Optimization of the Melanin Bleaching Method to Improve the Analysis of Malignant Melanoma

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ABSTRACT
Malignant melanoma is a highly aggressive tumor in which immunohistochemical (IHC) evaluation is often hindered by endogenous melanin, whose brown pigment mimics DAB staining and may lead to false-positive interpretation. An effective melanin bleaching method is therefore essential to ensure accurate IHC assessment. This study aimed to determine the optimal melanin bleaching protocol for melanoma, particularly for IHC analysis. This preliminary laboratory study used eighteen malignant melanoma samples. Initial bleaching with 0.5% H_2O_2 in PBS or Tris-HCl at 80°C for 4–12 minutes caused marked tissue damage. A second protocol using 5% H_2O_2 in PBS at 50°C for 60, 90, and 120 minutes was tested, followed by a 24-hour room-temperature protocol. Tissue integrity, bleaching efficacy, and subsequent HMB45 IHC staining were evaluated. High-temperature bleaching (protocol 1) produced significant morphological deterioration. Bleaching with 5% H_2O_2 at 50°C effectively removed melanin while preserving tissue structure at 60 and 90 minutes, whereas 120 minutes caused tissue detachment. Room-temperature bleaching for 24 hours also reduced melanin but resulted in partial tissue damage. HMB45 staining remained strong and interpretable after bleaching at 50°C for 60 and 90 minutes. In conclusion, the most favorable balance between melanin removal, tissue preservation, and IHC quality was achieved using 5% H_2O_2 in PBS at 50°C for 60 minutes. This protocol is recommended as the optimal melanin bleaching method for malignant melanoma tissue.
Keywords: melanin bleaching; immunohistochemistry; malignant melanoma

INTRODUCTION
Malignant melanoma is an aggressive neoplasm arising from melanocytes, the pigment-producing cells located within the basal layer of the epidermis, mucosal epithelium, and several other organs. This malignancy is broadly categorized into two major groups: melanoma associated with ultraviolet (UV) exposure and melanoma that develops independently of sun exposure. Based on its anatomical site, melanoma is further classified into cutaneous, mucosal, and ocular subtypes, each with distinct biological behavior and clinical implications [1,2].

The global incidence of melanoma has continued to rise over recent decades. According to Globocan 2022, melanoma ranks as the seventeenth most common cancer worldwide and is particularly prevalent in Western countries. The prevalence of cutaneous malignant melanoma reached 833,215 cases in 2021, reflecting a dramatic increase of 161.3% since 1990 [3]. Australia and New Zealand consistently report the highest incidence and mortality rates globally, underscoring the substantial public health burden associated with this malignancy [4].

Cutaneous melanoma is recognized as one of the most aggressive forms of skin cancer, with a high mortality rate that makes it a critical health concern requiring early and accurate diagnosis. Early detection is essential for improving patient outcomes, as advanced-stage melanoma is associated with a markedly increased risk of metastasis and death [5–7]. Histopathologically, melanoma is frequently characterized by abundant melanin pigment. Excessive melanin deposition can obscure cellular morphology and tissue architecture, complicating microscopic evaluation and potentially leading to diagnostic delay or misinterpretation. Yeh et al. reported that severe melanin accumulation may mask key morphological features of melanocytic lesions, thereby reducing diagnostic accuracy and hindering proper assessment [8].

Although histopathological examination remains the gold standard for melanoma diagnosis, immunohistochemical (IHC) markers are increasingly used to support diagnostic clarification, particularly when tumor differentiation is ambiguous on hematoxylin–eosin (HE) staining. IHC also plays an important role in distinguishing benign from malignant melanocytic lesions. The use of IHC in melanoma diagnosis has increased substantially, from 11% of cases in 2000 to more than 50% of cases diagnosed in 2017 [9]. Beyond diagnosis, IHC contributes to prognostic evaluation and is essential in research related to novel therapeutic strategies, including targeted therapy and immunotherapy [10,11]. However, the presence of melanin pigment poses a significant challenge in IHC interpretation. Melanin closely resembles the brown chromogenic product of diaminobenzidine (DAB), the most commonly used chromogen for visualizing antigen–antibody reactions in IHC. This similarity makes it difficult to distinguish true immunoreactivity from endogenous pigment, increasing the risk of false-positive results. Consequently, melanin bleaching has become a crucial preparatory step when processing heavily pigmented melanoma specimens. This technique improves the clarity of histopathological evaluation and enhances the reliability of IHC-based diagnostic and research applications. Despite the availability of numerous bleaching techniques, no standardized protocol has yet been established [12–14].

