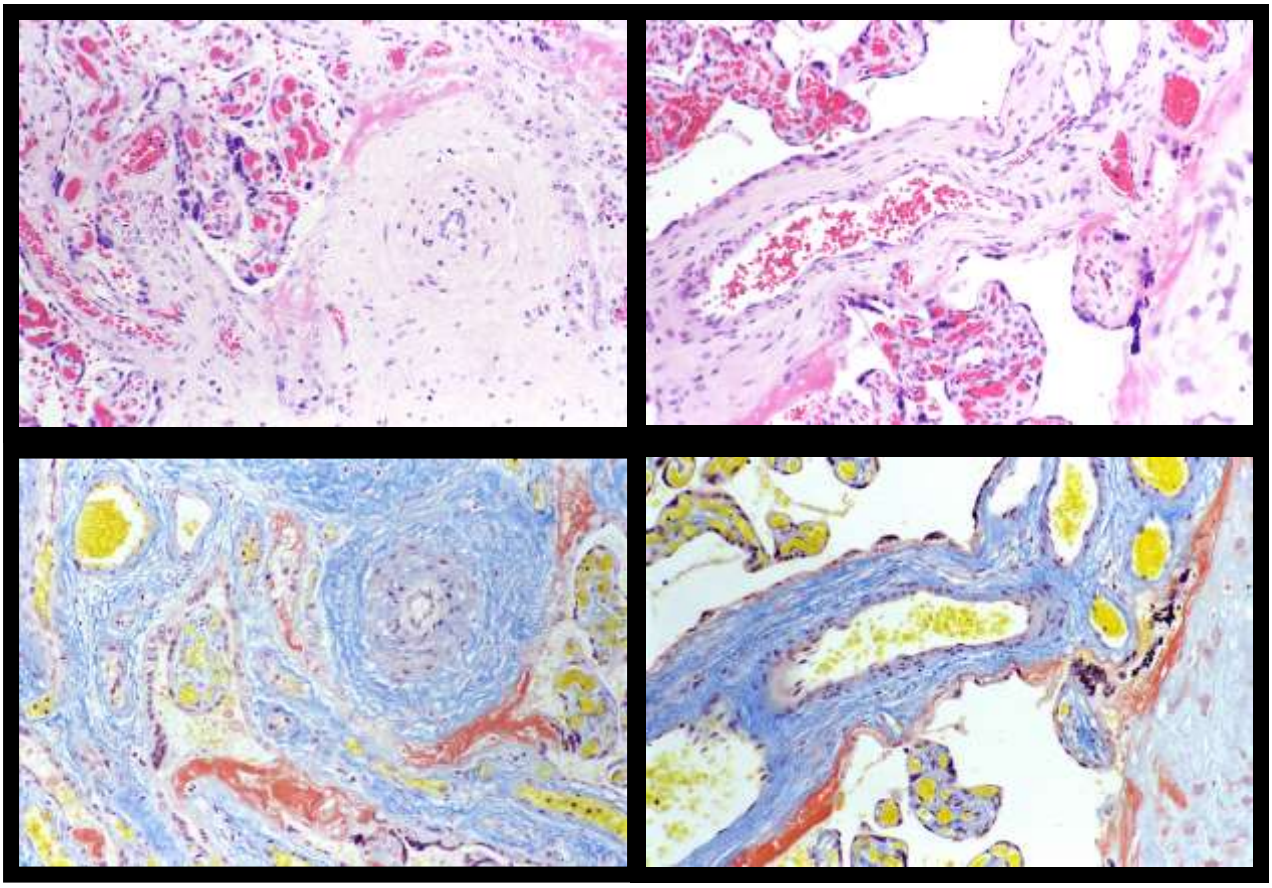
TISSUFIX™

A Routine Fixative for the Histopathology Laboratory:

Comparison of TISSUFIX™ and Neutral buffered Formaldehyde(NBF).



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INTRODUCTION.

The microscopical analysis of body tissue requires that tissue be sufficiently thin to be transparent and possess sufficient contrast to permit resolution of structural detail. Most tissues do not meet these requirements. It is therefore necessary to manipulate them. It is not sufficient for the histologist that a specimen meet just these requirements. In addition, the cells and extracellular materials must be preserved in such a way that there is as little alteration as possible to the structure and chemical composition of the living tissue. Without being spatially displaced, the structural proteins and other constituents of the tissue must be rendered insoluble in all of the reagents to which they will subsequently be exposed.

The single most important manipulation technique is stabilization of the tissue to prevent the degradation which can occur rapidly following cell death. In fact, whenever possible, it is preferable to commence tissue stabilization while simultaneously initiating cell death, in order to avoid agonal artifacts. In the case of experimental animals this may necessitate stabilization of the entire animal, or the organ system of interest, by vascular perfusion. Biopsy techniques offer a means of obtaining viable tissue samples which may be rapidly stabilized. Surgically removed tissue should have its viability maintained until stabilization is initiated. Harvesting of dead tissue samples should be done on an 'urgent' basis. Handling of the tissue, prior to stabilization, should be kept to a minimum and should always be as gentle as possible.

Subsequently, sectioning techniques will provide a representative slice of tissue, which will function as a matrix for the localization and visualization of tissue elements by histochemical reactions.

Histological sections manifest the Heisenberg effect, i.e. the act of observation unavoidably alters the behavior of that which is observed. The various manipulations of the tissue, which are necessary to obtain thin slices for microscopic analysis, introduce many artifacts. The fewer the number of these manipulations, the fewer the artifacts produced. However, the choice and careful application of each technique can minimize the artifacts or, at least, render them consistent and reproducible.

All histological techniques are highly interdependent and seemingly minor variations, uncritically introduced at any stage, can have far reaching effects on the final microscopic image and its analysis. Several thousand histological techniques have been published, most of them being merely empirical variations of previous work.
Many of these modifications prove to be of dubious value when applied to samples prepared in a careful, consistent and reproducible manner. Appropriate stabilization of fresh tissue is therefore the key factor in accurate histological analysis.
Immunohistochemical (IHC) techniques are particularly vulnerable to variations in tissue stabilization. No single stabilization technique exists to cover all eventualities. Therefore, in many cases, several techniques may need to be applied to different samples of the same tissue.

