Sample Preparation and Shipping Instructions for Frozen Applications

Preparation:

The fixative used to prepare histopathology and IHC samples is determined by the specific experimental goals, tissue type, and downstream applications. Below are some commonly used fixatives and how to prepare them:

1. **Formalin**: Formalin is the most used fixative for histopathology and Immunohistochemistry (IHC) samples.
    - To prepare fresh 10% neutral buffered formalin (also referred to as fresh neutral buffered 4% paraformaldehyde), dissolve 40g of formaldehyde in 900 ml of distilled water and add 100 ml of 10X phosphate-buffered saline (PBS). Adjust the pH to 7.4 using sodium hydroxide (NaOH) or hydrochloric acid (HCl) and bring the volume to 1 L with distilled water. Most commercial purchased 10% formalin contains 10-15 % methanol as a preservative.

2. **Ethanol**: Ethanol is a good fixative for preserving lipids and cellular membranes for Histopathology but not for IHC samples.
    - To prepare 95% ethanol fixative, mix 95 ml of absolute ethanol and 5 ml of distilled water.

3. **Methanol**: Methanol is a commonly used fixative for frozen sections and immunofluorescence and IHC assays.
    - To prepare methanol fixative, use absolute methanol.

4. **Bouin's fixative**: Bouin's fixative is used for preserving glycogen and elastic fibers. For Histopathology samples only
    - To prepare Bouin's fixative, mix 75 ml of saturated picric acid, 25 ml of 37% formaldehyde, and 5 ml of glacial acetic acid.

5. **Acetone**: Acetone is commonly used for preserving lipids and cellular membranes used for frozen and cell cultures for IHC and immunofluorescence assays.
    - To prepare acetone fixative, use absolute acetone.

There are a few specific fixation solutions for particular tissue such as Davidson Fixative for eyes only.

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Fixation:

There are different methods followed for various situations, below explained are two common methods of fixatives.

Perfusion fixation is a method of preserving biological tissues for further study or experimentation. The fixative solution is injected directly into the bloodstream by cardiac or vena cava puncture, allowing the fixative to permeate the tissues and preserve their structure and molecular content. Below explains the general steps taken to perform Perfusion fixation:

1. Anesthesia: The animal or organism is anesthetized to minimize pain and discomfort during the procedure.
2. Cannulation: A cannula (a thin, flexible tube) is inserted into a major blood vessel, usually into left ventricle of heart and also aorta or the vena cava.
3. Perfusion: A fixative solution, such as formalin, formaldehyde or paraformaldehyde, is pumped through the cannula into the bloodstream. The fixative is typically delivered at a slow and steady rate, usually between 5-10 ml per minute, to ensure uniform penetration throughout the tissues.
4. Post-fixation: After perfusion, the tissues are usually left in the fixative solution for an additional period of time to ensure complete fixation.
5. Tissue preparation: The fixed tissues are then typically dissected or sectioned for further analysis or experimentation.

It should be noted that the specifics of perfusion fixation can differ depending on the type of tissue being studied and the desired experimental outcome. The general steps outlined above, however, provide a basic overview of the technique.

Immersion Fixation: it involves the use of fixative solutions. Since the solution should completely diffuse through the sample, the size and density of the sample should be a major consideration. For best results, the sample should be immersed in the appropriate fixative at a minimum volume of 20 times greater than the sample. The general steps involved in immersion fixation are as follows:

1. Dissection: The tissues of interest are carefully dissected and isolated from the rest of the organism or sample.
2. Fixative preparation: A fixative solution, such as formaldehyde, is prepared according to the desired concentration and volume.
3. Immersion: The dissected tissues are then placed directly into the fixative solution and left to soak for a set period of time. The exact duration of immersion will depend on the type of tissue being studied and the desired level of fixation.
4. Post-fixation: After immersion, the tissues are typically left in the fixative solution for an additional period of time to ensure complete fixation.
5. Tissue preparation: The fixed tissues are then typically dissected or sectioned for further analysis or experimentation.
6. Everything should be done in 4C.

