

## **A comparison study of histochemical staining of various tissues after Carnoy's and formalin fixation**

Hussain Gadelkarim Ahmed, Ahmed Ibn idris Mohammed,  
Mohammed Omer Mohammed Hussein.

### **Abstract**

**Background:** The potential problems related to the use of formalin in histopathology, such as health hazards, deterioration of nuclei acids are well-known. The aim of this study was to evaluate the utilization of a Carnoy's solution fixation in comparison with formalin on subsequent tissue sectioning and histochemical staining.

**Materials and Methods:** Corresponding sections of 25 tissue biopsies of rabbit's different organs were fixed in Carnoy's solution and in 10% neutral buffered formalin. Samples were processed using the conventional method and then stained applying five histochemical methods. The degree of the quality of the staining was assessed for each method by scoring system (1-10) depending on comparison of the stained tissue sections with illustrated photomicrographs.

**Results:** For the quality of cutting, the best quality was obtained by Formalin (mean = 4.76) then Carnoy's fixative (mean = 3.84). The best quality of Haematoxylin and Eosin staining was obtained by formalin (mean = 5.28) then Carnoy's (mean = 4.00). For Alcian blue and Perl's Prussian blue, the best staining qualities were obtained by Formalin (mean = 4.76 and 5.64 respectively) followed by Carnoy's (mean = 2.88 and 3.92 respectively). For periodic Acid Schiff's the best staining quality was obtained following Carnoy's fixation (mean = 4.52) then, the formalin (mean = 3.76).

**Conclusion:** Although, Carnoy's fluid is a safe fixative and can rapidly penetrate the tissues, but it can't be a substitute for formalin.

**Keywords:** Haematoxylin, photomicrographs, Eosin staining.

**I**deal fixative must preserve cells and tissue constituents in as close as life-like state as possible while allowing them to undergo further preparative procedures without change<sup>1</sup>. With the advent of technology for developing staining techniques several reviews have discussed extensively the effects of fixation and fixatives on subsequent processing procedures<sup>2,3</sup>. Fixation depends on the coefficient of diffusibility of the fixative and the rate at which it reacts with the tissue components<sup>4</sup>. In general, the higher the coefficient of diffusibility the better the fixative. To facilitate more uniform penetration of the fixative, it is imperative to fix small volumes of tissues (5 mm to 1 cm). The volume of fixative should be in excess of 20 times the volume of the tissue<sup>5</sup>.

The speed of fixation depends on the rate of diffusion of fixative into the tissue and the rate of chemical reactions with various components<sup>6</sup>. In practice, it is assumed that these processes require at least one hour per mm of tissue thickness, but routinely the tissues are fixed for 24 to 48 hours. Although the relatively broad time range presumably has no effect on histopathology, the longer duration of fixation adversely affects the quality of tissue DNA<sup>7</sup>. The rate-limiting step in formaldehyde fixation is the binding of carbonyl formaldehyde to the tissues<sup>8</sup>. Immediate microwave irradiation of tissues for 1 to 2 minutes to 60°C has been shown to better preserve nucleic acids presumably because of reduced enzymatic degradation and enhanced fixation<sup>9</sup>. The average size of DNA extracted from tissues fixed in buffered formalin decreases with increasing fixation time. Tissues fixed in buffered formalin for 3 to 6 hours yield greater amounts of high-molecular weight DNA<sup>10</sup>.

---

Department of Histopathology and Cytology, Faculty of Medical laboratory Sciences, University of Khartoum, Khartoum, Sudan;

**Correspondence:** Hussaingad1972@yahoo.com

Aldehydes have various toxicity problems. Formaldehyde exposure was associated with an increased risk for Hodgkin lymphoma multiple myeloma and leukemia, particularly myeloid leukemia, that increased with peak and average intensity of exposure<sup>11</sup>. In an attempt to over-come these, there have been various efforts made to devise a non-aldehydes containing fixatives for general purposes. However, there have been several suggestions, but none has been satisfactory as a general purpose fixative for histopathology<sup>12</sup>.

Carnoy's fixative is rapid in action and may be used for urgent specimens for paraffin processing within five hours; tissue fixed in Carnoy's for one hour can be transferred to absolute alcohol to start tissue processing<sup>12</sup>. Therefore, in this study we tried the possibility of using Carnoy's as routine fixative for histopathology.

