MOHCCN Biospecimen Guidelines:
FFPE Nucleic Acid Isolation

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1. Introduction: FFPE Nucleic Acid Isolation

Nucleic acid isolation from formalin fixed paraffin embedded (FFPE) 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 will advise on the processes below based on data compiled from 11 sites across Canada.

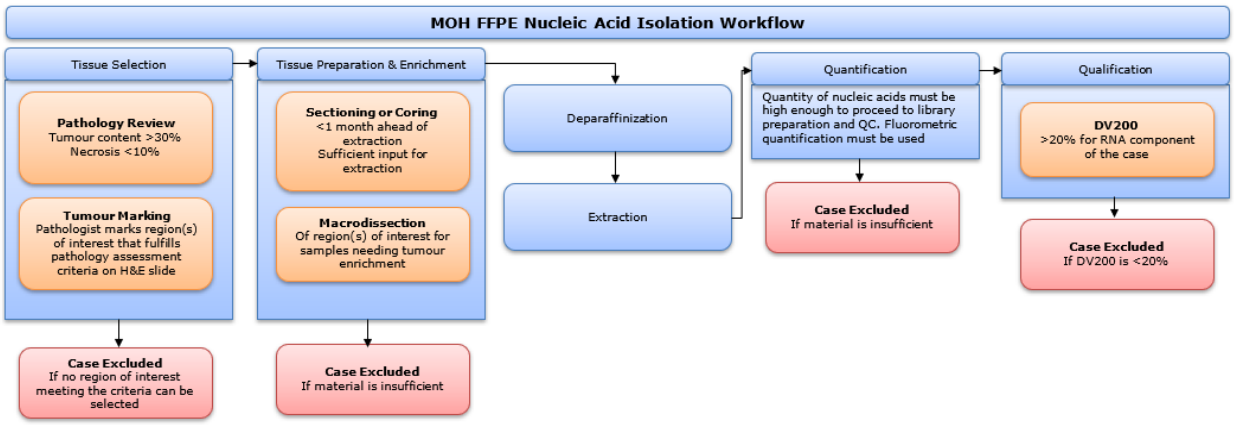
1. Tissue Selection
2. Tissue Preparation and Enrichment
3. Deparaffinization
4. Extraction
5. Quantification
6. Qualification

2. FFPE Biospecimen Workflow for WGTS

This workflow outlines the standard scenario for nucleic acid isolation ahead of library preparation. Please note that tissue collection, fixation and embedding is not within the scope of this document. As tissues are most often collected following the institution's pathology department protocols for diagnostic tissue, the ability to adjust variables such as ischemic time, fixation time and storage condition is limited. It is advisable to use blocks which are as recent as possible, with an ischemic time of less than 30min and a fixation time less than 48hr. However, the collection of these metrics and limiting of selected cases within these constraints is not feasible for most cohorts.

Tissue selection must be done with consultation from a pathologist, as the assessment of tumour cellularity will be crucial for a case's success after sequencing. This will limit the effort put towards samples that will ultimately be ineligible as part of the MOH cohort. Following selection, the tissue must be prepared either by coring or sectioning, and enrichment must be performed as needed. FFPE tissue is then deparaffinized in one of a few different ways depending on the protocol being followed. Extraction itself may occur in a semi-automated or automated way using a variety of different commercially available kits and protocols. Finally, the resulting nucleic acid must be quantified and qualified ahead of library preparation.

This process and decisions associated are illustrated below.



3. Tissue Selection Guidelines

Two main challenges were identified for sequencing of FFPE tissue for the 15K gold cohort. The first challenge is a high failure rate due to nucleic acid quality. 53% of cases failed RNA sequencing according to data provided from 3 institutions. This is due to the quality of nucleic acids, especially RNA, being compromised due to fixation inherent in the processing of the tissue. The second challenge was that following sequencing, the data provided little interpretable data due to a large amount of contaminating normal tissue.