STABILIZATION (FIXATION) OF TISSUE FOR ROUTINE HISTOLOGY.

Many fixation reagents are available. Choice of reagent is dependent upon the tissue component and the histochemical reaction selected. Inappropriate choice of the primary fixative may negate subsequent studies. No fixative currently available stabilizes tissue in a manner suitable for all histochemical techniques.
Stabilization of tissue therefore represents a compromise between classical morphology and the need to demonstrate structural or molecular components.

With few exceptions, most human tissues are stabilized in formaldehyde based fixatives. Formaldehyde fixation is the method of choice for the vast majority of histological techniques, including immunohistochemistry (IHC), for very good reasons. The mild cross-linking action with proteins provides tissue stabilization with minimum structural change, loss of less than 1% of protein [1], least loss of biological activity and the least change in chemical reactivity[2]. These fixation actions are largely reversible, making it possible to subsequently restore much of the structural change and the biological and chemical reactivity[3].

Tissue **optimally** stabilized in formaldehyde based fixatives will provide excellent morphology, at the light microscopical level, and submit to the broadest range of histochemical procedures. In order to optimize both morphology and histochemistry, it is necessary to standardize the fixation protocol. There are, unfortunately, many factors which affect optimum fixation. **The actions of formaldehyde are greatly modified by concentration, buffers and pH, osmolality of the fixative solution, added salts and the time and temperature of fixation[4].** Tissue size and density, the volume ratio between tissue and fixative and the duration of fixation are also important factors that are relatively easy to control. The duration of fixation is of particular concern for the following reasons;

1) While formaldehyde is one of the faster penetrating fixatives, it is one of the slowest in fixing action. Optimum fixation only occurs after 24 -72 hours, depending upon the temperature, although reproducible IHC results may be obtained after 18 - 24 hours at the same temperature[3].
2) For fixation times below 18 - 24 hours, reproducibility of IHC results becomes a major problem. This is because of inadequate exposure to the fixative and the ready reversibility of formaldehyde fixation. The shorter the initial exposure time, the more rapid and unpredictable the degree of reversal. In fact, for times less than approximately 8-12 hours, the tissue is largely fixed by the next reagent in the process, usually ethanol, a protein denaturing solvent which has marked effects on the secondary and tertiary structure of proteins[2,3]. Ethanol fixation can result in the loss of up to 40% of protein [1], is known to disrupt cellular membranes and may even strip cytoplasmic membranes completely. This can have disastrous consequences if the target antigen is located in such membranes, or is one of the proteins lost. In addition,

the effects of ethanol are irreversible, thus rendering "antigen retrieval" techniques largely ineffective or even deleterious to the tissue.

This variable admixture, of additive (formaldehyde) and coagulative (alcohol) fixation, accounts for the huge variability in both intra- and inter-laboratory IHC results [5].

However, there is currently **NO** standardized approach to this step. The time most laboratories currently allow for fixation to occur, in order to meet expected turn-around times for diagnosis, is more problematic. The advent of automatic tissue processing machines has taken a serious toll on the quality of fixation, a situation that is further compounded in hospital practice by efforts to produce a diagnosis as quickly as possible, with the intention of reducing the costs of patient care by decreasing hospital stay.
The "routine" handling of surgical specimens, by the majority of clinical laboratories, consists of allowing the tissues to fix in formaldehyde for variable periods of time, ranging from to 1-2 hours at the minimum, to several days (over a long weekend) at the maximum. This is mainly based upon allowing the tissue, received on any particular day, to fix only until the automated tissue processor commences the overnight run. On occasion, if fresh tissues are added after the processor has commenced the run, this minimum time may be as short as a few minutes, or even, for very late arriving, unfixed, samples placed directly on to the processor, formaldehyde may be missed completely.

Automatic tissue processors were never intended to be used for fixation, but were designed to replace the drudgery of hand changing all the solutions used for tissue processing. It was assumed that optimal fixation had already taken place. In the laboratory of today, the first and sometimes the second reagent on the processor is usually a formaldehyde solution, which is allowed to act for only 1-2 hours. There are consequently fewer locations for other processing reagents. This often leads to compromises in the quality of processing. This is done in order to reduce the time necessary to produce a report. Whilst this is a fairly successful strategy for those specimens which entail only morphological examination, for the approximately 10% of samples that require further investigation, particularly, IHC analyses, it means that the tissue is not optimally fixed and/or processed.
It is important to remember that adequate tissue morphology, based on H&E stained sections, does NOT correlate with adequate fixation for IHC.

One strategy, when it is suspected that IHC will be required, is to take an additional block of tissue for optimized handling. Another strategy, used to address this issue in some laboratories, is to designate certain specimens for special handling and allow them to fix overnight. For example, in many laboratories, lymphomas, pediatric tumors, bowel resections and breast tumor samples are designated as such specimens, but this is not necessarily the case. Smaller samples of breast tissue are often handled in the "routine" manner, in particular wedge, core and fine needle biopsies. This practice ensures that any IHC staining of breast tumors, such as for estrogen receptor protein (ER), progesterone receptor protein (PgR) and the HER2/neu protein (HER2), is performed on tissues which have been fixed in a widely diverse manner. This makes any intra- and inter-laboratory comparison of results problematic. Other tissues, such as small biopsies, routinely

handled specimens and cases that have been processed over a long weekend, may also require IHC. These samples will have wide variation in the quality of fixation. They also show the most variation in IHC results!

Immunohistochemical techniques rely on the steric interaction between an antibody and the target protein. In particular, recognition critically depends upon the structure of a portion of the target protein, the antigenic determinant or epitope, remaining unaltered. Monoclonal antibodies bind to only one epitope, whereas polyclonal antibodies bind to several different epitopes on the same target protein. Any alteration in the structure of the target protein which affects the epitope against which the antibody is directed would therefore have more serious consequences for monoclonal antibodies than for polyclonal antibodies. It is evident that variation in structural alteration during fixation/processing of tissue is the major source of inconsistency in results.

