KwaZulu-Natal Research Innovation and Sequencing Platform, UKZN

NEXTERA FLEX: WHOLE GENOME SEQUENCING OF nCoV-2019

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List of Abbreviations:

DNA	Deoxyribonucleic acid
EB	Elution Buffer
EtOH	Ethanol
PCR	Polymerase chain reaction
RT	Room temperature
RNA	Ribonucleic acid
UV	Ultraviolet
BLT	bead-linked transposomes
TB1	Tagmentation buffer
TSB	Tagment stop buffer
TWB	Tagment wash buffer
EPM	Enhanced PCR Mix
SPB	Sample Purification Beads
RSB	Resuspension Buffer

Documentation

QIAamp viral RNA Mini Handbook
Chemagic 360 TNA extraction from swab samples

1. Introduction

This protocol is describes a method for the whole genome sequencing of the 2019 novel Coronavirus (2019-nCoV) using a tiling PCR approach with overlapping primers. This method has proven to have a high success rate in the sequencing of viral genomes. Briefly, primers are designed to be 20-30bp in length and to generate 400bp amplicons with a 70bp overlap. The primers are designed using an online tool called Primal Scheme (http://primal.zibraproject.org/).

The amplicons generated can be sequenced on the on the Illumina MiSeq. This will produce next generation sequences covering the whole genome of the 2019-nCoV.

2. Purpose

The purpose of this document is to provide detailed instructions that should be followed when performing the sequencing of 2019-nCoV whole genomes from RNA samples using the NEXTFlex DNA Flex Library Kit.

3. Scope, Authority and Responsibility

This WI must be adhered to by all KRISP personnel involved in 2019-nCoV whole genome sequencing. Only trained and competent personnel shall conduct this assay.

4. Samples

- Viral RNA samples must be used for the whole genome sequencing procedure.
- Total nucleic acids (TNA) extracted using the CMG-1049 kit compatible with the automated Chemagic 360 Nucleic Acid Extractor (PerkinElmer), or TNA extracted using the QIAGEN manual
- These samples should have been tested on qPCR first and have a Ct between 18-35. If Ct is between 12-15, then dilute the sample 100-fold in water, and if Ct is between 15-18 then dilute 10-fold in water. This will help reduce PCR inhibition.

5. Materials

- Primers-specific for 2019-nCoV according to Primal Scheme
- Superscript IV (50rxn) Thermo 18090050
- dNTP mix (10 mM each) Thermo R0192
- Randon Hexamer (50uM) Thermo N8080127
- RNase OUT (125 rxn) Thermo 10777019
- Q5 Hot Start HF Polymerase NEB M0493S
- Ampure XP 60ml Beckman A63881
- Nextera DNA Flex Library Prep Kit
- (Illumina)
- Nextera™ DNA UD Indexes (96 Indexes, 96 Samples)
- MiSeq Reagent Nano Kit, v2 (500 cycles) MS-103-1003 (Illumina)
- Qubit dsDNA HS Reagent Q32854 (Thermofisher)
- Qubit Assay Tubes Q32856 (Thermofisher)
- DNA HiSens Reagent kit CLS760672 (Perkin Elmer)
- HT DNA 1K/12K/Hi Sens Labchip 760517 (Perkin Elmer)
- General PCR laboratory equipment and consumables

6. Procedure

6.1 cDNA synthesis

- Prepare the cDNA mastermix in the pre-PCR clean room.
- The mastermix hood must be decontaminated before and after use with 10% extran, and 70% ethanol, and sterilised with ultraviolet light (UV).
- Mix the following components in a labelled 1.5ml eppendorf tube:

Table 1. cDNA synthesis mastermix 1

Component	Volume (µl)
50µM Random Hexamers	1
10mM dNTPs mix (10mM each)	1
Template RNA	11
Total	**13**

- Gently mix by pipetting and pulse-spin the tube to collect the liquid at the bottom of the tube.
- Aliquot the mastermix in labelled PCR strip tubes.
- **Note:** PCR master mixes (shown in Tables 1 and 3) can be prepared at the same time, in the pre-PCR area before starting amplifications.

- Incubate the reaction as follows in a thermal cycler.

Table 2. PCR conditions

Temperature (°C)	Time
65	5 minutes
4	1 minute

- Spin down the tubes with the RNA and primers to get all liquid to the bottom.
- Prepare the following mastermix in the clean mastermix room.
- Mix the following components in a labelled 1.5ml eppendorf tube:

Table 3. cDNA synthesis mastermix 2

Component	Volume (µl)
SSIV Buffer	4
100mM DTT	1
RNaseOUT RNase Inhibitor	1
SSIV Reverse Transcriptase	1
Total	**7**

- The mastermix must be added to the 13µl denatured RNA for a 20µl total volume.
- Gently mix by pipetting and pulse-spin the tube to collect the liquid at the bottom of the tube.
- Incubate the reaction as follows in a thermal cycler.

