ORIGINAL ARTICLE

Validation of Fixation and Decalcification Protocols for Optimizing Immunohistochemical Staining for Bone Marrow Trephine Biopsy

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Background: Processing of bone marrow trephine biopsies involves the use of various fixatives and decalcifying agents, which can impact immunohistochemistry (IHC) results. However, a simultaneous analysis of both simultaneous fixatives and decalcifying agents has not been conducted.

Objective: To determine the optimal protocol that would yield superior IHC staining results.

Materials and Methods: Twenty reactive tonsillectomy specimens were collected. Forty-two tissue pieces of size 2×2×2 mm, from each tonsil were subjected to different fixation and decalcification protocols. The tested fixatives included 10% buffered formalin and aceto-zinc formalin solution (AZF), with fixation durations of 2, 4, 8, and 24 hours. Decalcification was performed using 5% nitric acid, “Decal II® (Surgipath)”, 10% formic acid, 20% ethylene diamine tetraacetic acid (EDTA) (pH 7.1), and 10% EDTA (pH 7.4). The effect of each protocol on staining quality was assessed using the tissue microarray (TMA) approach. The tested IHC staining panel included CD3, CD20, PAX5, CD30, CD5, cyclin D1, CD10, BCL6, and Ki67.

Results: Ten percent buffered formalin demonstrated significantly better IHC staining results compared to AZF, particularly for nuclear-stained antibodies. The duration of fixation of 2, 4, 8, or 24 hours, did not significantly affect the staining outcomes. Among the decalcifying agents, both 10% formic acid and 10% EDTA provided superior IHC staining results compared to the others. Both agents yielded similar staining outcomes without a significant difference.

Conclusion: For optimized IHC staining in bone marrow trephine biopsy, 10% buffered formalin is recommended as the preferred fixative, while either 10% formic acid or 10% EDTA can be used for decalcification.

Keywords: Bone marrow; Fixative; Fixation; Decalcification; Formic acid; EDTA; Immunostaining; Immunohistochemistry

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Immunophenotypic profiling plays a crucial role in the current classification of hematologic neoplasms according to the 2022 World Health Organization (WHO) classification[1]. Typically, formalin-fixed paraffin-embedded (FFPE) tissue allows for appropriate immunohistochemistry (IHC) staining. However, obtaining optimal results from decalcified marrow trephine biopsies can be challenging. To improve IHC staining outcomes, various fixation and decalcification protocols are currently utilized.

Ten percent buffered formalin is a widely used fixative, while some laboratories opt for B5 to achieve superior morphologic detail. However, concerns regarding toxic waste management have led many laboratories to prefer alternative fixatives[2], demonstrated that aceto-zinc formalin solution (AZF) provides comparable morphological detail to B5[2].

Decalcification is a critical step in processing bony tissue as it removes mineral deposits, allowing for adequate sectioning of bony tissue samples[3]. Decalcifying agents are primarily categorized as chelating agents and acids. Ethylene diamine tetraacetic acid (EDTA) is a widely used chelating agent due to its optimal IHC staining results and minimal DNA destruction[4-7]. However, EDTA requires a longer decalcification period, resulting in

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extended turnaround times. The prolonged duration is often considered unacceptable in many laboratories[8]. In Germany, EDTA is commonly used, whereas in United States, acid-based agents are preferred for shorter turnaround times. However, some IHC markers may show suboptimal results even with shorter decalcification periods.

At the Department of Pathology, Siriraj Hospital, a sizable number of trephine marrow biopsies, along with numerous IHC staining, are performed annually. These samples are fixed with AZF and decalcified using 10% formic acid, following the Hammersmith protocols[8]. However, subsets of cases exhibit suboptimal IHC staining results. Moreover, no study has simultaneously tested the effects of both fixation and decalcification on IHC staining outcomes. Therefore, we conducted this study to identify improved fixation and decalcification protocols that yield better IHC staining results for marrow trephine biopsies.

