MOHCCN Biospecimen Guidelines:
Nucleic Acid Isolation from Fresh Frozen Tumour Tissue V1

1. Table of Contents

1. Introduction: Nucleic Acid Isolation from Fresh Frozen Tumour Tissue

Nucleic acid isolation from fresh frozen (FF) tumour tissue is comprised of a series of activities from the selection and preparation of tissue to the qualification of DNA and RNA ahead of library preparation. These guidelines are based on data compiled from 4 sites across Canada actively participating in the Marathon of Hope Canadian Cancer Network (MOHCCN).

1. Tissue Preparation
2. Pathology Review
3. Tissue Enrichment
4. Extraction
5. Quantification
6. Qualification

2. Biospecimen Workflow for WGTS

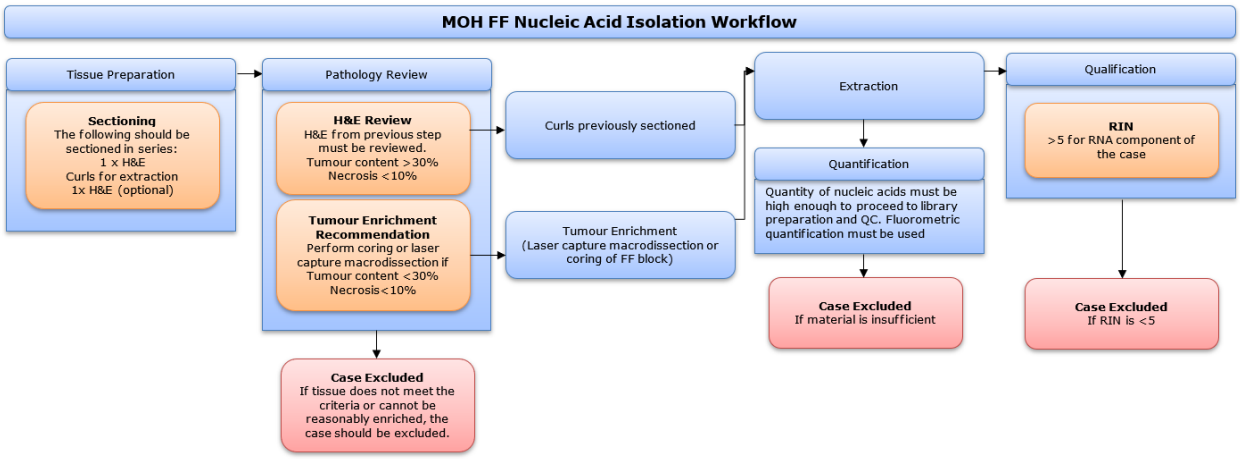
This workflow outlines the standard scenario for nucleic acid isolation ahead of library preparation. Please note that while tissue collection procedures are not within the scope of this document, these are critical for the success of downstream sequencing. Tissues are most often collected following existing institutional protocols and/or methods detailed as part of a clinical trial laboratory manual. While pre-analytical variables cannot be controlled for retrospectively accessed samples, for prospective collection, best practices should be outlined in laboratory manuals to enable high quality sample preservation for genomic and transcriptomic studies. These include guidance limiting ischemic time by immediate snap freezing, orienting cores within molds ahead of freezing, and storage of tissue at minimum -80°C or preferably in liquid nitrogen for long term use.

Core biopsies can be particularly challenging to work with. Freezing of these tissues in a tube is not recommended. Unlike the preservation of tissue chunks, the morphology of biopsy cores is easily damaged and can be difficult if not impossible to recover when removing these from a cryovial. The following video resources are recommended for describing how to appropriately freeze down biopsy cores for sequencing studies.

BCGSC Video 1
BCGSC Video 2

It is critical that the exact same tissue piece which is selected for sequencing is reviewed in consultation with a pathologist, as the tumour cellularity from that sample will be crucial for a case's success after sequencing. Doing this systematically, in ways that maintain tissue orientation, will ensure that effort is put towards samples that will ultimately be eligible for MOHCCN. Following review, some tissues may require tumour cell enrichment if they do not meet the required criteria for bulk sequencing. Once an adequate sample is obtained, extraction itself may occur using a variety of different protocols. Finally, the resulting nucleic acid must be quantified and qualified ahead of library preparation.