Table 4. PCR conditions

Temperature (°C)	Time
42	50 minutes
70	10 minutes
5	Hold

6.2 Primer pool preparation

- Primers must be diluted and pooled using nuclease free water in a clean mastermix hood. The mastermix hood must be decontaminated before and after use with 10% extran, and 70% ethanol, and sterilised with ultraviolet light (UV).
- If required, resuspend lyophilised primers at a concentration of 100µM each. 2019-nCoV primers for this protocol were designed using Primal Scheme to generate overlapping 400 nucleotide amplicons.
- To generate 100µM primer pool stocks, add 5µl of each primer pair (named pool 1 or pool 2) to a 1.5ml eppendorf tube labelled either “Pool 1 (100µM)” or “Pool 2 (100µM)”. Total volume will be 490ul for Pool 1 (100uM) and 490ul for Pool 2 (100uM). These are now 100uM stocks of each primer pool.
- Dilute the 100µM primer pool 1:10 in molecular grade water, to generate 10µM primer stocks. It is recommended that multiple aliquots of each primer pool are made in case of degradation or contamination.
- **Note:** Primers need to be used at a final concentration of 0.015µM per primer. In this case both pools have 98 primers in, so the requirement is 3.6µL primer pools (10µM) per 25µL reaction.

6.3 Tiling PCR

- Prepare the PCR mastermix in the clean mastermix room.
- The mastermix hood must be decontaminated before and after use with 10% extran, and 70% ethanol, and sterilised with ultraviolet light (UV).
- A mastermix for each pool must be made up in the mastermix hood.

- Mix the following components in a labelled 1.5ml eppendorf tube:

Table 5. PCR mastermix

Component	Pool 1 volumes (µl)	Pool 2 volumes (µl)
5X Q5 Reaction Buffer	5	5
10mM dNTPs	0.5	0.5
Q5 Hot Start DNA Polymerase	0.25	0.25
Primer Pool 1 or 2 (10µM)	3.6	3.6
Nuclease-free water	10.65	10.65
Total	**20.0**	**20.0**

- Aliquot the mastermix in labelled PCR strip tubes.
- Add 5µl of cDNA under the extraction hood or general lab hood, which has been decontaminated using with 10% extran, and 70% ethanol, and sterilised with ultraviolet light (UV).
- Gently mix by pipetting and pulse-spin the tube to collect the liquid at the bottom of the tube.
- Incubate the reaction as follows in a thermal cycler.

Table 6. PCR conditions

Step	Temperature (°C)	Time	Cycles
Heat Activation	98	30 seconds	1
Denaturation	98	15 seconds	35
Annealing	65	5 minutes	35
Hold	4	∞	1

- *Cycle number should be 25 for Ct 18-21, and up to a maximum of 35 cycles for Ct 35.

6.4 PCR Clean-Up

- Combine the entire contents of “Pool 1” and “Pool 2” PCR reactions for each biological sample into a single 1.5 ml eppendorf tube.
- Vortex Ampure beads thoroughly to ensure they are well resuspended; the solution should be a homogenous brown colour.
- Add an equal volume (1:1) of Ampure beads to the pooled sample tube and mix gently by either flicking or pipetting. For example, add 50µl Ampure beads to a 50µl reaction.
- Pulse centrifuge to collect all liquid at the bottom of the tube.
- Incubate for 5 minutes at room temperature.
- Place on magnetic rack and incubate for 2 minutes or until the beads have pelleted and the supernatant is completely clear.

- Carefully remove and discard the supernatant, being careful not to touch the bead pellet.
- Add 200µl of 70% ethanol (at room-temperature) to the pellet.
- Carefully remove and discard ethanol, being careful not to touch the bead pellet.
- Add 200µl of 70% ethanol (at room-temperature) to the pellet.
- Carefully remove and discard ethanol, being careful not to touch the bead pellet.
- Pulse centrifuge to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible using a P10 pipette.
- With the tube lid open incubate for 1 minute or until the pellet loses its shine (if the pellet dries completely it will crack and become difficult to resuspend).
- Resuspend pellet in 30µl Elution Buffer (EB), mix gently by either flicking or pipetting and incubate for 2 minutes.
- Place on magnetic stand and transfer sample to a clean 1.5mL eppendorf tube ensuring no beads are transferred into this tube.

6.5 PCR Amplicon Quantification

- Prepare the Qubit Working Solution by diluting the Qubit dsDNA HS Reagent with the Qubit dsDNA HS Buffer in a 15ml or 50ml conical tube, using the following formula:
- Qubit dsDNA HS Buffer = (no. of samples (n) x 199)
- Qubit dsDNA HS Reagent = 1 x no. of samples (n)
- Mix thoroughly by pulse vortexing to ensure a homogenous working solution (keep in dark till ready to use)
- Prepare assay tubes as follows:
- 10µl Standard (i.e. Standard 1 and Standard 2) + 190µl Working Solution.
- 1µl Sample + 199µl Working Solution.
- Vortex the tubes briefly and incubate at room temperature for at least 2 minutes prior to measuring the concentration. Make sure the tubes have no air bubbles.
- Turn the Qubit on and choose “DNA”, then choose “dsDNA High Sensitivity”. Choose “Yes” to read new standard.
- Place the Standard 1 tube in the Qubit, close the lid and press “Read”. Repeat with Standard 2. The Qubit will automatically generate a standard curve from the readings.
- Measure and record readings for each sample tube with volume set at 1µl and unit set at ng/µl.
- If the concentration is too high and falls above the Qubit standard curve, a diluted aliquot of the PCR amplicon sample must be made (e.g. 1:10 i.e. 1µl DNA sample + 9µl nuclease free water). The sample must then be re-aliquoted into a new assay tube with new working solution and read again.
- If the concentration is too low and falls below the Qubit standard curve, there is likely insufficient amplicon for successful sequencing and the sample should be omitted.
- Using the Qubit readings, calculate and record the concentration of each PCR amplicon.

