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A Fast and Automated Melanin-bleaching Method for Histopathologic Evaluation of Pigmented Melanoma Tissues

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Abstract: Histopathologic examination of highly pigmented melanoma tissues has always been a challenge for pathologists. The high concentration of melanin pigment is an obstacle for immunohistochemistry and the ensuing evaluation. Therefore, removing melanin has become a crucial step for processing heavily pigmented melanoma samples. Several bleaching techniques have been proposed in the past, however, the most commonly used methods are time-consuming and poorly standardized. In this study, we propose a new fast and fully automated bleaching method applicable to validated immunohistochemical panels already used in the diagnosis of melanocytic tumors. The proposed bleaching protocol is based on sample pretreatment with 0.5% hydrogen peroxide and a Tris base pH 10 solution for 8 minutes at 80°C before antigen retrieval. Immunohistochemistry with HMB45, MART-1, Ki-67, SOX10, S-100, Tyrosinase, and BRAF(V600E) antibodies showed that this pretreatment removed excess melanin without affecting the tissue antigenicity and cytoarchitecture. In conclusion, we propose a new fast and automated bleaching protocol, easily transferable to a routine setting with efficient results in specimens in which the melanin pigmentation could blunt the histopathologic examination.

Key Words: bleaching, melanin, multiplex, melanoma, immunohistochemistry

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Melanin, the major pigment in human skin and hair, is a complex and highly heterogeneous polymer consisting of monomeric units of dihydroxyphenyl-alanine and/or cysteinyl-dihydroxyphenyl-alanine. The main function of melanin is to protect skin from damage by ultraviolet radiation. Although this pigment plays a crucial role in protecting the human body, it represents a challenge for pathologists in evaluating highly pigmented formalin-fixed paraffin-embedded tissue samples from melanoma or pigmented skin tumors, in which abundant melanin may blind the cytomorphology of tumor cells and associated melanophages.[1]

To date, melanoma is one the major causes of cancer-related death and its incidence is increasing worldwide.[2,3] Diagnosis of melanoma is based on evaluations of cytoarchitectural characteristics on hematoxylin-eosin stained slide, confirmed with assessments on expression of specific markers highlighted by immunohistochemistry (IHC).[4] A high concentration of melanin represents an obstacle for IHC because of an impairment in antibody-antigen interactions.[5] In addition to this interference, melanin brown color appears similar to the detection reagent 3,3′-diaminobenzidine (DAB), which is one of the most common chromogens used in diagnostic pathology. This color interference between chromogen and melanin represents a challenge to the interpretation of IHC results. Similar issues persist even when another chromogen, such as Fast Red, is used. Several molecular assays, including polymerase chain reaction and reverse transcription-polymerase chain reaction, are also affected by the presence of melanin, thus leading to anomalies in the functioning of DNA polymerase during the running of assays.[6–8] Consequently, a lack of successful detection or misleading results for the detection of a BRAF mutation in pigmented melanoma specimens may reduce possibilities to treat patients with target therapies.[9]

Several bleaching techniques have been proposed in the past; however, the most common methods used are time-consuming, poorly standardized, manual or semi-automated protocols.[10–13] In this brief report, we propose a new, completely automated, bleaching method for IHC using Ventana Discovery ULTRA immunostainer (Ventana Medical Systems, Tucson, AZ). This new procedure can be easily applied to validated IHC panels commonly used in the diagnosis of melanocytic tumors, assuring cell

