

Title	Standard Operating Procedure (SOP) for Pancreatic cancer Fresh Tissue samples		
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I - PURPOSE

The purpose of this procedure is to describe the process for the collection and storage of pancreatic cancer patient tumour tissue in a biobank, so as to provide optimally preserved tissue that can be linked to relevant epidemiologic, genetic and/or clinical data.

II - SCOPE

This procedure is applicable to all individuals who want to perform a high quality collection and storage of pancreatic cancer fresh tissue that will be placed in a biobank.

III - DEFINITIONS

ASEPTIC PROCESS: Series of procedures that are performed under carefully controlled conditions and designed to prevent the introduction of unintended transfer or microorganisms.

LN2: liquid nitrogen, approximately -200° C

OCT: optimal cutting temperature. Embedding media (tissue-tek) for cryopreservation and cryosectioning of tissues.

OR: operating room

SNAP FREEZE: freezing tissue or cell pellet rapidly (optimally in of 10-20 seconds)

TRANSPORT MEDIUM: sterile isotonic solution into which a viable tissue sample is submerged during transport

WET ICE: crushed ice with a little water added to make slush

RLT Buffer: lysis buffer for lysing cells and tissues prior to RNA isolation and simultaneous RNA/DNA/Protein isolation.

RNAlater: aqueous, nontoxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA

FNA: Fine Needle Aspiration

IV – ORGANIZATIONAL ASPECTS

Sampling should be always performed under the guidance by pathologists/pathology trainee in order to preserve the characteristics of the resection specimen for further pathological examination.

SUBJECT SELECTION

Subjects potentially eligible are those with diseases of the pancreas who are scheduled for surgical resection. Subjects who have undergone radiation treatment prior to resection should be excluded in order to preclude the possibility of iatrogenic DNA sequence changes.

SAMPLES SUITABLE FOR BIOBANKING

- **Tissue samples:**
 - ⊖ Sample from normal pancreas
 - ⊖ Samples from pancreatic tumour

Tissue samples can be processed by a number of different methodologies, including snap freezing in liquid nitrogen, embedding the sample in OCT medium for subsequent frozen tissue sectioning, or immersion and refrigeration in an RNA-preserving reagent followed by snapfreezing 12 hours later. In addition, the Pathology Department will use a fraction of the sample to provide for paraffin-embedded samples.

- In the case of patients with unresectable tumours, it will be difficult to retrieve a sample for research. Obtaining biopsy (and even FNA samples) from ecoendoscopy should be valued upon approval by the patient, physicians and the endoscopists
- Also, when an exploratory laparotomy is carried out, obtaining tissue would be desirable.
- Pancreatic juice could be also sampled to further obtain DNA.

This SOP focuses on fresh tissue samples collection. Fresh tissue samples are valid for analysis of gene expression, protein structure and function, and other DNA mutation screening methodologies.

V – ETHICAL ISSUES

Retrieve the patient's informed consent (complying with the countries' ethical requirements). This consent must be signed prior to the date of surgical resection. The informed consent should clearly establish the purpose for pancreas samples biobanking, the patients' rights and

risks, as well as the benefits (see informed consent template). Also, appropriate institutional review board (IRB) (i.e. an independent ethics committee) approvals must be obtained prior to commencement of the collection of tissue samples for a specific research project.

VI - MATERIAL AND METHODS

1. Preparation for collection of pancreatic tissue samples

MATERIALS:

- Prepare cryotubes (at least, one for tumour and one for normal tissue samples).
- Prepare cryotubes containing 1 mL RLT buffer for intra-operative tissue sample samples.
- Prepare the liquid nitrogen (LN2) container in the pathology room.
- Also prepare:
 - o Safety glasses or face shield, freezer gloves and protective shoes
 - o Clean laboratory coat, surgical mask and sterile latex or nitrile surgical gloves
 - o Racks for vials/containers (also separated for tumor and normal tissue)
 - o Sterile disposable scalpels, forceps, dishes or plates, needles, etc.
 - o Sterile saline (PBS)
 - o Marking pen or pencil
 - o Chipped ice
 - o Sharps container for disposal of biohazardous sharps

2. Collection and processing of tissue samples

METHODS:

1. Ensure that supplies required for the collection procedure are available. Arrange pre-labeled vials (use of barcodes is recommendable) in a manner that allows for easy differentiation between vials intended for storage of tumor specimens and those for normal specimens. The liquid nitrogen transport container should be placed in a location in the pathology room where it can be easily accessed. Ensure that the vessel is secured in a manner that will prevent accidental spillage.

