# Pathology Services Core

## Frozen Tissue Preparation

**Disclaimer**

We recommend submitting a sample-pilot study to test the morphological viability of your preparation before submitting actual test samples.

**Materials/Reagents: Liquid Nitrogen/Isopentane Method**

- **Stainless steel bucket**
- **Liquid Nitrogen in appropriate container**
- **Isopentane**
- **Plastic staining rack with long handle**
- **OCT Compound**
- **Plastic cryomolds (size dependent on tissue type)**
- **A marking pen to identify specimen on Cryomold**
- **Aluminum foil**

**Materials/Reagents: Dry Ice/2-methylbutane Method**

- **Ice bucket with lid**
- **Stainless steel bucket**
- **Dry ice**
- **2-methylbutane (CAS# 78-78-4)**
- **Plastic staining rack with long handle**
- **OCT Compound**
- **Plastic cryomolds (size dependent on tissue type)**
- **A marking pen to identify specimen on Cryomold**
- **Aluminum Foil**

**Frozen Embedding of Tissue in OCT Compound – No Fixation**

## Liquid Nitrogen/Isopentane Method

1. Fill an appropriate container with liquid nitrogen (black bucket).
2. Immerse metal cup filled 3/4 full with isopentane into the liquid nitrogen. The levels of these two solutions should be the same for even freezing of your specimen.
3. The isopentane will look opaque (milky) white and will have a rim of frozen isopentane when it is chilled enough to snap freeze a specimen -150 degrees C (this takes approximately five minutes). *Keep adding liquid N2 into the container to keep the level of the two liquids equal while waiting for the isopentane to chill.

### Tissue Freezing

4. Label the sample ID on the surface of the Cryomold with a cryomarker or permanent marker.
5. Place few drops of OCT (Tissue Tek OCT Compound, #4583) depends on the size of the tissue to be embedded) onto the center of the bottom of cryomold. Be careful to select the proper size embedding mold according to the size of the tissues to be embedded (see Figure 1).
6. Gently blot excess liquid from tissue.
7. Place the unfrozen tissue sample in the cryomold and orient. Make sure that the side touching the bottom of the cryomold is the side to be sectioned first. Gently push the tissue with a forcep to ensure that the bottom surface of the tissue is evenly secured in the cryomold (see Figure 2). **Be very careful to orient the sample because it is important for the demonstration of proper morphology**.
8. Carefully drop more OCT onto the specimen until it is completely covered. None of the tissue should remain exposed.
9. Try to avoid the formation of air bubbles. Remove any bubbles inside the OCT by using a disposable-plastic dropper/pipette. This is important because the air bubbles will create problems when cutting sections.
10. Let OCT compound settle for 15-30 seconds to allow the OCT to completely wet the surface of the tissue. The settling can also help to ensure that the OCT covers the tissue evenly and, in some cases, give enough time for any bubbles to dissolve.
11. While holding cryomold between a pair of long handled forceps, carefully immerse it down into the metal cup containing the chilled isopentane. Do not let go of the mold into the isopentane. Let it freeze for approx. 20-50 seconds (depending on size/thickness of tissue) (see Figure 3).

12. After freezing of the OCT compound (between 20 – 50 seconds), wrap the OCT embedded block in foil, placed in a labeled zip bag, and store in a -80C freezer until ready to transport to the lab. OCT samples can be transported on dry ice in a Styrofoam container.

13. In the absence of Isopentane/liquid N2, dry ice can be used as a substitute in freezing (see Figure 4).

**Figure 1. Cryomold Size Examples**


**Figure 2. T issues Embedded in OCT Gel – Notice the tissues in (a) and (b) are centered within the molds and with a suitable amount of OCT gel. Tissue in (c) can be cryosectioned but a larger cryomold would have been more appropriate to ensure enough OCT gel for sectioning stability.**

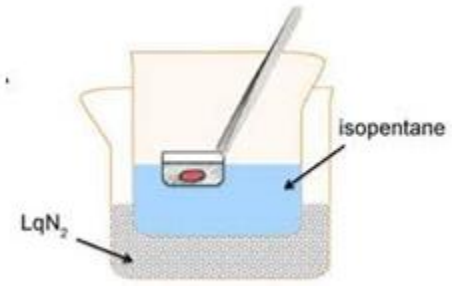


**Figure 3. Cryomold Isopentane/Liquid N2 Freezing Preparation sectioning stability.**


**Figure 4: Dry & Isopentane Freezing Methods**

**Standard OCT Preparation Protocol – Fixation Method**

* Excellent morphology compared to other methods.

