

Standard Operating Procedure (SOP)

SOP Title	MiSeq sequencing
SOP number	SEQ01
SOP Version	2

1. SCOPE

This SOP defines, step-by-step, how to load an Illumina MiSeq Instrument.

The MiSeq is used to sequence DNA libraries within a 24 hour timeframe. It is able to sequence read lengths from 25bp to 300bp and produces approximately 2-15 Gb of data.

NOTE: It is the assumption to MalariaGEN that Illumina has carried out onsite training on the correct use and maintenance of the instrument, and that a qualified technician familiar with the operation of the Illumina MiSeq is available at the site and/or has trained someone in the proper use of the machine. This SOP should only be used as a supplementary document to ensure key steps specific to sequencing libraries from the amplicon sequencing protocol are followed.

NOTE: Required Disk Space: The integrated instrument computer has approximately 550 GB of storage capacity. Before starting a run, the software checks available disk space. If there is not enough disk space for the run, a message indicating how much disk space is required appears. If prompted to make disk space available, move or delete older run folders as appropriate. After clearing adequate disk space, select Restart Check.

2. MATERIALS REQUIRED

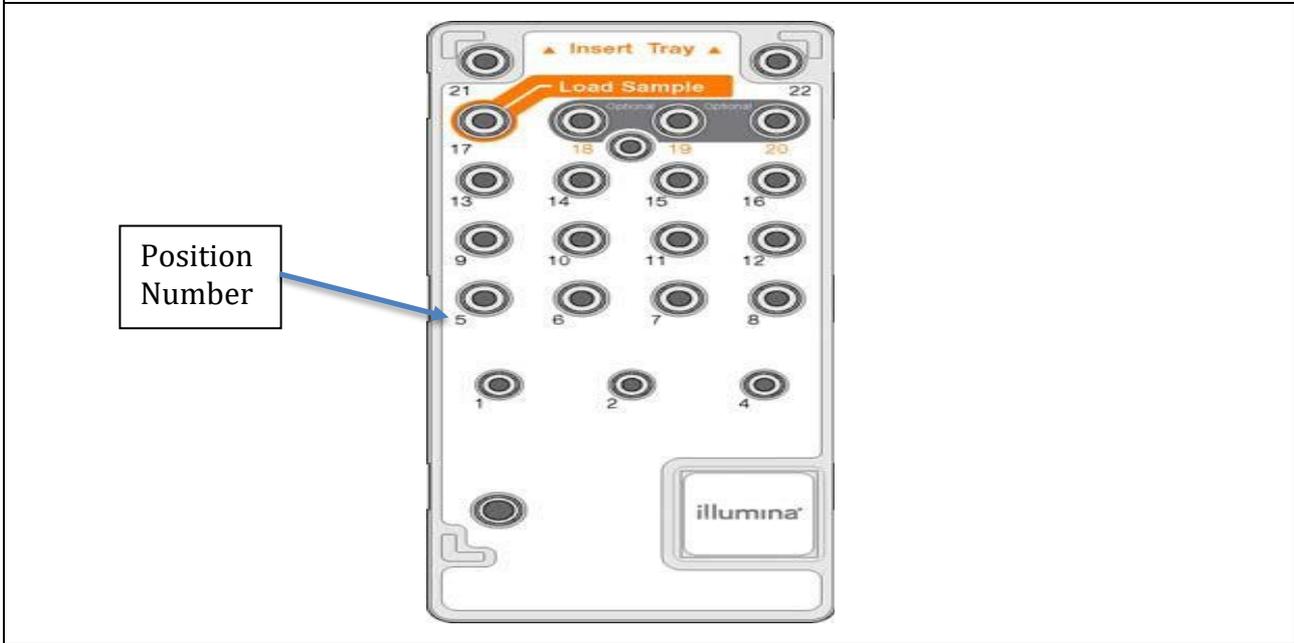
2.1 REAGENTS AND MATERIALS.		
<i>Library prep</i>		
Prepared library preparation (GRC1, GRC2, Speciation)		
<i>Materials</i>	<i>Supplier</i>	<i>Cat. No.</i>
MiSeq instrument	Illumina	
MiSeq reagent cartridge kit 150 PE MiSeq run – Reagent Kit v2 (300 cycle)	Illumina	MS-102-2002
MiSeq Wash bottle for cleaning (x2)	Illumina	
MiSeq Cartridge for cleaning (x2)	Illumina	
Sprout / Eppendorf centrifuge		
Azowipes <small>WIPES IMPREGNATED WITH 70% V/V SOLUTION OF ISOPROPYL ALCOHOL</small>	fisher scientific	15611944
Lint-free lens cleaning tissue (whatman)	MERCK	WHA2105841
<i>Reagents</i>	<i>Supplier</i>	<i>Cat. No.</i>
MiSeq reagent PR2	Illumina – comes with cartridge kit	
PR2 incorporation buffer	Illumina – comes with cartridge kit	
HT1 hybridization buffer	Illumina – comes with cartridge kit	
Index PCR (iPCR)*	See section 2.2 iPCR	
Sodium hydroxide solution (2N)	fisher scientific	10070190
Buffer Elution Buffer (Buffer EB, 10 mM Tris-Cl, pH 8.5)	Qiagen	19086
Sodium Hypochlorite (4-4.99%)	Honeywell	239305
10% Tween 20	MERCK	P7949-500ML