Materials and Methods

Recruitment of cases

The present study was approved by Siriraj Institutional Review Board, COA No. Si200/2016 (EC2). Twenty tonsillectomy specimens were included in the study, all of which were from patients diagnosed with chronic hypertrophic tonsillitis. The inclusion criteria required patients to be at least 18 years old. Specimens with any grossly suspected abnormality or prolonged ischemic time exceeding 20 minutes were excluded.

For each specimen, half of the tonsil tissue was submitted for routine histopathologic examination, while the remaining half was dissected into multiple small cubes of 0.2×0.2×0.2 cm in size. Forty tissue pieces were collected from each case to test various fixation and decalcification protocols.

To serve as non-decalcified controls, two pieces of tissue from each case were fixed in either 10% buffered formalin or AZF for 8 to 10 hours without undergoing any decalcification process. These control samples were used for comparison and reference throughout the study.

Fixation and decalcification

The chemical substances used in the present study are listed below. These chemical substances were utilized in the fixation and decalcification protocols during the study.

- 37% to 40% formaldehyde (Zenith, Thailand)
- Zinc chloride (Ajax Finechem Pty Ltd, New Zealand)
- Sodium hydroxide (SCHALAU, Spain)
- Sodium dihydrogen phosphate monobasic (SCHALAU, Spain)
- Glacial acetic acid (QReC, New Zealand)
- Nitric acid (RCI LAB SCAN, Thailand)
- Formic acid (SCHLAU, Spain)
- EDTA (Ajax Finechem Pty Ltd, Australia)
- "Surgipath Decal II®" (Leica, USA)
- 25% ammonium hydroxide (Merck, USA)

The working solutions used in the present study were prepared as follows:

1. 10% Millonig's phosphate-buffered formalin preparation (5,000 mL):
- Sodium hydroxide (210 g)
- Sodium dihydrogen phosphate (840 g)
- 37% to 40% formaldehyde (50 mL)
- Distilled water (4500 mL)
2. AZF preparation (1,157.5 mL):
- Zinc chloride (12.5 g)
- Glacial acetic acid (7.5 mL)
- 37% to 40% formaldehyde (150 mL)
- Distilled water (1,000 mL)
- 10% formic acid 5% formaldehyde preparation (1,000 mL)
3. Formic acid (100 mL):
- 37% to 40% formaldehyde (125 mL)
- Distilled water (775 mL)
- 5% nitric acid preparation (1,000 mL)
- 65% nitric acid (77 mL)
- Distilled water (923 mL)
4. 10% EDTA pH 7.4 preparation (1,000 mL):
- EDTA disodium salt (100 g)
- Distilled water (1,000 mL)
- 5M sodium hydroxide (6 mL) (used to adjust pH to 7.4 by adding NaOH)
- 20% EDTA pH 7.1 preparation (1,000 mL)
- EDTA disodium salt (200 g)
- Distilled water (1,000 mL)
- Ammonium hydroxide (used to adjust pH to 7.1)

These working solutions were prepared following the specified measurements and adjusted to the desired pH values. They were used in the fixation and decalcification processes during the study.

Each fixation protocol will involve the utilization of five pieces of tissue from each case. For containers 1, 2, 3, and 4, the tissue was fixed in 10% buffered formalin for 2, 4, 8, and 24 hours, respectively. For container 5, 6, 7, and 8, the tissue was fixed in AZF for 2, 4, 8, and 24 hours, respectively.

From each fixation protocol, one piece of

Figure 1. (A) All pieces of tissue from each case were arranged into a molding template, (B) The TMA block of one case.

tissue was selected and subjected to various decalcification protocols to simulate bone marrow trephine decalcification. The decalcification protocols used were as follows:

1. 5% nitric acid for 45 minutes
2. "Decal II® (Surgipath)" for 90 minutes
3. 10% formic acid 5% formaldehyde for 5 hours
4. 20% EDTA, pH 7.1 for 7.5 hours
5. 10% EDTA, pH 7.4 for 24 hours

In summary, each case underwent 40 different fixation and decalcification protocols. All pieces of tissue from the same case were arranged and constructed into an individual tissue microarray (TMA) block, as shown in Figure 1.

