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A melanin-bleaching methodology for molecular and histopathological analysis of formalin-fixed paraffin-embedded tissue

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Abstract

Removal of excessive melanin from heavily pigmented formalin-fixed paraffin-embedded (FFPE) melanoma tissues is essential for histomorphological and molecular diagnostic assessments. Although there have been efforts to address this issue, current methodologies remain complex and time-consuming, and are not suitable for multiple molecular applications. Herein, we have developed a robust and rapid melanin-bleaching methodology for FFPE tissue specimens. Our approach is based on quick bleaching (15 min) at high temperature (80 °C) with 0.5% diluted hydrogen peroxide (H₂O₂) in Tris-HCl, PBS, or Tris/Tricine/SDS buffer. Immunostaining for Ki-67 and HMB45 was enhanced by bleaching with 0.5% H₂O₂ in Tris/Tricine/SDS and Tris-HCl, respectively. In addition to histopathological applications, our approach also facilitates recovery of protein and nucleic acid from archival melanin-rich FFPE tissue sections. Protein extracted from bleached FFPE tissues was compatible with western blotting using anti-human GAPDH and AKT antibodies. Our bleaching condition significantly improved RNA quality compared with unbleached tissues without compromising the yield. Notably, the RNA/DNA obtained from

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

bleached tissues was suitable for end point PCR and real-time quantitative RT-PCR. In conclusion, this improved melanin-bleaching method enhances and simplifies immunostaining procedures, and facilitates the use of melanin-rich FFPE tissues for histomorphological and PCR amplification-based molecular assays.

Melanin is a complex and highly heterogeneous polymer of tyrosine derivatives that is primarily found in skin and hair, and provides protection against sunburn from UV-B radiation. Although melanin is beneficial in providing protection from harmful radiation, it presents significant challenges in biomedical applications. Melanin hinders histological assessment of melanocytic lesions by obfuscating morphology and negatively impacts immunohistochemical analysis of melanin-containing tissue samples by direct physical masking of antibody-antigen interactions.¹⁻³ Moreover, melanin pigment is very similar to the biproducts of 3,3'-diaminobenzidine (DAB), a commonly used chromogen for visualizing antigen-antibody reactions.^{2,4,5}

Other problems in the analysis of tissues containing melanin arise as a result of melanin's capacity to absorb a wide range of UV radiation, which can compromise RNA/DNA analysis by interfering with the photometric quantitation of nucleic acids.⁶ Furthermore, melanin also inhibits polymerase chain reactions (PCR) by binding to thermostable DNA polymerase, making amplification of isolated nucleic acids more difficult.⁶⁻⁸ Challenges in analyzing melanin-containing tissues have led to the development of several techniques to remove melanin from heavily melanin-pigmented tissue samples.

To date, two major melanin-bleaching methods have been widely utilized in histological and immunohistochemical analyses. These two methods, using either KMnO_4 /oxalic acid or dilute H_2O_2 with buffer, have certain advantages and disadvantages. Bleaching methods using KMnO_4 /oxalic acid are generally faster than those using dilute H_2O_2 ; however, many of the methods using KMnO_4 /oxalic acid tend to cause deterioration of tissue integrity.^{2,4,9} In contrast, H_2O_2 bleaching methods usually require > 1 day of incubation at room temperature. Although incorporation of heat during bleaching procedures significantly reduces the bleaching time in some H_2O_2 methods^{5,10}, incubation time with H_2O_2 is still relatively long (30–150 min). Furthermore, prior studies of bleaching methodologies exclusively focused on histological and immunohistochemical analysis, and did not address the quantity and quality of protein, RNA, and DNA for further use in molecular assays.⁹⁻¹² Therefore, development of a bleaching method that is compatible with both molecular profiling and histological diagnosis is needed.

In the present study, we developed a quick bleaching method using dilute H_2O_2 and high temperature, and we carefully evaluated different buffers (Tris-HCl, PBS, and Tris/Tricine/SDS) to find conditions that allow accurate histological diagnosis and yield high-quality/-quantity protein, RNA, and DNA. Biomolecules prepared with this method are compatible with western blotting and PCR analyses, which have been highly challenging for archival FFPE tissues containing melanin.

MATERIALS AND METHODS

Tissue Specimens

FFPE tissues were obtained anonymously and randomly from the Cooperative Human Tissue Network, and approved for use in research by the Office of Human Subjects Protection of the National Institutes of Health. Fifteen different tumor specimens were utilized, including melanomas that contained abundant melanin, as well as cases that lacked obvious melanin on H&E. All specimens had been fixed in neutral-buffered formalin and stored for at least 10 years at room temperature.