The FF nucleic acid isolation process and decisions associated with it are illustrated below.



3. Tissue Selection Guidelines

There were two main challenges identified by the Working Group in the process of sequencing FF tissue for the 15K gold cohort. The first of these was selection of tissue with sufficient tumour cellularity so that cases would be informative on the tumour specific genomics and transcriptomics. While best practices of various genomic cellularity assessment techniques is not within the scope of this document, these may be subsequently reported on by other working groups. To increase the probability of a case meeting desired informatics-based cellularity metrics, a minimum of >30% tumour cellularity is recommended, with >40% preferred for pathology review of FF samples undergoing bulk sequencing. The other challenge identified in the sequencing of FF tissue is the amount of input material for sequencing as FF tissue is most readily available from biopsies or requires enrichment due to low tumour cellularity, both of which result in very limited quantities of tissue.

The first challenge is best overcome by adherence to a strict tissue processing workflow whereby the tissue sequenced is reviewed for suitability and cases only continue to extraction steps if deemed to have sufficient tumour cellularity.

The following characteristics should be reviewed:

- Tumour content: should be >30% across the whole section, >40% is preferred
- Necrosis: recommended to be <10% within area used for extraction
- Inflammation: recommended for subjective evaluation (none, low, medium, high), used in conjunction with other characteristics to determine suitability, as this increases the non-tumour cell percentage within the area

It is recommended to enrich the tumor content using microdissection to achieve at minimum 30% tumour content before extraction. If this is not possible, the sample should not proceed in the workflow.

An example instruction sheet for pathology review is included in Appendix 2 and can be provided to pathologists ahead of MOH case review. A case review form, such as that shown in Appendix 3 can be used to collect relevant information during this pathology review.

Recommended sample selection QC Gate: **Tumour content >30% in whole section as per pathologist assessment. If <30%, tumour enrichment should be performed.**

4. Tissue Preparation and Enrichment

The processing of FF tissue should follow a similar workflow to what is done with FFPE tissue, in that sections should be taken in series, with sections at the beginning and optionally at the end of the series undergoing H&E and pathology review. The intermediate sections (will be referred to as curls thereafter) should be placed into a tube for purpose of extraction and frozen directly at -80°C either with or without lysis buffer including an RNase inhibitor.

It is very important for the RNA quality to prevent the curls from thawing ahead of RNase inhibitor addition. Some steps that should be taken is the cooling of tubes on dry ice or in the cryostat ahead of sectioning, the use of cooled instruments (for example: tweezers kept in the cryostat ahead of use), minimizing handling of the tubes and keeping these in the cryostat or on dry ice immediately after sectioning.

Unlike FFPE tissue, macrodissection of FF tissue is not recommended. Macrodissection of FF tissue cannot be done at room temperature, as this leads to rapid thawing of the tissue and degradation of RNA. Furthermore, thawed sections present with a gel-like texture, preventing accurate isolation of regions of interest as additional, unwanted adjacent tissue is pulled up with the scalpel blade. If sections are kept cold and macrodissected on dry ice or in the cryostat, a higher risk is posed to the operator's health and safety as they are working at cold temperatures and more likely to cut themselves during this process, risking exposure to pathogens within the tissue being processed.

Alternatives can be used to prepare the tissue so that the area used for sequencing contains a high proportion of tumour cells. If a tissue has an area of tumour, an area of adjacent tissue which has low tumour cellularity and there can be a straight line drawn between these to allow the tumour piece to then achieve >30% cellularity, then that portion of the tissue can be cleanly cut off ahead of sectioning. An example of such a case is shown to the right, where the dotted red line represents the location at which the block could be cut by using a single edge razor blade (Stanley,

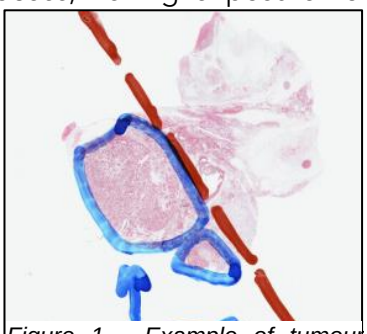


Figure 1 - Example of tumour tissue where there is an area of high tumour cellularity on the left (indicated in blue) which can be isolated by cutting the tissue along the red dashed line.

