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REVIEW

Optimization of Antigen Retrieval Solutions for Diagnostic Immunohistochemistry

Matthias Solomon Gamde

Plateau State University, Bokkos, Nigeria.

Abstract

Antigen retrieval is often used in immunohistochemistry. There are conflicting interests on the optimal retrieval technique for particular antigens. In many papers, EDTA and citric acid have been employed extensively. However, tissue damage, loss of structural integrity, and section loss especially in delicate specimens have resulted from higher pH and EDTA-based solutions, which have yielded inconsistent, even contradicting results and limits. This review study aimed to assess the effectiveness of several antigen retrieval techniques in immunohistochemistry, namely heat-induced epitope retrieval (HIER). The primary objective would be to assess how well antigen retrieval solutions enhanced antigen detection in paraffin-embedded tissue samples that have been preserved in formalin using published findings in a controlled experimental design to ensure consistency and dependability. The study would additionally evaluate the quality of antigen detection and the preservation of tissue morphology.

Keywords: Immunohistochemistry- Antigen Retrieval Solution- Epitope retrieval

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1. Introduction

Immunohistochemistry (IHC) is a laboratory technique that uses antibodies to locate proteins and other antigens within tissue sections. Because antibodies are highly specific, they only bind to the antigen of interest. The antibody-antigen interaction is then viewed using either a fluorescent dye (fluorescent detection) or a colored enzyme substrate (chromogenic detection). Fixing surgical specimens in formalin and embedding them in paraffin wax denatures tissue proteins and may obscure epitopes. Antigen retrieval is a technique that re-exposes hidden antigens in formalin-fixed and paraffin-embedded specimens so that antibodies can detect them [1].

Antigen retrieval is a procedure that can partially reverse the antigen-masking effects of fixation. Before immunohistochemical staining, the tissue sections are subjected to heat or proteolytic enzyme processing. The fixative, the length of fixation, and the target antigen all affect retrieval success. Over the past 60 years, immunohistochemistry research has grown significantly. In regular pathology, it is now a standard procedure. Unmasking antigens in formalin-fixed, paraffin-embedded tissues using an antigen retrieval (AR) procedure is one of the challenges associated with antigen detection [2]. These days, AR is an essential part of the widely utilized immunohistochemical staining procedure. Since it was thought to be comparable to the antigen unmasking mechanism under boiling conditions, a long AR step was initially employed in the labeling procedure. In subsequent research, however, the diagnostic yield in tumor-related antigens may either rise or fall depending on how long the oven or microwave is used [3].

Heat-induced epitope retrieval (HIER) and protease-induced epitope retrieval (PIER) are the two main techniques for retrieving antigens. Proteolytic enzymes like trypsin, pepsin, or protease are used in the protease-induced epitope retrieval (PIER) approach, which is antigen retrieval by enzymatic digestion of tissue sections [4]. Regardless of the enzyme used, longer fixation time requires longer enzymatic digestion, and this makes the enzymatic digestion procedure difficult to standardize. HIER is the more widely used technique that involves heating tissue sections to break the cross-links formed during fixation, thereby restoring the antigenic sites. This method can be performed using several

Corresponding Author:
Dr. Matthias Solomon Gamde
Plateau State University, Bokkos, Nigeria
Email: solomonmatthias85@gmail.com

appliances, including microwave ovens, pressure cookers, and water baths [5].

However, the type of tissue and antigen determine which approach is best. The majority of antigens react more favorably to heat-induced methods, particularly when combined with optimal buffer conditions. The most popular methods for efficiently retrieving antigens are EDTA and citrate buffers. The essential mechanism of these solutions is identical, even though their formulations may change slightly. They use formalin's steric hindrance and charge-blocking properties to remove proteins from cross-linked tissues. Furthermore, the lower pH of these solutions facilitates the elimination of hidden epitopes. We think that an examination of the immunohistochemistry literature is crucial given the widespread use of citrate and EDTA antigenic solutions [6].

