|  |
| --- |
| **LIBRARY PREPARATION FOR MISEQ ILLUMINA PLATFORM** |

1. **PROCEDURES**

# 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 ºGL disinfectant alcohol solution;
* Turn on the CSB UV for at least 15 minutes
* Check that all reagents are available before starting the procedure.

# 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).

# Reagents

* + 1. **For asepsis**
* MilliQ Water;
* Disinfectant alcohol solution at 81 ºGL;
* 2.0% sodium hypochlorite solution.

# 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 Extreded 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

# 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.

# CDNA Synthesis

* + - 1. **Solutions and reagents**
* Superscript IV
* mix dNTPs (10 mM each)
* Randon Hexamer (50 μM)
* RNase inhibitor

Stored in freezer (-25°C to -15°C)

# Description of the procedure

1. Separate and defrost the reagents in ice

(Table 1 and 2, column components);

1. Vortex and centrifuge (1000 x g for 10s) the reaction components before use;
2. Assemble a map with the order of the samples and identify a PCR plate of 96 wells with data + cDNA
3. 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 (μL)/ per sample** | **Volume (μL)/ 96 samples2** |
| Random Hexamers (50 μM) | 1 | 101 |
| dNTPs mix (10 mM) | 1 | 101 |
| RNA Template 1 | 11 | - |
| **Total** | **13** | **202** |

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

Source: ThermoFisher Scientific, 2022

1. Distribute 2 μL 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.

1. Add 11 μL of RNA to each of the PCR plate wells that will be used according to the map, in chapel and appropriate room;
2. Gently homogenize by pipetting (10x);
3. Seal the plate with optical adhesive;
4. Centrifuge at 1500 x g for 1 minute;
5. Place the plate on a thermocycler and save the condition (**Program: Incubação\_cDNA**):

|  |  |  |
| --- | --- | --- |
| **HOLD 1** | 1x | 65°C for 5 min |

1. Remove the plate from the thermocycler and place immediately in an ice bath;
2. 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 samples1** |
| SSIV Buffer | 4 | 403,20 |
| DTT (100 mM) | 1 | 101 |
| RNase Inhibitor | 1 | 101 |
| SSIV Reverse Transcriptase | 1 | 101 |
| **Total** | **7** | **706,2** |

1Calculation performed with an increase of 5%.

Source: ThermoFisher Scientific, 2022

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

|  |  |  |
| --- | --- | --- |
| **HOLD 1** | 1 x | 42°C for 50 min |
| **HOLD 2** | 1x | 70°C for 10 min |
| **HOLD 3** | ∞ | 4°C |

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

**Stop Point:** CDNA can Be stored In fashion freezer (-15°C) to -25°C) for long periods.

# Amplification reaction (PCR pool 1 and pool 2)

* + - 1. **Solutionsand 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

# Description of the procedure

1. In clean room, dilute the primer pools for concentration of use. For the ARTIC V4 primer set, dilute the stock solution to 100 μM, *from pool* 1 and 2 (in separate tubes) to 10 μM in nuclease-free water.  Ex: in a tube 1.5 mL, add 38 μL of pool 1 stock solution and complete with 342 μL of nuclease-free water, repeat the procedure for pool 2 (final volume of 380 μL of each pool at 10 μM);
2. 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 (μL)/ per sample** | **Volume (μL)/ 96 samples**1 |
| 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 **Pool 1** (10 μM) | 3,6 | 362,88 |
| **Total** | **20** | **2016** |

|  |  |  |
| --- | --- | --- |
| **Pool 2** | | |
| **Components** | **Volume (μL)/ per sample** | **Volume (μL)/ 96 samples**1 |
| 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 **Pool 2** (10 μM) | 3,6 | 362,88 |
| **Total** | **20** | **2016** |

1Hereit 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

1. Distribute 20 μL 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);
2. In the appropriate cabin and room, add 5 μL of cDNA (prepared in item 6.4.1) tothe corresponding plates of pool 1 and 2.
3. Gently homogenize by pipetting (10x);
4. Seal the plate with optical adhesive;
5. Centrifuge at 1500 x g for 1 minute;
6. Place the plates in thermocyclers and save the conditions (**COVID\_pool\_V4**):

|  |  |  |
| --- | --- | --- |
| **HOLD 1** | 1 x | 98°C for 30 s |
| **CYCLING** | 35x\* | 98°C for 15 s |
|  |  | 65°C for 5 min |
| **HOLD 2** | ∞ | 4°C |

\*The number ofcycles 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.

