Original Article

# The Optimal Immunohistochemical Staining Conditions for Engrailed 1 Protein

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***Abstract:***

***Background:*** *The optimal immunohistochemical staining condition is associated with the accuracy of evaluating an expression of tissue proteins.* ***Objective:*** *To standardise the immunohistochemical staining conditions of EN1 protein expression in invasive breast carcinoma.* ***Methods:*** *This study was to retrieve engrailed 1 (EN1) protein in the breast carcinoma tissue sections by using 1x10 mM sodium citrate buffer solution of pH 6.0 with a microwave antigen retrieval at 650, 700, and 750 watts for 5, 10, 15, and 20 minutes. Anti-human EN1 mouse monoclonal primary antibody (1°Ab) concentrations of 1.0, 2.5, 5.0, and 10.0 μg/mL were used for determining EN1 protein expression in the nuclei.* ***Results:*** *The results showed that normal mammary epithelial cells and malignant cells in the same tissue section microscopically revealed the greatest contrast between their nuclear immunoexpression of EN1 protein and nonspecific background immunostaining after microwaving.* ***Conclusion:*** *An expression of EN1 gene is distinctly detectable in the FFPE-TS-PIBC by MAR with SCBS at 700 watts for 10 minutes and using a 1°Ab concentration of 10.0 μg/mL.*

***Keywords:*** ● *Optimisation* ● *Immunohistochemistry* ● *Engrailed 1 protein* ● *Breast carcinoma*

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## นิพนธ์ต้นฉบับ

# สภาวะที่เหมาะสมของการย้อมอิมมูโนฮีสโตเคมีสำหรับโปรตีนเอนเกรล 1

เจตนา เรืองประทีป[1] รุจินี ปดิฐพร[1] ปุณยนุช จินดาธรรมานุสาร[2] และ ภัสรา อาณัติ[1]
[1]ภาควิชาพยาธิวิทยา วิทยาลัยแพทยศาสตร์พระมงกุฎเกล้า [2]หน่วยโลหิตวิทยาและมะเร็งวิทยา กองกุมารเวชกรรม โรงพยาบาลพระมงกุฎเกล้า

***บทคัดย่อ***

***ความเป็นมา*** *สภาวะที่เหมาะสมของการย้อมอิมมูโนฮีสโตเคมี เกี่ยวข้องกับความถูกต้องในการประเมินการแสดงออกของโปรตีนในเนื้อเยื่อ* ***วัตถุประสงค์*** *เพื่อวางมาตรฐานของการย้อมอิมมูโนฮีสโตเคมีในการประเมินการแสดงออกของโปรตีนในเนื้อเยื่อ* ***วิธีการ*** *การศึกษานี้ได้ทำการคืนสภาพโปรตีนเอนเกรล 1 (อีเอน 1) ในเนื้อเยื่อมะเร็งเต้านมชนิดคาร์ซิโนมาโดยใช้สารละลายบัฟเฟอร์โซเดียมซิเตรตที่มีความเข้มข้นหนึ่งเท่าของ 10 มิลลิโมลาร์และมีค่าพีเฮช 6.0 พร้อมกับให้ความร้อนจากเตาไมโครเวฟที่กำลังไฟฟ้า 650, 700 และ 750 วัตต์ โดยแต่ละค่าของกำลังไฟฟ้าจะใช้เวลาอบนาน 5, 10, 15 และ 20 นาที จากนั้นประเมินการแสดงออกของโปรตีนอีเอน 1 ที่นิวเคลียสของเซลล์ด้วยสารละลายแอนติบอดี้ที่ระดับความเข้มข้น 1.0, 2.5, 5.0 และ 10.0 ไมโครกรัม/มิลลิลิตร* ***ผลการวิจัย*** *พบว่าการใช้คลื่นไมโครเวฟที่กำลังไฟฟ้า 700 วัตต์ นาน 10 นาที และความเข้มข้นของสารละลายแอนติบอดี้ต่อโปรตีนอีเอน 1 ที่ระดับ 10.0 ไมโครกรัม/มิลลิลิตร มีความเหมาะสมที่สุดสำหรับการแยกความแตกต่างของการแสดงออกของโปรตีนอีเอน 1 ในนิวเคลียสของเซลล์เยื่อบุผิวของต่อมน้ำนมปกติและเซลล์มะเร็งที่อยู่ในชิ้นเนื้อเยื่อเดียวกัน* ***สรุป*** *การแสดงออกของโปรตีนอีเอน 1 สามารถตรวจพบได้โดยการย้อมอิมมูโนฮีสโตเคมี*

