May 16, 2022

Preparation of libraries (Metabarcoding) for Illumina sequencing - Genomer Platform

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The following detailed protocol is for the generation of paired-end sequencing reads of 16S or 18S (or any other marker) PCR amplicons with multiple barcodes (i.e.: “indices”) on the Illumina MiSeq machine using Nextera and V2 or V3 chemistry on the GENOMER platform. Catalog numbers are given when items from specific vendors (vs. generic choices) are required.

This work benefited from access to the GENOMER platform at Station Biologique de Roscoff.

The GENOMER Platform is one of Biogenouest Genomics platforms. GENOMER provides the scientific community with the necessary tools to complete sequencing projects. https://www.sb-roscoff.fr/en/research-and-training-centre-marine-biology-and-oceanography/services/technological-core-facilities/genomics-core-facility/about-genomics-core-facility

Protocol reference

- EB-BM-MO-022 Preparation of librairies (Metabarcoding) for Illumina sequencing - Plateform Genomer.pdf

List of supplemental files

- Supplemental material 1_DNA_5K_Quick_Guide.pdf
- Supplemental material 2_DNA-High-Sens-Quick-Guide.pdf
- Illumina_LibraryPool_Model.xlsx
- Primers_Illumina_1.0.xlsx

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protocol

S. Metz et al - Has been uploaded to ScholarOne.Diversity of photosynthetic picoeukaryotes in eutrophic shallow lakes as assessed by combining flow cytometry cell-sorting and high throughput sequencing - FEMS Microbiology Ecology, 2019, DOI: 10.1093/femsec/fiz038
S. Metz et al. Freshwater microeukaryotes: unveiling the unexplored in a large floodplain system. Environmental Microbiology , 2021, hal-03611307, doi.org/10.1111/1462-2920.15838

metabarcoding, sequencing, NGS, Illumina, MiSeq, diversity

protocol ,

Apr 12, 2018

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11464

MiSeq specifications

miseq-system-guide #15027617

AMPure beads/technical.

PRD-Automated-DNA-Sample-Analysis

miseq-denature-dilute-libraries-guide_#15039740

support.illumina/miseq-overclustering-primer.pdf

• **extraction for sorted cells**

Tris - Thermo-fisher - Ref AM9855G
EDTA - Thermo-fisher - Ref 15575020
Invitrogen™Ambion™ Nuclease-Free Water - Ref AM9937

• **MiSeq Reagent kit (cartridge, reagent & flow cell)**

•MiSeq Reagent Kit v2, 500 Cycles (**MS-102-2003**): Provides reagents for up to 525 cycles of sequencing, which is sufficient for up to 251-cycle paired-end run plus two eight-cycle reads.
•MiSeq Reagent Kit v2, 300 Cycles (**MS-102-2002**): Provides reagents for up to 325 cycles of

sequencing, which is sufficient for up to 151-cycle paired-end run plus two eight-cycle reads.
•MiSeq Reagent Kit v3, 600 Cycles (**MS-102-3003**): Provides reagents for up to 625 cycles of sequencing, which is sufficient for up to 301-cycle paired-end run plus two eight-cycle reads.
•MiSeq Reagent Kit v3, 150 Cycles (**MS-102-3001**): Provides reagents for up to 175 cycles of sequencing, which is sufficient for up to 76-cycle paired-end run plus two eight-cycle reads.

• **NGS Primers**

NGS Primers RP-HPLC - Eurogentec - 10 or 40nmol synthesis scale - dried or at 100µM TE concentration

• **Purification AMPure XP beads**

Agencourt AMPure XP beads - Beckman Coulter France - Ref A63881–60ml ; Ref A63880 - 5ml ; A6388 - 450ml

For 96 well format —Agencourt SPRIPlate 96 Ring Super Magnet Plate - Beckman Coulter - Ref A32782

Ethanol - Fisher Scientific - Ref 10342652

• **Nextera XT Index**

Nextera XT Index kit for 24 Indexes, 96 samples - Illumina Cambridge Ltd - Ref FC-131-1001

Nextera XT Index kit for 96 Indexes, 384 samples - Illumina Cambridge Ltd - Ref FC-131-2001; FC-131-2002; FC-131-2003; FC-131-2004 (obsolated Ref FC-131-1002)

2x KAPA HiFi HotStart ReadyMix - Roche Diagnostic France - KK2602

• **DNA Quantification**

LabChip:
DNA 5K - Perkin Elmer - Ref CLS760675
DNA 1K - Perkin Elmer - Ref CLS760673
DNA High sensitivity - Perkin Elmer - Ref CLS760672
Qubit:
DNA HS Assay Kit -ThermoFisher Scientific - Ref Q32854 (500 assays) or Q32851 (100 assays)
1x dsDNA HS Assay - Invitrogen - Ref Q33230

• **Divers**
UltraPure™ 1M Tris-HCl, pH 8.0 -Fisher Scientific - 15568-025
NaOH - Sigma Aldrich - Ref S8045-500G
PhiX Control - Illumina France - Ref FC-110-3001
Tween 20 Molecular biology - Sigma Aldrich - Ref P9416-50ML

General workflow

1 The general workflow is composed by 5 different parts:

1. DNA/RNA extraction protocols.

2. PCR amplification protocols.
Several genes amplification protocols are available on protocols.io for 18S rRNA, plastid 16S, bacterial

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16s, nifH etc

3. Library preparation protocols.

This protocol is dedicated to the preparation of libraries for sequencing on Genomer platform. The protocol will be more or less different in function of the sequencing platform.

4. Sequencing (Illumina)

5. Analysis

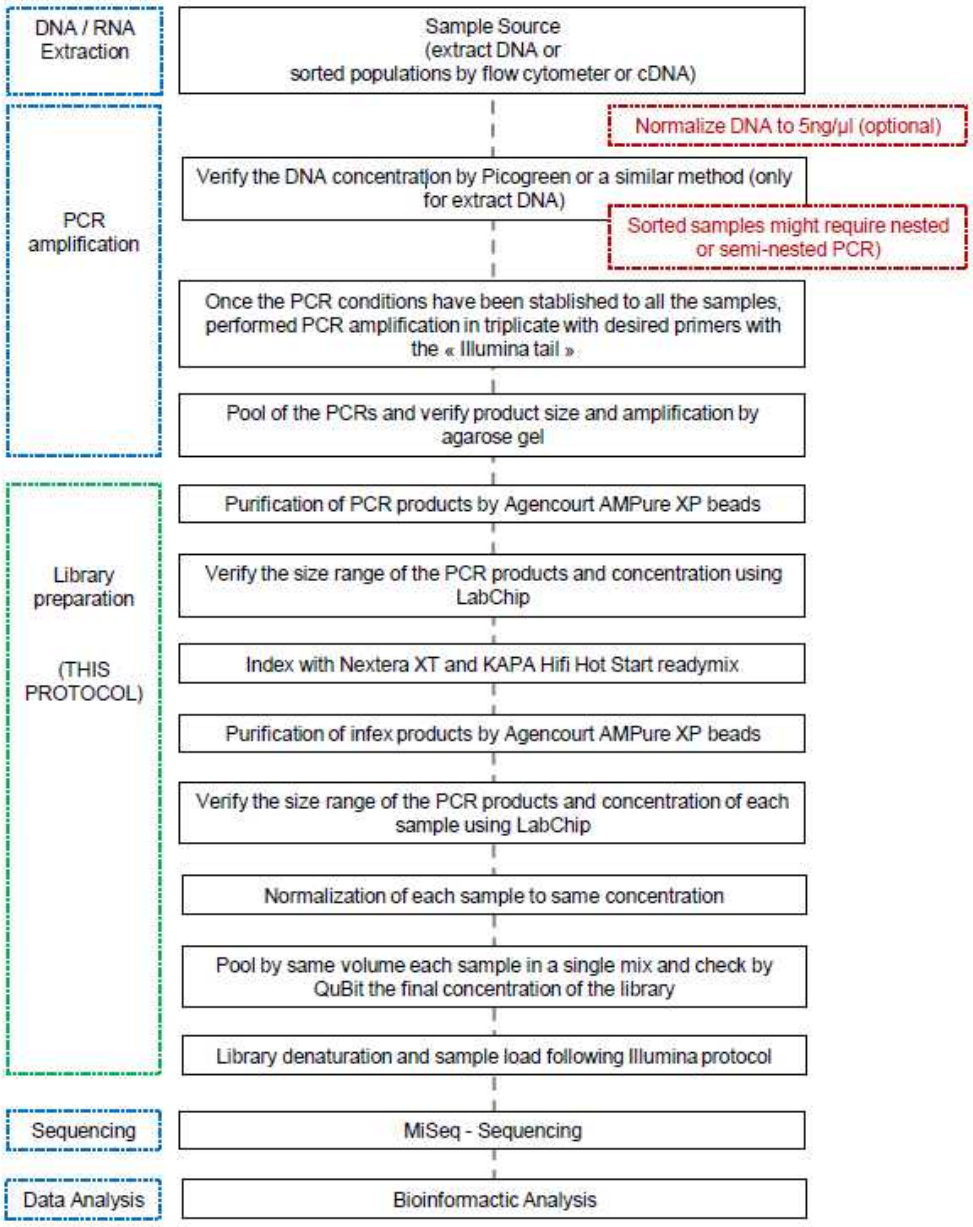

Figure 1: General Workflow.

