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SEQUENCING SERVICES

User Guide

Pacific Biosciences Technology

Version 7.0

Table of Contents

TABLE OF CONTENTS 2

GENERAL INFORMATION 3

SAMPLE PREPARATION 3

Starting Material 3
Sample Plating 5
Tubes 5
Plates and Adhesive Films 6
Requirements 6

SERVICE REQUEST FORM AND SAMPLE SUBMISSION 8

Service Request Form 8
Sample Submission 8

SAMPLE SHIPMENT PREPARATION 9

Waybill 9
Package Preparation 9
Samples Shipment 9

FOR MORE INFORMATION 10

Client Management Office 10
Version History 10

ADDITIONAL INFORMATION 11

Sequencing with Pacific Biosciences SMRT technology 11
Guidelines for amplicons with barcodes 11

General Information

This document describes the procedure to follow when requesting a sequencing service with Pacific Biosciences technology (also called PacBio). The detailed instructions for the preparation, the samples submission, the shipping requirements, as well as any additional information are all provided in this guide.

To avoid any delay in the processing of the request, the instructions provided in the present guide must be followed carefully.

The DNA must be of high molecular weight and of high quality. A band (no tail) of fragments of 45-50 kb or more indicates large DNA samples, which is desired for the generation of long insert size libraries.

Any damage to the genomic DNA (presence of nicks on a strand, abasic sites, modified bases or intra-strand and/or inter-strand bonds), as well as the presence of residual extraction contaminants or polysaccharides could harm sequencing with this technology. There is currently no quality control to detect such phenomena.

Total RNA is used as starting material for Iso-Seq protocols.

Note that delays in the processing of samples will vary depending on the size of the project. It is recommended to contact the Client Management Office for information regarding processing time.

Sample Preparation

Starting Material

The starting material for Pacific Biosciences technology is DNA or RNA.

Samples must not have been exposed to high temperatures (ex.: >65°C for 1 hour can cause a detectable decrease in sequence quality) and to extreme pH (< 6 or > 9).

Samples must not contain insoluble particles, chelating agents (ex.: EDTA), metal ions (ex.: Mg2+), denaturing agents (ex.: guanidinium salts, phenol) or detergents (ex.: SDS, Triton-X100). The presence of detergents can interfere with enzymatic reactions.

Samples should not contain contaminants from the tissue from which it was extracted (heme, humic acid, polyphenols, polysaccharides, etc.).

DNA

Single-stranded DNA is not compatible with the library preparation process. DNA samples should not contain traces of RNA.

Note that for microbial gDNA extraction, it is recommended to avoid incubation in complex or rich media. Additionally, harvesting multiple cultures (replicates) in the early/mid log phase of growth is preferable.

Using gel-extracted amplicon products may result in lower sequencing performance due to the damage inherently caused by intercalating dyes such as ethidium bromide and exposure to UV radiation. Sequencing amplicons stained with SYBR safe dyes is untested, and therefore cannot be recommended. If working with a gel-extracted product that has been stained with a dye, it is recommended to bring it through additional rounds of amplification to remove damage and/or dyes prior to library prep and sequencing.

See Pacific Biosciences technical note for recommended extraction kits, as well as tips and tricks for efficient high molecular weight DNA extraction.

See the Circulomics page of the Pacific Biosciences website for information on available extraction kits.

See https://extractdnaforpacbio.com/ for alternative DNA extraction methods.

When extracting DNA, follow these guidelines:

- It is preferable to extract from small volumes repeatedly rather than from large volumes in order to avoid the accumulation of high concentrations of potentially inhibiting secondary components.
- Phenol must be fresh (<3 months post-opening) and non-oxidized when the phenol-chloroform extraction method is used.
- Avoid the use of ethidium bromide and UV rays which damage DNA.
- Avoid the use of vortexes and narrow tips which fragment DNA. Recommend tube inversions, tapping and the use of wide-bore tips.
- Drying of the pellet of extracted samples should be done in the open air rather than by thermal drying or by a vacuum concentrator (speed-vac).
- Elute the DNA in a neutral buffered solution (ex. 10 mM Tris, pH 7.0-8.0). Do not elute DNA in water or unbuffered solutions, as this inhibits sequencing or does not allow long-term DNA stabilization.
- Allow DNA to resuspend in buffer at 25°C overnight.