***คำสำคัญ:*** ● *เงื่อนไขที่เหมาะสม* ● *อิมมูโนฮีสโตเคมี* ● *โปรตีนเอนเกรล 1* ● *มะเร็งเต้านมชนิดคาร์ซิโนมา*

***เวชสารแพทย์ทหารบก 2560;70:193-202.***

## Introduction

*Engrailed 1* (*EN1*) gene is located on chromosome 2q14.2 and has a role in controlling development of the central nervous system[1-4]. This gene is expressed in the nuclei of human epidermal neural crest stem cells[5]. In 2014, *EN1* gene has been reported to show gain in a human basal-like breast carcinoma cell line. Overexpression of this gene is also associated with promoting survival and resistance to chemotherapy of basal-like breast cancers[6]. Immunohistochemistry (IHC) is routinely used for the identification and demonstration of cellular prognostic and predictive biomarkers in human cancer tissue sections. These biomarkers are specific tumour antigens which are expressed de novo or upregulated in certain cancers[7]. The IHC has been applied for the evaluation of therapeutic response in invasive breast carcinoma according to the immunohistochemical expression of oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki67 proteins in the cancer tissues[8]. Since IHC is based on the specific interaction between antibodies and tissue antigens, formalin fixation of the tissues is associated with the formation of cross-linked antigenic proteins leading to variations in the reproducibility of immunohistochemical reactivity[8-12]. The formalin-induced cross-linkages can be reversed by placing the formalin-fixed tissue sections in different types of buffer solutions and heating to temperatures between 80 and 125°C. This antigen retrieval (AR) technique is most commonly used in pathology service and research. The current popular heat-induced antigen retrieval (HIAR) devices are microwave oven and pressure cooker. In addition, the most widely used HIAR solution is citrate buffer pH 6.0. Hence, any newer immunohistochemical antibodies will require standardisation before they are used for an immunostaining procedure. The optimal AR protocol and antibody concentration are primarily established to achieve the reproducible and reliable results of immunoexpression[8-26].

The aim of this study was to standardise the immunohistochemical staining conditions of EN1 protein expression in invasive breast carcinoma. The objective was to determine the optimal HIAR and primary antibody concentration for immunoreactivity of EN1 protein in formalin-fixed, paraffin-embedded (FFPE) tissue sections of primary invasive (infiltrating) breast carcinoma (PIBC) using a domestic 750-watt microwave oven with sodium citrate buffer solution.

## Materials and Methods

The FFPE-PIBC tissue block, which is stored in room temperature since 2014, was derived from Anatomical Pathology Division, Army Institute of Pathology, Bangkok, Thailand. It contained both normal mammary epithelial cells (NMECs) and malignant cells (MCs). There was ethical approval for the use of this tissue block [Institutional Review Board of the Royal Thai Army Medical Department (IRB, RTA.MD.) 307/2560].

The unstained sections of 3 μm FFPE-PIBC tissue were mounted onto Shandon Superfrost™ Plus positively charged microscopic slides (6776214, Thermo Scientific). After melting paraffin at 60°C in an air incubator for 24 hours, all sections were applied for deparaffinization in 2 changes of xylene and rehydration in 99%, 99%, and 95% ethyl alcohol for 3 minutes each. They were subsequently washed in running tap water for 5 minutes. The deparaffinized and rehydrated FFPE-TS-PIBCs were submerged by 1x 10 mM sodium citrate buffer solution (SCBS) of pH 6.0 and were performed on HIAR with a domestic 750-watt Samsung® microwave oven (GE711K) at 650, 700, and 750 watts for 5, 10, 15, and 20 minutes. The 10mM SCBS of pH 6.0 was prepared from the mixture of 2.94 grams of Tri-sodium

citrate (dihydrate), 1,000 mL of distilled water, 1N HCl for adjusting the final pH to 6.0, and 0.5 mL of TWEEN® 20 (P9416, Sigma®). Mouse and Rabbit Specific Horseradish Peroxidase (HRP) / 3,3'-Diaminobenzidine (DAB) [Avidin-Biotin Complex (ABC)] Detection Immunohistochemistry Kit (ab64264, abcam®) was used for determining EN1 protein expression in each individual slide.

For elimination of endogenous peroxidase activity, the sections were incubated with 200 μL of Hydrogen Peroxide Block in a humid chamber at room temperature for 10 minutes. Then they were incubated with 200 μL of Protein Block to reduce non-specific binding of primary antibody and Biotinylated Goat Anti-Polyvalent in a humid chamber at room temperature for 10 minutes. The sections were subsequently treated with 200 μL of anti-human EN1 mouse monoclonal primary antibody (clone number 1F5) (ab119111, abcam®) ($1^{\circ}$ Ab) concentrations of 1.0, 2.5, 5.0, and 10.0 μg/mL. The 50 mM Tris buffered saline (TBS) of pH 7.6 [BioUltra, 10 Tablets (For 500 mL/Tablet), 94158, Sigma®] solution was used as a diluent of primary EN1 antibody. The negative control sections were applied to 200 μL of TBS with no primary antibody (NPA). All sections were incubated overnight in a humid chamber at $4^{\circ}$C. The overnight incubated sections were reacted with 200 μL of Biotinylated Goat Anti-Polyvalent (secondary antibody) in a humid chamber at room temperature for 10 minutes. These sections were subsequently applied to 200 μL of Streptavidin Peroxidase in a humid chamber at room temperature for 10 minutes. All tissue sections were further incubated with 200 μL of DAB Substrate/Chromogen working solution for the development of final brown product in a humid chamber at room temperature for 10 minutes. This working solution was prepared from the mixture of 50x DAB Chromogen and DAB Substrate at a 1:50 ratio. Afterwards counterstaining was performed with Modified Haematoxylin Solution (Progressive Stain) (PATH.1, C.V. Laboratories, Thailand) for one minute. The immunostained tissue sections were subsequently dehydrated through 95%, 99%, and 99% ethyl alcohol then 2 changes of xylene for 3 minutes each. They were finally covered by Fisher Chemical™ Permount™ Mounting Medium (SP15-500, Fisher Scientific®).