Melanin Bleaching

A schematic diagram of the experimental study design of the presented study is shown in Figure 1. To examine the effects of different melanin-bleaching methods, four 5 μ m-thick FFPE serial tissue sections were cut from each of three different tumor specimens for each condition, adhered to positive-charged slides, and baked for 1 h at 60 °C. We investigated the use of dilute H₂O₂ with different buffers, temperatures, and times. We also tested pH effects of the conditions using human melanoma FFPE tissues. Dilute H₂O₂ solutions (0.1, 0.5, or 1.0%) in 1 $\times$ Tris-HCl (pH 10; Sigma-Aldrich, St. Louis, MO, USA), 1 $\times$ phosphate-buffered saline (PBS, pH 7.4; Lonza, Walkersville, MD, USA), or 1 $\times$ Tris/Tricine/SDS (pH 8.2; Sigma-Aldrich) buffer were added to each plastic coplin jar containing three FFPE slides and incubated for 2, 5, 10, 15, or 30 min at 80 °C. The pH effect for melanin bleaching in 0.5% dilute H₂O₂ with Tris-HCl buffer (pH 4, 7, or 10) was separately tested. For each condition, four melanin pigment-rich FFPE slides from each of three tumor specimens were placed into plastic coplin jars with diluted and buffered H₂O₂, and incubated with a steamer (Black & Decker, Towson, MD, USA) for variable periods of time. After incubation, the slides were removed, placed in a slide rack and washed in 1 $\times$ PBS for 3 min and left to air dry. When completely dry, the samples were scraped into microcentrifuge tubes and analyzed for protein, RNA, or DNA. After comprehensive analysis of various factors in melanin bleaching, the fully optimized protocol used 0.5% dilute H₂O₂ with Tris-HCl buffer (pH 10), PBS (pH 7.4), or Tris/Tricine/SDS (pH 8.2), and incubated for 15 min at 80 °C.

Histochemical and Immunohistochemical Evaluation

Immunohistochemical staining was performed on four 5 μ m-thick FFPE tissue sections from each of three different tumor specimens. After melanin bleaching, heat-induced antigen retrieval was performed for 20 min in an antigen-retrieval buffer of pH 6.0 (for Ki-67 and HMB45; Dako, Carpinteria, CA, USA) using a pressure cooker (Pascal, Dako). The endogenous peroxidase activity was blocked with 3% H₂O₂ (Dako) for 10 min with additional protein blocking (Dako) for 15 min to minimize non-specific staining. The slides were incubated with mouse anti-Ki-67 monoclonal antibody (Clone # MIB-1; dilution 1:250; Dako) or mouse anti-HMB45 monoclonal antibody (Clone # HMB45; dilution 1:100; Abcam, Cambridge, MA, USA) for 1 h at room temperature. Appropriate controls using an isotype-matched antibody instead of the primary antibody were performed. The antigen-antibody reaction was detected using a DAKO Envision⁺ peroxidase kit and visualized with

3,3-diaminobenzidine (Dako). Slides were lightly counterstained with hematoxylin, dehydrated in a graded ethanol series, cleared in xylene, and covered with coverslips.

The stained sections were digitized using the NanoZoomer 2.0 HT (Hamamatsu Photonics K.K., Japan) at $\times 40$ objective magnification ($0.23 \mu\text{m}/\text{pixel}$ resolution). Digital analysis of the stained sections was performed using Visiopharm software v4.5.1.324 (Visiopharm, Hørsholm, Denmark). The cytoplasm was further defined by outlining the defined nucleus. The mean intensity of DAB for each defined image was used for quantification, and was categorized as follows: 0 = negative, 1 = weak, 2 = moderate, and 3 = strong. The overall immunostaining score (histoscore) was calculated by the percentage of positive cells multiplied by their staining intensity (possible range 0–300).¹³ Ki-67 expression was evaluated according to the percentage of cells that were labeled. Bleaching efficacy was also assessed by quantification of melanin pigment after bleaching.

Protein Extraction and Western Blotting

Protein was extracted from unbleached- and bleached-FFPE tissue sections from each of the three different tumor specimens for each condition as previously reported.^{14–17} Briefly, four $5 \mu\text{m}$ -thick FFPE tissue sections were scraped into microcentrifuge tubes and homogenized using a Disposable Pellet Mixer in $200 \mu\text{l}$ protein-extraction solution ($1 \times$ high pH antigen-retrieval buffer (pH 9.9; Dako), 1% NaN_3 , 1% SDS, and 10% glycerol and protease inhibitor (1 tablet/25 ml, Roche)), followed by 15 min incubation at 115°C in a pressure cooker (Dako). After incubation, the tissue lysates were centrifuged at 13 000 r.p.m. for 30 min at 4°C . The supernatants were collected and stored at 4°C before analysis. Protein concentrations were determined using a BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA).

To examine protein quality, $10 \mu\text{g}$ of protein extracted from the bleached- and unbleached-FFPE tissue sections was resolved by 4–12% NuPAGE Novex Bis-Tris polyacrylamide gel electrophoresis, and electroblotted to a nitrocellulose membrane using the iBlot Dry Blotting System (Invitrogen, Carlsbad, CA, USA). The membranes were blocked with 5% non-fat dry milk in TBST (50 mM Tris, pH 7.5, 150 mM NaCl, and 0.05% Tween-20) for 1 h, washed, and incubated overnight at 4°C in TBST with mouse anti-AKT antibody (cat. 9272; dilution 1:1000; Cell Signaling Technology, Danvers, MA, USA) or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (clone # 6C5; dilution 1:3000; Calbiochem, Gibbstown, NJ, USA). Signals were detected with horseradish peroxidase-labeled anti-rabbit or anti-mouse secondary antibodies (Chemicon International, Temecula, CA, USA) and enhanced with the SuperSignal Chemiluminescence kit (Pierce Biotechnology). Quantitative analysis of the western blot was performed using ImageQuant (Ver. 5.2; Molecular Dynamics, Sunnyvale, CA, USA).