Hematoxylin and eosin (H&E) and immunohistochemical staining

In the present study, H&E staining was performed, along with IHC staining for several markers. The IHC included both membrane- and nuclear-stained markers, as listed in Table 1. The IHC staining protocols used in the study were previously reported by Pongpruttipan et al. in 2011[9]. These protocols were followed to ensure consistency and comparability of the staining techniques across the samples.

Evaluation of slides

In the present study, the signal intensity of the staining was assessed by two hematopathologists (TP and SS) using a scoring system. The fixation and decalcification protocols were blinded to the pathologists. The non-decalcified tissue was used as a control for comparison. The initial scoring system was as follows:

- Score 3+: The staining intensity was at least equal in quality to the controls.
- Score 2+: The staining intensity showed a mild decrease compared to the controls but was still adequate for interpretation.
- Score 1+: The staining intensity showed a noticeable decrease compared to the controls, which could potentially pose problems for interpretation.
- Score 0: No staining observed.

These scores were used to evaluate the staining results and assess the relative intensity of the signals obtained in the study.

Table 1. The immunohistochemical stains and protocols using in the study

Antibodies	Clone	Dilution	Provider	Staining platform
CD3	LN10	1:500	Novocastra	Ventana
CD5	4C7	1:300	Biogenex	Ventana
CD10	56C6	1:300	Novocastra	Ventana
CD20	L26	1:1,000	Dako	Ventana
CD30	Ber-H2	1:300	Dako	Dako
BCL6	LN22	1:200	Novocastra	Ventana
PAX5	SP34	1:300	Cell Marque	Ventana
Cyclin D1	SP4	1:250	Thermo Fisher Scientific	Ventana
Ki67	MIB1	1:200	Dako	Ventana

Novocastra, USA; Biogenex, Netherlands; Dako, USA; Cell Marque, USA; Thermo Fisher Scientific, USA; Ventana, USA

Statistical analysis

The authors used PASW Statistics, version 18.0 (SPSS Inc., Chicago, IL, USA) for statistical analysis. Fisher's exact test and two-way ANOVA were used for comparison among the tested protocols. A p-value less than 0.050 was considered statistically significant, indicating a significant difference or association. Additionally, a p-value less than 0.100

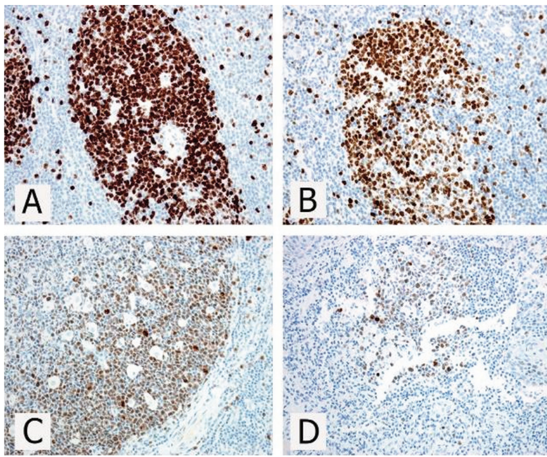

Figure 2. (A) The examples of staining results of Ki67, comparing to control, (B) Tested tissue with 3+ score, (C) Tested tissue with 2+ score, (D) Tested tissue with 1+ score (10X magnification).

was considered as a trend, suggesting a potential association that may warrant further investigation or monitoring. These thresholds were used to determine the significance of the statistical findings in the study.

Results

Out of the 40 fixation and decalcification protocols evaluated in the present study, 800 pieces of tissue were included. These tissues underwent H&E and immunohistochemical staining, resulting in 8,000 sections available for evaluation. However, 293 sections (3.7% of the total) had to be excluded due to either lack of lymphoid tissue or tissue loss during the staining process.

