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Melanin Bleaching with Warm Hydrogen Peroxide and Integrated Immunohistochemical Analysis: An Automated Platform

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Running Title: Melanin bleaching and immunohistochemical of automated platform

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Abstract

Diagnosing melanocytic lesions is among the most challenging problems in the practice of pathology. The difficulty of physically masking melanin pigment and the similarity of its color to commonly used chromogens often complicate examination of the cytomorphology and immunohistochemical staining results for tumor cells. Melanin bleach can be very helpful for histopathological diagnosis of heavily pigmented melanocytic lesions. Although various depigmentation methods have been reported, no standardized methods have been developed. This study developed a fully automated platform that incorporates hydrogen peroxide-based melanin depigmentation in an automated immunohistochemical analysis. The utility of the method was tested in one cell block of malignant melanoma cells in pleural effusion, ten ocular melanoma tissue samples, and ten cutaneous melanoma tissue samples. Our results demonstrated that the proposed method, which can be performed in only 3 hours, effectively preserves cell cytomorphology and immunoreactivity. The method is particularly effective for removing melanin pigment to facilitate histopathological examination of cytomorphology and for obtaining an unmasked tissue section for immunohistochemical analysis.

Keywords: melanin bleaching; immunohistochemistry; automated platform

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1. Introduction

Melanin is a naturally occurring complex oligometric material composed of various monomers that are the oxidation and tautomerization products of DOPA (dihydroxyphenylalanine) derived from the amino acid tyrosine[1, 2]. This pigment is typically brown-black (eumelanin), and the extent of melanin pigmentation differs by geographic region. By physical masking effect on cellular morphology, excessive melanin pigment can complicate histopathologic assessments of melanocytic lesions[3, 4]. Melanin pigment in tissues is brown-to-black and granular, and its distribution in tissues is often uneven[5]. The presence of these pigments can complicate analysis of immunohistochemical staining results for two reasons[6]. First, they have a direct physical masking effect on antigen-antibody interactions. Second, 3-3-diaminobenzidene (DAB), the most commonly used chromogen for demonstrating antigen/ antibody reactions, has a brown color that is difficult to distinguish from melanin pigments DAB[6]. Some laboratories use azure B melanin counterstaining, which can distinguish melanin granules from DAB chromogen[7, 8]. Immunoproducts with DAB are brown whereas melanin granules are green-blue. However, physical masking by excess melanin granules is still problematic in this approach.

Several melanin-bleaching methods have been developed to enable analysis of heavily pigmented melanocytic lesions. The two most popular bleaching methods are treatment with potassium permanganate method followed by oxalic acid ($KMnO_4$/oxalate)[5, 7, 9, 10] and treatment with dilute hydrogen peroxide (H_2O_2) (3% or 10% H_2O_2) [6, 11-14]. However, each bleaching method has disadvantages. For example, although the KMnO4/ oxalate bleaching method is faster (it can be performed in 1 hour) and is easier to incorporate in conventional daily immunostaining protocols, it reduces the antigenicity of some antigens used in IHC procedures[11]. In contrast, the dilute H_2O_2 method does not reduce antigenicity, but the process of bleaching melanin pigments at room temperature is relatively prolonged [11-14]. To achieve depigmentation without interfering with antigen-antibody reactivity, modified melanin bleaching methods using warm 10% H_2O_2 have been developed [6, 15, 16].

Orchard [6] reported that using 10% H_2O_2 in phosphate-buffered saline (PBS) at 60°C can reduce the duration of the bleaching procedure to 150 minutes. Another method reported by our research group [15] showed that elevating the temperature to 65°C reduces the reaction time to 30 min. In both of these studies, the proposed procedural modifications shorten the bleaching time without affecting antigenicity or cellular integrity. Notably, however, these methods require the bleaching step to be performed manually, and the procedures are difficult to standardize due to the variation in technical skill.

Therefore, this study developed a rapid, effective and fully automated melanin bleaching method that preserves tissue morphology and antigenicity and minimizes technical variation. Another objective was to integrate bleaching and immunostaining in the same platform.