RNA Extraction and cDNA Synthesis

RNA was extracted from four $5 \mu\text{m}$ -thick unbleached- and bleached-FFPE tissue sections from each of three different tumor specimens for each condition as previously reported.^{18–20} The specimens were rinsed briefly once in 100% ethanol, and then resuspended and ground in a solution of 4 M guanidine isothiocyanate, 20 mM sodium acetate, and 25 mM β -

mercaptoethanol (pH 5.5) followed by 72 h incubation at 65 °C with mild shaking. After incubation, RNA was isolated by phenol/chloroform extraction. The resulting RNA pellet was washed with ice-cold 75% ethanol and resuspended in diethyl-pyrocabonate-treated water.

To avoid problems associated with the contribution of melanin to the UV absorption spectrum, RNA quantity was assessed using a Quant-iT RiboGreen assay kit (Thermo Scientific, Waltham, MA, USA). Standard curves were generated using known amounts of RNA according to the manufacturer's instructions, and the quantity of RNA in each sample was calculated from the standard curve. Samples were diluted accordingly to fall within the linear range of each assay. The average quantity of duplicate measurements was used for subsequent assays. RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) with the RNA 6000 LabChip kit (Agilent Technologies). In addition, we also assessed RNA integrity by the paraffin-embedded RNA metric (PERM) number which is a novel metric for FFPE RNA.²¹

The extracted RNA was treated with 2 μ l TURBO DNase buffer, 4 units TURBO DNase (Invitrogen), and 40 units of RNase inhibitor (Promega, Madison, WI, USA) in a 100 μ l reaction volume to remove possible contaminating genomic DNA. Reverse transcription was performed with 5 μ g total RNA using Superscript II RNA H-Reverse Transcriptase (Invitrogen). For cDNA synthesis, 4 μ l 5 $\times$ first-strand buffer, 2 μ l DTT (0.1 M), 2 μ l dNTP (10 mM), 1 μ l RNasin (40 U/ μ l, Promega), 1 μ l random hexamers (50 μ M, Promega), and 1 μ l (200 U) SuperScript II reverse transcriptase were added to 9 μ l RNA. The mixture was incubated at 25 °C for 10 min and then at 42 °C for 90 min. The reaction was inactivated by heating to 70 °C for 15 min. To remove RNA complementary to the cDNA, 1 μ l RNase H (2 U) was added to the mixture and further incubated at 37 °C for 20 min. The synthesized cDNA was stored at -20 °C. Five microliters of fivefold diluted cDNA was used as a template for multiplex reverse-transcription-PCR (RT-PCR) reactions and real-time PCR.

Multiplex RT-PCR

Multiplex RT-PCR was performed using the multiplex polymerase chain reaction (MPCR) kit (Maxim Biotech, San Francisco, CA, USA) for the human housekeeping genes set-2. This kit was designed to detect five genes with amplicons of 242–612 bp. We performed multiplex RT-PCR according to the manufacturer's instructions in a 50 μ l reaction mixture. An initial pre-PCR step of 96 °C for 5 min was performed in the Bio-Rad iCycler PCR Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA), followed by 32 PCR cycles under the following conditions: two cycles of 94 °C for 1 min, 60 °C for 4 min, and 30 cycles of 94 °C for 1 min and 60 °C for 2 min. The final cycle was followed by an additional incubation 70 °C for 10 min to complete partial polymerizations. A MPCR-positive control (Maxim Biotech) and frozen mouse kidney RNA were included in each run. A negative control containing no nucleic acid was also included in each run to check for any PCR cross contamination.

A 1 μ l aliquot of the multiplex RT-PCR product was loaded onto the DNA 1000 kit (Agilent Technologies) and capillary electrophoresed in the Agilent 2100 Bioanalyzer. Agilent 2100 expert software was used to compare the electropherograms.

Real-Time Quantitative RT–PCR

Four micrograms of genomic DNA-free total RNA were reverse-transcribed into first-strand cDNA using a Quanti-Tect Reverse Transcription kit (Qiagen, Valencia, CA, USA). cDNA was generated from each of three replicates containing different bleaching solutions and frozen human RNA. We performed quantitative real-time PCR using TaqMan Gene Expression reagent (Applied Biosystems, Foster City, CA, USA). Briefly, quantitative real-time PCR was performed with 2 μ g cDNA assayed in a 20- μ l reaction volume. We examined the cycle threshold (Ct) value for the *actin* gene to assess RNA integrity in triplicate. The Mann–Whitney’s *U*-test was used to evaluate RNA integrity for each melanin-bleaching condition.

Assessment of DNA Quantity and Quality

DNA extraction from unbleached- and bleached-FFPE tissue sections was performed using a QIAamp DNA FFPE Tissue kit (Qiagen). DNA yield was determined using a NanoDrop ND-1000 UV spectrophotometer. In addition, DNA quantity was assessed using Quant-iT PicoGreen (Thermo Scientific). Standard curves were generated using known amounts of DNA according to the manufacturer’s instructions, and the quantity of DNA in each sample was calculated from the standard curve. The average quantity of duplicate measurements was used for subsequent assays.