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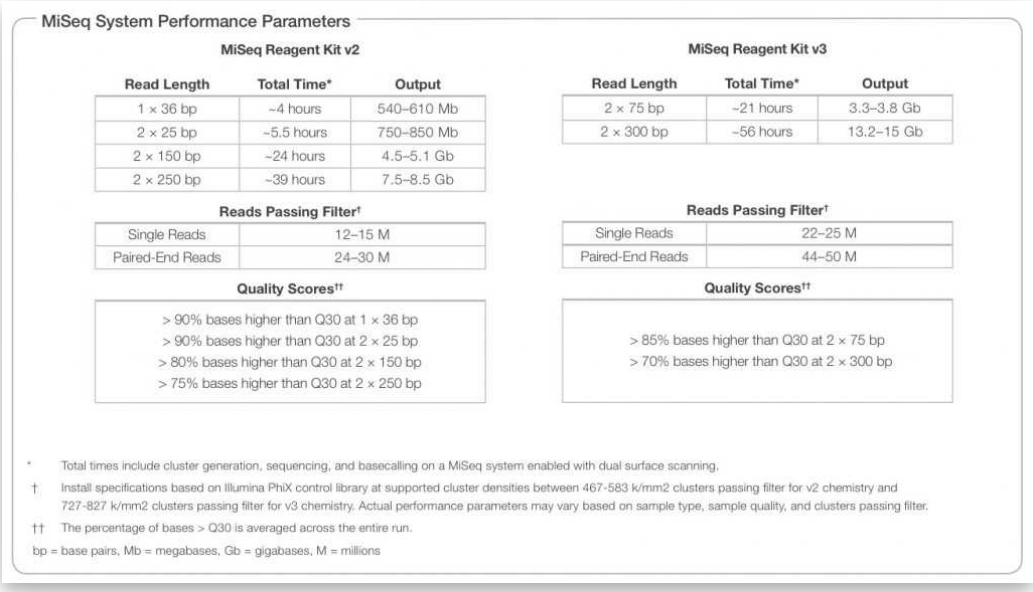
Before starting libraries

2

Choice of product

2.1 MiSeq System: Small genomes, amplicon, and targeted gene panel sequencing.
MiSeq specifications



MiSeq System Performance Parameters

MiSeq Reagent Kit v2

Read Length	Total Time*	Output
1 × 36 bp	~4 hours	540–610 Mb
2 × 25 bp	~5.5 hours	750–850 Mb
2 × 150 bp	~24 hours	4.5–5.1 Gb
2 × 250 bp	~39 hours	7.5–8.5 Gb

Reads Passing Filter†

Single Reads	12–15 M
Paired-End Reads	24–30 M

Quality Scores††

> 90% bases higher than Q30 at 1 × 36 bp
> 90% bases higher than Q30 at 2 × 25 bp
> 80% bases higher than Q30 at 2 × 150 bp
> 75% bases higher than Q30 at 2 × 250 bp

MiSeq Reagent Kit v3

Read Length	Total Time*	Output
2 × 75 bp	~21 hours	3.3–3.8 Gb
2 × 300 bp	~56 hours	13.2–15 Gb

Reads Passing Filter†

Single Reads	22–25 M
Paired-End Reads	44–50 M

Quality Scores††

> 85% bases higher than Q30 at 2 × 75 bp
> 70% bases higher than Q30 at 2 × 300 bp

* Total times include cluster generation, sequencing, and basecalling on a MiSeq system enabled with dual surface scanning.
† Install specifications based on Illumina PhiX control library at supported cluster densities between 467-583 k/mm2 clusters passing filter for v2 chemistry and 727-827 k/mm2 clusters passing filter for v3 chemistry. Actual performance parameters may vary based on sample type, sample quality, and clusters passing filter.
†† The percentage of bases > Q30 is averaged across the entire run.
bp = base pairs, Mb = megabases, Gb = gigabases, M = millions

Figure 2: MiSeq System Performance Parameters using v2 or v3 kits.

Different MiSeq Reagent Kit (for more details, see Materials) :
• kit v2, 500 Cycles : for up to 525 cycles => for up to 251-cycle paired-end run plus two eight-cycle reads.
• Kit v2, 300 Cycles : for up to 325 cycles => for up to 151-cycle paired-end run plus two eight-cycle reads.
• Kit v3, 600 Cycles : for up to 625 cycles => for up to 301-cycle paired-end run plus two eight-cycle reads.
• Kit v3, 150 Cycles : for up to 175 cycles => for up to 76-cycle paired-end run plus two eight-cycle reads.

NB: It is highly recommended to check on Illumina website for updates and changes concerning the products.

Illumina sequencing: How does it work?

2.2 Sequencing by synthesis method: https://youtu.be/fCd6B5HRaZ8

In principle, the concept behind NGS technology is similar to Sanger sequencing. DNA polymerase catalyzes the incorporation of fluorescently labelled deoxyribonucleotide triphosphates (dNTPs) into a DNA template strand during sequential cycles of DNA synthesis. During each cycle, at the point of incorporation, the nucleotides are

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identified by fluorophore excitation. The critical difference is that, instead of sequencing a single DNA fragment, NGS extends this process across millions of fragments in a massively parallel fashion. The sequencing by Illumina technology involves tree basic steps:

1. Library preparation:

When working with genomes, metagenomes or transcriptomes for example, this step involves the random fragmentation of the DNA or cDNA sample followed by selection of fragment size and 5' and 3' adapter ligation. In this case, the library preparation products as well as the protocols are different.
For metabarcoding purpose, this step involves the amplification by PCR of a given gene region (for example V4 or V9 region of 18S rRNA gene) or gene itself if its size does not exceed 600bp (the longest fragment size that can be sequenced by Illumina technology). The defined forward and reverse primers that are complementary upstream and downstream of the region of interest, needs to be designed with overhang adapters which will be use in a subsequent limited-cycle amplification step, in order to add the dual-index barcodes and Illumina flowcell adapters. Samples are normalized by equal concentration, pooled (more details of these steps below), and sequenced on the MiSeq **(Figure 3A)**.The final fragment contains the flow cell adapters (P5 and P7), barcodes indices (i5 and i7) and the adapter region for the sequencing step (adapter) **(Figure 3B)**.

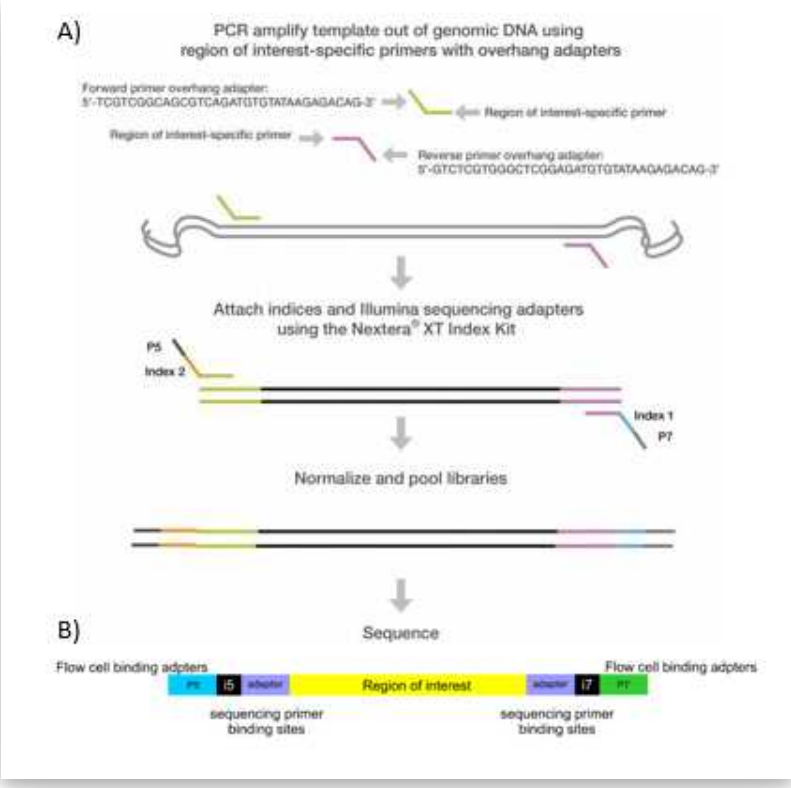

Figure 3: Metabarcoding steps a priori sequencing.
(A) PCR amplification using specific primers which are designed with overhang adapters (index barcodes and Illumina flowcell adapters).
(B) Final fragment with flow cell adapters (P5 and P7), barcodes indices (i5 and i7) and the adapter region for the sequencing step (adapter).

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2. Cluster generation

For cluster generation, the denaturated library is loaded into the flow cell where fragments are captured on a lawn of surface-bound oligos complementary to the library adapters (P5 and P7). Each fragment is then amplified into distinct, clonal clusters through bridge amplification. When cluster generation is complete, all fragments attached to P7 sites are cleaved off, leaving only fragments attached to the P5 sites. The sequencing processcan then start. For Illumina sequencing platform, Cluster density is a critically important metric that impacts run quality, reads passing filter and total data output. Under clustering generates high data quality however it results in lower data output. Alternatively, over clustering can lead to poor run performance, lower Q30 scores, the possible introduction of sequencing artifacts, and lower total data output since the majority of data can be with low Q30 score. Performing a run at optimal cluster density involves finding a balance between under- and over clustering. The goalis to sequence at high enough densities to maximize total data output, while maintaining low enough densities toavoid the negative effects of over clustering.

•How does over clustering affect Sequencing Data?
(this part has been adapted from the document: support.illumina/miseq-overclustering-primer.pdf)

I. **Lower Q30 Scores** — Due to overloaded signal intensities, the ratio of base intensity to background for each base is decreased. This decrease often results in ambiguity during base calling and leads to a decrease in data quality.

II. **Lower Clusters Passing Filter** — The percentage of clusters passing filter (%PF) is an indication of signal purity from each cluster. Over clustered flow cells typically have higher numbers of overlapping clusters. This leads to poor template generation, which then causes a decrease in the %PF metric.

III. **Lower Data Output** — Reduced yield (gigabases [Gb] per flow cell) is a by product of lower %PF.

IV. **Inaccurate Demultiplexing** — Index reads usually have low diversity by design, which can lead to poor base calling. Over clustering exacerbates the potential for poor base calling, which in turn, can lead to demultiplexing failures.

V. **Complete Run Failure** — In cases of extreme over clustering, focusing can fail and the run may terminate at any cycle.

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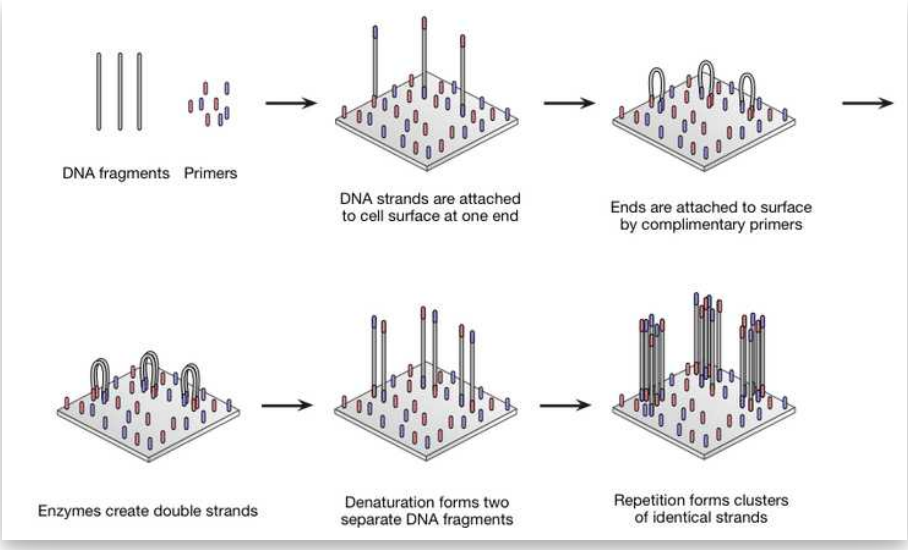

Figure 4: Cluster formation.