11-515), perpendicular to the block face. Curls would then be taken from this modified block and used for nucleic acid extraction.

If the tumour area is not easy to isolate alternatives to macrodissection that could be employed are coring the tissue or microdissection using laser capture. Both of these require the use of additional tissue past the curls that may have been taken during the original tissue sectioning process. Which method is selected will most heavily depend on the size of the region of interest, the expectation that histology will remain similar throughout the depth of the tissue and whether individual small clusters of cells need to be taken.

If the region of interest has an area of >2mm^2 and the tumour cellularity is expected to remain at >30% through the depth of the tissue, then coring should be used. Good candidates for this method include those tissue that have a well demarcated region of interest with high, dense tissue cellularity, but a large surface area of adjacent non-tumour tissue. Coring can be done in various sizes and should be taken from tumour rich areas of the tissue, as indicated on the pathologist marked H&E slide. A recommended protocol, used at the CHUM site, for frozen tissue coring is available in Appendix 4 of this document. Examples of tissue that may benefit from this technique are shown below.

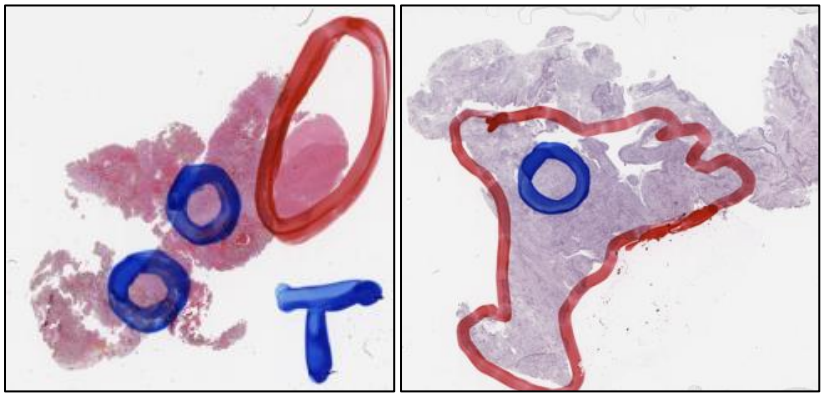


Figure 2 - Example of tumour tissue where coring would be recommended. On the left side, the tissue has a large amount of necrosis circled in red and the rest of the tissue has a high proportion (>60%) of normal cells. The areas circled in blue have a tumour cell proportion >30% and are recommended for coring. On the right side, the tissue has a large area of tumour, but it is has a lower tumour cell proportion due to a large amount of stroma. The area circled in blue has a high tumour cellularity, >80%, and is recommended for coring.

If the tissue has individual cell clusters, is quite sparse or the tumour cellularity is expected to change significantly through the depth of the tissue, then laser capture microdissection (LCM) should be used. Good tissue candidates for this method include pancreatic tissue or ductal carcinoma in situ. A pathologist will need to mark out representative areas of tumour on a guide H&E and serial sections will be dissected to isolate these regions of interest. An example of such mark-up is shown on the next page.

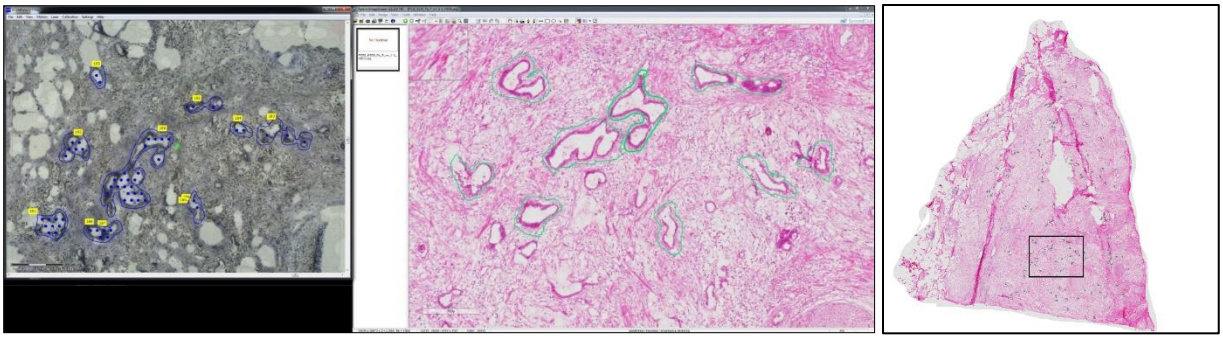


Figure 3 - Example of mark-up for LCM where a representative H&E section (right, field of view indicated by black box) has had tumour cells marked by a pathologist (center, green outline) and the tissue being dissected has been marked accordingly (left, blue outline) for microdissection.

