PATHOLOGICA 2025;117:145-152;
DOI: 10.32074/1591-951X-1065

Original article

One protocol to rule them all: a pilot study to identify the best fixation and decalcification approach for bone marrow biopsy immunohistochemistry

Michele Paudice[1,2*], Alessandro Gambella[2*], Simona Pigozzi[1,2], Michela Campora[3], Francesca Pitto[1,2], Silvia Bozzano[2], Luca Mastracci[1-2**], Federica Grillo[1,2**]

[1] IRCCS San Martino Policlinic Hospital of Genoa, Genoa, Italy; [2] Pathology Unit, Department of Surgical Sciences and Integrated Diagnostics (DISC), University of Genoa, Genoa, Italy; [3] Department of Pathology, Santa Chiara Hospital, APSS Trento, Trento, Italy
** MP and AG contributed equally to this study and shared first authorship; **FG and LM contributed equally to this study and shared last authorship*

Summary

Objective. Standardization of the pre-analytical phases of bone marrow trephine biopsy (BM) has yet to be achieved. In particular, several fixative and decalcifying reagents with specific benefits and drawbacks are described, but only a few direct comparisons are available. This study aims to test the most used fixation and decalcification protocols and evaluate their effect on tissue antigenicity via immunohistochemistry (IHC).

Methods. To avoid damaging and exhausting diagnostic BMs, we used "surrogate" BMs obtained from dedicated grossing of a non-pathologic spleen. Eleven fixation and decalcification protocols were tested, and their performances were evaluated via IHC protein expression of 25 biomarkers.

Results. The IHC yield varied based on the fixative and decalcifying reagents, but the overall quality is mainly related to the fixative rather than the decalcifying phases. The protocol with the lowest number of inadequate IHC stains (5 out of 25) combined commercially available B5-based fixative and EDTA-based decalcifying reagents. The worst metrics (8 inadequate IHC stains out of 25) were obtained with a protocol based on "in-house" B5-based and EDTA-based reagents.

Conclusions. We compared different protocols and found the best combination of fixative and decalcifying reagents for accurate IHC staining. These findings can improve bone marrow sample handling and standardization in pathology laboratories.

Key words: fixation, decalcification, pre-analytical phases, bone marrow

Received: August 8, 2024
Accepted: January 17, 2025

Correspondence
Alessandro Gambella
E-mail address: alessandro.gambella@gmail.com

How to cite this article: Paudice M, Gambella A, Pigozzi S, et al. One protocol to rule them all: a pilot study to identify the best fixation and decalcification approach for bone marrow biopsy immunohistochemistry. Pathologica 2025;117:145-152. https://doi.org/10.32074/1591-951X-1065

Introduction

Bone marrow trephine core biopsies (BMs) represent one of the most challenging specimens for the daily routine in pathology units: following tailored tissue fixation and processing, the diagnostic process of BMs is frequently based on the combination of several morpho-molecular approaches, including granular evaluation of morphological details, expression of multiple proteins via immunohistochemistry (IHC) , and cytogenetic and molecular genetic profiles [1]. Furthermore, this demanding workload requires optimal technical handling and well-established laboratory staff expertise to guarantee tissue adequacy for each of the assays mentioned above.

In particular, a critical phase is the balance between fixation and de-

calcification. Frequently used fixatives for BMs include 10% buffered formalin, acetic acid–zinc–formalin preparation (AZF), and mercuric chloride-based fixatives such as B5 or Zenker's fixative [1]. Each fixative has specific benefits and drawbacks regarding fixation time, toxicity, morphology yield, preservation of tissue antigenicity, and related IHC quality. Of note, zinc-formalin and B5-based fixation showed established advantages for morphological details, especially for cell nuclei. On the other hand, decalcification is required before tissue processing to allow the sectioning of bone spicules. Unfortunately, reagents used for bone decalcification are generally strong inorganic acids that present well-known detrimental effects, primarily on tissue morphology and antigenicity [2-4].

To date, several guidelines from international societies support pathology services for the management of most specimens, aiming to: (1) limit the impact of the pre-analytic phases and related technical problems, (2) standardize pathology reports, and (3) harmonize the related tissue management across different institutions [5]. Unfortunately, this is not the case for BMs. Only a few guidelines are available [1,6] and not widely implemented, thus resulting in institution-tailored and poorly standardized procedures for BMs fixation and decalcification.

In this context, we harnessed our expertise with pathology service quality assurance [7-12] to test several fixation and decalcification protocols and evaluate the subsequent IHC yield of multiple antigens commonly used for BMs in diagnostic routine.