**Fixation, Cryopreservation, and Tissue Freezing**

1. Dissect tissue accordingly and wash with 1xPBS.
2. Place in a 1:20 volume ratio of fixative (tissue: fixative), 10% NBF overnight at room temperature.
3. Wash the tissue 3x for 10 minutes with 1xPBS.
4. Place in 15% sucrose in 1×PBS at 4°C until tissue sinks, then transfer into 30% sucrose in 1×PBS at 4°C until tissue sinks. When initially placed in the mixture, the tissue should float at the top of the container. By the end of the 24-hour period, the tissue should have sunk to the bottom of the container (see Figure 5).
5. Place the unfrozen tissue sample in the cryomold and orient. Make sure that the side touching the bottom of the cryomold is the side to be sectioned first. Gently push the tissue with a forcep to ensure that the bottom surface of the tissue is evenly secured in the cryomold (see Figure 2). **Be very careful to orient the sample because it is important for the demonstration of proper morphology**.
6. Carefully drop more OCT onto the specimen until it is completely covered. None of the tissue should remain exposed.
7. Try to avoid the formation of air bubbles. Remove any bubbles inside the OCT by using a disposable-plastic dropper/pipette. This is important because the air bubbles will create problems when cutting sections.
8. Let OCT compound settle for 15-30 seconds to allow the OCT to completely wet the surface of the tissue. The settling can also help to ensure that the OCT covers the tissue evenly and, in some cases, give enough time for any bubbles to dissolve.
9. Follow Liquid Nitrogen/Isopentane Method or Dry Ice/2-methylbutane Method.
10. After freezing of the OCT compound (between 20 – 50 seconds), wrap the OCT embedded block in foil, placed in a labeled zip bag, and store in a -80C freezer until ready to transport to the lab. OCT samples can be transported on dry ice in a Styrofoam container.

## Dry Ice/2-methylbutane Method

1. Place the stainless-steel bucket inside the ice bucket and surround with dry ice. Fill the stainless-steel bucket with 2-methylbutane, approximately 4cm deep, and allow to cool with the lid on for 10 minutes. Note: this must be done in a fume hood!
2. Using the plastic staining rack, place the cryomold in the pre-chilled 2-methylbutane and allow to freeze completely (approx. 2-3 minutes; the OCT compound will become opaque when frozen).
3. Remove the cryomolds from the 2-methylbutane and shake off excess liquid. Store at -80 degrees Celsius wrapped in aluminum foil.

**Alternative OCT Preparation Protocol**

***Please note that there many protocols for frozen tissue preparation. This protocol is an alternative version to using 30% sucrose/PBS in which a 50:50 OCT: PBS mixture is substituted. In our experience, we've found that soaking tissues overnight in a sucrose/PBS or OCT/PBS mixture overnight in 4 degrees Celsius allows the OCT to better penetrate the tissue, reduces ice artifact as a result of uneven freezing, and augments the morphological representation of the tissue.

1. Dissect tissue accordingly and wash with 1xPBS.
2. Place in a 1:20 volume ratio of fixative (tissue: fixative), 4% paraformaldehyde or formalin for 4 hours, on ice, or in 4 degrees Celsius. *
3. Wash the tissue 3x for 20 minutes with 1xPBS (on ice).
4. Place in 50:50 OCT: PBS for 24 hours (overnight) in 4 degrees Celsius. When initially placed in the mixture, the tissue should float at the top of the container. By the end of the 24-hour period, the tissue should have sunk to the bottom of the container (see Figure 5).
5. Place in cryomolds molds filled with OCT and orient as desire, placing the desired tissue area to be sectioned at the bottom of the cryomold.
6. Place molds in -20 to -80 degrees Celsius for 30 minutes (can stay indefinitely until you are ready to bring to us).
7. When ready to transport to our lab, place on dry ice and bring to us, along with the order number.
8. When frozen sections are ready to be picked up, we will send you an email. Please bring a Styrofoam container with dry ice to transport your tissue and sections back to your lab. Unfortunately, we do not keep dry ice or Styrofoam containers.

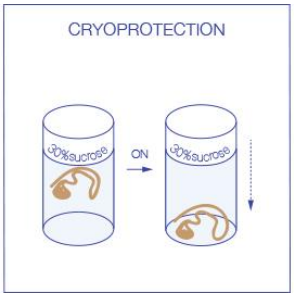


**Figure 5: Sinking of Tissue in Sucrose –**
**Tissue sinking in Sucrose or OCT/PBS will happen in a similar manner.**

Reference

https://www.jefferson.edu/content/dam/skcc/Sample%20prep-frozen%20embedding.pdf

http://www.cinj.org/sites/cinj/files/documents/C2ProcedureForFreezingTissueInOCTForCryocutting.pdf