centrifugation at 800 *g* for 5 min; the supernatant was decanted to obtain the cell sediment.

*Specimen Preparation*

Sediments (10 μL) of cultured RAJI cells were suspended in 5 mL of each fixative solution (red and blue) and stored at room temperature or refrigerated (8°C) for a specified period (1 h; 1 week; and 1, 3, and 6 months). The cell fixative solutions used for SP-LBC included gyne for gynecologic specimens and red and blue for nongynecologic specimens. We examined the latter in this study because we used a malignant lymphoma-derived cell line.

ICC was performed for the 1-h specimens at room temperature and used as a control. In addition, cultured cells were stored at −20°C for 1 week and examined. After the specified period, each fixative was centrifuged at 800 *g* for 10 min, and the fixation fluid was decanted. A mixture of the cell sediment with 300 μL of purified water was dispensed into a dedicated glass chamber slide and allowed to stand for 10 min. The slides were fixed in 95% ethanol for 10 min and immunostained.

*ICC*

Both CD20 (L26 monoclonal antibody, diluted antibody; Nichirei Bioscience Co., Ltd., Tokyo, Japan) and CD45 (leukocyte common antigen [LCA], clone: 2B11 + PD7/26 FLEX RTU diluted antibody; Agilent Technologies/Dako, Carpinteria, CA, USA) were used as cell membrane antigens, and Ki67 (clone: MIB-1 FLEX RTU diluted antibody; Agilent Technologies/Dako, Carpinteria, CA, USA) was used as a nuclear antigen. For Ki67 staining only, we also examined cultured cells stored at −20°C for 1 week and those stored at 8°C for a specified period (1 h; 1 week; and 1, 3, and 6 months). The slides were treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. After incubating with primary antibodies for 60 min at room temperature, the Histfine detection kit (Histfine Simple Stain MAX-PO; Nichirei Bioscience Co., Ltd., Tokyo, Japan) was used, and the specimens were incubated for 30 min. Peroxidase reaction was performed in a developing solution containing 3,3′-diaminobenzidine. Finally, the slides were counterstained with hematoxylin.

*Assessment of ICC*

Immunoreactive sites staining brown based on the type of antibody were considered positive, and those staining faintly or not staining were considered negative. CD20 and LCA exhibited positive staining on the cell membrane, whereas Ki67 staining was ev-

**Table 1.** Relationship between positivity rate (%) and storage time for each fixative

| RT | Fixative solution | | | |
|---|---|---|---|---|
| | red | | blue | |
| CD20 | | | | |
| 1 h | 60.1±7.9 | ($p < 0.001$)[a], ($p < 0.001$)[b], ($p < 0.001$)[c], ($p < 0.001$)[d] | 88.0±6.2 | ns |
| 1 week | 85.6±7.6 | ($p = 0.029$)[e], ($p = 0.005$)[f] | 88.7±5.7 | ns |
| 1 month | 91.8±4.0 | ns | 90.4±3.5 | ns |
| 3 months | 95.2±0.5 | ns | 90.6±2.4 | ns |
| 6 months | 97.5±2.3 | ns | 93.3±2.2 | ns |
| LCA | | | | |
| 1 h | 85.7±5.4 | ($p < 0.001$)[a], ($p < 0.001$)[b], ($p < 0.001$)[c], ($p < 0.001$)[d] | 96.7±3.6 | ns |
| 1 week | 94.0±2.7 | ($p = 0.001$)[f] | 99.2±1.2 | ns |
| 1 month | 98.4±2.1 | ns | 95.5±2.7 | ns |
| 3 months | 98.8±1.3 | ns | 98.8±2.0 | ns |
| 6 months | 99.7±0.8 | ns | 97.8±2.3 | ns |
| Ki67 | | | | |
| 1 h | 64.8±3.0 | ($p < 0.001$)[a], ($p < 0.001$)[b] | 88.2±2.3 | ($p < 0.001$)[a], ($p < 0.001$)[b] |
| 1 week | 1.5±0.7 | ns | 23.9±7.5 | ns |
| 1 month | 1.7±2.2 | ns | 29.6±4.5 | ns |
| 3 months | Nonspecific | nd | Nonspecific | nd |
| 6 months | Nonspecific | nd | Nonspecific | nd |

Steel-Dwass test ($p < 0.05$). RT, room temperature; nd, not done; ns, not significant. [a]Significant difference between 1 h and 1 week. [b]Significant difference between 1 h and 1 month. [c]Significant difference between 1 h and 3 months. [d]Significant difference between 1 h and 6 months. [e]Significant difference between 1 week and 3 months. [f]Significant difference between 1 week and 6 months.

ident in the nucleus. At least 300 cells in 6 randomly selected fields of view with a ×40 objective lens were counted, and the results were expressed as the percentage of positive cells. Statistically significant differences were analyzed by a Steel-Dwass test using the "R" statistical software (version 3.4.3; http://www.r-project.org/index.html). $p$ values <0.05 were considered statistically significant.

