

The aim of fixation

The aim of fixation is to preserve cells or tissues in as near a life like condition as possible, prevent autolysis and putrefaction, and protect the tissue from subsequent processing. Fixatives have different actions e.g. crosslinking, precipitative, coagulative etc. They also have different penetration rates, usually measured in depth penetrated per mm per hour. I normally recommend fixing in 20x the volume of fixative to tissue and fix for consistent periods of time- for example, I would normally fix samples 0.5 cm thick in at least one dimension for 24 hours in Neutral Buffered Formalin, or for 6 hours in Bouin's fluid. I would then transfer samples into 70% ethanol (the first stage in many routine paraffin processing protocols) prior to processing.

2. Phosphate buffered formalin

Formal calcium

Formal saline

Zinc formalin (unbuffered)

Zenker's fixative

Helly's fixative

B-5 fixative

Bouin's solution

Hollande's

Gendre's solution

Clarke's solution

Carnoy's solution

Methacarn

Alcoholic formalin

Formol acetic alcohol

Figure 1: A commercially available “concentrate” which, after dilution with water, produces a neutral buffered formalin solution.

Figure 1: Image of a commercially available “concentrate” which after dilution with water produces a neutral buffered formalin solution.

1. Phosphate buffered formalin

Formulation

40% formaldehyde: 100 ml

Distilled water: 900 ml

Sodium dihydrogen phosphate monohydrate: 4 g

Disodium hydrogen phosphate anhydrous 6.5 g

The solution should have a pH of 6.8

Fixation time: 12 – 24 hours

Recommended Applications

The most widely used formaldehyde-based fixative for routine histopathology. The buffer tends to prevent the formation of formalin pigment. Many epitopes require antigen retrieval for successful IHC following its use. Most pathologists feel comfortable interpreting the morphology produced with this type of fixative.

2. Formal calcium

Formulation

40% formaldehyde: 100 ml

Calcium chloride: 10 g

Distilled water: 900 ml

Fixation time: 12 – 24 hours

Recommended Applications

Recommended for the preservation of lipids especially phospholipids.

3. Formal saline

Formulation

40% formaldehyde: 100 ml

Sodium chloride: 9 g

Distilled water: 900 ml

Fixation time: 12 – 24 hours

Recommended Applications

This mixture of formaldehyde in isotonic saline was widely used for routine histopathology prior to the introduction of phosphate buffered formalin. It often produces formalin pigment.

4. Zinc formalin (unbuffered)

Formulation

Zinc sulphate: 1 g

Deionised water: 900 ml

Stir until dissolved then add –

40% formaldehyde: 100 ml

Fixation time: 4 – 8 hours

Recommended Applications

Zinc formalin solutions were devised as alternatives to mercuric chloride formulations. They are said to give improved results with IHC. There are a number of alternative formulas available some of which contain zinc chloride which is thought to be slightly more corrosive than zinc sulphate.

5. Zenker's fixative

Formulation

Distilled water: 950 ml

Mercuric chloride: 50 g

Potassium dichromate: 25 g

Glacial acetic acid: 50 ml

Fixation time: 4 – 24 hours

Recommended Applications

Gives good nuclear preservation but lyses red blood cells due to the presence of acetic acid. Has been recommended for congested specimens and gives good results with PTAH and trichrome staining. Produces mercury pigment which should be removed from sections prior to staining and can produce chrome pigment if tissue is not washed in water prior to processing. Is an intolerant agent so, after water washing, tissue should be stored in 70% ethanol.

6. Helly's fixative

Formulation

Distilled water: 1000 ml

Potassium dichromate: 25 g

Sodium sulphate: 10 g

Mercuric chloride: 50 g

Immediately before use add –

40% formaldehyde: 50 ml

Fixation time: 4 – 24 hours

Recommended Applications

Considered excellent for bone marrow, extramedullary haematopoiesis and

intercalated discs of cardiac muscle.

Produces mercury pigment which should be removed from sections prior to staining and can produce chrome pigment if tissue is not washed in water prior to processing. Is an intolerant agent so, after water washing, tissue should be stored in 70% ethanol. Because of the low pH of this fixative formalin pigment may also occur.

7. B-5 fixative

Formulation

Stock solution

Mercuric chloride: 12 g

Sodium acetate anhydrous: 2.5 g

Distilled water: 200 ml

Working solution, prepare immediately before use

B-5 stock solution: 20 ml

40% formaldehyde: 2 ml

Fixation time: 4 – 8 hours

Recommended Applications

Despite its mercury content and consequent problems with disposal this fixative is popular for fixation of haematopoietic and lymphoid tissue. It produces excellent nuclear detail, provides good results with many special stains and is recommended for IHC. Sections will require the removal of mercury pigment prior to staining. Tissue should not be stored in this fixative but placed in 70% ethanol.