3. sequencing

Illumina SBS (Sequencing by synthesis) technology uses a proprietary reversible terminator – based method that detects single bases as they are incorporated into DNA emplate strands. As all four-reversible terminator – bound dNTPs are present during each sequencing cycle, natural competition minimizes incorporation bias and greatly reduces raw error rates compared to other technologies. The result is highly accurate base-by-base sequencing that virtually eliminates sequence context – specific errors, even within repetitive sequence regions and homopolymers. The sequencing primers anneal to the adapter region and sequence the DNA fragment from the adapter. Then a second sequencing primer will sequence the opposite direction from the adapter, namely the index. Just one base is added to the sequence in each cycle. Each cycle the flow cell is flushed with a mix of all four fluorescent bases. The bases are reversibly terminated to ensure the incorporation of only one base per cycle and unincorporated bases are washed away. Then the fluorescence of the incorporated bases is recorded. Each cluster of clonally amplified DNA fragments, will show one base. After imaging, the fluorophore is cleaved there by eliminating fluorescence, the terminator is subsequently removed, and the cycle is repeated. We use the paired-end sequencing method. This means that instead of sequencing 300 consecutive nucleotides in one direction, 150 nucleotides are sequenced from both ends of the fragment.

Advanced preparation

2.3 1. Order the primers

Use existing primers or design your own custom gene primers with the proper Illumina indices and Nextera adaptor orientations (Primers_Illumina_1.0.xlsx in attached document).

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Illumina Forward tail (5'-3')	Illumina reverse tail (5'-3')
TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

Table 1: Illumina tail used with Nextera XT Index Kit & Illumina flow cell.

Target gene	Product size	Product size with the tail	Primer Forward name	Final Forward Primer	Primer reverse name	Final Reverse Primer
nifH	350	471	nifh1F_ILL_Genomer	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TGYGAYCCNAARGCNGA	nifh2R_ILL_Genomer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ADNGCCATCATYTCNCC
16S plastid	420	541	Pla491F_ILL_Genomer	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GAGGAATAAGCATCGGCTAA	PP936R_ILL_Genomer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG CCTTTGAGTTTCAYYCTTGC
18S-V4 Euk	406	527	V4F_ILL_Genomer	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCAGCASCYGCGGTAATTCC	V4R_Zig_ILL_Genomer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACTTTCGTTCTTGATYRATG
16S-V3V4 Bact	464	585	S-D-Bact-0341-b-S-17	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTACGGGNGGCWGCAG	S-D-Bact-0785-a-A-21	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GACTACHVGGGTATCTAATCC
16S-V4V5 Bact	411	474	515-Y-F_ILL_Genomer	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG GTGYCAGCMGCCGCGGTAA	926-R_ILL_Genomer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GTGYCAGCMGCCGCGGTAA

Table 2: Primers with tail used to Illumina sequencing, some examples.

It is recommended to order the primers with a good synthesis and purity parameters. We have order at EUROGENTEC the primers with RP-HPLC purity method, 10 or 40nmol synthesis scale and at 100µM TE concentration. We could also order in dried and dissolve them with nuclease free water or TE buffer to obtain 100µM stock solution (as described on the Eurogentec's technical data sheet).
Then we dilute them to 10 µM working concentration (1/10th the typical 100 µM working stock concentration for primers).
This proceed should be perform at the PCR hood and with molecular grade water.

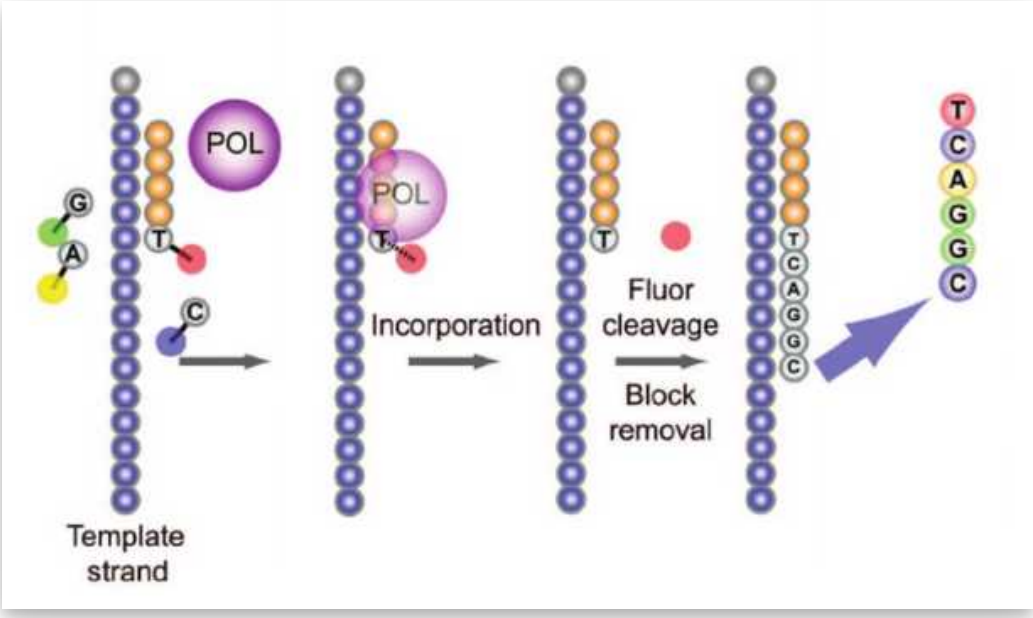

Figure 5: Sequencing by reversible dye terminators.

2. Acid nucleic extraction and quantification

Extract DNAs/RNA from the samples using the method/kit appropriate to the specific samples. By working with RNA, another step to transform the RNA in cDNA is

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necessary. Quantify and quality-check the final DNA via NanoDrop or Qubit/PicoGreen. The A280/260 ratios should be 1.8 or higher and once working with total community extracted DNAs, a concentration of at least 1 ng/µL is desirable to get consistent PCR results. Avoid secondary extractions or clean-ups for inhibitors, until PCRs have truly shown inhibition (example after trying the PCRs by diluting the original DNA and/or adding BSA to the final PCR reaction) – many suboptimal DNAs will still work in PCR. A gel can be run to verify integrity of the extracted material, but it is generally unnecessary for PCR-only studies. Some protocols suggest the normalization of the DNA samples concentration prior to the PCR. In our experience, this is not always possible given the low concentration of some samples. The volume of sample added to the PCR can be settled in function of a range of concentrations instead of normalizing all the samples to the same concentration.

Load in agarose gel 0.8%, 1µl DNA + loading buffer. The limited of DNA detection using EtBr gels is around 10ng.

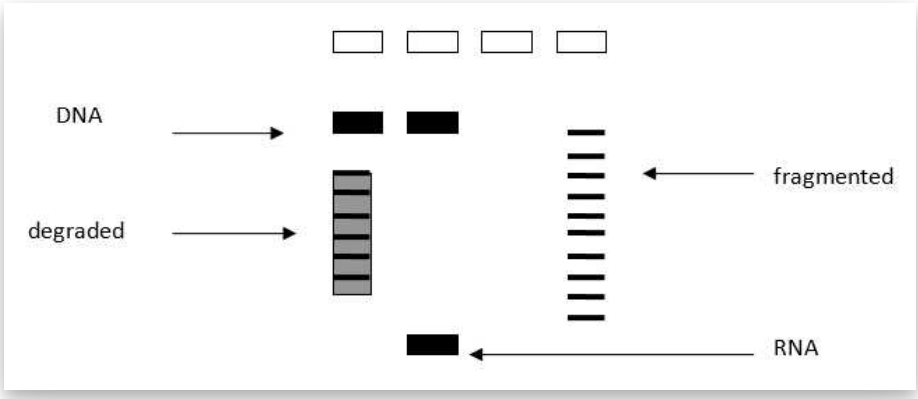

Figure 6: DNA/RNA detection in agarose gel.

Sorted populations by flow cytometer can be used as an input for the PCR reactions. Photosynthetic pico and nanoeukaryotes populations selected based on light scatter, orange phycoerythrin and red chlorophyll fluorescence are sorted in sterile eppendorf tubes containing Tris-EDTA lysis buffer (Tris 10mM, EDTA 1mM and Triton x-100 1.2%).The Tris, EDTA and Nuclease-Free water use should be molecular grade. A buffer containing Tris-HCl 10mM, pH 8.0 and NaCl 20mM and filtered at 0.22 µm should be used as sheath liquid for marine samples. Sheath fluid samples should be collected and analyzed as negative controls in all subsequent steps including sequencing, in order to test for contamination in the flow sorting process. After the sorting, samples can be stored at -80C. The DNA from sorted cells can be extracted by three cycles of freezing in liquid nitrogen and thawing in water.

Library preparation

3

PCR amplification

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3.1 There are some suggestion of protocols for 18S rRNA from sorted cell and total genomic DNA, plastidial 16S and nifH from sorted cells.

For that, consult the different protocols available on protocols.io :

- 18S-V4 rRNA amplification from total genomic DNA, dx.doi.org/10.17504/protocols.io.yxmvmxdmnl3p/v1
- 18S-V4 rRNA amplification from sorted cells (low-concentration DNA) for NGS Illumina sequencing (Metabarcoding), dx.doi.org/10.17504/protocols.io.81wgb71k1vpk/v1
- plastidial 16s (no published, shared on ECOMAP & AD2M protocols.io workspaces)
- Bacterial 16s V3-V4
- Bacterial 16S-V4V5 rRNA amplification for NGS Illumina sequencing (Metabarcoding), dx.doi.org/10.17504/protocols.io.n92ld9bzng5b/v1
- nifH amplication for Illumina sequencing, dx.doi.org/10.17504/protocols.io.bkipkudn
- viral genes

PCR products should be checked initially by gel electrophoresis for unspecific amplification and band size.
-Gel 1-1.5%
-Load 1 to 3 µl of each purified PCR product to see if the bands are weak or strong. Any samples with failed PCRs (or spurious bands) are re-amplified by optimizing the PCR (further template dilution to 1:100 or using BSA/other additives) to produce correct bands. Once correct bands have been obtained, organize the PCR products in appropriated PCR plates and record the plate sample order since this order will follow until the last step.

