GenomeQuébec CES | CENTRE D'EXPERTISE ET DE SERVICES

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SEQUENCING SERVICES

User Guide

Sequence Ready Libraries

Illumina technology

Version 09

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General Information

This document describes the procedure to follow when requesting library sequencing using Illumina's technology. The detailed instructions for the preparation, the libraries 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.

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

The requirements mentioned in this guide also apply for library quality control project.

The Illumina software that delineates the cluster boundaries on the flow cell and carries out the base calling is dependent upon sequence complexity at the ends, particularly the first dozen or so base pairs, at either end of the inserts being sequenced.
For this reason, any type of library which does not exhibit sufficient sequence complexity in these regions must be properly identified otherwise the sequencing data will be less than optimal. This includes but is not limited to:

- Amplicons
- BD Rhapsody Single-Cell libraries
- reduced genome representation methods such as Restriction site-associated DNA (RAD) marker libraries
- libraries with reduced nucleotide complexity such as bisulfate-converted libraries.

To overcome this issue with low complexity libraries, a control library could be spiked in (such as the control phiX174 library supplied by Illumina) at 10-50% of the lane, depending on the complexity of the initial library. The addition of phiX into the lane will results in lower reads number for the libraries of interest.

The same nucleotide complexity issue described above applies to the index sequence when multiplexing libraries. For best results, a minimum of 3 indexes should be used per lane when multiplexing libraries.

Sequencing results will be provided as is. The CES cannot be held responsible for problems related to the design, quality or sequence complexity of the libraries.

Library Preparation

Starting Material

The starting material is a library or a pool of libraries.

Custom sequencing primers (if required) should be sent in 1.5 mL tubes. Additional information about customizable sequences and use of custom sequencing primer can be found in the Additional information section.

Tubes must be properly identified:

- Principal Investigator last name and distinguishable primer name
- Primer concentration
- Sequencing read (Read 1, Read 2, Index 1 or Index 2)

Primer concentration must be at **100 μM**.

It is not possible to use custom primers on the NextSeq 2000.

Table 1 - Summary of custom primer requirements

Sequencing Type	100 µM Custom primer required volume per sequencing lane (µL)			
	Read 1	Index 1	Index 2	Read 2
MiSeq	≥ 5 µL	≥ 5 µL	N/A	≥ 5 µL
NovaSeq X Plus	≥ 30 µL	≥ 30 µL	≥ 30 µL	≥ 30 µL
NextSeq 2000	N/A	N/A	N/A	N/A

Library Plating

Libraries must be submitted in 96-well plates regardless of the number of libraries.

If libraries from multiple projects are submitted at once, they must be on different plates.

There should be one individual library or pool per well.

Plates and Adhesive Films

Recommended 96-well Plates

Pipelines have been optimized for high throughput processing of libraries. Introducing plates other than the specified models may lead to library loss and damage to the robotic liquid handlers. Therefore, libraries submitted in non-conforming plasticware will be re-plated in the proper plates. There will be additional fees for re-plating them.

The PCR plates must be full-skirted plates.

Five types of plates are accepted:

- BioRad Hard-Shell 96-Well PCR Plates, skirted, Cat# HSP9601
- Eppendorf twin.tec, Cat# 951020401
- Corning Thermowell GOLD, Cat# 3752
- Axygen 96-well PCR Microplate, Cat# PCR96FSC
- 96 well 0.2mL Skirted PCR plate - Grey ultra rigid frame, Cat# IST-601-096GCT

Unaccepted 96-well Plates

- 96-well cell culture plates
- No-skirt 96-well PCR plates
- Half-skirt 96-well PCR plates

Recommended Adhesive Films

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

- Aluminum adhesive film
  VWR Aluminum Foils for PCR and Cold Storage, Cat# 60941-074

Requirements

Library Volume and Concentration

The libraries should be diluted in Tris-HCl (10 mM) buffer or in nuclease-free water.
The libraries must not contain chelating agent (ex. EDTA) or the concentration must be reduce to 0.1mM.

Volume and concentration requirements are sequencing type dependent, see Table 2.