Many studies have attempted to improve the sensitivity of antibodies to detect their target antigen in FFPE sections, by utilizing either heat or proteolytic digestion as a fixation reversal pre-treatment prior to immunostaining, often with conflicting results. This is not surprising, since a predictable reversal is largely dependent upon a standardized fixation. There is no doubt that such measures can improve sensitivity of detection with some antibodies. Conversely, they can also destroy some epitopes or even create unwanted cross-reactions.

STABILIZATION OF TISSUE FOR IHC ANALYSIS.

Because of the dramatic effects of variable fixation on IHC results we have, for a number of years, used the strategies mentioned above to standardize our approach to patient samples which may require IHC stains. Our procedural control tissues are fixed in this standardized manner and the IHC stains are optimized using these standard controls. As a regional reference laboratory, we commonly have to deal with samples from other institutions which have been fixed and processed in a sub-optimal manner. We have also prepared tissue control samples fixed and processed, in a variety of ways, to accommodate this fact. It is therefore of some concern to us when a new fixative solution becomes commercially available.

This study was undertaken to compare a commercially supplied, formaldehyde based histological fixative, TISSUFIX™, with the in-house prepared neutral buffered formaldehyde (NBF) fixative, in current routine use.
The study was performed in four phases;

Phase I. **The effect of both fixatives on a variety of tissues was compared. Particularly, the effect on morphology and on a variety of special stains.**

Phase II. **The effect of both fixatives on common IHC markers was compared.**

Phase III. **The effect of both fixatives on prognostic and predictive IHC**

markers for breast tumours was compared.

Phase IV. The efficiency of fixation, using IHC for vimentin, was compared.

FIXATIVES.

Neutral buffered formaldehyde (NBF)
(4% formaldehyde in phosphate buffer pH 7.0-7.2)
The traditional formulations given in most texts on histological technique are appropriate for general use. However, they are usually slightly acidic, at pH 6.8, instead of neutral. We prefer to use formaldehyde at pH 7.0- 7.2, since there is always a slight drop in pH on addition of formalin to the buffer and also on the addition of tissue to the fixative.

Preparation
Sodium phosphate, monobasic, anhydrous (NaH_2PO_4) 4.02g
Sodium phosphate, dibasic, anhydrous (Na2HPO4) 9.1g
Dissolve the salts in 800 mL of warm water, add 100mL Formalin (37 - 40 % w/v Formaldehyde), and make up to 1000mL with water.
This fixative should be prepared at least 24 hours prior to use and remains stable for several weeks at room temperature.
Commercial Formalin also contains a variable amount of Methanol (7 – 15% v/v), which is added as a stabilizer to inhibit polymerization. The final solution therefore contains 4% Formaldehyde and 0.7-1.5% v/v Methanol, in a phosphate buffer solution.

TISSUFIX™
The exact formulation of TISSUFIX™ was not disclosed by **chaptec inc**. The MSDS reveals that it contains 6% w/v Formaldehyde, 2% v/v Methanol and 0.1% Trichloroacetic acid.
The components are dissolved in softened water, sodium phosphate is added to complete the buffer ingredients (trichloroacetic acid/ phosphate) and to obtain a final pH 7.2.
A 'proprietary' ingredient is added to speed fixation times (personal communication).

FIXATION PROTOCOL.

Adjacent slices (mirrored blocks) from fresh tissue samples, **3mm in thickness**, were placed in a sufficient volume of each fixative **(50:1, fixative to tissue)** for **a standard time** at room temperature. Following fixation, tissues were placed in appropriately labeled processing cassettes, washed briefly (1 minute) in tap water to remove excess fixative, and processed to paraffin wax.

PARAFFIN WAX PROCESSING OF ALL SAMPLES.

The tissue samples were all processed to paraffin wax in an identical fashion, using an MVP tissue processor and a standard processing procedure.

Dehydration was by several changes of alcohol, with increasing alcohol concentration up to 100%. Following dehydration, the alcohol, which is not miscible with paraffin wax, was replaced by the intermediate solvent (clearing agent) xylene, which is freely miscible with paraffin wax. This was followed by four changes of paraffin wax to replace the xylene. Finally, the wax infiltrated tissue was placed in a mold, filled with fresh molten wax, and allowed to solidify. The solid wax provides support for the tissue during the sectioning process. Thin, 2-4 μm, sections were obtained, floated on to warm water to flatten and subsequently picked up on slides.

TISSUE SAMPLES.

Phase I tissues.

The paired samples of; liver, large and small bowel, uterus, skin, kidney, skeletal muscle, placenta, spleen, lung and tonsil, were fixed for 24 - 48 hours, processed and sectioned. H&E staining, plus a variety of common special stains were performed on these sections.

Phase II tissues.

Because of the particular sensitivity of IHC techniques to fixation time, additional paired samples of tonsil were fixed for strategic time points. This was done in order to mimic the reality of daily surgical and anatomical pathology practice. These fixation time points ranged from a low of 2 hours, through 4 hours, 8 hours, 12 hours, 16 hours, 24 hours, 3 days, to a high of 7 days. These tissues were hand processed, using the same reagents as before. Finally, for each fixative, all the time point blocks from a single patient, were mapped and then re-embedded into a single large block. These multi-sample blocks (Timed set 1) allow simultaneous IHC staining to demonstrate the effects of fixation time. A selection of IHC markers was used on sections from these blocks.

Phase III samples.

The effect of fixation on the IHC demonstration of prognostic and predictive markers of breast tumours is well known. Several breast tumour cell lines, with known expression of estrogen receptor (ER), progesterone receptor (PgR) and HER2/neu proteins, were harvested from tissue culture and prepared as plasma clot suspensions. Paired plasma clots, from each cell line, were fixed in each fixative for 24 hours. These fixed clots were then processed to paraffin blocks and sectioned as before. The sections were stained for ER, PgR and HER2/neu proteins.

Phase IV tissues.

As a result of initial IHC staining, for CD3 and for the intermediate filament vimentin, during Phase II, it was observed that the tissues fixed in TISSUFIX™ (TF) appeared to stabilize more rapidly than in NBF. To test this observation, and to try to establish a consistent stabilization time, the supplier was asked to provide two additional samples of TISSUFIX™ (TF2). These samples were coded by the supplier as ‘A’ and ‘B’. Fresh

tonsil slices were prepared as for phase II, each paired slice was bisected resulting in 4 matched samples. These samples were fixed in NBF, TF, TF2A and TF2B, for the following time points; 2, 4, 6, 8, 10, 12, 14, 16, 20 and 24 hours.
Following processing to wax, mapped multi-sample blocks (Timed set 2) were made as for phase II.
This procedure was repeated using a second tonsil sample (Timed set 3).
IHC staining for vimentin was performed on sections from these blocks.

