นิพนธ์ต้นฉบับ • Original Article

เปรียบเทียบเทคนิคการกู้คืนแอนติเจนในงานอิมมูโนฮิสโตเคมี

เอมอร พนมศรี[1], นิษณา นามวาท[2], ศักดา วราอัศวปติ[1], รุ่งเพ็ชร ตั้งรัศมีประเสริฐ[2], สุพินดา คูณมี[1]
[1]ภาควิชาพยาธิวิทยา, [2]ภาควิชาชีวเคมี, คณะแพทยศาสตร์, มหาวิทยาลัยขอนแก่น, ขอนแก่น 40002

Comparison of Antigen Retrieval Techniques in Immunohistochemistry

Em-orn Phanomsri[1], Nisana Namwat[2], Sakda Waraasawapati[1], Roongpet Tangrassameeprasert[2], Supinda Koonmee[1]
[1]Department of Pathology and [2]Biochemistry, Faculty of Medicine, Khon Kaen University, Khon Kaen, Thailand, 40002

วัตถุประสงค์: การศึกษานี้ มีวัตถุประสงค์ในการหาสารละลายและแหล่งให้ความร้อนที่เหมาะสมที่สุดในการกู้คืนแอนติเจนในงานย้อมอิมมูโนฮิสโตเคมีสำหรับแอนติบอดีที่มีอยู่หลากหลายในห้องปฏิบัติการ

วัสดุและวิธีการ: กลุ่มผู้วิจัยทำการทดสอบสารละลาย 3 ชนิด ได้แก่ 10mM Tris-HCl + 1mM EDTA, pH 9, 0.05% citraconic anhydride solution pH 7.4 และ 10 mM citrate buffer pH 6 และใช้แหล่งให้ความร้อน 3 แบบ (Microwave, pressure cooker และ water bath) ในการกู้คืนแอนติเจน 21 ชนิด ในชิ้นเนื้อที่ดองด้วยฟอร์มาลิน

ผลการศึกษา: จากการศึกษาพบว่า สารละลายดัดแปลง Tris-HCl + EDTA buffer, pH 9 เป็นสารละลายที่เหมาะสมกับแอนติบอดีหลายชนิดและไม่ขึ้นกับแหล่งให้ความร้อน ขณะที่ citraconic anhydride, pH 7.4 และ citrate buffer, pH 6 จะมีความเหมาะสมปานกลางและต่ำในการเป็นสารละลายที่ใช้ในการกู้คืนแอนติเจน ตามลำดับและยังขึ้นกับแหล่งให้ความร้อนในการกู้คืนแอนติเจนอีกด้วย

สรุป: จากการทดลองนี้แสดงให้เห็นว่า การให้ความร้อนด้วย 10mM Tris-HCl + 1mM EDTA, pH 9 ในการกู้คืนแอนติเจนเหมาะสมกับงานย้อมอิมมูโนฮิสโตเคมีสำหรับแอนติเจนต่างๆ ในชิ้นเนื้อที่ดองด้วยฟอร์มาลินและฝังในพาราฟิน

คำสำคัญ: การกู้คืนแอนติเจน, อิมมูโนฮิสโตเคมี, citraconic anhydride, formaldehyde

Objective: The study aimed to find the best antigen retrieval buffer and heat source for the immunohistochemical staining of various kind of antibodies in our laboratory.

Materials and methods: We designed the method to evaluate the efficacy of three different retrieval solutions including 10mM Tris-HCl + 1mM EDTA, pH 9, 0.05% citraconic anhydride solution pH 7.4 and 10 mM citrate buffer pH 6, and 3 heat source pretreatment methods (Microwave, pressure cooker and water bath treatment) to retrieve twenty-one immunoreactivity in formalin-fixed, paraffin-embedded sections.