Materials and methods

Samples and dataset development

In this study, we tested 11 fixation and decalcification protocols and evaluated the related protein expression of 25 biomarkers via IHC assay. Considering the large amount of tissue sections needed to test each condition and biomarker, we created "surrogate" BMs (S-BMs) to prevent potentially permanent tissue damage and exhaustion of BMs collected for diagnostic purposes. S-BMs were developed by dedicated fresh grossing of one non-pathologic spleen specimen resected during a distal pancreatectomy (cold ischemia time: 20 minutes). A pathologist (LM) sectioned the spleen parenchyma and recreated 20-mm-long and 2-mm-wide lymphoid tissue cores, namely the S-BMs, to mirror the gross dimension of diagnostic BMs. Overall, 11 S-BMs were prepared to have one S-BM per fixation and decalcification protocol. All the procedures described in this study were in accordance with the ethical standards of the responsible institutional committee on human experimentation and with the World Medical Association Declaration of Helsinki of 1964 and later versions. All patients who underwent surgical resection in our Institution signed written informed consent for research purposes.

Fixation and decalcification: reagents used and protocol details

The fixation and decalcification protocols (labelled "A" to "M") were developed by matching different reagents and related S-BMs exposure times. Fixative reagents were both coupled with a tissue decalcification step (protocols A, C, E, G, H, I, and L) and tested alone (protocols B, D, F, and M). This way, we were able to specifically identify if variation in IHC yield was due to the fixative or the decalcifying phase. Of note, protocol M was characterized by buffered formalin fixation only and deprived from the decalcification step to mirror the fixation process generally used in the pathology service for most tissue specimens. We used protocol M as the reference for the IHC yield comparison across protocols. Protocol N, which combined buffered formalin fixation with the Mielodec B, was used to test the effect on routine formalin-fixed specimens. All protocols (except for the protocol M) included a final wash

Table I. List of reagents used for the fixation and decalcification protocols.

Preparation	Reagent	Procedure/Components
In-house	B5	1 liter of solution comprises 62.4 g of mercuric chloride ($HgCl_2$) in 800 ml deionized and distilled water. Subsequently, 13 g of sodium acetate anhydrous ($C_2H_3NaO_2$) is added as well as 200 ml of purified water
	Decalcifying agent	37 g of EDTA (ethylenediaminetetracetic acid), 70 ml of hydrochloric acid (HCl) at 37%, and deionized and distilled water added to reach 1 liter of solution. The solution is then filtered after 24 hours to eliminate any crystals that may have formed
	AZF	12.5 g of zinc chloride ($ZnCl_2$), 150 ml of 40% concentrated formalin, 7.5 ml glacial acetic acid ($C_2H_4O_2$), and distilled water was added to reach 1 liter
Industry, RTU (Bio-Optica spa, Milan, Italy)	B5	formalin-mercury II chloride used as per manufacturer's instructions
	MieloDec Kit	kit composed of 2 reagents: MieloDec A which is a B5 fixative; MieloDec B which is a decalcifying agent which contains EDTA in acid buffer. The kit, and each single reagent, were used as per the manufacturer's instructions

Table II. Details of the protocols developed and tested in this study.

Protocols	Fixation	Time (hrs)	Wash	Time (hrs)	Decalcifying agent	Time (hrs)	Wash
A	B5 in-house	2.5	70% ethanol	0.5	in-house	0.5	70% ethanol
B	B5 in-house	2.5	70% ethanol	0.5	/		70% ethanol
C	B5 Bio-Optica	2.5	70% ethanol	0.5	in-house	0.5	70% ethanol
D	B5 Bio-Optica	2.5	70% ethanol	0.5	/		70% ethanol
E	AZF	2.5	distilled water	0.5	in-house	0.5	70% ethanol
F	AZF	20-24	distilled water	0.5	/		70% ethanol
G	Mielodec A	1.5	70% ethanol	0.25	Mielodec B	1.5	70% ethanol
H	B5 in-house	2.5	70% ethanol	0.25	Mielodec B	1.5	70% ethanol
I	B5 Bio-Optica	2.5	70% ethanol	0.25	Mielodec B	1.5	70% ethanol
L	AZF	20-24	70% ethanol	0.25	Mielodec B	1.5	70% ethanol
M	Buffered formalin	20-24	/	/	/	/	/
N	Buffered formalin	20-24	/	/	Mielodec B	1.5	70% ethanol

with 70% ethanol to remove any reagents excess before tissue processing. Details of the reagents and fixation and decalcification protocols are reported in Table I and Table II, respectively.