An evaluation of immunohistochemical staining was performed on Nikon Eclipse E400 light microscope first using 4x objective lens (magnification of 40x) in order to scan and locate the histopathological appearances. Then the 10x and 40x objective lens (magnification of 400x) was subsequently applied for more detailed information on the staining[27,28]. The immunostaining results were assessed by Chetana Ruangpratheep (a Thai board-certified pathologist). The nuclear immunoreactivity of EN1 protein was evaluated from both NMECs and MCs of each PIBC tissue sections. An interpretation was based on the histochemical scoring (H-score) assessment incorporating both the staining intensity ($i$) and a percentage of stained cells at each intensity level ($P_i$). The $i$ values were indicated as 0 (no evidence of staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining). The $P_i$ values varied from 0% to 100%. The final H-score was derived from the sum of $i$ multiplied by $P_i$ as this equation: $(0 \times P_0) + (1 \times P_1) + (2 \times P_2) + (3 \times P_3)$. This score, therefore, was in the range of 0 to 300[29-31]. The most optimal immunostaining condition was considered according to the H-scores of NMECs and MCs with the quality of nuclear immunoexpression in both cells.

## Results

The different microwave antigen retrieval (MAR) conditions and concentrations of EN1 antibody yielded various H-scores of EN1 immunoexpression in NMECs and MCs. The immunoexpression of both cells was

inversely related to the power and time of MAR. The NMECs utterly showed poor immunoreactivity according to the higher heating power with the longer duration of MAR and the lower concentration of primary EN1 antibody. Similarly, an obvious decrease in EN1 immunoreactivity was present in MCs (Table 1). After microwaving at 700 watts for 10 minutes and using a 10.0 μg/mL of 1° Ab, NMECs and MCs microscopically revealed the greatest contrast between their nuclear immunoexpression of EN1 protein (H-scores of 300 and 140, respectively) and nonspecific background immunostaining (Figure 1).

**Discussion**

Immunohistochemistry (IHC) is a simple technique for demonstrating the presence and location of EN1 protein in the formalin-fixed, paraffin-embedded tissue sections of primary invasive (infiltrating) breast carcinoma (FFPE-TS-PIBC). Although 10% neutral buffered formalin solution is a common tissue fixative reagent used in the diagnostic histopathology laboratory[32], it leads to poor immunohistochemical reactivity due to the cross-linking of cellular proteins with the reduction in the recognition of antigenic epitopes and an inefficient antibody binding[33,34]. Hence, AR is an essential step in immunohistochemical staining of FFPE tissue sections[26]. The reliable and reproducible immunoexpression of each antigenic protein depend on the optimal protocol of AR and the optimal concentration of each 1° Ab[8,11,12]. HIAR is commonly used for recovery of antigenicity by disruption to cross-linkages between formalin and protein[35-39]. Microwave heating is the most popular AR method in many pathological laboratories because of a cost-effective, uncomplicated application. There are different brands of domestic microwave oven used in the previous studies, i.e. Toshiba (operating at a frequency of 2.45 GHz with the highest power setting of 720 W, model ER-855BT)[35], Emerson (operating at a frequency of 245 MHz with the highest power setting of 600 W, model MT3410TG, North Bergen, New Jersey)[40], Philips (Mod AVM 600/WH, maximum output 900 W)[41], Philips (M742, The Netherlands; Bauknecht MCID 1125 Duo, Germany)[42], Hitachi (600W, MR-6000AL, Tokyo, Japan)[43], Sharp (900 W, 2450 MHz, model R-4A46)[17], and Panasonic (1,000 W, NN-S65B)[44]. The aforementioned microwave ovens yielded various immunoreactivities according to the retrieval medium, the time of exposure to microwave irradiation, and the dilution of 1° Ab. This study applied a domestic 750-watt Samsung® microwave oven (GE711K) instead of a laboratory oven for HIAR of EN1 protein in FFPE-TS-PIBC because it is affordable and has the heating power control expressed in watts.

Basically, microwaves are electromagnetic waves with frequencies between 300 MHz and 300 GHz, corresponding to wavelengths between 1 m and 1 mm, respectively. All domestic microwave ovens operate at 2.45 GHz, corresponding to a wavelength in vucuo of 12.2 cm[45]. The possible mechanisms of MAR are (a) breaking the formalin-induced cross-links between epitopes and unrelated proteins, (b) extraction of diffusible blocking proteins, and (c) precipitation of proteins and rehydration of the tissue section allowing better penetration of antibody and increasing accessibility of epitopes[46]. SCBS of pH 6.0, which was used in this study, is the most popular solution for HIAR[47,48] and probably causes the dissociation of $Ca^{2+}$ complexes by high concentrations of $H^{+}$ ions and/or the breaking up of cross-links from formalin fixation[49]. However, SCBS is previously reported that it more often produces poor immunostaining intensity and low number of positive cells. The 1 mM ethylenediaminetetraacetic acid (EDTA) of pH 8.0 is the most efficient AR solution for numerous 1° Ab because it independently achieves good intensity of intranuclear, intracytoplasmic, or membrane-bound

