

## LIBRARY PREPARATION FOR MISEQ ILLUMINA PLATFORM

### 1. PROCEDURES

#### 1.1 Care

- Observe the safety procedures inherent to the respective reagents and samples, using the necessary EPIs (lab coats, gloves without talc, mask and cap);
- The steps of preparation of *mastermixes and primers dilution* should be performed in a clean room with ideal aseptic conditions, to avoid contamination;
- All plastics used must be new, free of RNase and DNase;
- Clean the biological safety cabine *or workstation* with a 2.0% sodium hypochlorite solution, followed by sanitization with distilled water and 81 °GL disinfectant alcohol solution;
- Turn on the CSB UV for at least 15 minutes
- Check that all reagents are available before starting the procedure.

#### 1.2 Materials and Equipments

- Gauze or paper towel;
- PPE (lab coat, gloves without talcum powder, mask and cap);
- Tips with rnase-free filter and DNase of varying volumes;
- 96-well PCR plates;
- Optical adhesive for 96-well plate;
- Magnetic rack (for tube and plate);
- Microtubes of 1.5 mL and 2.0 mL;
- Tube of 15 mL and 50 mL;
- Qubit tubes;
- Micropipettes (varied volumes );
- Centrifuge (for tube and plate);
- Thermoblock (if necessary);
- Vortex agitator (for tube and plate);
- Thermocycler;
- LabChip (Elmer Perkin);
- Qubit Fluorometer (ThermoFisher);
- MiSeq Platform (Illumina).

#### 1.3 Reagents

##### 1.3.1 For asepsis

- MilliQ Water;
- Disinfectant alcohol solution at 81 °GL;
- 2.0% sodium hypochlorite solution.

##### 1.3.2 To carry out the procedure

- Set of *primers* specific to the pathogen of interest

- *Superscript IV: First strand synthesis system (ThermoFisher)*
- *Q5 Hot Start High Fidelity DNA polymerase (NEB)*
- *Qubit dsDNA HS Assay Kit (ThermoFisher)*
- *DNA Chip Extended Range HT (Perkin Elmer)*
- *DNA HiSens Reagent Kit CLS760672 (Elmer Perkin )*
- *Illumina DNA Prep Kit (Illumina)*
- Nuclease-free water
- ETHANOL PA for molecular biology
- Sodium Hydroxide (NaOH) 1N

#### 1.4 Library preparation

To exemplify the procedure of genomic library preparation and sequencing, the amplification reaction will be performed using the ARTIC V4 primer set for sequencing the complete genome of SARS-CoV-2.

##### 1.4.1 CDNA Synthesis

###### 1.4.1.1 Solutions and reagents

- Superscript IV
  - mix dNTPs (10 mM each)
  - Random Hexamer (50  $\mu$ M)
  - RNase inhibitor
- Stored in freezer (-25°C to -15°C)

###### 1.4.1.2 Description of the procedure

- Separate and defrost the reagents in ice (Table 1 and 2, column components);
- Vortex and centrifuge (1000 x g for 10s) the reaction components before use;
- Assemble a map with the order of the samples and identify a PCR plate of 96 wells with data + cDNA
- In a cleanroom, combine the volumes of the components of table 1 into a 1.5 mL tube, with **the exception** of RNA (multiply the volumes by the number of samples that will be processed plus an increase of 5% dead volume):

TABLE 1  
CDNA Mastermix 1

Components	Volume ( $\mu$ L)/ per sample	Volume ( $\mu$ L)/ 96 samples <sup>2</sup>
Random Hexamers (50 $\mu$ M)	1	101
dNTPs mix (10 mM)	1	101
RNA Template <sup>1</sup>	11	-
<b>Total</b>	<b>13</b>	<b>202</b>

<sup>1</sup>Add rna in chapel and appropriate room; <sup>2</sup>Calculation performed with an increase of 5%.

