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Appendix D to Agreement on Commissioned DNA Extraction Project

Input Material Guidelines and Extraction Methods

Never send any material without prior agreement with NGI-UGC.

Please take note that NGI-UGC is not equipped to handle input material potentially infectious or in other ways harmful to human health (as also stated in the agreement).

COLLECTING AND STORING YOUR INPUT MATERIAL

- Remove unwanted tissue before shipping your sample. What you ship is what we use for extraction unless otherwise agreed upon **prior** to shipping.

- Consider if parts of your sample contains contaminating DNA (e.g. guts, skin, etc.) and remove them if possible. For smaller organisms, microbiotic and food content can be reduced by lack of feeding prior to harvesting. However, starvation is not recommended if your aim includes studies of DNA modifications.

- Never ship or store fresh material directly on ice as this might transiently freeze the sample surface and result in cell damage. In-depth shipping instructions can be found in Appendix C.

- Fresh material is always better for obtaining HMW-DNA, however this may not always be an option, or at all necessary, depending on your sequencing needs. It should also be considered that some species and tissues harbor very aggressive nucleases. In these cases, flash freezing with liquid nitrogen as soon as possible, followed by storage at -80° and shipping on dry ice, is highly recommended to minimize DNA damage. Even a few hours of aggressive nuclease exposure can result in very fragmented DNA.

- Always handle input material in a manner that minimizes nuclease exposure and activity, e.g. wear clean gloves when handling input material, storage tubes etc., use nuclease-free buffers and tubes and keep the input material as cold as possible during handling.

- Liquid nitrogen is the best way to preserve your sample for HMW-DNA extraction as it immediately reduces nuclease activity to a minimum and increases the proportion of intact cells during the freezing process - those are the key factors for preserving DNA structure. Freeze-thaw cycles should be avoided as this will promote cell breakage, and it is recommended to remove unwanted tissue before freezing your sample. Therefore, we suggest freezing several aliquotes of the sample and working with one aliquot at the time. For optimal storage, samples should be kept as cold as possible and turbulence should be avoided (e.g., minimize shaking, bumping, dropping etc.).

- When liquid nitrogen is not an option, various buffers or 95% ethanol can be used. Best option depends on your sample, which you presumably are the expert on. Some buffer components may be hard to remove and can interfere with extraction chemistry. The Circulomics Nanobind kits now also include pre-treatment protocols for samples stored in RNAlater and ethanol, however fast freezing is still the recommended approach.

- If using buffer/ethanol it is highly recommended to dissect your sample prior to storage and shipping as we have seen several sample types partially dissolve into the storage liquid, leading to decreased DNA yield and quality. If prior dissection is not possible and you suspect a high level of contaminants in certain sample parts, we recommend freezing as fast and cold as possible without buffer. Dry-ice can be a good way of freezing samples solid faster.

RECOMMENDATIONS AND EXPECTATIONS FOR DIFFERENT SAMPLE TYPES

How much material is needed

To get an estimate of the amount of input material needed to reach specific sequencing requirements, the following calculation can be helpful, especially if you have an idea about the number of cells in your sample. If not, we recommend looking at general amount of DNA previously obtained from similar samples.

*DNA amount (pg/cell) = Ploidy * Genome size in Mb * 1.096e-3*

For a standardized human cell this would look like this:

*2 * 3200 mb * 1.096e-3 = 7 pg/cell*

Meaning that in a best case scenario, you would need around 1.43e5 cells to obtain 1 µg of DNA. This however does not take into account that DNA from damaged cells is most often lost when doing HMW-DNA extractions and thus more material may be needed, depending on input material quality.

Blood
Blood is one of the easiest sample types to handle and extract high quality HMW-DNA from. If collected in blood preservation tubes with EDTA, most samples can be stored for up to 10 days at 3-5°C without significant deterioration. However, flash freezing for longer-term storage also provides excellent results. For larger volumes, consider freezing in aliquots to avoid unnecessary freeze thaw cycles. For nucleated blood, only small sample volumes are usually required to provide adequate amounts of HMW-DNA for most Long Read applications.