8. Bouin's solution

Formulation

Picric acid saturated aqueous soln. (2.1%): 750 ml

40% formaldehyde: 250 ml

Acetic acid glacial: 50 ml

Fixation time: 4 – 18 hours

Recommended Applications

Gives very good results with tissue that is subsequently trichrome stained. Preserves glycogen well but usually lyses erythrocytes. Sometimes recommended for gastro-intestinal tract biopsies, animal embryos and endocrine gland tissue. Stains tissue bright yellow due to picric acid. Excess picric should be washed from tissues prior to staining with 70% ethanol. Because of its acidic nature it will slowly remove small calcium deposits and iron deposits.

9. Hollande's

Formulation

Copper acetate: 25 g

Picric acid: 40 g

40% formaldehyde: 100 ml

Acetic acid: 15 ml

Distilled water: 1000 ml

Dissolve chemicals in distilled water without heat.

Fixation time: 4 – 18 hours

Recommended Applications

Recommended for gastro-intestinal tract specimens and fixation of endocrine tissues. Produces less lysis than Bouin. Has some decalcifying properties.

Fixative must be washed from tissues if they are to be put into phosphate buffered formalin on the processing machine because an insoluble phosphate precipitate will form.

10. Gendre's solution

Formulation

95% Ethanol saturated with picric acid: 800 ml

40% formaldehyde: 150 ml

Acetic acid glacial: 50 ml

Fixation time: 4 - 18 hours

Recommended Applications

This is an alcoholic Bouin solution that appears to improve upon ageing. It is highly recommended for the preservation of glycogen and other carbohydrates. After fixation the tissue is placed into 70% ethanol. Residual yellow colour should be washed out before staining.

11. Clarke's solution

Formulation

Ethanol (absolute): 75 ml

Acetic acid glacial: 25 ml

Fixation time: 3 – 4 hours

Recommended Applications

Has been used on frozen sections and smears. Can produce fair results after conventional processing providing fixation time is kept very short. Preserves nucleic acids but lipids are extracted. Tissues can be transferred directly into 95% ethanol.

12. Carnoy's solution

Formulation

Ethanol absolute: 60 ml

Chloroform: 30 ml

Acetic acid glacial: 10 ml

Fixation time: 1 – 4 hours

Recommended Applications

Is rapid acting, gives good nuclear preservation and retains glycogen. It lyses erythrocytes and dissolves lipids and can produce excessive hardening and shrinkage.

13. Methacarn

Formulation

Methanol absolute: 60 ml

Chloroform: 30 ml

Acetic acid glacial: 10 ml

Fixation time: 1 – 4 hours

Recommended Applications

Similar properties to Carnoy but causes less shrinkage and hardening.

14. Alcoholic formalin

Formulation

40% Formaldehyde: 100 ml

95% Ethanol: 900 ml

0.5 g calcium acetate can be added to ensure neutrality

Fixation time: 12 - 24 hours

Recommended Applications

Combines a denaturing fixative with the additive and cross-linking effects of formalin. Is sometimes used during processing to complete fixation following incomplete primary formalin fixation. Can be used for fixation or post-fixation of large fatty specimens (particularly breast), because it will allow lymph nodes to be more easily detected as it clears and extracts lipids. If used for primary fixation specimens can be placed directly into 95% ethanol for processing.

15. Formol acetic alcohol

Formulation

Ethanol absolute: 85 ml

40% formaldehyde: 10 ml

Acetic acid glacial: 5 ml

Fixation time: 1 – 6 hours

Recommended Applications

A faster acting agent than alcoholic formalin due to the presence of acetic acid that can also produce formalin pigment. Sometimes used to fix diagnostic cryostat sections. If used for primary fixation specimens can be placed directly into 95% ethanol for processing.

Proprietary fixative solutions

During the last few years there have been an increasing number of proprietary fixatives developed for use in histopathology and medical research. They are generally marketed as less hazardous replacements for traditional formalin fixatives or as less toxic substitutes for fixative mixtures containing mercury such as B5.

Even though an MSDS must be provided the exact composition of these reagents is not usually published and a potential user has to make do with a general description of the reagent. Those recommended as substitutes for B5 and Zenker's (which are commonly used to fix lymphoid and hemopoietic tissues) usually contain zinc or barium salts and a low percentage of formaldehyde, while direct formalin substitutes often contain glyoxal and other components. It is in the latter group that reagents recommended for microwave-assisted fixation are found (see Part 5). Ethanol, methanol and isopropanol are included in some formulations.⁵

Figure 2: Two examples of proprietary fixative solutions. According to the

manufacturer "Fix-All" containing, alcohol, barium chloride and 10% formalin, is recommended for fixation of all types of tissues and as a substitute for mercury containing B-5 fixative. "O-Fix" contains alcohol, formalin and acetic acid and can also be used to fix all types of tissues but is particularly recommended for highlighting lymph nodes during dissection.

Figure 2: Two examples of proprietary fixative solutions showing Surgipath Fix-All and O-Fix. According to the manufacturer "Fix-All", containing alcohol, barium chloride and 10% formalin, is recommended for fixation of all types of tissues.