Hydrogen peroxide (H_2O_2) is one of the most commonly used bleaching agents due to its effectiveness in pigment removal and its compatibility with routine staining workflows. However, H_2O_2 has relatively weak bleaching capacity and often requires prolonged incubation. Other agents, such as potassium permanganate ($KMnO_4$) and hypochlorous acid (HClO), offer faster bleaching but are more aggressive and may compromise tissue integrity [15–17]. Several bleaching protocols have been proposed. Conventional methods typically employ varying concentrations of H_2O_2 at room temperature for 24 hours or longer [15]. Alternative approaches use very low concentrations of H_2O_2 at high temperatures (80°C), with neutral or alkaline pH, for short durations ranging from 4 to 30 minutes [18]. Additional methods apply low-concentration H_2O_2 at moderately elevated temperatures (50°C to 65°C) for extended periods of 60 to 360 minutes [19]. Given the essential role of IHC in diagnostic, prognostic, and therapeutic decision-making for melanoma, and considering that most melanoma lesions contain substantial melanin pigment that interferes with IHC interpretation, an optimized bleaching method is urgently needed. The absence of a standardized protocol highlights the importance of identifying the most effective and tissue-preserving melanin bleaching approach for IHC evaluation. This study aims to determine the optimal melanin bleaching method for melanoma analysis, with particular emphasis on achieving accurate and reliable immunohistochemical staining.

METHODS
This study was designed as a laboratory-based preliminary investigation aimed at the initial optimization and early validation of melanin bleaching methods prior to the main research phase. The study population consisted of all histopathologically confirmed cases of cutaneous malignant melanoma

diagnosed at the Diagnostic Center of Anatomical Pathology, Faculty of Medicine, Universitas Andalas. The study samples were selected from this population based on predefined inclusion criteria, namely cases of malignant melanoma confirmed by histopathological examination and subsequently reviewed by two pathologists, who then identified cases exhibiting diffuse melanin pigmentation. Cases lacking melanin pigment or showing only focal pigmentation were excluded. A total of eighteen samples met the eligibility criteria and were included in the analysis.

The variables examined in this study comprised independent and dependent variables. The independent variables were the variations in melanin bleaching protocols previously described in the literature, including differences in bleaching solution concentration, pH, temperature, and incubation duration. The dependent variables were the degree of melanin expression and the immunohistochemical expression of HMB45 following bleaching.

The study was conducted at the Diagnostic Center of Anatomical Pathology, Faculty of Medicine, Universitas Andalas, from July to November 2025. Data were obtained from patient records of previously diagnosed malignant melanoma cases, followed by reevaluation of hematoxylin–eosin (HE) slides. Paraffin blocks were sectioned at a thickness of 3 µm and mounted on glass slides. Deparaffinization was performed using xylene, followed by rehydration through graded alcohols (100%, 90%, and 70%) and rinsing under running water until all alcohol residues were removed. The slides were then subjected to melanin bleaching according to three established protocols.

Protocol 1 utilized 0.5% hydrogen peroxide (H_2O_2) at 80°C in phosphate-buffered saline (PBS) and in Tris-HCl buffer, with incubation durations of 4, 8, and 12 minutes [18]. Protocol 2 employed 5% H_2O_2 in PBS at 50°C for 30, 60, and 90 minutes [19]. Protocol 3 applied 5% H_2O_2 in PBS at room temperature for 12 and 24 hours [15]. Following bleaching, immunohistochemical staining with HMB45 was performed. The evaluation of melanin expression and HMB45 immunoreactivity after bleaching was conducted by the primary investigator and an anatomical pathologist. To minimize interpretation bias, quantitative assessment was subsequently performed using ImageJ software, generating ratio-based measurements for both melanin reduction and IHC expression.