H&E and IHC staining

In the present study, staining results were evaluated based on a scoring system. Tissues with a score of 2+ or 3+ were considered adequate staining results, while those with score of 0 or 1+ were categorized as poor staining results. Examples of tissues with scores of 1+, 2+, and 3+ are shown in Figure 2.

H&E staining across all fixation and decalcification protocols demonstrated similar adequate staining without any significant differences. Examples of immunostaining on the tested tissues are shown in Figure 3. Fixation duration of 2, 4, 8, and 24 hours, using the same fixatives did not affect the staining results, as indicated in Table 2. Therefore, fixation duration was not considered in the subsequent analysis.

When comparing 10% buffered formalin and AZF solution, 10% buffered formalin yielded significantly better immunostaining results of 94% versus 78% ($p<0.001$). Consequently, only tissues fixed with 10% formalin were included in the subsequent analysis.

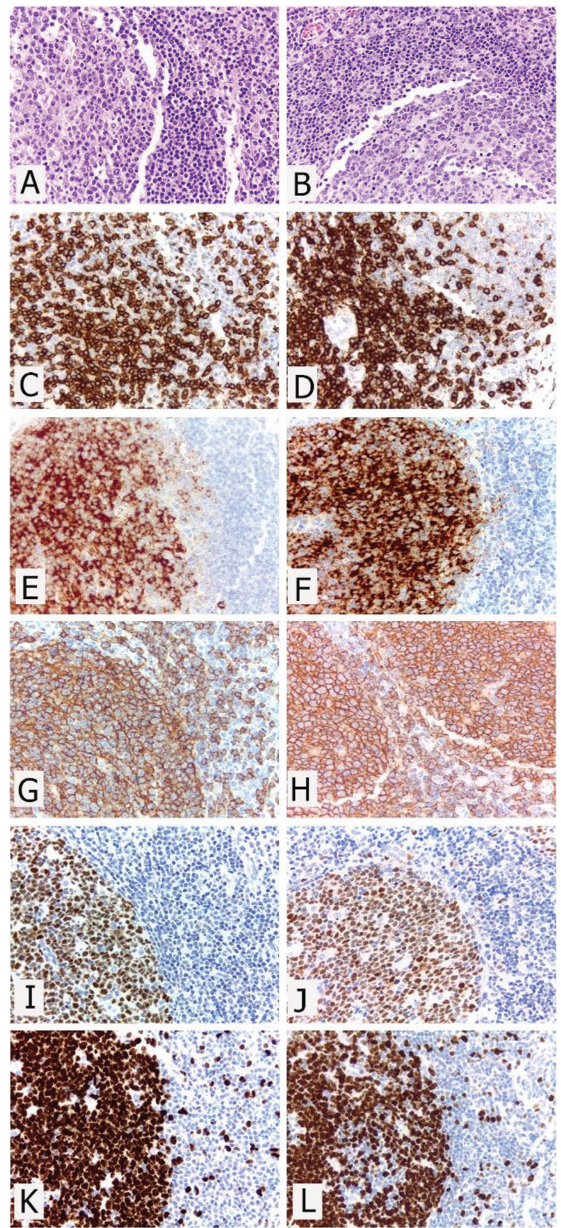

Figure 3. The examples of immunohistochemistical staining, comparing between 10% formalin (left column) and AZF (right column). (A, B) H&E, (C, D) CD3, (E, F) CD10, (G, H) CD20, (I, J) BCL6, (K, L) Ki67. In most tested tissue, there is no significant difference between these 2 fixatives (10X magnification).