#### Materials and Methods

Twenty five Parallel tissue biopsies of similar dimensions,  $2 \times 2 \times 0.3$  cm were taken from rabbit's different organs (Spleen, Liver, Intestine, Kidney and others) and fixed in Carnoy's fixative and 10% neutral buffered formalin. Carnoy's fixative is composed of ethanol, chloroform and glacial acetic acid. All samples were immersed in fixative within 30 minutes of surgery. Immersion time was similar for both fixatives for each specimen (12 hours for Carnoy's and 24 hours for formalin). Carnoy's and Formalin-fixed samples were then processed using a conventional 24 hours schedule tissue processing method (A tissue processing machine (Leica 2000)). Paraffin-wax embedded tissue blocks were produced and sectioned by a Rotary Microtome to obtained 5 micron tissue sections. The quality of tissue sectioning was assessed for each tissue block depending on the following criterion; softness, releasing of serial sections, complete section, absence of scratching and thickness. Each 25 tissue sections (obtained from 25 tissue biopsies of different organs) were stained separately using one of the following histochemical staining methods; H and E, Alcian blue, Perl's Prussian blue and PAS.

This means that 50 sections (25 fixed in formalin and 25 in Carnoy's) were stained by each staining method. The degree of the quality of the staining was assessed for each method by scoring system (1-10) depending on comparison of the stained tissue sections with illustrated photomicrographs<sup>13</sup>.

The study was approved by Sudan University for Science and Technology, Faculty of Medical Laboratory Science Research Board.

#### Results

In this study, the subsequent qualities of tissue sectioning and histochemical staining following 10% neutral buffered Formalin and Carnoy's fixatives were assessed in tissues obtained from different organs of a rabbit.

The mean count for each procedure with its corresponding fixative was calculated from 50 sections. For quality of cutting, the best quality was obtained by buffered Formalin (mean = 4.76) then Carnoy's fixative (mean = 3.84). The quality of (H and E) staining was obtained by buffered formalin (mean = 5.28) then Carnoy's (mean = 4.00). For the Alcian blue and pearl's Prussian blue, the best staining qualities were obtained by Formalin (mean = 4.76 and 5.64 respectively) followed by Carnoy's (mean = 2.88 and 3.92 respectively). For periodic Acid Schiff's the best staining quality was obtained following Carnoy's fixation (mean = 4.52) then, the buffered formalin (mean = 3.76), as shown in Table.1.

Table 1: Distribution of fixatives efficiency means count by subsequent procedures

Fixative subsequent procedures	F	C
Quality of cutting	4.76	3.84
Quality of staining by H&E	5.28	4.00
Quality of staining by Alcian blue	4.76	2.88
Quality of staining by PAS	3.76	4.52
Quality of staining by Perl's Prussian blue	5.64	3.92

F= Formalin, C= Carnoy's.

In regard to the degree of the quality of the procedure following fixation, very good

qualities of sectioning were obtained in 9 (36%) and 5(20%) after Formalin and Carnoy's fixatives respectively. Hence, good sectioning qualities were identified in 3 (12%) and 6(24%) after formalin and Carnoy's correspondingly. Furthermore, acceptable sectioning qualities were detected in 13(52%) and 14(56%) following formalin and Carnoy's fixative in this order, as indicated in Figure. 1.

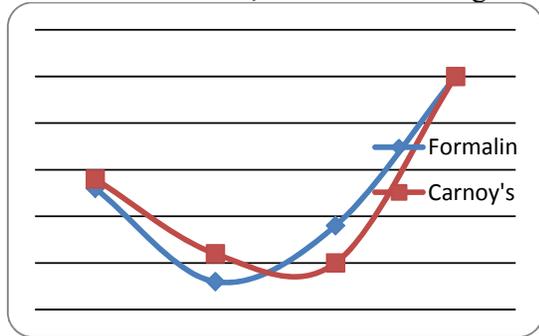


Figure1. Degrees of the quality of tissue sectioning and (H&E) after 10% neutral buffered formalin and Carnoy's fixatives.

Very good qualities of staining by Haematoxylin and Eosin (H & E) were obtained in 10 (40%) and 7(28%) after Formalin and Carnoy's fixatives respectively. Hence, good staining qualities were identified in 4 (16%) and 2(8%) after formalin and Carnoy's correspondingly. Acceptable staining qualities were detected in 11(44%) and 16(64%) following formalin and Carnoy's

fixative in this order, as indicated in Figure2.