Tissue processing and IHC staining

Following fixation and decalcification protocols, all 11 S-BMs were processed with the Leica Biosystems ASP6025 automatic processor (Leica Biosystems, Nußloch, Germany), and then paraffin-embedded individually. From each S-BMs tissue block, several sections were collected for IHC stains, mounted on Superfrost slides (Thermo Scientific, Braunschweig, Germany) to prevent section detachment [13], and then used right after sectioning to prevent confounding factors related to delayed staining and long-term storage [14].

IHC was performed with the BenchMark Ultra® automated immunostainer (Roche Tissue Diagnostics, Basel, Switzerland) using the following antibodies: CD2 (mouse monoclonal, clone MRQ-11, catalogue #760-4377), CD3 (rabbit monoclonal, clone 2GV6, catalogue # 790-4341), CD4 (rabbit monoclonal, clone SP35, catalogue # 790-4423), CD5 (rabbit monoclonal, clone SP19, catalogue # 790-4451), CD7 (rabbit monoclonal, clone SP94, catalogue # 790-4558), CD8 (rabbit monoclonal, clone SP57, catalogue # 790-4460), CD10 (rabbit monoclonal, clone SP67, catalogue # 790-4506), CD20 (rabbit monoclonal, clone SP67, catalogue # 790-4506), CD23 (rabbit monoclonal, clone SP23, catalogue # 790-4408), CD34 (mouse monoclonal, clone QBend/10, catalogue # 790-2927), CD43 (mouse monoclonal, clone L60, catalogue # 760-2511), CD44 (rabbit monoclonal, clone SP37, catalogue # 790-4537), CD68 (mouse monoclonal, clone KP-1, catalogue # 790-2931), CD79α (rabbit monoclonal, clone SP18, catalogue # 790-4432), CD138 (mouse monoclonal, clone B-A38, catalogue # 760-4248), Bcl2 (rabbit monoclonal, clone SP66, catalogue # 790-4464), Cyclin D1 (rabbit monoclonal, clone SP4-R, catalogue # 790-4508), Kappa light chain (rabbit polyclonal, catalogue # 760-2514), Lambda light chain (rabbit polyclonal, catalogue # 760-2515), CD61 (mouse monoclonal, clone 2f2, catalogue # 760-4249), MPO (rabbit polyclonal, catalogue # 760-2659), Ki-67 (rabbit monoclonal, clone 30-9, catalogue # 790-4286), CD45 RO (mouse monoclonal, clone UCHL-1, catalogue # 790-2930), S100 (mouse monoclonal, clone 4C4.9, catalogue # 790-2914), and CD117 (rabbit monoclonal, clone EP10, catalogue # 790-7061). All antibodies were developed ready-to-use by Roche (Roche Tissue Diagnostics, Basel, Switzerland) and then associated with the ultraView Universal DAB Detection Kit (Roche Tissue Diagnostics, Basel, Switzerland) for staining detection.

IHC evaluation and scoring systems

Considering the need to evaluate both morphology and IHC for BMs diagnostic purposes, we developed a specific semi-quantitative score, namely the Q-score, that combined IHC cytological details (D), signal intensity (I), and background (B). Details of the Q-score and related definitions are reported in Table III.

A final Q-score value was calculated using the following formula: **Q = (D + I) – B.** A Q-score > 4 was considered acceptable, a value < 2 was inadequate, and a value of 3 was considered borderline. Two expert pathologists (LM and FG) evaluated all IHC slides and assessed the related Q-score. In case of disagreement, a consensus was reached by joint review and discussion. Representative images of the Q-score are reported in Figure 1.

Table III. Details of the Q-score and related definitions.

Category	Definition
Cytological detail (D)	
0	Complete loss of cytological details
1	Cytological details are poorly maintained; subcellular compartments (nucleus, cytoplasm, and cell membrane) are challenging to visualize and distinguish
2	Fairly good cytological details with sufficient but suboptimal visualization of the different cellular compartments
3	Good cytological details with clear visualization of the different cellular compartments
***Signal intensity* (I)**	
0	No staining
1	Weak intensity
2	Moderate intensity
3	Strong intensity
Non-specific background staining (B)	
0	No non-specific background
1	Weak non-specific background; no interference with overall interpretation
2	Moderate non-specific background; partial interference with staining interpretation;
3	Severe non-specific background; complete interference with staining interpretation

Results

IHC markers presented an overall adequate yield with notable exceptions

The first phase of our study aimed to assess the overall yield of IHC biomarkers before considering the effect of each fixation and decalcification experimental protocol. In this study, we were interested in evaluating the stain quality per se and the related tissue cito-morphological details. To this end, we developed a specific semi-quantitative score, namely the Q-score, that considered IHC signal intensity, background "noise" (non-specific staining), and cytological details and combined them in a single value representative of the overall IHC stain quality.