While the first challenge is difficult to overcome due to the nature of formalin fixed tissue and the limited control over the way the tissue is initially collected and fixed, the second challenge can primarily be overcome by pathologist review of the sample's H&E ahead of proceeding with nucleic acid isolation.

The following characteristics should be reviewed:

- Tumour content: should be 30% within area used for extraction
- 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 macrodissection to achieve at minimum 30% tumour content before extraction. If this is not possible, the sample should not proceed in the workflow.

Samples that have undergone decalcification should be avoided as this process greatly degrades already compromised RNA.

Details on pathology review requirements for various centers are included in the comparison tables in Appendix 1. An example instruction sheet for pathology review is included in Appendix 2 and can be provided to pathologists ahead of MOH case review.

Recommended sample selection QC Gate: **Tumour content >30% and tumour necrosis <10% in region of interest, as per pathologist assessment.**

4. Tissue Preparation and Enrichment

FFPE tissue can be prepared in two ways: sectioning or coring.

Sections should be cut no more than one month ahead of extraction. It is recommended they be dried at room temperature on uncharged slides. If intended for extraction more that one month after sectioning, paraffin dipping the slide may assist in preventing degradation.

Coring can be done in various sizes and should be taken from tumour rich areas of the tissue as indicated on a slide marked by the pathologist.

Tumour enrichment should be done by macrodissection and may either precede or succeed deparaffinization as advised by the chosen extraction method. A guide H&E which has been marked by the pathologist should be used to assist in guiding macrodissection. This is easiest to do using a marked physical glass slide, but a digitized H&E slide may also be used if necessary. An example of macrodissected tissue is illustrated below in Figure 1.

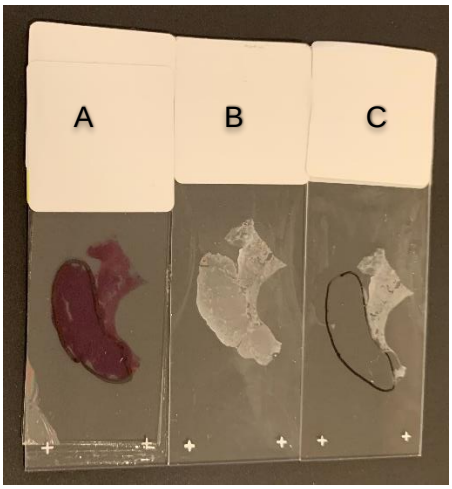


Figure 1 - Macrodissection of tumour tissue. A H&E section with region of interest marked by pathologist. B Deparaffinized unstained slide. C Macrodissected slide where tissue removed has been captured for extraction.

The amount of tissue prepared for 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. Suggested inputs for various extraction methods used by the contributing sites are outlined in Appendix 1.

5. Deparaffinization

Regardless of the protocol used, the tissue must be deparaffinized during nucleic acid isolation from FFPE. Several different reagents can be used for this including, Qiagen Deparaffinization Solution (Qiagen, 19093), xylene, mineral oil, CitriSolv (Decon Labs, 1601) or CitriSolv Hybrid (Decon Labs, 1601H). Depending on the extraction protocol, deparaffinization may be done ahead of or succeeding dissection. Further details are provided in Appendix 1.

6. Extraction

A comparison of extraction methods across all sites responding to the information request revealed that a variety of methods were being used, the most common being the AllPrep FFPE Kit (Qiagen, 80234). Full details are available in the comparison tables in Appendix 1 and no one extraction method is recommended over another. Briefly, 4 centers are using AllPrep FFPE Kit (silica column based) as their base protocol. This protocol could either be run manually or in a semi-automated manner using the QIACube (Qiagen, 9002864). The manual method is advantageous as it does not require large, specialized equipment and it yields DNA and RNA from the same input material.

One center reported using a combination of RNeasy FFPE Kit (Qiagen, 73504) and QIAmp DNA FFPE Tissue Kit (Qiagen, 56404) protocols (both silica column based). These methods could also be run manually or on the QIACube, but they require separate material inputs. This means that separate sections or cores need to be used for DNA and RNA isolations. To overcome differences that could appear due to tissue heterogeneity, it is advised to that sections be cut serially, and be inputted alternately into the two isolations. If using cores, the homogenate should be split between the two extraction methods.