RESULTS.

Phase I.

Following fixation, all tissue samples exhibited the change of colour typical of formaldehyde fixation. This change in colour could be largely reversed by treatment in 70% alcohol, again typical of formaldehyde fixation. It was noted that tissues fixed in TISSUFIX™ had a slightly firmer, more rubbery, consistency compared to the same tissues fixed in NBF.
Sections, ranging from 2 to 4 micrometers in thickness, were easily obtained from all tissue sample blocks and no difficulties were noticed during microtomy. When stained by H&E, all paired samples had a very similar morphological appearance. Nuclear and cytoplasmic details were comparable and the level of staining was similar for each fixative **(figures 1a and 1b).**
The following special stains were performed on these samples;
Masson trichrome, Gomori one step trichrome, MSB trichrome, Miller's elastic, Gordon and Sweet's reticulin, PAS, and Atwood's stain (alcian blue/phloxine/tartrazine). All special stains tested gave similar excellent results with both fixatives. A critical comparison of both the MSB trichrome **(figure 2a and 2b)** and the Atwood's stains revealed a very slight increase in brilliance with the tissues fixed in TISSUFIX™.

Phase II.

IHC staining for the following CD markers was performed on tonsil sections, timed set 1, from both fixatives. All sections were batched together and stained simultaneously, in order to minimize run-to-run variation.

CD45.
This marker has proven to be very robust in our hands, and has exhibited reliable staining over a wide range of fixation conditions. Excellent and comparable staining was achieved with both fixatives across all time points. **Figures 3a and 3b** illustrate the staining obtained at the 24 hour time point for both fixatives.

CD20.
This B cell marker has also exhibited reliable staining over a wide range of fixation conditions. Again, comparable staining was achieved with both fixatives across all time

points. **Figures 3c, 3d and figures 5c, 5d,** illustrate the staining obtained at the 24 hour and 7 day fixation time points respectively.

CD3.

This T cell marker exhibits some interesting effects depending upon fixation conditions. The polyclonal antibody used requires pre-treatment of the sections with a proteolytic enzyme prior to staining. The time of proteolysis and type of enzyme used, is dependent upon the degree of fixation. As such, the methodology used to obtain successful staining gives an indirect indication of the quality of fixation. Our standard protocol for this marker is to use trypsin for 30 minutes at 37º C. This has been optimized to un-mask tissues fixed in formaldehyde for 24 - 48 hours, but is insufficient for tissues fixed for longer time periods. These longer fixed tissues require the use of protease XXIV (pronase) to obtain a reliable demonstration of CD3. Fixation of tissues in formaldehyde for times shorter than approximately 8 hours leaves proteins susceptible to over-digestion by proteolytic enzymes, whereas tissues fixed for longer than 8 – 12 hours are more resistant. Such was the case with the tissues in timed set 1. Examination of these sections revealed the expected pattern of CD3 staining for tissues in both fixatives for 24 hours. **Figures 3e and 3f** illustrate this finding. Tissues fixed with both fixatives, for 3 days (not shown) and 7 days, required the use of pronase pre-treatment. **Figures 5c and 5d** illustrate the expected pattern of CD3 staining, after pronase pre-treatment, following 7 days fixation.

For fixation periods of 8 hours or less, the NBF fixed tissues exhibited the classic appearance of 'nuclear meltdown' that results from proteolytic over-digestion. Nuclear chromatin threads appear to dissolve and coalesce into a diffuse pattern. The shorter the fixation time period, the more severe and noticeable the effect. In the sections fixed in TISSUFIX™, this effect was also present at fixation times of 2 and 4 hours, but was not readily seen in tissue fixed for 8 hours. This would indicate an increased resistance to proteolytic over-digestion at between 4 and 8 hour fixation times. **Figures 11a-11f** illustrates this finding.

CD5.

The CD5 antigen is a cell surface glycoprotein, mainly expressed on T cells but is also expressed on a subset of B cells located primarily in the mantle zones of normal secondary lymphoid follicles.

CD5 can be difficult to demonstrate, as it appears to be sensitive to formaldehyde fixation. Heat induced epitope recovery (HIER) is necessary for the demonstration of this marker following formaldehyde fixation. Longer fixation times may necessitate longer HIER times, in some hands. Our standard time for HIER is 40 minutes at 98ºC. This seems to cover all the fixation time points as CD5 was successfully demonstrated across all time points with both fixatives.

Figures 4a and 4b and figures 5e and 5f, illustrate the staining after 24 hours and 7day fixation respectively.

CD23.

The CD23 antigen is a membrane glycoprotein which functions as the low affinity IgE receptor found on B cells. CD23 can be difficult to demonstrate, as it is often lost following fixation in formaldehyde for times less than 12 hours (not shown), longer fixation in formaldehyde, 24 - 48 hours, improves retention of this marker (personal observation). CD23 was successfully demonstrated following both fixatives. However, fixation in TISSUFIX™ appeared to reduce both the number of cells demonstrated, and the intensity of the reaction, following our standard protocol. The reason for this is not clear but it may be that extending the HIER time, changing the HIER buffer used, or re-optimizing the antibody dilution, will correct the situation. This effect is illustrated in **figures 4c and 4d and figures 6a and 8b,** after 24 hours and 7 day fixation respectively.

Other CD markers.
Successful staining with both fixatives was also achieved for the following; CD4, CD8, CD15, CD34, CD57 and CD79a (not shown).

Other IHC markers.

Fascin.
Human fascin is a 55 – 58 kD actin bundling protein, the actin binding activity of which is regulated by phosphorylation. In normal lymphoid material, staining is restricted to the interdigitating reticulum cells in T cell zones, with variable reactivity in follicular dendritic cells. Following fixation in formaldehyde, HIER is necessary for the complete demonstration of the reticular network. **Figures 4e and 4f and 6c and 6d,** illustrate the staining after 24 hours and 7 day fixation respectively.