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2. Results

2.1 Effective melanin bleaching of heavily pigmented cutaneous and ocular melanomas on automated platform

Melanin bleaching was successfully achieved in all heavily pigmented cutaneous and ocular melanomas (Table 1). The obscuring effect of melanin pigment was completely eliminated or at least minimized. The tissue integrity and cytomorphology were well preserved after automated bleaching for up to 40 minutes. At 30 minutes, melanin was completely removed in three cases of orbital melanoma and in eight cases of cutaneous melanoma. In the remaining cases, melanin bleaching was successfully achieved at 40 minutes. At 50 minutes, slight interference of the cytomorphology including suboptimal H&E staining and disrupted cytoplasmic details was observed in three cases. Therefore, we conclude that a 40-minute duration of automated bleaching with 10% hydrogen peroxide at 65 °C is optimal for both cutaneous and ocular melanomas with heavy melanin pigmentation (Fig.1).

Table 1. Determination of the optimal time for H_2O_2 in melanin bleaching

	Duration of incubation time (min)		
	30	40	50
Cutaneous melanoma	8*	10*	10*
Orbital melanoma	3*	10*	10*

*The number depicts cases with complete depigmentation

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Figure 1

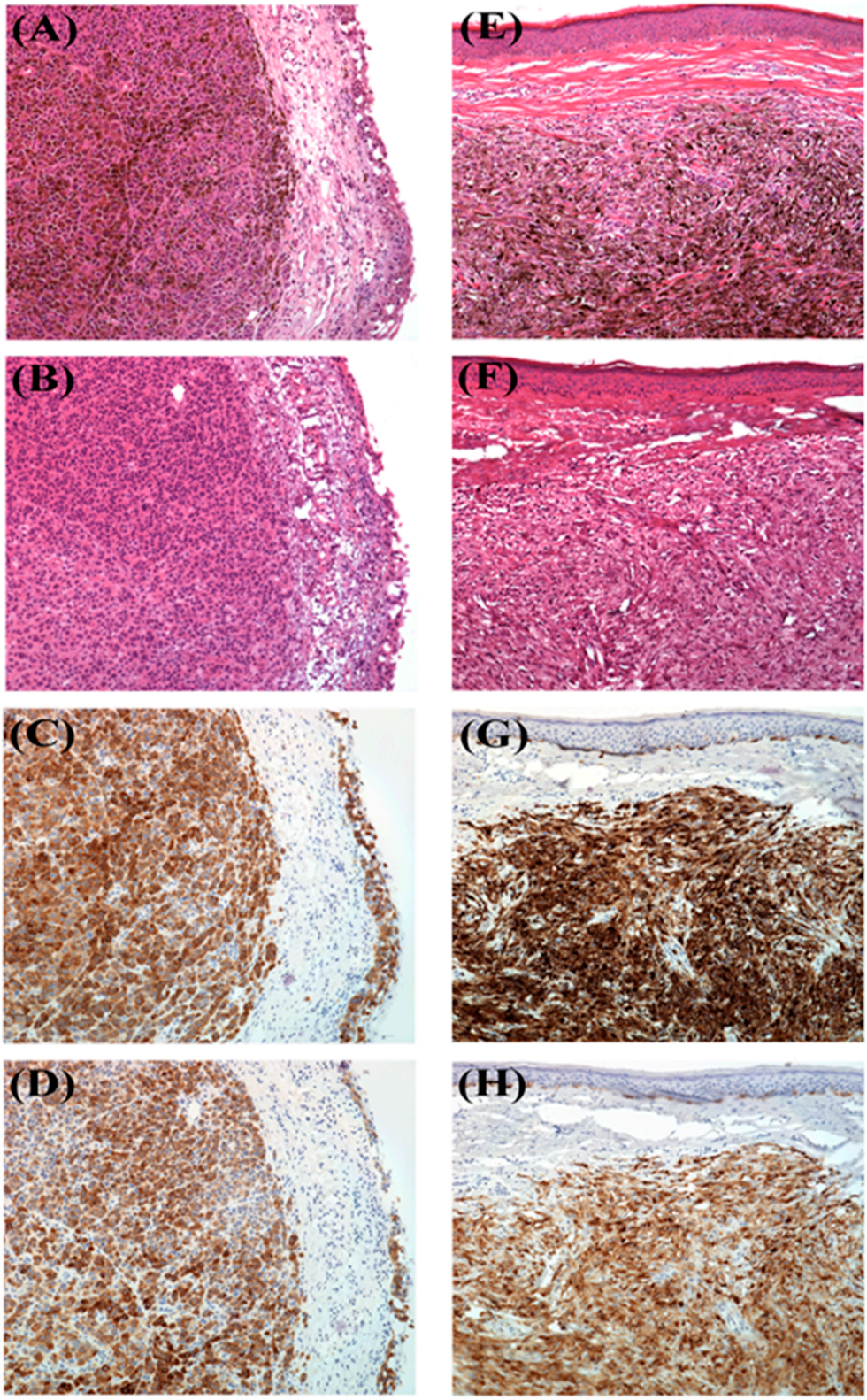

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Figure 1. Heavily pigmented ocular (A, B , C, D)and cutaneous melanomas (E, F, G, H) bleached using warm H2O2 protocol by the BOND ™ Polymer Refine Detection (original magnification, ∗100). A, B, E and F were hematoxylin and eosin stain. C, D, G and H were Melan-A stain. A, C, E and G without melanin bleach. B, D, F and H were melanin bleach. A, B, C and D were ocular melanoma. E, F, G and H were cutaneous melanoma.

2.2 Immunoreactivity of heavily pigmented cutaneous and ocular melanomas after automated melanin bleaching

After optimizing the duration of automated melanin bleaching for depigmentation of heavily pigmented cutaneous and ocular melanomas, immunohistochemical staining of Melan-A was integrated in the automated protocol. Starting with an unstained section from FFPE, both melanin depigmentation and Melan-A immunohistochemistry were completed in a single 3-hour automated protocol. In all test cases, Melan-A immunostaining showed strong and diffuse immunoreactivity, which suggested that immunoreactivity was unaffected by the automated bleaching protocol (Fig. 1).

2.3 Automated melanin bleach and Melan-A immunohistochemitry for cell block preparation from malignant pleural effusion of melanoma

To test the utility of the integrated protocol for melanin bleaching and immunohistochemistry, one example of cell block preparation from malignant pleural effusion of melanoma was subjected to the automated protocol. Melanin depigmentation and Melan-A were successfully performed with no negative effects on morphological detail or immunoreactivity at 20 min of bleaching. The metastatic tumor cells were highlighted by immunoreactivity to Melan-A and were easily identifiable among the reactive mesothelial cells. Therefore, we conclude that the automated melanin bleach protocol is applicable for cell block preparation (Fig. 2).