Purification of PCR products by AMPure beads

3.2 PCR products should be purified from free primers, nucleotides and primer dimer species.This step uses AMPure XP beads. This purification systems utilize Agencourt's solid-phase paramagnetic bead technology for high-throughput purification of PCR amplicons and an optimized buffer to selectively bind PCR amplicons 100bp and larger to paramagnetic beads. Excess primers, nucleotides, salts, and enzymes can be then removed by a washing procedure.

For more details please look: AMPure beads/technical.

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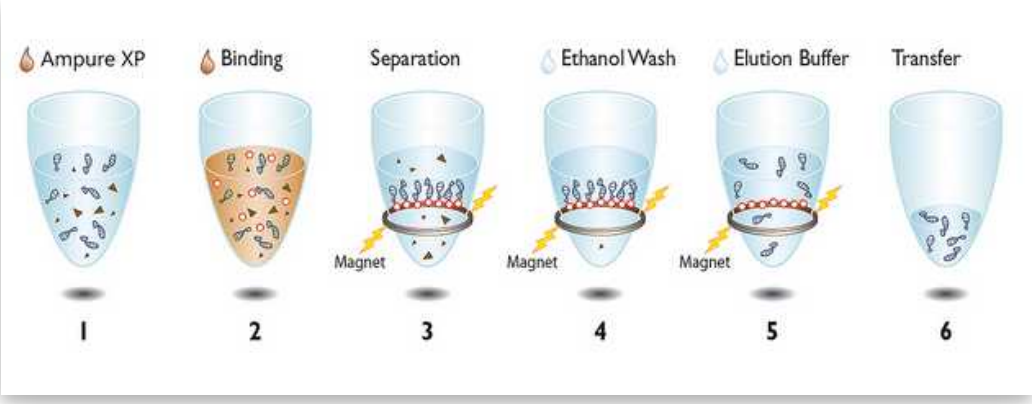

Figure 7: Agencourt AMPure XP beads workflow.

Protocol for 96 Well Format using DNA hood:

Before starting:

- Fresh 70% ethanol should be prepared for optimal results (35ml of ethanol and 15ml of nuclease free water).
The ethanol and the water should be molecular grade.

- 10mM TRIS pH 8.0 (molecular grade) for DNA elution.

- Determine whether or not a plate transfer is necessary. If the PCR reaction volume multiplied by 2.8 exceeds the volume of the PCR plate, a transfer to a 300 µL round bottom plate is required.

1. Gently shake the Agencourt AMPure XP bottle to resuspend any magnetic particles that may have settled.
Pour a volume Agencourt AMPure XP in a reservoir.
Add Agencourt AMPure XP according to the PCR reaction volume following equation:
(Volume of Agencourt AMPure XP per reaction) = 1.8 x (Sample Reaction Volume).

2. Mix reagent and PCR reaction thoroughly by pipette mixing 10 times.
Cover the plate with an adhesive seal.
Let the mixed samples incubate for 5 minutes at room temperature for maximum recovery.
This step binds PCR products larger than 100bp to the magnetic beads. Pipette mixing is preferable as it tends to be more reproducible. The color of the mixture should appear homogenous after mixing.

3. Place the reaction plate onto an Agencourt SPRIPlate 96 Super Magnet Plate for completely separation of the beads from the solution which usually takes about 10 minutes.
Wait for the solution to clear before proceeding to the next step.

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Figure 8: Agencourt SPRIPlate 96 Super Magnet Plate.

4.Aspirate the cleared solution from the reaction plate and discard.**This step must be performed while the reaction plate is situated on the Agencourt SPRIPlate96 Super Magnet Plate. Do not disturb the ring of separated magnetic beads. If beads are drawn out, leave a few microliters of supernatant behind** (~5µL).

5. With the reaction plate situated on an Agencourt SPRIPlate 96 Super Magnet Plate, dispense 200 µL of fresh prepared 70% ethanol to each well of the reaction plate and incubate for 30 seconds at room temperature.
Aspirate out the ethanol using an multichannel pipette and discard.
Repeat for a total of two washes. Do not disturb the separated magnetic beads.

6. Remove all of the ethanol from the bottom of the well. The same tips can be use twice with the same sample to completely aspirate the ethanol. It is important that to pass the tips by a kimwipes between the aspiration to avoid carry over of ethanol. Change the kimwipes and NEVER touch where the previous samples have touched and left ethanol.
Despite the fact that some protocols indicate that the beads can be let to dry, in our experience, after the beads are dried (the beads are dry when the bead ring appears cracked), it is very hard to recover the purified material leading to a significantly decrease in the elution efficiency.

7. Remove the reaction plate from the magnet plate and add 40 µL of 10mM TRIS pH 8.0 to each well of the reaction plate and pipette mix 10 times. Incubate for 2 minutes. In addition, we obtained better results by shaking the plate for 2'/ 1800 rpm.
The liquid level will be high enough to contact the magnetic beads at a 40µL elution volume. A greater volume of elution buffer can be used, but using less than 40µL will require extramixing (to ensure the liquid comes into contact with the beads) and may not be sufficient to elute the entire PCR product.

8. Place the reaction plate onto an Agencourt SPRIPlate 96 Super Magnet Plate for 1 minute to separate beads from the solution or as long the solution is clear.

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9. Transfer the eluant, about 32 –35µL to a new plate.

NB: Place the new plate onto the Agencourt SPRIPlate 96 Super Magnet Plate and check there are no beads.

Store at 4°C.

Reservoir should be cleaned with water then ethanol.

LabChip for size and concentration

3.3 The LabChip GX assays are based on traditional gel electrophoresis principles that have been transferred to a chip format. The chip format dramatically reduces separation time and provides automated sizing and quantitation information in a digital format.It has the same principle as the bioanalyzer, however 96 samples can be run within 2 hours with the labchip and for a low cost when compared to the bioanalyzer. The chip contains an interconnected set of microchannels that join the separation channel and buffer wells. One of the microchannels is connected to a short capillary that extends from the bottom of the chip at a 90-degree angle. The capillary sips sample from the wells of a microplate during the assay. The following protocol can be use with either the DNA 5K **(Supplemental material 1)** or DNA HS kits **(Supplemental material 2)**. In our experience to obtain reliable concentrations, the concentration range per fragment of each kit should be respect.
For more information please check the the link: PRD-Automated-DNA-Sample-Analysis

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Specifications and Ordering Information for LabChip GX Touch Kits

	DNA 1K	DNA 5K	DNA 12K	DNA High Sensitivity	Genomic DNA
Sizing Range	25 bp – 1000 bp	100 bp – 5000 bp	100 bp – 12000 bp	50 bp – 5000 bp	50 – 40,000+ bp
Sizing Resolution[1]	±15% from 25 – 100 bp ±10% from 100 – 150 bp ±5% from 150 – 700 bp ±10% from 700 – 1000 bp	±15% from 100 – 150 bp ±10% from 150 – 500 bp ±15% from 500 – 1500 bp ±20% from 1500 – 5000 bp	± 10% from 150 – 1000 bp ± 15% from 1000 – 2000 bp ± 20% from 2000 – 8000 bp ± 25% from 100 – 150 bp, 8000 – 12000 bp	± 5% from 100 – 500 bp ± 10% from 50 – 100 bp, 500 – 1000 bp ± 15% from 1000 – 3000 bp ± 22% from 3000 - 5000 bp	
Sizing Accuracy	±10%	±10%	±10%	±10%	±20% (Up to 10 kb, based on ladder)
Sizing Precision	5%	5%	5%	5%	20% CV (Up to 10 kb, based on ladder)
Linear Concentration Range	0.1 ng/µL – 50 ng/µL 0.2 Per fragment	0.25 ng/µl – 50 ng/µL Per fragment	0.25 ng/µl – 50 ng/µl Per fragment	10 pg/µL – 500 pg/µL Per fragment[4]	0.2 - 5 ng/µl 2.0 - 50 ng/µl (diluted)
Sensitivity	0.1 ng/µL	0.25 ng/µL	0.25 ng/µL	Down to 5 pg/µL[5]	0.1 ng/µL
Maximum Total DNA Concentration	80 ng/µL, 50 ng/µl Per fragment	80 ng/µl, 50 ng/µL Per fragment	60 ng/µl, total 50 ng/µl Per fragment	5 ng/µL, 500 pg/µL Per fragment	
Quantification Precision	20% from 25 – 500 bp 10% from 500 – 1000 bp	20% CV	20% CV from 100 – 5000 bp 25% CV from 5000 – 12000 bp	20% CV	20% CV
Maximum Salt Concentration	125 mM	125 mM	125 mM	10 mM Tris. 1 mM EDTA	
Additives[3]	BSA/detergents should not exceed 0.05 mg/mL/0.01% v/v	BSA/detergents should not exceed 0.05 mg/mL/0.01% (v/v)	BSA/detergents should not exceed 0.05 mg/mL/0.01% (v/v)	BSA/detergents should not exceed 0.05 mg/mL/0.01% (v/v)	
Chip Lifetime[2]	2000 samples/chip	2000 samples/chip	2000 samples/chip	2000 samples/chip	500 samples/chip
Reagent Kit Lifetime	Up to 10 chip preps for HTS or 20 chip preps for small batch runs	Up to 10 chip preps for HTS or 20 chip preps for small batch runs	Up to 10 chip preps for HTS or 20 chip preps for small batch runs	Up to 10 chip preps for HTS or 20 chip preps for small batch runs	5 chip preps
Max Samples/Chip Prep	384 samples (4 96-well plates or 1 384-well plate)	384 samples (4 96-well plates or 1 384-well plate)	384 samples (4 96-well plates or 1 384-well plate)	96 samples	48
Standard Assay: Specifications are Defined for This Assay	DNA 1K Standard: For sizing of DNA fragments in 25 – 1000 base pair range; analysis time/sample=68 seconds	DNA 5K Standard: For sizing of DNA fragments in 100 to 5000 base pair range. Fastest analysis time/sample compared with all available assays; analysis time/sample=28 sec	DNA 12K Standard: for sizing of DNA fragments in 100 to 12000 base pair range; analysis time/sample=65 sec	DNA High Sensitivity Standard: Analysis time/sample=68 sec	gDNA Quality Analysis time/sample =150 sec
Extra Assays	DNA 1K High Resolution: For sizing of DNA fragments in 25 to 1000 base pair range. Greater resolution with longer analysis time per sample; analysis time/sample=120 sec		DNA 12K High Resolution: For sizing of DNA fragments in 100 to 12000 base pair range. Greater resolution with longer analysis time/sample; analysis time/sample=130 sec) DNA 12K Extended Time: To be used only if peaks are cut off using the standard DNA 12K script (occurs in some high salt sample buffer; analysis time/sample=80 sec		

Table 3: LabChip kits specifications.