1. 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 oneof 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 m room temperature.

# Genomic DNA tagmentation reaction

* + - 1. **Solutions and reagents**
         * BLT (*Enrichment BLT*)c
         * TB1 (*Buffer Tagmentation*)d
         * Nuclease-free water

c Storedin a refrigerator (2°C to 8°C)

d Stored in freezer (-25°C to -15°C)

# Tagmentation procedure

1. Homogenize and centrifuge (1500 x g for 1 min) the plates containing the pools (item 6.4.2.2);
2. On a new 96-well PCR card, add 10 μL of pool 1 and 10 μL from pool 2;
3. Vortex BLT and TB1 for 10 s until completely resuspended. DO NOT centrifuge the BLT after homogenizing.
4. 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 |
| **Total** | **36** | **3456** |

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

Source: ILLUMINA, 2021b

1. Homogenize the mastermix of pipetting tagment (10x);
2. Transfer 30 μL of the tagmentation mastermix to each plate well containing the pools;
3. Homogenize by pipetting (10x);
4. Place the plate in a thermocycler and save the conditions

|  |  |  |
| --- | --- | --- |
| **HOLD 1** | 1x | 55°C for 5 min |
| **HOLD 2** | ∞ | 10°C |

1. Remove the thermocycler plate and proceed to item 6.4.4 immediately.

# Purification post tagmentation reaction

* + - 1. **Solutions and reagents**
         * TSB (*Tagment Stop Buffer*) and
         * TWB (*Tagment Wash Buffer*)and
         * Nuclease-free water

andStored in a refrigerator (2°C to 8°C).

# Purification procedure

1. Centrifuge the plate containing the tagmentation reaction (500 x g for 10s);
2. Homogenize the TSB 10x by inversion and add 10 μL (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;

1. Homogenize by pipetting (10x);
2. Incubate the plate at room temperature for 5 min;
3. 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.

1. Using a multichannel pipette , remove and discard the entire overnor;
2. Perform two washes with TWB, as described in the steps from "h" to "l";
3. To put the TWB into a channel to facilitate distributionon the board. Remove the plate from the magnetic bookcase, add 100 μL of TWB directly into the *beads*;
4. Carefully homogenize by pipetting (10x), ensuring that  *the beads*

were completely resuspended;

1. Place the plate back on the magnetic shelf and esperar until the sobrenator is completely clear (approximately 2 min);
2. Using a multichannel pipette , remove and discard the entire overnor;
3. Repeat steps "h" and "i";
4. Keep the plate off the magnetic shelf while preparing the "mastermix of the amplification reaction of the tagmentated amplicons" (table 5, item 6.4.5.2).

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

# Amplification reaction of tagmentated amplicons

* + - 1. **Solutions and reagents**
         * EPM (*Enhanced PCR Mix*)f
         * *Index Adapters*f
         * Nuclease-free water

fStored in freezer (-25°C to -15°C)

# Amplification procedures

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

TABLE 5

Mastermix of amplification reaction of the tagmentated amplicons

|  |  |  |
| --- | --- | --- |
| **Components** | **Volume (μL) per sample** | **Volume (μL) 96 samples** |
| EPM | 24 | 2304 |
| Nuclease-free water | 24 | 2304 |
| **Total** | **48** | **4608** |

Source: ILLUMINA, 2021b

1. Vortexar and centrifuge (500 x g for 10 s) the mastermix of amplification reaction;
2. 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);
3. Remove and discard the supernatant (TWB) from the plate;
4. With the aid of a 20 μL multichannel pipette, remove all residual TWB;
5. Remove the plate from the magnetic bookcase ;
6. Immediately add 40 μL of amplification reaction mastermix in each well, directly on *bead*;
7. Carefully homogenize by pipetting (10x) until the *beads* are completely resuspended;
8. 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.