To partially reduce the impacts of the presence of potential contaminants/inhibitors, perform DNA purification with AMPure® beads. Ensure that there are no residual beads with the samples as these may affect sequencing.

If highly contaminated DNA is suspected, purify the DNA with:

- The DNeasy PowerClean Pro Cleanup Kit (Product Information) with the modifications suggested below. Be careful not to overload the columns. DNA recovery is low (10-30%) following this purification.
    - After adding the CU Solution (Step 2), mix quickly and add the IR Solution (Step 3). Minimize exposure time to CU Solution to prevent DNA damage.
    - During the last elution step (step 13), elute with 10 µL of buffer and centrifuge for one second (quick spin) in the microcentrifuge to wet the filter and then incubate for 1 minute at room temperature. Add the remaining elution volume to the membrane and incubate for 15 minutes at room temperature. Then proceed as indicated in the protocol.
- The high-salt phenol-chloroform cleaning protocol for samples with unusual colors or suspected polysaccharide contamination.

Store DNA at 4°C for short term (up to 4 weeks) and at -20°C or -80°C for long term (>4 weeks). The samples must not have undergone several freeze-thaw cycles.

Submit samples of the highest quality and purity possible. The 260/280 optical density ratio must be between 1.8 and 2.0, while the 260/230 ratio must be equal to or greater than 2.0.

RNA

Recommendations for handling RNA samples:

- Wear new gloves and always keep samples on ice.
- Resuspend samples in commercial RNase-free water.
- Avoid water treated with DEPC (diethylpyrocarbonate).
- If Trizol is used to extract RNA samples, it is recommended to perform a final cleaning (ex. with the Qiagen mRNA cleaning kit) before submission.
- Avoid excessive pipetting and vortexing when working with RNA.
- Do not expose RNA samples to fluorescent intercalating dyes or ultraviolet radiation. SYBR dyes do not damage RNA, but ethidium bromide should be avoided.

Lyophilyzed RNA is not accepted.

The 260/280 optical density ratio must be ~2.0, while the 260/230 ratio must be equal to or greater than 2.0.

The extraction kits listed in Table 1, have not been tested or validated by PacBio but are listed here as examples of third-party kits used by other PacBio customers for isolating total RNA for Iso-Seq SMRTbell library preparation.

Table 1 - RNA extraction kit options for Iso-Seq SMRTbell library preparation

Kit type	Product name
mRNA isolation	Ambion Poly(A) Purist MAG Kit
Total RNA isolation	Qiagen RNeasy Plus Kits
Total RNA isolation	Sigma Spectrum Plant Total RNA Kit
Total RNA isolation	iNtRON Easy Spin Total RNA
Total RNA isolation	TRIzol Reagent can be used to isolate total RNA from tissues or cells, including lipid-rich and difficult samples
RNA stabilization & storage	RNALater is an aqueous, nontoxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA

Always store RNA at -80°C, regardless of storage time.

Sample Plating

For a shipment of less than 12 samples, put the samples in 1.5 mL tubes. Otherwise, send the samples in plate.

Tubes

Recommended Tubes

- 1.5 mL tubes

Any type of tube with a volume of 1.5 mL with the lid attached (snap cap)
Example: *DNA LoBind Tubes*, 1.5 mL, *PCR clean, colorless*; Eppendorf, Cat # 022431021

Unaccepted Tubes

- 0.5 mL tubes

Plates and Adhesive Films

Each plate must contain only the samples belonging to a single project. The plates must be correctly sealed.