Chip Preparation

1. Allow the chip and reagents to equilibrate to room temperature for about 20 minutes before use. The Dye Concentrate must be completely thawed and vortexed before use. One vial of DNA Gel Matrix (red cap) is good for 4 Low-throughput chip preparations (for up to 48 samples) or 2 High-throughput chip preparations (for up to 96 samples).

2. Prepare Gel-Dye by adding 13 µL DNA Dye Concentrate (blue cap) to 1 vial of DNA Gel Matrix (red cap). Vortex and transfer mixture into two spin filters (approximately 550 µL per spin filter). Centrifuge at 9200 rcf for 10 minutes at room temperature. Ensure that all of the gel has passed through the filter and then discard the filter. The Gel-Dye can be stored for up to 3 weeks in the dark at 4°C. Please check for available

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gel matrix with dye at the platform.

3. Rinse and aspirate each active well (1, 3, 4, 7, 8 and 10) twice with molecular biology grade water with the pump that is set up at the platform.

4. Using a Reverse Pipetting Technique add gel-dye to chip well 3, 7, 8 and 10 as shown in **Figure 9A** (Low-throughput) or **Figure 9B** (High-throughput).

5.Add DNA Marker (green cap) to chip well 4 as shown in **Figure 9A** (Low-throughput) or **Figure 9B** (High-throughput).

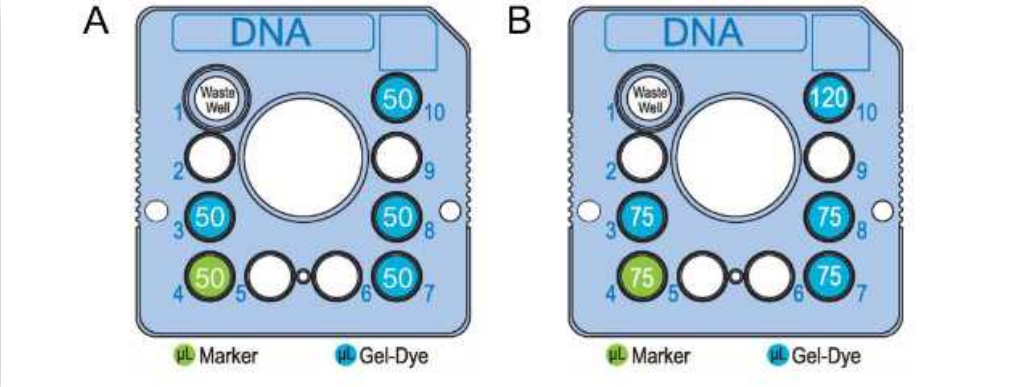

Figure 9: LabChip reagent configuration. A) Low-throughput, up to 48 samples and B) High-throughput, up to 96 samples.

6.Clean both sides of the chip window with kimwipe.

DNA Sample, Ladder and Buffer Preparation (Standard Sample Workflow)

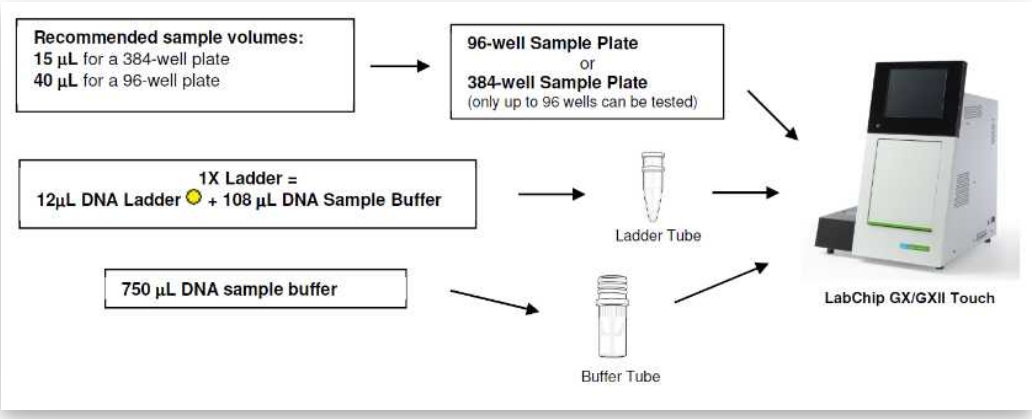

Figure 10: Standard sample worflow.

Preparation Notes:

•This workflow requires only 2µL of PCR sample respecting the average concentration per fragment of each kit. Despite the original protocol **(Figure 10)** works with 40µl final volume. **We have use 30µl.**
•To ensure a buffer match, the buffer for the Buffer Tube is diluted in a similar manner

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as the sample. Then Ladder is diluted in the diluted buffer.We have use TE1X.
•Sample plate should be tested soon after preparation to minimize evaporation and sipping samples more than once is not recommended.
•Quantitation given by the Labchip GX software should be multiplied by the dilution factor, for example, by adding 2µl in 28µl of TE, the dilution factor is 15X.
•If you notice any problem with the ladder check performed by the labchip in the beginning of the run, refer to the "troubleshooting" section in the Labchip manual near the instrument.

INDEX PCR

3.4 This step attaches dual indices and Illumina sequencing adapters using the Nextera XT Index Kit. By using Illumina Experiment Manager, organize your plate and dual indices per sample based on the NEXTERA features you have purchased and include the negatives controls as independent samples.

Sample plate preparation

1. Open IEM (Illumina Experiment Manager) software

2. **Create a sample plate** (or edit a sample sheet if you want to copy another sample sheet for example) **(Figure 11A**).

3. **Choose the Nextera XT kit** you are currently using. There are 4 different sets of indices which allows you to run more than 96 samples with the same primer or same gene target (NEXTERA XT set A to D). If the same set of PCR samples has been generate from different gene target, the same indices can be used and the reads per sample will be separated a posteriori based on the indices and primer sequence (**Figure 11B**).

4. Create a plate name based (Project_gene_date) and **index reads 2** (**Figure 11C**)

5. Copy and paste the samples name and samples ID and **apply default index layout** (**Figure 11D**).

6. Check if the samples order and indices are correct. Print the list of samples, plate position and indices or the plate graphic to help during the index PCR step.

7. Click on **finish** and save the "Project_gene_date.**nexxt28.plt**" file in the folder of the project. This file will be use later. **If working with more plates, repeat these steps and choose the appropriate Nextera set kit in order to create one .plt file for each plate.**

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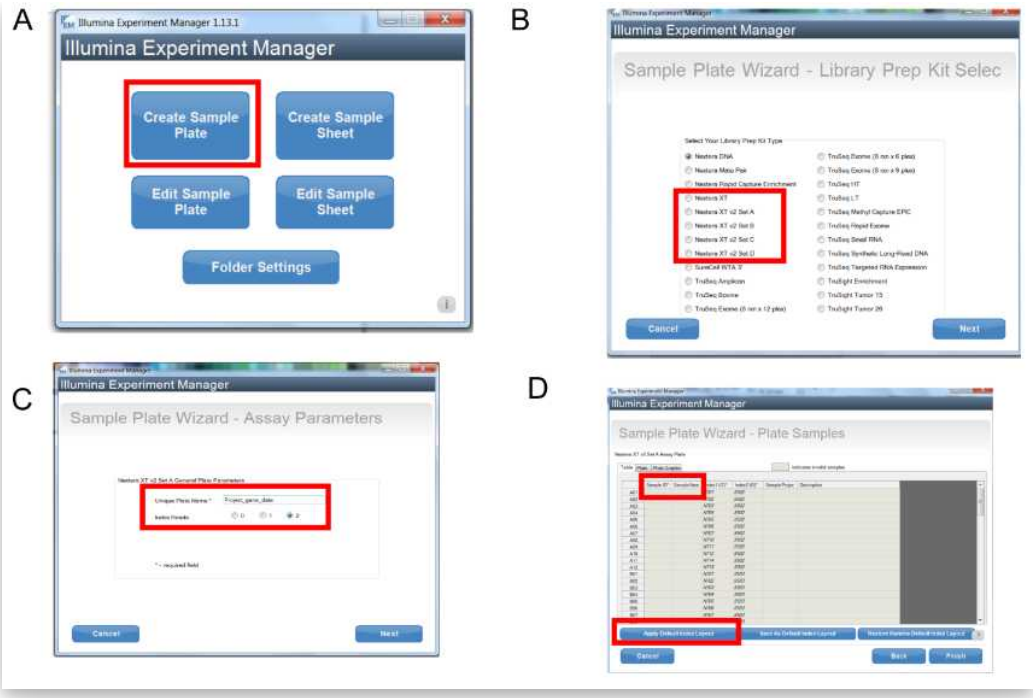

Figure 11: Creating sample plate.

PCR Index (nextera)

The following protocol has been modified for half of the proposed reaction. We have successfully used half reaction for concentrated and low concentrated PCR products.

1. Place a new the 96-well PCR plate in the rack as **Figure 12.**
Make a mix with 2x KAPA HiFi HotStart ReadyMix and PCR Grade water for the whole plate.
For 1 reaction, add 12.5µL of 2x KAPA HiFi HotStart ReadyMix, and 5µL PCR Grade water.
With a multichannel pipette dispense the mix of water and KAPA.The volume of water can be adjusted to add more or less DNA.

2. Arrange the Index 1 and 2 primers in a rack, with Index 2 primer tubes (white caps) vertically, aligned with rows A through H and Index 1 primer tubes (orange caps) horizontally, aligned with columns 1 through 12.

3. With a multichannel pipette add 2.5µL indices 2 from A to H columns in each column of the new plate. It is not necessary to change tips in this step but make sure all the columns have received the correspondent index.