Table 2 – Concentration in nM and volume in µL to submit depending on the sequencing type

Sequencing Type	Required concentration (nM)	Required Volume (µL)
NovaSeq	≥ 3 nM	≥ 25µL
NextSeq	≥ 2 nM	≥ 25µL
MiSeq	≥ 2 nM	≥ 25µL
QC only	N/A	≥ 25µL

* Library: individual library or pool (1 well from your sample submission)

Quantify library concentration using a qPCR-based method rather than purely spectrophotometric- (e.g., Nanodrop) or fluorometric-based (e.g., picogreen) methods to ensure the presence of sequences required for sequencing (P5, P7).

Library concentration should not exceed 60 ng/µL or 120nM. Libraries that are too concentrated will be diluted and QC redone. Additional fees may apply.

Should the library volume be inferior to the minimum volume, it will be diluted to an appropriate volume without notice.

Volume exceeding 110 µL will be discarded without notice.

It is recommended to send an aliquot of your library. Sequence-ready libraries will not be returned and will be discarded 3 months after the completion of the project without notice.

Replacement libraries must meet total requirements. Partial replacement (« top-ups») will not be accepted. Additional fees will be applied for the QC of each replacement library.

Identification

All plates must be identified, and this identification must match what is written on the submission form ("Plate Name" field). The identification must be clear, specific to that plate and preferably concise.

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 libraries.

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.

   - **Pool/Lib Only**: Choose Lib Only for an Individual Library. Choose Pool for pooled libraries that need to be demultiplexed. For the latter option, a new window opens to define pool attributes. Once the entry completed, the window allows to define to attributes of each library within the pool. Note that the first line of the sample submission contains information on the pool whereas subsequent lines define each library that is part of the pool. New libraries or pools can be added one after the other in the template.

- **Number / Fraction of Sequencing Units**: fraction of the lane which will be attributed to the pool or library. For instance, if 4 libraries are to be sequenced on 1 lane, the sequencing unit for each library is 0.25.

  This field is automatically filled according to the General Informations section and is editable to adjust pooling proportion and reads number requirement.

- **Adaptor**: select the sequencing adaptor used during library preparation from a dropdown menu. Adapter names are from commercial kits. See Additional information for details on adaptors and indices. Do not hesitate to contact your Client Management Office representative for help if you believe that your adapters might be custom.

- **Index 1**: i7 index added to DNA fragment of interest during library preparation (see Additional information).

  This is an autocomplete field. When a few digits have been entered, Nanuq will list choices that contain these digits.

  You can enter either enter the Index Name or Sequence.

  Autocomplete options are based on the choice of Adapter. If indices are absent from the menu, chances are that a wrong selection has been made when selecting the Adapter. Select another Adaptor and see if indices used appear in the menu.

- **Index 2**: i5 index added to DNA fragment of interest during library preparation. See **Index 1** for explanations.

Contact the Client Management Office for remaining questions on how to fill this Form, especially regarding the adaptors, index or if custom index has been used for the library preparation.

Make sure the volume and concentration meet the requirements specified in Table 2.

The volume entered must exactly match the physical volume in the plate.

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 libraries to the request or to add replacement libraries.

Due to the large numbers of samples that are processed by the unit service, it is not guarantee that specific loading schemes can be honored.

The unit service reserves the right to sequence lanes over multiple runs as deemed appropriate and without prior notice. Specific schemes must be entered in the Comments column of the Sample Submission Sheet.

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

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

The library plates must be sent in dry ice. If the package contains heavy items that can damage the contents during transportation (ex.: block of dry ice, ice pack) it is recommended to protect it from those impacts.

The package must contain enough dry ice to keep the libraries frozen until they arrive at their destination. If the librairies thaw during transportation, it can cause the seal on the plates to unstick, which may cause a loss of library volume or cross contamination.

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

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 from [species]", "For research use only", and "No commercial value" on the commercial invoice will help expedite customs clearance.