LCM is a laborious technique requiring expensive, specialized equipment. While this may be suitable for certain studies, it may not be reasonable to perform ad hoc LCM outside of a particular prospective study. For additional consultation and guidance on performing LCM, please contact one of these laboratories:

Dr. George Zogopoulos' Laboratory
The Research Institute of the McGill University Health Centre
Alicia Gomez Mendez, Research Laboratory Manager
Email: alicia.gomezmendez@affiliate.mcgill.ca

UHN Biospecimen Services
Princess Margaret Cancer Center
Heidi Wagner, Head of Operations
Email: info@uhnbiospecimenservices.com

Tissue Portal
Ontario Institute for Cancer Research
Ilinca Lungu, Project Manager
Email: ilungu@oicr.on.ca

The amount of tissue prepared from dissection should be in accordance with the extraction method. This will depend on the capacity of the extraction method and output requirements for nucleic acid input to library preparation.

5. Extraction

A comparison of extraction methods across all sites responding to the information request revealed that a variety of methods were being used. These protocols are summarized in Table 1 below.

Table 1 - Extraction Protocols for FF Nucleic Acid Extraction

Protocol Name	Manufacturer	Catalog Number	Protocol Type	Nucleic Acid Eluted
DNeasy	Qiagen	69504	Column - Manual	DNA
Gentra Puregene (modified)*	Qiagen	158667	Precipitation - Manual	DNA
QIAcube RNeasy Mini	Qiagen	74116	Column - Semi-Automated	RNA
RNeasy Mini	Qiagen	74104	Column - Manual	RNA
RNeasy Micro	Qiagen	74004	Column - Manual	RNA
PicoPure RNA*	PicoPure	KIT0204	Column - Manual	RNA
All Prep DNA/RNA Micro	Qiagen	80284	Column - Manual	DNA and RNA
All Prep DNA/RNA Mini	Qiagen	80204	Column - Manual	DNA and RNA
EvoPure RNA Isolation and in-house DNA Isolation	Aline/Various	R-907T	Magnetic Bead - Semi-Automated	Total Nucleic Acid
DNA Multi-Sample Ultra 2.0	Thermo Fisher	A36570	Magnetic Bead - Semi-Automated	DNA and RNA

**Used exclusively for LCM inputs*

Sites tended to use a combination of methods. Dual extractions were most common and used for initial nucleic extraction. These yielded either total nucleic acid, or both DNA and RNA fractions within the same run. If top-up was needed due to insufficient yield, then a separate extraction method yielding only DNA or only RNA was used. Separate extractions were also recommended for very low inputs (such as those for LCM material).

For the collection of quality control data and future comparisons, it is recommended that the extraction kit used is tracked at the sample level.

6. Quantification

Fluorometric quantification is the gold standard for NGS and is essential for verification of sufficient quantity of nucleic acid ahead of sequencing. As library preparation inputs differ based on technique, it is up to each center to validate what this cut off needs to be based on their library preparation protocols, but a fluorometric (as opposed to absorbance) based quantification technique must be used.

If an unusually low quantity of RNA for the input is noted after extraction, but DNA material was as expected without any suspected operator error, this may be used in conjunction with qualification data to justify the exclusion of this case as library preparation is likely to fail. This is often observed if the tissue has been left at room temperature for an extended period of time instead of being snap-frozen immediately after removal from the patient.

Recommended sample quantification QC Gate: **Resulting nucleic acid content is sufficient for library preparation. This amount may differ between DNA and RNA, as necessary.**

7. Qualification

Qualification of nucleic acids after extraction can be done to screen samples and assess the probability of success through library prep and sequencing.

Very few DNA or RNA failures are reported for appropriately collected FF tissue. As with FFPE, RNA determines the success of a case and it may be advisable to prepare, qualify and sequence the RNA library ahead of proceeding with DNA for the case.