1.1 Statement of the Problem

There is still debate over the best retrieval method for different antigens, despite the fact that antigen retrieval is frequently employed in IHC. Citric acid and EDTA have been the main tools used in earlier studies and the literature. Higher pH and EDTA-based solutions, however, have produced uneven, even contradictory outcomes and limits, such as tissue injury, loss of structural integrity, and section loss, especially in sensitive specimens. Because current antigen retrieval techniques are difficult to standardize, they may yield inconsistent results in the same or different laboratories [7]. As a result, it is still vital to compare the current methods and carefully and thoroughly develop alternative antigen retrieval solutions for immunohistochemistry applications.

2.0 Antigen Retrieval

In order to improve the accessibility of antigens in formalin-fixed, paraffin-embedded tissue samples, antigen retrieval is an essential pre-treatment procedure in immunohistochemistry. Re-exposing buried antigens in paraffin-embedded and formalin-fixed specimens allows antibodies to identify them [8]. In order to preserve their morphology, most surgically removed tissue samples in medicine are stored in paraffin and fixed in formalin (10% neutral buffer formalin). To make a diagnosis, pathologists will analyze them using stains and microscopy. However, formaldehyde can alter proteins (antigens) chemically in immunohistochemistry, making them less detectable. By cross-linking proteins and nucleic acids, these fixatives make it more difficult for antibodies to successfully identify their targets. Antigen retrieval breaks these cross-links and restores the antigenic sites, improving the specificity and sensitivity of IHC staining [3]. Several factors, such as the target antigen, the antibody employed, the kind of tissue, and the fixation technique and duration, determine whether antigen retrieval is required [9].

Furthermore, the antigen retrieval process is an integral part of standard laboratory protocols, and without it, many immunohistochemical markers would be difficult, if not impossible, to visualize. Research into optimizing antigen retrieval methods continues to be a significant area of interest for improving diagnostic accuracy, as well as advancing our understanding of cellular processes at the molecular level. In addition to IHC, AR is used in TUNEL tests, immunoelectron microscopy (IEM), in situ hybridization (ISH), immunofluorescence, and flow cytometry [10], imaging mass spectrometry (IMS), protein, RNA, and DNA extraction. Advances in the development of new buffers, enzymes, and heating methods hold the promise of making antigen retrieval even more efficient and tailored to specific diagnostic and research needs. By enhancing the overall performance of IHC, antigen retrieval directly contributes to more accurate disease detection, improving patient outcomes and advancing medical research. In conclusion, antigen retrieval plays a vital role in maximizing the potential of immunohistochemistry as a diagnostic tool and research methodology.

2.1.0 Methods of Antigen Retrieval

Antigen retrieval techniques aim to disrupt the formaldehyde-induced cross-links between proteins, thus unmasking the antigenic sites, thereby restoring the antigen's ability to bind to its corresponding antibody. The type and method of antigen retrieval that works best depends on the tissue type and primary antibody [9]. The two most important methods for antigen retrieval are Protease-Induced Epitope Retrieval (PIER) and Heat-Induced Epitope Retrieval (HIER).

2.1.1 Protease-induced Epitope Retrieval (PIER)

Antigenic determinants masked by formalin fixation and paraffin embedding may be exposed by enzymatic digestion. This can, however, not be used with frozen sections or cells that are not paraffin-embedded. The beneficial effects of protease treatment are presumably related to cleavage of the molecular cross-links by the proteolytic enzyme, allowing the antigen to return to its normal conformation, which serves for more effective antibody binding. Antigen retrieval by enzymatic digestion of tissue sections is achieved by the use of proteolytic enzymes such as trypsin, proteinase K, pepsin, pronate, ficin, and others to cleave the cross-linked protein structures, thus revealing the antigenic epitopes [11]. Though less common now, enzyme digestion was previously used to improve antigen detection, but it has largely been replaced by heat-induced AR. Regardless of the enzyme used, longer fixation time requires longer enzymatic digestion, which makes the enzymatic digestion procedure difficult to standardize [4]. This method is especially beneficial for tissues that are heavily fixed or when heat-induced epitope retrieval (HIER) is less effective. Enzymatic retrieval offers a more selective approach compared to HIER, and the choice of enzyme, along with its concentration and incubation time, should be optimized based on the specific tissue and antigen being studied. Both techniques can be used independently.