## Results

### *Relationship between the Positivity Rate (%) and Storage Period for Each Fixative*

#### Cell Membrane Antigen

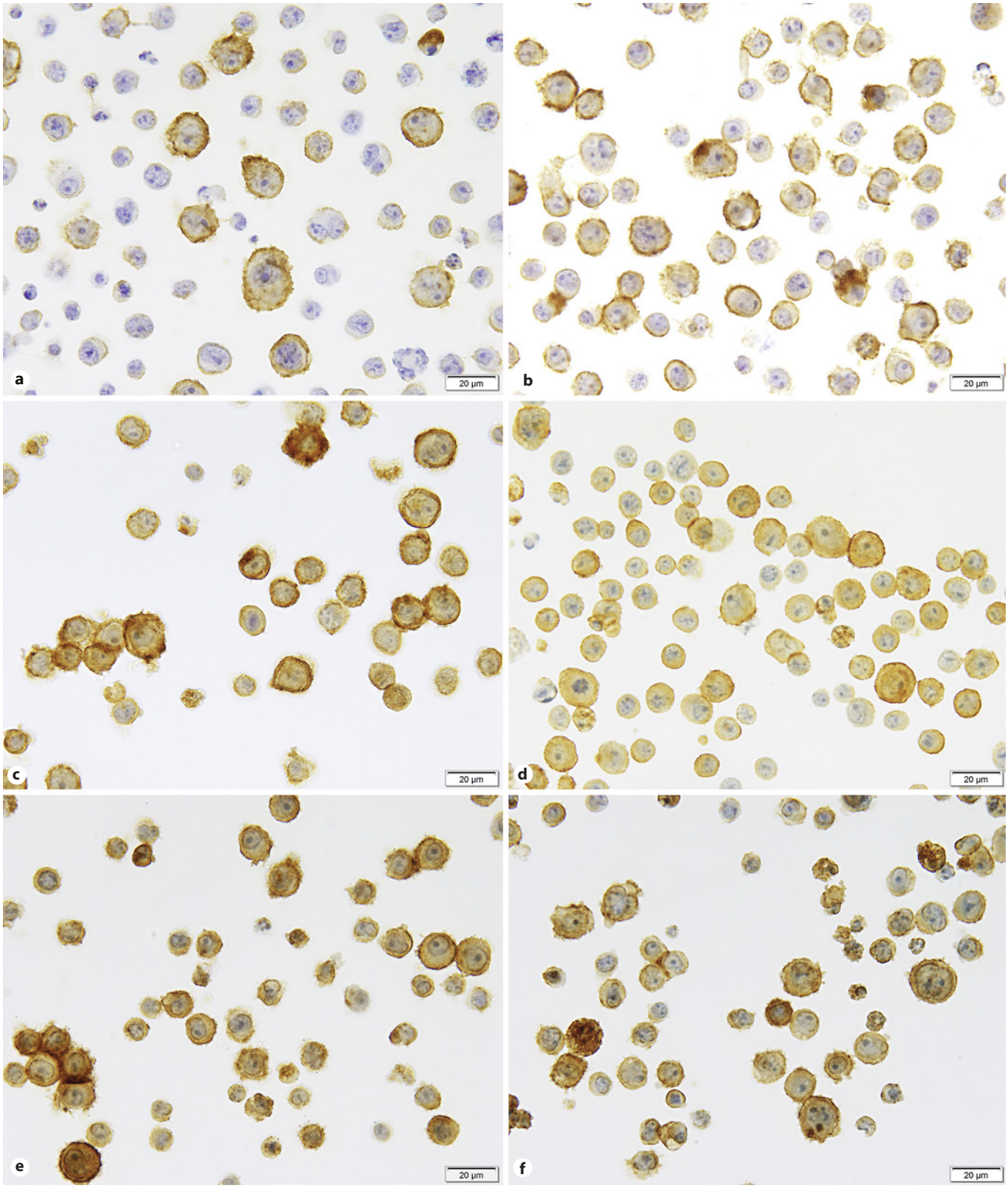
*CD20.* The components of red and blue fixatives vary [19]. The red fixative comprises isopropanol (23.3%), methanol (10.0%), formaldehyde (0.4%), etc., and has hemolytic and protein aggregation inhibitory effects. On the other hand, the blue fixative comprises ethanol (44.0%), methanol (5.0%), etc., and has no hemolytic and protein aggregation inhibitory effects.