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Figure 2

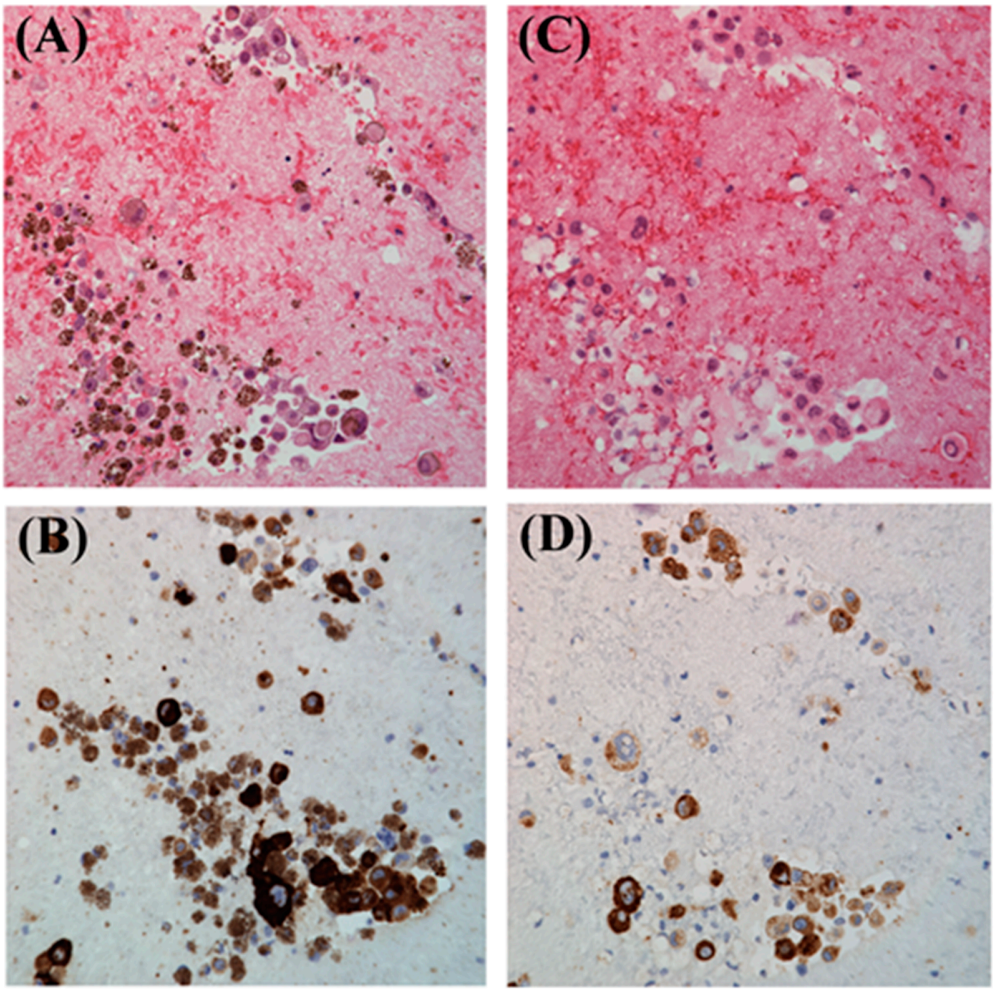

Figure 2. Cell block specimen melanin bleaching using warm H2O2 protocol by the BOND ™ Polymer Refine Detection (original magnification, ∗200). A and C were hematoxylin and eosin stain. B and D were Melan-A stain. A and B without melanin bleaching. C and D were melanin bleaching.

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3. Discussion

In pathology practice, melanocytic lesions may be difficult to diagnose and classify because they are often obscured by melanin pigment and because the cytomorphological details may be masked during the histopathological examination. Physical masking and misidentification as a chromogen currently used in immunohistochemistry are additional problems of diagnosing melanocytic lesions. Therefore, depigmentation of melanin is a potentially useful technique for heavily pigmented melanocytic lesions.

To the best of our knowledge, however, melanin bleaching methods developed to date require manual handling and lack standardization. Some protocols require up to 24 hours to complete, and some cause loss of immunoreactivity [14]. We previously demonstrated that 10% hydrogen peroxide at an elevated temperature can be used for manual bleaching of melanin pigment of cutaneous melanomas in 30 minutes. This study is the first to evaluate the use of an automated protocol for using warm hydrogen peroxide for melanin depigmentation integrated with IHC staining. The entire process can be performed in a single scheduled protocol starting with an unstained slide freshly cut from an FFPE block or cell block. The entire protocol can be performed in about 3 hours. For histopathological examination, the bleached slide can also be retrieved without immunostaining and stained using the standard H&E protocol. That is, bleached H&E staining and IHC staining of heavily pigmented melanocytic lesions can be completed in a single day. The automated platform was based on a platform originally designed for automated immunohistochemical staining but with some additional heating and washing steps for depigmentation. We assume that this minor modification can also enable use of the proposed protocol in other commercially available immunostaining platforms.

The proposed automated melanin bleaching system has several notable advantages. First, the system can be standardized to reduce manual handling that can cause variations in results obtained by different technicians. Another advantage is that the duration of warm hydroxide bleaching can be easily adjusted and controlled to meet the specific requirements of various tissue samples and the density of melanin pigment. Notably, the experiments in this study indicate that ocular melanomas may require a longer duration of bleaching compared to cutaneous melanomas. Increasing the duration of bleaching may reduce tissue integrity, which would affect the histopathological examination results. We strongly suggest another H&E stain accompanied with immunohistochemical stain using the same bleaching duration to evaluate the effectiveness of depigmentation and the preservation of tissue integrity and cytomorphology.