Source: ThermoFisher Scientific, 2022

- Distribute 2  $\mu$ L of mastermix 1 in pcr plate wells ;

**NOTE:** Step L of step 1 (Preparation of cDNA mastermix 2) can be performed at that time and stored at 4°C.

- Add 11  $\mu$ L of RNA to each of the PCR plate wells that will be used according to the map, in chapel and appropriate room;

- g. Gently homogenize by pipetting (10x);
- h. Seal the plate with optical adhesive;
- i. Centrifuge at 1500 x g for 1 minute;
- j. Place the plate on a thermocycler and save the condition (**Program: Incubação\_cDNA**):

<b>HOLD 1</b>	1x	65°C for 5 min
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- k. Remove the plate from the thermocycler and place immediately in an ice bath;
- l. In a clean room, combine the volumes of the components of mastermix 2 (Table 2), in a 1.5 mL tube (multiply by the number of samples that will be processed):

TABLE 2  
CDNA Mastermix 2

Components	Volume (µL)/ per sample	Volume (µL)/ 96 samples <sup>1</sup>
SSIV Buffer	4	403,20
DTT (100 mM)	1	101
RNase Inhibitor	1	101
SSIV Reverse Transcriptase	1	101
<b>Total</b>	<b>7</b>	<b>706,2</b>

<sup>1</sup>Calculation performed with an increase of 5%.

Source: ThermoFisher Scientific, 2022

- m. Distribute 7 µL of mastermix 2 on the plate portions containing denatured RNA (mastermix 1) to obtain final volume of 20 µL;
- n. Repeat steps g, h, and i of item 6.4.1.2;
- o. Place the plate on a thermocycler and save the conditions (**Program: SSIV\_cDNA**):

<b>HOLD 1</b>	1 x	42°C for 50 min
<b>HOLD 2</b>	1x	70°C for 10 min
<b>HOLD 3</b>	∞	4°C

- p. Remove the thermocycler plate and centrifuge (1500 x g for 1 minute).

<b>Stop Point:</b> CDNA can Be stored In fashion freezer (-15°C) to -25°C for long periods.
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## 1.4.2 Amplification reaction (PCR pool 1 and pool 2)

### 1.4.2.1 Solutions and reagents

- ARTIC V4 nCoV-2019 Panel IDT primer set (100 µM) – Pool 1 and Pool 2
- 5X Q5 Reaction Buffer
- dNTPs (10 mM)
- Q5 Hot Start DNA Polymerase
- Nuclease-free water

### 1.4.2.2 Description of the procedure

- a. In clean room, dilute the primer pools for concentration of use. For the ARTIC V4 primer set, dilute the stock solution to 100  $\mu\text{M}$ , *from pool 1* and 2 (in separate tubes) to 10  $\mu\text{M}$  in nuclease-free water. Ex: in a tube 1.5 mL, add 38  $\mu\text{L}$  of pool 1 stock solution and complete with 342  $\mu\text{L}$  of nuclease-free water, repeat the procedure for pool 2 (final volume of 380  $\mu\text{L}$  of each pool at 10  $\mu\text{M}$ );
- b. Still in a clean room, prepare the amplification mix of each pool separately, combining the volumes of the components listed in table 3 (multiply by the number of samples that will be processed).

TABLE 3  
Amplification Mastermix (pool 1 and pool 2)

Pool 1		
Components	Volume ( $\mu\text{L}$ )/ per sample	Volume ( $\mu\text{L}$ )/ 96 samples <sup>1</sup>
Nuclease-free water	10,65	1073,52
5X Q5 Reaction Buffer	5	504
dNTPs (10 mM)	0,5	50,4
Q5 Hot Start DNA Polymerase	0,25	25,2
Primer <b>Pool 1</b> (10 $\mu\text{M}$ )	3,6	362,88
<b>Total</b>	<b>20</b>	<b>2016</b>

Pool 2		
Components	Volume ( $\mu\text{L}$ )/ per sample	Volume ( $\mu\text{L}$ )/ 96 samples <sup>1</sup>
Nuclease-free water	10,65	1073,52
5X Q5 Reaction Buffer	5	504
dNTPs (10 mM)	0,5	50,4
Q5 Hot Start DNA Polymerase	0,25	25,2
Primer <b>Pool 2</b> (10 $\mu\text{M}$ )	3,6	362,88
<b>Total</b>	<b>20</b>	<b>2016</b>

<sup>1</sup>Hereit was carried out with an increase of 5%. Make each of the mastermixes in 2 tubes of 2.0 mL (divide the volumes by 2).