**Table 1** Immunostaining H-scores of normal mammary epithelial cells and cancer cells according to MAR at **(A)** 650, **(B)** 700, and **(C)** 750 watts for 5, 10, 15, and 20 minutes with EN1 antibody concentrations [Ab] of 1.0, 2.5, 5.0, and 10.0 μg/mL

(A)

| Normal mammary epithelial cells | | | | | | | | Cancer cells | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Microwave | | [Ab] (μg/mL) | Immunostaining percentage | | | | H-score | Microwave | | [Ab] (μg/mL) | Immunostaining percentage | | | | H-score |
| Power (Watts) | Time (Minutes) | | None (0) | Weak (1) | Moderate (2) | Strong (3) | | Power (Watts) | Time (Minutes) | | None (0) | Weak (1) | Moderate (2) | Strong (3) | |
| 650 | 5 | 10.0 | 1 | 1 | 98 | 0 | 197 | 650 | 5 | 10.0 | 95 | 5 | 0 | 0 | 5 |
| | | 5.0 | 1 | 5 | 89 | 5 | 198 | | | 5.0 | 94 | 5 | 1 | 0 | 7 |
| | | 2.5 | 39 | 40 | 20 | 1 | 83 | | | 2.5 | 100 | 0 | 0 | 0 | 0 |
| | | 1.0 | 94 | 5 | 1 | 0 | 7 | | | 1.0 | 100 | 0 | 0 | 0 | 0 |
| | 10 | 10.0 | 1 | 5 | 44 | 50 | 243 | | 10 | 10.0 | 94 | 1 | 5 | 0 | 11 |
| | | 5.0 | 30 | 65 | 5 | 0 | 75 | | | 5.0 | 100 | 0 | 0 | 0 | 0 |
| | | 2.5 | 99 | 1 | 0 | 0 | 1 | | | 2.5 | 100 | 0 | 0.1 | 0 | 0.2 |
| | | 1.0 | 100 | 0 | 0 | 0 | 0 | | | 1.0 | 100 | 0 | 0 | 0 | 0 |
| | 15 | 10.0 | 1 | 5 | 54 | 40 | 233 | | 15 | 10.0 | 80 | 10 | 5 | 5 | 35 |
| | | 5.0 | 1 | 20 | 74 | 5 | 183 | | | 5.0 | 99.9 | 0 | 0 | 0.1 | 0.3 |
| | | 2.5 | 95 | 5 | 0 | 0 | 5 | | | 2.5 | 100 | 0 | 0 | 0 | 0 |
| | | 1.0 | 100 | 0 | 0 | 0 | 0 | | | 1.0 | 100 | 0 | 0 | 0 | 0 |
| | 20 | 10.0 | 1 | 1 | 49 | 49 | 246 | | 20 | 10.0 | 25 | 50 | 20 | 5 | 105 |
| | | 5.0 | 1 | 1 | 49 | 49 | 246 | | | 5.0 | 60 | 30 | 5 | 5 | 55 |
| | | 2.5 | 100 | 0 | 0 | 0 | 0 | | | 2.5 | 100 | 0 | 0 | 0 | 0 |
| | | 1.0 | 100 | 0 | 0 | 0 | 0 | | | 1.0 | 100 | 0 | 0 | 0 | 0 |

(B)

| Normal mammary epithelial cells | | | | | | | | Cancer cells | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Microwave | | [Ab] (μg/mL) | Immunostaining percentage | | | | H-score | Microwave | | [Ab] (μg/mL) | Immunostaining percentage | | | | H-score |
| Power (Watts) | Time (Minutes) | | None (0) | Weak (1) | Moderate (2) | Strong (3) | | Power (Watts) | Time (Minutes) | | None (0) | Weak (1) | Moderate (2) | Strong (3) | |
| 700 | 5 | 10.0 | 50 | 49 | 0 | 1 | 52 | 700 | 5 | 10.0 | 98 | 1 | 1 | 0 | 3 |
| | | 5.0 | 0 | 0 | 1 | 99 | 299 | | | 5.0 | 98 | 1 | 0 | 1 | 4 |
| | | 2.5 | 93 | 5 | 1 | 1 | 10 | | | 2.5 | 97 | 1 | 1 | 1 | 6 |
| | | 1.0 | 99 | 1 | 0 | 0 | 1 | | | 1.0 | 100 | 0 | 0 | 0 | 0 |
| | 10 | 10.0 | 0 | 0 | 0 | 100 | 300 | | 10 | 10.0 | 30 | 30 | 10 | 30 | 140 |
| | | 5.0 | 5 | 40 | 50 | 5 | 155 | | | 5.0 | 99 | 0 | 0.5 | 0.5 | 2.5 |
| | | 2.5 | 5 | 5 | 45 | 45 | 230 | | | 2.5 | 99 | 0 | 1 | 0 | 2 |
| | | 1.0 | 30 | 70 | 0 | 0 | 70 | | | 1.0 | 100 | 0 | 0 | 0 | 0 |
| | 15 | 10.0 | 1 | 0 | 10 | 89 | 287 | | 15 | 10.0 | 30 | 20 | 20 | 30 | 150 |
| | | 5.0 | 0 | 0 | 70 | 30 | 230 | | | 5.0 | 84 | 5 | 10 | 1 | 28 |
| | | 2.5 | 85 | 10 | 5 | 0 | 20 | | | 2.5 | 100 | 0 | 0 | 0 | 0 |
| | | 1.0 | 100 | 0 | 0 | 0 | 0 | | | 1.0 | 100 | 0 | 0 | 0 | 0 |
| | 20 | 10.0 | 0 | 0 | 1 | 99 | 299 | | 20 | 10.0 | 30 | 70 | 0 | 0 | 70 |
| | | 5.0 | 20 | 78 | 1 | 1 | 83 | | | 5.0 | 99.9 | 0 | 0.1 | 0 | 0.2 |
| | | 2.5 | 100 | 0 | 0 | 0 | 0 | | | 2.5 | 100 | 0 | 0 | 0 | 0 |
| | | 1.0 | 98 | 1 | 1 | 0 | 3 | | | 1.0 | 100 | 0 | 0 | 0 | 0 |