Among the tissues fixed with 10% formalin, both 10% formic acid and 10% EDTA showed superior immunostaining results at 98.8% versus 98.5%,

Table 2. Comparison of 10% formalin and AZF solution treated by various decalcifying agents effecting on immunohistochemical staining results

Fixation duration	10% formic acid		10% EDTA		5% nitric acid		20% EDTA		Decal II®		All decalcifying agents	
	Formalin	AZF	Formalin	AZF	Formalin	AZF	Formalin	AZF	Formalin	AZF	Formalin	AZF
	Percentage of good staining results (total number of valid tests)											
2 hours	99 (180)	88 (169)	99 (176)	94 (177)	97 (177)	74 (174)	91 (170)	80 (177)	83 (175)	58 (169)	94 (878)	79 (866)
4 hours	98 (178)	90 (166)	99 (177)	93 (165)	98 (173)	71 (171)	96 (180)	72 (162)	81 (177)	69 (176)	94 (885)	79 (840)
8 hours	97 (180)	89 (162)	98 (170)	90 (160)	93 (168)	71 (176)	96 (167)	79 (173)	79 (178)	66 (177)	92 (863)	79 (848)
24 hours	100 (169)	86 (166)	97 (179)	89 (174)	97 (179)	71 (177)	98 (175)	73 (166)	89 (173)	63 (174)	96 (875)	76 (857)
Total	99 (707)	88 (663)	98 (702)	91 (676)	96 (697)	72 (698)	95 (692)	76 (678)	83 (703)	64 (696)	94 (3,501)	78 (3,411)
p-value	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001	

EDTA=ethylene diamine tetraacetic acid; Formalin=10% Millonig's phosphate buffered formalin; AZF=aceto-zinc formalin solution
Good staining results, the staining with at least adequate for interpretation (score 2+ and 3+)
All fixation duration (2 to 24 hours) of both 10% formalin and AZF shows no significant difference on staining results (p=0.960 and 0.970, respectively)
10% formalin, when compare to AZF, shows superior immunostaining results (94% vs. 78%, p<0.001)
p-value from the comparison of formalin versus AZF of each decalcification protocol

Table 3. The immunohistochemical staining results of the 10% formalin fixed tissue treated with various decalcifying reagents

Immunostaining	10% formic acid	10% EDTA	5% nitric acid	20% EDTA	Decal II®	p-value
	Percentage of good staining results (total number of valid tests)					
CD3	100 (79)	100 (79)	100 (79)	100 (80)	100 (80)	1
CD5	100 (79)	100 (79)	100 (80)	100 (80)	100 (80)	1
CD10	95 (77)	96 (74)	99 (76)	92 (72)	95 (76)	1
CD20	100 (79)	100 (79)	100 (80)	100 (80)	100 (80)	1
CD30	100 (79)	100 (79)	100 (76)	94 (79)	99 (79)	0.003
BCL6	95 (77)	92 (77)	92 (72)	99 (69)	78 (74)	0.001
PAX5	99 (79)	100 (79)	94 (78)	94 (78)	60 (78)	<0.001
Cyclin D1	100 (79)	100 (79)	95 (79)	100 (80)	88 (80)	<0.001
Ki67	99 (79)	97 (78)	87 (77)	77 (74)	25 (76)	<0.001
Total	98.8 (707)	98.5 (703)	96.7 (697)	95.6 (692)	84.5 (703)	0.008

EDTA=ethylene diamine tetraacetic acid
Good staining results, the staining with at least adequate for interpretation (score 2+ and 3+)

respectively, as shown in Table 3. All decalcification protocols resulted in similar adequate staining for CD3, CD20, CD5, and CD10. However, the remaining IHC markers, CD30, BCL6, PAX5, cyclin D1, and Ki67, exhibited variable results among the decalcification protocols, with Ki67 showing the most variability in staining results.

Discussion

The tested fixatives and decalcifying agents in the present study were 10% buffered formalin and AZF solution. Both fixatives are formaldehyde-based and function effectively by cross-linking amino acids between peptide chains. In addition to formaldehyde, AZF also contains zinc and acetic acid. Zinc is believed to provide partial protection to proteins and DNA during acid decalcification[2]. AZF, with its low-concentration acetic acid, is thought to enhance morphologic details on H&E slides and act as a mild decalcifying agent[2,8]. The authors' experience with using AZF as a fixative for bone marrow biopsy suggests that it offers better morphologic details. However, all tested fixative and decalcifying protocols in the present study yielded similar H&E staining without any significant differences.