6.6 Fragment Analysis Using the LabChip GX Touch- 384 Well Plate

- Remove the chip and reagents from the fridge and allow to equilibrate to room temperature for 20 minutes prior to use.
- To prepare the gel dye; ensure that the dye concentrate is completely thawed and vortexed before use. Add 13µl DNA dye concentrate (blue cap) to 1 vial of DNA HiSens Gel matrix (red cap). Vortex till liquid in tube is light blue in colour and transfer mixture to two spin columns (550µl each). Centrifuge at 9 200 rcf for 10 minutes at room temperature. **Note:** Gel dye can be stored up to 3 weeks in the dark at 4^{O}C.

- To prepare the chip and the LabChip GX Touch instrument:

  - Select the LabChip GX Touch icon and select 'unload chip'.
  - Use a lint-free wipe with 70% ethanol to gently clean the stage of the Labchip GX Touch.
  - Use a Q-tip provided with kit to clean the electrodes and O-rings with nuclease free water.
  - Select purge to flush tubings.
  - Use a vacuum pump to rinse and aspirate all wells of the chip three times with 100µl nuclease-free water. On the final rinse ensure that there is no residual water.
  - Use a lint-free wipe to clean both sides of the chip window and the top of each well with 70% ethanol.
  - Use a reverse pipetting technique to add gel dye to wells 3, 7, 8 and 10 (Figure 1).
  - For a low-throughput (1-48 samples), add 50µl of Gel dye to wells 3, 7, 8, 10. Add 100ul DNA HiSens marker to well 4 (Figure 1).
  - For a high-throughput (49-96 samples), add 75µl of Gel dye to wells 3, 7, 8 and 120µl to well 10 (Figure 2).
  - Add 100µl DNA HiSens marker to well 4 (Figure 2).

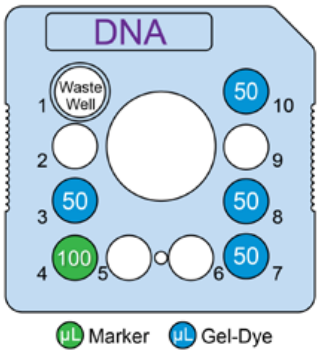

Figure 1. Low-throughput

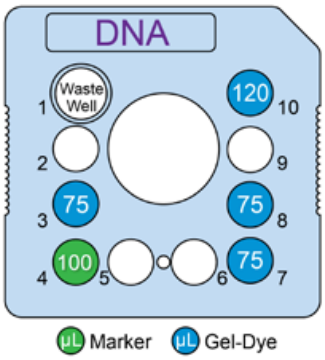

Figure 2. High-throughput

  - Run the limited sample workflow for samples: This step must be performed just prior to loading the machine to prevent evaporation of sample.
  - Prepare sample by adding 8µl of nuclease-free water to each well on the plate to be used.
  - Add 2µl of DNA to the wells.
  - Seal plate with a foil plate cover, briefly vortex and centrifuge plate to remove all bubbles.

- Make up a 1X ladder by adding 12µl of DNA ladder (yellow cap) to 108µl DNA storage buffer in the provided ladder tube. Pipette mix volume.
- Make up the running buffer in the provided buffer tube by adding 150µl DNA storage buffer to 600µl nuclease-free water