(C)

| Normal mammary epithelial cells | | | | | | | | Cancer cells | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Microwave | | [Ab] (μg/mL) | Immunostaining percentage | | | | H-score | Microwave | | [Ab] (μg/mL) | Immunostaining percentage | | | | H-score |
| Power (Watts) | Time (Minutes) | | None (0) | Weak (1) | Moderate (2) | Strong (3) | | Power (Watts) | Time (Minutes) | | None (0) | Weak (1) | Moderate (2) | Strong (3) | |
| 750 | 5 | 10.0 | 80 | 20 | 0 | 0 | 20 | 750 | 5 | 10.0 | 100 | 0 | 0 | 0 | 0 |
| | | 5.0 | 99.8 | 0.1 | 0.1 | 0 | 0.3 | | | 5.0 | 100 | 0 | 0 | 0 | 0 |
| | | 2.5 | 100 | 0 | 0 | 0 | 0 | | | 2.5 | 100 | 0 | 0 | 0 | 0 |
| | | 1.0 | 100 | 0 | 0 | 0 | 0 | | | 1.0 | 100 | 0 | 0 | 0 | 0 |
| | 10 | 10.0 | 80 | 20 | 0 | 0 | 20 | | 10 | 10.0 | 100 | 0 | 0 | 0 | 0 |
| | | 5.0 | 90 | 10 | 0 | 0 | 10 | | | 5.0 | 100 | 0 | 0 | 0 | 0 |
| | | 2.5 | 100 | 0 | 0 | 0 | 0 | | | 2.5 | 100 | 0 | 0 | 0 | 0 |
| | | 1.0 | 100 | 0 | 0 | 0 | 0 | | | 1.0 | 100 | 0 | 0 | 0 | 0 |
| | 15 | 10.0 | 10 | 80 | 10 | 0 | 100 | | 15 | 10.0 | 100 | 0 | 0 | 0 | 0 |
| | | 5.0 | 10 | 90 | 0 | 0 | 90 | | | 5.0 | 100 | 0 | 0 | 0 | 0 |
| | | 2.5 | 99 | 0 | 1 | 0 | 2 | | | 2.5 | 100 | 0 | 0 | 0 | 0 |
| | | 1.0 | 100 | 0 | 0 | 0 | 0 | | | 1.0 | 100 | 0 | 0 | 0 | 0 |
| | 20 | 10.0 | 100 | 0 | 0 | 0 | 0 | | 20 | 10.0 | 100 | 0 | 0 | 0 | 0 |
| | | 5.0 | 5 | 90 | 5 | 0 | 100 | | | 5.0 | 98.9 | 0 | 1 | 0.1 | 2.3 |
| | | 2.5 | 100 | 0 | 0 | 0 | 0 | | | 2.5 | 100 | 0 | 0 | 0 | 0 |
| | | 1.0 | 100 | 0 | 0 | 0 | 0 | | | 1.0 | 100 | 0 | 0 | 0 | 0 |