* Essential component specific to Amplicon Sequencing Protocol

2.2 iPCR.	
iPCR details as follows:	
NAME	iPCRTagseq
BASE COUNT	35
SEQUENCE	5' AAGAGCGGTTTCAGCAGGAATGCCGAGACCGATCTC 3'
SYNTHESIS SCALE	1 μ mole
PURIFICATION	HPLC
FORMAT	DRIED
<p>Upon receipt re-suspend to be at 100μM. iPCR can be ordered from either IDT or Sigma/Merck</p>	

2.3 THE MISEQ CARTRIDGE.

The MiSeq reagent cartridge is a single-use consumable consisting of several reservoirs, each prefilled with a specific reagent. Each reservoir on the cartridge is numbered. The following table lists the reagent in each numbered position (150 PE MiSeq run – Reagent Kit v2 (300 cycle)).



<i>Position</i>	<i>Reagent name</i>	<i>Description</i>
1	IMF	Incorporation mix
2	SRE	Scan mix
4	CMF	Cleavage mix
5	AMX1	Amplification mix
6	AMX2	Read 2 amplification mix
7	LPM	Linearization premix
8	LDR	Formamide
9	LMX1	Linearization mix
10	LMX2	Read 2 Linearization mix
11	RMF	Resynthesis mix
12	HP10	HP10 read 1 primer mix
13	HP12	HP12 index primer mix
14	HP11	Read 2 primer mix
15	PW1	Water
16	PW1	Water
17	Sample	Your denatured and diluted library (or multiplexed pool)
18	Custom	Spare port for custom primer.
19	Custom	Spare port for custom primer.
20	Custom	Spare port for custom primer.
21	PW1	Water
22	Empty	

3. METHODOLOGY

Reagent storage.

The MiSeq kit is stored in two parts:

- **Freezer (-20°C)** – The Reagent Cartridge (Synthesis by Sequencing and clustering reagents in one unit) and a tube of HT1 (hybridization buffer).
- **Fridge (4°C)** – The Flowcell component and PR2 buffer bottle.

MiSeq – Reagent Kit Overview.

The MiSeq Reagent Kit is a single-use reagent kit required to perform a sequencing run. It is available in different types and sizes. Each type of MiSeq Reagent Kit includes a kit-specific flow cell type and all reagents required for performing a run. The flow cell, PR2 bottle, and reagent cartridge provided in the kit use radio-frequency identification (RFID) for accurate consumable tracking and compatibility. Always use the reagent cartridge associated with your flow cell type. If the reagent cartridge is not compatible, a message appears during run setup that prompts you to load a compatible reagent cartridge.

Loading concentration.

Library loading concentration can be optimised for the MiSeq according to the results you are seeing from the study/library type.

If **low cluster density** is observed then increasing the loading concentration is recommended. For example, if a library (loading conc. of 8pM) produced clusters of 400k, with a high (>90%) %PF and Q30 scores then increasing the loading concentration to 16pM for the next MiSeq run would be recommended.

Alternatively, if **high cluster density** is observed, then decreasing the loading concentration is recommended. For example, if a library (loading conc. of 8pM) produced clusters of ~1300k, with a poor (<80%) %PF and Q30 scores then decreasing the loading concentration to 4pM for the next MiSeq run would be recommended.