A noted limitation of this study is the sample size, which may not represent the full spectrum of melanin pigmentation density. Since the analysis did not consider melanocytic tissues other than eye and skin tissues, the optimal conditions for other sites require further analysis. Our previous study also demonstrated that the thermal conductivity of the container may affect the effectiveness of warm hydrogen peroxide bleaching [15]. Similarly, the bleaching conditions applied in our previous study (10% H_2O_2 in PBS for 30 min at 65 °C) may have decreased in terms of the effectiveness of the bleaching procedures in this study (100% vs. 80%, respectively). The variation assumedly resulted from differences between manual and automated procedures and from differences in thermal conductivity. Therefore, the bleaching duration should be adjusted

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for different tissues and for different automated systems. Further validation in clinical cases is also needed before routine clinical application in individual laboratories.

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4. Material and methods

4.1 Tissue samples and slide preparation

Twenty-one samples of heavily pigmented melanocytic lesions, including ten cutaneous melanomas, ten ocular melanomas, and one cell block of malignant pleural effusion from cutaneous melanoma were obtained from the tissue archives at our institute. After reviewing representative specimens, formalin-fixed paraffin-embedded (FFPE) blocks of tissues and cell block containing heavily pigmented melanoma tumor cells were selected. All tissue acquisitions were approved by the Institutional Review Board of Kaohsiung Medical University hospital (KMUH-IRB-20140183).

Serial 3μm-thick sections were adhered to glass microscope slides (DAKO, Denmark) by oven heating at 65°C for 2 hours. All slides were bleached and/or stained within 24 hours after preparation. The un-bleached slides were dewaxed in xylene before rehydration with graded alcohols, rinsed with running tap water, and stained with hematoxylin and eosin (H&E).

4.2 Procedure for melanin bleaching with dilute H_2O_2 and assessment of optimal diluent

A modified melanin bleaching technique was performed using 10% warm hydrogen peroxide (H_2O_2) (30%)(SIGMA-ALDRICH) diluted with PBS(0.05M,ph7.4)(SIGMA-ALDRICH) at 65°C in the BOND ™ Polymer Refine Detection system as described in our previous study[15]. Figure 3 shows a flowchart of the procedure for optimizing the parameters for the ten cutaneous melanomas, ten ocular melanomas, and one cell block of metastatic pleural effusion from cutaneous melanoma.

Figure 3

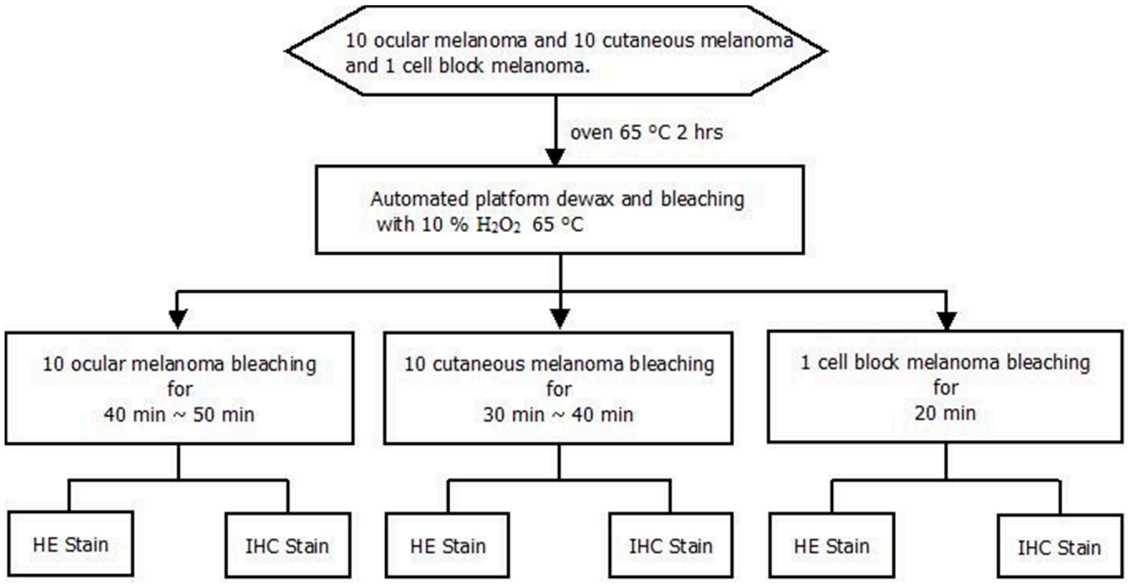

Figure 3. Flow-chart shows the procedure for optimizing the parameters for the ten cutaneous melanomas, ten ocular melanomas, and one cell block.