2.1.2 Heat-induced Epitope Retrieval (HIER)

Protein structure is necessary for antigen-antibody recognition. The antigenicity of proteins in formalin-treated tissue may be impacted by a conformational shift in a protein brought on by formalin fixation that obscures the epitope [12]. The three-dimensional protein structure is restored to something close to its original state as a result of the antigen retrieval, which causes renaturation or at least partial restoration of the protein structure [13].

The most used technique is antigen retrieval by HIER, which entails putting tissue pieces in a container with an antigen retrieval solution (buffer like citrate or Tris-EDTA) and heating at 900–1100 °C (depending on tissue). This method, which was first described using microwaves as a heat source, has also been shown to be successful when using various heat sources, such as autoclaves, home pressure cookers, hot water baths, and vegetable steamers [14]. This technique restores the protein's structure to enable antibody binding by breaking the cross-links that formalin has with proteins. Strong alkaline treatment or heating above 100°C may break the cross-links between formalin and protein. The heat-induced AR technique was created in 1991 using this understanding of high-temperature heating as a possible retrieval strategy [13].

HIER is especially time, temperature, buffer, and pH-sensitive, and the best method must be determined empirically. HIER involves heating the tissue section in a buffer of specific pH, usually citrate buffer (pH 6.0), EDTA buffer (pH 8.5), or Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA solution, pH 9) at high temperature [15].

The choice of antigen retrieval method depends on several factors, including the type of antigen, the tissue being studied, and the fixation protocol used. Depending on these variables, researchers can apply either heat or enzyme treatments individually or in combination to enhance receptor accessibility. For particularly challenging tissues or antigens, a sequential application of heat followed by enzyme treatments often yields the best results. Optimizing the retrieval conditions is crucial for achieving strong and specific staining while minimizing background noise. However, be cautious with heat and enzyme exposure, as excessive treatment can lead to antigen degradation or non-specific staining. Therefore, careful adjustment of retrieval parameters is necessary to attain optimal outcomes. In summary, the mechanisms underlying antigen retrieval focus on reversing the structural changes that tissue fixation induces.

2.1.3 Antigen retrieval using a domestic vegetable steamer or a water bath

Preheat the steamer or water bath with a Coplin jar filled with an antigen retrieval solution of choice until the temperature reaches 95°C-100°C. Immerse slides in the Coplin jar and place the lid loosely on the jar. Incubate for 30-60 min and turn off the steamer or water bath. Place the Coplin jar at room temperature and allow the slides to cool for 20 min.

2.1.4 Antigen Retrieval using the Microwave Method

Before the beginning of the immunostaining protocol, slides are always placed in a Coplin jar filled with an antigen retrieval solution of choice and heated in a commercial microwave oven operating at a frequency of 2.45 GHz and a 600 W power setting. After two heating cycles of 5 min each, slides should be allowed to cool at room temperature and thoroughly washed in PBS [16].

3.0 Effect of Antigen Retrieval on IHC Staining

Successful antigen retrieval is important for immunohistochemistry (IHC) staining to be effective. Studies show that antigen retrieval increases antigen visibility, which raises the sensitivity of antibody detection. Antigen retrieval solutions achieve this by dissolving formalin-induced cross-links and restoring the immunoreactivity of tissue-bound antigens [17].

However, changes in the retrieval process, including temperature, pH levels, and solution concentration, might have a big impact on the staining result. For example, the efficiency of antigen exposure depends critically on the concentration of retrieval solutions. Researchers have found that low concentrations of retrieval buffers might not break the crosslinks sufficiently, leading to weak or non-specific staining. Conversely, overly concentrated solutions may result in tissue damage or non-specific binding, compromising the quality of the staining. A balanced concentration of antigen retrieval solutions [18]. It is crucial to find the right concentration for each specific tissue type and antigen.