One center is using the FFPE Genepure Kit (Aline Biosciences, FP-2001) as their routine protocol. This is a silica magnetic bead-based method, using the Hamilton MicroLab Nimbus96 liquid handler (Hamilton Company, 3368416) for automation. This is a total nucleic acid extraction, yielding both DNA and RNA in the same elution. While this method requires specialized equipment to be run, it offers the advantage of a higher throughput and a lower probability of error during handling steps.

The quantity of tissue input into the extraction varies depending on the extraction protocol and desired quantity of nucleic acids used for DNA and RNA library preparation. Detailed information is available in the comparison tables in Appendix 1 for sections, curls and cores.

7. 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.

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

8. Qualification

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

In collecting data from 703 samples across four centers, it was found that there were very few DNA failures which reported, and thus qualification is focused mainly on RNA. DV200 values for these samples are tabulated in Appendix 3. Each center had their own specific criteria of determining failure during library qualification and sequencing, these metrics are beyond the scope of this guideline.

As RNA often determines the success of a case, it may be advisable to prepare and sequence the RNA library ahead of proceeding with DNA for the case.

There is not one recommended method of qualification, but review of DV200 is advised. Centers reported the use of three different qualification methods as described in the two comparison tables in Appendix 1. The Bioanalyzer (various models, Agilent), TapeStation (various models, Agilent) or Fragment Analyzer (various models, BioLabTech) were all in use to help qualify RNA ahead of library preparation. It was found that less than 20% of samples with a DV200 lower than 30% were successful at passing both library preparation and sequencing methods. As such, it is strongly recommended to avoid proceeding with such samples especially if yields are lower than anticipated based on the same input.

Recommended sample qualification QC Gate: **RNA should have a DV200 value greater than 20% to proceed to library preparation. If yields are lower than those expected based on input amounts and DV200 is on the low end, it is recommended these samples do not proceed to library preparation.**

