NCI Biospecimen Evidence-Based Practices	SNAP-FREEZING OF POST-SURGICAL TISSUE BIOSPECIMENS				
Author	Biorepositories and Biospecimen Research Branch			Revision #	
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1.0 PURPOSE

The purpose of this document is to provide evidence-based guidance for the proper snap-freezing of human tissue biospecimens. This guidance is intended to support the development and execution of evidence-based Standard Operating Procedures (SOPs) for human biospecimen collection, processing, and storage operations.

2.0 SCOPE

This evidence-based best practice document is applicable to all human tissues that are to be preserved by snap-freezing. Biospecimens preserved under these procedural guidelines are suitable for downstream analysis of DNA, RNA, protein, and morphology endpoints. Additional analytical endpoints, including but not limited to cell viability, cell sorting, drug sensitivity testing, or use as donor specimens for xenografts or primary tissue culture, do not fall within the scope of this document.

3.0 DEFINITIONS

3.1 **Organ** – the complete or partial organ that is removed from the patient for dissection.

3.2 **Module** – the portion(s) of the organ that is/are specifically removed for the creation of segments or aliquots.

3.3 **Segment** – the component(s) that is/are dissected from the module that will be used to create the aliquot(s) for final labeling and submission.

3.4 **Aliquot** – the final tissue component(s) that is/are dissected directly from the organ or the segment according to protocol.

3.5 **Surgical Warm Ischemia Time** – the length of time a biospecimen is retained at physiological temperature, commencing with instrument-obstructed blood flow and terminating upon removal from the patient.

3.6 **Surgical Cold Ischemia Time** –the length of time elapsed between the time of removal of the tissue from the patient and the time the tissue is preserved by freezing, placement in formalin, or other stabilization method.

3.7 **Post-Mortem Interval (PMI)**–the length of time elapsed between the time of non-beating heart death and the time the tissue is preserved by freezing, placement in formalin, or other stabilization method.

4.0 ENVIRONMENTAL HEALTH & SAFETY

4.1 Universal Precautions (CDC-1978) are used for all phases of organ/tissue dissection and handling. Reference 9.1.1.

5.0 RECOMMENDED MATERIALS/EQUIPMENT

5.1 Plastic-backed absorbent bench paper.

5.2 New disposable dissecting equipment for each organ.

5.3 Liquid Nitrogen (LN2).

5.4 Dewar flask.

5.5 Cryogenic specimen storage container (cryovial, cryostraw, cryosette®, cryomold, or equivalent storage container designed for temperatures at or below -190°C), LN2 storage container or, in the event of immediate shipment, LN2 dry shipper.

5.6 Should LN2 be unavailable, alternative freezing media may include: isopentane pre-cooled with LN2; isopentane cooled with dry ice; dry ice alone; -80°C freezer. When utilizing dry ice or -80°C for freezing and storing at -80°C, suitable cryogenic specimen storage containers designed for temperatures at or below -80°C will be acceptable, and shipment may be performed on dry ice.

6.0 PROCEDURAL GUIDELINES

6.1 Recording of biospecimen preacquistion data

6.1.1 Whenever possible, extensive data should be recorded relating to preacquisition conditions that may affect the integrity of the biospecimen. Such data may include patient information (including age, gender, diagnosis and treatment) as well as details relating to surgery and

biospecimen acquisition (including the use of anesthesia, warm ischemia time, and surgical procedure and duration).

6.2 Preparation of freezing containers and bench space.

6.2.1 Pre-labeled cryogenic specimen storage containers for each organ being dissected should be identified and arranged before the organ is available for dissection.

6.2.2 Specimen containers should be appropriately labeled and organized, and tissues of different anatomic sites as well as tumor and normal tissues should be segregated to the extent possible.

6.2.3 Clean disposable scalpels and forceps should be used when cutting different tissue types of the same patient and specimens from different patients. Contact with absorbent materials which may contaminate dissected research tissues or where capillary action may draw fluid from tissue samples should be avoided.

6.3 Post-collection storage of tissue specimens on wet ice.

6.3.1 Specimens may be placed in a sterile closed container on wet ice until dissection (See 8.1).

6.4 Minimizing cold ischemia time.

6.4.1 Dissection should be accomplished soon after the specimen is released by the supervising physician. Cold ischemia time should be minimized as much as possible, optimally less than 20 min but no more than 1 hour (See 8.2). Cold ischemia time should be documented for every module or segment and for each subsequent aliquot.

6.4.2 For tissue specimens collected postmortem, PMI should be minimized as much as possible, optimally less than 2 hours, but no more than 6 hours (See 8.3). This time should be documented for every patient and attached to the module and its aliquots.