Vimentin.
Vimentin is a 57kD intermediate filament protein present in cells of mesenchymal origin. The monoclonal vimentin antibody(clone V9) when used without HIER, has long been utilized as a 'housekeeping' antibody for IHC, due to the fact that vimentin is very susceptible to being masked by cross-linking during formaldehyde fixation. In fact, the progressive loss of IHC reaction for vimentin can be used as an indicator of the degree of cross-linking, i.e. the completeness of formaldehyde fixation.
Vimentin is fixed in non-masked form by alcohol. Even after greatly prolonged fixation in alcohol, vimentin is readily stained by IHC using the same antibody without HIER. In practice, tissues which have been fixed in formaldehyde for less than 24 hours are secondarily fixed by the alcohol used during processing. This variable admixture, of masked (formaldehyde) and non-masked (alcohol) fixation of vimentin, allows IHC staining for vimentin to work without HIER. For tissues that are formaldehyde fixed for 24 hours or longer, HIER pre-treatment completely reverses the masking of vimentin due to cross-linking and the immunoreactivity is restored.

With both fixatives, when tonsil sections from timed set 1 were stained for vimentin without HIER, the progressive loss of immunoreactivity over time was readily demonstrated. **Figures 7a and 7b,** illustrate the degree of immunoreactivity obtained following 24 hours of fixation. For the NBF fixed sample, the overall level of reactivity was low but staining of the capillary endothelium is still evident. However, for the

TISSUFIX™ fixed sample there is almost complete loss of immunoreactivity. It appears that masking occurred earlier than in samples fixed in NBF. This finding suggests that cross-linking may occur in a shorter time. If that is so, it would support the manufacturer's claim of faster fixation.

Figures 8a, 8c and 8e, illustrate the level of vimentin immunoreactivity, obtained without HIER, on tonsil fixed in Alcohol, NBF and TISSUFIX™ for 7 days respectively. **Figures 8b, 8d and 8f,** illustrate the level of vimentin immunoreactivity, obtained with HIER, on tonsil fixed in Alcohol, NBF and TISSUFIX™ for 7 days respectively. It should be noted that immunoreactivity is slightly reduced following HIER on the alcohol fixed sample, whereas, in both the NBF and TISSUFIX™ samples, immunoreactivity is fully restored, and is comparable to that seen in figure 8a.

Figures 9a - 9f, illustrate the progressive loss of vimentin immunoreactivity, over 2 to 8 hours in both fixatives. **Figures 10a -10f,** illustrate the progressive loss of vimentin immunoreactivity, over 12 to 24 hours in both fixatives. It should be noted that the degree of vimentin immunoreactivity seen in TISSUFIX™ at 16 hours, is comparable to that seen in NBF at 24 hours.

Successful staining with both fixatives was also achieved with several other robust IHC markers, these included; S-100 protein, wide spectrum cytokeratins, desmin, α-smooth muscle actin, and immunoglobulins (not shown).

Phase III.

The effects of fixation on the IHC demonstration of the prognostic and predictive markers of breast tumours, ER, PgR and HER2 are well known. In particular, fixation with alcohol based fixatives compromises the IHC immunoreaction, as does fixation for less than 12 – 16 hours in formaldehyde[11]. To compare the two fixatives, three different cell lines were harvested from tissue culture and prepared as plasma clot suspensions. Paired plasma clots, from each cell line, were fixed in each fixative for 24 hours.
The IHC demonstration for ER, PgR and HER2, was performed on the fixed plasma clots of the following cell lines:

MCF-7.
This breast tumour cell line is a known expresser of estrogen receptor protein (ER). It also expresses low to moderate levels of progesterone receptor protein (PgR). It therefore provides a useful positive control target for ER and PgR IHC. The lower levels of expression of PgR provide a sensitive target to assess loss of this marker during fixation. This cell line has a normal (non-amplified) expression of the HER2/neu protein. It therefore provides a useful negative control target for HER2 IHC.
As illustrated in **figures 13a -13f,** the demonstration of ER and PgR was not compromised by either fixative. In fact, the demonstration following TISSUFIX™ appeared slightly superior to NBF for both markers. As expected, staining for HER2 was negative following both fixatives.

SKBR3.
This breast tumour cell line is a known high level over-expresser of the HER2/neu protein. It therefore provides a useful positive control target for this marker. The cell line does not express detectable levels of either ER or PgR. It therefore provides a useful negative control target for these markers (not shown).
As illustrated in **figures 14a and 14b,** the demonstration of over-expression of HER2 protein was not compromised by either fixative.

MDA-175.
This breast tumour cell line is a low level over-expresser of HER2 protein. It therefore provides a useful positive control and a sensitive target to assess loss of this marker during fixation. Unfortunately, this cell line proved very difficult to grow and despite initial success on NBF fixed test blocks, we were unable to demonstrate any HER2 protein in any subsequently harvested cells in either fixative. Consequently, these staining results are not shown.

Phase IV.

Sections from the 4 blocks of timed set 2, the 4 blocks of timed set 3, together with repeat sections from the 2 blocks of timed set 1, were stained for vimentin as in phase II. All sections were grouped and stained simultaneously, in order to minimize run-to-run variation.
On careful examination of all the stained sections, there was little or no difference in vimentin immunoreactivity between the three samples of TISSUFIX™. As in the original stains on timed set 1, a progressive loss of vimentin immunoreactivity was seen over time **(figures 9a-9f, and figures 10a-10f).** For all the NBF fixed samples, in all three timed sets, 24 hours fixation appeared to be the stabilization point. For all the TISSUFIX™ fixed samples, in all three timed sets, 16 hours fixation appeared to be the stabilization point. The level of vimentin immunoreactivity following 16 hours in TISSUFIX™, appeared to be the same as, or less than, that produced by 24 hours in NBF.
Figures 12a-12e), illustrate the difference in vimentin immunoreactivity, between NBF and TISSUFIX™, at 16 hours fixation time, across all three timed sets.

DISCUSSION.

Background information on the actions of Formaldehyde a fixative.