Source: PILLAY, S.; GIANDHARI, J.; OLIVEIRA, T, 2021

- c. Distribute 20  $\mu\text{L}$  of pool 1 and pool 2 mastermixes on two mirrored cards. Assemble a map with the order of the samples and identify the plates with the date and pool (e.g. 02022022\_Pool1);
- d. In the appropriate cabin and room, add 5  $\mu\text{L}$  of cDNA (prepared in item 6.4.1) to the corresponding plates of pool 1 and 2.
- e. Gently homogenize by pipetting (10x);
- f. Seal the plate with optical adhesive;
- g. Centrifuge at 1500 x g for 1 minute;
- h. Place the plates in thermocyclers and save the conditions (**COVID\_pool\_V4**):

<b>HOLD 1</b>	1 x	98°C for 30 s
<b>CYCLING</b>	35x*	98°C for 15 s 65°C for 5 min
<b>HOLD 2</b>	$\infty$	4°C

\*The number of cycles will depend on the CT of the sample, it is recommended: 25 cycles for samples with CT between 18 - 21 and the maximum of 35 cycles for samples with TC up to 35.

- i. Remove the thermocycler plate, centrifuge (1500 x g for 1 minute).

**Stop Point P: Pools can** Be stored In fashion freezer (-25°C to -15°C) for up to 3 Days.

**NOTE:** The average size of the amplicons generated after cad amplification reaction to one of the samples can be analyzed in labchip (PerkinElmer), according to the manufacturer's recommendations.

**WARNING:** Wash the MiSeq (Illumina) equipment according to the manufacturer's recommendations, defrost the cartridge and HT1 (item 6.4.10.1) and in room temperature.

### 1.4.3 Genomic DNA tagmentation reaction

#### 1.4.3.1 Solutions and reagents

- BLT (*Enrichment BLT*)<sup>c</sup>
- TB1 (*Buffer Tagmentation*)<sup>d</sup>
- Nuclease-free water

<sup>c</sup> Stored in a refrigerator (2°C to 8°C)

<sup>d</sup> Stored in freezer (-25°C to -15°C)

#### 1.4.3.2 Tagmentation procedure

- a. Homogenize and centrifuge (1500 x g for 1 min) the plates containing the pools (item 6.4.2.2);
- b. On a new 96-well PCR card, add 10 µL of pool 1 and 10 µL from pool 2;
- c. Vortex BLT and TB1 for 10 s until completely resuspended. DO NOT centrifuge the BLT after homogenizing.
- d. In a clean room, prepare the mastermix of the DNA tagmentation reaction as described in Table 4 (multiply by the number of samples that will be processed):

TABLE 4  
Mastermix tagmentation

Components	Volume (µL) per sample	Volume (µL) 96 samples
BLT	4	384
TB1	12	1152
Nuclease-free water	20	1920
<b>Total</b>	<b>36</b>	<b>3456</b>

Make the mastermix in 2 tubes of 2.0 mL (divide the volumes by 2).

Source: ILLUMINA, 2021b

- e. Homogenize the mastermix of pipetting tagment (10x);
- f. Transfer 30  $\mu$ L of the tagmentation mastermix to each plate well containing the pools;
- g. Homogenize by pipetting (10x);
- h. Place the plate in a thermocycler and save the conditions

<b>HOLD 1</b>	1x	55°C for 5 min
<b>HOLD 2</b>	$\infty$	10°C

- i. Remove the thermocycler plate and proceed to item 6.4.4 immediately.

#### 1.4.4 Purification post tagmentation reaction

##### 1.4.4.1 Solutions and reagents

- TSB (*Tagment Stop Buffer*)<sup>and</sup>
- TWB (*Tagment Wash Buffer*)<sup>and</sup>
- Nuclease-free water  
<sup>and</sup>Stored in a refrigerator (2°C to 8°C).

##### 1.4.4.2 Purification procedure

- a. Centrifuge the plate containing the tagmentation reaction (500 x g for 10s);
- b. Homogenize the TSB 10x by inversion and add 10  $\mu$ L (slow tonot bubble) in all wells containing the tagmentation reaction (item 6.4.3.2).