DNA quality was assessed using a Bioscore Screening and Amplification kit (Enzo Life Sciences, Farmingdale, NY, USA), as previously reported.²² In addition, we also performed real-time PCR using the TaqMan Gene Expression reagent (Applied Biosystems). Briefly, quantitative real-time PCR was performed with 1 μ g DNA in a 20 μ l reaction volume. The reactions were incubated for 2 min at 50 °C and 10 min at 95 °C for initial denaturing, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min in the 7500 real-time PCR system (Applied Biosystems). We determined the Ct-value for the *ACTB* gene to assess DNA integrity in triplicates. The Mann–Whitney’s *U*-test was used to evaluate DNA integrity for each fixative condition.

RESULTS

Bleaching Effect is Facilitated by High Temperature and High pH

To establish a rapid and reliable melanin-bleaching methodology that can be applied to molecular profiling of archival melanin-pigmented melanoma FFPE tissues, we first examined the effect of temperature and H₂O₂ concentration on bleaching efficiency. Because our initial testing indicated that completion of bleaching was observed within 30 min at 80 °C (data not shown), we examined the bleaching efficiency of a highly pigmented FFPE tissue using different dilutions of H₂O₂ (0.1, 0.5, and 1.0%) with Tris-HCl (pH 10), PBS (pH 7.4), or Tris/Tricine/SDS (pH 8.2) buffer at five different time points (Figure 1). As shown in Figure 2a, increasing H₂O₂ concentration significantly decreased the time for completion of bleaching as measured by the percentage of melanin pigment in H&E-stained tissue slides using image analysis. Completion of bleaching was achieved with only 15 min incubation at 80 °C when using H₂O₂ at 0.5% or higher regardless of buffer composition (Figure 2b).

Next, we analyzed the effect of pH on the efficiency of melanin bleaching from FFPE tissues, given that the pH effect of antigen-retrieval buffer in immunohistochemistry of archival FFPE tissue sections is well-documented.²³ We treated FFPE tissues with melanin-bleaching solutions containing 0.5% H₂O₂ in Tris-HCl at three different pH conditions (pH 4, 7, and 10) for 15 min and assessed the bleaching efficacy using image analysis. As shown in Figure 3, high pH (pH 10) resulted in 5.2- and 6.9-fold increased bleaching efficacy compared with pH 7 and pH 4 Tris-HCl-buffered 0.5% H₂O₂ solution, respectively. Subsequent experiments using Tris-HCl-diluted H₂O₂ were conducted at pH 10. Overall, higher temperature and higher pH resulted in greater bleaching efficacy among the tested bleaching conditions without affecting histomorphology.

Antigenicity of Ki-67 and HMB45 Can Be Preserved in Bleached FFPE Tissue

To assess protein quality after melanin bleaching, we performed immunohistochemistry for Ki-67 and HMB45. Representative immunohistochemical staining of Ki-67 and HMB45 is shown in Figure 4a. Ki-67 staining was observed in the nucleus, whereas HMB45 was expressed in the cytoplasm of the cells. Ki-67 staining in the sample prepared in 0.5% H₂O₂ with Tris/Tricine/SDS appeared to be the most sensitive, with the highest mean percentage of Ki-67-positive value among the tested melanin-bleaching conditions (Figure 4b). In detail, the mean percentage of Ki-67-positive value in 0.5% H₂O₂ in Tris/Tricine/SDS ($86.46 \pm 5.5\%$) was significantly higher than the slide in 0.5% H₂O₂ with Tris-HCl ($72.06 \pm 7.7\%$, $P < 0.001$), and the one in 0.5% H₂O₂ with PBS ($54.66 \pm 17.3\%$, $P < 0.001$; Figure 4b). For HMB45, 0.5% H₂O₂ with Tris-HCl produced the highest mean histoscore. The mean histoscore of HMB45 in 0.5% H₂O₂ with Tris-HCl (211.5 ± 7.3) was significantly higher than the one in 0.5% H₂O₂ with PBS (168.4 ± 6.5 , $P < 0.001$), and the one in 0.5% H₂O₂ with Tris/Tricine/SDS (127.2 ± 3.1 , $P < 0.001$; Figure 4b).

While melanin bleaching enabled good-quality antibody staining of highly pigmented tissue specimen, we also examined the antigenicity preservation during the bleaching process. For this purpose, we performed immunohistochemistry for Ki-67 and HMB45 before and after the usage of the bleaching technique on a melanoma tumor specimen without visible melanin pigment (Figures 4c and d). Relative expression signal of both Ki-67 and HMB45 are normalized to that of the unbleached melanoma tissue specimen. As shown in Figure 4c, 0.5% H₂O₂ with Tris-HCl bleaching condition (1.12 ± 0.15 -fold) demonstrated a similar mean percentage of Ki-67-positive value to the slide from the unbleached. Among tested bleaching methods, 0.5% H₂O₂ with PBS (0.76 ± 0.28 -fold, $P = 0.046$) showed significantly lower Ki-67-positive value than the unbleached slide, whereas the 0.5% H₂O₂ with Tris/Tricine/SDS (1.36 ± 0.28 -fold, $P < 0.001$) showed significantly higher value than the unbleached one (Figure 4c). Furthermore, the mean histoscore of HMB45 in 0.5% H₂O₂ with Tris-HCl (1.03 ± 0.66 -fold) and the slide in 0.5% H₂O₂ with PBS (0.92 ± 0.12 -fold) was similar to that of the unbleached tissue, whereas the slide in 0.5% H₂O₂ with Tris/Tricine/SDS (0.65 ± 0.23 -fold, $P < 0.001$) was significantly lower than the unbleached one (Figure 4c). These data suggest that the antigenicity of both Ki-67 and HMB45 antigen can be preserved during the melanin bleaching.