Results: We found that, modified retrieval solution, 10mM Tris-HCl + 1mM EDTA buffer, pH 9 is the most efficient for a large variety of antibodies and not depending on heat sources. On the other hand, 0.05% citraconic anhydride solution and 10mM citrate buffer are moderate and poor retrieval solutions, respectively. Moreover, these two solutions are heat source-dependent.

Conclusion: These results demonstrate that 10mM Tris-HCl + 1mM EDTA, pH 9 and heat-pretreatment is useful for the immunohistochemistry of many antigens in aldehyde-fixed, paraffin-embedded tissues.

Keywords: antigen retrieval, immunohistochemistry, citraconic anhydride, formaldehyde

ศรีนครินทร์เวชสาร 2552; 24(2): 130-6. • Srinagarind Med J 2009; 24(2): 130-6

Introduction

Preservation of tissue morphology and restoration of immunoreactivity of tissue antigen are the most important processes in immunohistochemical (IHC) study using formalin fixed, paraffin-embedded (FFPE) tissues. The loss of antigenicity is often caused by sequential processes of fixation, dehydration and embedding in paraffin-embedded tissue[1]. Formaldehyde is a commonly used fixative for tissue preservation in pathological laboratories due to it is inexpensive and it produces morphology that pathologists are familiar to. Formaldehyde preserves mainly peptides and the general structure of cellular organelles. It also interacts with nucleic acids but has little effect on carbohydrate. Formalin containing calcium can be a good preservative of lipids[2]. However, a major adverse effect of this fixative is the hiding of tissue antigens by protein cross-linking[3]. The restoration of antigenicity by heat treatment was firstly reported by Shi and co-worker[4]. After this finding, heat-induced antigen retrieval (HIAR) becomes a common technique in IHC study. This method can revert the reaction between formalin and proteins that blocks antibody recognition and heat by microwave irradiation has a simplified immunohistochemical staining protocols[5]. The mechanisms of HIAR have been described that heat cleaves crosslinks (methylene bridges) and adds methylol groups into formaldehyde-fixed proteins and extends polypeptides to unmask epitopes hidden in the inner portion of antigens or covered by adjacent macromolecules[6]. Although heat is believed to be the primary mechanism in retrieval of antigens, a variety of chemical solutions and heat source have been tested to define an optimal antigen retrieval (AR) solution for influencing the quality of immunostaining[7]. Citrate buffer, pH 6.0 with microwave irradiation (the conventional protocol) is effective for many antibody/antigen recognitions[8]. In general, pathological laboratory may often have a lot of antibodies used for diagnosis and research. Variation in kind of antibodies may produce an effect of false-positive or false-negative result when AR method is inappropriate. While citrate buffer that is the most commonly used protocol is effective for many antibody/antigen recognitions, but in some occasions, this standard approach is unsuccessful. Under these circumstances, a successful antigen retrieval protocol may still be established by varying key conditions in the antigen retrieval process[8]. Variation in the solution media, buffer equilibrium, temperature and heat source have been used to adjust for successful AR-IHC by different laboratories. Several investigators showed that, Tris-HCl buffer, pH 9 in pressure cooker is satisfactory for AR of most antibodies employed in routine surgical pathology[9, 10]. In addition, using 1 mM EDTA, pH 8.0 also showed the good result including staining intensity, number of positive cells and number of antibodies[11]. Recently, Namimatsu reported a novel retrieval protocol that citraconic anhydride (a reversible protein cross-linking agent) solution with heating under optimal condition was able to satisfactorily retrieve a wide variety of antigens for IHC[3]. This new protocol has advantage including superior morphological preservation, greater reproducibility, and more intense staining after retrieval. Those previous studies demonstrates the importance of comparing various AR protocols to obtain maximal IHC for standardization and for quantitative IHC[3, 12, 13]. In this study, we modify an antigen retrieval method and compare this method with 2 different antigen retrieval techniques for immunoperoxidase staining of paraffin-embedded tissue that suitable with several antibodies.