**NOTE:** If the TSB is hasty, heat to 37°C for 10 minutes and homogenize by inversion;

- c. Homogenize by pipetting (10x);
- d. Incubate the plate at room temperature for 5 min;
- e. Place the plate on an appropriate magnetic shelf and wait until the sobrentan is completely clear (approximately 2 min);

**Reminder:** Remove index *adapters* from freezer and leave room temperature.

- f. Using a multichannel pipette, remove and discard the entire overnor;
- g. Perform two washes with TWB, as described in the steps from "h" to "i";
- h. To put the TWB into a channel to facilitate distribution on the board. Remove the plate from the magnetic bookcase, add 100  $\mu$ L of TWB directly into the *beads*;
- i. Carefully homogenize by pipetting (10x), ensuring that *the beads* were completely resuspended;
- j. Place the plate back on the magnetic shelf and esperar until the sobrenator is completely clear (approximately 2 min);
- k. Using a multichannel pipette, remove and discard the entire overnor;
- l. Repeat steps "h" and "i";

- m. Keep the plate off the magnetic shelf while preparing the "mastermix of the amplification reaction of the tagmented amplicons" (table 5, item 6.4.5.2).

**NOTE:** TWB should remain in the wells to avoid dryness of the *beads*.

#### 1.4.5 Amplification reaction of tagmented amplicons

##### 1.4.5.1 Solutions and reagents

- EPM (*Enhanced PCR Mix*)<sup>f</sup>
- *Index Adapters*<sup>f</sup>
- Nuclease-free water

<sup>f</sup>Stored in freezer (-25°C to -15°C)

##### 1.4.5.2 Amplification procedures

- a. Defrost the EPM in ice bath and *index adapter* at room temperature;
- b. Homogenize the EPM by inversion and centrifuge (500 x g for 10 s);
- c. In a clean room, combine the volumes of the components described in table 5 "mastermix of the amplification reaction of the tagmented amplicons" (multiply by the number of samples that will be processed):

TABLE 5  
Mastermix of amplification reaction of the tagmented amplicons

Components	Volume (μL) per sample	Volume (μL) 96 samples
EPM	24	2304
Nuclease-free water	24	2304
<b>Total</b>	<b>48</b>	<b>4608</b>

Source: ILLUMINA, 2021b

- d. Vortexar and centrifuge (500 x g for 10 s) the mastermix of amplification reaction;
- e. Place the plate (item 6.4.4.2, step "m") on a magnetic shelf and wait until the sobrendante is completely clear (approximately 2 min);
- f. Remove and discard the supernatant (TWB) from the plate;
- g. With the aid of a 20 μL multichannel pipette, remove all residual TWB;
- h. Remove the plate from the magnetic bookcase ;
- i. Immediately add 40 μL of amplification reaction mastermix in each well, directly on *bead*;
- j. Carefully homogenize by pipetting (10x) until the *beads* are completely resuspended;
- k. Homogenize and centrifuge the plate containing *the index adapters* and add 10 μL of each *index adapters* to the plate wells containing the samples.

**NOTE:** Stick the *index adapters plate adhesive* with the tips during pipetting.

- l. Homogenize again, as step "j";
- m. Place the plate on a thermocycler and save the conditions

<b>HOLD 1</b>	1x	72°C for 3 min
<b>HOLD 2</b>	1x	98°C for 3 min
<b>CYCLING</b>	10x	98°C for 20 s
		60°C for 30 s
		72°C for 1 min
<b>HOLD 3</b>	1x	72°C for 3 min
<b>HOLD 4</b>	∞	10°C

- n. Remove the plate from the thermocycler and proceed to the next step.

**NOTE:** Remove the SPB from the refrigerator and rsb from the freezer (item 6.4.6.1).

### 1.4.6 Purification of the tagmented amplicons amplification

#### 1.4.6.1 Solutions and reagents

- SPB (*Sample Purification Beads*)<sup>g</sup>
- RSB (*Resuspension Buffer*)<sup>h</sup>
- Nuclease-free water
- 80% fresh ethanol

<sup>g</sup>Stored in a refrigerator (2°C to 8°C)

<sup>h</sup>Stored in freezer (-25°C to -15°C)

#### 1.4.6.2 Purification procedures

- a. Defrost the RSB;
- b. Prepare 2mL ethanol at 80% ;