There is not one recommended method of qualification, but a review of RIN is advised. Centers reported that while a RIN greater or equal to 7 was expected for fresh frozen tissue, they routinely prepare libraries from RNA where RIN was as low as 5. Some centers would forgo this qualification entirely as failed library preparation from FF tissue was very rare.

Recommended sample qualification QC Gate: **RNA should have a RIN value greater than 5 to proceed to library preparation. If yields are lower than those expected based on input amounts and RIN is on the low end, it is recommended these samples do not proceed to library preparation.**

Document Revision History

Developed by	Reviewed by	Endorsed by	Effective Date	Policy Version	Summary of revisions
BWG	Steering Committee	Network Council	February 1, 2024	V1	n/a

Authors

Name	Institution	Title
Anne-Marie Mes-Masson (Chair)	CHUM	Associate Scientific Director, Basic and Translational Research
Trevor Pugh (Chair)	U of T	Senior Investigator & Director, Genomics
Madeleine Arseneault	McGill	Research Assistant
Veronique Barres	CHUM	
Jonathan Bush	UBC / BCCH	Clinical Associate Professor
Christine Caron	CHUM	Research Assistant
Jennifer Chan	U of C	Director, Associate Professor, Pathology and Laboratory Medicine
Stephanie Crapoulet	Vitalite Biobank	Lead
Sidney Croul	NSHA	Lead
Viktor Deineko	Northern Health	Research Program Manager
Carrie Hirst	BCGSC	Production Coordinator
Sachin Katyal	CCMB	Senior Scientist
Ilinca Lungu	OICR	Research Technician
Liliane Meunier	CHUM	Research Assistant
Andy Mungall	BCGSC	Group Leader, Biospecimen & Library Cores / Staff Scientist
Danielle Simonot	U of C	Associate
Thomas Sontag	CHU Sainte-Justine Research Centre	Biobank Manager
Emily Van de Laar	UHN	Project and Program Management
Suzanne Vercauteren	UBC / BCCH	Clinical Associate Professor
Ben Wang	UHN	Head, Immune Profiling Team

8. Appendix A – Method Comparison Tables

Item / Protocol	1 (CHUM)	2 (CHUSJ)
Pathology review requirement	Path review is performed to ensure tumour is present, no specific metrics	H&E before and after curls extracted are reviewed for % surface of tumour tissue / total tissue area. Excludes necrotic areas, interstitial space – ex. Fat / lifted tissue
Input types accepted	Tissue pieces	Curls, Core biopsies
Preparation of tissue ahead of extraction	Tissue kept in RNA later on ice	Curls are kept in RNA later at 4°C until extraction
Macrodissection performed?	No	No
Manual/Automated/Semi-automated	Semi-automated	Manual
Base Protocol	Qiagen D Neasy (DNA) / QI Acube Rneasy Mini (RNA), QIAcube All prep DNA/RNA mini (Dual)	Ambion mirVana miRNA isolation (RNA), Qiagen AllPrep DNA/RNA, mini or micro
Extraction Type	Dual extraction (yields both DNA and RNA from the same input, eluted in different fractions)	Dual extraction (yields both DNA and RNA from the same input, eluted in different fractions)
Homogenization	QIAshredder (if necessary)	QIAshredder
Elution buffer used	Kit Elution Buffer	Kit Elution Buffer / NFW
Quantification method	Tape Station	Qubit + Nanodrop
Storage after extraction	-20°C for DNA and -80°C for RNA	-80°C
Item / Protocol	**3 (BC)**	**4 (OICR)**
Pathology review requirement	Tumour cellularity, overall cellularity, surface area, % necrosis	Path review of >40% tumor cellularity, <10% necrosis and >25mm2 surface area – whole slide (for tumour tissue chunk/curls only)
Input types accepted	Frozen biopsies (16 – 20 x 50um sections or whole tissue used), 4 x 50um sections of OCT-embedded tissue, frozen cell pellets	Curls, Core biopsies, Bulk tumour (tissue chunk or homogenate), LCM ed material, Cell pellets
Preparation of tissue ahead of extraction	Sections place into lysis buffer and rocked overnight	If curls cut at OICR, these are place in extraction buffer immediately after sectioning. For tissue chunk bulk extraction (rare), RNA later Ice overnight is used.
Macrodissection performed?	No. Obvious normal tissue is removed during grossing.	No
Manual/Automated/Semi-automated	Semi-automated (can be done manually as last resort, but not recommended)	Manual
Base Protocol	EvoPure RNA Isolation Kit + In house DNA	Qiagen Gentra Puregene (w significant modification), Qiagen AllPrep DNA/RNA, Applied Biosystems PicoPure RNA, Qiagen RNeasy
Extraction Type	Magnetic bead; total nucleic acid elution	Dual extraction (yields both DNA and RNA from the same input, eluted in different fractions) or individual extract as needed + for low input
Homogenization	NA	Bullet blender (for cells and bulk tumour only)
Elution buffer used	dH20	Kit Elution Buffer / TE (DNA) / NFW (RNA)
Quantification method	Qubit	Qubit
Storage after extraction	-80°C	-20°C (short term) -80°C (long term)