We performed 25 single-plex IHC stains per protocol, thus totalling 275 IHC slides available for evaluation. We observed adequate results (identified by a Q-score > 4) in the majority of cases (188 out of 275 IHC stains, 68.4%). Few stains (28 out of 275, 10.2%) resulted in borderline (Q-Score = 3), but a non-negligible number of stains (59 out of 275, 21.4%) were inadequate (Q-score ≤ 2).

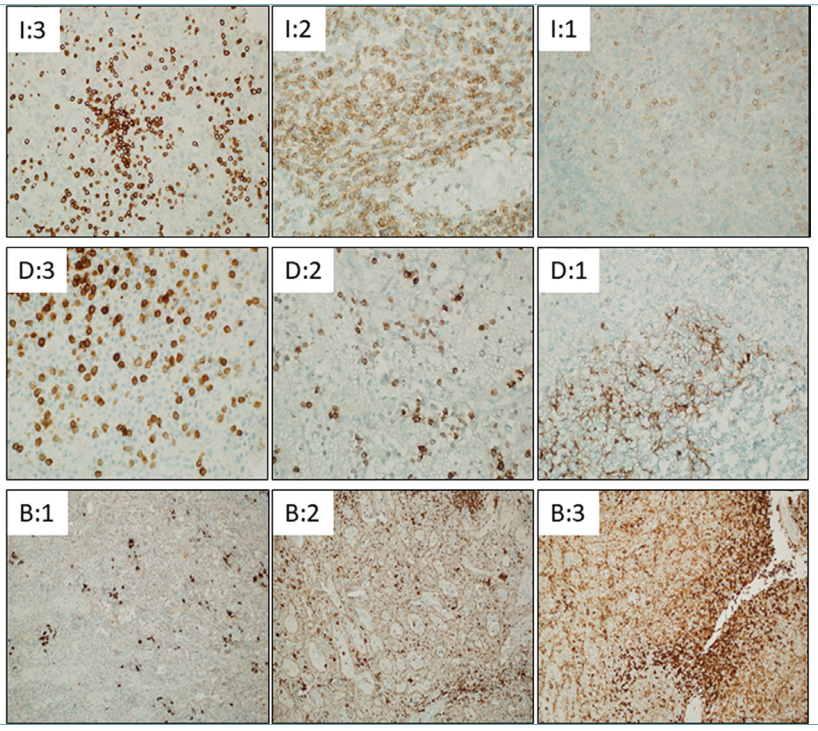

Figure 1. Representative images of IHC intensity (I), cytological detail (D), and non-specific background (B) and the relative Q-score. I:3) CD10, protocol M (magnification 40x); I:2) CD2, protocol M (magnification 40x); I:1) CD3, protocol M (magnification 40x); D:3) CD20, protocol H (magnification 60x); D:2) CD7, protocol H (magnification 60x); D:1) CD5, protocol C (magnification 60x); B:1) CD20, protocol L (magnification 20x); B:2) Bcl2, protocol F (magnification 20x); B:3) CD4, protocol F (magnification 20x).

Then, we decided to focus on each biomarker and evaluate the specific performance across protocols. We observed that seven of the 25 biomarkers (CD3, CD5, CD8, CD34, CD68, CD138, and CD61) showed adequate Q-score in all 11 protocols. Differently, three antibodies (Cyclin D1, CD10, and Kappa light chain) presented inadequate Q-score in almost all protocols, except protocol M (i.e., standard formalin fixation deprived of the decalcification step) where, instead, adequate Q-score was observed. The remaining 16 biomarkers presented heterogeneous Q-scores across different protocols. Details of all IHC biomarker Q-scores are reported in Table IV.

Overall, these findings suggested a putative effect of the different fixative and decalcifying reagents on some of the evaluated biomarkers and supported the need for a more granular protocol-specific analysis.