A “**good**” MiSeq run should produce clusters of around 1000k. In addition, the %PF score should be high (>85%) with Q30 score also high >90% (the higher the better). This is not always achievable if the library is low complexity.

See current guidelines - section 6.1.

3.1 MAKING THE NaOH/EB MIX.

3.1.01	<p>From the freezer remove an aliquoted tube of sodium hydroxide (2N NaOH) and EB buffer.</p> <p>N.B: Make sure the correct tube of NaOH is selected (the 2N not the 0.1N). It is good practice to aliquot the sodium hydroxide stock into 13-15µL aliquots. This will minimise the risk of contaminating the stocks.</p>
3.1.02	Defrost, vortex and spin the contents of the tube before use.
3.1.03	Take a new 1.5 mL Eppendorf tube & label it NaOH/EB mix.
3.1.04	Pipette 10µl of the 2N NaOH into the tube labelled NaOH/EB mix.
3.1.05	Pipette 190µl of EB Buffer into the same tube.
3.1.06	It is important to vortex this mix well. Then spin using a mini centrifuge.

3.2 LIBRARY MiSEQ CALCULATIONS.

The following calculations are dependent on the concentration of the library. This protocol assumes the library is at 4nM. New libraries are loaded at 8pM when using the V2 kit. The calculations below are for diluting the library from 4nM to 8pM in a volume of 1000 µL.

Library dilution:

- Target pM / (library concentration/2) x Volume required.
- = Volume of diluted library required.
- **Example for a 4nM library:**
- $8\text{pM} / (4000\text{pM}/2) \times 1000\mu\text{L} = 4\mu\text{L}$
- = 4µL final volume to be denatured.

Library denaturing (3.2.04):

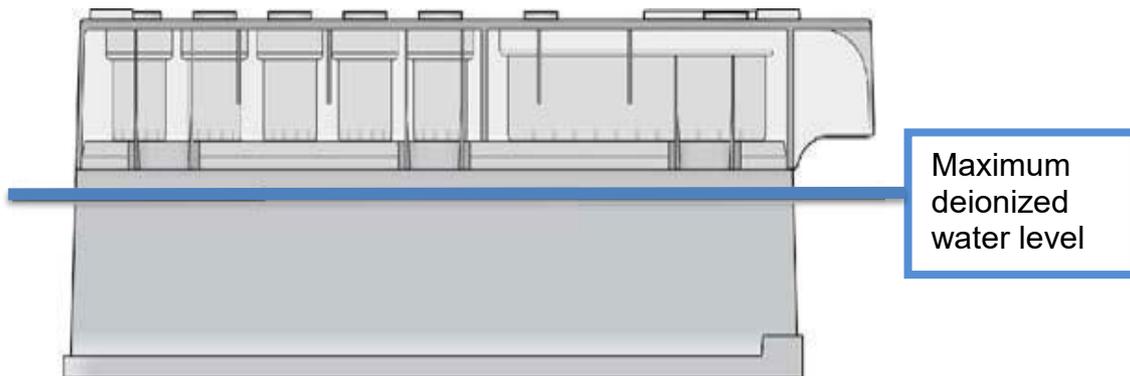
- Final volumes for dilution of the library:
- **Required Volume 4µL**
- Therefore, the library will be diluted 1:1 with NaOH/EB mix:
- 2µL library + 2µL of NaOH/EB mix.

Final dilution (3.2.06):

- Dilute library to 8pM loading concentration.
- **Final Dilution:**
- 2000pM to 8pM
- 4µL library Preparation + 1000µL of HT1.