Appendix B – Pathology Review Instructions

The guidelines below do not an exhaustive list of features to be observed and a pathologist must rely on their training to determine which areas of a tissue are best suited for sequencing.

It is essential that the area used for sequencing have a proportion greater than 30% tumour cells and less than 10% necrosis. A section in its entirety must meet this criteria. If it does not, laser capture microdissection or coring may be used. For coring, the region of interest should be demarcated on the H&E to allow for an accurate punch to be taken.

Tumour Marking

1. Place the slide on the microscope stage.
2. Select a low magnification objective and scan the whole section, noting any areas of densely packed, hypochromatic cells.
3. Move the stage so one of these areas is on view and select the preferred objective (10x or 20x recommended).
4. Cells should be observed on a higher power, noting nuclear appearance and arrangement. Nuclear features indicative of malignancy (but not always) include the following:
   - Irregular nuclear membrane;
   - Irregular chromatin pattern/distribution within the nucleus (clearings within the chromatin/grainy 'salt and pepper' appearance);
   - Nuclear pleomorphism;
   - Hypochromasia;
   - Loss of polarity (i.e., cells do not uniformly face the same direction);
   - Disorganization/nuclear over-crowding;
   - Increased nuclear to cytoplasmic ratio;
   - Presence of one or more nucleoli.

   Other features associated with invasive tumours include inflammatory exudates, necrosis, and lack of a membrane around clusters of tumour cells.
5. Once satisfied that the whole section has a high enough tumour cell proportion (>30%) and low enough proportion of necrosis (<10%), this case should proceed to extraction.
6. If the above criteria is not met, then two options for tumour cell enrichment may be evaluated:
   - Laser capture microdissection may be used if available and small clusters of cells are visible in an otherwise sparse stroma, consult the appropriate lab for review/marking instructions.
   - Coring may be used if there is an area <2mm where tumour cellularity is >30% and this is expected to continue through the depth of the tissue, mark this area.
7. If there is doubt about the presence of a tumour within the section, the sample should be excluded.

9. Appendix C – Pathology Review Form

MOHCCN Fresh Frozen Pathology Review Form

General Information			
Project Name		Request Date	
PI		Contact Name	
Patient ID		Contact Email	
Surgical Pathology Number			
Histological Diagnosis			
Tissue Site			

Service Request *to be completed by receiving lab*	
☐1 x H&E stained slide	☐10 x 10um curls in _____ buffer

Pathology Review *to be completed by Pathologist*		
Tumour Present	☐YES ☐NO	
Estimated Surface Area in Region of Interest	____mm x ____mm = ____mm^2	
Estimated Total Tumour Cellularity in Region of Interest	☐Very Low (<100 cells) ☐Low (100 – 4,000 cells) ☐Intermediate (4000-10,000 cells) ☐High (> 10,000 cells) ☐Very High (> 50,000 cells)	
Percentage Neoplastic Content within Region of Interest (tumour %)		
Percentage Necrosis within Region of Interest (necrosis %)		
Pathologist Name	Pathologist Signature	Date (dd/mmm/yyyy)
Additional comments:		

Samples and review form must be returned to the contact listed after review. Thank you!

10. Appendix D – Tumour Cell Enrichment by Coring FF Tissue Blocks

Recommended punches: Disposable Biopsy Punches (1mm), Robbins instruments, RBP-10P.