In the red fixative, the positivity rate significantly increased with time when comparing the specimen of 1 h storage (60.1%) with that of 1 week (85.6%; $p < 0.001$), 1 month (91.8%; $p < 0.001$), 3 month (95.2%; $p < 0.001$), and 6 month (97.5%; $p < 0.001$) storages (Table 1; Fig. 1a–c). The positivity rate at 1 week was significantly lower than that at 3 and 6 months ($p = 0.029$ and $p = 0.005$, respectively). In the blue fixative, no significant differences were observed when comparing the specimen of 1 h storage (88.0%) with that of 1 week (88.7%; $p = 0.903$), 1 month (90.4%; $p = 0.939$), 3 month (90.6%; $p = 0.999$), and 6 month (93.3%; $p = 0.999$) storages (Table 1; Fig. 1d–f).

*LCA.* In the red fixative, the positivity rate significantly increased for the specimen of 1 h (85.7%) storage compared with that of 1 week (94.0%; $p < 0.001$), 1 month (98.4%; $p < 0.001$), 3 month (98.8%; $p < 0.001$), and 6 month (99.7%; $p < 0.001$) storages (Table 1; Fig. 2a–c). In addition, there was a significant difference between the specimens of 1 week and 6 month storages. However, for the blue fixative, no significant differences were observed when comparing the specimen of 1 h storage (96.7%) with that of 1 week (99.2%; $p = 0.405$), 1 month (95.5%; $p = 0.908$), 3 month (98.8%; $p = 0.572$), and 6 month (97.8%; $p = 0.935$) storages (Table 1; Fig. 2d–f).

#### Nuclear Antigen

*Ki67.* In the red fixative, the Ki67 positivity rate was the highest at 1 h (64.8%) and then markedly de-

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creased with time at 1 week (1.5%; $p < 0.001$) and 1 month (1.7%; $p < 0.001$) (Table 1; Fig. 3a, b). Similarly, in the blue fixative, the positivity rate was highest at 1 h (88.2%) and significantly decreased with time at 1 week (23.9%; $p < 0.001$) and 1 month (29.6%; $p < 0.001$) (Table 1; Fig. 3c, d). For both fixatives, the cytoplasm showed nonspecific reactions at 3 and 6 months at room temperature. Furthermore, in the red fixative, the positivity rate at 8°C significantly decreased at 1 week (2.3%; $p < 0.001$) and 1 month (4.1%; $p < 0.001$) compared with that at 1 h (64.8%) (Table 2; Fig. 3e). In the blue fixative, the positivity rate at 8°C significantly decreased at 1 week (75.7%; $p < 0.001$) and 1 month (32.1%; $p < 0.001$) compared with that at 1 h (88.2%)(Table 2; Fig. 3f). Treatment with both fixatives resulted in nonspecific reactions in the cytoplasm at 3 and 6 months at refrigerated (8°C) storage.

*Relationship between the Positivity Rate (%) and Each Fixative at Various Storage Times*

Cell Membrane Antigen

*CD20.* The positivity rates in red and blue (red vs. blue) fixatives were as follows: 1 h (60.1% vs. 88.0%), 1 week (85.6% vs. 88.7%), 1 month (91.8% vs. 90.4%), 3 months (95.2% vs. 90.6%), and 6 months (97.5% vs. 93.3; Table 3). The positivity rate in the blue fixative at 1 h was significantly higher than that in the red fixative at 1 h ($p < 0.001$); however, the positivity rates in the blue fixative at 3 and 6 months were significantly lower than those in the red fixative at 3 and 6 months ($p = 0.009$ and $p = 0.003$, respectively).

*LCA.* The positivity rates in red and blue (red vs. blue) fixatives were as follows: 1 h (85.7% vs. 96.7%), 1 week (94.0% vs. 99.2%), 1 month (98.4% vs. 95.5%), 3 months (98.8% vs. 98.8%), and 6 months (99.7% vs. 97.8%; Table 3). There were no significant differences in the positivity rates among these storage times.

Nuclear Antigen

*Ki67.* The positivity rate at 1 h (64.8% vs. 88.2%; $p < 0.001$), 1 week (1.5% vs. 23.9%; $p < 0.001$), and 1 month (1.7% vs. 29.6%; $p < 0.001$) was significantly higher in the blue fixative than that in the red fixative (Table 3). Results for the storage temperature of 8°C indicated that at 1 week (2.3% vs. 75.7%; $p < 0.001$) and 1 month (4.1% vs. 32.1%; $p < 0.001$), the positivity rate in the blue fixative was significantly higher than that in the red fixative (Table 3).