High-Quality Protein Can Be Recovered From the Bleached FFPE Tissue Sections

It has been demonstrated that protein extracted from FFPE tissues can be used for western blotting with a relatively large amount of protein loading.¹⁵ We extracted protein from unbleached- and bleached-human melanoma FFPE tissue, and subjected them to western blot analysis using antibodies against human AKT and GAPDH. Unbleached human melanoma FFPE tissue was used as a negative control (Supplementary Figure 1, Figure 5). Analysis of protein quantity revealed that the bleaching conditions examined in this study did not decrease protein yield; in fact, 0.5% H₂O₂ with Tris/Tricine/SDS ($185.0 \pm 17.5 \mu\text{g}$) significantly increased yield compared with the non-bleaching condition ($131.9 \pm 24.6 \mu\text{g}$; Figures 5a and b). This result was concordant with our previous finding that the incorporation of detergent in protein extraction from FFPE tissue vastly improved the protein-recovery yield.¹⁵ Although the protein extracted from the 0.5% H₂O₂ with Tris-HCl showed the highest AKT and GAPDH signals in western blotting, no significant difference in quality was detected among tested bleaching conditions based on relatively similar intensities of the bands (Figure 5c).

RNA Integrity Was Retained in the Bleached FFPE Tissue

We next assessed the quality of RNA retrieved from unbleached- and bleached-FFPE tissue sections. It has been widely demonstrated that melanin hampers photometric quantification of nucleic acid because of its absorbance of UV light over the whole spectrum of 200–400 nm.⁶ To exclude any possible contribution of melanin to the UV absorption spectrum, RNA quantity was assessed using a Quant-iT RiboGreen assay kit. The RNA-recovery yield from 0.5% H₂O₂ with Tris-HCl ($4.12 \pm 0.37 \mu\text{g}$) was higher than that of 0.5% H₂O₂ with PBS ($3.69 \pm 0.29 \mu\text{g}$) or 0.5% H₂O₂ with Tris/Tricine/SDS ($3.33 \pm 0.73 \mu\text{g}$; Figure 6a). However, no significant difference was detected among the bleaching conditions tested in the present study, or between bleached and non-bleached FFPE tissue specimens.

The RNA quality was examined by PERM which is based on a formula that approximates a weighed area-under-the curve approach (Figures 6b and c), because of RNA fragmentation in FFPE tissues.²¹ The mean PERM number of 0.5% H₂O₂ with Tris-HCl (36.5 ± 0.4 , $P < 0.001$) and 0.5% H₂O₂ with PBS (31.7 ± 1.4 , $P = 0.021$) was significantly higher than that of the non-bleached condition (28.2 ± 1.3). Although the PERM value of the 0.5% H₂O₂ with Tris/Tricine/SDS (30.9 ± 0.7) was also higher than that of the non-bleached sample, this difference was not significant (Figure 6c).

To further evaluate the quality of extracted RNA, we performed multiplex RT-PCR using a MPCR kit for Human housekeeping genes set-2. It is well known that melanin potently inhibits PCR by binding to thermostable DNA polymerase. As shown in Figure 6d, we observed two peaks (242 and 312 bp) that correspond to the exact sizes of the targets in bleached conditions, whereas no detectable bands were found in the non-bleached condition. The band intensity of 0.5% H₂O₂ with Tris-HCl was higher than that of 0.5% H₂O₂ with PBS or Tris/Tricine/SDS (Figure 6d). Furthermore, we performed real-time RT-PCR using primers detecting *ACTB* transcripts to evaluate the RNA integrity. The Ct-value of the quantitative RT-PCR amplification was 17.5 ± 0.2 for the *ACTB* gene using RNA prepared from frozen tissue. As shown in Figure 7, the Ct-value for 0.5% H₂O₂ with Tris-HCl was

32.2 ± 0.9 , whereas the values for 0.5% H_2O_2 with PBS and Tris/Tricine/SDS were 35.0 ± 0.8 and 35.8 ± 1.2 , respectively. The Ct-value of RNA isolated from non-bleached tissues was the highest (mean, 45.1 ± 1.1).