Antibody non-specific binding may be reduced with the aid of antigen retrieval. The staining becomes more accurate, and the tissue morphology is clearer with less background noise, which can normally interfere with interpretation, when the antigens are optimally unmasked. Optimizing antigen retrieval (AR) is crucial for achieving the best results in immunohistochemistry. Systematic testing involves systematically testing various combinations of factors such as buffer solutions, pH, heating times, and heating methods to determine the optimal conditions [4].

The pH level at which antigen retrieval operates is an important factor that determines the effectiveness of the process. This is because pH can alter the chemical mechanisms involved in breaking down cross-links and restoring antigenicity. Depending on the target, both acidic and alkaline conditions can be used, and different pH levels can impact AR's effectiveness. The ability of a strong alkaline solution to hydrolyse and denature proteins, breaking down nuclear and cell membranes and disintegrating the cross-links created by formalin fixation, may determine how successful it is [16].

Acidic conditions (pH below 6) are commonly used in antigen retrieval. The cross-links created by formalin can be broken by low pH, increasing the accessibility of antigens. However, several possible problems may arise if antigen retrieval is carried out using a buffer that is too acidic (i.e., with a pH below the ideal range). Although alkaline conditions (pH above 9) are less commonly used, they can be useful for certain antigens because they can break down the methylene bridges in tissues that have been fixed with formalin, which makes antigen retrieval easier. However, using a buffer that is too basic (i.e., has a pH above the ideal range) can also result in several problems that can negatively impact the success of your immunohistochemistry (IHC). Excessively basic or acidic environments can harm tissue structures, causing cellular morphology loss, protein denaturation, decreased signal, and epitope destruction. These effects can lead to false-negative or unreliable immunohistochemistry (IHC) results because the antibodies may not bind well. These may cause results to be misinterpreted or make it difficult to obtain useful data.

Temperature also plays a significant role in antigen retrieval. In the heat-induced antigen retrieval (HIER) process, tissues are exposed to high temperatures, often between 95°C and 100°C. Studies have shown that higher temperatures can help break the formalin-induced crosslinks more efficiently, but excessive heat may cause tissue damage or denaturation of proteins [11]. Many of the active pioneers were practicing pathologists who understood the need to improve IHC's capabilities on FFPE tissues to preserve the essential morphologic features that serve as the basis for diagnostic histopathology [19]. These studies showed that heating over 100°C might break the cross-links between formalin and protein [20]. Established an AR technique for combining IF and FISH labeling in formalin-fixed paraffin-embedded (FFPE) sections using a microwave oven at a low power level of 4 (40%) for 3 cycles × 5 min, with a 1-minute break between each cycle. The temperature reached 100°C in the jar, and the method gave satisfactory IF and FISH staining signals with a clean background [20]. Therefore, the duration and intensity of heat application must be carefully optimized to avoid tissue degradation while achieving the desired antigen retrieval. Additionally, the duration of the antigen retrieval process can affect the quality of the staining. Prolonged exposure to retrieval solutions, especially when using high-pH or high-concentration solutions, can lead to tissue overexposure, which may cause a reduction in antigen specificity or cause other artifacts in the tissue.

First, for more than one hundred years, FFPE tissues have served as the standard tissue preparation method in surgical pathology, providing the basis for most of the criteria for pathological diagnosis in "routine" hematoxylin and eosin–stained FFPE tissue sections [21]. The tissue fixation technique can also affect how well the antigen is retrieved. Effective antigen retrieval may be affected by over- or under-fixation, which can change antigenicity. Because under-fixation can lead to antigen loss and over-fixation can result in excessive crosslinking, studies have shown that antigen retrieval is most effective when tissues are fixed in neutral-buffered formalin for a typical length of 24 to 48 hours. Other fixatives, such as acetone and alcohol, can lead to protein loss and different IHC results [11].