## Discussion

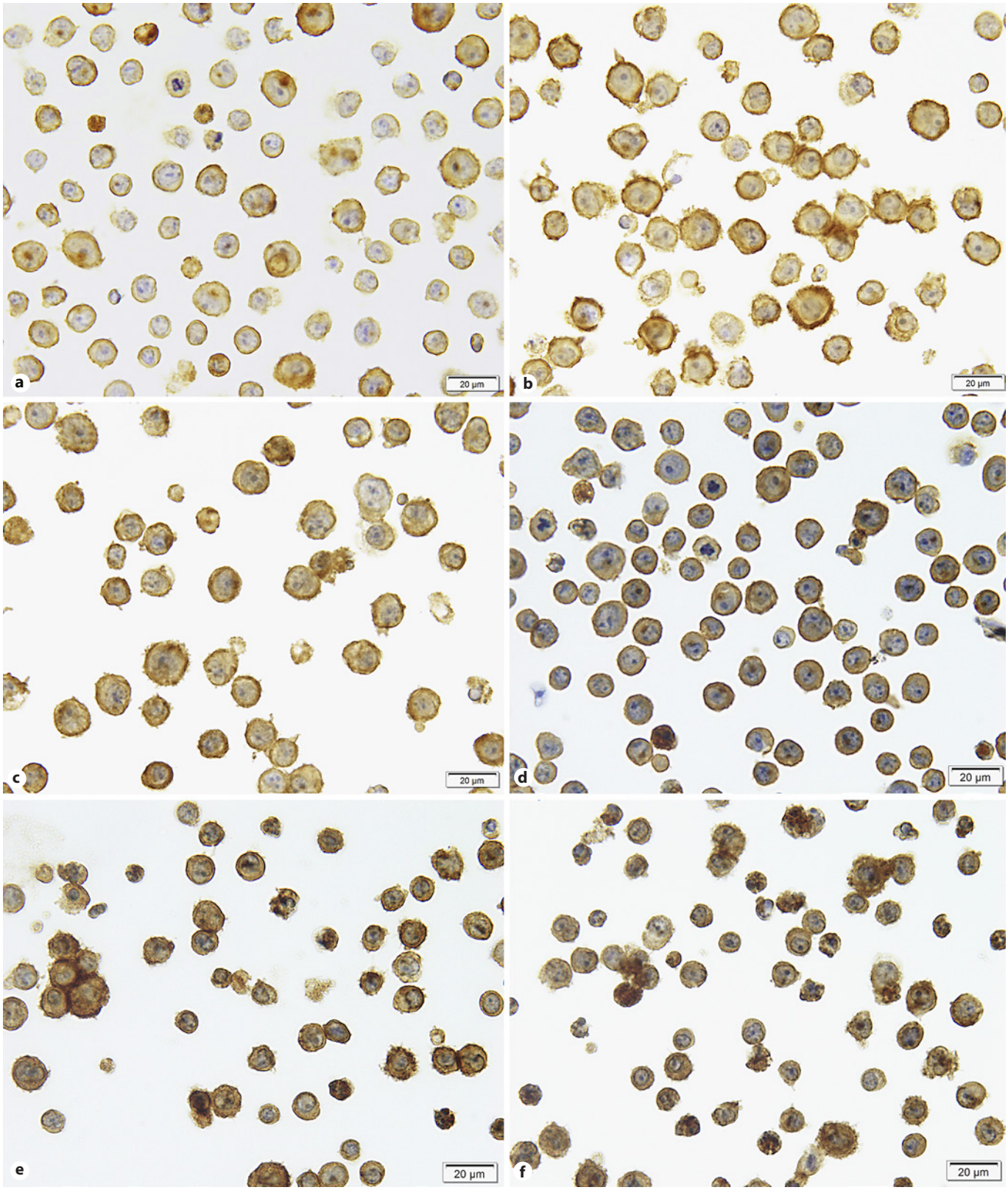
Cultured cells used to investigate LBC usually represent the epithelial and nonepithelial (muscular system) systems. To our knowledge, there have been no studies using a hematopoietic system-derived cell line. Therefore, in the present study, we investigated the antigenici-

**Table 2.** Relationship between the Ki67 positivity rate (%) and each fixative for various storage periods at 8°C

| 8°C | Fixative solution | | | |
|---|---|---|---|---|
| | red | | blue | |
| Ki67 | | | | |
| 1 week | 2.3±2.9 | ($p < 0.001$)[a] | 75.7±6.9 | ($p < 0.001$)[a] |
| 1 month | 4.1±1.4 | ns | 32.1±4.0 | ns |
| 3 months | Nonspecific | nd | Nonspecific | nd |
| 6 months | Nonspecific | nd | Nonspecific | nd |

Steel-Dwass test ($p < 0.05$). nd, not done; ns, not significant. [a] Significant difference between 1 week and 1 month.

**Fig. 1.** Immunocytochemical staining of CD20 using red and blue fixatives in various storage periods at room temperature. **a–c** The positivity rate in the red fixative increased with time compared with that in the 1 h control. **d–f** The positivity rate in the blue fixative was higher at 1 h and maintained at this level throughout the storage period. **a** Red fixative for 1 h. **b** Red fixative for 1 week. **c** Red fixative for 1 month. **d** Blue fixative for 1 h. **e** Blue fixative for 1 week. **f** Blue fixative for 1 month (ICC, ×400, objective, counterstained with hematoxylin).