Materials and Methods

Reagents and tissue specimen

Table 1 shows the antibodies used in this study and their detail. Human tissue from various organ including tonsil, breast, thymus and positive tumor for each antibody were obtained from the Department of Pathology, Faculty of Medicine, Khon Kaen University, Thailand. All specimens were fixed in 10% buffer formalin and embedded in paraffin for routine diagnosis. Serial sections were obtained from each block cut a thickness of 3 micron and attached to silan-coated slides. The sections were deparaffinized and rehydrated in a graded series of ethanol and soaked in 3% hydrogen-peroxide in methanol for 5 min to block endogenous enzyme activity and washes for 5 min with PBS.

Antigen retrieval (AR) method

After wash in PBS, antigen retrieval method was performed using following conditions:

Microwave treatment - 10 mM citrate buffer, pH 6.0, 10 mM Tris-HCl buffer + 1mM EDTA, pH 9, 0.05% citraconic anhydride solution, pH 7.4 were used as retrieval

solution. Hydrated sections were immersed in each solution and process in microwave oven set power at level 10 for 3 min and level 3 for 10 min : 200 ml of each retrieval solution was put into a plastic jar and the same number slide (n=20) were always placed. After the completion of cycle, sections were allowed to cool for 20 min at room temperature and then rinse in running tap water, distilled water and PBS.

Pressure cooker - One liter of each solution was placed in a stainless steel 6-litre capacity pressure cooker with an operating pressure of 10 psi and brought to the boil on the 1500 w electric hotplate. Hydrate sections were plated in metal racks and lowered into the boiling retrieval solution.

The pressure cooker was then sealed and brought to full pressure. The heating time began when full pressure was reached and heating time was 3 min. After that the cooker was depressurized and cool under running tap water; the lid was then removed and sections were allowed to cool for 20 min at room temperature. The cooled sections were washed in running tap water, distilled water and PBS, prior to immunohistochemical staining.

Water bath - 200 ml of each solution was put into a plastic jar placed into water bath that contained distilled water. Retrieval solution level was equal to the water level in a bath and then set temperature of water bath at 95°C. In constant temperature (95°C), hydrated sections were immersed into retrieval solution and incubate for 45 min. The sections were allowed to cool for 20 min at room temperature and then washed in running tap water, distilled water and PBS, prior to immunohistochemical staining.

Immunohistochemistry

After pretreatment and washes with PBS, the sections were treated with PBS containing 3% normal horse serum at room temperature for 20 min, and then incubation with the primary antibodies for 1 hr. After washing with PBS, the sections were incubated with labeled polymer horseradish peroxidase-conjugated supersensitive kit (Biogenex, San Ramon, CA) for 50 min and the color was developed with 0.1% diaminobenzidine tetrahydrochloride (DAB) solution. The sections were then counterstained lightly with Mayer's Haematoxylin, rinsed in water for 3-4 min, dehydrated, cleared and mounted in Permount®.

Interpretation

Interpretation of immunostained sections were performed blindly without the knowledge of AR methods used. We defined the immunostaining by the mean percentage of immunostained area and classified as 0%, -; <5%, +/-; 5-25%, 1+; 26-50%, 2+; and >50%, 3+. The intensity of staining was judged as weak, moderate and strong.

Results

Table 2 and Table 3 summarize the results of immunostaining with different antibodies by different AR solution and heat source.