The Melanin Bleaching Method Enabled DNA Analysis in Archival FFPE Tissue

We then performed similar analyses for DNA quality assessment after melanin bleaching. To exclude any contribution of melanin to the UV absorption spectrum, DNA quantity was assessed using a Quant-iT PicoGreen kit. The DNA extraction yield using 0.5% H_2O_2 with Tris-HCl (mean, $1.55 \pm 0.10 \mu\text{g}$) and 0.5% H_2O_2 with PBS (mean, $1.39 \pm 0.17 \mu\text{g}$) was similar to that of the non-bleached condition (mean, $1.65 \pm 0.09 \mu\text{g}$), whereas DNA recovery yield prepared from the 0.5% H_2O_2 with Tris/Tricine/SDS (mean, $1.04 \pm 0.23 \mu\text{g}$, $P = 0.008$) was significantly lower than that of the non-bleached condition (Figure 8a). In addition, the quality of the isolated DNA was assessed in terms of with the values indicating co-purification of protein (A_{260}/A_{280}), and chaotropic salt and organic solvent value (A_{260}/A_{230}) using a spectrophotometer. The A_{260}/A_{280} value of bleached conditions was significantly higher than that of the unbleached condition. The A_{260}/A_{230} value of DNA prepared from the bleached conditions was similar to that in non-bleached condition (Figure 8b). We then further examined the DNA quality using a Bioscore Screening and Amplification Kit.²² Approximately $5.61 \pm 0.57 \mu\text{g}$ of high-quality DNA for nucleic acid array analysis was amplified using 100 ng of DNA template extracted from the sample prepared with 0.5% H_2O_2 with Tris-HCl. In addition, the DNA-amplification yield of 0.5% H_2O_2 with PBS ($4.73 \pm 0.35 \mu\text{g}$) and Tris/Tricine/SDS ($3.93 \pm 0.76 \mu\text{g}$) was also significantly higher than that of the non-bleached condition ($2.94 \pm 0.08 \mu\text{g}$; Figure 8c).

Finally, we performed real-time PCR using primers detecting *ACTB* DNA to assess the effect of different bleaching methods on DNA integrity. As shown in Figure 8d, the mean Ct-value for dilute 0.5% H_2O_2 with Tris-HCl (27.1 ± 0.5) was significantly lower than that of the non-bleached condition (40.2 ± 0.8), whereas mean Ct-values of dilute 0.5% H_2O_2 with PBS and Tris/Tricine/SDS were 27.9 ± 0.5 and 29.2 ± 0.8 , respectively. Overall, these data suggest that our bleaching method can retain DNA of a quality compatible with PCR and real-time PCR.

DISCUSSION

Biomolecules (protein, RNA, and DNA) isolated from melanin-bleached FFPE tissues have potentially important applications, including a diagnostic assay combined with molecular profiling. It is therefore critical to have a rapid and reliable bleaching method that will not cause deterioration of the quantity and quality of the biomolecules extracted from bleached-FFPE tissue while retaining compatibility with current histological and immunohistochemical analysis methods. The most widely used bleaching methods are based on incubation with KMnO_4 /oxalic acid or dilute H_2O_2 ; the advantages and disadvantages of these two methods have been reported in previous studies.^{2,4,9} Although the KMnO_4 /oxalic acid-based bleaching method is fast and effective, subsequent immunostaining quality is compromised, especially with a high concentration of KMnO_4 , because of deterioration of tissue integrity.^{11,24,25} Alternatively, immunostaining of bleached FFPE tissue with the

dilute H₂O₂ method showed excellent quality comparable to that of unbleached control tissues. However, a major disadvantage of the dilute H₂O₂ method is that it requires a minimum of 24 h, and possibly up to 2 weeks, for complete removal of the pigments at room temperature or lower.^{24,26,27} Prior studies demonstrated that increasing the temperature reduced the bleaching time with 10% H₂O₂ to 30 min at 65 °C and 150 min at 60 °C.^{5,11,12} Recently, Manicam *et al*¹¹ demonstrated that 10% H₂O₂ diluted in PBS bleaches melanin most effectively at 65 °C for 120 min. Previous studies suggest that the use of temperatures exceeding 75 °C in the bleaching procedure cause deterioration of protein quality of FFPE tissue.^{11,12} In contrast, our approach does not affect the quality of histomorphology (Figure 2). This discrepancy might be explained by the use of a lower concentration of H₂O₂ (0.5% instead of 10%), different diluent, and different incubation time. In the present study we observed a loss of tissue integrity and cellular morphology using 1% H₂O₂ diluted with PBS and Tris/Tricine/SDS buffers (data not shown). These data suggest that heat, incubation time, and the buffer used as diluent are critical factors in the dilute H₂O₂-based bleaching technology.