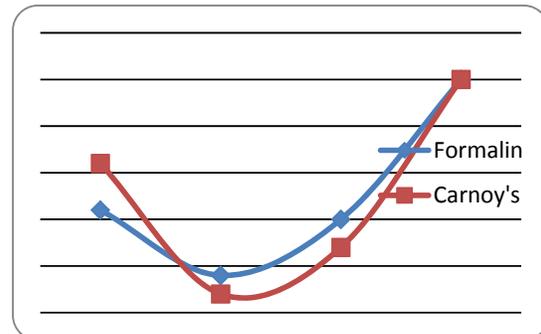


Figure.2. Degrees of the quality (H&E) staining after 10% neutral buffered formalin and Carnoy's fixatives.

Very good qualities of staining by Alcian blue were obtained in 10 (40%) and 3(12%) after Formalin and Carnoy's fixatives respectively. Hence, good staining qualities were identified in 1 (4%) and 4(16%) after formalin and Carnoy's correspondingly. Acceptable staining qualities were detected in 14(56%) and 18(72%) following formalin and Carnoy's fixative in this order, as shown in Figure3. Also, very good qualities of staining by Perl's Prussian blue were obtained in 12 (42%) and 0 (0%) after Formalin and Carnoy's fixatives respectively. Hence, good staining qualities were identified in 2(8%) and 7(28%) after formalin and Carnoy's correspondingly, as shown in Figure.4.

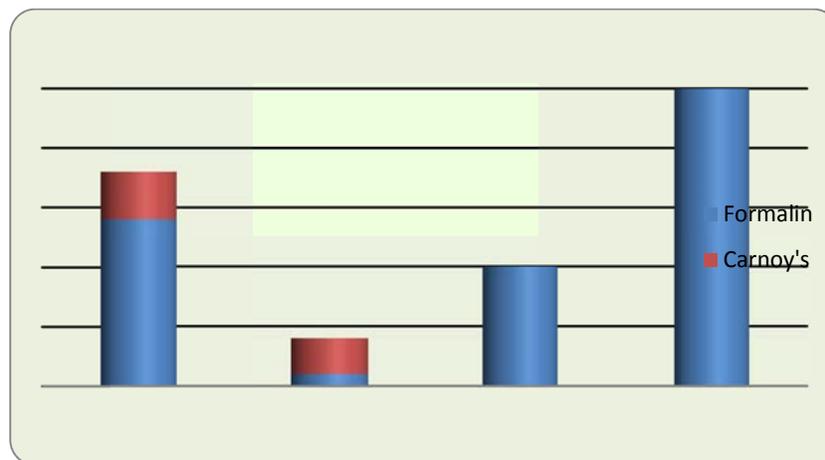


Figure. 3. Degrees of the staining quality of Alcian blue after 10% neutral buffered formalin and Carnoy's fixatives.

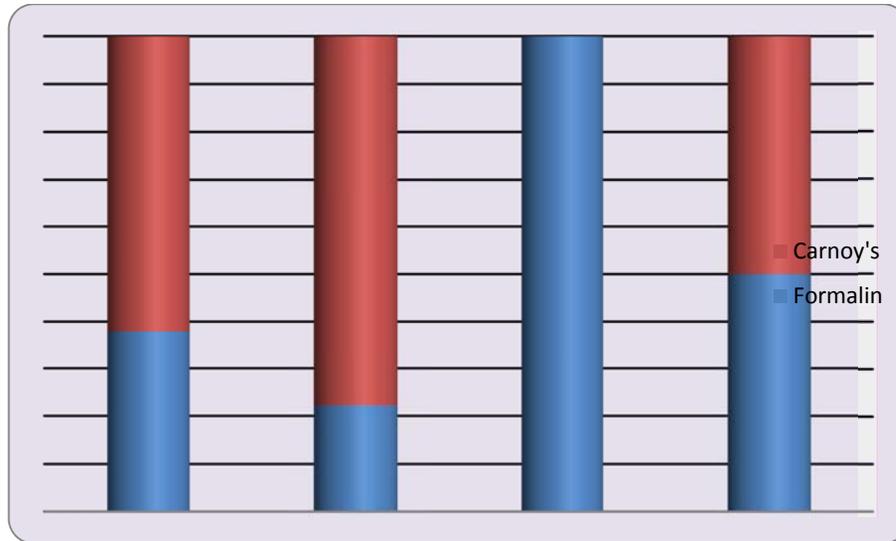


Figure.4. Degrees of the staining quality of Perl's Prussian blue after 10% neutral buffered formalin and Carnoy's fixatives.