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ty-retaining ability of fixatives using a malignant lymphoma-derived cell line.

For the cell membrane antigens, CD20 and LCA, the positivity rate for specimens stored in the red fixative significantly increased with time compared with that of the control. The positivity rate was highest at 1 h (room temperature) and was consistent at a high level throughout the storage period. Comparison of the CD20 positivity rates between red and blue fixatives indicated that the rate after 1 h storage was significantly higher in the blue fixative than in the red fixative ($p < 0.001$), whereas it significantly decreased in the blue fixative at 3 months and thereafter; however, these rates (red 95.2 vs. blue 90.6 and red 97.5 vs. blue 93.3; $p = 0.009$ and $p = 0.003$, respectively) represented small differences. Regarding LCA, no differences were observed between red and blue fixatives for any time period. On the basis of these results, it can be said that the membrane antigen-retaining abilities of red and blue fixatives are almost equivalent, and long-term room temperature storage of cell membrane antigens may be possible.

There are 2 types of fixatives: chemical fixatives and coagulation precipitants. The former includes aldehyde-based fixatives, such as formalin, that generate methylene bridges within or between proteins [23, 24]. Overfixation with an aldehyde-based fixative may reduce antigenicity [25, 26]. The latter includes organic solvent solutions such as ethanol. Hydrogen bonds between amino acids in the tertiary structure of the protein are disrupted by the formation of hydrogen bonds between amino acids and the organic solvent through dehydration. This destroys the higher order structure resulting in degeneration and precipitation [27].

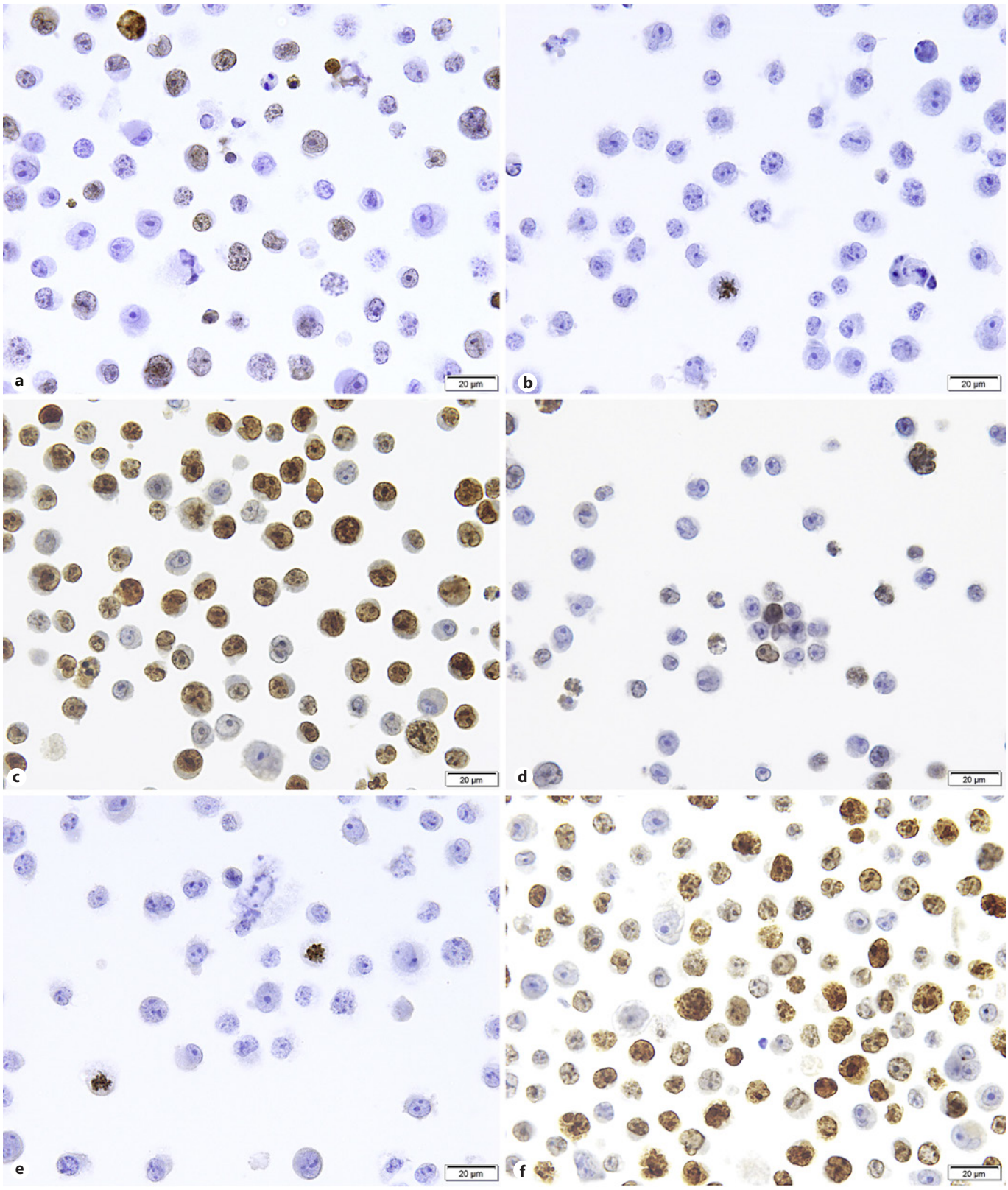
It is generally believed that tissues are stable for long periods not only because they are rapidly fixed but also because the protein components and other elements that make up the tissue may remain inside for a long time. The state of the preservation of DNA, RNA, and protein fixed in ethanol is excellent compared with that in neutral-buffered formalin [28, 29]. The molecular weight of CD20 is 33–37 kDa, and it belongs to the MS4A family of phosphoproteins, which contains 4 transmembrane domains [30]. CD45, also known as LCA, is a leukocyte-specific transmembrane protein and a receptor-like protein tyrosine phosphatase [31]. Our results revealed that the use of ethanol-based fixative is recommended for this membrane antigen to retain antigenicity. This suggests that the interrelationship between the structural components of