Dilution Calculations.	
3.2.01	<p>Using the formulae above calculate the following:</p> <ul style="list-style-type: none"> • Library dilution • Library denaturing. • Final dilution.
Library denaturing.	
3.2.02	Thaw out the library. Once the library has thawed, spin down, vortex and spin down again before use. Place library on ice.
3.2.03	Write the library ID onto a new 1.5mL Eppendorf tube.
3.2.04	<p>Dilute the library 1:1 with prepared NaOH/EB mix as per the calculation above:</p> <p>Example:</p> <ul style="list-style-type: none"> • Add 2μL of the library to the labelled Eppendorf. • Add 2μL of the NaOH/EB mix to the labelled Eppendorf.
3.2.05	Allow to denature for 5 minutes .
Final dilution.	
<p>NOTE: Accurate pipetting is key to consistent and reproducible results. When pipetting the library try to keep the pipette tip nearer the top of the liquid level, rather than further down. This minimizes the amount that gets transferred on the outside of the pipette tip.</p>	
3.2.06	Once the 5-minute incubation has finished add 1mL of HT1 - Hybridization Buffer to the labelled Eppendorf.
3.2.07	Vortex this tube well and spin down.
3.2.08	The library is now ready to be used and can be left at room temperature until needed.
3.2.09	Return the undiluted stock library to the freezer.
3.2.10	Continue to next section "Preparing the Reagent Cartridge".

3.3 PREPARING THE REAGENT CARTRIDGE.



3.3.01 Thawing out the reagents can be done one of two ways.

3.3.02 A **On the day of use** prepare a water bath at room temperature containing only enough deionized water to submerge the base of the reagent cartridge. N.B. Do not allow the water to exceed the maximum water line.

- i. Remove the reagent cartridge and HT1 Hybridisation Buffer tube from freezer (-20°C).
- ii. Place the reagent cartridge in the water bath for approximately 60 minutes (300 cycle cartridge). Do not allow the water to exceed the maximum water line.
- iii. Remove the cartridge from the water bath and gently tap it on the bench. To dislodge water from the base of the cartridge and reduce air bubbles in the reagents.
- iv. Dry the base of the cartridge using paper towel.
- v. Invert the reagent cartridge ten times to mix the thawed reagents.

OR

3.3.02 B **The day before** remove the reagent cartridge from the freezer (-20°C) and place in a fridge (4°C).

- i. Place the reagent cartridge and HT1 Hybridisation Buffer tube from the freezer (-20°C) into separate plastic bags and place in the fridge.
- ii. Leave the reagents overnight to thaw.
- iii. Remove the cartridge from the fridge and gently tap it on the bench then invert the reagent cartridge ten times to mix.

3.3.03 Visually inspect the IMF reagent (purple solution in port 1). Make sure that it is fully mixed and free of precipitates.

3.3.04	If precipitates are present then leave the IMF reagent at room temperature until precipitates are fully solubilised.
3.3.05	Place the reagent cartridge on ice or set aside at 2°C to 8°C until you are ready to load your diluted denatured library in to the reagent cartridge and set up your run.
Index PCR (iPCR) primer (Steps 3.3.05 - 3.3.08 are essential for the amplicon sequencing protocol).	
3.3.06	Vortex and spin down the iPCR (stock @ 100µM).
3.3.07	Pierce the foil on Position 13 'HP12 Index Primer' on the MiSeq cartridge using a small pipette tip (10µL). Make sure the hole is as small as possible and to one side.
3.3.08	To Position 13 add the 3.4µL of your iPCR primer to the side of the well.
3.3.09	Cover the well with a little Parafilm and whilst holding the Parafilm tight to the well invert the cartridge 5 times to mix the primers.
<p>NOTE: Ensure there is no residual reagent at the top of the well or on the Parafilm. If so carefully using a pipette transfer reagent from around the top of well back into the well. When adding iPCR primer to the cartridge ensure you make only a very small hole in the foil. A larger hole in the foil increases the chance of losing the contents of the well</p>	
Loading the diluted denatured library to the reagent cartridge.	
3.3.10	Pierce the foil on Position 17 and add the total volume of your diluted denatured library into Position 17 (~1000µL).

3.4 MISEQ SETUP, LOADING AND RUN.

Please follow guidelines specific to the version of the software installed on the MiSeq as provided by the manufacturer. For the MiSeq pre-run checks, flowcell setup and loading, reagent loading and run setup please follow guidelines provided by Illumina during installation and training.