An excellent description of the properties of formaldehyde may be found in Kiernan J.A., *Histological and Histochemical Methods: Theory and Practice*, 3rd Edition, 1999. Oxford: Butterworth-Heinemann. ISBN # 0-7506-3106-6.

Formaldehyde is a gas with the formula $H_2C{=}{=}{=}O$. It is available to the histologist as a solution (Formalin) containing 37-40% w/v gas in water, and as a solid polymer, paraformaldehyde. As commercial Formalin contains between 10-15% v/v methanol and small amounts of formic acid, it is often recommended that paraformaldehyde be used to

generate formaldehyde for research applications. There is no concrete proof that the small amount of methanol present in the diluted solutions is deleterious. For fixation purposes, formalin and paraformaldehyde are identical.

The concentration of formaldehyde used for fixation has been the subject of much confusion. This is due to commercial formalin solutions being referred to, and thought of, as 100%. The content of formaldehyde in a fixative is stated as the percentage by weight of the gas, rather than as a percentage of the formalin used to prepare it. The concentration of formaldehyde used in various fixatives varies widely (0.5 - 15%). The majority of fixatives, using formaldehyde as the sole fixative agent, contain between 2.5% and 8% w/v.

In aqueous solution, formaldehyde rapidly becomes hydrated to form methylene glycol (methylene hydrate). Hardly any (<0.1%) true formaldehyde is present in the solution [6]. The chemical reactions of methylene hydrate are essentially those of formaldehyde in the presence of water, so it is usual to speak and write of aqueous solutions as if they contained formaldehyde.

The equilibrium between methylene glycol and formaldehyde lies so far in favour of methylene glycol, that conversion of methylene glycol to formaldehyde by the removal of formaldehyde is well known to physical chemists as an example of a “clock” reaction [7]. When tissues are placed in solutions of formaldehyde, they are penetrated rapidly by methylene glycol and the small fraction of existing formaldehyde. The formaldehyde is consumed by tissue binding and more formaldehyde is then formed from dissociation of methylene glycol. This reaction can be used as a “real-time” clock, measurable in hours. This equilibrium, between methylene glycol and formaldehyde explains the paradox of formaldehyde fixatives being rapid in penetration of tissue (methylene glycol), but slow in fixation(formaldehyde).

Formaldehyde fixes not by coagulation, but by addition and condensation. The reactions of formaldehyde with macromolecules are numerous, complex and occur slowly over time in a sequence of steps. Initial reactions are with basic amino acids (primarily lysine and arginine) and other side chains with active hydrogen groups, to form hydroxymethyl adducts. These addition compounds change the tertiary and quaternary structure of the proteins, making other side chains available to form more hydroxymethyl adducts. These addition compounds subsequently condense with other active groups to form cross-linking “methylene bridges”. It is this cross-linking which confers stability to the proteins without changing their secondary structure [8]. It is also the reversibility of many of these weak cross-links which allows restoration of tertiary structure and successful IHC [9]. Although formaldehyde is one of the fastest penetrating fixatives, as explained above, it is really slow to form addition compounds.

The penetration rate of formaldehyde fixatives has been extensively studied, often with conflicting results. The penetration of non-coagulating fixatives into tissue is difficult to measure. The original experiments of Medawar[10] utilized plasma clots. Using indicators to measure the depth of penetration, he reported that aqueous formaldehyde had a

Medawar constant of 5.5. Medawar showed that fixatives obey the diffusion laws, that is, the depth penetrated was proportional to the square root of time. The Medawar constant is the coefficient of diffusibility of the fixative. In the equation, $d= K\sqrt{t}$, where d is distance penetrated in mm, *t* is time and K is the Medawar constant, a constant depending on the fixative used. In practical terms, using this value, aqueous formaldehyde would penetrate 27 mm in 24 hours. Plasma clots are easier for fixatives to penetrate than solid tissues, so the real penetration rate is probably less.
Baker[3] chose a gelatin/albumen gel to more closely mimic solid tissue and obtained K = 3.6, or 17.5 mm in 24 hours. Baker pointed out that the actual penetration into tissue would be considerably less, possibly due to the resistance of lipid containing cell membranes. He quotes the data of Tellyesniczky who, using tissue samples, indicated a more conservative K value of 0.78 for formaldehyde. That would translate to 3.8 mm in 24 hours. It is likely that the Tellyesniczky K number is reflective of the 'true' time to achieve fixation, i.e. not the penetration rate but the binding time.

From the equation $d= K\sqrt{t}$, it follows that fixatives penetrate more quickly into small pieces than large pieces of tissue. The initial rate of penetration into tissue is extremely rapid; the first layer of cells (20μm) may be 'penetrated' in a second or so. If K=3.6 that translates to greater than 70 mm/hour. Whereas, according to $d= K\sqrt{t}$, fixative will not reach cells 3.6 mm below the surface for another 59 minutes 59 seconds.
So for a large spherical sample, 5 cm in diameter, only a layer 3.6 mm thick at the surface will be 'penetrated'. For a smaller spherical sample, only 5 mm in diameter, 3.6mm far exceeds the radius of the sphere and central cells will actually be 'penetrated' in 30 minutes. Obviously, the smaller the sphere, the faster the penetration rate. This has practical importance for the fixation of tissue. Tissue samples are seldom taken as spheres, but as slices 'X' mm thick. The same observation applies, since these slices can be penetrated from all sides. The smaller 'X', the faster the penetration. A core biopsy of breast tumour, 1.5 mm thick will be penetrated in 5 minutes; a 5 mm thick slice of the tumour in 30 minutes.

The time required for the covalent binding of formaldehyde, to form addition compounds with tissue elements, has proven more difficult to study. Baker[3] quotes experiments that, after 8 hours at 70°C, indicate only 50% of the eventual binding of formaldehyde was complete and even after 24 hours, at that elevated temperature, only 90%. Baker even suggested that the only complete fixation by formaldehyde occurred when the tissue was also stored in it! Pearse[11] states that "It is clear that in using neutral 10% formalin at room temperature, and even more so at 4°C, we are making relatively little use of its capacity to form addition compounds and bridges".

