Protocol for 16S and ITS amplicon sample preparation from microbial DNA contaminated with plant material

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Author: Erika Yashiro, Ph.D.

Version 1.0

(This protocol has been updated from 190501_Ebstd_Nextera Amplicon Sequencing v1.6.docx)

Introduction

This protocol describes how to make amplicon sequencing libraries of the prokaryotic 16S rRNA gene hypervariable region and eukaryotic ITS1 of the rrn operon.

The 16S rRNA gene and ITS region are used as universal phylogenetic markers for distinguishing and classifying bacteria, archaea, fungi, and oomycetes. Targeting the 16S and ITS regions enables whole bacterial, archaea, fungi, and oomycete community analysis with a resolution ranging from taxonomic family to species level depending on the variable region used and the phylogenetic branches investigated. The library preparation is PCR based and relies on PCR primers targeting conserved sequence regions between the hypervariable regions in the prokaryotic 16S rRNA gene and eukaryotic ITS1 region. This means that the analysis will not be able to capture microbial taxa whose sequences deviate from the consensus sequences of the targeted conserved regions.

The protocol for bacteria has been optimized for reducing amplification from mitochondria and plastid DNA when using DNA samples containing high concentrations of plant material. Typical strategies used to reduce host plant organelle amplification include discriminating PCR primers (that do not amplify host plastids), addition of peptide nucleic acids (PNAs) targeting and blocking amplification of plant organelles, addition of blocking primers similar to PNAs, or a combination of these strategies. This protocol covers two strategies:

- The V4 bacterial primers + mPNA (PNA to block mitochondria) + pPNA (PNA to block chloroplast). Both mPNA and pPNA target the same region as the bacterial V4 region.
- The V5-V7 bacterial primers (discriminates against chloroplast) + m57PNA (PNA to block mitochondria in the V5-V7 region)

If host organelle content is to be used to quantify microbial load (e.g. in Humphrey et al. 2020), use the protocol with the V4 bacterial primers without PNAs.

Materials needed

Instruments

- Infinite M2000 PRO (Tecan) or similar
- Thermocycler
- Magnetic stand for bead cleanup

- Tapestation 2200 (Agilent), alternatively BioAnalyzer OR standard electrophoresis gel capable of 3% agarose gels
- Pipet (Range 1 ul to 1000 ul, with filter tips)
- Multichannel pipet (Range 1 ul to 200 ul, with filter tips)
- Microcentrifuge
- 96-well plate compatible centrifuge
- Water bath

Reagents/consumables

- DNAse free tips (1 ul, 10 ul, 200-300 ul, 1000 ul) *
- DNAse free microcentrifuge tubes (1.5 mL)
- 96-well PCR plates
- PCR strip caps (compatible with the 96-well PCR plates), **alternatively** silicon plate seals
- Sterile aluminum PCR plate seals (have a couple on reserve)
- Plate seal rollers or thick knitted fabric
- Reagent reservoir (e.g. tip tub)
- OptiPlate-96 Black (#82006-664, VWR)
- Nuclease free water (e.g. Qiagen)
- Qubit dsDNA HS assay kit (Thermo Fisher Scientific)
- Qubit dsDNA BR assay kit
- PCRBIO Ultra 2x mix (PCRBIO vendor Nippon Genetics Europe)
- PCRBIO HiFi polymerase (PCRBIO vendor Nippon Genetics Europe)
- dNTP mix
- Tailed 16S/ITS PCR primers (1uM of each forward and reverse)
- Barcoded Nextera adapter mixes (1 uM of each forward and reverse adaptor)
- Agencourt AMPure XP (Beckman Coulter)
- EtOH 99%
- D100 Screentapes (Agilent) for Tapestation, **alternatively** agarose and TBE/TAE
- Genomic DNA Screentapes (Agilent) for Tapestation, **alternatively** agarose and TBE/TAE
- Standard positive control samples (made in-house and tested prior to deployment)
    - DNA extracted using same methods as experimental samples
    - Ampure bead cleaned PCR product
    - Ampure bead cleaned amplicon library

* If filter tips are unavailable due to Covid-19, use non-filtered tips. Low-binding pipet tips are highly recommended for accurate quantification of DNA using the Qubit system.

Oligos

Below is the list of the standard primers (right of the hyphen) with the tails (left of the hyphen) that allow the Nextera adapters to bind during the second PCR. Here, the tailed primers will be named Nex-[primer name]. The references refer to the primers without the Nextera tails.