Therefore, proper fixation procedures are essential for ensuring the effectiveness of antigen retrieval techniques. Moreover, the type of tissue examined can also determine the choice of retrieval solution. For example, soft tissues, such as liver and kidney, may be more responsive to antigen retrieval solutions than harder tissues like bone, which may require more intense retrieval methods. Certain tissues, such as those with high lipid content or dense connective tissue, may also require specialized antigen retrieval protocols. FFPE tissue sections provide superior morphology and increasingly give equivalent or better IHC staining results compared to frozen sections. Thus, it is not surprising that AR-IHC immunostaining results have increasingly been accepted as the "gold standard" [3]. Antigen retrieval is a multifaceted process that significantly impacts the quality and reliability of immunohistochemical staining. By carefully selecting retrieval solutions, optimizing solution concentrations, and controlling temperature and timing, researchers can improve the visibility of target antigens in tissues. It is essential to consider various factors, such as tissue type, fixation methods, and antibody compatibility, to achieve the best results.

4.0 Solutions/Buffers In Antigen Retrieval

Antigen retrieval solutions are used to reverse the effects of formalin fixation on tissue samples, which modifies the structure of proteins, making it difficult for antibodies to bind. The use of various retrieval solutions plays a pivotal role in determining the effectiveness of the process. One of the most widely used antigen retrieval solutions is citrate buffer, which has become a standard choice due to its ability to break formalin-induced cross-links. According to Gustafsson et al. [22], who compared several AR solutions, the citrate acid AR method is an essential step in performing the proteome analysis for FFPE tissue.

Citrate buffer works very well when retrieving antigens from tissues that have undergone standard formalin fixation and paraffin embedding. It restores the antigen's ability to interact with the antibody by reducing the formalin-induced cross-links that prevent antibody binding [22].

High pH buffers, such as EDTA-containing solutions, provide better antigen recovery than citrate-based retrieval buffers; however, treated tissues may exhibit higher tissue damage. In tissues with high calcium or magnesium concentrations, EDTA functions by chelating metal ions that may stabilize antigen-antibody complexes. Compared to a more acidic solution, this buffer can prevent severe tissue destruction, which is especially helpful for antigens that need a softer retrieval process [13].

Conversely, high pH solutions typically between 8.0 and 9.0 are used for more reliable antigen retrieval, particularly for antigens that are particularly resistant to conventional retrieval methods or that are firmly buried in tissue structures. Numerous comparison studies have examined the efficacy of these retrieval solutions. While EDTA might be better for nuclear or membrane-bound antigens, citrate buffer frequently yields better results for cytoplasmic and membrane antigens. Research has shown that the success rates of various solutions vary based on the type of antigen and the tissue being studied. For instance, EDTA is shown to be more appropriate for epithelial tissues, whereas citrate buffer is frequently more beneficial for smooth muscle tissue [21].

Antigen retrieval is also a complicated procedure that necessitates the careful selection of retrieval solutions depending on the tissue, antigen type, and particular experimental conditions. EDTA and high-pH buffers are essential for some applications, although citrate buffer is still a popular and flexible option. The increasing amount of empirical data emphasizes that retrieval techniques must be tailored to produce the best outcomes for various tissues and antigens. Researchers can increase the sensitivity and specificity of immunohistochemical staining, which will improve diagnostic results in clinical and research settings, by further investigating and improving these methods. Examining the distinctive qualities and efficacy of the buffers you have listed is essential when evaluating antigen retrieval solutions in immunohistochemistry (IHC), particularly when employing the heat retrieval method. In IHC, the heat-induced epitope retrieval (HIER) technique is frequently employed to reveal antigens and enhance antibody binding. This is an overview of the buffers and their application in heat retrieval.