2. Arrive at the operating theatre prior to the expected time of completion of the surgical resection. Wear a clean lab coat, and bring personal protective equipment and all supplies required for the procedure.
3. Bring a copy of the signed consent form for the patient.
4. **Collecting the tissue should be always done under the guidance of the pathologist.** Tissue has to be collected from tumor and uninvolved tissue that is removed during the surgical resection and would otherwise be discarded.
5. Immediately after its removal, it must be transported it in an unfixed state (in a closed sterile container surrounded by wet ice) to the Pathology department. Operating procedures to minimise warm ischemia time and tissue degradation are essential.
6. Processing of tissue samples must be done as soon as possible after excision. Although in the case of pancreas cancer tissue limited warm ex-vivo ischemia and long-term storage seems to not negatively influence RNA quality (Rudloff et al. 2010), the tissues should be frozen within 30 min according to the Tubafrost recommendations (Mager et al. 2007). This is the desired standard for human tissue preservation.

PROCEDURES FOR PROCESSING OF SAMPLES (in the Pathology room)

Pathologist

Supply research technician with sample(s) for cryostorage – representative parts of the tumour and normal tissue:

Site priority for tumour tissue (in order of decreasing priority)

- a. Central area of the tumor
- b. Tumor margin

Site priority for normal tissue (in order of decreasing priority)

- a. Distant, grossly uninvolved pancreas
- b. Perilesional uninvolved pancreas (normal tissue adjacent to the tumor)
- c. Surrounding stroma

Technician

1. Prepare the tissue sample for freezing on a clean surface and using clean/sterile instruments—change instruments between preparing normal and tumour tissue.
2. Using a sterile needle or forceps, transfer the specimens provided by the pathologist to separate pre-labeled sterile dishes. Use separate forceps/needles for each specimen

to avoid cross-contamination. Check that each dish is correctly labeled with the tissue type (normal/tumor).

3. The minimum size of tissue for freezing is approximately 0.5 cm³ (0.5 x 0.5 x 0.5 cm), though the amount of tissue available will differ depending upon the sample site. Smaller fragments should still be frozen and stored in the tissue bank. If there is sufficient material, freeze duplicate samples.
4. All tissue must be handled as if potentially infectious. Some safety precautions are:
 - a. All human samples must be treated as if they are infectious for Hepatitis Viruses, Human Immunodeficiency virus, and other known and unknown infectious agents.
 - b. Personal protective equipment must be used at all times while working with human tissue (latex or nitrile gloves, face shield, protective laboratory coat, and covered protective shoes).
 - c. Gloves should be immediately removed and replaced in the event that they become torn or perforated.
 - d. All waste must be disposed of prior to leaving the work area. Biohazardous sharps must be properly disposed of in an approved "sharps" container.
 - e. After completion of work, all work surfaces must be disinfected.
 - f. Any injuries or exposure to human tissue or potentially infectious biologic agents must be reported promptly.

Flash or snap-freezing in LN2

Flash freezing in LN2 provides excellent specimen integrity and a wide array of options for tissue analysis. All procedures should be carried out in accordance with the local codes of practice. Working with liquid nitrogen is hazardous—all procedures must comply with local safety rules specific to this chemical. Take precautions to avoid accidental spillage or spattering of liquid nitrogen.

1. Label cryotubes with a barcode, also distinguishing with labels for tumour and normal tissue.
1. Put the specimens into the sterile cryotubes, which can be then tightly capped and submerged in liquid nitrogen for "flash freezing".
2. Take into consideration that snap freezing of all tissue samples should be undertaken within 30 minutes of excision from patient (tissue subject to a delay of up to 2 hours should still be collected and the delay noted within the local inventory database).

Embedding in OCT

Embedding the tissue samples in optimal cutting temperature (OCT) compounds followed by snap freezing not only preserves DNA, RNA, and protein integrity, but also enables sectioning of the frozen tissue.

2. Label cryomolds with a barcode, also distinguishing with labels for tumour and normal tissue.
3. The OCT compound is poured into the cryomolds. It is important to avoid formation of air bubbles, and to ensure that the top surface of the OCT compound is completely level.
4. Using a sterile forceps or needle, transfer the specimen to the OCT-filled cryomold and gently submerge the tissue into media until it is completely covered. None of the tissue should remain exposed.
5. The tray can be then placed on dry ice or in the vapor phase of liquid nitrogen for freezing.
6. After hardening of the OCT, the tray is transferred to a liquid nitrogen storage container (or alternatively a -80 C freezer for short-term storage).
7. The frozen OCT containing the specimen can be placed into a cryo-microtome chuck and sectioned. Frozen sections can be mounted on microscope slides and stained to characterize the tissue sample and determine the tumor/normal ratio, and if need be microdissected to isolate specific cell populations.