Library 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

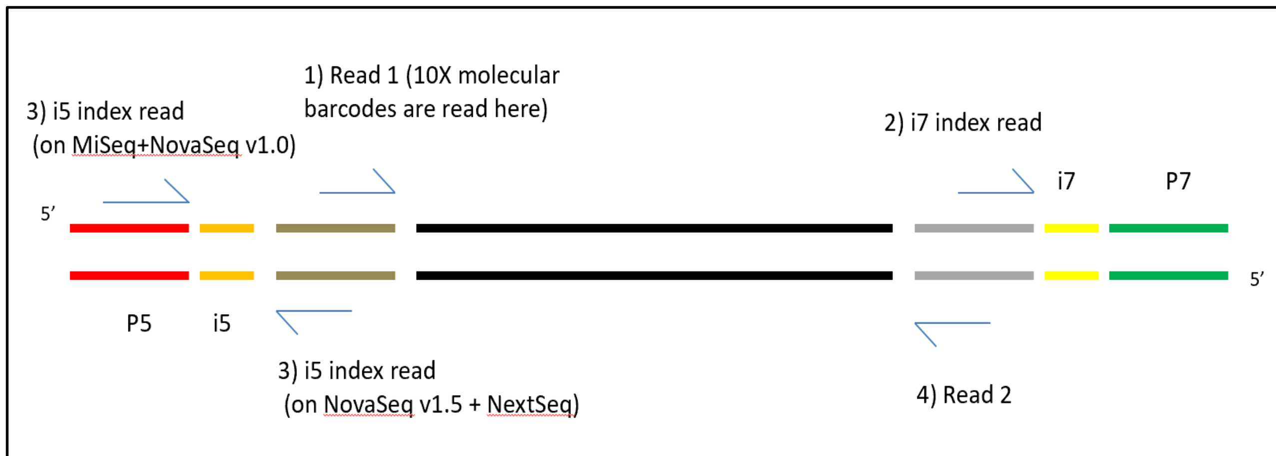
Client Management Office

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

Additional Information

The library preparation can be performed using commercial kits. It is possible to use an adapted protocol using custom primers sequence.

Figure 1 - Library construction for Illumina sequencing



Library adapters are 58-64 nucleotides located at the extremities of the insert (black line). They contain:

- Sequences required for linking the library to the flow cell (P5 forward in red; P7 reverse in green)
- Sequencing primers (brown and gray)
- Index i5 (orange) and i7 (yellow) sequences

Flow cell adapters: 25-30 nucleotides bound to the flow cell. Their sequence is complementary to library adapters.

Sequencing primers: 30-35 nucleotides. Required for sequencing Read 1 and Read 2 as well as index 1 and 2. Sequencing primers can be standard (present in the Illumina sequencing reagents) or custom (need to provide custom primer).
There is no option for a custom Index 2 (i5) primer in MiSeq sequencing since the template uses the grafted P5 primer on the surface of the flow cell.

Index: generally 6-10 nucleotides long. Indices are required for assigning reads to the right sample. Sample multiplexing can be performed using 2 index reads: Index 1 (or i7) is located on adapter P7 (reverse) and index 2 (or i5) is located on adapter P5 (or forward). Both are sequenced on separate reads from the main sequence. To perform multiplexing using single index, i7 must be used.

Note that this is distinct from in-line barcode which is not located in the adapter but within read 1 or read 2 or from molecular barcodes. In-line barcodes cannot be used to distinguish reads from various libraries sequenced together on a lane.

Molecular barcode (UMI): 8-10 bases sequence added to the DNA fragment during the ligation step of library preparation. UMI are required for distinguishing independent DNA fragments from PCR duplicates. UMI are generally located just after Index 1.

Table 3 - Adapter and primer sequences required for Illumina sequencing

	P5 adapter	**index i5** (6, 8 or 10 bases)	**Read 1 seq primer** (Customizable sequence, need to provide custom primer)
Dual index			
Nextera	AATGATACGGCGACCACCGAGATCTACAC	NNNNNNNN	TCGTCGGCAGCGTCAGATGTGTATAAGAGACA
TruSeq HT		NNNNNNNN	ACACTCTTTCCCTACACGACGCTCTTCCGATCT
Single index			
TruSeq LT	AATGATACGGCGACCACCGAGATCT		ACACTCTTTCCCTACACGACGCTCTTCCGATCT
TruSeq Small RNA			ACACGTTCAGAGTTCTACAGTCCGACGATC
NEB Small RNA			ACACGTTCAGAGTTCTACAGTCCGACGATC

	P7 adapter	**index i7** (6, 8 or 10 bases)	**Read 2 seq primer** (Customizable sequence, need to provide custom primer)
Dual index			
Nextera	CAAGCAGAAGACGGCATACGAGAT	NNNNNNNN	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA
TruSeq HT		NNNNNNNN	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Single index			
TruSeq LT	CAAGCAGAAGACGGCATACGAGAT	NNNNNN	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
TruSeq Small RNA		NNNNNN	GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA
NEB Small RNA		NNNNNN	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT