KwaZulu-Natal Research Innovation and Sequencing Platform, UKZN

NEBNEXT: 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

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 using the NEBNext Ultra II Library Preparation Kit.

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.

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 extraction

- 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
- NEBNext® Ultra™ II DNA Library Prep Kit,
- NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs), New England Biolabs
- MiSeq Reagent Nano Kit, v2 (500 cycles) MS-103-1003 (Whitehead Scientific)
- 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 it's 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).

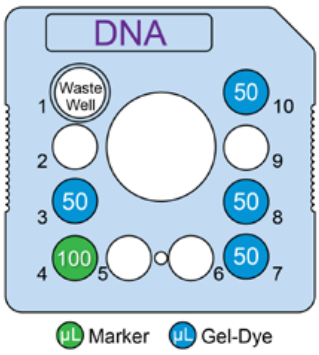

Figure 1. Low-throughput

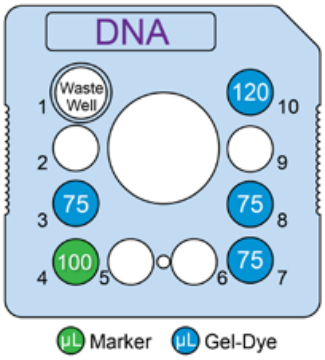

Figure 2. High-throughput

  - 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

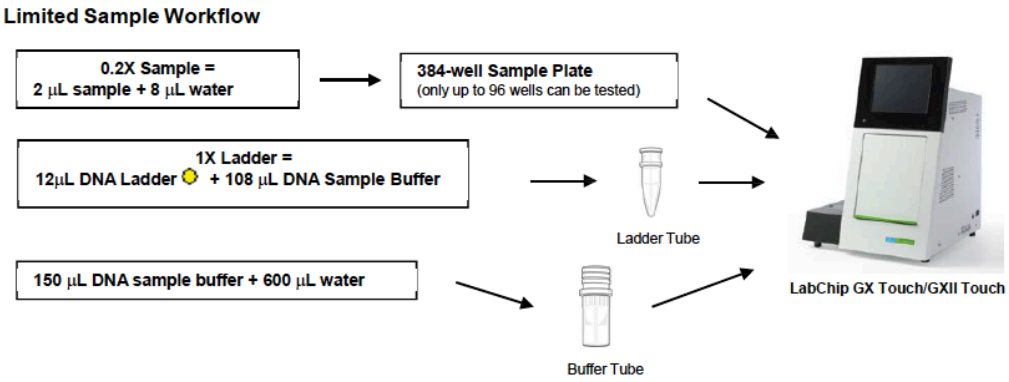

Figure 3. Limited sample workflow

- 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^{o}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.

Starting Material: *Cleaned-up DNA diluted to 1 – 5 ng/µl, in at least 50 µl volume.*

6.7.1 NEBNext END PREP

Mix the following components in a sterile nuclease-free tube:

Table 7. NEBNext End Prep Master Mix

	1x reaction (µl)
NEBNext Ultra II End Prep Enzyme Mix (green top)	3.0
NEBNext Ultra II End Prep Reaction Buffer (green top)	7.0
Total	**10.0**

- Add 50µl of DNA to the respective wells, for a total reaction volume of 60 µl.
- Mix well on a vortex mixer or by pipetting up and down 10 times.
- Perform a quick spin to collect all liquid from the sides of the tube.

Note: **It is important to mix well. The presence of a small amount of bubbles will not interfere with performance.**

- Place in a thermocycler, with the heated lid set to ≥75°C, and run the following program:

Table 8. NEBNext End Prep PCR conditions

Time	**Temperature (°C)**
30 minutes	20
30 minutes	65
Hold	4

Note: **If necessary, samples can be stored at –20°C; however, a slight loss in yield (~20%) may be observed. We recommend continuing with adaptor ligation before stopping.**

6..1 ADAPTOR LIGATION

Note: **Adaptor ligation has been modified to use TruSeq indexes.**

Make up the following Mastermix and add 31µl to the respective wells.

Table 9. Adaptor Ligation Master Mix

	1x reaction (µl)
NEBNext Ultra II Ligation Master Mix (red top)	30.0
NEBNext Ligation Enhancer (red top)	1.0
	31.0

- Add 35µl of the End Prep DNA to the respective wells.
- Add 2.5µl of NEBNext Adpaters for Illumina to the respective wells.
- Mix well on a vortex mixer or by pipetting up and down 10 times.
- Perform a quick spin to collect all liquid from the sides of the tube.
- Incubate at 20°C for 15 minutes in a thermocycler with the heated lid **open**.