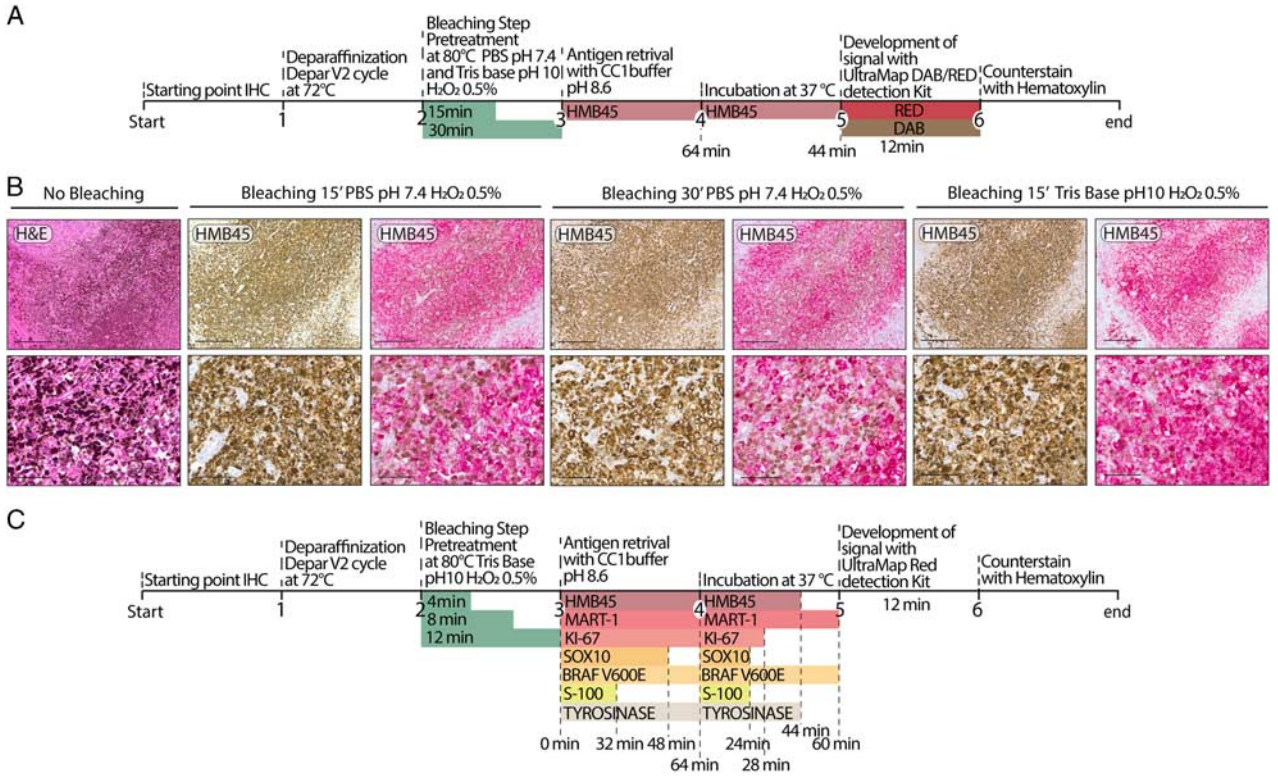

FIGURE 1. A, Schematic representation of automated bleaching protocols with phosphate buffered saline pH 7.4 solution in comparison with Tris base pH 10 solution in Ventana Discovery Ultra immunostainer. B, Effects of different bleaching buffer and treatment time on protein immunogenicity. Representative immunohistochemistry of a pigmented melanoma tissue for HMB45, after 15′ and 30′ of bleaching. Magnification ×20, inset ×200 (Scale bars: 1 mm, inset 100 μm). C, Schematic representation of automated bleaching protocols with Tris base pH 10 solution, after 4′, 8′, and 12′ in Ventana Discovery Ultra immunostainer.

morphology preservation and antigenicity in heavily pigmented tissue specimens.

MATERIALS AND METHODS

Tissue Samples

Pigmented tissue specimens representative of primary cutaneous melanoma (n = 6), uveal melanoma (n = 3), lymph node melanoma metastasis (n = 3), pigmented epithelioid melanocytoma (n = 3), containing abundant melanin pigment, were selected for the purpose of the study. Hematoxylin and eosin stained sections were reviewed to assess pathology tissue quality control.

IHC

Representative 3-μm thick formalin-fixed paraffin-embedded tissue sections were selected for IHC analysis. Sample processing was performed with automated immunostainer (Ventana Discovery ULTRA; Ventana Medical Systems). The sections were deparaffinized in EZ prep (950–102; Ventana Medical Systems), and antigen retrieval was achieved by incubation with cell-conditioning solution 1 (950–124; Ventana Medical Systems). After these 2 initial steps, bleaching treatment was applied as follows: sections were pretreated with 0.5% H_2O_2 phosphate buffered saline pH 7.4 or with 0.5% H_2O_2 Tris base pH 10 solution for 4, 8, 12, 15, and 30 minutes at 80°C. Sections were then incubated with the following primary antibodies: antimelanosome HMB45 (#790-4366 mouse monoclonal, clone HMB45, ready to use; Ventana Medical Systems) anti-MART-1/MelanA (#790-2990, mouse monoclonal, clone A103, ready to use; Ventana Medical Systems) anti-Ki-67 (#790-4286 rabbit monoclonal, clone 30-9, ready to use; Ventana Medical Systems) anti-SOX10 (#760-4968, rabbit monoclonal, clone SP267, ready to use; Ventana Medical Systems), anti-S100 (#790-2914, mouse monoclonal, clone 4C4.9, ready to use; Ventana Medical Systems), anti-Tyrosinase (#790-4365, mouse monoclonal, ready to use; Ventana Medical Systems) and anti-BRAF V600E (#790-5095, mouse monoclonal, clone VE1, ready to use; Ventana Medical Systems) (Fig. 1). For IHC, the signal was developed with UltraMap Red or Chromomap DAB antimouse or antirabbit detection kit (Ventana Medical Systems). For multiplex IHC, we combined our bleaching procedure with the previously described protocols to obtain two different double staining for KI67/BRAF V600E and SOX10/BRAF V600E. The signal was developed with the UltraMap Red detection kit and Discovery Green HRP RUO detection kit (Ventana Medical Systems). Sections were counterstained with hematoxylin. Stained tissue sections were digitally scanned at ×400 magnification with Aperio AT2 platform (Leica Biosystems, Wetzlar, Germany).

RESULTS

Schematic representation of automated bleaching protocols used are shown in Figure 1.

To investigate the efficacy of automated bleaching protocol, IHC staining with HMB45 was performed using 15 and 30 minutes of pretreatment with the two different buffers