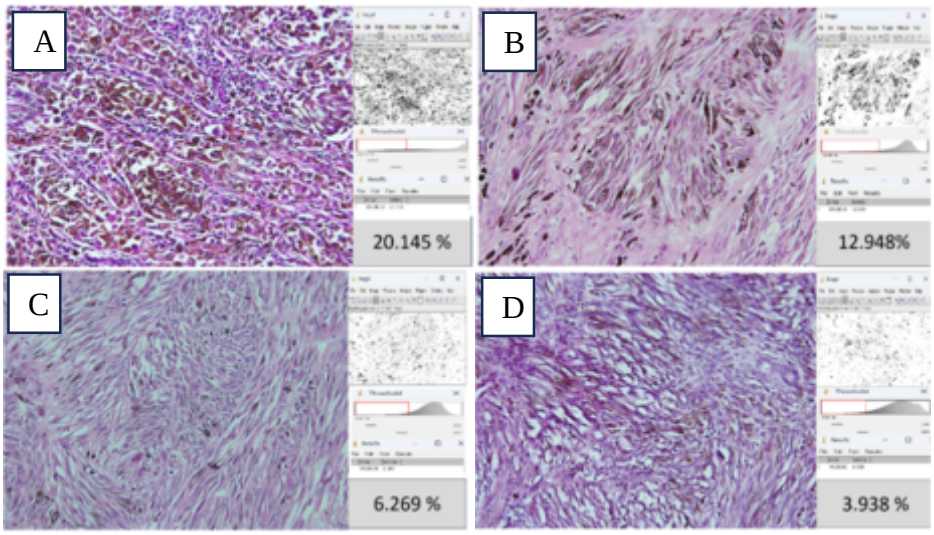

Figure 1. Photomicrographs of melanin expression before (A) and after melanin bleaching according to Protocol 1 using 0.5% H_2O_2 at 80°C for 4 minutes in PBS (B), 8 minutes in PBS (C), and 4 minutes in Tris-HCl (D). HE stain, 200×