Fox *et.al.*[6] used ^{14}C labeled formaldehyde to study the covalent binding time for rat kidney tissues. At a temperature of 25°C, the amount of formaldehyde bound to tissue increased with time until equilibrium was achieved at 24 hours. At 37°C the reaction was faster and equilibrium was reached at 18 hours. A later study by Helander[12] also used ^{14}C labeled formaldehyde to study binding time for the fixation of rabbit liver. At 25°C, half maximal binding was reached at 100 minutes, and equilibrium achieved at 25 hours. In addition to binding time, this study also examined reversibility of binding. For tissue

fixed 24 hours and subsequently stored in water, binding was reduced to 50% after 17 hours. Even for tissue fixed for 6 days, 90% of binding was reversed on prolonged storage in water. Unfortunately, reversibility was not studied in tissues fixed for shorter time periods than 24 hours. It would have been interesting to observe the reversal times after the half maximal point.

The correlation of results between these two studies is impressive. Particularly in view of the fact that Helander[12] used **4 mm** cubes of fresh rat liver, whereas Fox *et.al.*[6] used **16µm** thick sections of fresh rat kidney. The virtually identical equilibrium times achieved by each study indicate that penetration time is not a factor in the kinetics of the reaction. Despite the fact that thin slices of tissue will be penetrated faster than thicker slices, it would seem that the binding time is the limiting factor for tissue stabilization. In a further study also by Helander[13], using rat brain and kidney, equilibrium was not achieved until 50 hours. However, the tissue in Helander's latest study was twice the thickness (8mm) of the original study, a factor to be taken into account when comparing the data.

For both 1 mm thick biopsies and 4mm thick tissue slices, the minimum stabilization time is 24 -25 hours at ambient temperatures. The minimum stabilization time does not, unfortunately, denote complete fixation time. The initial cross-links are still relatively weak and reversible; stronger cross-linking continues to occur over time. Complete fixation is thought to take at least 7 days. Even after this time cross-links continue to form slowly.

Werner [14], quoting the two papers above, considers cross-linking complete in 24 -48 hours, but also expresses concern about the 'over-fixation' due to excessive cross-linking, which may occur if fixation is allowed to exceed 24-48 hours. We agree with Werner, in that excessive cross-linking may mask some epitopes, but in our experience this does not occur with the vast majority of antibodies in use until 5 -7 days of fixation. Even then, providing the IHC has been optimized, with the majority of antibodies, fixation up to 4 weeks is acceptable. A far more serious problem is short <24 hour fixation. Formaldehyde fixation begins at the periphery of the tissue. The initial layers of cells bind all of the available formaldehyde (<0.1%) and start the 'clock'. Methylene glycol continues to rapidly penetrate the tissue and, over hours, more formaldehyde is generated from methylene glycol. If this process is interrupted before completion, the formation of addition compounds will be incomplete, easily reversed and full stabilization by cross-linking will not occur. Depending upon the time of interruption, the periphery of the tissue may show adequate cross-linking, whereas the remainder of the tissue is fixed by coagulant alcohol. This may have disastrous effects upon IHC staining. This effect will occur whether the tissue is a small biopsy or a 3 mm slice.

We believe that this effect is readily seen with some antibody binding sites. For example, in breast tumour samples that have been short(<24 hour) fixed, demonstration of HER-2neu protein by IHC often reveals a heterogeneous staining pattern or edge staining with no central staining, or even negative staining despite over-amplification demonstrated by FISH. In addition, a curious 'ring staining' artifact is sometimes seen. In this artifact,

positive staining of the stroma, immediately surrounding 'nests' of tumour cells is seen. The tumour cells may have either partial or no intact membrane staining. The staining is specific for HER-2neu protein. It is neither a cross-reaction nor is it background stain, but truly represents the location of the protein. A theory that this artifact is due to delayed fixation is attractive, however, we have seen this artifact in needle core biopsies that were fixed immediately after they were obtained. We believe that the artifact is a result of short fixation and have some evidence pointing in this direction[15]. We suggest the following scenario; Tumour cells at the periphery of the section bind sufficient formaldehyde to stabilize their surface protein. Deeper cells may be preserved by methylene glycol but no cross-linking has occurred. Fixation is then interrupted by the ethanol used in processing and coagulation fixation occurs. HER-2neu protein is stripped from the cell surface by the coagulant fixative and either removed by the solvent or, in some cases the stripped protein is attracted to stromal proteins by hydrophobic interaction and becomes deposited. We have been able to duplicate the stripping effect of ethanol on HER-2neu positive cultured cells fixed in plasma clots. In addition, a formaldehyde fixation time series, on similar cell clots, revealed an edge to center gradient of positive to negative HER-2neu staining that was not seen after 18 hours of fixation.

Comparison of NBF and TISSUFIX™.

The results of this study indicate that TISSUFIX™, acts in a similar way to other buffered formaldehyde fixatives. Adequate cross-linking is essential to optimum stabilization performance, and the preservation and restoration of protein configuration. The length of time a formaldehyde fixative is allowed to act is crucial. This has particular importance in relation to IHC reactivity. It would appear that, compared to NBF, faster cross-linking is produced by fixation in TISSUFIX™, as the manufacturer claims. Some users may have concerns regarding this faster cross-linking and the potential for so-called 'over-fixation'. The results of IHC staining of tissues, fixed for 7 days, should alleviate those concerns. In fact, faster cross-linking translates to faster optimum fixation. This is a distinct advantage in cases that require IHC for fixation sensitive markers. Standardized, optimum, reproducible fixation is becoming increasingly necessary for the prognostic markers that may influence the patient's treatment.

When considering the faster cross-linking produced by TISSUFIX™, it should be remembered that, "The actions of formaldehyde are greatly modified by concentration, buffers and pH, osmolality of the fixative solution, added salts and the time and temperature of fixation[4].", whether this apparent increase in fixation speed is due to the concentration of formaldehyde, the unusual buffer constituents, or to the 'proprietary' ingredient remains to be discovered.

SUMMARY.

A study was undertaken to compare a commercially supplied, formaldehyde based histological fixative, TISSUFIX™, with in-house prepared, neutral buffered formaldehyde (NBF). Particular attention was addressed to the reactions of

immunohistochemical (IHC) stains to TISSUFIX™. The routine and special histological stains performed reacted well to TISSUFIX™. The morphological appearance of tissues was essentially identical to that produced by formaldehyde. Extensive testing of IHC stains, including prognostic and predictive breast tumour markers, revealed no significant problems with the use of TISSUFIX™. In short, we would have no concerns in making TISSUFIX™ our fixative for routine histological analysis.