**Table 3.** Relationship between positivity rate (%) for each fixative and storage period

| | 1 h | 1 week | | 1 month | | 3 months | | 6 months | |
|---|---|---|---|---|---|---|---|---|---|
| | RT | RT | 8°C | RT | 8°C | RT | 8°C | RT | 8°C |
| CD20 | | | | | | | | | |
| Red | 60.1±7.9 | 85.6±7.6 | nd | 91.8±4.0 | nd | 95.2±0.5 | nd | 97.5±2.3 | nd |
| Blue | 88.0±6.2 | 88.7±5.7 | nd | 90.4±3.5 | nd | 90.6±2.4 | nd | 93.3±2.2 | nd |
| *p* value | **<0.001** | ns | nd | ns | nd | **0.009** | nd | **0.003** | nd |
| LCA | | | | | | | | | |
| Red | 85.7±5.4 | 94.0±2.7 | nd | 98.4±2.1 | nd | 98.8±1.3 | nd | 99.7±0.8 | nd |
| Blue | 96.7±3.6 | 99.2±1.2 | nd | 95.5±2.7 | nd | 98.8±2.0 | nd | 97.8±2.3 | nd |
| *p* value | ns | ns | nd | ns | nd | ns | nd | ns | nd |
| Ki67 | | | | | | | | | |
| Red | 64.8±3.0* | 1.5±0.7 | 2.3±2.9 | 1.7±2.2 | 4.1±1.4 | Nonspecific | Nonspecific | Nonspecific | Nonspecific |
| Blue | 88.2±2.3 | 23.9±7.5 | 75.7±6.9 | 29.6±4.5 | 32.1±4.0 | Nonspecific | Nonspecific | Nonspecific | Nonspecific |
| *p* value | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | nd | nd | nd | nd |

Scheffe test ($p < 0.05$). RT, room temperature; nd, not done; ns, not significant.

**Fig. 2.** Immunocytochemical staining of LCA using red and blue fixatives in various storage periods at room temperature. **a–c** The positivity rate in the red fixative increased with time compared with that in the 1 h control. **d–f** The positivity rate in the blue fixative was higher at 1 h and maintained at this level throughout the storage period. **a** Red fixative for 1 h. **b** Red fixative for 1 week. **c** Red fixative for 1 month. **d** Blue fixative for 1 h. **e** Blue fixative for 1 week. **f** Blue fixative for 1 month (ICC, ×400, objective, counterstained with hematoxylin). LCA, leukocyte common antigen.

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antigens and fixative solution has a significant effect on the retention of antigenicity.

With respect to CD20, positivity rate after 1 h storage was significantly higher in the blue fixative than in the red fixative, suggesting that fixation during short-term storage in the red fixative is inferior to that in the blue fixative. This may result from differences in alcohol concentration (red: approximately 33% vs. blue: approximately 50%). In addition, the CD20 positivity rate observed in the red fixative was significantly higher at 1 week than that at 1 h; the rate in the blue fixative was significantly lower than that in the red fixative after long-term storage for ≥3 months. A reason for this is that the red fixative contains 0.4% formaldehyde; the addition of formaldehyde may have increased the antigen-retaining ability because of its synergistic effects compared with alcohol alone.

In short-term storage, the antigenicity-retaining ability is dependent on the alcohol concentrations, whereas in long-term storage, the antigenicity-retaining ability is maintained when formaldehyde is present. However, for the intranuclear antigen Ki67, the positivity rate in the control (1 h at room temperature) was the highest in red and blue fixatives, which subsequently decreased markedly with time. The decrease was smaller in the blue fixative than in the red fixative, suggesting that the antigenicity-retaining ability of the blue fixative is higher for intranuclear antigens.