Effect of antigen retrieval solutions on HIAR

Immunostaining results from paraffin-embedded sections of various tissues by using three different antigen retrieval buffers; shown in Table 2. 10mMTris-HCl + 1mM EDTA pH 9 demonstrated that, 21 of 21 (100%) antibodies were shown the best result (the best result in term of both staining intensity and the number of positive cell). One antibody, TdT shown modulates intensity. In the other buffers, 10 mM citrate buffer pH 6 show 15 of 21 (71%) for the best results, 3 antibodies show weak positive whereas Bcl-6, TdT and CD4 were negative. 0.05% citraconic anhydride solution pH 7.4 show 17 of 21 (81%) for the best results, 3 antibodies show weak positive and 1 antibody, CD23 was negative. When we divided antigens to three groups according to localization of antigens expressed including nuclear antigens, cytoplasmic and intermediate filament antigen and membrane antigens, we found that all of cytoplasmic and intermediate filament antigens are effectively unmask by using three solutions. On the other hand, nuclear antigens and membranes antigens need the optimal buffer and heat source to restore the antigenicity. 10mM Tris-HCl + 1mM EDTA pH 9 produced the best efficient with the large variety of antigens. It is indicate the efficiency of 10mMTris-HCl + EDTA pH 9 to unmask antigen independently of location of the target molecule (nuclear antigen, cytoplasmic antigen or membrane antigen).

Effect of heat sources on HIAR

In different heat source (MW, PC and WB), we found that the result of AR with 10 mM Tris-HCl buffer + 1mM

Table 1 List of antibody

Antibody	Clone/species	Type of antibody	Source	Antibody dilution
BCL-6	PG-B6p	Mono (mouse)	Dako	1:100
Ki-67	MIB1	Mono (mouse)	Dako	1:1000
TdT		Poly (rabbit)	Cell marque	1:100
PCNA	PC10	Mono (mouse)	Dako	1:5000
CD3	Code A0452	Poly (rabbit)	Dako	1:500
CD4	NCL-CD4-368	Mono (mouse)	Novocastra	1:400
CD8	C8/144B	Mono (mouse)	Dako	1:200
CD10	NCL-cd10-270	Mono (mouse)	Novocastra	1:200
CD20	L26	Mono (mouse)	Dako	1:1000
E-cadherin	NCH-38	Poly (rabbit)	Dako	1:500
EMA	E29	Mono (mouse)	Dako	1:1000
CK7	OV-TL 12/30	Mono (mouse)	Dako	1:500
CK20	Ks20.8	Mono (mouse)	Dako	1:500
CK8	35bH11	Mono (mouse)	Dako	1:500
CK pan	AE1/AE3	Mono (mouse)	Dako	1:500
CD7	CBC37	Mono (mouse)	Dako	1:200
CD23	MHM6	Mono (mouse)	Dako	1:150
TFF-1	8G7G3/1	Mono (mouse)	Dako	1:500
Desmin	NCL-DES-ERII	Mono (mouse)	Novocastra	1:1000
Vimentin	3B4	Mono (mouse)	V9	1:1000
BCL-2	124	Mono (mouse)	Dako	1:1000

Table 2 Comparison of immunostaining using different antigen retrieval methods

Antibody	Citrate buffer	Tris-HCL + EDTA	Citraconic anhydride
Bcl-6	+/-,w	+++,s	++,m
Ki-67	+++,m	+++,s	+++,s
TdT	+/-,w	+++,m	+++,s
PCNA	+++,s	+++,s	+++,s
CD23	++,m	+++,s	+,w
CD3	+++,s	+++,s	+++,s
CD4	+/-,w	+++,s	+++,m
CD8	+++,s	+++,s	+++,s
CD10	+++,m	+++,s	+++,s
CD20	+++,s	+++,s	+++,s
E-cadherin	+++,s	+++,s	+++,s
EMA	+++,s	+++,s	+++,s
CK7	+++,s	+++,s	+++,s
CK20	+++,s	+++,s	+++,s
CK8	+++,s	+++,s	+++,s
CK pan	+++,s	+++,s	+++,s
TFF-1	+++,m	+++,s	+++,s
CD7	++,w	+++,s	++,w
BCL-2	+,w	+++,s	++,w
Desmin	+++,s	+++,s	+++,s
Vimentin	+++,m	+++,s	+++,s

w = weak intensity; m = moderate intensity; s = strong intensity; CK = cytokeratin