On the basis of the principle of heat-induced antigen-retrieval techniques, we previously demonstrated that the use of antigen-retrieval buffer (pH 10) containing 1% SDS combined with high temperature resulted in excellent protein-extraction yield from archival FFPE tissue sections, without changing the antigenicity.¹⁹ Therefore, we used antigen-retrieval buffer (Tris-HCl, pH 10) and Tris-based buffer containing detergent (Tris/Tricine/SDS, pH 8.2) as test bleaching buffers in addition to PBS as a control to investigate their effect on melanin-bleaching efficiency and downstream applications. All three alkaline diluents for H₂O₂ showed good quality of histomorphology (Figure 2). As expected, the protein-extraction yield from bleached FFPE tissues with 0.5% H₂O₂ diluted with Tris/Tricine/SDS buffer was the highest among the tested conditions, which is consistent with our previous data.¹⁵ Notably, Tris-HCl (pH 10) was a better diluent for H₂O₂ in preserving nucleic acid quality after bleaching than PBS and Tris-based buffer containing detergent. Earlier studies demonstrated that the dilute H₂O₂-based bleaching technology is compatible with classic immunohistochemistry using FFPE tissue.^{5,10,24} We similarly confirmed that the immunoreactivity of both Ki-67 and HMB45 antigen was preserved using our bleaching method. Immunohistochemistry on bleached tissue is easily optimized and generates staining patterns of nearly identical features compared with unbleached tissue (Figures 4c and d). In the present study, we evaluated Ki-67 and HMB45 by staining the nuclear and cytoplasmic compartments, respectively. Although we neither performed a systematic review of immunohistochemical conditions, nor perform a rigorous validation, we were able to demonstrate identical staining pattern between unbleached- and bleached-tissue specimen for the two antigens tested. However, the action mechanism of H₂O₂ in the bleaching procedure is still unknown. Li *et al*²⁴ proposed that H₂O₂ may oxidize certain radicals on melanin molecules with or without disintegration of the melanin protein. We also previously demonstrated that the possible oxidation of endogenous and exogenous water is a key factor in the loss of antigenicity during storage of FFPE tissue.²⁸ On the basis of our histomorphological observations of over-bleached FFPE tissue sections (data not shown), the H₂O₂-based bleaching method may be linked with tissue oxidation. Clearly, the exact mechanism of the H₂O₂-based bleaching procedure is an area for further study. Furthermore,

the effect of the diluent for H₂O₂ on antigenicity may be dependent on the quality of the antibody, and further studies are required to assess the effect of this approach on the immunoreactivity of other markers. In addition, our method resulted in good protein-extraction yield and quality from bleached FFPE tissues while retaining immunoreactive molecules. Moreover, our method has the advantage of being compatible with archival melanin pigment-containing FFPE tissues and permits tissue protein analysis and profiling paired with proteomic tool.

Importantly, our method is also applicable to the generation of high-quality DNA and RNA, which is compatible with quantitative PCR from FFPE melanoma tissues, without loss of yield. Despite the need to isolate good-quality DNA and RNA from highly pigmented FFPE tissues for molecular profiling, previous melanin-bleaching methods have not been extensively tested for this purpose. To our knowledge, the present study is the first attempt to establish a robust and rapid bleaching method suitable for both histomorphological and molecular assays. Previously reported melanin-removal methods from melanin-rich nucleic acid include purification using column matrices²⁹ and cetyltrimethylammonium bromide (CTAB)-based precipitation.³⁰ Although these post-isolation clean-up methods allow efficient removal of melanin and generates high-quality nucleic acid, the DNA/RNA yield is extremely poor with a loss of 25–30% (column matrices),²⁹ and 20–75% (CTAB)³⁰ during the process. In addition, these published approaches were mainly tested for frozen tissues or cultured cells but not for FFPE tissues, for which tissue material is often highly limited, thus making these approaches not the most ideal. Our method circumvents these issues by simplifying the steps of melanin-removal procedure, and generates DNA and RNA that can be readily used in molecular assays based on PCR amplification.

In conclusion, the use of 0.5% H₂O₂ diluted with high-pH antigen-retrieval buffer (Tris-HCl, pH 10), PBS, or Tris-based buffer containing detergent (Tris/Tricine/SDS, pH 8.2) at 80 °C for 15 min allows rapid removal of melanin pigments from archival FFPE tissue section while preserving cellular structure. Among the tested diluents for H₂O₂, the high-pH Tris-HCl buffer showed the best quality for PCR amplification molecular methods. As a fundamental molecular pathological tool, this bleaching method enables the use of protein and nucleic acids isolated from melanin-pigmented FFPE tissue for molecular assays in addition to enhancing diagnostic pathology within the current framework of pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic diagram of the experimental procedure to test the effects of bleaching techniques using different buffers (Tris-HCl (pH 10), PBS (pH 7.4), or Tris/Tricine/SDS (pH 8.2)), dilutions of H₂O₂ (0.1%, 0.5%, or 1.0%), and incubation periods (2, 5, 10, 15, or 30 min) at 80 °C.

Figure 2. Melanin pigment concentration over time for different dilutions of H_2O_2 (0.1, 0.5, and 1.0%) with different buffered bleaching solutions (Tris-HCl, PBS, or Tris/Tricine/SDS) at 80 °C. (a) Effect of melanin bleaching using 0.1, 0.5, or 1.0% H_2O_2 in Tris-HCl (pH 10), PBS, or Tris/Tricine/SDS buffer. Melanin pigments were measured using image analysis software (Visiopharm). Black triangles indicate melanin pigment. (b) H&E images of 0.5% H_2O_2 diluted with Tris-HCl, PBS, or Tris/Tricine/SDS buffer (scale bar: 100 μm).

Figure 3.