4.1 Hydrochloric Acid (HCl)

Hydrochloric acid is a strong acid and is made of hydrogen and chlorine. Hydrochloric acid (HCl) is sometimes used in immunohistochemistry (IHC) as an antigen retrieval and decalcification solution, as well as in pretreatment procedures to improve staining. To reveal hidden antigenic sites, it denatures DNA and unmasks epitopes; however, its application requires careful optimization to prevent tissue damage (Wakayama et al., 2014). During research, Wakayama et al. [23] also discovered that Tris-HCl buffer combined with heat-induced antigen retrieval (HIAR) is more effective than HCl alone in maintaining tissue shape and enhancing staining quality. Likewise, Shi et al. discovered that 20 mM Tris-HCl with 2% SDS was the best protein extraction buffer, outdoing several commercial solutions. Although decalcification with HCl is essential for IHC analysis of bone metastases, it can also impact the expression of specific biomarkers; research indicates that decalcification with HCl can result in a categorical decrease in the expression of the estrogen receptor (ER) and progesterone receptor (PR) in breast tumors [24]. Antigens that are resistant to milder acidic buffers can be successfully recovered using heat retrieval techniques that include HCl. Adequate concentration, duration, and temperature optimization are necessary when using HCl in IHC to reduce tissue damage and preserve antigenicity.

4.2 Glacial Acetic Acid (CH_3COOH)

In antigen retrieval techniques, glacial acetic acid is occasionally used to assist in revealing antigens in tissues fixed in paraffin. Although it is not as frequently employed as some of the other buffers, it may be useful in specific situations, especially when combined with heat. Antigen detection in IHC can be improved by using fixatives that contain glacial acetic acid. The majority of studied proteins were able to recognize antigens in ovarian tissue using a fixative containing acetic acid without the need for antigen retrieval [25].

Acetic acid is frequently employed in more specialized antigen retrieval applications, such as those that target nucleic acids, and functions by interfering with protein-protein interactions [26]. Due to its acidic nature, glacial acetic acid should be used with caution, just like formic acid. In conclusion, the specific antigen and tissue type under study determine which buffer is best for the heat-induced antigen retrieval method.

4.3 Formic Acid (HCOOH)

Formic acid is a weak, colorless, acidic liquid that fumes and has a strong, acrid smell. Its chemical formula is HCOOH, and it possesses both reducing and acidic properties. In immunohistochemistry (IHC), formic acid is an acidic antigen retrieval solution that is mostly used to restore antigenicity in tissues that are highly cross-linked. It is often utilized to enhance immunostaining results when combined with heat or other techniques [27]. To improve antigen retrieval, formic acid is commonly used together with microwave heating. This combination has been widely used as a method for enhancing immunohistochemistry analysis.

Compared to enzymatic or no pretreatment, microwave heat treatment has been proven to improve the staining of intraneuronal Aβ (beta amyloid) in Alzheimer's disease [27]. Furthermore, Kitamoto et al. [28] also found that formic acid pretreatment enhances immunostaining of cerebral and systemic amyloids. Although formic acid works well, it's vital to remember that variables like pH and heating temperature might affect how well the antigen is retrieved. Extended exposure may result in tissue damage and over-digestion.

4.4 Water (H_2O)

Water is a substance made up of the elements oxygen and hydrogen. In immunohistochemistry (IHC), water is used in antigen retrieval, solution preparation, and washing processes, among other procedures. This simple technique of boiling formalin-fixed paraffin-embedded (FFPE) tissue sections in water has played a major role in extending the reach and use of immunohistochemistry (IHC) in FFPE tissues [19].

In immunohistochemistry (IHC), water can be utilized as an antigen retrieval solution, especially when using heat-induced epitope retrieval (HIER) techniques. Although water isn't as frequently utilized as other solutions like citrate buffer or Tris-EDTA, it has certain benefits and can work well in some situations. According to studies, the combination of heat and water can aid in simple retrieval protocols, but it is often less effective for tougher epitopes that need particular pH conditions to unmask [29].