- = complete negative; +/- = immunostaining area < 5%; + = immunostaining area 5-25%; ++ = immunostaining area 25-50%; +++ = immunostaining area >50%

Table 3 Comparison of heat source

Antidodies	Citrate buffer			Tris-EDTA			Citraconic anhydride		
	MW	PC	WB	MW	PC	WB	MW	PC	WB
BCl-6	+/-,w	+++,s	++,w	+/-,w	+++,s	+++,s	+,w	-	++,w
Ki-67	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s
TdT	-	+,w	+,w	+,w	+++,m	+++,m	++,w	+,w	+++s
PCNA	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s
CD23	++,m	++,m	++,s	+,s	+++,s	+++,s	-	-	+/-,w
CD3	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++.s	-	+++,s
CD4	+/-,w	++,w	+,w	++,w	+++,s	+++,s	++,w	-	++,m
CD8	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+,w	+++,s
CD10	+++,m	++,w	+++,m	+++,m	+++,s	+++,s	+++,w	-	+++,s
CD20	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s
E-cadherin	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s
EMA	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s
CK7	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s
CK8	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s
CK20	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s
CK pan	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s
TFF-1	+++,m	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s
CD7	+,w	-	++,s	+++,m	+++,s	+++,s	+/-,w	+/-,w	++,w
BCL-2	+,w	++,w	+,w	+++,m	+++,s	+++,s	++,w	+,w	++,w
Desmin	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s
Vimentin	+++,m	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s	+++,s

MW = microwave; PC = pressure cooker; WB = water bath; w = weak intensity; m = moderate intensity; s = strong intensity; CK = cytokeratin
- = complete negative; +/- = immunostaining area < 5%; + = immunostaining area 5-25%; ++ = immunostaining area 25-50%; +++ = immunostaining area >50%

EDTA, pH 9 showed similar results in term of positive area and intensity when we used PC and WB, but in MW BCL-6, CD4 and CD23 showed weak positive. Thus, MW is unsuitable for unmasking these antigens. 0.5% citraconic anhydride solution, pH7.4 in WB showed the best results and similary to in MW but in PC, all of nuclear and membrane antigens showed weak positive or negative, indicated that 0.05% citraconic anhydride solutions in unsuitable for overheat treatment (over than 100°C). On the other hand, 10 mM citrate buffer, pH6 in different heat sources depends on antigen that we detected (Table 3). For BCL-6 antigen, it is suitable with citrate and Tris-HCl buffer when we used PC as heat source. However, most methods are suitable for cytoplasmic and intermediate filament antigens.

Different groups of antigens such as nuclear antigens, membrane antigens and cytoplasmic and intermediate filament antigens showed enhanced reaction. Also, several difficult to detect antigens including, BCL-6, CD4, CD23, TdT, CD7 and BCL-2 revealed distinct staining results when using different conditions.

Discussions and conclusions

Although several fixatives have been used in routine pathology laboratory, but formaldehyde is a commonly used because it is inexpensive and produces morphology that pathologists are familiar to. Formaldehyde preserves mainly peptides and the general structure of cellular organelles; it also interacts with nucleic acids but has little effect on carbohydrate. It is a good preservative of lipids if the fixative contains calcium[2]. However, the defect of this fixative is protein cross-linking. In solution form, formaldehyde is capable of binding the following amino acids: lysine, tyrosine, asparagines, histidine, arginine, cysteine, and glutamine. The basic mechanism of fixation with formaldehyde is the formation of addition products between the formalin and uncharged reactive amino groups (-NH or NH_2), forming

cross-links. The cross-linking of critical side chains of amino acids is thought to result in selective masking of different epitopes of different antigens, with subtle alterations in the overall 3-dimensional shape of the protein molecule. In addition to inducing cross-linking, formaldehyde also disrupts hydrogen bonds and other electrostatic interactions that affect the configuration of proteins, further increasing the possibility of important alterations to epitopic targets. As antibodies are mostly raised to proteins in their native conformation state, they may not bind effectively to target polypeptides that have such structural modification, so-called antigen masking[14].