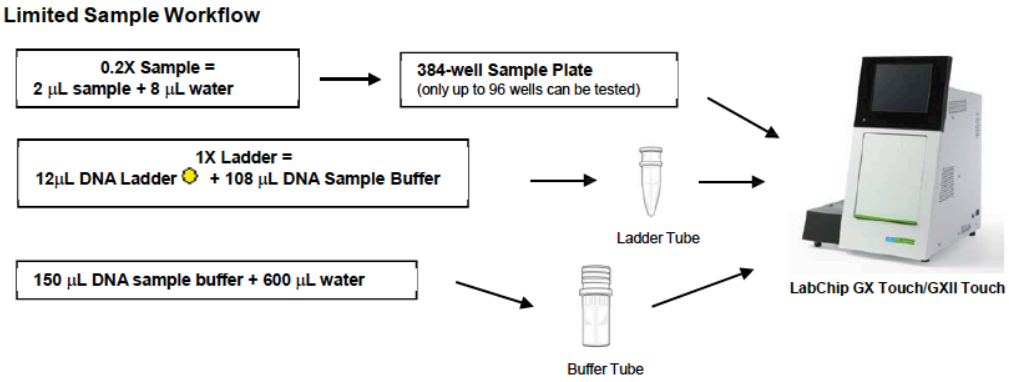

Figure 3. Limited sample workflow

- On the home screen, select 'unload plate' and place the buffer tube and ladder tube in the designated area in the machine plate holder.
- Remove foil cover and add the sample plate to the machine. Select 'unload plate' to retract plate.
- Place the loaded chip on the machine and close the door.
- On the pop-up window select HT DNA High Sensitivity, then OK. Select "run" on the home screen. Select the correct wells to be tested and the correct plate being used. Fill in:
  - Operator: Initials,
  - File Prefix: Date,
  - Project Name: Date and project name
- Click Start the Run.
- Click green arrow, check all the information entered and correct where necessary.
- Click Start.
- The machine will automatically perform a wash.
- After the run is completed transfer the .gxd data file (My computer/Program Files/Labchip GX Touch/data/Run folder (date of run)/.gxd file) onto a USB and analyze the data.
- Purge the machine.
- Clean the chip by using the vacuum pump to rinse and aspirate all wells of the chip with 100µl nuclease-free water three times.
- Add 100µl of storage buffer to all active wells and place the chip back onto the machine. Ensure that the buffer tube is filled with 750µl storage buffer or molecular grade water and select Wash.
- After the wash, remove the chip from the machine, cover all wells with parafilm, place in its container and store at 4°C.
- Close the LabChip GX Touch software.
- To analyse the data: Open up the LabChip Reviewer software by double clicking on the icon on the Desktop.
  - On the menu bar, select File - New Workspace and import data from the .gxd file.

- Select the correct wells in which the samples are in. The fragment sizes for each will be displayed in the upper right-hand side panel.
- To obtain the fragments size, take the highest peak that is most representative of majority of the fragments. The workspace can be saved for future record.

6.7 Sequencing on the MiSeq

Recommendation:

- For a 400bp insert, use 200ng input DNA. Input amounts lower than those specified results in low yield and increased duplicates.

6.7.1 Tagment Amplicon DNA

Table 7. Preparation of reagents

Item	Storage	Instructions
BLT(bead-linked transposomes)	2°C to 8 °C	Bring to room temperature. Vortex to mix. Do not centrifuge before pipetting.
TB1(Tagmentation buffer)	-25 °C to -15 °C	Bring to room temperature. Vortex to mix.

- Add 2–30 µl DNA to each well of a 96-well PCR plate / 0.2ml strip tubes so that the total input amount is 100–500 ng.
- If DNA volume < 30 µl, add nuclease-free water to the DNA samples to bring the total volume to 30 µl.
- Vortex BLT (yellow cap) vigorously for 10 seconds to resuspend.
- Vortex in between adding as necessary.
- Prepare the tagmentation master mix.
- Multiply each volume by the number of samples being processed

Table 8. Tagmentation Master Mix

Component	Volume (µl) per sample
BLT	11
TB1	11
Total	**22**

- Vortex the tagmentation master mix thoroughly.
- Transfer 20 µl tagmentation master mix to each well of the plate containing a sample.
- Use fresh tips for each sample column.
- Resuspend by pipetting each sample 10 times.
- Seal the plate with a plate sealer, place on the preprogrammed thermal cycler, and run the tagmentation program.

Table 9. PCR – Tagmentation conditions

Temperature (°C)	Time
55°C	15 minutes
10	Hold

6.7.2 Post Tagmentation Cleanup

Table 10. Preparation of Reagents

Item	Storage	Instructions
TSB (Tagment stop buffer)	15 °C to 30 °C	If precipitates are observed, heat at 37°C for 10 minutes, and then vortex until precipitates are dissolved. Use at room temperature.
TWB (Tagment wash buffer)	15 °C to 30 °C	Use at room temperature.

Table 11. PTC (Post Tagmentation Cleanup) programme

Temperature (°C)	Time
37°C	15 minutes
10	Hold

- Add 10 µl TSB to the tagmentation reaction.
- Resuspend the beads by slowly pipetting each well/ tube 10 times.
- Seal the plate with / tubes, place on the preprogrammed thermal cycler, and run the PTC program.
- Place the plate on the magnetic stand for approximately 3 minutes until liquid is clear.
- Using a multichannel pipette, remove and discard supernatant.
- Wash two times as follows:
    1. Remove the sample plate from the magnetic stand and use a deliberately slow pipetting technique to add 100 µl TWB directly onto the beads.
    2. This slow pipetting technique minimizes the potential of TWB foaming to avoid incorrect volume aspiration and incomplete mixing.
    3. Slowly pipette until beads are fully resuspended.
    4. Place the plate on the magnetic stand for approximately 3 minutes until liquid is clear.
    5. Using a multichannel pipette, remove and discard supernatant.
    6. Remove the plate from the magnetic stand and use a deliberately slow pipetting technique to add 100 µl TWB directly onto the beads.
    7. Slowly pipette each well/tube to resuspend the beads.
    8. Place the plate on the magnetic stand for approximately 3 minutes until liquid is clear.

9. Keep on the magnetic stand until step 4 of the Procedure section in Amplify Tagmented DNA.
10. The TWB remains in the wells to prevent overdrying of the beads.

6.7.3 Amplify Tagmented DNA

Table 12. Preparation of Reagents

Item	Storage	Instructions
EPM (Enhanced PCR Mix)	-25°C to-15°C	Thaw on ice. Invert to mix, then briefly centrifuge.
Index Adapters Tubes/plates	-25°C to-15°C	Thaw at room temperature. [Tubes] Vortex to mix, then centrifuge briefly. [Plates] Spin briefly before use.