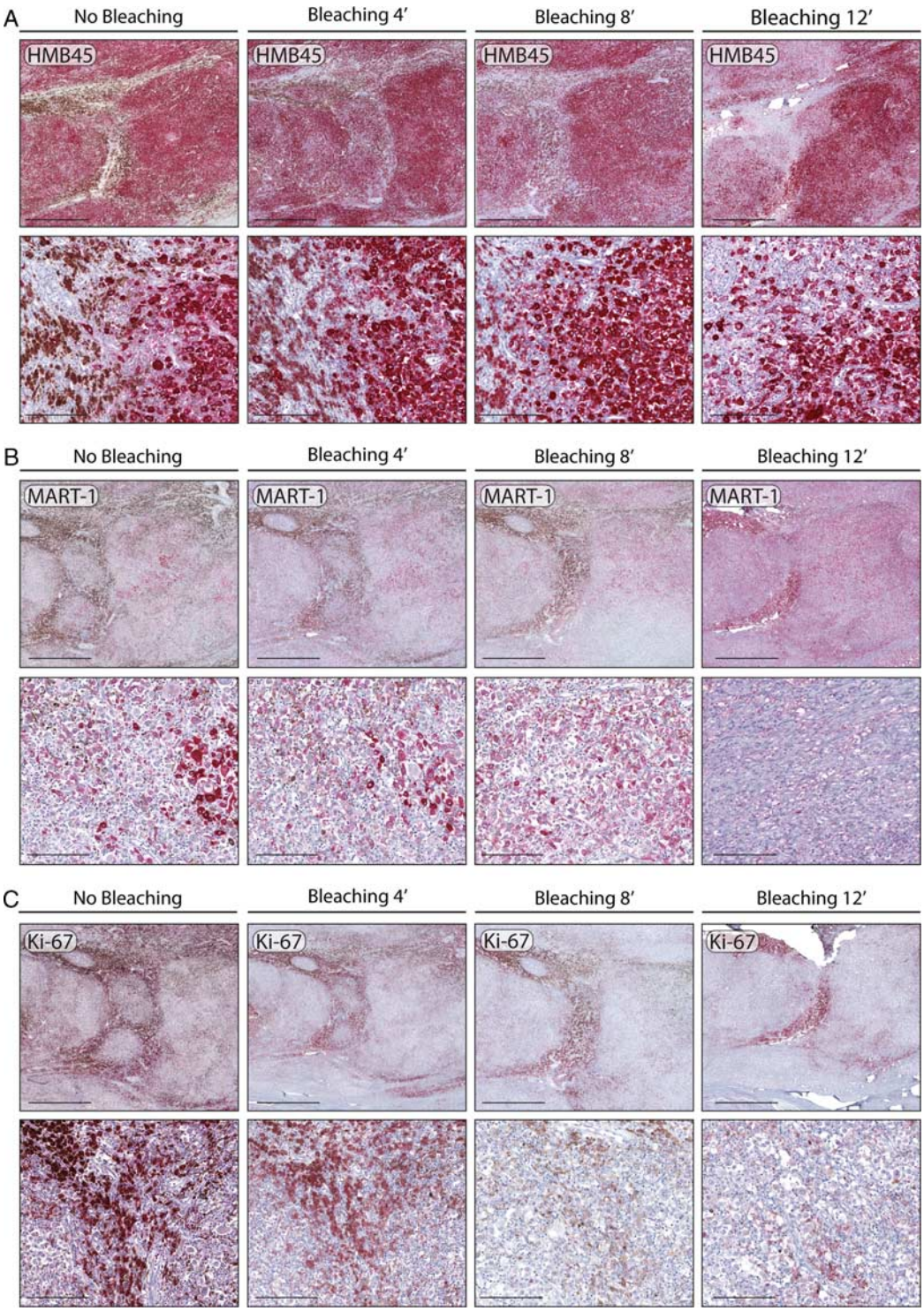

FIGURE 2. Effects of different bleaching treatment time on protein immunogenicity. Representative immunohistochemistry of a pigmented melanoma tissue for HMB45 (A), MART-1 (B) and KI-67 (C) after 4′, 8′, and 12′ of bleaching. Magnification ×20, inset ×200 (scale bars: 1 mm, inset 100 μm).

described (Fig. 1A). Signal was developed using UltraMap Red or Chromomap DAB detection kit. The 0.5% H_2O_2 phosphate buffered saline pH 7.4 has a reduced bleaching efficacy in comparison to 0.5% H_2O_2 Tris base pH 10 protocol, both at 80°C 15 minutes of treatment (Fig. 1B). Furthermore, we compared the results obtained with the 2 different chromogens, Red and DAB. As shown in Figure 1B Red chromogen allowed a better contrast for better IHC visualization, reducing the risk of improper interpretations caused by the residual melanin pigment. Although we obtained a satisfying bleaching effect using 0.5% H_2O_2 Tris base pH 10 solution for 15 minutes (Fig. 1B) and 30 minutes (data not shown, complete loss of antigenicity), this pretreatment turned out to be too aggressive for tissues, changing the cytoarchitecture and reducing the antigenicity. For these reasons we create 3 additional experimental protocols for bleaching pretreatment (4, 8, and 12 min) (Fig. 1C).

Indeed, in order to assess the quality of staining and the morphologic and cytoarchitectural tissue details, IHC staining with HMB45, MART-1, and Ki-67 antibodies in different pigmented tissue specimens by using 4, 8, and 12 minutes of time of pretreatment with bleaching protocol, was performed (Fig. 2). Results showed that the pretreating of slices with 0.5% H_2O_2 Tris base pH 10 solution at 80°C reached optimal performance at 8 minutes of treatment. In fact, pretreatment at 8 minutes achieved optimal bleaching by reducing the melanin content without affecting the tissue cytoarchitecture (Fig. 2). Different times yielded opposite results: 4 minutes bleaching was not effective, resulting in a persistently high content of melanin; conversely, after 12 minutes bleaching, severe tissue morphologic and cytoarchitecture alterations appeared, thus affecting the interpretation of IHC results (Fig. 2).