6.5 Dissection notes.

6.5.1 Dissection should be performed one organ at a time. Final aliquots should be no thicker than 0.4 cm and placed into the proper cryogenic specimen storage containers. If morphological analysis is anticipated then specimens can be surrounded by OCT medium prior to freezing; however, the use of OCT is not optimal for some specific molecular analysis methods (See 8.4).

6.6 Freezing of tissues.

6.6.1 Optimally, the tightly sealed cryogenic specimen storage container should be frozen in LN2 vapor. This can be achieved by suspending a stainless steel beaker inside a bench top Dewar flask pre-filled with LN2 (See Figure 7.1). The specimen storage container should then be placed inside the steel beaker for 2 minutes or less depending on the size of the specimen (See 8.5). Common alternatives to freezing in LN2 vapor may include freezing by immersion in LN2 or immersion in isopentane pre-cooled to -80°C or below (See 8.6).

6.6.2 If LN2 is unavailable at the physical site where specimens are collected and preserved, alternative freezing methods may used, and include immersion in isopentane pre-cooled with dry ice, placement on dry ice, or placement in a -80°C freezer. Freezing specimens directly on dry ice should be avoided if they are to be used for morphological analysis (See 8.7).

6.7 Transfer and storage of frozen biospecimens.

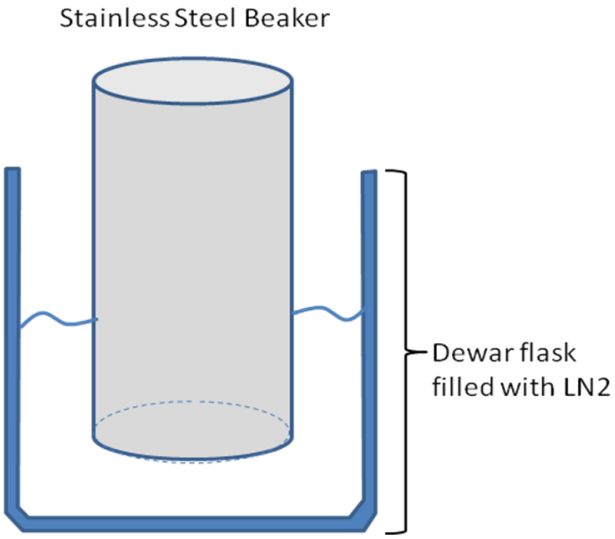
6.7.1 After freezing, the cryogenic specimen storage container should be transferred for storage in a LN2 vapor freezer. Should LN2 be unavailable, specimen storage containers may be stored at -70°C or colder (See 8.8).

6.7.2 Alternatively, the frozen specimens may be placed directly into a LN2 dry shipper for immediate transport (See 8.8). Specimen containers frozen in LN2 and destined for storage in LN2 should be held in LN2 vapor before and during transfer to repository/long term storage. Should LN2 be unavailable, specimen storage containers may be shipped on dry ice.

Specimen containers destined for storage at -80°C should be held on dry ice before and during transport.

7.0 FIGURES

7.1 Experimental set-up for snap-freezing in LN2 vapor



8.0 SUMMARIES OF LITERATURE EVIDENCE

8.1 Incubation of specimens on wet ice as opposed to room temperature reportedly delays the onset of ischemia-induced effects for RNA analyses [1-3], although data conflict as to whether incubation on wet ice [4] or at room temperature in the absence of buffer [5] is the optimal ischemic condition for protein preservation.

8.2 Cold ischemia has elicited quick and selective alterations in RNA transcript levels [6-8] and protein expression [4] after as little as 15-20 min at room temperature. Importantly, studies investigating similar timepoints extend the window of stability citing significant and selective changes after 30 min [5, 9], 60 min [10], 120 min [11], 6 h [1], or no change after 2 h [12, 13] or 5 h [14]. Other analytical endpoints appear to be more robust, as a cold ischemia time of 60 min or less did not affect yields of DNA [9], RNA [15, 16] or protein [9], PCR amplification of

DNA targets [9], or DNA quality [9, 14] and evidence of protein degradation was first reported after 24 h at room temperature [11]. Similarly, cellular analysis by flow cytometry was not significantly affected by ischemia time of 4 h or more [17]. While reports conflict as to whether RNA quality is adversely affected by progressive ischemia the earliest reported onset of significant RNA degradation as determined by RIN was after 45 min at room temperature in thyroid and colon [18]. Additional variables confounding investigation of ischemia-induced effects on RNA quality include: (a) tissue-specific differences in the timing and magnitude of effect as significant RNA degradation has also been reported after 12 h in liver and 18 h in an ovarian carcinoma specimen [2], (b) increased variability among RNA integrity numbers (RIN) following 30 to 120 min of ischemia [8, 19], (c) tissue composition-dependent differences in RIN, with lower RINs reported for specimens rich in connective tissue [20], (d) manual versus automated extraction methods [21], (e) and analysis [22, 23].