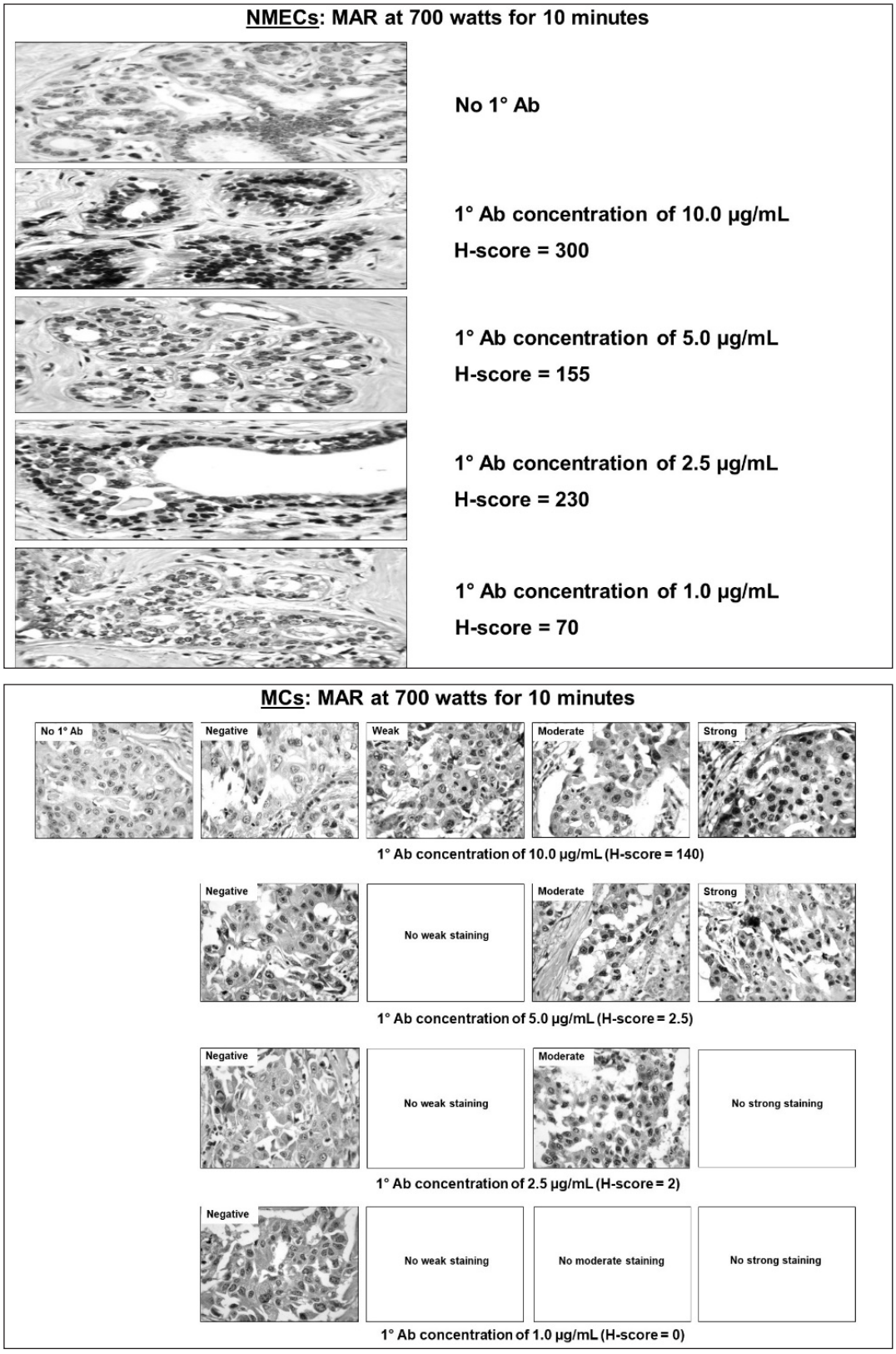


**Figure 1** Immunoexpression of EN1 protein in **(A)** normal mammary epithelial cells (NMECs) and **(B)** malignant cells (MCs) according to MAR at 700 watts for 10 minutes and EN1 antibody ($1^{\circ}$ Ab) concentrations of 1.0, 2.5, 5.0, and 10.0 µg/mL

immunoexpression of the target protein in the tissue section[42]. On the other hand, SCBS yields a higher nuclear immunostaining intensity of ER, PR, and Ki67 proteins and there is least background staining[40]. Regarding nuclear expression of EN1 protein[5], SCBS was used as AR solution in this study. If IHC without AR is performed, no positive staining is usually seen[40]. This study utterly applied MAR with NPA instead because the heating power and time of MAR and the concentration of 1° Ab were determined their optimal conditions of IHC.

For the formalin-fixed tissues, a higher temperature during HIAR causes pronounced breakage of the cross-links but tissue deterioration may also be developed[34,50,51]. The longer times of microwave heating, up to a maximum of 20 minutes possibly give a better immunostaining intensity. However, this likely leads to nonspecific background staining and some degradation in tissue architecture and morphology[40]. Hence, this study revealed that poor immunoexpression of both NMECs and MCs was correlated with the higher heating power with the longer duration of MAR and the lower concentration of 1° Ab. Since the FFPE tissue block used in this study was stored in room temperature for 3 years prior to the immunostaining, immunoreactivity might be affected by antigenic degradation during tissue storage. However, the effect on immunohistochemical staining of stored FFPE tissue sections depends on the types of antigen[52].

The immunohistochemical reactivity is actually influenced by many factors. The optimisation is the most important procedure for achieving the maximum immunostaining result[12,25,26,52]. This study first shows the optimal immunohistochemical staining conditions for the evaluation of *EN1* gene expression in the FFPE-TS-PIBC based on HIAR with a domestic 750-watt Samsung® microwave oven and 1x 10 mM SCBS of pH 6.0.

## Conclusion

An expression of *EN1* gene is distinctly detectable in the FFPE-TS-PIBC by MAR with SCBS at 700 watts for 10 minutes and using a 1° Ab concentration of 10.0 μg/mL.