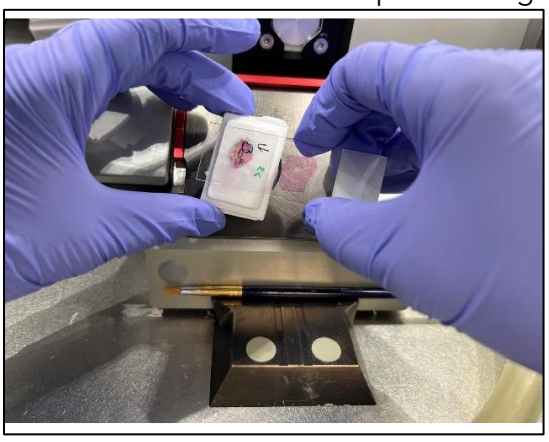
Important notes:

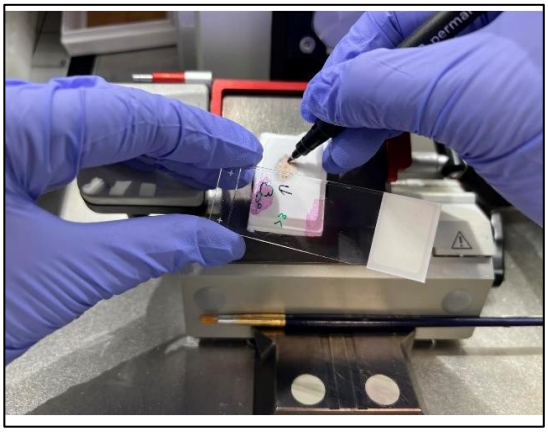
The tissue being punched should allow a minimum of 2 cores to be taken (the region of interest should have a minimum surface area of $2mm^3$)

It is recommended to cut an H&E after punching to compare with the initial H&E ensuring the correct area was punched.

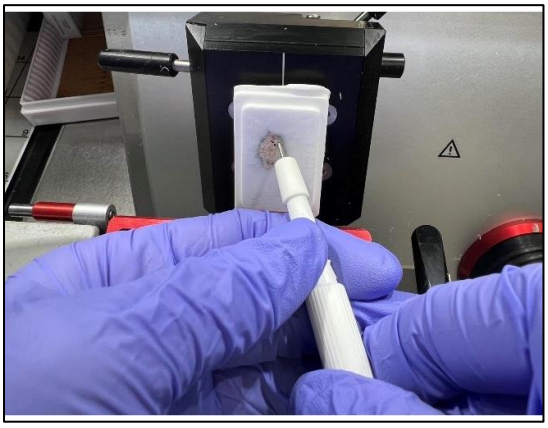
Procedure :

1. Make a preparation of the extraction buffer to be used and place in the homogenization tube of choice.
2. If the extraction buffer freezes in the cryostat, add it immediately after the cores instead of pre-filling the tube. Alternately, place the filled tubes on wet ice beside the cryostat.
3. Let the tube cool in the cryostat while performing the coring.
4. Align the H&E slide with the tissue and mark the area to be punched with a sharpie (it is recommended that a sharpie be assigned and used only at the cryostat),

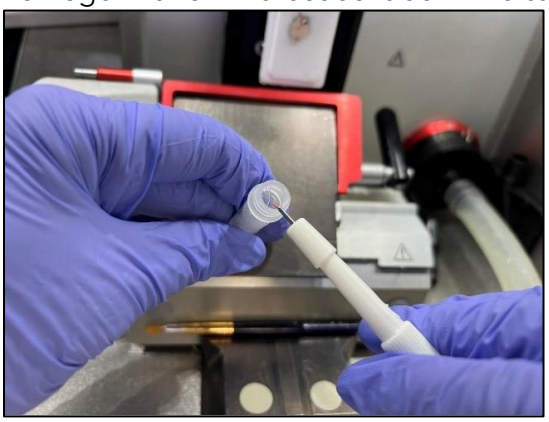




5. Use the marking to position the biopsy punch and take 2-6 punches of the tissue within the area of interest.




6. Place the cores into the homogenization tube and place on ice and proceed to homogenization in a beads tube in the same day



7. Recommended: Cut a section for H&E and stain using the standard institutional protocol. Use this to confirm that the region of interest was indeed the area cored.