4.5 Citric Acid ($C_6H_8O_7$)

Citric acid is an organic compound; it is a colorless, weak organic acid that occurs naturally in citrus fruits. Citric acid has the ability to restore antigenicity while maintaining tissue shape, which makes it one of the most commonly used antigen retrieval solutions. It works by lowering the pH, which helps to break protein cross-links formed during formalin fixation, exposing the antigens and improving antibody binding and staining quality [30]. The solution is usually heated to around 95–100°C for 10–20 minutes. Gustafsson et al. [22] also summarized that the citrate acid AR method is an important step in being able to fully analyze the proteome for FFPE tissue. According to Shi & Taylor [3], boiling sections in a citric acid buffer of pH 6.0 for 20 minutes was one heat-induced AR protocol used in a study of FFPE cell block sections of metastatic breast cancer. It is a common choice for heat-induced retrieval and also has the advantage of shortening antibody incubation times.

4.6 Oxalic Acid ($C_2H_2O_4$)

Oxalic acid is a strong dicarboxylic acid found in plants and fungi, with the ability to form oxalate salts due to its metal-chelating potential. Oxalic acid's primary mechanism of action is the acidic breakdown of protein cross-links caused by formalin; its strong chelating properties also aid in the removal of calcium deposits, which may sometimes hinder antigen recognition. However, it may cause tissue damage if not properly optimized. In immunohistochemistry, calcium ions are often removed during decalcification using acids and chelating agents. As a potent calcium chelator, oxalic acid can be used to prepare calcified tissues, including bone, for IHC analysis to ensure proper antigen detection. Additionally, a mixture of potassium permanganate and oxalic acid is used as a pretreatment method; to improve reactivity with melanoma-specific monoclonal antibodies, this pretreatment is notably identified in the examination of canine melanomas, which reacted with HMB-45 and MEL-1 antibodies [31].

4.7 EDTA ($C_{10}H_{16}N_2O_8$)

EDTA, or ethylenediaminetetraacetic acid, is a chelating agent. It may create many connections with just one metal ion. In immunohistochemistry (IHC), EDTA is commonly used as an antigen retrieval (AR) solution, especially for epitopes that are challenging to expose using acidic solutions like citric acid. Calcium ions that are affixed to tissue are eliminated by EDTA. It reveals epitopes by eliminating these calcium ions, suggesting that some antigens are hidden by the complexing of calcium ions with proteins during formaldehyde fixation [16]. Furthermore, a second trypsin treatment is not necessary before or after the HIER as long as the EDTA solution is used [11]. Similarly, skin samples seem to react better with EDTA at pH 8.0, according to Yang et al. [32]. Many antigens, including those on cell membranes and in the nucleus, are frequently extracted using EDTA. Strong alkaline solutions work well because they can hydrolyze and denature proteins, which helps break down cell and nuclear membranes and break up cross-links that could conceal antigens [3].

Hydrochloric acid and glacial acetic acid both performed well in retrieval among the acid-based solutions, regularly obtaining high scores, especially for PR and CD20. This implies that well-optimized acid-based solutions can be useful substitutes in situations when standard buffers are unavailable. Overall, the results unequivocally show that the best buffers for retrieving antigens in immunohistochemistry (IHC) are citrate and EDTA, with hydrochloric acid buffer coming in second. Furthermore, the Quick Score approach is a useful and repeatable way to assess how well antigen retrieval techniques work. Additionally, this work supports ongoing attempts to standardize IHC techniques, which are crucial for improving diagnostic precision and repeatability among labs.

In conclusion, in immunohistochemistry, antigen retrieval solutions are essential, particularly for tissue that has been paraffin-embedded and formalin-fixed. Antigen exposure and antibody binding are significantly enhanced by the right retrieval solution and variables. These results show that buffer selection significantly affects IHC results and that the ideal antigen retrieval should be tailored to the right pH, concentration, antigen of interest, and tissue type.

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Statement of Transparency and Principles:

• The authors declare no conflict of interest.

• The study was approved by the Research Ethics Committee of the authors' affiliated institution.

• The study data are available upon reasonable request.

• All authors contributed to the implementation of this research.

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