Recommended 96-well Plates

- Half-skirt PCR plate
  All types of half-skirt clear 96-well PCR plates.
  Example: *Thermo-Fast 96 PCR detection plate with flat deck: Life Technologies, Cat # AB1400L*

Unaccepted 96-well Plates

- 96-well cell culture plates
- Full-skirt 96-well PCR plates
- No-skirt 96-well PCR plates
- Opaques 96-well PCR plates

Recommended Adhesive Films

- Clear adhesive film
  Example: Life Technologies MicroAmp® Clear Adhesive Film, Cat# 4306311

Requirements

Samples Volume and Concentration

Additional quality control charges will apply to each replacement sample sent. The decision to proceed with library prep and/or sequencing with suboptimal sample quantity or quality comes with the client's acceptance of all inherent risks.

DNA

The quantity and concentration of samples to submit are dependent on the type of library, see Table 2. If necessary, dilute the DNA in a neutral, buffered solution (ex. 10 mM Tris, pH 7.0-8.0).

Table 2 – DNA quantities required for the construction of PacBio libraries

Nucleic Acid Source	Library Type	Quantity	Volume (µL)

gDNA, amplicons	short Fragments	2 µg	30-60
gDNA	small genomes (up to 100 Mb)	4 µg	40-120
gDNA	large genomes (> 100 Mb)	6 µg	60-120
cDNA	Iso-Seq	160-500 ng*	30-50

* Samples with concentrations >40 ng/µL may result in suboptimal data due to cDNA overamplification. Optimal libraries can be obtained by repeating cDNA generation starting with less RNA input or by decreasing the number of PCR cycles.

The minimum volume accepted per sample is 30 µL. Anything less will be diluted to the required volume. The sample not respecting this requirement could be refused.

The maximum volume should not exceed the volume listed in Table 2. Any larger volume will be concentrated to the required volume using AMPure XP bead purification. Note that there is an additional charge for the concentration step.

The volume of each sample must be indicated in the Sample Submission form.

A standard agarose gel can give an indication of DNA integrity but does not allow good separation for high molecular weight fragments (≥ 20KB). It is recommended to check DNA size using one of the following methods, if possible:

- pulsed-field gel (Pippin Pulse System (Sage Science)
- CHEF Mapper XA System (Bio-Rad)
- Femto Pulse or Fragment Analyzer System (Agilent)

It is recommended to quantify the DNA using a fluorometric method for double-stranded DNA, for example the PicoGreen method, and not UV absorbance.

RNA

Table 3 – RNA quantities required for the construction of Iso-Seq PacBio libraries

Application	Minimum quantity (ng)	Minimum concentration (ng/µL)	Minimum volume (µL)	RIN*
Iso-Seq standard	500	45	15	>7.0

* RIN = RNA Integrity Number

A lower RIN is an indicator that the sequences could be limited to the 3' region of the genes.

Samples with an RIN <7.0 can be processed, but the risk of significant underperformance or even failure is greatly increased.

The RIN value may be decreased with the presence of abundant chloroplast ribosomal RNA even if the RNA is intact.

Identification

The identification of each sample must correspond exactly to what is indicated in the Sample Submission.

All plates should be clearly identified, for example, with the submission number, the principal investigator's name (PI) and the shipment date.

Service Request Form and Sample Submission

All service request forms and sample submissions must be done on the web through Nanuq by using a user's account. To get an account contact the Client Management Office.

Work in the laboratory will only start once all the documentation is submitted. An incomplete documentation will cause delays.

Service Request Form

1. Open a session in Nanuq.
2. Click on "Add new request" in the section "Request" and follow the instructions.

   The option "new request" does not need to be used to complete an already existing request.

   Do not use the "Back" button in your browser to go back to the previous pages. Use the menu on the left to navigate through the form.

   Click on "Next" to go to the next page of the request.

   It is always possible to save the information by clicking "Save and continue later". The drafts are accessible through "My request lists" in the section "Request". The request will stay in draft until it gets submitted. To modify a request in draft, click on "Modify" in the menu on the left.