## References


1. *Kohler A, Logan C, Joyner AL, Muenke M. Regional assignment of the human homeobox-containing gene EN1 to chromosome 2q13-q21. Genomics. 1993;15:233-5.*
2. *Sgaier SK, Lao Z, Villanueva MP, Berenshteyn F, Stephen D, Turnbull RK, et al. Genetic subdivision of the tectum and cerebellum into functionally related regions based on differential sensitivity to engrailed proteins. Development. 2007;134:2325-35.*
3. *Alves dos Santos MT, Smidt MP. En1 and Wnt signaling in midbrain dopaminergic neuronal development. Neural Dev. 2011;6:23-4.*
4. *EN1 engrailed homeobox 1 [Homo sapiens (human)]. 2017; Available at: https://www.ncbi.nlm.nih.gov/gene/2019. Accessed February 2017.*
5. *Narytnyk A, Verdon B, Loughney A, Sweeney M, Clewes O, Taggart MJ, et al. Differentiation of human epidermal neural crest stem cells (hEPI-NCSC) into virtually homogenous populations of dopaminergic neurons. Stem Cell Rev. 2014;10:316-26.*
6. *Beltran AS, Graves LM, Blancafort P. Novel role of Engrailed 1 as a prosurvival transcription factor in basal-like breast cancer and engineering of interference peptides block its oncogenic function. Oncogene. 2014;33:4767-7.*
7. *Duraiyan J, Govindarajan R, Kaliyappan K, Palanisamy M. Applications of immunohistochemistry. J Pharm Bioallied Sci. 2012;4(Suppl 2):S307-9.*
8. *Walker RA. Immunohistochemical markers as predictive tools for breast cancer. J Clin Pathol. 2008;61:689-96.*
9. *Bunea M, Zarnescu O. New current aspects on the immunohistochemical techniques. Roum Biotechnol Lett. 2001;6:177-206.*
10. *Ramos-Vara JA. Technical aspects of immunohistochemistry. Vet Pathol. 2005;42:405-26.*
11. *True LD. Quality control in molecular immunohistochemistry. Histochem Cell Biol. 2008;130:473-80.*
12. *Ramos-Vara JA. Principles and methods of immunohistochemistry. Methods Mol Biol. 2011;691:83-96.*
13. *Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem. 1991;39:741-8.*

14. *Boon ME, Kok LP. Microwaves for immunohistochemistry. Micron. 1994;25:151-70.*
15. *Taylor CR, Shi SR, Chaiwun B, Young L, Imam SA, Cote RJ. Strategies for improving the immunohistochemical staining of various intranuclear prognostic markers in formalin-paraffin sections: androgen receptor, estrogen receptor, progesterone receptor, p53 protein, proliferating cell nuclear antigen, and Ki-67 antigen revealed by antigen retrieval techniques. Hum Pathol. 1994;25:263-70.*
16. *Vitarelli E, Sippelli G, Tuccari G, Barresi G. The use of microwave irradiation for immunohistochemistry: a new methodological proposal. Histol Histopathol. 1995;10:35-8.*
17. *Taylor CR, Shi SR, Chen C, Young L, Yang C, Cote RJ. Comparative study of antigen retrieval heating methods: microwave, microwave and pressure cooker, autoclave, and steamer. Biotech Histochem. 1996;71:263-70.*
18. *Pileri SA, Roncador G, Ceccarelli C, Piccioli M, Briskomatis A, Sabattini E, et al. Antigen retrieval techniques in immunohistochemistry: comparison of different methods. J Pathol. 1997;183:116-23.*
19. *Krenacs L, Krenacs T, Raffeld M. Antigen retrieval for immunohistochemical reactions in routinely processed paraffin sections. Methods Mol Biol. 1999;115:85-93.*
20. *Ramos-Vara JA, Beissenherz ME. Optimization of immunohistochemical methods using two different antigen retrieval methods on formalin-fixed paraffin-embedded tissues: experience with 63 markers. J Vet Diagn Invest. 2000;12:307-11.*
21. *Shi SR, Cote RJ, Taylor CR. Antigen retrieval techniques: current perspectives. J Histochem Cytochem. 2001;49:931-7.*
22. *Tacha D, Teixeira M. History and overview of antigen retrieval: methodologies and critical aspects. J Histotechnol. 2002;25:237-42.*
23. *Yamashita S. Heat-induced antigen retrieval: mechanisms and application to histochemistry. Prog Histochem Cytochem. 2007; 41:141-200.*
24. *D'Amico F, Skarmoutsou E, Stivala F. State of the art in antigen retrieval for immunohistochemistry. J Immunol Methods. 2009; 341:1-18.*
25. *Qadir A, Mushtaq S, Sharif MA, Mamoon N, Khadim MT. Comparison of heat induced antigen retrieval techniques for immunehistochemical estrogen receptor expression in ductal carcinoma of breast in females. J Coll Physicians Surg Pak. 2010;20:519-23.*
26. *Shi SR, Shi Y, Taylor CR. Antigen retrieval immunohistochemistry: review and future prospects in research and diagnosis over two decades. J Histochem Cytochem. 2011;59:13-32.*
27. *Nicholson RI, Bouzubar N, Walker KJ, McClelland R, Dixon AR, Robertson JF, et al. Hormone sensitivity in breast cancer: influence of heterogeneity of oestrogen receptor expression and cell proliferation. Eur J Cancer. 1991;27:908-13.*
28. *Keller E, Goldman RD. Light microscopy. In: Spector DL, Goldman RD, editors. Basic Methods in Microscopy: Protocols and Concepts from Cells: A Laboratory Manual China: Cold Spring Harbor Laboratory Press; 2006:1-42.*
29. *McCarty KS, Jr, Miller LS, Cox EB, Konrath J, McCarty KS S. Estrogen receptor analyses. Correlation of biochemical and immunohistochemical methods using monoclonal antireceptor antibodies. Arch Pathol Lab Med. 1985;109:716-21.*
30. *McCarty KS, Jr, Szabo E, Flowers JL, Cox EB, Leight GS, Miller L, et al. Use of a monoclonal anti-estrogen receptor antibody in the immunohistochemical evaluation of human tumors. Cancer Res. 1986;46(Suppl 8):s4244-8.*
31. *Tadrous PJ. Breast. Diagnostic Criteria Handbook in HISTOPATHOLOGY: A Surgical Pathology Vade Mecum England: John Wiley & Sons; 2007:258-66.*
32. *Eltoum I, Fredenburgh J, Myers RB, Grizzle WE. Introduction to the Theory and Practice of Fixation of Tissues. J Histotechnol. 2001;24:173-90.*
33. *Arnold MM, Srivastava S, Fredenburgh J, Stockard CR, Myers RB, Grizzle WE. Effects of fixation and tissue processing on immunohistochemical demonstration of specific antigens. Biotech Histochem 1996;71:224-30.*
34. *Eltoum I, Fredenburgh J, Grizzle WE. Advanced Concepts in Fixation: 1. Effects of Fixation on Immunohistochemistry, Reversibility of Fixation and Recovery of Proteins, Nucleic Acids, and other Molecules from Fixed and Processed Tissues. 2. Developmental Methods of Fixation. J Histotechnol. 2001;24:201-10.*
35. *Shi SR, Key ME, Kalra KL. Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem. 1991;39:741-8.*
36. *Werner M, Von Wasielewski R, Komminoth P. Antigen retrieval, signal amplification and intensification in immunohistochemistry. Histochem Cell Biol. 1996;105:253-60.*
37. *Bussolati G, Leonardo E. Technical pitfalls potentially affecting diagnoses in immunohistochemistry. J Clin Pathol. 2008;61:1184-92.*
38. *D'Amico F, Skarmoutsou E, Stivala F. State of the art in antigen retrieval for immunohistochemistry. J Immunol Methods. 2009;341: 1-18.*
39. *Shi SR, Shi Y, Taylor CR. Antigen retrieval immunohistochemistry: review and future prospects in research and diagnosis over two decades. J Histochem Cytochem. 2011;59:13-32.*