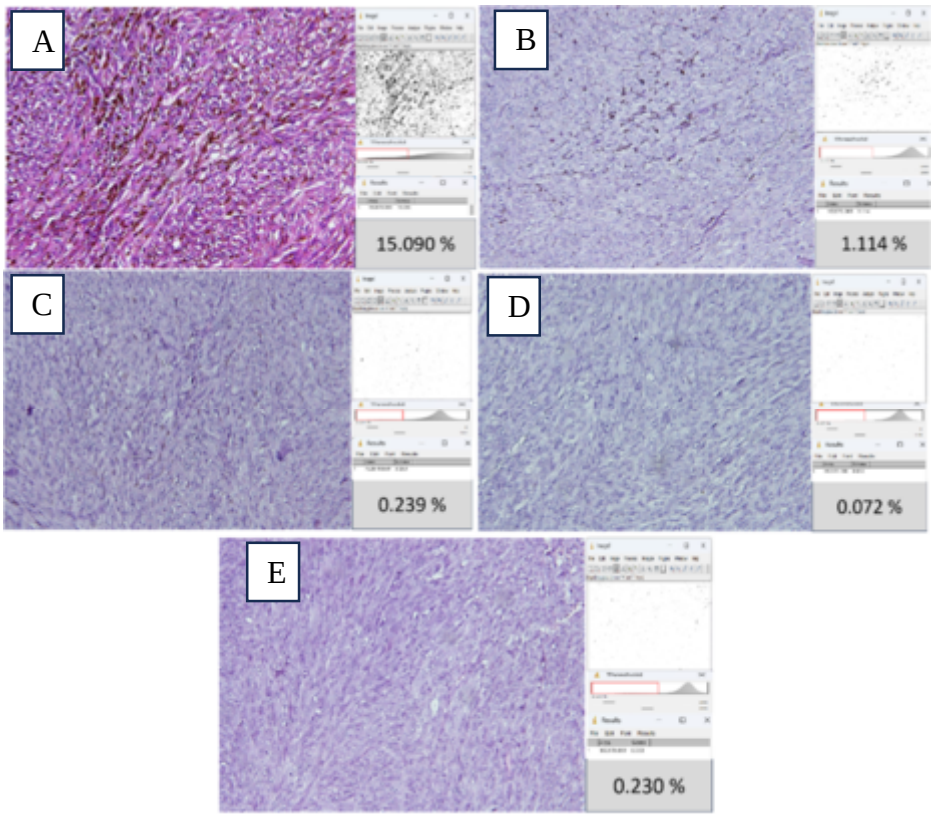

Figure 2. Photomicrographs of melanin expression before (A) and after melanin bleaching according to Protocol 2 using 5% H_2O_2 in PBS (neutral pH) at 50°C for 30 minutes (B), 60 minutes (C), and 120 minutes (D), and according to Protocol 3 at room temperature for 24 hours (E). HE stain, 200×

RESULTS

Optimization of melanin bleaching using identical concentration and temperature but different durations and pH (Protocol 1)

Table 1. Percentage of tissue area expressing melanin before and after melanin bleaching according to protocol 1

Duration (minute)	Melanin expression (%)	
	PBS (% area)	Tris HCl
0	20.145	20.145
4	12.948	3.938
8	6.269	–
12	–	–

The optimization of melanin bleaching under Protocol 1 is illustrated in Figure 1. ImageJ analysis demonstrated a significant reduction in melanin expression in samples treated with PBS at 4 and 8 minutes; however, these changes were accompanied by noticeable tissue damage. More extensive morphological deterioration was observed at 12 minutes, as shown in Table 1. All samples treated with Tris-HCl exhibited marked tissue damage, although melanin reduction could still be assessed at 4 minutes, with a substantial decrease in melanin expression recorded.

These findings indicate that while PBS-based bleaching at high temperature effectively reduces melanin, it also results in considerable tissue damage. Similarly, Tris-HCl produces a meaningful reduction in melanin expression but causes severe structural disruption, limiting its applicability for further histopathological or immunohistochemical evaluation.

Optimization of melanin bleaching using identical concentration and ph but different durations and temperatures (Protocols 2 and 3)

Table 2. Percentage of tissue area expressing melanin after melanin bleaching according to protocols 2 and 3

Duration	Temperature	Melanin expression (%)
0 minutes	Room	15.090
60 minutes	temperature	1.114
90 minutes	50°C	0.239
120 minutes	50°C	0.072
24 hours	50°C	0.230

Table 3. Percentage of tissue area expressing HMB45 immunoreactivity after optimal melanin bleaching according to protocols 2 and 3

Protocol	HMB45 expression (%)
50°C for 60 minutes	8.916
50°C for 90 minutes	7.854
Room temperature for 24 hours	–

Optimization under Protocols 2 and 3 is shown in Figure 2. ImageJ analysis revealed a marked reduction in melanin expression in samples treated with 5% H_2O_2 in PBS at 50°C, with optimal tissue preservation observed at 60 and 90 minutes. At 120 minutes, however, extensive tissue detachment and structural loss were noted. In contrast, bleaching at room temperature for 24 hours resulted in minimal tissue damage while still achieving substantial melanin reduction, as presented in Table 2. These findings indicate that optimal bleaching occurs at 50°C for 60 and 90 minutes, as well as at room temperature for 24 hours, although the latter may still produce mild structural alterations.

Immunohistochemical expression following optimal melanin bleaching

The immunohistochemical (IHC) expression following optimal bleaching conditions is shown in Figure 3. ImageJ analysis demonstrated clear HMB45 immunoreactivity in tissues treated with 5% H_2O_2 at 50°C for 60 and 90 minutes. In contrast, bleaching at room temperature for 24 hours resulted in partial tissue degradation, limiting the interpretability of IHC staining, as reflected in Table 3. These findings indicate that the most reliable IHC expression following melanin bleaching is achieved at 50°C for 60 and 90 minutes.