Furthermore, acceptable staining qualities were detected in 11(44%) and 18(72%) following formalin and Carnoy's fixative in this order. Moreover, very good qualities of staining by Periodic Acid Schiff's (PAS) Diastase were obtained in 9 (36%) and 6(24%) after Carnoy's and Formalin fixatives respectively. Hence, good staining qualities were identified in 2 (8%) and 3(12%) after Carnoy's and formalin correspondingly. Acceptable staining qualities were detected in 14(56%) and 16(64%) following Carnoy's and formalin fixative in this order, as shown in Figure.5.

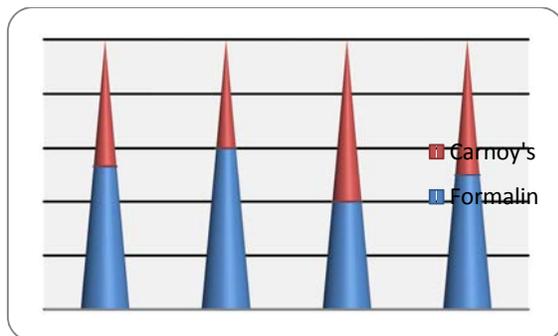


Figure.5. Degrees of the staining quality of PAS-Diastases after 10% neutral buffered formalin and Carnoy's fixatives.

**Discussion**

The aim of fixation is to preserve tissue components from the effects of autolysis, putrefaction and changes caused by the reagents used in various histological processes. In routine histotechnology laboratories, fixatives are chosen according to the intended properties, e.g., preservation of tissue, speed of penetration, and prevention of rearrangement or loss of soluble constituents. Formalin is widely used in routine histopathology laboratories, since it penetrates rapidly with minimal shrinkage of the tissue. Carnoy's fixative was created for histological procedures to balance shrinkage and distortion in tissues fixed in absolute alcohol, and acetic acid. It consists of chloroform, alcohol, and acetic acid. Some studies suggest Carnoy's fixative is best for optimal preservation of nucleic acids in tissues. When compared to neutral buffered formalin fixation, RNA was found to be easily extractable from Carnoy's-fixed mouse liver and well preserved with only a slight degradation of high-molecular weight RNA<sup>7</sup>. Although, formalin has better properties on tissues, but its use have many disadvantages, such as health hazards, deterioration of nuclei acids and cross-linking

of proteins<sup>14-16</sup>. Formalin gives off an unpleasant vapor that causes irritation to the respiratory system and eyes<sup>17</sup>. There have been many attempts in recent years to introduce alternative methods of tissue fixation and processing<sup>18, 19</sup>. Most have impractical aspects for routine laboratory use; e.g. the requirement for fixation and/or processing at or below  $-4^{\circ}\text{C}$ , or the lack of potential for high throughput automation. With few exceptions, these methods and reagents have not been evaluated or validated for tissue studies<sup>20,21</sup>.

Therefore, in this study we tried to evaluate the utilization of a Carnoy's solution fixation in comparison with formalin on subsequent tissue sectioning and histochemical staining. Tissue biopsies from different organs of a rabbit were initially fixed in 10% neutral formalin or Carnoy's solution and subsequently stained applying; H and E, Alcian blue, Perl's Prussian blue and PAS. Although, there are many fixatives that can give more acceptable results with particular tissue components, but still formalin is replacing them in most of their applications due to its popularity. However, the current findings of this study favor the wide popularity of formalin as a routine fixative that permits the possibility of application of many subsequent procedures e.g. tissue sectioning, tissue staining methods etc. This explain why formalin was found to be superior with quality of tissue sectioning and quality of tissue staining with the Haematoxylin and Eosin (the routine staining method) and other investigated staining techniques in the present study with exception of PAS method. Formalin fixes tissue by irreversibly cross-link primary amino group in protein with other nearby nitrogen atom in other protein, it dose not harden the tissue and retains cellular constituents in their vivo relationship thus producing sections with very good quality<sup>12</sup>. Notably, there are many formalin solutions, some of which have deleterious effects on certain tissue varieties and some can affect the tissue subsequent stainability (e.g. Acid formalin). Therefore, in this study we applied 10% neutral buffered

formalin to avoid the effects of formalin acidity. The problem with 10% buffered formalin is the slowly increasing concentration of methanol. Methanol promotes clumping of proteins, instead of the cross-linking of proteins that formaldehyde performs. A methanol-free fixative will give the best preservation, particularly if you plan to use the tissue for antibody staining at a later time.