**NOTE:** 2mL ethanol 80 % for each library of 96 samples.

- c. Place the plate containing the amplification reaction of the taglineed amplicons (item 6.4.5.2) on the magnetic shelf until the surface rummage is clear (approximately 2 minutes);
- d. Transfer 5 µL of the overnof each well of the amplification plate to a strip of 8 wells (change the tips at each column), which will result in 60 µLper p oiof the strip;
- e. Homogenize and transfer 55 µL of each strip well to a 1.5 mL tube (55 µL x 8 wells = 440 µL pool);
- f. Homogenize the SPB in vortex and centrifuge (500 x g for 10 s);
- g. Add the SPB volume according to the final volume of the pool x 0.9. Ex: For a library with a final volume of 440 µL, add 396 µL of SPB (440 µL x 0.9 = 396 µL);



- h. Incubate at room temperature for 5 minutes;
- i. Place the tube on an appropriate magnetic shelf for approximately 3 minutes, until the supernatant is completely clear;
- j. Remove and discard the supernatant;
- k. Wash twice with 80% ethanol, as described in steps "l", "m", "n", "o" and "p";
- l. Add 1mL of 80 % ethanol to the tube;
- m. Incubate for 30 s;
- n. Without touching the pellet, remove and discard the supernatant;
- o. Repeat the steps "l", "m" and "n";
- p. With a pipette of 20 µL, discard the residual ethanol;
- q. Wait for the ethanol to evaporate and the SPB gets the opaque staining ;
- r. Remove the tube from the magnetic shelf and add 55 µL of the RSB (vortex before use);
- s. Homogenize by pipetting (10x);
- t. Incubate at room temperature for 2 min;
- u. Place the tube on an appropriate magnetic shelf for approximately 3 minutes until the supernatant is completely clear;
- v. Transfer 50 µL of the supernatant to a new tube.

**Stop PointP:** The tube Can Be Stored In the freezer (-25°C) to -15°C) is Up to 30

## 1.4.7 Library Quantification

### 1.4.7.1 Solutions and reagents

- Qubit dsDNA HS<sup>cap i</sup>
- Qubit dsDNA HS Reagent \*200X<sup>i</sup> Stored at room temperature (15°C to 30°C)

### 1.4.7.2 Quantification procedures

- a. Prepare the Qubit work solution:  
**Working solution** : (199 µL of dsDNA HS buffer x number of samples) + (1 µL of dsDNA Reagent x number of samples).
- b. Homogenize the working solution in vortex until smooth;
- c. Distribute the working solution on each tube and add the sample and standards to the Qubit tubes as described below:  
**Standard**: 190 µL of the working solution + 10 µL of the standard (Standard 1 and Standard 2 in separate tubes );  
**Sample**: 198 µL of working solution + 2 µL of sample.
- d. Vortex the tubes and incubate at room temperature for 1 minute;
- e. Turn on the *Qubit Fluorometer*, select the option "dsDNA", then select the kit to use "*dsDNA High sensitivity*";

- f. Place the tube with standard 1 in the Qubit, close the lid and tighten "Read standards". Repeat the same procedure for pattern 2;
- g. Quantify the sample by clicking on the "Run samples" option, select the sample volume used (2 µL) and the unit (ng/µL).

**NOTE:** If the sample concentration is too high, dilute 1:10. In this case, the diluted sample should be pipetted into a new tube containing the working solution (prepared as described in steps "a", "b", "c" and "d") and quantified.

#### 1.4.8 Library normalization

##### 1.4.8.1 Solutions and reagents

- RSB (*Resuspension Buffer*)<sup>j</sup>
  - Nuclease-free water
- <sup>j</sup>Stored in freezer (-15°C to -25°C).

##### 1.4.8.2 Normalization procedure

- a. Calculate molarity and normalize the library to 4nM (use the RSB buffer to dilute the library);
- b. To calculate molarity, use the following formula:

$$\frac{Cb \left( \frac{ng}{\mu l} \right) \times 10^6}{660 \frac{g}{mol} \times ta (pb)} = \text{Molaridade (nM)}$$

where:

- **Cb**: concentration of the library in ng/µL (quantified in item 6.4.7.2);
- **Ta**: Average size of the amplicons generated, obtained by the analysis in labchip (PerkinElmer), according to the manufacturer's recommendations.

