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# ORIGINAL ARTICLE

## EFFECT OF DIFFERENT STORAGE TEMPERATURES ON ENZYME-ANTIBODY CONJUGATE USED IN IMMUNOHISTOCHEMISTRY

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**ABSTRACT:** The usefulness of an enzyme-antibody conjugate in detecting antigen or antibody depends upon the degree to which that conjugate can act. It is important to check storage conditions for specificity before making interpretations of Immunohistochemistry results. Optimal conditions for long-term storage of conjugate for use in staining have been investigated with particular emphasis on the preservation. Conjugates were preserved at different temperatures (room, 4, -70° C) without any preservatives. The activity of the conjugates was evaluated at regular intervals throughout storage. The best results were obtained with conjugate stored at 4° C and retained excellent reproducibility in immuno-staining after 2 years also. In storage at –70° C repeated freezing and thawing has destroyed the conjugate while at room temperature conjugate got contaminated. The results have led us that conjugate is the backbone and storage conditions must be of great attention.
**KEYWORDS:** Temperature, Conjugate, Staining.

**INTRODUCTION:** Since 1955, when Coons and colleagues[1] revolutionized the identification of tissue antigens using a direct fluorescence method, Immunohistochemistry (IHC), has been used as an important method of investigation in both diagnostic and research.[2,3] In all the IHC procedures three basic regents-antibody, enzyme and cross-linking are of major importance. Above the entire pivotal reagent common to all immunohistochemical techniques is the antibody-enzyme conjugate.[4,1] Titer and dilutions, as well as incubation time and temperature are tightly interwoven in their effect on the quality of staining. These factors can be changed independently, or as is more often the case, in complementary fusion, to bring about marked differences in the quality of staining. Time, temperature and titer are interdependent; a change in one factor will affect the others. Generally, when making any changes, the overriding goal should be the achievement of optimal specific staining accompanied by minimal interference from background staining.[5]

To get the quality of staining the antibody-enzyme conjugate which is the backbone of all the results produced, its storage conditions must be of great attention. So, far no attempts have been known to see storage conditions in order to obtain a maximal stability and activity. Most authors treat the stability of the conjugates casually, given no specific data.[6] In this paper, our purpose is to compare the staining efficiency of Horseradish peroxidase- Antibody conjugation prepared by two-step glutaraldeyde method[7,8] and kept at three different temperatures. The conjugates kept at different temperatures were tested by Immunohistochemical staining (Direct method) on the tissue sections obtained from routine reports.

**MATERIALS AND METHODS:** Preparation of Conjugate: Horseradish Peroxidase (HRP) was employed because enzyme is commercially available in relatively pure form and is mostly used in all the IHC purposes.[9,10] The monoclonal antibody which was prepared by known hybridoma technique

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against Hela cells (Human Carcinoma Cervix)-AntiHela was linked by the enzyme HRP by two-step glutaraldeyde method.[11,12]

The reactivity of the antibody-conjugate was assessed by testing with Hela cells and also tested against the paraffin embedded tissue sections obtained from routine histological reports.

Storage of Conjugate: The conjugate was separately kept in sterilized glassware vials (one ml per vial) at room temperature (between 18° to 40° C), 4 and –70° C. Conjugates stored were tested after ever three months for their contaminations and positivity. Storage conditions have been tested for 24 months. Repeated freezing and thawing was avoided. No preservative was added in any of the vial.

Testing of Conjugates: The conjugate was tested by Immunohistochemical staining (Direct method).[13,14] Because this method utilizes only one antibody, it can be completed quickly, and nonspecific reactions are limited. The tissue sections obtained from routine histological reports were assessed (a) Carcinoma cervix sections grade I to III, (b). Non-Malignant Cervix and (c) Hela Cells against the conjugates. Diamine-benzidine (DAB) was used as substrate. A yellow-brown colour was developed. Protein content estimation of each conjugate was done by Lowry method.[15,16]

**DISCUSSION:** IHC staining methods are so widely used today that they are referred as just another special stain. Reagent and procedure used in these procedures using the Horseradish peroxidase (HRP), the Peroxidase-anti-peroxidase (PAP), the Avidin-biotin complex (ABC), or the Alkaline phosphatase-anti-alkaline-phosphatase (APAAP) for detection of antigen-specific antibodies in frozen or paraffin sections are necessary for the validation of the staining results.[17,18] Correct reagents contribute to the quality of staining if they are not prepared accurately and handled properly. They are often unstable when not in their native environment.[19] Each conjugate may have specific requirements once it is made and purified. If these requirements are not satisfied, the conjugate rapidly lose its ability to perform specific functions.

Several important practical steps are recognized in the process and many compounds are found to be added to lengthen shelf life, which are not satisfying and leads to inactivation in some cases or instability in others.