The most common way to avoid methanol in a formaldehyde solution is to make the solution up fresh from crystalline paraformaldehyde. Paraformaldehyde can be quite hazardous to handle and it is often difficult to get it to go into solution<sup>22</sup>.

Formalin favors the staining of acidic structure (nucleus) with basic dyes and diminishes the effect of acidic dyes on basic structure (cytoplasm)<sup>17</sup>. These facts render formalin suitable with most subsequent staining methods, which is line with the findings of the current study. However, development of quantitative molecular assays using formalin fixed paraffin wax processed tissue has many difficulties. Beside inherent problems in creating standards in tissue-based assays, formalin-fixed tissues require different methodological approaches compared to fresh samples, e.g., for immunohistochemistry it requires antigen recovery techniques<sup>23</sup>.

Fixation of tissue for glycogen study should be prompt as there is an initial sharp loss of glycogen postmortem. Fixatives having faster penetration rates always give better preservation of glycogen and since, there are some fixatives with faster penetrating capabilities, they can produce better results with glycogen. Carnoy's is one of the best fixatives that produce good results in subsequent tissue processing procedures. The chemical composition of Carnoy's changes with time and stored solution has the fruity smell of ethyl acetate<sup>24</sup>. It interacts by hydrogen bond formation with each other and with various groups in tissues. These association compounds apparently stabilize tissue structures and prevent or minimize shrinkage<sup>25</sup>. It is rapid in action and may be

used for urgent biopsy specimens for paraffin processing within 5 hours. This explains why we got better results with PAS staining method for glycogen. Fixation on Carnoy's for more than 18 hours can result in hydrolysis of nucleic acids with loss of RNA; this effect can be suppressed by using 5 ml instead of 10 ml acetic acid<sup>26</sup>. Carnoy's fluid is the fixative of choice for studies of fibrous proteins and associated carbohydrate by histochemical and special staining techniques<sup>25</sup>. Substitution of methanol for ethanol in Carnoy's fixative gives methacarn, which has been shown to be an excellent fixative for preserving tissue RNA<sup>27</sup>. Methacarn fixation is superior to NBF in retaining antigen immunoreactivity and does not require antigen retrieval. It has been recommended for prospective immunohistochemical studies assessing mitotic indices in tissues<sup>28, 29</sup>.

This is the first study from the Sudan that compare the most frequently used procedures in Sudanese histotechnology laboratories following buffered formalin and Carnoy's fixatives. There are few un-published previous studies in this context in the Sudan. These studies compared formalin with other fixatives such as Omni-fix and microwave which are not widely applicable for routine work.

In conclusion: Although, Carnoy's fluid is a safe fixative and can rapidly penetrate the tissues, but it cannot be a substitute for buffered formalin. Though, formalin has credited the best results, but it might not be suitable with tissues requiring faster penetrating rates such as glycogen and other important carbohydrates.

Indeed, there exists a great neediness for human tissues for research and development. Efforts are required toward developing standardized methods of tissue fixation and processing for more accurate comparison of all intended diagnostic molecules from multiple tissue varieties. Such an exercise will help in the identification of the best fixation procedure to develop an ideal tissue template for histochemical and molecular identifications.