To estimate the protein conservation levels after bleaching procedures, IHC for HMB45, MART-1, KI-67, SOX10, S100, tyrosinase, and BRAF V600E was performed. Representative staining of pigmented tissues is shown in Figure 3. Data showed that antigenicity was highly conserved for nuclear antigens KI-67 and SOX10

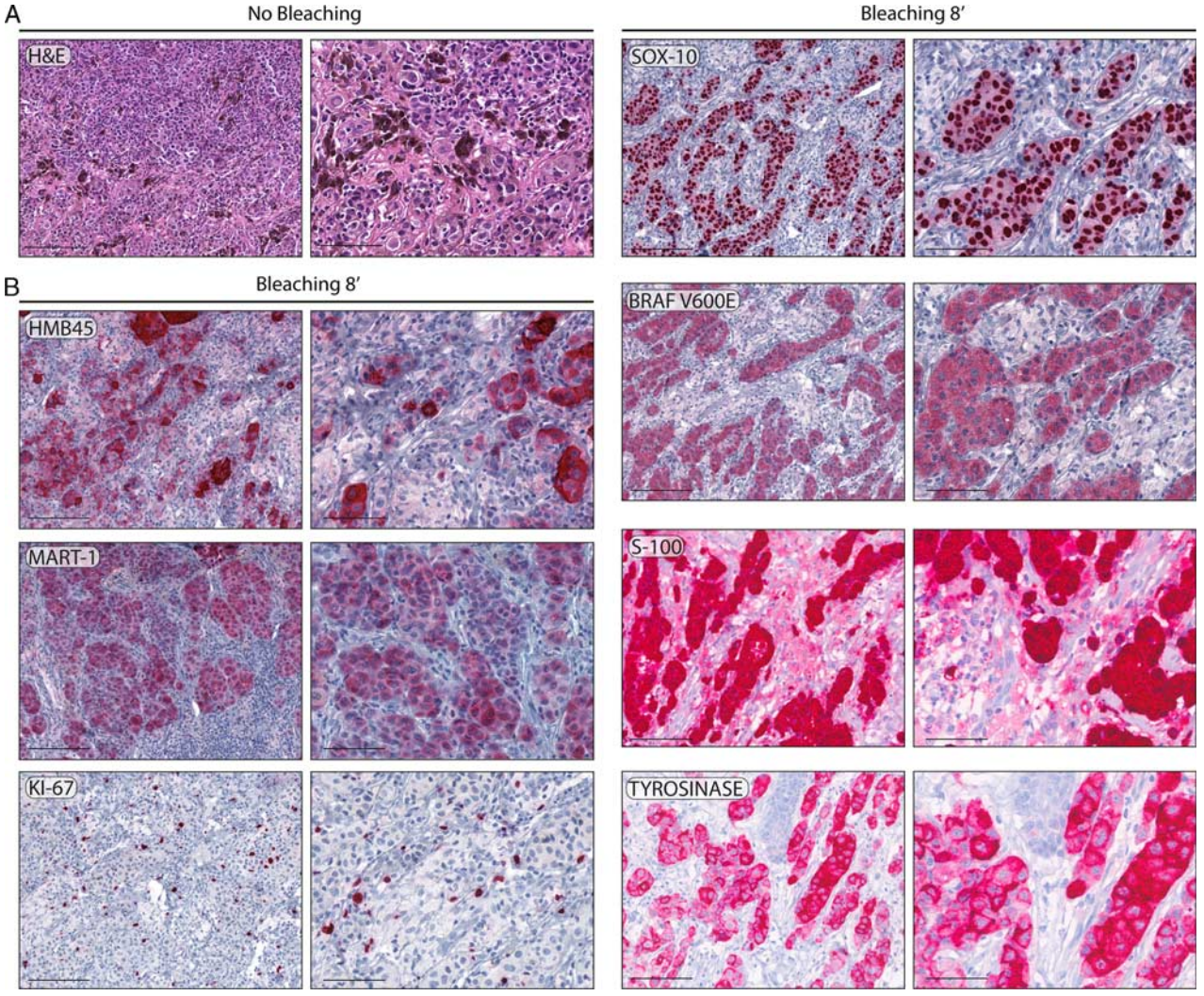

FIGURE 3. Representative photomicrographs reporting hematoxylin and eosin of pigmented melanoma tissue (A) and immunohistochemistry for HMB45, MART-1, KI-67, SOX10, S-100, Tyrosinase, and BRAF V600E after 8′ of bleaching (B). Magnification ×200, inset ×400 (scale bars: 100 μm, inset 50 μm).