Table 13. PCR Master Mix

Component	Volume (µl) per sample
EPM	22
NFW (Nuclease Free Water)	22
Total	**44**

1. Combine the following volumes to prepare the PCR master mix. Multiply each volume by the number of samples being processed.
2. Reagent overage is included in the volume to ensure accurate pipetting.
3. Vortex and centrifuge the PCR master mix at 280 × g for 10 seconds.
4. With the plate on the magnetic stand, use a 200 µl multichannel pipette to remove and discard supernatant. (from step 9 of post tagmentation clean-up)
5. Foam that remains on the well walls does not adversely affect the library.
6. Remove from the magnet.
7. Immediately add 40 µl PCR master mix directly onto the beads in each sample well/ tube.
8. Pipette mix until the beads are fully resuspended.
9. Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
10. Seal the sample plate and centrifuge at 280 × g for 3 seconds.
11. Add 10 µl the appropriate index adapters to each sample.
12. Using a pipette set to 40 µl, pipette 10 times to mix.
13. Alternatively, seal the plate/ tubes and use a plate shaker at 1600 rpm for 1 minute.
14. Centrifuge at 280 × g for 30 seconds.
15. Place on the thermal cycler and run the BLT PCR program.

Table 13. PCR Conditions

Temperature (°C)	Time	
68°C	3 minutes	
98°C	3 minutes	
98°C	45 seconds	8 cycles
62°C	30 seconds	
68°C	2 minutes	
68°C	1 minute	
10°C	Hold	

SAFE STOPPING POINT: If you are stopping, store at 2°C to 8°C for up to 3 days

Clean Up Libraries

Table 14. Preparation of Reagents

Item	Storage	Instructions
SPB (Sample Purification Beads)	2°C to 8°C	Let stand at room temperature for 30 minutes. Vortex and invert to mix.
RSB (Resuspension Buffer)	-25°C to-15°C	Thaw and bring to room temperature. Vortex to mix.

- Prepare fresh 80% EtOH from absolute ethanol.
- Centrifuge at 280 × g for 1 minute to
- Place the plate/ tubes on a magnetic stand for approximately 5 minutes until liquid is clear.
- Transfer 45 µl supernatant from each well of the PCR plate/ tubes to the corresponding well of a new plate/ tubes.
- Vortex and invert SPB multiple times to resuspend.
- Add 40 µl nuclease-free water to each well/ tube.
- Add 45 µl SPB to each well/ tube.
- Mix well by pipetting 10 times.
- Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.
- Incubate at room temperature for 5 minutes.
- Place on the magnetic stand for approximately 5 minutes until the liquid is clear.
- During incubation, thoroughly vortex the SPB (undiluted stock tube), and then add 15 µl to each well of a new plate/tubes.
- Transfer 125 µl supernatant from each well of the first plate/ tubes into the corresponding well of the second plate/ tubes (containing 15 µl undiluted SPB).
- Mix well by pipetting 10 times.
- Alternatively, seal the plate and use a plate shaker at 1600 rpm for 1 minute.

- Discard the first plate/ tubes.
- Pipette each well 10 times to mix.
- Incubate at room temperature for 5 minutes.
- Place on the magnetic stand for approximately 5 minutes until the liquid is clear.
- Without disturbing the beads, remove and discard supernatant.
- Wash two times as follows:

    - Add 200 µl freshly prepared 80% ethanol with the plate on the magnetic stand.
    - Incubate for 30 seconds.
    - Without disturbing the beads, remove and discard the supernatant
    - Use a 20 µl pipette to remove any residual ethanol.
    - Air-dry on the magnetic stand for 5 minutes.
    - Remove from the magnetic stand.
    - Add 32 µl RSB to each well/ tube.
    - Resuspend by pipette mixing.
    - Incubate at room temperature for 2 minutes.
    - Place the plate/ tubes on the magnetic stand for approximately 2 minutes
    - Transfer 30 µl supernatant to a new 96-well PCR plate/ tubes.

SAFE STOPPING POINT If you are stopping, seal the plate, and store at -25°C to -15°C for up to 30 days.

6.7.6 Normalization of DNA

- Quantify the DNA as described in 6.5 using the Qubit and determine the fragment length using the LabChip as described in 6.6.
- Using the Qubit concentrations and fragment length normalize the libraries to equimolar 4nM by diluting with RSB buffer.
- Calculate appropriate amount of diluent in an excel sheet to add to respective sample libraries in order to achieve a 4nm library concentration, using the following formula; Nanomolar concentration = (ng/µl /660 x 500) x 10^6.
- Pipette mix 5 times.
- Use a multi-channel pipette to transfer 5µl of the diluted sample library to an 8 strip-tube and spin briefly.
- Pool the library samples from the 8-strip tubes to a labelled Pooled Amplicon Library (PAL) 2ml eppendorf tube.
- Proceed to library denaturation.

6.7.7 Library Denaturation

- Remove the tube of HT1 (Hybridization Buffer) from the freezer (-15°C to -25°C) and set aside at room temperature to thaw.
- When thawed, store at 2°C to 8°C until you are ready to dilute denatured libraries.