## References

1. Leong ASY, James CL, Thomas AC. Handbook of Surgical Pathology. New York: Churchill Livingstone 1996; 321: pp viii.
2. O'Leary TJ. Standardization in immunohistochemistry. Appl Immunohistochem Mol Morphol 2001;9:3-8.
3. Shi SR, Cote RJ, Taylor CR. Antigen retrieval techniques: current perspectives. J Histochem Cytochem 2001;49:931-937.
4. Start RD, Cross SS, Smith JH. Assessment of specimen fixation in a surgical pathology service. J Clin Pathol 1992;45:546-547.
5. Bancroft J D and Gamble M. Theory and practice of histological technique. fifth edition ed. London: Churchill living stone 2002; 2: pp 63-76.
6. Start RD, Layton CM, Cross SS et al. Reassessment of the rate of fixative diffusion. J Clin Pathol 1992;45:1120-1121.
7. Foss RD, Guha-Thakurta N, Conran RM et al. Effects of fixative and fixation time on the extraction and polymerase chain reaction amplification of RNA from paraffin-embedded tissue. Comparison of two housekeeping gene mRNA controls. Diagn Mol Pathol 1994;3:148-155.
8. Boon ME, Gerrits PO, Moorlag HE et al. Formaldehyde fixation and microwave irradiation. Histochem J 1988;20:313-322.
9. Leong ASY, James CL, Thomas AC: Handbook of Surgical Pathology. New York, Churchill Livingstone, 1996, pp viii, 321.
10. Douglas MP, Rogers SO. DNA damage caused by common cytological fixatives. Mutat Res 1998;401:77-88.
11. Freeman LE, Blair A, Lubin JH et al. Mortality From Lymphohematopoietic Malignancies Among Workers in Formaldehyde Industries: The National Cancer Institute Cohort. JNCI Journal of the National Cancer Institute 2009 101(10):751-761.
12. Bancroft JD, Stevens A. Theory and Practice of Histological Techniques. 4th ed. Edinburgh: Churchill Livingstone 1996; pp 766.
13. Curran RC. Color Atlas of Histopathology. 3rd ed. Harvey Miller Publisher, oxford university press, London 1995;pp 12-220.
14. Werner M, Chott A, Fabiano A et al. Effect of formalin fixation and processing on immunohistochemistry. Am J Surg Pathol 2000;24:1016-1019.
15. Nadji M, Gomez-Fernandez C, Ganjei-Azar P, et al. Immunohistochemistry of estrogen and progesterone receptors reconsidered: experience with 5,993 breast cancers. Am J Clin Pathol 2005;123:21-7.
16. Chen J, Byrne GE Jr, Lossos IS. Optimization of RNA extraction from formalin-fixed, paraffin-embedded lymphoid tissues. Diagn Mol Pathol 2007;16:61-72.
17. Drury R A B and Weilngton E A. Carleton Histological Techniques. fifth ed. London: Oxford University press 1980; 3: pp 36-44.

18. Warmington AR, Wilkinson JM, Riley CB. Evaluation of ethanol-based fixatives as a substitute in a diagnostic clinical laboratories. *J Histotechnol* 2000;23:299-308.
19. Gillespie JW BC, Bichsel VE, Cole KA et al. Evaluation of non-formalin tissue fixation for molecular profiling studies. *Am J Pathol* 2002;160:449-57.
20. Uneyama C, Shibutani M, Masutomi N et al. Methacarn fixation for genomic DNA analysis in microdissected, paraffin-embedded tissue specimen. *J Histochem Cytochem* 2002;52:903-913.
21. Delfour C, Roger P, Bret C et al. RCL2, a new fixative, preserves morphology and nucleic acid integrity in paraffin-embedded breast carcinoma and microdissected breast tumor cells. *J Mol Diag* 2006;8:157-169.
22. Kiernan J A. *Histological and histochemical methods, theory and practice*. third ed. London: Oxford University 1999; 2: pp 27.
23. Specht K, Richter T, Muller U et al. Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. *Am J Pathol* 2001;158:419-428.
24. Gregory R E. Stain Technology. *J Histochem* 1980;55:143-160.
25. Puchtler H, Faye S W, Coner H M et al. Carnoy's fixation, practical and theoretical consideration. 1986;16:361-371.
26. Vincek V, Nassiri M, Nadji M et al. A tissue fixative that protects macromolecules (DNA, RNA, and protein) and histomorphology in clinical samples. *Lab Invest* 2003;83:1-9.
27. Shibutani M, Uneyama C, Miyazaki K et al. Methacarn fixation: a novel tool for analysis of gene expressions in paraffin-embedded tissue specimens. *Lab Invest* 2000;80:199-208.
28. Beckstead JH. A simple technique for preservation of fixation-sensitive antigens in paraffin-embedded tissues. *J Histochem Cytochem* 1994;42:1127-1134.
29. Borrebaeck CA. Tapping the potential of molecular libraries in functional genomics. *Immunol Today* 1998;19:524-527.