In most of the conjugates commercially prepared for IHC sodium azide, an antibacterial agent is present which can prevent binding of the peroxidase enzyme to its substrate and inhibit colour development. Thus the use of sodium azide is not recommended. Cryoprotectants such as glycerol or ethylene glycol are added to help to stabilize by preventing the formation of ice crystals at –20° C that also destroy structure of the combining antibody.[3] Protease inhibitors stops proteolytic cleavage of antibody while reducing agents like dithiothreitol (DTT) and 2-mercaptoethanol (2-ME) also prevents oxidation in the conjugate. Drying, lypolization and freezing of conjugates are also criteria in some IHC manufactured kits.[20] Since in the antibody-enzyme conjugates, the antibody comprise both hydrophilic and hydrophobic amino acids and are flexible polymeric molecules which results in the antibody spontaneous folding in aqueous solution.[21,22,23] These phase changes (repeatedly freezing and thawing) also effect interaction forces like electrostatic, H-bond, Vander Waals, chelation and disulfide bonds.[24,25]

Some of the IHC kits assembled and shipped also uses stabilizing diluent or solution for stabilization for conjugates. While aqueous solution is the natural functioning state for conjugates, this is not their state of greatest stability. Antibody becomes more flexible and prone to conformational changes.

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The frequency of collision with other molecules and /or the cotainer surface increases. The possibility of microbial contamination increases and antibody used to conjugate becomes more susceptible to oxidation.[26,27,28]

This study insures consistently high levels of activity for both the enzyme and the antibody following long-term storage. This storage at 4°C greatly improves the signal to noise ratios which increases the sensitivity and offers significantly increase of shelf life. This condition also provides resistance to various shipping conditions and storage temperatures for the groups who provide conjugates as well as improve day-to-day IHC work.

**CONCLUSIONS/RESULTS:** The usefulness of an enzyme-antibody conjugate in detecting antigen or antibody depends upon the degree to which that conjugate can be bind quantitatively to the antigen or can exhibit proportional loss of binding to fixed antigen by known amounts of free antigen. The success of staining depends on the quality of the conjugate, which, in turn, is a function of (1) the coupling process, (2) particular enzyme used, (3) purity of antibody and (4) the stability of the linkage. Since the quality and quantity of the antibody to be used are identical, so the conjugate is very useful and needs extra attention. Each antibody-enzyme conjugate suggest that it is important to check storage conditions specificity before making interpretations of immunohistochemical results.

Immunohistochemical methods acts as a valuable tools for both routine biochemical and research permanence of the reaction product and usefulness in fixed tissue sections, together with the facility for simultaneous pathological diagnosis, make the immunoperoxidase method the technique of choice in laboratory at the present time.

Our work emphasizes the importance of IHC of selecting the appropriate antibody-enzyme conjugate storage conditions before drawing conclusions about the specificity of new conjugate and the distribution of unknown antigens and suggests the conjugate of HRP prepared by two-step glutaraldeyde method kept at 4°C gave best result uptill two years. The cojugate showed positive reaction with Hela cells. The amount of protein in freshly prepared conjugate was 6.9 mg/dl and decreases with time. The presence or absence of specific immunostaining due to the storage conditions of antibody-conjugate are shown in Tables-I and II. Intensity of immunoreactivity was scored semi-quantitatively by classifying the sections as not detectable expression (-), very low (±), low (+), moderate (++) or high staining intensity (+++).

Freezing and thawing of the conjugate also proved to affect stability. After multiple freezing and thawing cycles antibody activity was decreased in conjugate produced in the laboratory.

| Temp °C | 0 day | 3 months | 6 months | 9 months | 12 months | 15 months | 18 months | 24 months |
|---|---|---|---|---|---|---|---|---|
| RT | +++ | ++ | ++ | ++ | + | ± | - | - |
| 4 | +++ | +++ | ++ | ++ | ++ | ++ | ++ | + |
| -70 | +++ | +++ | ++ | + | ± | ± | - | - |

Table I: Intensity and activity of antibody-conjugate

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| Temp | 0 day | 3 months | 6 months | 9 months | 12 months | 15 months | 18 months | 24 months |
|---|---|---|---|---|---|---|---|---|
| RT | 6.9 | 6.2 | 6.2 | 4.5 | 3.2 | **Contaminated** | | |
| 4 | 6.9 | 6.5 | 6.2 | 5.9 | 5.6 | 4.2 | 3.1 | 2.8 |
| -70 | 6.9 | 6.6 | 6.0 | 5.6 | 3.8 | 3.0 | 2.5 | 1.6 |

Table II: Protein Contents of antibody-conjugate in mg/dl

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