- Prepare 500µl of 0.2 N NaOH by combining the following volumes in a 1.5ml microcentrifuge tube: 490µl laboratory-grade water and 10µl Stock 1.0 N NaOH. Refer to the formula below:
  1M = 1N
  10N (x) = (0.2) (500)
  x= 10µl NaOH + 490µl laboratory-grade water

  Note: A fresh dilution of 0.2N NaOH is required for the denaturation process in preparing sample DNA and a PhiX control.

Litmus blue can be used to check the pH of the NaOH. Add 20ul of NaOH to the litmus blue strip and do not use if it does not remain blue.

- Invert the tube several times to mix.
- Combine the following volumes of pooled sample DNA and freshly diluted 0.2 N NaOH in a micro-centrifuge tube, by adding 5µl of 4nM sample DNA to 5µl of 0.2N NaOH.
- Discard the remaining dilution of 0.2 N NaOH or set aside to prepare a PhiX control within the next 12 hours.
- Vortex briefly to mix the sample solution, and then centrifuge the sample solution to 280 × g for 1 minute.
- Incubate for 5 minutes at room temperature to denature the DNA into single strands.
- Add 10µl denatured DNA to 990µl of pre-chilled HT1. The result is a 20pM denatured library in 1 mM NaOH.
- Place the denatured DNA on ice or at 4^{o}C until you are ready to proceed to the final dilution.

6.7.8 Dilution of Denatured Library

- Use the following instructions to dilute the 20pM DNA further to give 600µl of the desired input concentration.
- Dilute the denatured DNA to the desired concentration using the following example (if using 5% PhiX):

Table 15. Dilution of denatured library to desired concentration

Final Concentration	20pM denatured DNA	5% PhiX	Pre-chilledHT1
12 pM	356.4µl	3.6µl	240µl

- Invert several times to mix and then pulse centrifuge.
- As a guide the fragment size helps to determine the amount of library to load:

Table 16. Recommended concentrations for fragment sizes

- To dilute PhiX to 4nM concentration, combine the following volumes in a micro-centrifuge tube:
- 2µl of 10nM PhiX library

- 3µl of 10mM Tris-Cl, pH 8.5 with 0.1% Tween 20
  Note: If not prepared within the last 12 hours, prepare a fresh dilution of 0.2 N NaOH.
- Combine the following volumes in a micro-centrifuge tube.
- 5µl of 4 nM PhiX library
- 5µl of 0.2 N NaOH
- Vortex briefly to mix.
- Centrifuge at 280 × g for 1 minute.
- Incubate at room temperature for 5 minutes.
- Dilute denatured PhiX to 20pM by adding pre-chilled HT1 to the denatured PhiX library as follows:
- 10µl denatured PhiX library
- 990µl pre-chilled HT1
- Invert to mix.
- Combine Library and PhiX Control

- Mix this solution well and briefly centrifuge. Keep on ice or at 4°C until it is ready to be loaded onto the MiSeq reagent cartridge.

6.7.8 MiSeq Run Preparation

The Sample Sheet provides the necessary information required by the MiSeq Control Software (MCS) to perform your run and organize your data output. It is recommended to prepare your sample sheet prior to starting the Index PCR.

- Prepare the sample sheet as follows:

  Open the IEM software. IEM is available for download at: http://support.illumina.com/sequencing/sequencing_software/experiment_manager/downloads.html. For further instructions, refer to the following Illumina support page, and scroll down for the "Illumina Experiment Manager" training video (http://support.illumina.com/sequencing/sequencing_instruments/miseq/training.html)

  - Create a Sequencing Sample Sheet
  - On the IEM main screen, select Create Sample Sheet.
  - On the Instrument Selection screen, select MiSeq, and then select Next.
  - On the MiSeq Application Selection screen, select Other, then FastQ, and then select Next.
  - On the Workflow Parameters screen, do the following:
    - In the Reagent Cartridge Barcode field, enter the barcode number of the MiSeq reagent cartridge.
    - From the Sample Prep Kit drop-down menu, select Nextera DNA Flex.
    - In the Index Reads field, Nextera DNA CD Indexes (96 Index plate).
    - Type an experiment name, investigator name, and description.
    - Select the date.
    - Select the number of cycles for each read in your sequencing run, plus 1. For example, for a 500 cycle kit select 251 cycles.
    - Select or clear the Workflow-Specific Settings checkboxes, as desired.
    - Click Next.
    - Add samples to the sample sheet
    - Click Finish and save the sample sheet file.

- When prompted, select No to exit the sample sheet wizard without reviewing the sample sheet.
- When you are ready to start your run, use a USB drive to transfer the file to the MiSeq desktop.
- Alternatively, the sample sheet can be named using the barcode from the MiSeq cartridge being used. This will automatically link your sample sheet to your run during the run set-up. Otherwise, the sample sheet must be manually selected for the run.

6.7.9 MiSeq Reagent Cartridge

- Remove a MiSeq v2 Nano 500 cycle cartridge from the freezer and thaw using a room temperature water bath. Ensure that the water does not pass the Max Water Line on the cartridge. About 60 minutes is required to completely thaw the cartridge.
- Note: The reagent cartridge can be thawed earlier, preferably during the start of library normalization. If it takes longer than 60 minutes to load the cartridge, place the reagent cartridge at 2°C to 8°C until ready to load, or on ice for up to 6 hours. For best results, proceed directly to loading the sample and setting up the run.
- Once thawed, invert the cartridge 10 times to gently mix the reagents. It is very important that there are no bubbles at the bottom of the reagent wells. Lightly tap the cartridge on the bench to remove any bubbles.
- Visually inspect the bottom of the cartridge to ensure that there are no bubbles remaining.