Appendix A – Method Comparison Tables

Item/Protocol	1 (CHUM)	2 (CHUSJ)	3 (BC)	4 (McGill Genome Centre)	5 (OICR)
Pathology review requirement	Not specified	Not specified	Not specified, but Tumour Content (%) is requested as part of accessioning	Not specified	Yes, required submission is >40% tumour, >25mm2 surface area, ROI marked by pathologist for macrodissection
Input types accepted	1-2 20um sections	Not specified (likely sections)	5 x 10um sections (minimum 120mm2 x 10um), if surface area <120mm2 up to 3 s curls/scrolls, or up to 2 x 1-3mm cores (2.5mm x 1-3mm)	Up to 4 X 10µm sections with a maximum surface area of 150mm² each (max of 600mm² total)	10 x 10um sections
Preparation of tissue ahead of extraction	Remove as much paraffin from around the tissue as possible, cut sections the morning of extraction	Tissue scraped into tube	Samples should be kept at -80*C ahead of submission, submission should be in matrix tubes	Surface area determined by using ImageJ, trim excess paraffin from block ahead of cutting, clean microtome with RNase Zap between samples, extraction on freshly cut material, sections should be provided in Safe-Lock microcentrifuge tube	Pathology review is necessary, recommendation for tissue to be cut fresh or paraffin dipped immediately after sectioning if being banked for more than a few months. Tissue is macrodissected according to ROI. Maximum of 6000mm3 is accepted, following macrodissection tissue can be frozen at -80*C until extraction.
Manual/Automated/Semi-automated	Semi-automated using QIAcube	Manual	Semi-automated using Hamilton Nimbus liquid handler	Manual	Manual
Column/Beads	Column based using Qiagen AllPrep DNA/RNA FFPE Kit	Column based using AllPrep DNA/RNA FFPE Kit	Magnetic bead based using ALINE FFPE GenePure Purification Kit	Column based using the Qiagen AllPrep DNA/RNA FFPE kit (cat#80234)	Column based using AllPrep DNA/RNA FFPE Kit with non-kit components (different pro-K)
Deparaffinization details	Qiagen deparaffinization solution (n-Hexadecane), vortexed and incubated at 56*C for 15 min	Qiagen deparaffinization solution (n-Hexadecane), vortexed and incubated at 56*C for 3 min	Mineral oil, incubate at 90*C for 8 min	following step 4a from Qiagen protocol with the deparaffinization solution (Qiagen, cat#19093)	2 changes of citrisolv, 2 changes of ethanol prior to macrodissection.
Homogenization	NA	NA	using vortex/spin protocol for cores only	NA	NA
Reverse cross-linkage	2hr at 90*C	2hr at 90*C	2hr at 90*C	DNA: 2h at 90°C RNA: 1hr at 80°C	2hr at 90*C
Elution buffer used	RNA: NFW, DNA: Qiagen kit elution buffer (10 mM Tris-Cl, pH 8.5)	RNA: NFW, DNA: Qiagen kit elution buffer (10 mM Tris-Cl, pH 8.5)	Kit buffer (composition not specified, may be TE)	DNA: EB buffer (Qiagen, cat#19086) RNA: RNase-free water from kit	RNA: NFW, DNA: Qiagen kit elution buffer (10 mM Tris-Cl, pH 8.5)
Quantification method	Not specified	Qubit (BR for RNA, HS or BR for DNA) uses 2uL stock to 198uL working solution	Qubit or Qant-iT, full protocol exists but was not submitted	DNA: Qubit HS and gDNA Tapestation RNA: Qubit BR and Nano Bioanalyzer	Qubit or Quant-it (HS for RNA, HS for DNA) uses 1uL stock to 199uL working solution
Storage after extraction	RNA: -80*C, DNA: 4*C	RNA: -80*C, DNA: 4*C	Total Nucleic Acid: -80*C	DNA: -80°C RNA: -80°C	RNA: -80*C, DNA: -80*C

Item/Question	Is your site actively sequencing FFPE tissue?	Are there any QC metrics you feel are important in deciding whether to proceed to library preparation?	Do you do a pathology review to decide which cases to sequence at your site? If so, what metrics are you asking to be assessed (ex: tumour cellularity, surface area, marking done for regions of interest, % necrosis, etc.)
1 (CHUM)	No	NA	Yes, requirement is >30% tumour content.
2 (CHUSJ)	Performs DNA-seq, but have piloted RNA-seq and it looks promising.	NA	There is a confirmation from the pathologist, but specific criteria are not defined.
6 (OICR)	Yes.	No prescriptive QC cut offs.	Surface area is recommended to be >25mm2, minimum 15mm2. >40% tumour cellularity in the region of interest. Region of interest is pathologist marked for enrichment.
7 (MOH-Q)	Some of the partner sites perform FFPE RNAseq, a pilot project was performed.	For library preparation, if using TruSeq Gold, fragmentation is adjusted based on Bioanalyzer profile (not DV200). For NEB protocol, fragmentation time adjusted on RIN value.	Digital H&E is assessed by pathologist to determine tumour cellularity (%). Estimation of the surface area of tumour / surface area of tissue. Fat/lifted tissue will be excluded from estimate. Result catagories are 0 (no cancer on slide), <30 % requires enrichment, >=30% is acceptable and >=50% is preferable. <30% will be marked up to facilitate enrichment. Necrosis is quantified as surface area of necrotic tissue / total tissue area. This estimation will be performed on the whole tissue section (includes fat and folds). Inflammation is subjectively evaluated (+, ++, +++, -). Important information specific for the tissue is also noted , such as presence of remodeling, tissue quality, etc. (ex: metaplasia, presence of mucin, fibrosis, etc.). Enrichment is used to get all tissue extracted to >75% tumour content.
8 (Moncton NB)	Yes	NA	Sample must have >10% tumour cellularity, regions of interest are marked on H&E, <10% necrosis and decalcification treatment must be avoided.
9 (ACC Halifax)	No, but will start.	NA	Tumour cellularity estimate, necrosis estimate and regions of interest are marked for enrichment.
10 (Sick Kids)	Yes	NA	Pathology review to select sections/blocks with good tumour content, minimal necrosis, dense tissue. If there are multiple tissues per case, the larger one is preffered. If there are multiple tissue pieces in the same block, only one is used, they are not combined.