40. *Taylor CR, Shi SR, Chaiwun B, Young L, Imam SA, Cote RJ. Strategies for improving the immunohistochemical staining of various intranuclear prognostic markers in formalin-paraffin sections: androgen receptor, estrogen receptor, progesterone receptor, p53 protein, proliferating cell nuclear antigen, and Ki-67 antigen revealed by antigen retrieval techniques. Hum Pathol. 1994;25:263-70.*
41. *Vitarelli E, Sippelli G, Tuccari G, Barresi G. The use of microwave irradiation for immunohistochemistry: a new methodological proposal. Histol Histopathol. 1995;10:35-8.*
42. *Pileri SA, Roncador G, Ceccarelli C, Piccioli M, Briskomatis A, Sabattini E, et al. Antigen retrieval techniques in immunohistochemistry: comparison of different methods. J Pathol. 1997;183: 116-23.*
43. *Ehara H, Deguchi T, Koji T, Yang M, Ito S, Kawada Y, et al. Autoclave antigen retrieval technique for immunohistochemical staining of androgen receptor in formalin-fixed paraffin sections of human prostate. Acta Histochem Cytochem. 1996;29:311-8.*
44. *Abreu CC, Nakayama PA, Nogueira CI, Mesquita LP, Lopes PF, Varaschin MS, et al. Domestic microwave processing for rapid immunohistochemical diagnosis of bovine rabies. Histol Histopathol. 2012;27:1227-30.*
45. *Kok LP, Boon ME. Microwaves for microscopy. J Microsc. 1990;158(Pt 3):291-322.*
46. *Boon ME, Kok LP. Microwaves for immunohistochemistry. Micron. 1994;25:151-70.*
47. *Tacha D, Teixeira M. History and overview of antigen retrieval: methodologies and critical aspects. J Histotechnol 2002;25:237-42.*
48. *Yamashita S. Heat-induced antigen retrieval: mechanisms and application to histochemistry. Prog Histochem Cytochem. 2007; 41:141-200.*
49. *Morgan JM, Navabi H, Jasani B. Role of calcium chelation in high-temperature antigen retrieval at different pH values. J Pathol. 1997;182:233-7.*
50. *Krenacs L, Krenacs T, Raffeld M. Antigen retrieval for immunohistochemical reactions in routinely processed paraffin sections. Methods Mol Biol. 1999;115:85-93.*
51. *Dapson RW. Macromolecular changes caused by formalin fixation and antigen retrieval. Biotechnic & Histochemistry. 2007;82:133-40.*
52. *Ramos-Vara JA, Webster JD, DuSold D, Miller MA. Immunohistochemical evaluation of the effects of paraffin section storage on biomarker stability. Vet Pathol. 2014;51:102-9.*