WARNING!
This set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Wear protective equipment, including eye protection, gloves, and a laboratory coat. Handle used reagents as chemical waste and discard in biohazard boxes.

6.7.10 MiSeq Flow Cell

- Using the thumb and forefinger, remove the flow cell from the storage container only touching the edge.
- Using laboratory-grade water, rinse the flow cell to remove excess salts. This includes the glass surface as well as the surrounding plastic. Take extra care to ensure the glass surface and black gaskets have been thoroughly rinsed.
- Using a lint-free tissue, gently pat excess water from the area around the gasket and adjacent to the glass. Lean the flow cell against a clean surface, making sure nothing is touching the glass or the black gaskets and allow to air-dry.
- Once the flow cell is dry, hold it up to the light and visually inspect it to ensure that there is no dust or debris on the glass surface. If required, gently wipe the imaging area with a lint-free tissue moistened with distilled water. Then repeat the rinse and dry steps.
- Allow the flow cell to air dry till no smears of liquid can be seen.

6.7.11 Loading the Cartridge

- Using a clean P1000 tip, pierce the foil on the “Load Samples” well (#17) of the MiSeq Cartridge.
- Using a new tip, load the full contents (600µl) of the DAL+ PhiX eppendorf tube into the “Load Sample” well. Take care to ensure that your pipette tip does not touch the foil. Note: Do not invert the cartridge once the library has been added!
- The cartridge is now ready to be placed onto the MiSeq machine.

6.7.11 Starting the MiSeq Run

- Perform pre-run wash if a wash has not been performed in the past 7 days.
- Launch the MiSeq Control Software (MCS). From the Welcome screen, select “Sequence”.
- Follow the MCS prompts to load the MiSeq:
- Confirm that the flow cell and platform are clean. Load the flow cell securely onto the platform. Allow MCS to read the barcode.
- Invert the PR2 buffer bottle to mix, then place in the reagent compartment, and lower the sipper. Allow MCS to read the barcode.
- Empty the contents of the waste bottle into the appropriate waste container.
- Slowly lower the sipper handle.
- Select Next.
- Load the Reagent Cartridge
  - Open the reagent chiller door.
  - Hold the reagent cartridge on the end with the Illumina label, and slide the reagent cartridge into the reagent chiller until the cartridge stops.
  - Close the reagent chiller door.
  - Close the reagent compartment door.
- Browse and select the correct sample sheet, previously created in the IEM software.
- Select Next.
- Review Run Parameters
- Review Experiment Name, Analysis Workflow, and Read Length. These parameters are specified in the sample sheet.
- Review the folder locations in the lower-left corner.
- Select Next.
- Review Pre-Run Check. Select Start Run.
- During the run, monitor run progress, intensities, and quality scores that appear on the Sequencing screen. The Sequencing screen is view-only.
  - When the run is complete, the Next button appears. Review the results on the Sequencing screen before proceeding.

  *NOTE

  The Sequencing screen remains viewable until Next is selected. After you select Next, it is not possible to return to the Sequencing screen.
- Select Next to exit the Sequencing screen and proceed to a post-run wash.
- Run Metrics

  After sequencing begins, the following metrics appear at the indicated cycles:

  -Cycle 1-7: Intensity

  -Cycle 8-25: Intensity and Cluster Density

-Cycle 26 through run completion: Intensity, cluster Density, %PF, Yield and Q scores

6.7.12 Perform a Post-Run Wash

- Prepare fresh wash solution with Tween 20 and laboratory-grade water, as follows.
  - Add 5ml 100% Tween 20 to 45ml laboratory-grade water. These volumes result in 10% Tween 20.
  - Add 25ml 10% Tween 20 to 475ml laboratory-grade water. These volumes result in a 0.5% Tween 20 wash solution.
  - Invert 5 times to mix.
- Prepare the wash components with fresh wash solution as follows.
  - Add 6ml wash solution to each reservoir of the wash tray.
  - Add 350ml wash solution to the 500ml wash bottle.
- Select Start Wash on the instrument.
- Open the reagent compartment door and reagent chiller door, and remove the used reagent cartridge from the chiller.
- Slide the wash tray into the reagent chiller until it stops, and then close the reagent chiller door.
- Raise the sipper handle in front of the PR2 bottle and waste bottle until it locks into place.
- Remove the PR2 bottle and replace it with the wash bottle.
- Remove the waste bottle and discard the contents appropriately. Return the waste bottle to the reagent compartment.
- Slowly lower the sipper handle, making sure that the sippers lower into the wash bottle and waste bottle.
- Close the reagent compartment door.
- After the post-run wash, leave the used flow cell, wash tray, and wash bottle on the instrument.
- The sippers remain in the down position to prevent the sippers from drying out and prevent air from entering the system.