Heat-induced antigen retrieval (HIAR) techniques can reverse reactions between formalin and proteins that block antibody recognition. Interactions between antibodies and antigens are further enhanced by microwave irradiation, which has simplified immunohistochemical staining protocols[5]. The mechanisms of HIAR have been suggested that heating cleaves crosslinks (methylene bridges) and adds methylol groups in formaldehyde-fixed proteins and nucleic acids and extends polypeptides to unmask epitopes hidden in the inner portion of antigens or covered by adjacent macromolecules[6]. Citrate buffer, pH 6.0 with microwave irradiation (the conventional protocol) is effective for many antibody/antigens recognition[8]. Variation in the solution media, buffer equilibrium, temperature and heating source have been used to adjust for successful AR_IHC by different laboratories.

In this study, we compared AR solution and heat sources to test a wide variety of antibodies, we found that, 10 mM Tris-HCl + 1 mM EDTA buffer, pH 9 by any heat sources are suitable for retrieving various antibodies in our laboratory. This result agreed with a previous report. Torlakovic et al, used heat-induced epitope retrieval in an alkaline buffer, 0.1 mmol/L of Tris-EDTA buffer, pH 9, which was seemed mandatory for the optimal detection of CyD1 expression[15] EDTA-Tris buffers with pH 9.0 is better than other low pH retrieval buffers for the antigen retrieval of Ki-67, P504S and P63[16]. Emoto and co-worker demonstrated the effect of ionic strength on HIAR, they found that 10 mM Tris-HCl + 25 mM NaCl show stronger immunostaining than NaCl-free Tris-HCl buffer in basic condition[9]. Other other report, sections heated in Tris-HCl buffer, pH9 exhibited much strong immunoreaction than other low pH retrieval buffers[16, 10]. On the other hand, when compared Tris-HCl buffer with EDTA buffer in basic condition, its showed the good result including staining intensity, number of positive cells and number of antibodies in EDTA buffer than Tris-HCl buffer[17]. Our report demonstrated that when we added EDTA into Tris-HCl buffer in alkaline condition it enhanced the restore of any antigenicity. EDTA is the most well known chelating agent, and its AR enhancing effect was shown to be mediated by chelation of Ca^{2+}[18]. When heating paraffin sections in solution contain $CaCl_2$, the coordinate bonds between calcium ions and the methyol groups of protein introduced by formaldehyde treatment are formed a cage-like structure and preventing antigen-antibody interactions demonstrated by Ki-67 immunostaining being suppressed. However, when heated these sections with solution containing EDTA Ki-67 immunoreactivity were restored. In addition, Arber et al., used decalcifying agents, EDTA before detecting mRNA polyadenylated sequences performed on non-neoplastic and neoplastic decalcified, paraffin-embedded tissues by In situ hybridization (ISH) using a poly d(T) oligonucleotide probe to assess the efficacy of this molecular diagnostic tool on decalcified tissue samples. ISH can be performed with success on decalcified, paraffin-embedded tissues when using, an EDTA-based agent when compared with the results from the nondecalcified control samples[19]. However, whether the effect of combined use of EDTA and salt is related to the Ca^{2+}-chelating effect of EDTA is not certain[17]. Recently, Namimatsu reported a novel retrieval protocol that citraconic anhydride (a reversible protein cross-linking agent) solution with heating under optimal condition was able to satisfactorily retrieve a wide variety of antigens for IHC[3]. In our study, citraconic anhydride solution at neutral pH is a second best retrieval solution when used with water bath and set temperature to 95°C. In our condition there is no temperature control probe, thus the temperature may not constant in this study. As Namimatsu's condition, they used an electric kitchen pot, capable of maintaining a constant temperature and set constant warm temperature at 98°C. This reason may cause the result of our group not in agreement with previous report. We found citraconic anhydride solution be suitable for WB and MW but not for PC. It indicated that, in high temperature (more than 100°C) citraconic anhydride is a poor retrieval solution. In our study, conventional retrieval buffer, 10mM citrate, pH 6 is