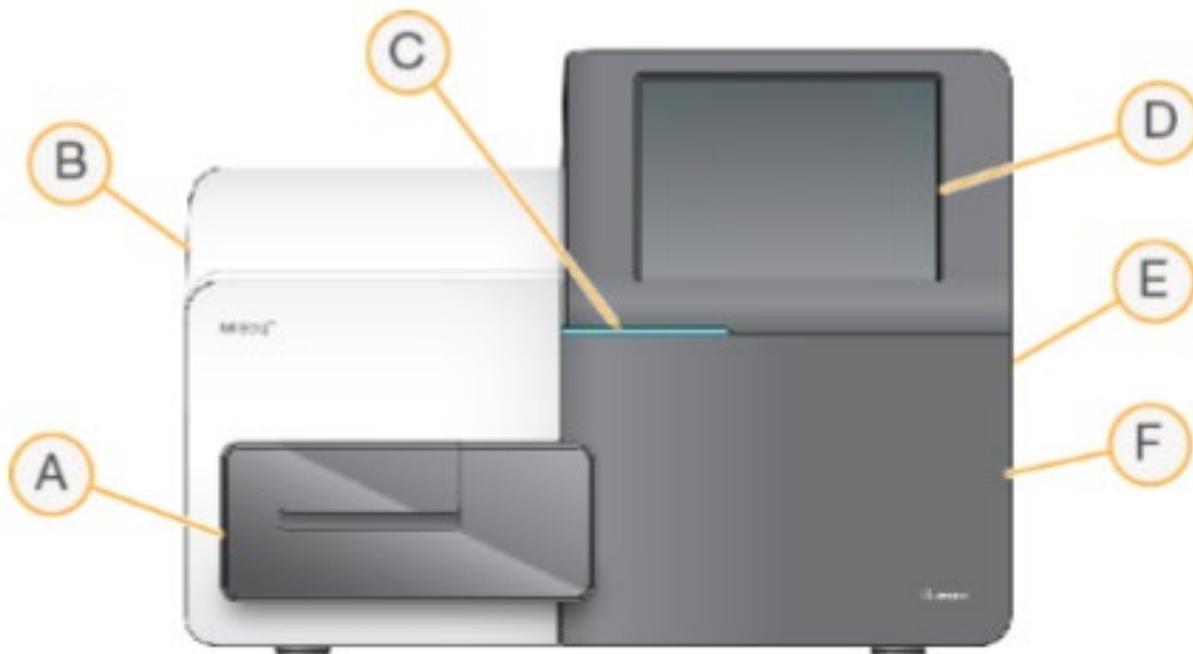
4. QUALITY CONTROL

4.1 CURRENT GUIDELINES.	
<p>Amplicons included in the two-step PCR protocol. The processes are designed to capture a narrow size range (190-250bp inclusive of priming sites) to complement the sequencing length of the MiSeq v2 300 kit (Illumina, San Diego, CA), whilst enabling efficient Ampure XP size selection away from contaminating smaller off-target amplification products.</p>	
<p>For the GBS runs we have seen in the Data QC team so far over the last 12 months or so we can generally expect to see the following:</p>	
4.1.01	Cluster density at around 900-1200k. Anything above 1200k can result in over-clustering which impacts upon quality.
4.1.02	Yield is usually around 4Gb for a 316 cycle run but obviously this is dependent upon the cluster density so will vary to some extent.
4.1.03	Q30 scores are good for the forward read (90% approx.). We tend to see a drop in quality on the reverse read to 70-80%. This is seen on MiSeq runs in general and not only GBS.
4.1.04	We tend to see lots of missing/under-represented tags within a GBS pool – this is partly due to pools being very highly plexed (1000+ samples on some runs) and partly due to how the pools are put together.
4.1.05	GBS call rates/pass rates can be very variable – we will flag if vast majority of these rates are poor but main function of QC role is to check the sequencing run has worked and not to interpret the call/pass rates.