1. Homogenize again, as step "j";
2. Place the plate on a thermocycler and save the conditions

|  |  |  |
| --- | --- | --- |
| **HOLD 1** | 1x | 72°C for 3 min |
| **HOLD 2** | 1x | 98°C for 3 min |
|  |  | 98°C for 20 s |
| **CYCLING** | 10x | 60°C for 30 s |
|  |  | 72°C for 1 min |
| **HOLD 3** | 1x | 72°C for 3 min |
| **HOLD 4** | ∞ | 10°C |

1. 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).

# Purification of the tagmentated amplicons amplification

* + - 1. **Solutions and reagents**
         * SPB (*Sample Purification Beads*)g
         * RSB (*Resuspension Buffer*)h
         * Nuclease-free water
         * 80% fresh ethanol

gStored in a refrigerator (2°C to 8°C)

hStored in freezer (-25°C to -15°C)

# Purification procedures

1. Defrost the RSB;
2. Prepare 2mL ethanol at 80% ;

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

1. 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);
2. 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;
3. Homogenize and transfer 55 μL of each strip well to a 1.5 mL tube (55 μL x 8 wells = 440 μL pool);
4. Homogenize the SPB in vortex and centrifuge (500 x g for 10 s);
5. 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);
6. Incubate at room temperature for 5 minutes;
7. Place the tube on an appropriate magnetic shelf for approximately 3 minutes, until the overnor is completely clear;
8. Remove and discard the overnor;
9. Wash twice with 80% ethanol, as described in steps "l", "m", "n", "o" and "p";
10. Add 1mL of 80 % ethanol to the tube;
11. Incubate for 30 s;
12. Without touching the pellet, remove and discard the overnator;
13. Repeat the steps "l", "m" and "n";
14. With a pipette of 20 μL, discard the residual ethanol;
15. Wait for the ethanol to evaporate and the SPB gets the opaque staining ;
16. Remove the tube from the magnetic shelf and add 55 μL of the RSB (vortex before use);
17. Homogenize by pipetting (10x);
18. Incubate the ambient temperature for 2 min;
19. Place the tube on an appropriate magnetic shelf for approximately 3 minutes until the overnage is completely clear;
20. Transfer 50 μL of the overnatant to a new tube.

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

# Library Quantification

* + - 1. **Solutions and reagents**
         * Qubit dsDNA HScap i
         * Qubit dsDNA HS Reagent \*200Xi iStored at room temperature (15°C to 30°C)

# Quantification procedures

1. 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).

1. Homogenize the working solution in vortex until smooth;
2. Distribute the working solution on each tube and add the sample and patterns 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.

1. Vortex the tubes and incubate at room temperature for 1 minute;
2. Turn on *the Qubit Fluorometer,* select the option "dsDNA", then select the kit to use "*dsDNA High sensitivity*";
3. Place the tube with standard 1in the Qubit, close the lid and tighten "*Read standards".*  Repeat the same procedure for pattern 2;
4. 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 quantifiednovame nte.

# Library normalization

* + - 1. **Solutions and reagents**
         * RSB (*Resuspension Buffer*)j
         * Nuclease-free water

jStored in freezer (-15°C to -25°C).

# Normalization procedure

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



where:

-**Cb**: concentration of theiblyteca b in ng/μL (quantified in item 6.4.7.2);

-**Ta**: Average size of the amplicons generated, as 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/ 660x380 \*106= 80.54 nM

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



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.