4. Add the indices 1 from columns 1 through 12 also with the multichannel pipette. **Change the tips each time to avoid carry over of the indices 2 to the stock tube of indices 1.**

NB: remove caps of index primers and throw away, put new caps at the end.

5. Using a multichannel pipette, transfer 2.5µL from the sample the PCR index plate. The remaining volume of purified PCR can be stored at -20C for other uses.

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6. Mix the plate, seal and spin for a minute.

7. Perform PCR on a thermal cycler using the following program:

• 95°C for 3 minutes
• 8 cycles of:
—95°C for 30 seconds
—55°C for 30 seconds
—72°C for 30 seconds
• 72°C for 5 minutes
• Hold at 4°C

8. Once the PCR is done, perform the purification of the products as **item 3.2** and labchip for size and concentration as **item 3.3 of this protocol**. The size of the fragments should increase an average of 200 bp after the addition of the indices and adapters.

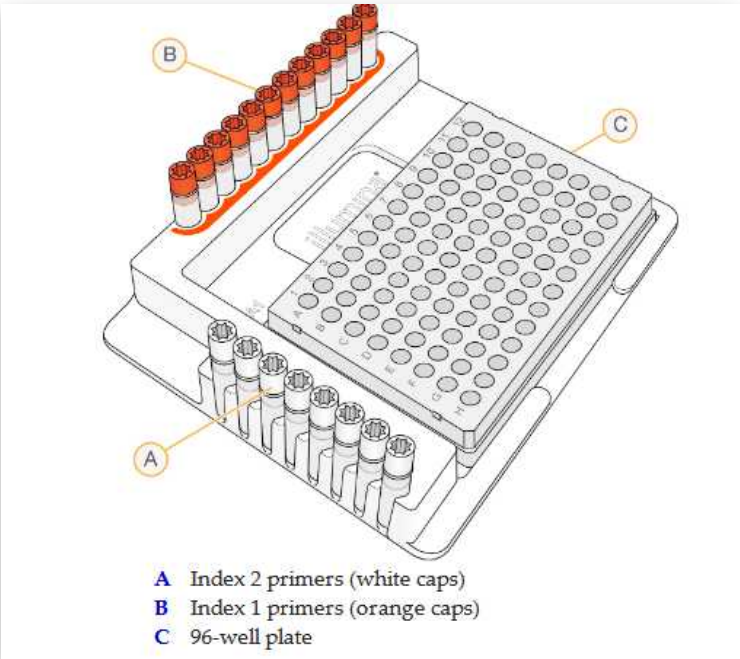

Figure 12: Index Plate Fixture.

Library Quantification, Normalization, and Pooling

3.5 Calculate DNA concentration in nM, based on the size of DNA amplicons and ng/µl as determined by labchip trace:

(concentration in ng/µl) / (660 g/mol × average library size) × 106= concentration in nM

Dilute the index-PCR samples, individually, to the same concentration using 10mM Tris pH 8.0. The illumina protocol for “library denaturation and sample load” works with two initial library concentrations : 4 and 2nM.

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For concentrated samples, we have stablished an intermediate concentration of 20nM or 10nM that will be subsequently diluted to either 4nM or 2nM respectively.

For samples with low concentrations, we have adapted the “library denaturation and sample load” protocol to work with a library of 1nM.

To calculate the dilutions, please look the **excel file: Illumina_LibraryPool_Model.xlsx**.
The volumes used in Illumina_LibraryPool_Model.xlsx are just examples, please adapted to your samples and ALWAYS avoid to pipette very small volumes.

Aliquot 5µl of diluted DNA from each sample with unique indices to a final pool containing all the diluted samples and controls in one eppendorf tube. Control of DNA concentration of pool with QuBit.

Qubit fluorometer with ds DNA HS Assay Kit (range 0.2-100ng)

Preparation of the QuBit solution : 597µl buffer (provided) + 3µl dye (provided)

190µl QuBit solution + 10µl standart1 (provided), vortex, centrifuge
190µl QuBit solution + 10µl standart2 (provided), vortex, centrifuge

196µL QuBit solution +4µL samples pool, vortex, centrifuge

incubate 2 minutes RT
vortex and centrifuge just before the measure

Measure the standart then the samples pool following the instructions on Genomer Platform.

NB: 1ng/µL = approximately 500pb = approximately 3nM

Sample sheet file

3.6 1. Open IEM (Illumina Experiment Manager) software and Create a sample sheet (**Figure 13A**).

2. Choose **MiSeq** (**Figure 13B**)

3. Select category **others** and then **FastaQ only**

4. Write the barcode of the cartridge (it is written on the cartridge). If the samples have been prepared with one unique nextera set kit, choose the appropriate kit from A to D. If several plates will be load in the same run, choose **Nextera XT v2** (**Figure 13C**).

5. Choose **Index reads 2**

6. Add experiment name, data, choose paired end and number of cycles for read 1 and

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for read 2. For example, if the kit is for 500 cycles (2 x 250), 251 will be read 1 and 251 for read 2.

7. Choose **Use adapter trimming**

8. Choose **select plate** (**Figure 13D**) and open the file "Project_gene_date.**nexxt28.plt**" saved during the sample plate preparation step. The plate should appear at the region 1.

9. Check the order and indices of the samples and **select all** or only the samples to be loaded on the run by clicking in the samples row. Then click on **selected samples**. The list of samples with their respective barcode will appear in the region 2 (**Figure 13E**).

10. If the different plates are from different primers, you just need to add one plate to the run (the one which has more samples). The de-replication will be done with bioinformatic tools.

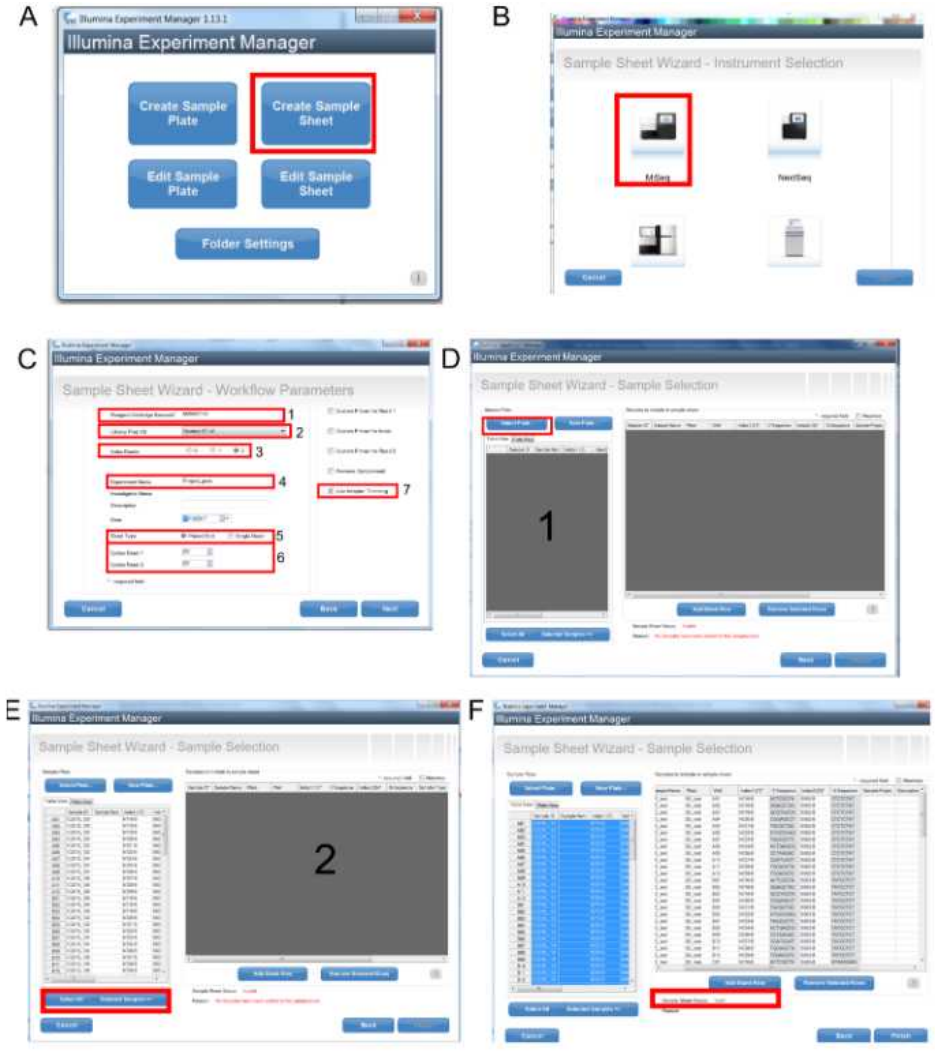

Figure 13: Creating sample sheet file.

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11. Check if the sample sheet status is **valid** (**Figure 13F**) and click on **Finish**. Save the .csv file that will be load in the MiSeq.
If not, read the reason why it is invalid and correct that.

Denaturation of the library and PhiX control

3.7 This step is required to denature and dilute libraries after sample preparation to prepare them for loading into the MiSeq cartridge. Here we explain the different steps for 3 libraries concentrations: 4nM, 2nM and 1nM.
For more details concerning 4 and 2nM libraries, please look [miseq-denature-dilute-libraries-guide_#15039740](#) (protocol A).

✓ **Prepare solutions**

Prepare HT1

The tube of HT1 (Hybridization Buffer) is used to dilute denatured libraries before loading libraries onto the reagent cartridge for sequencing.

-Remove the tube of HT1 from -15°C to -25°C storage and thaw at room temperature.
-When thawed, store at 2°C to 8°C until you are ready to dilute denatured libraries.

Prepare a Fresh dilution of NaOH

Using freshly diluted NaOH is essential in denaturing libraries for cluster generation on the MiSeq. A fresh dilution of NaOH is required for the denaturation process in preparing sample DNA and preparing a PhiX control. After preparing the sample DNA, you can set aside remaining NaOH if you plan to prepare a PhiX control within the next 12 hours. Otherwise, discard the remaining dilution of NaOH.

A stock solution of 1N NaOH is necessary. The stock solution should be also prepared in advance or bought. The platform has a stock that can be eventually use.