RNAlater

This substance protects RNA degradation in fresh samples. It eliminates the need to immediately process or freeze samples, but it impairs cutting at the cryostat and denatures proteins. It is only recommendable when RNA-analysis (gene expression) is the only application.

1. Label *RNAlater* containers with a barcode , also distinguishing with labels for tumour and normal tissue.
2. Cut tissue to be less than 0.5cm in at least one dimension, then submerge tissue in 5 volumes of *RNAlater* (e.g. a 0.5 g sample requires about 2.5ml of *RNAlater*). It is imperative that height and width of the specimen not exceed 0.5 x 0.5 cm, as this tissue perfusion of the RNA stabilizing reagent is significantly impaired in larger specimens. Some considerations are:
 - a. Use sterile and separate instruments for each specimen to avoid cross contamination

- b. Ensure that the specimen is completely embedded
3. Incubate the specimens in the RNAlater reagent overnight at 2-8 C.
4. After a minimum of 12 hours of refrigeration, transfer the specimen using a sterile forceps to a sterile labeled cryovial (with a barcode assigned).

Formalin Fixation

Formalin is used to stabilize protein in fresh tissue, and prevent autolysis and putrefaction. Preserving the tissue sample in formalin enables the embedding of specimens into paraffin blocks. The advantage of this method is that sections obtained from the block can be stained with hematoxylin and eosin (H&E) for optimal tumor characterization. In addition, sections can be used for immunohistochemical analyses.

Specimens intended for formalin fixation should be processed after the completion of other fresh tissue procedures, such as flash freezing, embedding in OCT compound, and submersion in RNA stabilizing reagent.

Working with formalin is hazardous—all procedures must comply with local safety rules specific to this chemical.

1. Prior to the procedure, aliquot formalin to specimen containers. The volume of formalin should be a minimum of 15-20 times the volume of the tissue sample - e.g., 20 ml of formalin per 1 cm³ of tissue. A 15 ml sterile centrifuge or conical tube can be used, or alternatively, a urine container or other capped, leak-proof container.
2. Label vials/containers with a barcode, also distinguishing with labels for tumour and normal tissue.
3. Tissue specimens to be fixed should not be bigger than 1.5 x 1 x 0.5cm. Fix specimens (using sterile and separate instrumental for each specimen) in fresh 10% neutral buffered formalin (NBF) Blue for a minimum of 4 and a maximum of 48 hours. Ensure that the specimen is completely submerged in the formalin fixative.
4. The tissue should be fixed at room temperature for a minimum of 24 hours (maximum of 48 hours), at which point the tissue can be transferred to a histology lab for embedding in paraffin (following conventional techniques).

Other fixatives:

- Fixation media such as Bouin's containing picric acid should be avoided, as this compound interferes with subsequent PCR analysis of extracted nucleic acids.

- PAXgene fixation may be used as an alternative to formalin (it offers some advantages concerning molecular analysis). However, the effect on long-term storage using this alternative fixative is not known.

3. Storage of samples

LN2 snap frozen and OCT embedded samples

Transfer the frozen samples from the temporary LN2 transport container to a liquid nitrogen storage tank for long-term storage, or a locked -80°C freezer. For storage longer than 5 years, liquid nitrogen is recommended.

- Store duplicate samples in a different storage facility if this is available.
- Check the back-up system for the storage repository—either a back-up freezer running constantly or adequate supplies of liquid nitrogen.
- Record storage details in the inventory database and check earlier data that were entered. At a minimum the information recorded should include: i) inventory number (local sequential code); ii) location; iii) pathology number; iv) type of tissue (site and also whether the sample is tumour/normal); v) lag time between excision and freezing; and vi) date. A barcode system is recommendable.
- Transfer details to the computerized database system.
- Update the database when samples are moved or depleted.

RNAlater-treated tissue

They can be stored at 4°C for one month, at 25°C for one week or at -20°C for indefinite time. For RNA isolation, simply remove the tissue from *RNAlater* and process. Specimens can be then transferred to a liquid nitrogen freezer or a mechanical -80°C freezer.

Blocks and slides

Formalin embedded tissues and slides should be stored under climate controlled conditions (temperature, humidity and sunlight). Frozen sections must be stored in a freezer.

V - REFERENCES

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