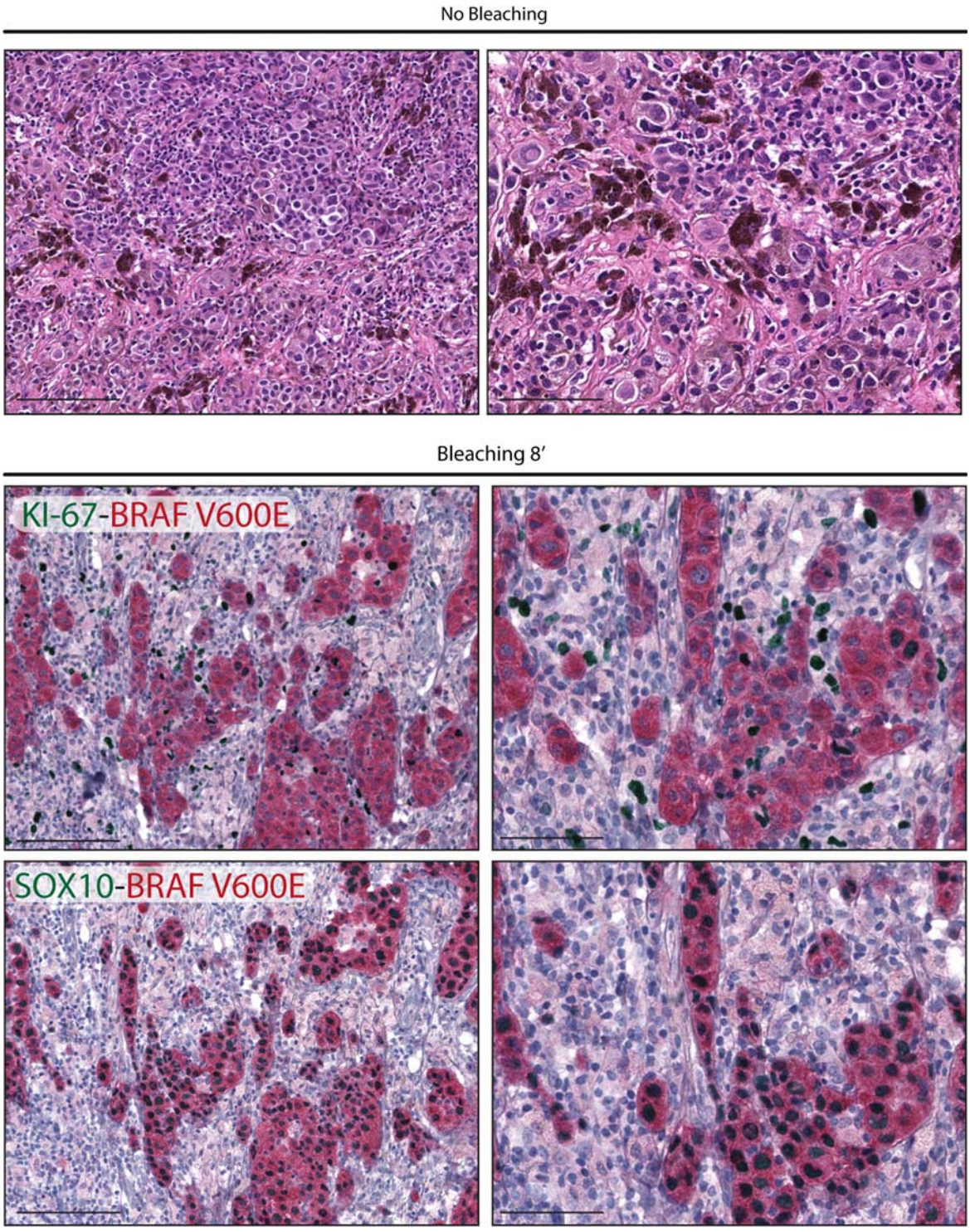

FIGURE 4. Representative multiplex immunohistochemistry for KI-67/BRAF V600E and SOX10/BRAF V600E after 8′ of bleaching. Magnification ×200, inset ×400 (scale bars: 100 μm, inset 50 μm).

revealing a clear staining with no loss of specificity and intensity (Fig. 3). However, cytoplasmatic antigens HMB45, MART-1, and BRAF V600E were more affected by the bleaching procedure, showing a slight loss of intensity with paler staining compared with samples tested without bleaching. Cytoplasmatic staining for S-100 and tyrosinase maintain high specificity and intensity (Fig. 3).