Note: **The Ligation Master Mix and Ligation Enhancer can be mixed ahead of time and is stable for at least 8 hours @ 4°C. We do not recommend adding adaptor to a premix in the Adaptor Ligation Step.**
Caution: **The NEBNext Ultra II Ligation Master Mix is very viscous. Care should be taken to ensure adequate mixing of the ligation reaction, as incomplete mixing will result in reduced ligation efficiency. The presence of a small amount of bubbles will not interfere with performance).**
Note: **Samples can be stored overnight at –20°C.**

6..2 CLEAN-UP OF ADAPTOR-LIGATED DNA

Note: **Allow AMPure XP Beads to warm to room temperature for at least 30 minutes before use.**

- Vortex AMPure Beads thoroughly to resuspend.
- Add 57µl (~0.8X) of resuspended beads to the adaptor ligation reaction.
- Mix well by vortexing for 3 – 5 seconds or by pipetting up and down 10 times.
- Centrifuge briefly. Be sure to stop the centrifugation before the beads start to settle out.
- Incubate samples on bench top for at least 5 minutes at room temperature.
- Place the tube/plate on an appropriate magnetic stand for 5 minutes to separate the beads from the supernatant.
- Carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
- Incubate at room temperature for 30 seconds.
- Carefully remove and discard the supernatant without disturbing the pellet.
- Add 200µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

- Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Caution: **Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.**

- Remove the tube/plate from the magnetic stand.
- Add 17µl of nuclease free water to elute the DNA target from the beads.
- Mix well on a vortex mixer or by pipetting up and down 10 times.
- Incubate at room temperature for at 2 minutes.
- Quickly spin to collect the liquid from the sides of the tube or plate wells.
- Place the tubes/ plate on the magnetic stand and incubate for at least 5 minutes at room temperature.
- Transfer 15µl (i.e. 7.5µl twice) of the supernatant to a new tubes/ plate for amplification.

Note: **Samples can be stored at –20°C.**

6.7.4 PCR ENRICHMENT OF ADAPTOR-LIGATED DNA

Prepare the following master mix:

Table 10. Enrichment Master Mix

	1x reaction (µl)
NEBNext Ultra II Q5 Master Mix (blue top)	25.0
Universal PCR primer (blue top)	5.0
	30.0

- Add 30µl of the master mix into newly labelled tubes/ plate.
- Add 15.0µl of adaptor-ligated DNA to the respective wells.
- Mix well on a vortex mixer or by pipetting up and down 10 times.
- Perform a quick spin to collect all liquid from the sides of the tube.
- Place the tubes/plate on a thermocycler and perform PCR using the following conditions:
-

Table 11. Enrichment PCR Conditions

	Temperature (°C)	Time	Cycles
Initial Denaturation	98	3 minutes	1
Denaturation	98	10 seconds	8
Annealing	60	30 seconds	
Extension	65	45 seconds	
Final Extension	65	5 minutes	1
Hold	4	Hold	

Note: **The PCR will take approximately 30 minutes**

6.7.5 CLEANUP OF PCR ENRICHMENT PRODUCT

Note: **If using AMPure XP Beads, allow the beads to warm to room temperature for at least 30 minutes before use**

- Vortex AMPure Beads thoroughly to resuspend.
- Add 45µl (0.9X) resuspended AMPure beads to the PCR reaction.
- Mix well by vortexing for 3 – 5 seconds or by pipetting up and down 10 times.
- Centrifuge very briefly. Be sure to stop the centrifugation before the beads start to settle out.
- Incubate samples on bench top for at least 5 minutes at room temperature.
- Place the tube/plate on an appropriate magnetic stand for 5 minutes to separate the beads from the supernatant.
- Carefully remove and discard the supernatant. Be careful not to disturb the beads that contain the DNA targets.
- Add 200µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand.
- Incubate at room temperature for 30 seconds.
- Carefully remove and discard the supernatant without disturbing the pellet.
- Add 200µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.
- Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Caution: **Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack, they are too dry.**

- Remove the tube/plate from the magnetic stand.
- Add 33µl of nuclease free water to elute the DNA target from the beads.
- Mix well on a vortex mixer or by pipetting up and down 10 times.
- Quickly spin to collect the liquid from the sides of the tube or plate wells.
- Incubate the tubes/ plate for 2 minutes at room temperature.
- Place the tubes/ plate on the magnetic stand and incubate for at least 5 minutes.
- Transfer 30µl (i.e. 15.5µl twice) of the supernatant to a new tubes/ plate.
- Assess the concentration of the libraries using a Qubit fluorometer.
- Assess the library fragments using the LabChip GX Touch.

Note: **Samples can be stored at –20°C after clean-up.**

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°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 1% PhiX):

Table 12. Dilution of denatured library to desired concentration

Final Concentration	20pM denatured DNA	1% 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:

- 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:

- Download a MiSeq sample sheet, NEBNext Direct GS Barcodes file, and refer to the “Sample Sheet Guidelines” located in the “Protocols, Manuals & Usage” tab on the NEBNext Direct GS Target Enrichment product page at **www.neb.com/E9530**. Do not use the Illumina Experiment Manager to generate a sample sheet.
- Fill in the sample sheet with your sample and barcode information.
- Transfer the sample sheet file (*.csv) to the MiSeq and save the file in D:\Illumina\Miseq Control Software\SampleSheets\

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.