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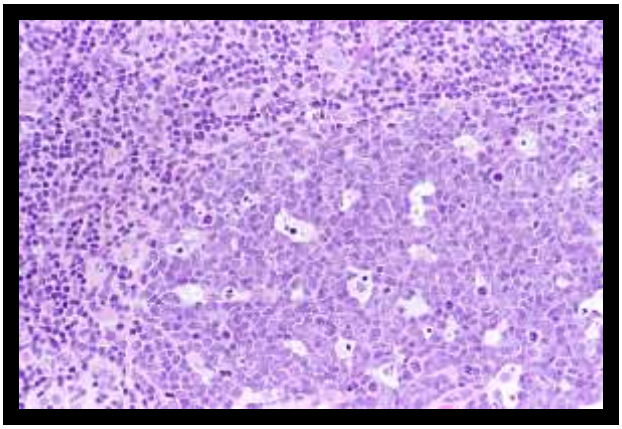
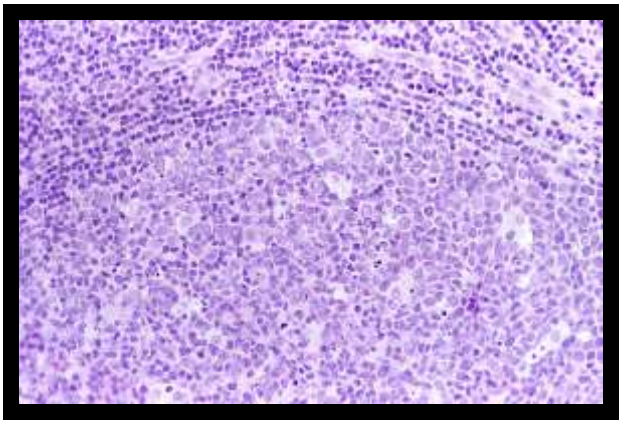
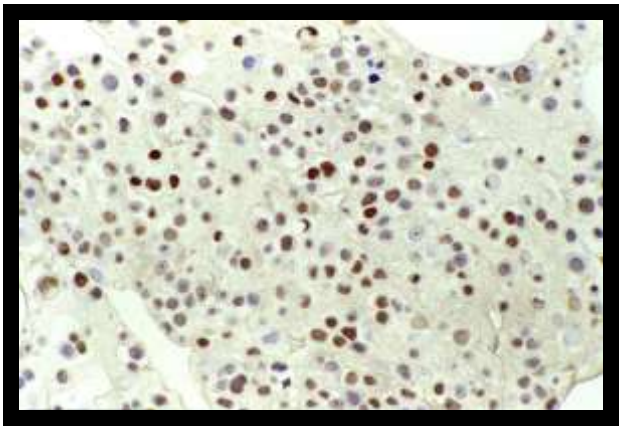
Comparison of TISSUFIX™ and Neutral buffered formaldehyde (NBF).



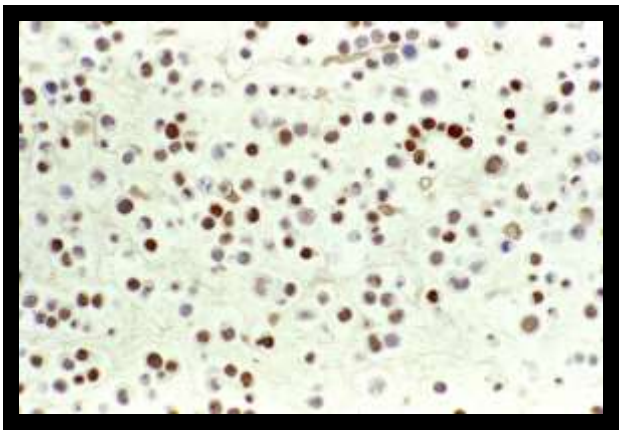
Tonsil, NBF, 2µm section, H&E



Tonsil, TISSUFIX™, 2µm section, H&E



MCF7 cells, NBF, ER



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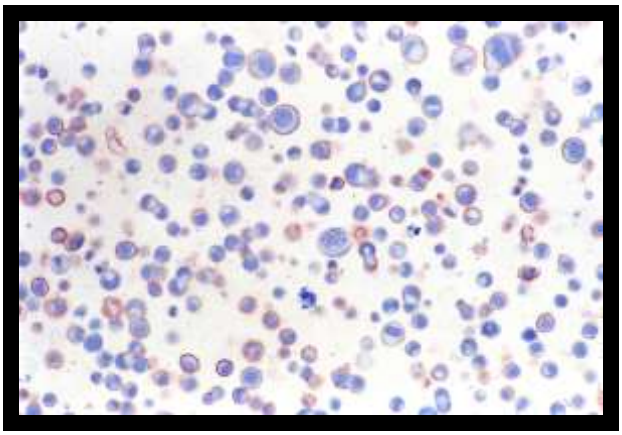
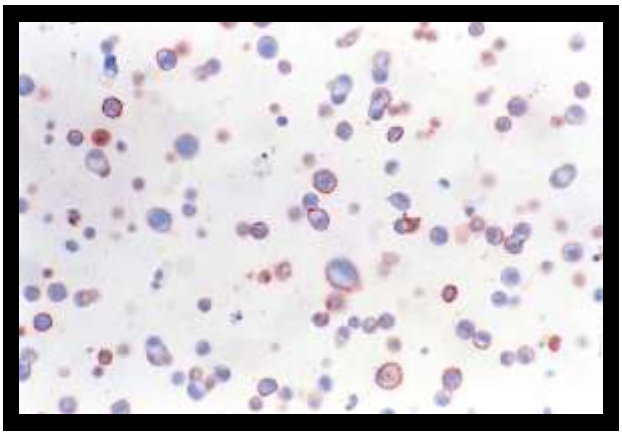


SKBR3 cells, NBF, HER2



SKBR3 cells, TISSUFIX™, HER2