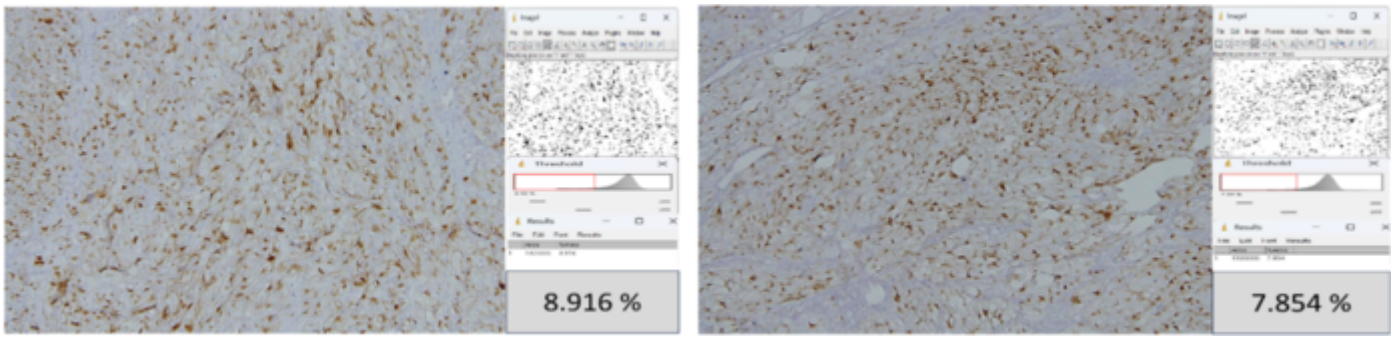

Figure 3. Photomicrographs of HMB45 immunohistochemical expression following optimal melanin bleaching according to Protocols 2 and 3, using 5% H_2O_2 in PBS (neutral pH) after 60 minutes (A) and 90 minutes (B). IHC stain, 200×.

DISCUSSION

Melanin pigment is insoluble in most organic solvents due to the strong chemical bonds between melanin and its associated protein components. The use of strong oxidizing agents, such as permanganate, chlorate, chromic acid, peroxides, and acetic acid can effectively bleach melanin. The darker the melanin pigment, the longer the bleaching time required. Methods employing acetic acid or 0.25% potassium permanganate followed by 2% oxalic acid have been shown to work effectively; however, these agents often exert detrimental effects on tissue quality and may damage antigenic epitopes, thereby compromising subsequent immunohistochemical or immunocytochemical analyses. For this reason, one of the preferred approaches is the use of dilute hydrogen peroxide (H_2O_2), which offers a more balanced profile between bleaching efficacy and tissue preservation [20].

In this study, the use of 0.5% H_2O_2 at 80°C (Protocol 1) in PBS, compared with Tris-HCl, demonstrated a significant reduction in melanin expression following bleaching in PBS, although this was accompanied by notable tissue damage. A similar reduction in melanin expression was observed with Tris-HCl; however, the degree of tissue destruction was markedly more severe. These findings are consistent with the work of Ugolini et al., who reported that melanin bleaching using 0.5% H_2O_2 in Tris buffer (pH 10) is highly aggressive toward tissue. Their study showed that this solution can alter cytoarchitecture and reduce antigenicity when applied for longer durations, although they concluded that effective bleaching can be achieved with very short incubation times. This highlights the importance of buffer environment and pH control in determining bleaching efficacy [12].

Nordio et al. employed 10% H_2O_2 in Tris-buffered saline (pH 7.4) to bleach heavily pigmented tissue sections, demonstrating that commonly used buffers contribute to solution stability and overall bleaching performance [19]. Momose et al. further re-evaluated melanin bleaching using low-concentration H_2O_2 at warm temperatures dissolved in various high-pH buffer systems, including Tris-HCl (pH 10), PBS, and Tris-based buffers containing detergents (Tris/Tricine/SDS, pH 8.2), across different temperatures and incubation times. Among the tested diluents, high-pH Tris-HCl produced the best results for molecular applications. Their findings emphasize that the choice of diluent significantly influences optimal bleaching conditions, suggesting that chemical properties such as buffering capacity and pH play crucial roles in determining bleaching outcomes [19].