Table IV. Q-score of the 25 IHC biomarkers assessed. Protocol A: in-house B5 and in-house decalcification; Protocol B: in-house B5 and no decalcification; Protocol C: Bio-Optica B5 and in-house decalcification; Protocol D: Bio-Optica B5 and no decalcification; Protocol E: AZF and in-house decalcification; Protocol F: AZF and no decalcification; Protocol G: Mielodec A and Mielodec B; Protocol H: in-house B5 and Mielodec B; Protocol I: Bio-Optica B5 and Mielodec B; Protocol L: AZF and Mielodec B; Protocol M: buffered formalin and no decalcification.; Protocol N: buffered formalin and Mielodec B.

	Protocols											
Antibodies	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**L**	**M**	**N**
CD 2	3	5	3	3	1	4	3	3	3	3	4	4
CD 3	4	6	5	5	5	5	5	4	4	5	6	6
CD 4	2	5	1	5	3	4	2	5	3	3	6	4
CD 5	4	6	6	4	5	6	6	6	5	5	6	5
CD 7	4	6	5	5	5	4	6	4	4	3	6	n.a.
CD 8	5	5	5	4	6	5	5	6	6	5	6	3
CD 10	0	2	-1	-1	-2	0	-2	1	0	-1	4	2
CD 20	4	5	5	5	5	6	5	3	4	4	6	3
CD 23	3	6	5	5	1	4	5	4	4	5	6	6
CD 34	6	5	6	6	6	6	6	6	6	6	6	5
CD 43	4	5	6	4	3	4	2	4	3	2	6	2
CD 44	3	6	5	4	4	4	2	4	3	4	6	n.a.
CD 68	6	6	5	4	5	6	4	6	5	5	6	6
CD 79α	3	6	5	5	4	5	4	4	6	5	6	6
CD 138	6	6	5	4	4	5	5	5	4	5	6	6
Bcl-2	1	4	1	4	1	3	1	1	2	2	6	2
Kappa	2	3	1	1	2	0	3	2	0	-1	4	1
Lambda	6	4	4	3	4	2	5	6	5	4	6	1
CD 61	4	5	4	6	6	4	6	4	6	5	6	5
MPO	6	4	5	5	4	2	5	6	5	4	6	4
Ki-67	0	0	0	5	5	5	0	0	0	0	6	4
CD 45 RO	6	6	4	4	4	5	5	6	3	4	5	n.a.
S 100	1	4	5	2	3	2	4	4	3	2	5	5
CD117	-1	4	4	1	3	4	3	0	4	3	5	5
Cyclin D1	0	0	-2	2	0	2	-1	0	-1	-2	4	2

Comparison of S-BMs protocols without decalcification: formalin provided the best IHC yield

Initially, we focused on the protocols deprived of the decalcification step. This way, we could clearly evaluate the specific role of the fixation phase on the tissue antigenicity and related IHC quality.

Protocol M (standard formalin fixation) presented maximum Q-score metrics with no inadequate or borderline IHC stains. In addition, 19 of the 25 biomarkers obtained the maximal Q-score (Q-score = 6; Tab. IV).

Protocols B (fixation with in-house prepared B5 fixative), D (Bio-Optica B5), and F (AZF) presented heterogeneous results with a non-negligible representation of inadequate and borderline IHC stains. Considering these results and the analogy of protocol M with the fixation process commonly used in the pathology service for other tissue specimens, we used protocol M as the reference procedure for further comparison.

Focusing on protocol B (in-house B5), three biomarkers were considered inadequate and one borderline. Nine biomarkers presented maximum Q-score and only one biomarker (i.e., CD45RO) showed superior yield with protocol B (Q-score = 6) compared to protocol M (Q-score = 5). Cytologic details were exceptionally well preserved with this protocol.

Protocol D (Bio-Optica B5) hindered tissue antigenicity and presented inadequate and borderline IHC quality with five and two biomarkers, respectively. Similar to protocol B, cytologic details were well preserved, but only two biomarkers had maximum Q-score (CD34 and CD61), and no biomarkers showed a superior yield compared to protocol M.

Protocol F (AZF fixation) presented inadequate and borderline yield with six and one biomarkers, respectively. While four biomarkers showed maximum Q-score, none was superior to those with protocol M. Details of the Q-score across the protocols are reported in Table V.

These results showed that, prior to the decalcification step, the IHC yield can vary based on the fixative reagents used, as expected.

Table V. Protocols and related Q-scores used in our study. Protocol A: in-house B5 and in-house decalcification; Protocol B: in-house B5 and no decalcification; Protocol C: Bio-Optica B5 and in-house decalcification; Protocol D: Bio-Optica B5 and no decalcification; Protocol E: AZF and in-house decalcification; Protocol F: AZF and no decalcification; Protocol G: Mielodec A and Mielodec B; Protocol H: in-house B5 and Mielodec B; Protocol I: Bio-Optica B5 and Mielodec B; Protocol L: AZF and Mielodec B; Protocol M: buffered formalin and no decalcification.

	Protocols											
Q-score	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**L**	M	N
Inadequate (≤ 2)	8	3	6	5	6	6	7	6	5	7	0	6
Borderline (= 3)	4	1	1	2	4	1	3	2	6	4	0	2
Adequate (≥ 4)	13	21	18	18	15	18	15	17	14	14	25	14

Comparison of protocols with decalcification: the effect of decalcifying reagents greatly varied based on the matched fixative

Once we established the role of the different fixative reagents on biomarkers quality, we proceeded by evaluating the protocols that added the decalcification step, namely protocols A (in-house B5 fixative combined with in-house decalcifying agent), C (Bio-Optica B5 and in-house decalcifying), E (AZF and in-house decalcifying), G (Mielodec A fixative and Mielodec B decalcifying), H (in-house B5 and Mielodec B decalcifying), I (Bio-Optica B5 and Mielodec B decalcifying), and L (AZF and Mielodec B decalcifying).

The protocol with the lowest number of inadequate biomarkers was protocol I (n = 5), but it contemporarily presented a high number of borderline (n = 6) biomarkers. Protocol C presented a relatively high number of inadequate (n = 6) but only minimal (n = 1) borderline biomarkers, together with the highest number of adequate (n = 18) IHC stains. On the other hand, the protocol that showed the worst Q-score metrics was protocol A, presenting eight inadequate, four borderline, and only 13 adequate IHC stains (Tab. IV).

To evaluate the direct effect of decalcifying agents on IHC stains, we compared the Q-scores of protocols with different decalcification steps but the same fixative. First, we focused on the in-house B5 fixative. Compared to its use alone (protocol B), the best results were obtained with the addition of Mielodec B (protocol H; six inadequate and two borderline) rather than the in-house decalcifying reagent (protocol A; eight inadequate and four borderline). Different results were observed for the Bio-Optica B5 and the AZF. Compared to their use alone (protocols D and F, respectively), both fixatives presented better results in combination with the in-house fixative (protocols C and E) rather than with the Mielodec B (protocols I and L).

Protocol G, which combined Mielodec A (fixative) with Mielodec B (decalcifying), showed intermediate Q-score performances (seven inadequate and three borderline) compared to the other protocols.

Overall, these findings showed that adding a decalcifying agent worsened the IHC yield across all protocols but to different degrees depending on the initial fixation step. Furthermore, they demonstrated that the quality of IHC stains is mainly related to the fixative reagent rather than the decalcifying solution.

Discussion

In this study, we demonstrated that the IHC yield of biomarkers for BM diagnostic routine largely depends on the tissue fixative and decalcifying reagents. We tested several fixative and decalcifying conditions commonly used by pathology services, evaluated the related antigen protein expression yield of 25 biomarkers, and eventually identified the combination of Bio-Optica B5 fixative and Mielodec B decalcifying agent (protocol I) as the procedure with the overall best IHC yield defined by the lowest number of inadequate biomarkers' staining.

BM diagnostic process requires the preparation of specimens with optimal preservation of tissue morphology. For decades, BMs were processed and embedded in epoxy resin to obtain semithin sections with exquisite morphologic details [15]. With the advent of IHC and its increasing relevance in BM diagnostics [16,17], pathology services progressively abandoned epoxy-resin-embedding and semithin-sectioning, and switched to BMs fixation, decalcification, and paraffin-embedding [1,6]. As this new approach allowed faster and more feasible IHC staining of BMs, it also led to new drawbacks: pre-analytical phases such as fixation and decalcification can be performed with several different reagents, thus resulting in poor standardization across institutions and studies.

In our study, we decided to evaluate the performance of different protocols by directly comparing the IHC yield of biomarkers commonly used in the BM diagnostic process. The highest number of adequate IHC

stains were obtained with protocol C, which combined a commercially available B5-based fixative and an EDTA-based decalcifying reagent prepared in our institution. However, we preferred the results provided by protocol I, which combined commercially available reagents for both the fixation (B5-based) and decalcification (EDTA-based) phases. There are multiple reasons behind this choice. First, protocol I showed the lowest number of inadequate IHC staining. The adjudicator pathologists considered this criterion the most relevant: inadequate IHC stains were deemed useless, whereas borderline IHC, while suboptimal, still allowed to evaluate antigen/biomarker expression and formulate a diagnostic opinion. Furthermore, protocol C is partially based on in-house reagents (specifically, the decalcifying step). While advocating in-house formulation may be tempting, we preferred supporting commercially available reagents as they facilitate regulation of tissue management across institutions.