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It is important to note that immersion fixation can result in uneven penetration of the fixative solution, resulting in incomplete fixation or tissue structure distortion. Some researchers may use agitation or other techniques to ensure uniform distribution of the fixative solution to mitigate these effects. Furthermore, the specifics of immersion fixation can vary depending on the type of tissue under investigation and the desired experimental outcome. The general steps outlined above, however, provide a basic overview of the technique.

Promptness & Volume of Fixative:

Prompt fixation is critical to preventing tissue sample degradation caused by autolysis and other factors. Tissues should be fixed as soon as possible after being collected, ideally within minutes. The longer the time between tissue collection and fixation, the greater the risk of tissue damage and degradation, which can jeopardise histopathological analysis's accuracy and reliability.

The amount of fixative needed for histopathology samples is determined by the size and type of tissue being studied, as well as the desired level of fixation. To ensure adequate penetration of the fixative solution, the volume of fixative should be at **least 20 times the volume of the tissue to be fixed**. **Perfusion fixation may be required for larger tissues**, such as organs or whole bodies, to ensure complete fixation.

It is also critical to select the correct fixative solution for the tissue sample.
Different fixatives have different properties and may be better suited to specific tissues or analyses. Although formalin is a widely used fixative that is effective for a wide variety of tissues, other fixatives such as **ethanol** or **Bouin's** solution may be more appropriate for specific tissue types or research questions.

To summarise, both prompt fixation and an adequate volume of fixative are required for high-quality histopathology samples.

The specific requirements for fixative promptness and volume will vary depending on the tissue type and research question, but in general, tissues should be fixed as soon as possible after collection, and the volume of fixative should be at least **20 times** the volume of the tissue being fixed.

Length of Fixations:

The amount of fixation time required for histopathology and immunohistochemistry (IHC) will vary depending on the type of tissue being studied and the specific experimental goals. Fixation times can vary from a few hours to overnight or longer.

The length of fixation should be sufficient for **histopathology** to ensure optimal preservation of tissue morphology and cellular structures. Formalin fixation is commonly used in histopathology, with fixation times ranging from several hours to overnight depending on the size and type of tissue being studied.

Over-fixation can cause tissue hardening and antigenic site masking, whereas under-fixation can result in poor cellular structure preservation.

For IHC, the fixation time should be long enough to preserve antigenicity while also preserving tissue morphology. Over-fixation can result in antigenicity loss, while under-fixation can result in poor tissue morphology preservation. Shorter fixation times are generally preferred for IHC to preserve antigenicity. Fixation times of 6-24 hours (maximum 48 hrs) are commonly used for IHC using formalin-fixed paraffin-embedded (FFPE) tissue to preserve both tissue morphology and antigenicity.
On the other hand, it is important to note that the length of fixation may also be affected by the specific antibody used for IHC, as some antibodies may necessitate longer fixation times to maintain antigenicity.

Furthermore, the type of fixative used may influence fixation times. Ethanol-based fixatives, for example, require shorter fixation times than formalin-based fixatives.

Cryoprotection of Tissues:

Once tissue has been fixed, the tissues must be then cryoprotected. Cryoprotectant agents prevent ice formation, which causes freezing damage to the biological tissue. Follow the general steps to cryoprotect your tissues:

1. Once remaining fixative is removed, transfer tissue to 15% sucrose at 20X volume compared to tissue.
2. Store in fridge (4C) until sample sinks to the bottom of container (24-48 hours).
3. Remove 15% sucrose and transfer tissue to 30% sucrose at 20X volume compared to tissue.
4. Store in fridge (4C) until sample sinks to the bottom of container (24-48 hours).

Freezing Fixed Tissue in Freezing Medium:

After the tissue is cryoprotected, tissue can then be shipped directly to us (shipping instructions further below) or can be embedded immediately. There are two methods to embedding fixed tissue in freezing medium, the dry ice or liquid nitrogen method.