##### Example:

Dosage: Too high (Dilute 1:10 = 5 µL from library + 45 µL RSB) Dilute

dosage: **Cb**= 20.2 ng/µL

**Ta**= 380 bp

$$20.2 / 660 \times 380 \times 10^6 = 80.54 \text{ nM}$$

- c. Then calculate the required volume of library and RSB to obtain the concentration of 4nM, using the following formula:

$$Ci \times Vi = Cf \times Vf$$

where:

- **Ci**: Initial concentration of the library in nM (calculated in step "b");

- **Vi**: Library volume required to obtain the desired concentration;
- **Cf**: Final concentration of the library (concentration of interest 4nM);
- **Vf**: Final library volume at 4nM.

**NOTE:** Normalize at least 30 µL of library at 4nM.

- Subtract the final volume (Vf) by the initial volume (Vi) to know how much RSB will be required;

**Example:**

$$C_i \times V_i = C_f \times v_f$$

$$80.54 \text{ nM} \times V_i = 4 \text{ nM} \times 30$$

$$V_i = 1.5 \text{ µL diluted library}$$

$$V_f - V_i = \text{RSB Volume}$$

$$30 \text{ µL} - 1.5 \text{ µL diluted library} = \mathbf{28.5 \text{ µL RSB}}$$

- After dilution, homogenize the sample into vortex and centrifuge (500 x g by 10 f). Keep the diluted library at 4nM in a refrigerator (2°C to 8°C) until use.

#### **1.4.9 Preparation of *Flow cell* and MiSeq equipment**

Start the procedure described in item 6.4.10 after the cartridge has completely thawed and the equipment ready for sequencing, with the washing(s) performed and the running prepared.

##### **1.4.9.1 Solutions and reagents**

- *PE MiSeq Flow cell* (FC)<sup>k</sup>
- PR2 (*Buffer Incorporation*)<sup>k</sup>
- *Cartridge - V3 600 cycles*<sup>l</sup>
- Nucleases-free water<sup>k</sup> Stored<sup>d</sup> in a refrigerator (2°C to 8°C) <sup>l</sup>Stored in freezer (-15°C to -25°C)

##### **1.4.9.2 Preparing the equipment**

- Start this procedure after the equipment has been undergone by the recommended washing(s);
- Remove the HR and PR2 from the geladeira;
- Using the index finger and thumb, remove the HR from the stock solution by touching only the plastic edge;
- Rinse with water all glass surface and surrounding FC plastic. Hold the HR by the plastic part and do not touch the glass part;
- Gently knock the plastic part (gray) of the FC on a paper to remove excess water;

- f. Tilt the HR over a clean surface without touching the glass. Allow the FC to dry outdoors or clean with a paper that does not loosen lint;
- g. After drying, hold the HR against the light and check for dirt or debris on the surface of the glass (especially on the black central lines). If present, pass lint-free paper dampened with water and repeat the previous steps ("d" to "g").
- h. Homogenize the defrosted cartridge by inversion (10x). Check that the reagents located in the center of the cartridge are precipitated, if so, leave the cartridge in warm water for 5 min;
- i. Knock the cartridge on the countertop to drain all water generated during thawing and/or incubation;
- j. Homogenize PR2 by inversion (5x);
- k. **Preparing the race:** Click on the **Local Run Manager** software • **Create Run** • Fill in the **race name** and **select the** library preparation kit and index **reads set** used. In the **trimming adapter option**, select ON. Fill in the **number of** samples that will be **sequenced and identify each of them** - **Save**. Another option is to prepare a spreadsheet with all this data and import by clicking **Sheet samples**.
- l. **Preparing the equipment:** Click on the **MiSeq Control Software** • **Sequence** • **Select the option used to prepare the race**. Follow all the controls for preparing the equipment: **Place the HR** • **place the PR2** and the empty waste in the **indicated** compartment and lower the sipper.