Effect of pH using 0.5% H_2O_2 in Tris-HCl buffer at 80 °C for 15 min in FFPE tissue specimens of human malignant melanoma, (a) Representative H&E images obtained for 0.5% H_2O_2 in Tris-HCl buffer with pH 4, 6, or 10. The boxed regions are displayed at high magnification in the right panel (scale bar: 100 μm). (b) Bar graph of melanin pigment after bleaching. Melanin pigments were measured using image analysis software (Visiopharm). After bleaching, the highest pH (10) showed the lowest histoscore compared with pH 7 ($P < 0.001$) and pH 4 ($P < 0.001$). Data are depicted as mean $\pm$ s.d. from three experiments.

Figure 4.

The effects of different buffered bleaching methods on immunogenicity. We performed immunohistochemistry for Ki-67 and HMB45 after the usage of the bleaching technique with heavily melanin-pigmented melanoma FFPE tissues. **(a)** Representative immunohistochemical images of Ki-67 and HMB45 after melanin bleaching (scale bar: 100 μ m). **(b)** Immunogenicity measured using mean percentage of Ki-67 and mean histoscore of HMB45 indicated that Tris/Tricine/SDS and Tris-HCl (pH 10) yielded the highest signals for nuclear staining (all $P < 0.001$) and cytoplasmic staining (all $P < 0.001$). We also performed

immunohistochemistry for Ki-67 and HMB45 before and after the usage of the bleaching technique with melanoma tumor specimen without melanin pigment. (c) Relative expressional signal of both Ki-67 and HMB45 was normalized to that of unbleached melanoma tissue specimen. The antigenicity of Ki-67 and HMB45 was relatively well-preserved after the bleaching methodology using Tris-HCl (pH 10) buffer. NB, non-bleaching. (d) Representative immunohistochemical images of Ki-67 and HMB45 before and after melanin bleaching (scale bar: 100 μ m).

Figure 5.

Protein quantity and quality from different melanin-bleaching methodologies, (a) Quantity of protein extracted under each condition was measured using a BCA Protein Assay kit. The protein extraction yield is expressed as the mean of three replicates (mean $\pm$ s.d.). Black bars and clear bars represent the result of non-bleaching (■) and bleaching (□) method, respectively, (b) Protein integrity under different melanin-bleaching conditions was assessed by gel electrophoresis. Proteins extracted after the use of different bleaching solutions were separated by 4–12% reducing SDS-PAGE and the gel was stained using a silver staining kit.

(c) Proteins (10 μ g) were subjected to 4–12% gradient SDS-PAGE, transferred to nitrocellulose membrane, and subjected to western blotting with anti-AKT (■) and anti-GAPDH (□) antibodies. The signal was detected with a SuperSignal Chemiluminescence kit. NB, non-bleaching; M_r , protein molecular marker (kDa).

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Figure 6.

RNA quantity and quality with different melanin-bleaching methods. **(a)** Quantity of RNA extracted from melanin-bleached tissue specimens was measured using the RiboGreen kit. RNA-extraction yield is expressed as the mean of three replicates (mean $\pm$ s.d.). Black bars and clear bars represent the result of no bleaching (■) and bleaching (□) methods, respectively. **(b)** To compare the quality of RNA extracted under each condition we used an electropherogram to overlay four different conditions, including non-bleaching. **(c)** RNA integrity value was represented by the PERM. Black bars and clear bars represent the result of non-bleaching (■) and bleaching (□) methods, respectively. **(d)** Representative electropherogram of multiplex PCR amplification. The human housekeeping genes set-2 was used for multiplex PCR reactions. Insets show high magnification of the areas indicated with boxes. NB, non-bleaching.

Figure 7.

RNA integrity with different melanin-bleaching methods. Gene expression levels (ie, enzymatic amplification efficiency) of a housekeeping gene (*ACTB*) were examined for RNA extracted from human malignant melanoma FFPE tissues under different melanin-bleaching conditions. Mean Ct-values from quantitative real-time RT-PCR are shown as a box plot. NB, non-bleaching; FF, fresh-frozen human tissue (positive control).

Figure 8.

Comparison of DNA-extraction yields and quality among the different bleaching buffer solutions, (a) Amount of DNA extracted from each specimen was measured using the PicoGreen kit. DNA-extraction yield is expressed as the mean of three replicates (mean $\pm$ s.d.). Black bars and clear bars represent the result of non-bleaching (■) and bleaching (□) methods, respectively, (b) Analysis of the quality of the DNA extracted from bleached human malignant melanoma FFPE tissues using a Nanodrop spectrophotometer. DNA quality was assessed by distribution of the ratio values (A_{260}/A_{280} and A_{260}/A_{230}). Purity of DNA is expressed as average ratio values of three replicates (mean $\pm$ s.d.). (c) Amplification yield of DNA extracted from samples with different melanin-bleaching methodologies. One hundred nanograms of extracted DNA from each sample was amplified using a BioScore Amplification kit (Enzo Life Science). Black and clear bars represent the result of non-bleaching (■) and bleaching (□) methods, respectively. (d) Enzymatic amplification efficiency of a housekeeping gene (*ACTB*) was examined for DNA extracted from human malignant melanoma FFPE tissues under different melanin-bleaching conditions. Mean Ct-

values from quantitative real-time RT–PCR are shown as a box plot. The bars indicate standard deviation. NB, non-bleaching; FF, fresh-frozen human tissue (positive control).

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