APPENDIX 1 – OVERVIEW

NOTE: Adapted from Illumina reference documents

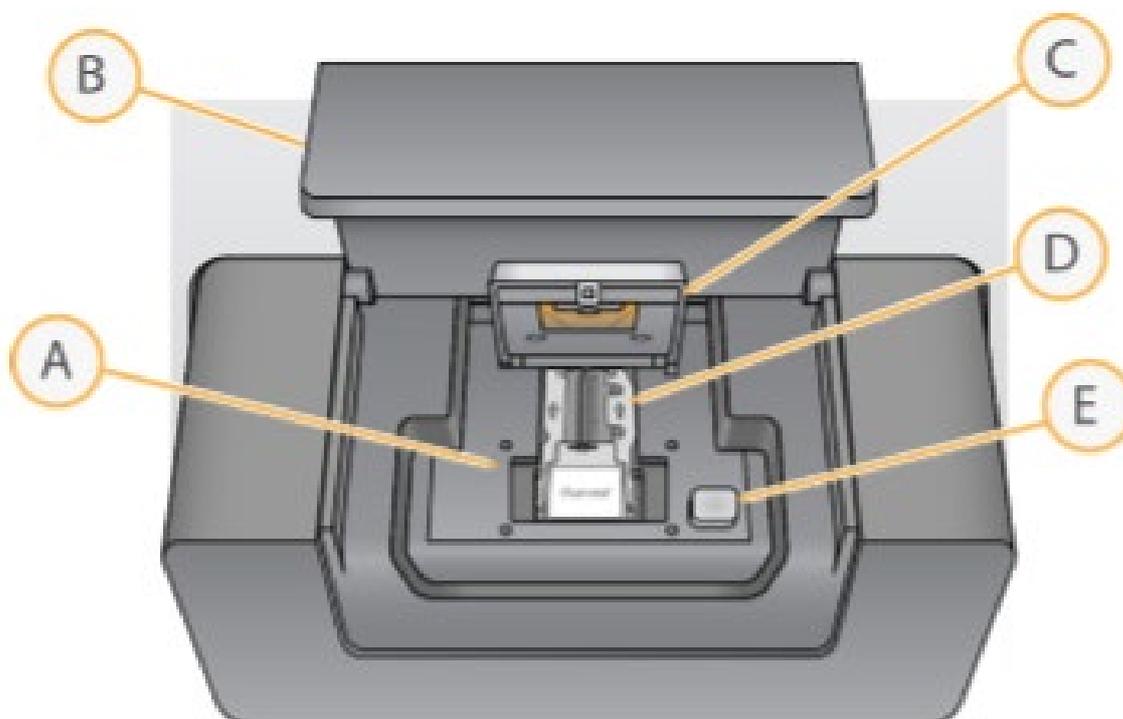
Components



A	Flow cell compartment — Contains the flowcell stage that houses the flowcell throughout the run. Flowcell stage motors move the stage out of the enclosed optical module for flowcell loading and returns the stage when the run begins.
B	Enclosed optics compartment — Contains optical components that enable imaging of the flowcell.
C	Status bar — Indicates flowcell status as ready to sequence (green), processing (blue), or needs attention (orange).
D	Touch screen monitor — Displays the control software interface for system configuration and run setup.
E	External USB ports — Facilitates the transfer of files and data to the instrument computer from the touch screen monitor.
F	Reagent compartment — Contains reagents at proper temperatures, wash solutions, and a bottle for used reagents. A magnetic latch secures the reagent compartment door.