The standardization of BM management from pathology services has yet to be achieved. Several groups advocated the advantages of their institutional-tailored protocol or fixative and decalcifying reagents [18-21], and only a few international recommendations regarding pre-analytical steps for BMs are available [1,6]. In 2008, the International Council for Standardization in Haematology (ICSH) published guidelines for BMs' tissue handling [1]. Following an international call of European centers [22], additional ICSH guidelines on BM IHC staining were published soon after [6]. These guidelines highlighted the toxic nature of B5-based fixatives and the related need for dedicated hazardous waste, and recommended the use of formalin or AZF as the fixative reagents. In our study, AZF-based protocols did not show superior metrics, but they still provided valid IHC yields. We recognize the environmental and safety concerns related to the mercuric chloride content of B5-based fixatives [18]. Furthermore, decalcifying agents may differ for decalcification efficiency. However, in our pilot study we aimed to evaluate the IHC yield alone, and it was behind the purpose of our analysis to perform cost-effective, safety, or decalcification efficiency evaluations of each reagent. The exploratory nature of our study also required the use of spleen-based surrogates of BMs to prevent damage and exhaustion of BM diagnostic specimens. Despite these limitations, we believe that we obtained satisfactory preliminary results that allowed us to select and test the protocols with the best metrics on a prospective, larger cohort of "real" BMs, allowing us to test additional crucial metrics of additional decalcifying agents. In conclusion, several fixative and decalcifying reagents can be used to manage BM specimens in the pathology service, but they have different effects on tissue morphology and antigenicity. Here, we provided valuable insights on different protocols and identified the combination of commercially available B5-based fixative and EDTA-based decalcifying reagents as the most promising for adequate IHC stains. Implementation and further validation of our findings will help standardize BM pre-analytical phases and specimen management in the daily pathology routine.

Acknowledgments

LM is supported by grants from the Italian Ministry of Health (5×1000–2022-2024).

Conflicts of interest statement

The Authors do not have any conflict of interest to disclose.

Funding

None.

Authors' contributions

Conceptualization: Luca Mastracci, Federica Grillo; Methodology: Michele Paudice, Alessandro Gambella, Luca Mastracci, Federica Grillo; Formal analysis and investigation: Michele Paudice, Simona Pigozzi, Michela Campora, Francesca Pitto, Silvia Bozzano; Writing - original draft preparation: Alessandro Gambella; Writing - review and editing: All Authors; Supervision: Federica Grillo, Luca Mastracci.

Ethical consideration

All the procedures described in this study were in accordance with the ethical standards of the responsible institutional committee on human experimentation and with the World Medical Association Declaration of Helsinki of 1964 and later versions. All patients who underwent surgical resection in our Institution signed written informed consent for research purposes.

References

1 Lee SH, Erber WN, Porwit A, et al, International Council for Standardization. In: H. ICSH guidelines for the standardization of bone marrow specimens and reports. Int J Lab Hematol. 2008;30(5):349-64. https://doi.org/10.1111/j.1751-553X.2008.01100.x

2 Singh VM, Salunga RC, Huang VJ, et al. Analysis of the effect of various decalcification agents on the quantity and quality of nucleic acid (DNA and RNA) recovered from bone biopsies. Ann Diagn Pathol. 2013;17(4):322-6. https://doi.org/10.1016/j.anndiagpath.2013.02.001

3 Savi FM, Brierly GI, Baldwin J, et al. Comparison of Different Decalcification Methods Using Rat Mandibles as a Model. J Histochem Cytochem. 2017;65(12):705-722. https://doi.org/10.1369/0022155417733708

4 Miquelestorena-Standley E, Jourdan ML, Collin C, et al. Effect of decalcification protocols on immunohistochemistry and molecu-

lar analyses of bone samples. Mod Pathol. 2020;33(8):1505-1517. https://doi.org/10.1038/s41379-020-0503-6