Studies have suggested that zinc in formalin helps preserve antigens[10]. However, the present study showed that 10% formalin provides better immunostaining results in all decalcifying protocols compared to AZF (Table 2). The reason for the decreased staining quality when using AZF remains unclear. It is possible that zinc, acetic acid, or both may be responsible.

In routine histopathology, the recommended fixation time ranges from 6 to 72 hours. Fixatives penetrate tissue slowly, at a rate of 0.5 to 1 mm per hour, depending on the tissue type. Fat or adipose tissue is more challenging to penetrate than

other tissues. When collecting tissues for routine pathological examination, immediate fixation in a fixative like 10% buffered formalin is necessary to prevent autolysis and ensure optimal IHC stains[11]. However, in the case of bone marrow biopsies, with their small diameter of only 1.5 mm, the penetration of formalin may require a shorter fixation duration compared to larger tissues. Therefore, a 2-hour fixation is not recommended for other larger tissues.

Some laboratories use B5 fixative for bone marrow biopsies, which contains formaldehyde, mercuric chloride, and sodium acetate. However, it is not widely preferred due to the toxic waste produced by mercury. Additionally, Righi et al. have demonstrated that formalin fixation improves the performance of tested antibodies compared to B5 fixation[12].

Decalcification is a process used to remove mineral deposits from bone or calcified tissues, allowing for thin sectioning. The recommended volume of decalcifying reagents is usually similar to the volume of the fixative used. The duration of decalcification depends on the type of reagents and the thickness of the bone. For compact bone that requires a longer decalcification duration, a 5% to 10% nitric acid solution is preferred. However, using such a strong inorganic acid may not be suitable for bone marrow biopsy specimens.

In the present study, all decalcifying protocols showed similar staining results for membrane-stained antibodies. However, for nuclear-stained antibodies such as BCL6, PAX5, cyclin D1, and Ki67, both 10% formic acid and 10% EDTA yielded significantly better results compared to other protocols. This suggests that nuclear antigens may be more susceptible to the effects of decalcifying agents. The intensity of staining can be influenced by factors, including tissue thickness, antibody concentration, antigen retrieval methods, and other variables[13]. However, the present study utilized a TMA approach, staining all tissues on the same slides, which helped eliminate potential confounding factors.

Among decalcifying agents, 10% EDTA is widely used but requires a longer decalcification period compared to acid-based decalcifiers. Schrijver et al. suggested the use of EDTA as a decalcifying agent for FISH analysis[14]. However, the drawback of 10% EDTA is its slow decalcification process and extended turnaround time. Nath et al. recommended the use of 20% EDTA for a faster decalcification process[15], but there are no corresponding IHC results available for this reagent. Limited studies have explored the effects of different decalcifying agents on IHC staining results.

Decal II® (Surgipath) is a commercial solution used for rapid decalcification. It contains hydrochloric acid. No studies have mentioned this reagent in relation to IHC staining results. In the present study, Decal II® showed inferior results for nuclear-stained antibodies.

It has been documented that the aging of blocks and slides significantly affects antigen decay[16]. In the present study, the tissue blocks used for IHC staining were less than three months old and freshly cut sections were utilized, minimizing the potential effects of block and slide aging.

Conclusion

In conclusion, when dealing with small biopsies requiring decalcification, such as bone marrow biopsies, 10% buffered formalin is recommended as the fixative. Both 10% formic acid and 10% EDTA are preferred decalcifying agents that yield superior results in IHC staining. These choices can help ensure optimal tissue preservation and staining outcomes in pathological analysis.

What is already known on this topic?

Ten percent buffered formalin is recommended as the fixative.

What does this study add?

Both 10% formic acid and 10% EDTA are preferred decalcifying agents that yield superior results in IHC staining.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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