Cells/Bacteria
In general, cells are relatively easy to obtain high quality HMW-DNA from. Most cell types also have the advantage that they can be shipped fresh in culture. However, this is very rarely necessary, as frozen cell pellets give excellent results when stored and treated properly. For bacteria with thick outer cell walls an extra step may be required to enzymatically break this barrier while avoiding damage to the DNA. Depending on the cell wall composition, this step may require optimization.

Tissue
Soft non-fibrous tissue low in contaminants and fat is the best option to obtain high quality HMW-DNA. For fibrous tissue or tissues high in contaminants, optimization may be needed to obtain optimal purity. For larger pieces of tissue, it is recommended to divide and freeze into 100-500 mg pieces to aid fast freezing and avoid freeze thaw cycles.

Insects
High quality HMW-DNA extraction from insects can be compromised by several factors such as high levels of pigments, chitin and tissue/gut contaminants. The small size of many insects make tissue isolation difficult and single individual yield of pure HMW-DNA is often inadequate for Long Read applications and sample pooling will be required. Best options for obtaining high quality HMW-DNA are colorless pupae/larvae or isolation of body segments low in contaminants.

Plants and filamentous fungi
Plants and microscopic fungi possess multiple challenges due to high levels of polysaccharides, phenolic compounds, pigments, and a variety of secondary metabolites in the starting material. Polysaccharides often co-precipitate with the DNA and polyphenols irreversibly bind to the DNA, which both negatively affect purity. Young and dark-treated plant material provide the best option to obtain high quality HMW-DNA. If freezing, we recommend dividing into 2-5g aliquots to avoid freeze thaw cycles.

GENERAL DNA REQUIREMENTS FOR LONG-READ TECHNOLOGIES

For more specific information regarding sequencing options and requirements, request a meeting with a project coordinator through the NGI portal.

PacBio

- Purity is very important as impurities can have detrimental effect on throughput.
- The longer the desired insert size, the more DNA is required, e.g. 10 µg for 10 kb library, 20 µg for 30kb and so on.
- One library can be used for several SMRT cells.

Oxford Nanopore Technologies

- Purity is very important as impurities can have detrimental effect on throughput.
- DNA amount required for one library on one flow cell is up to 6 µg.
- The more flow-cells per sample, the more DNA is required.

Linked-Reads

- Requires less input, e.g. 0.5-1 µg.
- Indications that purity may affect outcome.

BioNano Genomics (not available at NGI)

- Purity is very important as impurities can have detrimental effect on throughput.
- Best option for shipping is embedded in Agarose plugs (In situ extraction).
- Library preparation and staining require specific plug DNA concentration.

EXTRACTION METHODS OFFERED BY NGI-UGC

Best option for extraction method depends on both starting material available and sequencing aim.

For PacBio sequencing, bigger is not always better and aiming to obtain Mb DNA for a 30kb library may actually decrease sequencing success, due to difficulties with library prep of highly viscous and heterogeneous DNA. If extracting ultra HMW-DNA, it may also be more difficult to obtain a highly pure extraction. Consider aiming for “standard” HMW-DNA for any projects below 50kb length.

Nanopore sequencing may be more efficient in sequencing long reads even for a library sheared to 30kb. It may therefore be of more value to extract ultra HMW-DNA for this technology, but the sequencing success is still highly dependent on input DNA purity.

Length and quality of the genomic DNA obtained will vary depending on sample type, quality of input material, and processing parameters.

Please note that NGI-UGC cannot guarantee specific quality metrics.

Circulomics Nanobind kits
Currently first choice extraction method at NGI-UGC unless circumstances dictate otherwise. In our experience the Nanobind kits provide superior recovery, length and purity compared to other kits tested at NGI-UGC.
Several sample type kits exist:

- **CBB kit for Cells, Blood and Bacteria**
  Protocols for obtaining either standard HMW-DNA (50 - 300+ kb) or ultra HMW-DNA (50 kb - 1+ Mb). However, obtaining uHMW-DNA may come at the expense of purity when working with non-standard samples.