1. Subtract the final volume (Vf) by the initial volume (Vi) to know how much RSB will berequired;

**Example:**



80.54 nM x Vi = 4 nM \* 30

**Vi = 1.5 μL diluted library**

*Vf - Vi = RSB Volume*

30 μL - 1.5 μL diluted library = **28.5 μL RSB**

1. 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. **Preparation of *Flow cell*  and MiSeq equipment**

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

# Solutions and reagents

* + - * + *PE MiSeq Flow cell* (FC)k
        + PR2 (*Buffer Incorporation*)k
        + *Cartridge - V3 600 cyclesl*
        + Nucleases-free water k Storedin a refrigerator (2°C to 8°C) lStored in freezer (-15°C to -25°C)

# Preparing the equipment

1. Start this procedure after the equipment has been undergone by the recommended washing(s);
2. Remove the HR and PR2 from the geladeira;
3. Using the index finger and thumb, remove the HR from the stock solution by touching only the plastic edge;
4. Rinse with water all glass surface and surrounding FC plastic. Hold the HR by the plastic part anddo not touch the glass part;
5. Gently knock the plastic part (gray) of the FC on a paper to remove excess water;
6. 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;
7. 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 damled with water and repeat the previous steps ("d" to "g").
8. 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;
9. Knock the cartridge on the countertop to drain all water generated during thawing and/or incubation;
10. Homogenize PR2 by inversion (5x);
11. **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 identifyeach of them** - **Save**. Another option is to prepare a spreadsheet with all this data and import by clicking **Sheet samples**.
12. **Preparing the equipment**: Click on **the MiSeq Control Software** • **Sequence** • **Select the optionused 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.

# Denaturation of the library

* + - 1. **Solutions and reagents**
         * HT1 (*Hibridization Buffer*)m
         * **NaOH 1N** = **NaOH 1M** (Sodium hydroxide at 1 normal = 1 molar)
         * Nuclease-free water

mStored in freezer (-15°C to -25°C)

# Denaturation procedure

1. Prepare 500 μL of NaOH 0.2N (mix 400 μL of water with 100 μL of NaOH 1N and homogenize 10x per inversion);
2. Homogenize the 4nM per pulse library in the vortex and add 5 μL in a new 1.5 mL tube;
3. Proceed to step "d" **AFTER** HT1 defrosts completely;
4. Add 5 μL of NaOH 0.2N to the tube containing the library;
5. Quickly vortex the solution containing the library and the NaOH, and then centrifuge (500 x g for 10 s);
6. Incubate at room temperature for 5 minutes for denaturation of double DNA tape;
7. Homogenize HT1 by inversion (10x);
8. Add 990 μL of HT1 in 10 μL of denatured library to obtain a denatured library at 20 pM;
9. Place the denatured DNA in ice or in a refrigerator (2°C to 4°C) until the nextstep is started.

# Denatured library dilution

* + - 1. **Solutions and reagents**
         * HT1 (*Hibridization Buffer*)n
         * Nuclease-free water

nStored in freezer (-15°C to -25°C).

# Description of the procedure

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



20 pM x Vi = 9 pM x 600

V= 270 μL library at 20 pM + 330 μL HT1 (final volume of 600 μL)

# Phix denaturation and dilution (OPTIONAL)

* + - 1. **Solutions and reagents**
         * HT1 (*Hibridization Buffer*)o
         * NaOH 0.2N (prepared in item 6.4.10.2)
         * Nuclease-free water
         * PhiX 10 nMo

oStored in freezer (-15°C to -25°C).

# Phix denaturation and dilution procedure

1. Homogenize PhiX rapidly in vortex;
2. In a new tube, dilute the PhiX to 4 nM (2 μL of PhiX + 3 μL of water);
3. Add 5 μL of NaOH 0.2N to the tube containing PhiX at 4 nM;
4. Quickly vortex the solution containing PhiX and NaOH 0.2N, and then centrifuge (500 x g for 10s);
5. Incubate at room temperature for 5 minutes for denaturation;
6. Homogeinize HT1 by inversion (10x)
7. Add 990 μL of HT1 in 10 μL of denatured PhiX to obtain PhiX at 20 pM;
8. 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;
9. 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.

# Loading the cartridge

1. After finishing the procedure of denaturation and dilution of the library at 9 pM, homogenize the cartridge again by inversion;
2. With the aid of a 1000 μL tip, drill the aluminum present in the well

***cartridge load samples*** ;

1. 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.

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

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