Flow Cell Compartment

NOTE: Adapted from Illumina reference documents

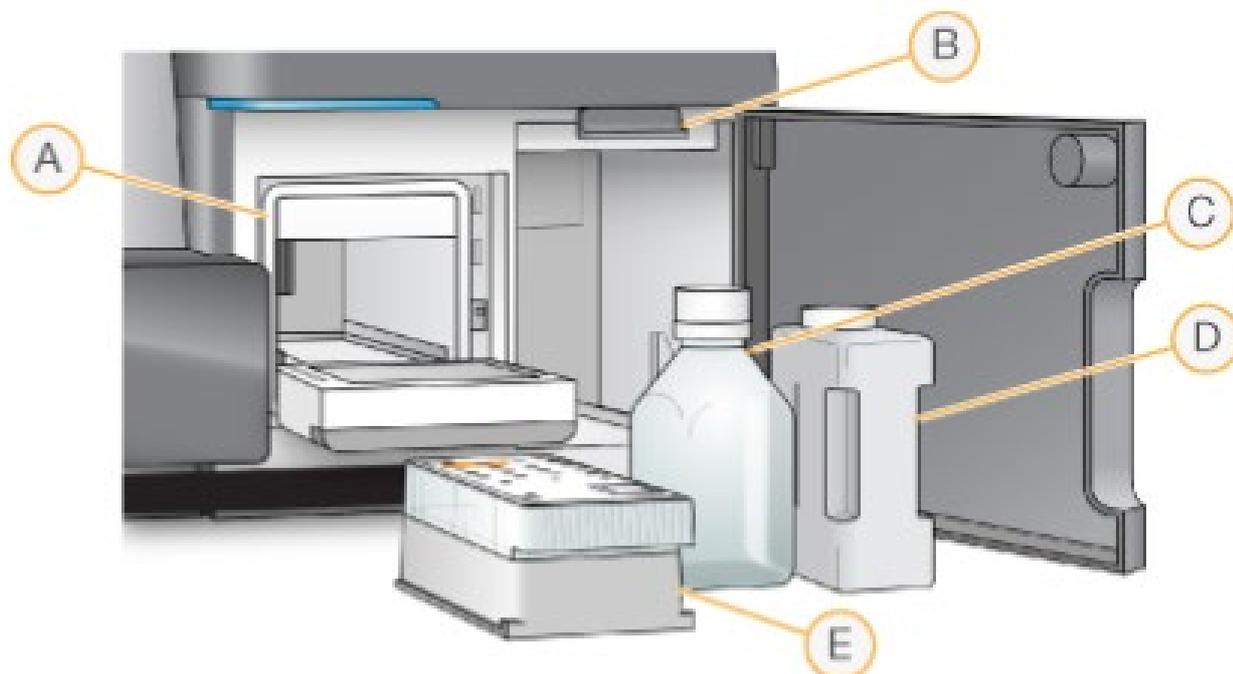


The flow cell compartment contains the flow cell stage, thermal station, and fluidics connections to the flow cell. The flow cell stage holds the flow cell and the flow cell clamp secures and positions the flow cell. When the flow cell clamp closes, two pins near the clamp hinge auto-position the flow cell. The thermal station, located beneath the flow cell stage, controls changes in flow cell temperature required for cluster generation and sequencing.

A	Flow Cell Stage.
B	Flow Cell Compartment Door.
C	Flow Cell Clamp.
D	Flow Cell.
E	Flow Cell Clamp Release Button.

Reagent Compartment

NOTE: Adapted from Illumina reference documents



The reagent compartment contains the reagent chiller, and positions for the wash buffer (PR2) bottle and the waste bottle. To maintain a consistent temperature, open and close the reagent chiller only when instructed.

NOTE The required temperature range of the reagent chiller is 2°C to 11°C. During the run, the reagent chiller holds a single-use reagent cartridge. During the instrument wash, the reagent chiller holds the wash tray. The software automatically lowers sippers into each reservoir of the reagent cartridge at the appropriate time during a run depending on the process being performed. To the right of the reagent chiller are form-fitted slots for the PR2 bottle and the waste bottle. The sipper handle locks the bottles in place and lowers the appropriate sipper into each bottle. Reagents are pumped through the sippers and fluidics lines, and then to the flow cell. Reagent waste is delivered to the waste bottle throughout the process.

A	Reagent Chiller.
B	Sipper Handle (shown in raised position).
C	PR2 Bottle.
D	Waste Bottle.
E	Reagent Cartridge.

APPENDIX 2 – LOADING CONCENTRATIONS

Other loading concentrations & volumes.

The guidelines below can be used if the library is at a different concentration (i.e. not 4nM) or if a different loading concentration is required (see section 3. 2 library MiSeq calculations).

As stated before, new libraries are loaded at 8pM for V2 kits.

The lower the concentration of your library the more of it is required for the MiSeq run. Please see the below for examples of variations in preparing the libraries.

NOTE: ensure that the volume of diluent is not less than 600µL

Volume required

- Target pM / (library concentration/2) x Volume required.
- = Volume of diluted library prep required.
- **Example for a low concentration (2nM):**
- $8\text{pM} / (2000\text{pM}/2) \times 1000\mu\text{L} = 8\mu\text{L}$
- = 8µL final volume to be denatured.
- **Example for using a lower loading volume coupled with a low concentration:**
- $8\text{pM} / (1000\text{pM}/2) \times 700\mu\text{L} = 11.2\mu\text{L}$
- = 11.2µL final volume to be denatured.
- **Example for using a higher loading concentration:**
- $10\text{pM} / (2000\text{pM}/2) \times 700\mu\text{L} = 7\mu\text{L}$
- = 7µL final volume to be denatured.

Subsequent calculations should be adjusted accordingly to take into account the changes in volume.

Once the calculations have been completed, the process of creating the denatured library can continue.

APPENDIX 3 – FLOW DIAGRAM