Item/Question	1 (CHUM)	2 (CHUSJ)	4 (McGill Genome Centre)	6 (OICR)	11 (ACCI Calgary)
How many cores do you recommend and at what size? (for example: 2 cores, 2mm diameter)	4 cores of 0.6mm.	At least 2 cores of 2mm diameter.	NA (not used for MOH, but normally use 1-2 cores, 1mm, for DNA extraction only for other studies).	3 x 1mm.	Cores should contain 1mm x 6mm of tissue minimum. The paraffin portion of the core should not be counted within the quantity. For example, if a block is thin with a depth of only 1.5mm of tissue, 4 cores should be submitted. These must all have a minimum of 40% tumour cells.
How many sections do you recommend, at what thickness and at what surface area for the ROI? (for example: 10 x 10um, minimum 25mm2 ROI)	NA	8 x 10 um for minimum 400mm2.	10um thickness used. The number of sections varies depending on surface area and other cohort information - year of blocks, tissue type, etc.	10 x 10um with a minimum ROI of 25mm2.	5 sections for ROIs of 100mm2. 20 sections for ROIs of 25mm2. All sections should be cut at 10um, be unbanked and unstained.
How many curls do you recommend, at what thickness? (for example: 10 curls, 5um)	2 to 4 x 10um.	8 curls of 10um.	1-4 curls depending on tissue surface area at 10µm thickness.	Curls are not accepted.	Curls only used if tumour cell content (i.e. the % of nucleated cells that are neoplastic) in the entire block face is estimated to be at least 40%, and preferably greater than 60%. For a surface area of 100mm2, 5 curls should be used. For a surface area of 25mm2, 20 curls should be used. All curls should have a 10um thickness.
Are there any other kinds of input that you accept?	NA	The whole FFPE block will be accepted and our site sections curls or cut it in small pieces of 1-2mm.	NA	Blocks will be accepted and our site will cut sections.	NA
How much DNA (in ng) do you aim for from the extraction?	1000ng.	Between 200 and 5000ng.	No set amount (as long as it is sufficient for QC and lib prep).	Minimum 130ng.	NA
How much DNA (in ng) do you use for library preparation?	NA	200 to 300ng.	500ng (250ng sheared DNA). Protocol allows for less if necessary.	100ng.	NA
How much RNA (in ng) do you aim for from the extraction?	500ng.	Between 200 and 15000ng.	No set amount (as long as it is sufficient for QC and lib prep).	Minimum 275ng.	NA
How much RNA (in ng) do you use for library preparation?	NA	1000ng if DV200 is good.	250ng.	200ng	NA
Other comments	None.	None.	None.	None.	In general, aim for an input of at least 5mm3 (or ~5mg) of tissue regardless of input type.

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.