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The Automatic Staining Module, including heating steps, was modified for use in melanin bleaching. The steps of the module were as follows: (1) Bond Dewax deparafinization solution for 1.5 min at 72°C; (2) 95% alcohol for 1.5 min; (3) Bond Wash solution for 5 min at 35°C; (4) 10% H_2O_2 for 20, 30, 40 or 50 min at 65°C to determine the optimal duration for bleaching; (5) Bond Wash solution for 5 min; (6) deionized water for 5 min. The following durations of melanin depigmentation were tested: tissue blocks: 30, 40, 50 min; cell block: 15, 20 min. The slides were then subjected to H&E staining or immunohistochemical analysis.

4.3 Immunohistochemistry

Immunostaining was automatically performed in the BOND ™ Polymer Refine Detection Automated Staining Module. The steps of the module were as follows: (1) Bond Demax deparaffinization solution for 1.5 min at 72°C; (2) 95% alcohol for 1.5 min; (3) Bond Wash solution for 5 min at 35°C; (4) Bond ER 2 solution for 30min at100°C; (5) Bond Wash solution for 4.5 min at 35°C; (6) Melon-A (DAKO, Denmark) for 15min; (7) Bond Wash solution for 2 min; (8) post primary for 8 min; (9) Bond Wash solution for 6 min; (10) polymer for 8 min; (11) Bond Wash solution; (12) Peroxide block (3%H_2O_2) for 5 min; (13) Bond wash solution; (14) Mixed DAB Refine for 10 min; (15) deionized water for 2 min ; (16) hematoxylin for 5 min; (17) deionized water for 2 min.

In conclusion, the fully automated platform developed in this study integrates melanin depigmentation with automated immunohistochemistry in a single protocol that can be completed in only 3 hours, and cytomorphology and immunoreactivity are well preserved. The use of this method for rapid and efficient removal of melanin pigment facilitates histopathological examination of cytomorphology and provides an unmasked tissue section for immunohistochemistry. Although this study indicated that the method is applicable in ocular and cutaneous melanocytic lesions, its use in other sites requires further study and evaluation.

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Author Contributions

Chia-Hsing Liu and Kun-Bow Tsai conceived and designed the experiments; Chia-Hsing Liu and Chih-Hung Lin performed the experiments; Chia-Hsing Liu, Sheau-Fang Yang, and Kun-Bow Tsai analyzed the data; Yu-Hsuan Chen and Sheau-Fang Yang contributed reagents/materials/analysis tools; Chih-Hung Lin wrote the paper; Chia-Hsing Liu and Min-Jan Tsai helped to complete the manuscript; Kun-Bow Tsai as well as all authors read and approved the final manuscript.

Conflict of Interest

The authors have no conflict of interest to declare.

C.-H.L. and C.-H.L. contributed equally.

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Legends

Figure 1. Heavily pigmented ocular (A, B , C, D)and cutaneous melanomas (E, F, G, H) bleached using warm H2O2 protocol by the BOND ™ Polymer Refine Detection (original magnification, ∗100). A, B, E and F were hematoxylin and eosin stain. C, D, G and H were Melan-A stain. A, C, E and G without melanin bleach. B, D, F and H were melanin bleach. A, B, C and D were ocular melanoma. E, F, G and H were cutaneous melanoma.

Figure 2. Cell block specimen melanin bleaching using warm H2O2 protocol by the BOND ™ Polymer Refine Detection (original magnification, ∗200). A and C were hematoxylin and eosin stain. B and D were Melan-A stain. A and B without melanin bleaching. C and D were melanin bleaching.

Figure 3. Flow-chart shows the procedure for optimizing the parameters for the ten cutaneous melanomas, ten ocular melanomas, and one cell block.