a poor retrieval property. However, heat source or temperature is an important key when we used conventional buffer. In conclusion, modified retrieval solution, Tris-HCl + EDTA buffer, pH 9 is the most efficient for a large variety of antibodies and independent on heat source and localization of antibody. On the other hand, citraconic anhydride solution and citrate buffer are intermediate and poor retrieval solution, respectively. However, these two solutions are depending on heat source and localization of antibodies.

References

1. Ezaki T. Antigen retrieval on formaldehyde-fixed paraffin sections: its potential drawbacks and optimization for double immunostaining. Micron 2000; 31:639-49.
2. Ramos-Vara JA. Technical aspects of immunohistochemistry. Vet Pathol 2005; 42:405-26.
3. Namimatsu S, Ghazizadeh M, Sugisaki Y. Reversing the effects of formalin fixation with citraconic anhydride and heat: a universal antigen retrieval method. J Histochem Cytochem 2005; 53:3-11.
4. 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.
5. Long DJ, 2nd, Buggs C. Microwave oven-based technique for immunofluorescent staining of paraffin-embedded tissues. J Mol Histol 2008; 39:1-4.
6. Yamashita S. Heat-induced antigen retrieval: mechanisms and application to histochemistry. Prog Histochem Cytochem 2007; 41:141-200.
7. Shi SR, Imam SA, Young L, Cote RJ, Taylor CR. Antigen retrieval immunohistochemistry under the influence of pH using monoclonal antibodies. J Histochem Cytochem 1995; 43:193-201.
8. Shi SR, Cote RJ, Liu C, Yu MC, Castelao JE, Ross RK, et al. A modified reduced-temperature antigen retrieval protocol effective for use with a polyclonal antibody to cyclooxygenase-2 (PG 27). Appl Immunohistochem Mol Morphol 2002; 10:368-73.
9. Emoto K, Yamashita S, Okada Y. Mechanisms of heat-induced antigen retrieval: does pH or ionic strength of the solution play a role for refolding antigens? J Histochem Cytochem 2005; 53:1311-21.
10. Yamashita S, Okada Y. Application of heat-induced antigen retrieval to aldehyde-fixed fresh frozen sections. J Histochem Cytochem 2005; 53:1421-32.
11. 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.
12. Shi SR, Liu C, Young L, Taylor C. Development of an optimal antigen retrieval protocol for immunohistochemistry of retinoblastoma protein (pRB) in formalin fixed, paraffin sections based on comparison of different methods. Biotech Histochem 2007; 82:301-9.
13. Takizawa T, Eguchi H, Namimatsu S, Jeschke U, Fuchs R, Robinson JM. Histochemistry for placenta research: theory and application. J Nippon Med Sch 2007; 74:268-73.
14. Leong TY, Leong AS. How does antigen retrieval work? Adv Anat Pathol 2007; 14:129-31.
15. Torlakovic E, Nielsen S, Vyberg M. Antibody selection in immunohistochemical detection of cyclin D1 in mantle cell lymphoma. Am J Clin Pathol 2005; 124:782-9.
16. Du J, Shi XY, Zheng J, Zhou M, Cui X. [Antigen retrieval immunohistochemistry under the influence of pH value and time]. Beijing Da Xue Xue Bao 2005; 37:195-7.
17. Kim SH, Kook MC, Song HG. Optimal conditions for the retrieval of CD4 and CD8 antigens in formalin-fixed, paraffin-embedded tissues. J Mol Histol 2004; 35:403-8.
18. 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.
19. Arber JM, Weiss LM, Chang KL, Battifora H, Arber DA. The effect of decalcification on in situ hybridization. Mod Pathol 1997; 10:1009-14.