The efficiency of melanin bleaching is also strongly dependent on H_2O_2 concentration. Tang et al. reported that low concentrations of H_2O_2 (0.3–0.5%) may paradoxically enhance melanin synthesis and melanosome transfer. Achieving effective bleaching typically requires higher concentrations, which must be carefully balanced with temperature and incubation duration, as these variables are interdependent [14]. Wan et

al. similarly concluded that high concentrations or prolonged exposure to H_2O_2 may lead to tissue damage, limiting its applicability in histopathological workflows [22].

The use of H_2O_2 at elevated temperatures, such as 80°C, has been shown to be effective for bleaching pigmented histopathology samples, particularly melanoma, prior to immunohistochemical evaluation. The primary rationale for applying high temperature is to accelerate the bleaching reaction without compromising tissue integrity or antigenicity. Ugolini et al. developed a rapid and automated bleaching protocol using 0.5% H_2O_2 in alkaline Tris buffer (pH 10) for 8 minutes at 80°C, demonstrating successful melanin removal without adversely affecting antigenicity or cytoarchitecture. The use of 80°C significantly shortens processing time, making this approach suitable for routine laboratory workflows [18].

In this study, bleaching with 5% H_2O_2 at 50°C (Protocol 2) across varying incubation times, as well as bleaching at room temperature for 24 hours (Protocol 3), demonstrated that optimal melanin removal occurred at 50°C for 60 and 90 minutes, and at room temperature for 24 hours. Momose et al. specifically evaluated warm dilute H_2O_2 at temperatures of 50°C, 55°C, and 60°C with incubation times ranging from 0.5 to 3 hours. Their findings indicated that 50°C is effective for melanin bleaching, although efficiency depends on H_2O_2 concentration, diluent type, and incubation duration [18]. While increasing temperature accelerates chemical reactions, excessively high temperatures may increase the risk of tissue damage or antigen loss [14,18].

Incubation time must also be carefully considered, as lower temperatures may require longer exposure to achieve comparable bleaching results. H_2O_2 concentration must be optimized in conjunction with temperature, and the choice of diluent is equally important for maintaining pH stability and preventing tissue degradation [19]. Bleaching at room temperature using H_2O_2 is a conventional method, but one of its major limitations is the prolonged duration required to achieve adequate pigment removal. Wang and several other studies have noted that H_2O_2 has relatively weak bleaching capacity at room temperature, often requiring 24 hours or more to achieve sufficient depigmentation [13,14]. Under neutral pH and in the absence of metal ions, melanin exhibits high resistance to oxidation by H_2O_2, suggesting that bleaching efficiency at room temperature is inherently limited unless additional catalytic factors are present. Numerous studies have demonstrated that increasing temperature significantly accelerates the bleaching reaction [14].

The optimal immunohistochemical expression observed in this study occurred at 50°C for 60 and 90 minutes. Successful melanin bleaching requires not only effective pigment removal but also preservation of antigenicity for IHC. Recent studies emphasize the need for caution during bleaching to avoid damaging antigens or generating false-positive staining results [22]. The use of warm temperatures aims to accelerate bleaching while maintaining cellular morphology and antigen reactivity for subsequent IHC analysis. Several studies have also developed automated platforms integrating H_2O_2-based melanin bleaching into automated IHC workflows, further supporting the feasibility of controlled bleaching conditions in diagnostic practice [12].

CONCLUSION

This study demonstrates that variations in H_2O_2 concentration, pH, temperature, and incubation duration produce distinct differences in both melanin expression and tissue quality following melanin bleaching. The most favorable balance between effective melanin reduction and preservation of tissue morphology was achieved using 5% H_2O_2 in PBS at 50°C for 60 and 90 minutes, as well as at room temperature for 24 hours. Among these conditions, the optimal protocol for subsequent immunohistochemical evaluation was bleaching with 5% H_2O_2 in PBS at 50°C for 60 minutes, which provided the best combination of pigment removal, tissue integrity, and antigen preservation for reliable IHC staining.

Ethical consideration, competing interest and source of funding

-This study received ethical approval from the Research Ethics Committee of the Faculty of Medicine, Universitas Andalas, under approval number 706/UN.16.2/KEP-FK/2025.
-There is no conflict of interest related to this publication.
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