Finally, we applied the bleaching method to a multiplex IHC protocol. Representative multiplex staining of a pigmented melanoma analyzed for KI-67/BRAF and

SOX10/BRAF is shown in Figure 4. Although the multiplex protocol included additional steps that further affected tissue stability, the bleaching method described here did not affect the results of multiple staining.

DISCUSSION

Bleaching techniques facilitate pathologists in histopathologic examination of tissue samples with abundant melanin pigment.[10–14] Several manual and semiautomated protocols have been proposed, including: (i) potassium permanganate (or potassium dichromate) followed by oxalic acid ($KMnO_4$/oxalate), which is faster (1 h), but reduces antigenicity in IHC; (ii) diluted hydrogen peroxide (3% or 10% H_2O_2 or 10% H_2O_2 in PBS at 60°C); and (iii) trichloroisocyanuric acid (TCCA) for 30 minutes after IHC (Benchmark XT).[9,10,14]

In this study, we describe a simple and completely automated method for melanin bleaching, with the specific purpose of translating this procedure into routine diagnostic use. For this reason, based on the protocols recommended we decided to automate and standardize the use 0.5% H_2O_2 Tris base pH 10 as bleaching buffer. This because it guaranteed an excellent compromise between bleaching efficacy, processing time and antigenic conservation compared with the other proposed buffers.[10–14]

In contrast with previously reported bleaching methods,[11] this procedure was designed to translate bleaching techniques into a fully automated system, the Ventana Discovery ULTRA (Ventana Medical Systems). Although we have used 0.5% H_2O_2 Tris base pH 10 solution as a buffer for bleaching, as reported by Chung and colleagues, we introduced the use of the red chromogen, which, in our experience, provides better results in comparison with the chromogen DAB. Our results show that red chromogen coupled with bleaching treatment allowed a clear contrast for IHC evaluations. The use of this chromogen may reduce the risk of improper interpretations, which have resulted from DAB chromogen intensifying the color of residual melanin pigment.[12] A limitation of our methodology could be the partial melanin removal that we found in a few very heavily pigmented lesions, such as uveal melanomas.

A point of strength of our procedure is the total automation of the process.[14] Compared with previous studies that used a manual or semiautomated bleaching protocol,[10–13] in the present study we used a more standardized automated IHC, making the method easier and faster to use, also eliminating variability because of manual steps. The use of the proposed bleaching protocol did not affect the nuclear antigens, showing univariate staining intensity of no bleaching samples. Although a slight decrease in staining intensity was observed for cytoplasmatic antigens, this mild reduction did not compromise the IHC evaluations. Furthermore, the bleaching protocol made both the nuclear and cytoplasmatic staining more visible.

A point weakness of the study could be represented by the limited number of samples tested. This limitation is because of the difficult availability of tissues, furthermore, RNA and DNA degradation caused by bleaching technique, could represent a criticism for in situ hybridization and DNA/RNA scope assays. Nevertheless, the primary purpose of our study was to completely automate and improve a methodology that has been already validated manually,[11] in addition, the automatic bleaching pretreatment is directly coupled with IHC process preventing further changes to the protocol.

Moreover, we validated for the first time the bleaching protocol for VE1 (BRAF V600E) antibody, which is a fundamental IHC screening test for differential diagnosis of melanocytic tumors as well as predictive purposes in melanoma patients. By using a double staining protocol, we showed well-preserved staining for BRAF V600E and the Ki-67/SOX10, together with preservation of tissue and cytomorphology integrity.

In conclusion, we propose a new, fast automated bleaching protocol easily transferable to a routine setting with efficient results in specimens in which the melanin pigmentation could blunt the histopathologic examination, thus leading to a misclassification of melanocytic lesions and inaccurate screening of melanoma patients for predictive purposes.

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