Note: Always use laboratory-grade water in preparing wash reagents for the instrument

- Once a run is successfully completed, the output data must be stored in an alternate location and removed from the MiSeq in order to make room for subsequent runs.
- To retrieve the *.fastq.gz files, navigate to; Data (D:)\Illumina\MiSeqOutput\“run folder name by date”\Data/Intensities\ BaseCalls.

6.7.13 Maintenance Wash

- Before you begin the maintenance wash, make sure a used flow cell is loaded on the instrument. The maintenance wash takes approximately one hour and consists of a series of three steps.
- Perform the first wash.
  - Select Perform Wash, then
  - Select Maintenance Wash.
  - Select Next.

- Prepare fresh wash solution by adding 25ml of 10% Tween 20 to 475ml laboratory grade water. Invert several times to mix.
- Fill each reservoir of the wash tray with 6ml wash solution. Fill the 500ml wash bottle with 350ml wash solution.
- Load the wash tray and wash bottle onto the instrument.
- Open the reagent compartment door, then open the reagent chiller door.
- Remove the used reagent cartridge or wash tray from the chiller. Slide the wash tray into the chiller until it stops. Close the reagent chiller door.
- Raise the sipper handle until it locks into place. Replace the PR2 bottle with the wash bottle.
- Remove the waste bottle and empty it in an appropriate waste container. Return the waste bottle to the reagent compartment. Slowly lower the sipper handle. Make sure the sippers lower into the wash bottle and waste bottle. Close the reagent compartment door.
- Select Next. The first wash begins.
- Perform the second wash.
- Open the reagent compartment door and the reagent chiller door.
- Remove the used wash tray from the chiller. Raise the sipper handle and remove
- the wash bottle. Discard the used wash solution from the wash tray and wash
- bottle.
- Prepare fresh wash solution by adding 25ml 10% Tween 20 to 475ml laboratory grade water. Invert several times to mix.
- Refill each reservoir of the wash tray with 6ml fresh wash solution. Refill the wash bottle with 350ml of fresh wash solution. Slide the wash tray into the reagent chiller until it stops. Close the reagent chiller door.
- Load the wash bottle and slowly lower the sipper handle. Close the reagent compartment door.
- Click Next. The second wash begins.
- Perform the third wash.
- Open the reagent compartment door and the reagent chiller door.
- Remove the used wash tray from the chiller. Raise the sipper handle and remove the wash bottle. Discard the used wash solution from the wash tray and wash bottle in an appropriate waste container.
- Prepare fresh wash solution by adding 25ml 10% Tween 20 to 475ml laboratory grade water. Invert several times to mix.
- Refill each reservoir of the wash tray with 6ml fresh wash solution. Refill the wash bottle with 350ml of fresh wash solution. Slide the wash tray into the reagent chiller until it stops. Close the reagent chiller door.
- Load the wash bottle and slowly lower the sipper handle. Close the reagent compartment door. Click Next. The final wash begins.
- After the wash, leave the used flow cell, wash tray, and wash bottle on the
- instrument.
- The sippers remain in the down position to prevent the sippers from drying out and prevent air from entering the system.
- When the wash is complete, a message appears on the screen. Select Done.
- Leave the used flow cell, wash tray, and waste bottle on the instrument.

6.7.13 Standby Wash

- Perform a standby wash if the instrument will be not be used within the next seven (7) days. Repeat the standby wash every 30 days the instrument remains idle.
- Allow approximately two hours to complete the standby wash.
- Prepare fresh wash solution by adding 25ml 10% Tween 20 to 475ml laboratory grade water. Invert several times to mix.
- Fill each reservoir of the wash tray with 6 ml fresh wash solution. Fill the 500ml wash bottle with 350ml fresh wash solution.
- Select Perform Wash. Then, select Standby Wash. Before you begin the standby wash, make sure a used flow cell is loaded on the instrument.
- Select Next.
- Load the wash tray and wash bottle onto the instrument.
- Open the reagent compartment door and reagent chiller door. Remove the used wash tray from the chiller. Slide the wash tray into the chiller until it stops. Close the chiller door. Raise the sipper handle until it locks into place.
- Replace the PR2 bottle with the wash bottle. Remove the waste bottle and empty it in an appropriate waste container. Return the waste bottle to the reagent compartment. Slowly lower the sipper handle. Make sure the sippers lower into the wash bottle and waste bottle. Close the reagent compartment door.
- Select Next. The first wash begins.
- Perform the second wash. When the first wash is complete, remove
- the wash tray and the wash bottle.
- Discard the remaining wash solution in an appropriate waste container.
- Discard the used wash solution from the wash tray and wash bottle in an appropriate waste container.
- Prepare fresh wash solution by adding 25ml 10% Tween 20 to 475ml laboratory grade water. Invert several times to mix.
- Refill each reservoir of the wash tray with 6ml fresh wash solution. Refill the wash bottle with 350ml of fresh wash solution. Slide the wash tray into the reagent chiller until it stops. Close the reagent chiller door. Load the wash bottle and slowly lower the sipper handle. Close the reagent compartment door.
- Click Next. The second wash begins.
- After the wash, leave the used flow cell, wash tray, and wash bottle on the
- instrument.
- The sippers remain in the down position to prevent the sippers from drying out and prevent air from entering the system.
- When the wash is complete, a message appears on the screen. Select Done.
- Leave the used flow cell, wash tray, and waste bottle on the instrument.