5 Misra S, Moberg-Aakre K, Langlois M, et al. How Useful are Laboratory Practice Guidelines? EJIFCC. 2015;26(3):190-6.

6 Torlakovic EE, Brynes RK, Hyjek E, et al. ICSH guidelines for the standardization of bone marrow immunohistochemistry. Int J Lab Hematol. 2015;37(4):431-49. https://doi.org/10.1111/ijlh.12365

7 Bragoni A, Gambella A, Pigozzi S, et al. Quality control in diagnostic immunohistochemistry: integrated on-slide positive controls. Histochem Cell Biol. 2017;148(5):569-573. https://doi.org/10.1007/s00418-017-1596-y

8 Grillo F, Bruzzone M, Pigozzi S, et al. Immunohistochemistry on old archival paraffin blocks: is there an expiry date? J Clin Pathol. 2017;70(11):988-993. https://doi.org/10.1136/jclinpath-2017-204387

9 Ridolfi M, Paudice M, Salvi S, et al. Agar pre-embedding of small skin biopsies: real-life benefits and challenges in high throughput pathology laboratories. J Clin Pathol. 2019;72(6):448-451. https://doi.org/10.1136/jclinpath-2018-205680

10 Mastracci L, Gambella A, Bragoni A, et al. Coping with formalin banning in pathology: under vacuum long-term tissue storage with no added formalin. Histochem Cell Biol. 2019;151(6):501-511. https://doi.org/10.1007/s00418-018-1765-7

11 Grillo F, Campora M, Pigozzi S, et al. Methods for restoration of ki67 antigenicity in aged paraffin tissue blocks. Histochem Cell Biol. 2021;156(2):183-190. https://doi.org/10.1007/s00418-021-01987-w

12 Grillo F, Paudice M, Gambella A, et al. Evaluating mismatch repair deficiency in colorectal cancer biopsy specimens. Histochem Cell Biol. 2023;160(2):113-125. https://doi.org/10.1007/s00418-023-02202-8

13 Gambella A, Porro L, Pigozzi S, et al. Section detachment in immunohistochemistry: causes, troubleshooting, and problem-solving. Histochem Cell Biol. 2017;148(1):95-101. https://doi.org/10.1007/s00418-017-1558-4

14 Grillo F, Pigozzi S, Ceriolo P, et al. Factors affecting immunoreactivity in long-term storage of formalin-fixed paraffin-embedded tissue sections. Histochem Cell Biol. 2015;144(1):93-9. https://doi.org/10.1007/s00418-015-1316-4

15 Krenacs T, Bagdi E, Stelkovics E, et al. How we process trephine biopsy specimens: epoxy resin embedded bone marrow biopsies. J Clin Pathol. 2005;58(9):897-903. https://doi.org/10.1136/jcp.2004.023788

16 Gatter KC, Heryet A, Brown DC, et al. Is it necessary to embed bone marrow biopsies in plastic for haematological diagnosis? Histopathology. 1987;11(1):1-7. https://doi.org/10.1111/j.1365-2559.1987.tb02604.x

17 Kremer M, Quintanilla-Martinez L, Nahrig J, et al. Immunohistochemistry in bone marrow pathology: a useful adjunct for morphologic diagnosis. Virchows Arch. 2005;447(6):920-37. https://doi.org/10.1007/s00428-005-0070-8

18 Bonds LA, Barnes P, Foucar K, et al. Acetic acid-zinc-formalin: a safe alternative to B-5 fixative. Am J Clin Pathol. Aug 2005;124(2):205-11. https://doi.org/10.1309/29DA-CY9K-BHNW-4BG6

19 Naresh KN, Lampert I, Hasserjian R, et al. Optimal processing of bone marrow trephine biopsy: the Hammersmith Protocol. J Clin Pathol. 2006;59(9):903-11. https://doi.org/10.1136/jcp.2004.020610

20 Fend F, Tzankov A, Bink K, et al. Modern techniques for the diagnostic evaluation of the trephine bone marrow biopsy: methodological aspects and applications. Prog Histochem Cytochem. 2008;42(4):203-52. https://doi.org/10.1016/j.proghi.2007.10.002

21 Anagnostopoulos I, Lenze D, Hummel M, et al. Bone Marrow work-up: report of a pilot study. Recent Results Cancer Res. 2015;199:95-105. https://doi.org/10.1007/978-3-319-13957-9_10

22 Torlakovic EE, Naresh K, Kremer M, et al. Call for a European programme in external quality assurance for bone marrow immunohistochemistry; report of a European Bone Marrow Working Group pilot study. J Clin Pathol. 2009;62(6):547-51. https://doi.org/10.1136/jcp.2008.063446