8.3 PMI does not significantly alter DNA [24], RNA [24-28], or protein [24, 28] yields with a few notable tissue-specific exceptions in frontal cortex [24, 29] and spleen [30]. Although RNA degradation has been reported in autopsy specimens [26, 31], reports conflict as to whether there is no clear relationship between the degree of degradation and PMI [25, 28, 30-35] or whether a weak but significant negative correlation is present [29]. The majority of DNA and RNA [25, 26, 28, 30, 31, 34, 36, 37] and protein expression analyses [28], as well as protein methylation activity [38], do not appear to be affected by a PMI of 1-5 days or less.

8.4 DNA and RNA yields, RNA quality, PCR and RT-PCR analyses and immunohistochemistry and Western blot analyses generated equivalent results in OCT-embedded specimens immersed in isopentane pre-cooled with LN2 and case-matched controls preserved by immersion in LN2, suggesting these molecular and immunoassays are not adversely affected by the presence of OCT or isopentane [39]. Although OCT-embedding has been reported to interfere with subsequent PCR analysis of amplicons longer than 280 bp [40], a more recent study employing a column-based extraction method observed no deleterious effects of OCT on PCR analysis of amplicons ranging in length from 267-927 bp when compared to untreated controls snap frozen in LN2 [39].

Receptor binding capability as determined by radioligand-binding and ligand titration also produced equivalent results between OCT-embedded and unembedded controls [41], while interference due to OCT has been reported for the dextran-charcoal and Lowry Protein assays [42]. While OCT has also been shown to interfere with mass spectrometry-based proteomic analyses in an animal model [43], a recent study using a human cell line demonstrated that OCT compound can be successfully removed by ether-methanol precipitation or filter-aided sample preparation [44].

8.5 Superior morphology was observed when specimens were frozen in LN2 vapor using a double-walled vessel either alone [45] or in media [46] compared to those directly immersed in LN2 [45, 46] or on dry ice with a cooling device [45]. While cellular dehydration and extra- and intracellular ice crystal formation and their resultant artifacts occurred more prominently at cooling rates slower than LN2 (an estimated 2000°C/min) [47], direct immersion in LN2 can result in the Leidenfrost effect, the creation of a insulating vapor layer upon contact with a substance hotter than the liquid's boiling point [48].

8.6 Specimens frozen by immersion in LN2 or isopentane pre-cooled to -80°C produced comparable DNA, RNA and protein yields; RNA purity and integrity; and RNA and protein expression levels [39]. However, morphology was modestly superior among specimens immersed in isopentane pre-cooled to -80°C than those immersed in LN2 [39], but morphology was equivalent among specimens frozen in isopentane pre-cooled with either LN2, dry ice, or a -100 °C freezer [49].

8.7 Specimens frozen by placement in a -70°C freezer or immersion in LN2 produced comparable RNA quality and were successfully used in the construction of a cDNA library [27]; and specimens frozen in a -20°C or -70°C freezer displayed epidermal growth factor receptor (EGFR) activity that was comparable to specimens immersed in LN2 [50]. However, freezing specimens on dry ice using a cooling device [45] or by the carbon dioxide quick freeze method [39] compromised morphology in comparison to LN2 due to the formation of macro- and microscopic cracks. Similarly, microscopic and ultrastructural damage due to ice crystal formation were more prevalent among specimens embedded in OCT

and frozen in a -20°C cryostat compared to those immersed in isopentane pre-cooled to -60°C [51, 52] or -112°C [53], although effects may be influenced by tissue type [51].

8.8 Potential effects of storage temperature on DNA and RNA analyses have not been investigated. Although EGF-R activity was reduced by the initial freeze, short term storage for up to 21 days at -20°C, -70°C, or LN2 produced equivalent results [54]. Specimens stored in a LN vapor freezer or at -80°C displayed similar gross morphology and equivalent levels of rubidium, iron, and zinc [55].

8.9 Potential effects of the temperature and conditions of shipment on DNA, RNA, and morphological analyses have not been investigated. Shipment of breast cancer specimens on dry ice, as opposed to in a LN2 dry shipper, resulted in a reduction in ER binding and a subsequently lower incidence of ER-positive cases [56].

9.0 REFERENCES

9.1 Laboratory Guidelines

9.1.1 Universal Precautions (CDC, 1987): http://www.cdc.gov/mmwr/preview/mmwrhtml/00000039.htm; Tools for Protecting Healthcare Personnel, CDC: http://www.cdc.gov/HAI/prevent/ppe.html.

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