   To request the return of samples once the project is completed, indicate it under the "Sample Information" tab and complete the requested information.
3. You must click on "Submit" so that your request can be approved by the Client Management Office. Requests that are not submitted will not be processed.

Sample Submission

Once the service request is complete and submitted, submit the samples.

1. Open a session in Nanuq.
2. If applicable, find the request using "My request list" and click to open it.

3. Click on the tab "Sample submission", and then on "Add new samples".

4. Follow the instructions on the screen.

5. Verify that the status of the submission is at "Submitted" under the "Sample submissions" tab in the Service request.

Follow the same steps to add new samples to the request or to add replacement samples.

Sample Shipment Preparation

Waybill

After the sample submission, return to the tab "Sample submission", select the sample submission(s) related to the package being prepared, and click on "Print waybill". By default, only one copy will print, but two are required.

Package Preparation

One copy of the waybill must accompany the samples. Make sure that the waybill stays dry by placing it in a sealed plastic bag (type of Ziploc).

The plates must be properly sealed and placed in a Ziploc bag.

The tubes must be placed in a container resistant to transport.

If the shipment contains heavy objects that may damage the contents during transport (ex. blocks of dry ice, ice pack), it is recommended to protect the samples against impacts.

Samples crossing the Canadian border should be sent at the beginning of the week to avoid the risk of them being stored at the carrier's warehouse over the weekend. The use of clear phrases such as: "non-biohazardous biological samples", "Purified DNA or RNA from [species]", "For research use only", and "No commercial value" on the commercial invoice will help expedite customs clearance.

DNA

If shipping from Canada for an overnight or same day trip and the samples are not frozen, include enough ice packs to keep the contents cold. However, if the samples are already frozen, ship the contents on enough dry ice to keep the samples frozen until destination to minimize freeze-thaw cycles.

If shipping from outside of Canada, the package must contain enough dry ice to keep the samples frozen until they arrive at their destination. If the samples thaw during transportation, it can cause the seal on the plates to unstick, which may cause a loss of sample volume or cross contamination.

RNA

RNA samples should be sent on dry ice. The shipment must contain enough dry ice to keep the samples frozen until destination or quality may be compromised.

Samples Shipment

The delivery address and the directives concerning the delivery will be found on the waybill.

One copy of the waybill must be visible on the outside of the package. It can be glued to the package or placed in a transparent enveloped and glued to the package.

For More Information

Client Management Office

Telephone: 514-398-7211
Email: infoservices@genomequebec.com

Version History

Version	Summary of modifications	Author	Effective date (aaaa-mm-jj)
06	Identification of the document and use of template Add requirements for HiFi and Iso-Seq libraries Update informations and specifications for sample preparation DNA and RNA	G. Geneau	2022-06-03
07	Update requirements table 2 – DNA quantities and add note about gel-extracted amplicons for sample preparation	G. Geneau	2023-06-29

Additional information

Sequencing with Pacific Biosciences SMRT technology

Unlike other next-generation sequencing technologies, Pacific Biosciences' (PacBio) Single Molecule Real Time Sequencing (SMRT) technology does not require amplification during library preparation. The technology uses the natural process of DNA replication to sequence long fragments of native DNA to produce highly accurate long sequences or HiFi sequences. As such, a high quality, high molecular weight starting genomic DNA (gDNA) will result in longer libraries and better performance during sequencing.

If small plasmids or small extra-chromosomal elements are present in the samples, please note that they may be under-represented or may not even be detected with the PacBio technology since the library preparation targets fragments of ~15- 20kb.

Guidelines for amplicons with barcodes

A set of 384 barcodes, each consisting of 16 bp, is custom designed for the PacBio system. By adding these barcodes to PCR primers, users can perform parallel or multiplex sequencing using SMRT Analysis v1.4 or later. This barcode set is designed for optimal discrimination with SMRT Sequencing technology.

See the multiplexing sequencing section of the Pacific Biosciences website for more information on designing barcoded PCR primers.