For the dry ice method:

1. Crush Dry Ice into powder and put into Styrofoam container.
2. Quickly rinse the tissue in TBS and dab dry on a paper towel.
3. Place the specimen in an appropriate mold which has been filled with embedding solution.

Caution: During embedding air bubbles may be trapped in the tissue, causing the tissue to rise before the embedding is done. Air bubbles can also cause problems during sectioning as stretching of the section during pick-up may be compromised.

4. Push the specimen below the surface and ensure that the tissue does not touch any of the side walls.

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5. Allow 10 min. for the embedding medium (Refer to Section 5.1 Purchased Reagents) to infiltrate the specimen.
6. Sink the mold into the powered Dry Ice and hold the mold firm in the Dry Ice with large forceps until freezing occurs.
7. Do not allow any Dry Ice to fail into the embedding medium as this will cause air bubbles to form in the embedding medium.
8. Make sure that the specimen is completely submerged and top up the mold with embedding medium if necessary.

Note: Not enough embedding medium the mold might cause the specimen to melt during mounting on the chuck and might damage the specimen in the process.

9. Once the block is completely frozen remove from Dry Ice.
10. Invert the mold and tap the back of the mold.
11. If the block does not release from the mold, pry it from the mold. Warming the bottom of the mold with the palm of your hand may expediate the release of the block.
12. Place the block on the shiny side of the prelabelled piece of aluminium foil and tightly wrap the foil around the block with the label visible.
13. Store in a -80C freezer.

For the liquid nitrogen method:

1. Quickly rinse the tissue in TBS and dab dry on a paper towel.
2. Place the specimen in an appropriate mold which has been filled with embedding medium.

Caution: During embedding air bubbles may be trapped in the tissue, causing the tissue to rise before the embedding is done. Air bubbles can also cause problems during sectioning as stretching of the section during pick-up may be compromised.

3. Push the specimen below the surface and make sure the tissue does not touch any of the side walls.
4. Cover the mold if the specimen contains a fluorescent probe.
5. Let the embedding medium infiltrate the specimen for at least 10 minutes, 15 minutes is maximum.

Caution: Be careful not to leave the specimen in the embedding medium too long as it may disrupt morphology.

6. Pour liquid nitrogen in the Dewar vessel. Always use insulated gloves and safety glasses when using liquid nitrogen.
7. Pour 2 to 3 mm of 2-Methylbutane in a Copper Embedding Pot and chill the Pot in liquid nitrogen.

8. Once boiling ceases, place Copper Embedding Pot on the table.
9. Make sure the specimen is completely submerged and top up the mold with Embedding solution if necessary.

    Note: Not enough embedding solution in the mold now might cause the specimen to melt during mounting on the chuck and might damage the specimen in the process.

10. Place the mold in the liquid nitrogen chilled 2-Methylbutane. Cover the Copper Embedding Pot if the specimen contains a fluorescent probe.
11. After two minutes check if the Embedding medium is completely solid.
12. If it isn't solid yet, chill the Copper Embedding Pot in the liquid nitrogen until the boiling stops. Remove from liquid nitrogen and place on the table. Wait another minute before attempting to release the tissue block from the mold.
13. Remove mold from the Pot: invert the mold and tap the back of the metal mold.

    Note: If the tissue block does not come out this way, place mold back in Copper Embedding Pot and chill the Pot in liquid nitrogen until boiling stops. Remove from liquid nitrogen and wait another minute before trying to remove the block.