•To prepare a **stock solution of 1N NaOH** from powder: add 0.4g of NaOH molecular grade to 10ml of molecular water in a falcon tube. Invert the tube several times to mix

•To prepare 1ml of **0.2N NaOH** combine the follow the following volumes in a microcentrifuge tube: 800µl of Molecular grade water and 200µl of stock 1.0N NaOH. Invert the tube several times to mix

•To prepare 1ml of **0.25N NaOH** combine the follow the following volumes in a microcentrifuge tube: 750µl of Molecular grade water and 250µl of stock 1.0N NaOH. Invert the tube several times to mix

✓ **Denaturation of the library**

Starting with 4nM library

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Requires a 4 nM library. Supports high library concentrations (10–20 pM) and rResults in a 20pM DNA solution in 1 mM NaOH.

1. Combine the following volumes of sample DNA and freshly diluted 0.2N NaOH in a microcentrifuge tube: 5µl of **4nM sample DNA** and 5µl of 0.2N NaOH. Set aside the 0.2N of NaOH to futher prepare the PhiX control

2. Vortex briefly to mix the sample solution, and then centrifuge the sample solution to 280 × g for 1 minute.

3. Incubate for 5 minutes at room temperature to denature the DNA into single strands.

4. Add the following volume of pre-chilled HT1 to the tube containing denatured DNA: 10µl of Denatured DNA and 990µl of Pre-chilled HT1. **The result is a 20pM denatured library in 1mM NaOH**.

5. Place the denatured DNA on ice until you are ready to proceed to final dilution.

6. Use the following instructions to dilute the 20pM DNA further to give 600µl of the desired input concentration.

We have successfully worked with 6pM libraries; however this concentration should be adjusted to the type of samples.

Concentration	6 pM	8 pM	10 pM	12 pM	15 pM	20 pM
20 pM library	180 µl	240 µl	300 µl	360 µl	450 µl	600 µl
Prechilled HT1	420 µl	360 µl	300 µl	240 µl	150 µl	0 µl

Table 4: Dilute Denatured 20pM Library to the desired concentration using the following volumes.

7. Invert several times to mix and then pulse centrifuge the DNA solution.

8. Place the denatured and diluted DNA on ice until you are ready to load your samples onto the MiSeq reagent cartridge.

Starting with 2nM library

1. Combine the following volumes of sample DNA and freshly diluted 0.2N NaOH in a microcentrifuge tube: 5µl of **2nM sample DNA** and 5µl of 0.2N NaOH. Set aside the 0.2N of NaOH to futher prepare the PhiX control

2. Vortex briefly to mix the sample solution, and then centrifuge the sample solution to 280 × g for 1 minute.

3. Incubate for 5 minutes at room temperature to denature the DNA into single strands.

4. Add the following volume of pre-chilled HT1 to the tube containing denatured DNA: 10µl of Denatured DNA and 990µl of Pre-chilled HT1. **The result is a 10pM**

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denatured library in 1mM NaOH.

5. Place the denatured DNA on ice until you are ready to proceed to final dilution.

6. Use the following instructions to dilute the 10pM DNA further to give 600 µl of the desired input concentration.

We have successfully worked with 6pM libraries; however this concentration should be adjusted to the type of samples.

Concentration	6 pM	8 pM	10 pM
10 pM library	360 µl	480 µl	600 µl
Prechilled HT1	240 µl	120 µl	0 µl

Table 5: Dilute Denatured 10pM Library to the desired concentration using the following volumes.

7. Invert several times to mix and then pulse centrifuge the DNA solution.

8. Place the denatured and diluted DNA on ice until you are ready to load your samples onto the MiSeq reagent cartridge.

Starting with 1nM library

1. Combine the following volumes of sample DNA and freshly diluted 0.2N NaOH in a microcentrifuge tube: 6µl of **1nM sample DNA** and 4µl of 0.25 N NaOH. Discard the remaining dilution of 0.25N of NaOH and set aside the 0.2N of NaOH to futher prepare the PhiX control.

2. Vortex briefly to mix the sample solution, and then centrifuge the sample solution to 280 × g for 1 minute.

3. Incubate for 5 minutes at room temperature to denature the DNA into single strands.

4. Add the following volume of pre-chilled HT1 to the tube containing denatured DNA: 10µl of Denatured DNA and 990µl of Pre-chilled HT1. **The result is a 6pM denatured library in 1 mM NaOH**.

5. Place the denatured and diluted DNA on ice until you are ready to load your samples onto the MiSeq reagent cartridge. No further dilution is necessary.

✓ **Preparing PhiX Control**

Low diversity libraries are libraries where a significant number of the reads have the same sequence. This lack of variation shifts the base composition because the reads are no longer random. Low diversity can occur in low plexity amplicon pools such as those originated from sorted cells, adapter dimer, or bisulfite sequencing, for example. A higher concentration spike-in of PhiX helps balance the overall lack of sequence diversity.

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20 pM PhiX Control (required for v3 kit)

1. Dilute PhiX to 4nM:
Combine the following volumes to dilute the PhiX library to 4 nM:
- 2µl of 10nM PhiX library
- 3µl of 10mM Tris-Cl, pH 8.0 with 0.1% Tween 20 **(prepare fresh solution with molecular grade Tris and water)**.
The result is 4nM PhiX control solution.

2. Denature PhiX Control:
Combine the 5µl of 4 nM PhiX control and 5µl of freshly diluted 0.2N NaOH in a microcentrifuge tube.
The result is 2nM PhiX denaturated control solution.

3. Vortex briefly to mix the 2nM PhiX library solution.

4. Centrifuge the template solution to 280 × g for 1 minute.

5. Incubate for 5 minutes at room temperature to denature the PhiX library into single strands.

6. Dilute Denatured PhiX to 20pM:
Add the 990µl of **pre-chilled HT1** to the tube containing 10µl of the **denatured PhiX library** to **result in a 20 pM PhiX library**.
The denatured 20 pM PhiX library can be store up to 3 weeks at -15° to -25°C. After 3 weeks, cluster numbers tend to decrease. Check if there is a library already diluted at the platform.

12,5pM PhiX Control (required for v2kit)

1. Combine 375µL of **denatured 20pM PhiX library** with 225µl **pre-chilled HT1**.

2. Invert several times to mix the solution and discard the remaining dilution of 0.2N NaOH.
The result is a 12,5pM PhiX library.

Combine Sample Library and PhiX Control

3.8 Illumina recommends a low-concentration PhiX control spike-in at 1% for most libraries. For low diversity libraries, increase the PhiX control spike-in to at least 5%.

We have used 1% for libraries prepared from filter samples and 5% for sorted samples.

1. Combine the following volumes of denatured PhiX control library and your denatured sample library.

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	Most Libraries (1% Spike-In)	Low-Diversity Libraries (≥ 5% Spike-In)
Denatured and diluted PhiX	6 µl	30 µl
Denatured and diluted library (from protocol A, B, C, or D)	594 µl	570 µl

Table 6: Combine sample library and PhiX control.

2. Set the combined sample library and PhiX control aside on ice until you are ready to load it onto the MiSeq reagent cartridge.

For more details of the following steps please look the MiSeq System User Guide: [miseq-system-guide #15027617]

Thaw & Mix Reagent Cartridge

3.9 For more details, please follow the instruction at [miseq-system-guide #15027617/ chap3. sequencing].

1. Remove the reagent cartridge from -25°C to-15°C storage.

2. Place the reagent cartridge in a waterbath containing enough room temperature deionized water to submerge the base of the reagent cartridge. (Do not allow the water to exceed the maximum waterline printed on the reagent cartridge.)

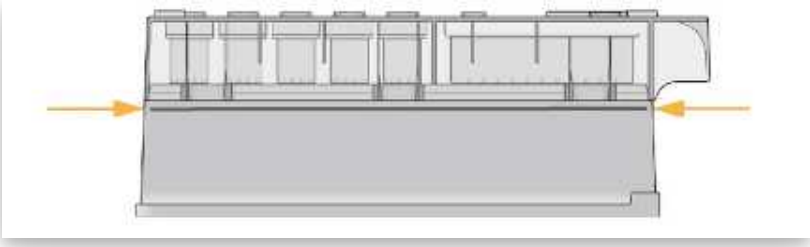

Figure 14: Maximum water line.

3. Thaw in the room temperature waterbath until it is thawed completely.
• MiSeq v3 cartridges—~ 60-90 minutes.
• MiSeq v2 cartridges—~ 60 minutes.

4. Remove the cartridge from the waterbath and gently tap it on the bench to dislodge water from the base of the cartridge. Dry the base of the cartridge

5. Invert the reagent cartridge 10 times to mix the thawed reagents, and then inspect that all positions are thawed.

6. Inspect the reagents in positions 1, 2, and 4 to make sure that they are fully mixed and free of precipitates.

7. Gently tap the cartridge on the bench to reduce air bubbles in the reagents.

Store at 4°C until the loading (max 6 hours).

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Load Sample Libraries

3.10 For more details, please follow the instruction at [miseq-system-guide #15027617 / chap.3 sequencing]

1. Clean the foil seal covering the reservoir labeled
Load Samples with a low-lint lab tissue.

2. Pierce the foil seal with a clean 1 ml pipette.

3. Pipette 600 µl prepared libraries into the reservoir "Load Samples". Avoid touching the foil seal.

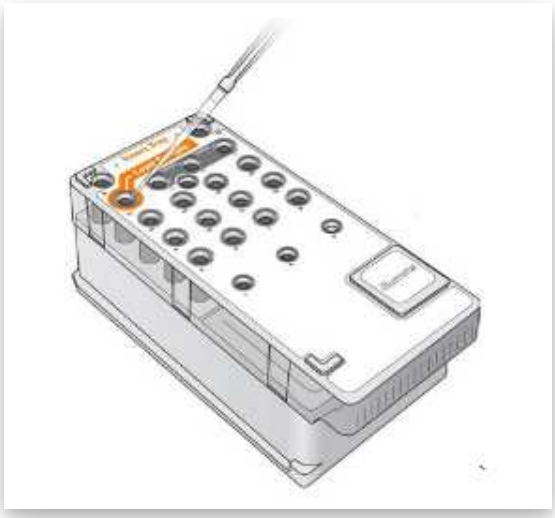

Figure 15: Load libraries in Illumina Cartdridge.

4. Proceed directly to the run setup steps using the MiSeq Control Software (MCS) interface

Set Up a Run Using MiSeq Control Software (MCS)

3.11 MiSeq Control Software controls instrument operation.

The software interface guides you through the steps to load the flow cell and reagents before beginning the run, post-run and maintenance wash. An overview of quality statistics appears as the run progresses.

1. If the MiSeq is not already on, reach around the right side of the instrument to locate the power switch on the back panel. It is in the lower corner directly above the power cord.