- **Plant kit for intact plant nuclei**
  Protocol for obtaining HMW-DNA (50 – 300+ kb). Prior isolation of clean, intact nuclei is required. Be aware that nuclei isolation can be time-consuming and require optimization for optimal output, however this may be well worth it in terms of sequencing success.

- **Tissue kit for a wide variety of tissues**
  Protocol for obtaining HMW-DNA (50 – 300+ kb). For best results, fresh frozen tissue is recommended, however pre-treatment protocols for RNAlater- and ethanol-preserved tissues exist. Several application notes for specific tissues are also available.
  Released Nov 2019 and is yet to be tested by NGI-UGC.

- **Insect kit – Beta version**
  Protocols for obtaining HMW-DNA (50 - 400+ kb) from drosophila, cricket, mosquito, harvestman, and Colorado potato beetle. For insects that are big enough it is recommended to only use the parts that are high in DNA containing cells and low in contaminants. If possible, larval stage specimens are recommended as they contain less chitin.
  Beta version is based on Tissue kit and is yet to be tested by NGI-UGC.

CTAB/SDS with Phenol-Chloroform extraction
Recommended for samples displaying purity issues using other extraction methods. Especially useful for samples high in polysaccharides, phenols, fats and/or pigments. For this type of extraction, NGI-UGC tailors extraction workflow to each specific sample. Be aware that this approach can be time-consuming and require optimization for optimal output, however this may be well worth it in terms of sequencing success. Depending on input sample type and quality HMW-DNA of 30-100+ kb can be obtained.

In situ Agarose Plug Extraction
Only recommended for BioNano Genomics or if you truly need Mb-sized DNA. Not recommended for Nanopore sequencing, as agarose remnants in the DNA solution may block the pores in the Flow Cell. Offers the possibility of obtaining Mb DNA and is well suited for blood, cells, bacteria and other samples containing little to no contaminants. Isolating pure nuclei from samples high in contaminants can be hard and require extensive optimization. Especially suited for Bionano optical mapping and samples requiring shipping as the agarose protects the DNA from fragmentation.

QIAGEN MagAttract HMW kit
Only recommended by NGI-UGC for samples expected to be low in contaminants. This is a less expensive option and can provide 100 - 200kb fragments for high quality material. It is especially suitable for blood, bacteria, cells and soft tissues intended for bulk sequencing with “shorter” libraries of 10-20kb.

Clean-up protocols
HMW-DNA with subpar purity can be subjected to an extra clean-up step most often involving phenol/chloroform. This will, however, reduce both yield and length. Recovered HMW-DNA is usually 20-50 kb, depending on sample type and initial fragment length.

USEFUL RESOURCES FOR HMW-DNA EXTRACTION
Nanopore Sequencing Book: DNA extraction and purification methods:
http://lab.loman.net/2018/05/25/dna-extraction-book-chapter/#__RefHeading___Toc505877559
Bionano Genomics kits and extraction protocols:
https://bionanogenomics.com/support/kits-and-consumables/
Circulomics Nanobind kits and protocols:
https://www.circulomics.com/store
Protocols.io group for HMW-DNA extraction from all kingdoms:
https://www.protocols.io/groups/high-molecular-weight-dna-extraction-from-all-kingdoms
10xGenomics genome and exome sample prep:
https://support.10xgenomics.com/genome-exome/sample-prep/doc/demonstrated-protocol-hmw-dna-extraction-from-whole-blood
PacBio Guidelines for Using a Salt:Chloroform Wash to clean up gDNA:
https://www.pacb.com/wp-content/uploads/2015/09/Shared-Protocol-Guidelines-for-Using-a-Salt-Chloroform-Wash-to-Clean-Up-gDNA.pdf