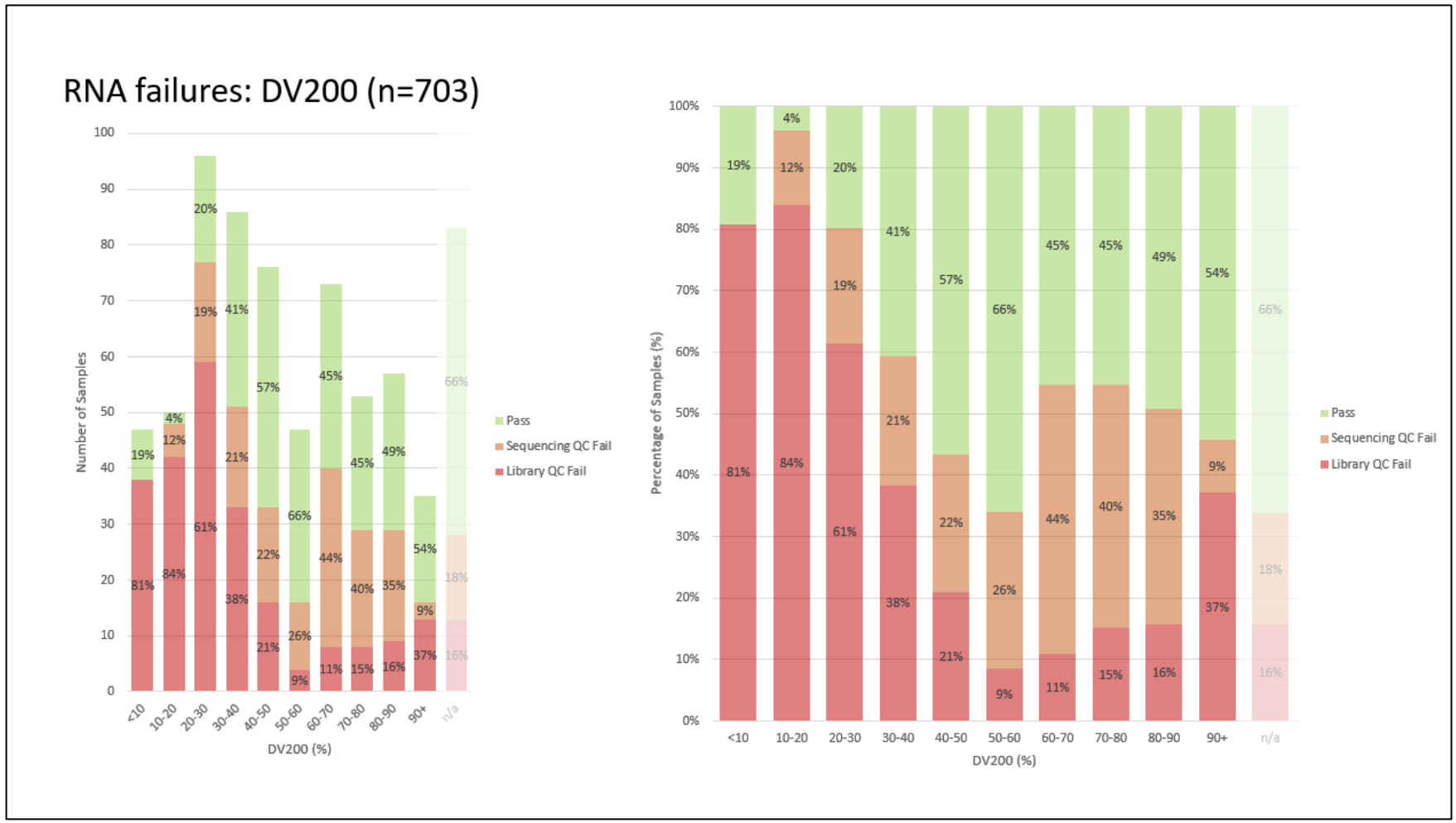
It is essential that the area used for sequencing have a proportion greater than 30% tumour and less than 10% necrosis. A section in its entirety may meet this criteria or a region of interest may need to be demarcated to allow for tumour enrichment by macrodissection.

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 10x objective.
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 there exists an area of tumour with high enough tumour cell proportion (>30%) and low enough proportion of necrosis (<10%), this should be marked using a fine-tip, alcohol resistant, permanent marker.
   - If the full section fulfills this criteria, the entire section should be circled
   - If there is only an area of the section which fulfills this criteria, carefully mark the outline of this area to serve as a guideline for macrodissection.
6. If there is any doubt about the presence of a tumour within a section, the sample should be excluded.
7. If there are no areas of tumour present within the section which have >30% tumour content and <10% necrosis, the sample should be excluded unless tumor tissue enrichment methods can be applied.

Appendix C – DV200 Data



Document revision history

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Biospecimens WG	Steering Committee	Network Council	June 1, 2023	V1	n/a