Regarding the composition of each fixative [19], the red fixative contains isopropanol (23.3%), methanol (10.0%), and formaldehyde (0.4%). The alcohol concentration is low in many fixatives used in LBC, such as those described above. Owing to the gradual increase in the fixation time and low fixative concentration, intracellular components leech out over time [32]. The blue fixative contains approximately 50% alcohol, which is a higher percentage, and includes ethanol (44.0%) and methanol (5.0%). This suggests that the antigenicity-retaining ability of the blue fixative is excellent for Ki67 as it prevented the outflow of Ki67 protein.

Positivity was also noted with respect to nuclear division as a characteristic of positive cells. The Ki67 antigen is expressed in the active phase (G1, S, G2, and M phases) of the cell cycle, whereas it is absent in resting cells (G0 phase). Ki67 exhibits varying patterns of nuclear localization corresponding to the cell cycle. The localization and DNA binding affinity of Ki67 in the M phase is influenced by the phosphorylation of Ki67, and a complex regulatory mechanism has been reported [33], suggesting that these cellular states influence the localization of positive staining.

In addition, Ki67 was positive in the cytoplasm at 3 and 6 months, indicating a nonspecific reaction. Regarding alcohol fixation of protein, ethanol-based fixative is generally suitable for the retention of insoluble antigens such as cell surface markers and cytoskeletal proteins, whereas aldehyde-based fixatives are excellent for soluble proteins in the cytoplasm and nucleus because they are eluted by ethanol in the dehydration and degreasing fixation process [32, 34]. Therefore, the Ki67 protein likely eluted into the cytoplasm during long-term storage for ≥3 months, and this process may have strongly influenced the positivity of the cytoplasm.

Preservation and room temperature storage are unsatisfactory for the retention of antigenicity of the intranuclear antigen; we assessed it under a refrigerated (8°C) storage condition. In the red fixative, the positivity rate was low at 1 week and 1 month, exhibiting no improvement. The rate significantly increased at 1 month compared with that at 1 week ($p < 0.001$), and this might have resulted from the synergistic effects of formaldehyde. In the blue fixative, the positivity rate at 1 week improved in refrigerated storage compared with that in room temperature storage, but it significantly decreased at 1 month ($p < 0.001$). For both red and blue fixatives, nonspecific reactions were noted at 3 months and thereafter, as observed at room temperature. The abovementioned findings show that refrigerated storage was also unsuitable; therefore, we evaluated storage at −20°C for 1 week. The results indicated that the positivity rate markedly improved to 87.1% in red and 91.7% in blue fixatives, and no nonspecific reactions were observed (Fig 4). The reason for this improvement in intranuclear antigen preservation at −20°C includes the large influence of temperature on protein stability. When protein is exposed to high

**Fig. 3.** Immunocytochemical staining of Ki67 using red and blue fixatives in various storage periods. **a–d** The positivity rate at 1 h was the highest in both red and blue fixatives and markedly decreased with time. **e** The positivity rate in the red fixative at 8°C for 1 week was low. **f** The positivity rate in the blue fixative for 1 week improved at 8°C compared with that at room temperature. **a** Red fixative at room temperature for 1 h. **b** Red fixative at room temperature for 1 week. **c** Blue fixative at room temperature for 1 h. **d** Blue fixative at room temperature for 1 week. **e** Red fixative at 8°C for 1 week. **f** Blue fixative at 8°C for 1 week (ICC, ×400, objective, counterstained with hematoxylin).