#### 1.4.10 Denaturation of the library

##### 1.4.10.1 Solutions and reagents

- HT1 (*Hybridization Buffer*)<sup>m</sup>
  - **NaOH 1N = NaOH 1M** (Sodium hydroxide at 1 normal = 1 molar)
  - Nuclease-free water
- <sup>m</sup>Stored in freezer (-15°C to -25°C)

##### 1.4.10.2 Denaturation procedure

- m. Prepare 500 µL of NaOH 0.2N (mix 400 µL of water with 100 µL of NaOH 1N and homogenize 10x per inversion);
- n. Homogenize the 4nM per pulse library in the vortex and add 5 µL in a new 1.5 mL tube;
- o. Proceed to step "d" **AFTER** HT1 defrosts completely;
- p. Add 5 µL of NaOH 0.2N to the tube containing the library;
- q. Quickly vortex the solution containing the library and the NaOH, and then centrifuge (500 x g for 10 s);
- r. Incubate at room temperature for 5 minutes for denaturation of double DNA tape;

- s. Homogenize HT1 by inversion (10x);
- t. Add 990 µL of HT1 in 10 µL of denatured library to obtain a denatured library at 20 pM;
- u. Place the denatured DNA in ice or in a refrigerator (2°C to 4°C) until the next step is started.

#### 1.4.11 Denatured library dilution

##### 1.4.11.1 Solutions and reagents

- HT1 (*Hibridization Buffer*)<sup>n</sup>
  - Nuclease-free water
- <sup>n</sup>Stored in freezer (-15°C to -25°C).

##### 1.4.11.2 Description of the procedure

- a. Dilute the library at 20 pM to 9 pM at 600 µL (desired input);
- b. Calculate by following the formula described below:

$$C_i \times V_i = C_f \times v_f$$

$$20 \text{ pM} \times V_i = 9 \text{ pM} \times 600$$

$$V = 270 \text{ µL library at } 20 \text{ pM} + 330 \text{ µL HT1 (final volume of } 600 \text{ µL)}$$

#### 1.4.12 Phix denaturation and dilution (OPTIONAL)

##### 1.4.12.1 Solutions and reagents

- HT1 (*Hibridization Buffer*)<sup>o</sup>
  - NaOH 0.2N (prepared in item 6.4.10.2)
  - Nuclease-free water
  - PhiX 10 nM<sup>o</sup>
- <sup>o</sup>Stored in freezer (-15°C to -25°C).

##### 1.4.12.2 Phix denaturation and dilution procedure

- a. Homogenize PhiX rapidly in vortex;
- b. In a new tube, dilute the PhiX to 4 nM (2 µL of PhiX + 3 µL of water);
- c. Add 5 µL of NaOH 0.2N to the tube containing PhiX at 4 nM;
- d. Quickly vortex the solution containing PhiX and NaOH 0.2N, and then centrifuge (500 x g for 10s);
- e. Incubate at room temperature for 5 minutes for denaturation;
- f. Homogenize HT1 by inversion (10x)
- g. Add 990 µL of HT1 in 10 µL of denatured PhiX to obtain PhiX at 20 pM;
- h. Dilute PhiX at 20 pM to 9 pM (as described in item 6.4.11.2): 270 µL of PhiX at 20 pM + 330 µL HT1;
- i. Mix 5% (30 µL) phix at 9 pM at 570 µL of the denatured teak biblio diluted in item 6.4.11.2.

**NOTE:** Removes 30 µL from the library at 9 pM and adds 30 µL of PhiX to 9 pM (30 µL of PhiX to 9 pM + 570 µL from the diluted denatured library to 9 pM).

**Additional step - OPICIONAL:**

To ensure denaturation, incubate the library at 9pM, containing or not PhiX in a dry bath at 96°C for 3 min, and then leave it in an ice bath for 5 min. Keep the library in the ice bath until the time of applying to the cartridge.

**1.4.13 Loading the cartridge**

- a. After finishing the procedure of denaturation and dilution of the library at 9 pM, homogenize the cartridge again by inversion;
- b. With the aid of a 1000 µL tip, drill the aluminum present in the well **cartridge load samples** ;
- c. With a new tip of 1000 µL, load 600 µL from the library in the **well load samples of** the cartridge (apply the sample slowly and on the well wall to prevent the formation of bubbles);

**WARNING:** Do not invert the cartridge after the library is added.

- d. Bearthe cartridge in the refrigerated compartment of the equipment;
- e. Then select the **previously created run** and start **sequencing (START RUN)."**

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