2. Turn the power switch to the ON position. The integrated instrument computer starts.

3. Wait until the operating system has finished loading. When the system is ready, the MCS launches and initializes the system automatically. After the initialization step is complete, the **Home screen appears**.

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4. Performa stand by wash or maintenance accordingly to the platform.

5. From the Home screen, select **Sequence** to begin the run setup steps. The **BaseSpace Options screen opens**.

6. From the BaseSpace Options screen, clear the Use BaseSpace for storage and analysis/Use BaseSpace Onsite for storage and analysis check boxes.
When you select Sequence on the Home screen, a series of run setup screens open inthe following order: BaseSpace Option, Load Flow Cell, Load Reagents, Review, andPre-Run Check.

Cleaning and loading flow cell, and reagents

3.12 For more details, please follow the instruction at [miseq-system-guide #15027617 / chap.3 sequencing](#).

Clean the Flow Cell

1. Put on a new pair of powder-free gloves.

2. Grip the flow cell by the base of the grey plastic cartridge and remove it from the flow cell container (Figure 16A).

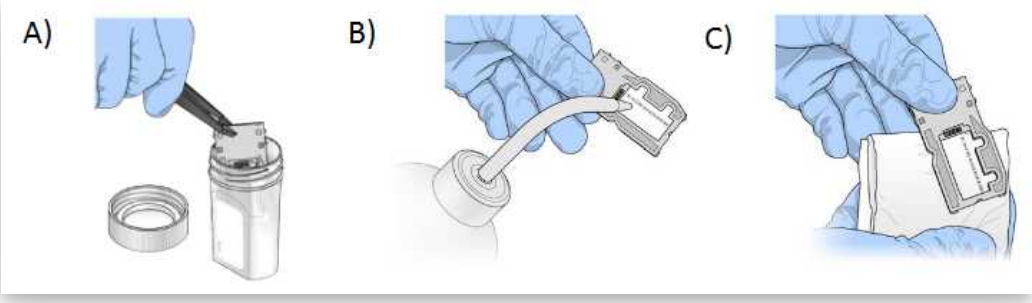

Figure 16: Clean & dry the flow cell.

Figure 17: Holding the flow cell by the edges.

3. Rinse the flow cell with milliQ water until both the glass and plastic cartridge are thoroughly rinsed of excess salts (Figure 16B).

4. Using care around the black flow cell port gasket, thoroughly dry the flow cell and cartridge with a kimwipe tissue. Gently pat dry in the area of the gasket and adjacent glass (Figure 16C).

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The flow cell must be dry and clean.

Load the Flow Cell

1. Open the flow cell compartment door, and then press the button to the right of the flow cell clamp (Figure 18A).
The flow cell clamp opens.

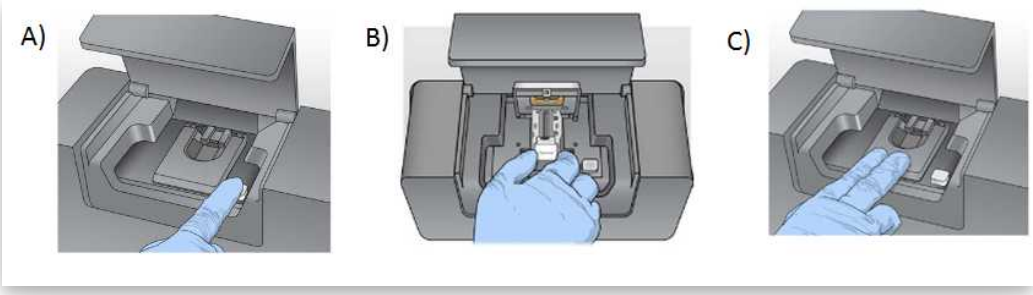

Figure 18: Load the flow cell.

2. Make sure that the flow cell stage is clean. If not, carefully wipe the surface of the flow cell stage until it is clean and dry.

3. Place it on the flow cell stage (Figure 18B).

4. Gently press down on the flow cell clamp to close it over the flow cell (Figure 18C). An audible click indicates that the flowcell clamp is secure.

5. If the software does not identify the flow cell RFID, see the intructions in User guide.

6. Close the flow cell compartment door.

7. Select **Next**.

Load Reagents

1. Remove the bottle of PR2 from 2° to 8°C storage. Invert to mix, and then remove the lid.

2. Open the reagent compartment door.

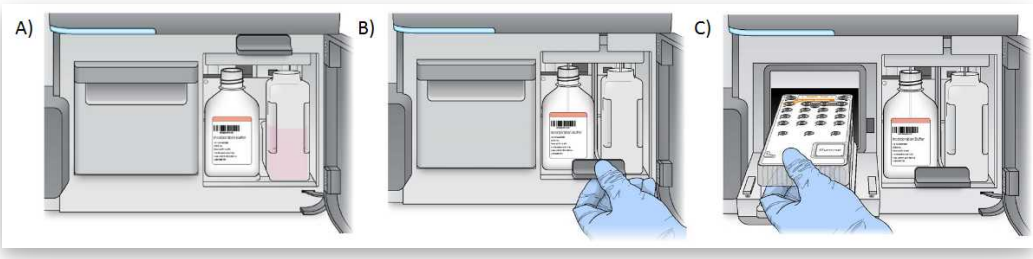

Figure 19: Load reagent and cartdridge

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3. Raise the sipper handle until it locks into place (Figure 19B).

4. Remove the wash bottle and load the PR2 bottle (Figure 19A).

5. Empty the waste bottle into the appropriate waste container.

6. Slowly lower the sipper handle. Make sure that the sippers lower into the PR2 and waste bottles.

7. If the software does not identify the RFID of the PR2 bottle, see the intructions in User guide.

8. Select **Next.**

Load the Reagent Cartridge

1. Open the reagent chiller door. (Do not leave it open for a long time.)

2. Hold the reagent cartridge on the end with the Illumina label, and slide the reagent cartridge into there agent chiller until the cartridge stops (Figure 19C).

Select **Back** to load the appropriate reagent cartridge or Home to return to the Home screen.

3. Close the reagent chiller door.

4. If the software does not identify the RFID of the reagent cartridge, see the intructions in User guide.

5. Close the reagent compartment door.

6. Select **Next**

Starting the Run

3.13 1. After loading the flow cell and reagents, 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.
If any changes are needed, select **Change Folders**.
When the changes are complete, select **Save**, and then select **Next**.

2. The Pre-Run Check screen opens. The system performs a check of all run components, disk space, and network connections before starting the run. If any items do not pass the pre-run check, a message appears on the screen with instructions on how to correct the error.

3. When all items successfully pass the pre-run check, select **Start Run**. Make sure to close all files on the MiSeq before starting a run, and do not open files during a run. **The MiSeq is sensitive to vibration. Touching the instrument after starting a**

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run could adversely affect sequencing results.

4. After selecting Start Run, do not open the flow cell compartment or the reagent compartment doors, or touch the instrument monitor except to pause the run, if necessary.

Monitoring the Run

3.14 During the run, monitor run progress, intensities, and quality scores that appear on the Sequencing screen. The Sequencing screen is view-only.

Four parameters will inform if the run will be successful or not, and they will be available after cycle 25 :

Q-Score All Cycles — Shows the average percentage of bases greater than Q30, which is a quality score (Q-score) measurement. A Q-score is a prediction of the probability of a wrong base call.

Phred Quality Score	Probability of Incorrect Base Call	Base Call Accuracy
10	1 in 10	90%
20	1 in 100	99%
30	1 in 1,000	99.9%
40	1 in 10,000	99.99%
50	1 in 100,000	99.999%

Table 7: Quality scores & base calling accuracy

Cluster Density (K/mm²) — Shows the number of clusters per square millimeter for the run.
• MiSeq v2 reagents accommodate an optimal raw cluster density of 865–965 K/mm²
• MiSeq v3 reagents accommodate an optimal raw cluster density of 1200–1400 K/mm²

Clusters Passing Filter (%) — Shows the percentage of clusters passing filter based on the Illumina chastity filter, which measures quality.

Estimated Yield (Mb) — Shows the projected number of bases called for the run, measured in megabases. These parameters will differ depending on the kit use, for more information please look the link: MiSeq specifications

Perform a Post-Run Wash WITHOUT template line wash

3.15 For more details, please follow the instruction at miseq-system-guide #15027617 / chap.3 sequencing.

Always perform an instrument wash after completing a run.

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Follow the software prompts to load the wash components and perform the wash.The **post-run wash** takes approximately 20 minutes.

Leave the used flowcell on the instrument. A flowcell must be loaded on the instrument to perform an instrument wash.

1. Prepare fresh wash solution with Tween 20 and laboratory-grade water:
a. Add 5 ml 100% Tween 20 to 45 ml laboratory-grade water. These volumes result in 10% Tween 20.
b. Add 25 ml 10% Tween 20 to 475 ml laboratory-grade water. These volumes result in a 0.5% Tween 20 wash solution.
c. Invert 5 times to mix.

2. Prepare the wash components with fresh wash solution:
a. Add 6 ml wash solution to each reservoir of the wash tray.
b. Add 350 ml wash solution to the 500 ml wash bottle.

3. When the run is complete, select **Start Wash**. The software automatically raises the sippers in there agent chiller. **Do not select** Perform optional template line wash on the Post-Run wash screen.

4. Open the reagent compartment door and reagent chiller door, and slide the used reagent cartridge from the chiller.

5. Slide the wash tray in to the reagent chiller until it stops, and then close the reagent chiller door.

6. Raise the sipper handle in front of the PR2 bottle and waste bottle until it locks into place.

7. Remove the PR2 bottle and replace it with the wash bottle. NOTE Discard the PR2 bottle after each run.

8. Remove the waste bottle and discard the contents as chemical waste (formamide) in accordance with the safety standards. Return the waste bottle to the reagent compartment.

9. Slowly lower the sipper handle, making sure that the sippers lower into the wash bottle and waste bottle.

10. Close the reagent compartment door.

11. Select **Next**.
The post-run wash begins.
When the wash is complete, leave the used flowcell, wash tray, and wash bottle containing the remaining wash solution on the instrument

NB: The template line wash with sodium hypochloritesolution (NaOCl) requires a different procedure.

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Run performance

3.16 For details in the run performance open the run folder in the **Illumina Sequencing Analysis Viewer (SAV)** which can be downloaded and installed in your personal computer.

For the parameters to analyze, look **support.illumina/miseq-overclustering-primer.pdf**

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