14. If the tissue block is still stuck, pry it from the mold. Warming the bottom of the mold with the palm of a hand may expedite the release.
15. Place tissue block on the shiny side of the corresponding aluminum foil and wrap foil so that Work Order # and/or Study #, and Sample ID are still visible.
16. Store box in -80C freezer

Freezing Fresh Tissue in Freezing Medium:

Tissue samples can also be embedded into freezing medium without fixation. Follow this procedure to fresh freeze tissue:

1. This procedure works best with two people but can be done by one experienced person. The procedure must be performed in a chemical hood or a very well-ventilated area because of the isopentane. The freezing of the tissue must be done beside the mouse during dissection. The removal of tissue and chilling of the isopentane must be done together for the best results.
2. After collecting tissue quickly wash it in ice cold PBS to remove blood, and extra junk.
3. As one person is collecting the tissue, the other will carefully pour the liquid nitrogen in a Styrofoam container or Dewar flask, use large tongs or forceps to lower a stainless steel, copper, Pyrex, or polypropylene container of isopentane (half full) into the liquid nitrogen. The isopentane starts to become opaque as it nears freezing.
4. Take the isopentane out of the liquid nitrogen when it starts to look opaque do not let it freeze.
5. Immediately, one tech will place a mold with a small amount of embedding medium covering the bottom of the mold into the chilled isopentane so that bottom half of the mold is

submerged into the isopentane. Use a large pair of forceps to do this. Do not submerge the mold into the isopentane.

6. The second tech will quickly remove the tissue from the cold PBS and roll the tissue around on a Kim wipe to dry the surface. Make sure that all liquid is removed from the surface. Water and OCT are slow to mix. Be careful not to damage the tissue.
7. As soon as the embedding medium starts to freeze (becomes white) which will be quick add another layer of freezing medium. Do not let the freezing medium completely freeze before adding the fresh freezing medium because the two surfaces will not melt together and will separate when sectioning.
8. Take the mold from the isopentane and quickly place the tissue into the freezing medium in the correct orientation before it freezes. Add enough freezing medium to completely cover the tissue.
9. Quickly submerge the lower half of the mold back into the isopentane.
10. Once the freezing medium is completely frozen remove the sample block (mold + sample) and wrap the block with aluminum foil tightly to prevent any drying of the freezing medium and quickly place the sample block into -80 C or on Dry Ice.
11. Rechill the isopentane again after 2 samples have been frozen. It is very important that the isopentane must be kept very cold. If the technicians are working slowly the isopentane may require cooling between samples. If in doubt about the temperature of the isopentane rechill it before moving to the second sample. The sample blocks are stored in the -80C freezer.

Shipping:

Histopathology and IHC samples must be shipped and packaged with special care to ensure that they are not damaged during transit and arrive in usable condition. Below is a general approach to shipping and packaging these kinds of samples:

1. Samples for frozen application for immunohistochemistry and immunofluorescence assays can be shipped in PBS instead of formalin depending on timelines.
2. If samples embedded in OCT are being shipped, ensure to package enough dry ice within the package.
3. Use leak-proof and sturdy packaging materials such as plastic bags, plastic containers, or cardboard boxes. The packaging materials should be resistant to temperature and humidity changes.
4. Select a reliable courier or shipping service that can deliver the samples quickly and safely. (Most labs have their preferred method of delivery and courier service)
5. Label the samples with all relevant information, including the sample name, the date of collection, the type of sample, and the test requested.
6. For liquid samples, add absorbent material such as paper towels or cotton to prevent leaks. Could use paraffin wax for the lids of sample containers to ensure no leaks occur.
7. Place the labeled and appropriately packaged samples in a secondary container, such as a plastic bag, Styrofoam cooler, or a plastic container, to further protect them during transit.
8. Add cushioning material such as bubble wrap, packing peanuts, or shredded paper to fill any empty spaces and prevent movement of the samples during transit.

9. Seal the primary and secondary packaging securely with tape or plastic seals.
10. Ship the package using a reliable courier or shipping service and track the shipment to ensure that it reaches its destination in a timely manner.
11. Ship samples early in the week to avoid delays and hold ups over the weekend.

Overall, the type of sample, the required temperature conditions, and the shipping method must all be carefully considered when shipping and packaging histopathology and IHC samples. Proper sample labelling, packaging, and cushioning can help ensure that the samples arrive in good condition for further analysis.

If you have further questions regarding preparing or shipment of tissue, contact info@waxitinc.com or call 604-822-1591

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