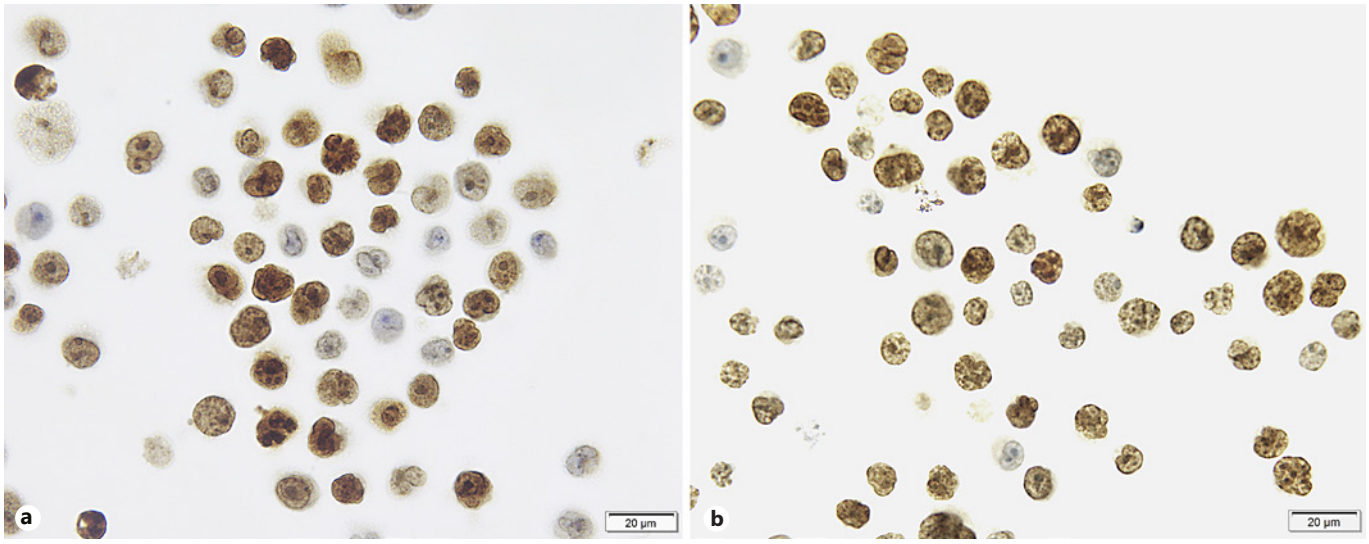


**Fig. 4.** Immunocytochemical staining for Ki67 at −20°C for 1 week. In both red (**a**) and blue (**b**) fixatives, the positivity rate was high. (ICC, ×400, objective, counterstained with hematoxylin).

temperature, weak interactions maintaining the functional structure of protein are broken, resulting in denaturation.

Regarding the storage temperature, storages at −20°C and −74°C for preparations using the ThinPrep-LBC fixative have been reported to maintain the immunoactivity of ER and PgR for 6 months [35]. However, in our study, antigenicity was maintained although the specimens were stored in a vial. Therefore, these can be prepared as needed in an efficient manner.

Compared with LBC specimens, formalin-fixed paraffin-embedded tissue specimens showed degradation of antigenicity over time, which might be because of the significant effect of environmental conditions during storage. The reasons for antigenic deterioration (antigenic instability) include temperature, oxidation, UV irradiation, and humidity [36–42]. Long-term storage at room temperature is reportedly nonoptimal. Therefore, improved antigen activation and detection methods, paraffin coating, and low temperature storage have been proposed [37–42]. In particular, several studies have reported that storage at 4°C maintained antigenicity for several months to years [37–42] and that storage at −20°C or −80°C [39, 41, 42] was also effective. In addition, storage in dark (to remove the effects of light irradiation) [40] and in presence of desiccators (to remove the effects of moisture [41, 42]) was useful. Humidity and temperature can reportedly cause structural changes in epitopes and decrease immunoreactivity in PD-L1 protein, which can be analyzed by combining immunostaining and mass spectrometry using multiple antibodies of different epitopes under set environmental conditions [42]. In this study, we showed that antigenic activation was not necessary, and antigenicity could be retained for long if the specimens were stored in vials containing fixed storage solution at −20°C. The reason for this is that vials can be stored at low temperatures to protect them from oxidation and light irradiation, and vials are more versatile than specimens.

Although long-term storage at room temperature using SP-LBC in both red and blue fixatives is not recommended for intranuclear antigens, the deterioration of antigenicity may be inhibited at ultralow temperatures such as −20°C. However, as only one antibody was used in this study, it is essential to include other intranuclear antigens in further investigations on long-term storage at ultralow temperatures.

## Conclusion

Our study findings indicate that suitable fixative type and storage temperature differ depending on antigen location. This finding may contribute to the proper use of ICC using SP-LBC for clinical specimens in the future. For example, the antigen-retention ability of SP-LBC may be used for immunological phenotyping and genotyping required for the WHO classification of hemato-

poietic tumors. Thus, we expect that the introduction of SP-LBC will increase the usefulness of cytological diagnosis in a wide range of clinical domains, including hematopoiesis.

## Statement of Ethics

The authors have no ethical conflicts to disclose. In this study, cultured cell lines obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB Cell Bank, Kanagawa, Japan) as research materials were used. Therefore, approval from the ethics committee is not required.

## Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.


## Funding Sources

The study was supported by a grant from the 34th Hokkoku Cancer Foundation of Japan.


## Author Contributions

Hiaki Sato designed the study, performed the experiments, analyzed the data, and wrote the manuscript. Yoshiaki Norimatsu conceptualized, designed and supervised the study, and revised the manuscript. Satoshi Irino contributed to the data analysis. Takeshi Nishikawa analyzed the data. All authors read and approved the final manuscript.

## Data Availability Statement

The data that support the findings of this study are openly available in Hokuriku University Institutional Repository at https://hokuriku.repo.nii.ac.jp/.

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