Name	**Bacteria 16S V4**	**Ref.**
Nex-515F-Y	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-GTGYCAGCMGCCGCGGTAA	[1]
Nex-806RB	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GGACTACNVGGGTWTCTAAT	[1]
Name	**Bacteria 16S V5-V7**	**Ref.**
Nex-779F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-AACMGGATTAGATACCCKG	[2]
Nex-1193R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-ACGTCATCCCCACCTTCC	[2]
Name	**Fungi ITS1**	**Ref.**
Nex-ITS1-F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CTTGGTCATTTAGAGGAAGTAA	[3]
Nex-ITS2	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GCTGCGTTCTTCATCGATGC	[3]
Name	**Oomycetes ITS1**	**Ref.**
Nex-ITS1-O	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CGGAAGGATCATTACCAC	[2]
Nex-5.8s-O-Rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-AGCCTAGACATCCACTGCTG	[2]
Name	**Archaea**	**Ref.**
tbd		
Name	**Peptide Nucleic Acids (PNAs)**	**Ref.**
mPNA (V4)	GGCAAGTGTTCTTCGGA	[4]
pPNA (V4)	GGCTCAACCCTGGACAG	[4]
m57PNA (V5-7)	tbd	

[1] Walters W., E. R. Hyde, D. Berg-Lyons, G. Ackermann, G. Humphrey, *et al.*, 2015 Improved Bacterial 16S rRNA Gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Surveys. *mSystems* 1: e00009-15. https://doi.org/10.1128/mSystems.00009-15

[2] Thiergart T, Durán P, Ellis T, Vannier N, Garrido-Oter R, Kemen E, et al. Root microbiota assembly and adaptive differentiation among European Arabidopsis populations. *Nat Ecol Evol* 2020; **4**: 122–131.

[3] Bellemain E, Carlsen T, Brochmann C, Coissac E, Taberlet P, Kauserud H. ITS as an environmental DNA barcode for fungi: an in silico approach reveals potential PCR biases. *BMC Microbiology* 2010; **10**: 189.

[4] Lundberg DS, Yourstone S, Mieczkowski P, Jones CD, Dangl JL. Practical innovations for high-throughput amplicon sequencing. *Nat Methods* 2013; **10**: 999–1002.

The current maximum amplicon length for MiSeq is ca. 550bp.

Primer pair	**Amplified region**	**Amplicon length (bp)**	**Library length (bp)**
515F-Y & 806RB	V4 (Bacteria)	253-290*	391-425*
799F & 1193R	V5-V7 (Bacteria)	377	515
ITS1-F & ITS2	ITS1 (Fungi)	230-357	350*-495
ITS1-O & 5.8s-O-Rev	ITS1 (Oomycetes)	?	?

Ref: Francioli D, Lentendu G, Lewin S, Kolb S. DNA Metabarcoding for the Characterization of Terrestrial Microbiota—Pitfalls and Solutions. *Microorganisms* 2021; **9**: 361.

* The approximate value was empirically obtained previously by Erika Yashiro.

Step by step procedure

Planning samples barcoding

1. Assign barcoded adapters to samples
2. Use the spreadsheet (file name: Amplicon barcode cheatsheet.xlsx)

Input sample material and QC

DNA samples must be extracted using the exact same procedure if they are to be compared. For example, use the PowerSoil 96-well kit for all samples, and do not mix single tube and 96-well PowerSoil kits in the same project.

The PCR settings and cleanup steps depend on the target amplicon and its origin. Be sure to check the PCR steps of the protocol in order to ensure that they are compatible with your sample type.

For >5 samples out of every 96 samples:

1. Nanodrop UV-Vis Quality and Concentration Check.
    a. Follow vendor recommended protocol
    b. A260/280 should be 1.8-2.0. A2600/230 2.0 to 2.2. However most times, the environmental samples are too dirty to obtain such perfect scores on the Nanodrop. Don't worry excessively if this is the case, but do record your data and screen shot the Nanodrop curves for safe-keeping.
2. Tapestation OR electrophoresis gel.
    a. Use Genomic DNA Screentapes OR 0.8% agarose gel that has been cooled in a 50°C water bath for 30 minutes prior to pouring into the cast. Follow standard guidelines.
    b. For Tapestation: Qualitative range of the tapes is 3-300 ng/ul. Dilution might be necessary.
    c. For Tapestation: Remember to add the ladder to one of the wells.
    d. For agarose gel: Remember to add the ladder to either end of each row of wells and one in the middle of the row if you are loading many samples across a row of wells. The ladder must be able to resolve at 5-10kb.
    e. The majority of the DNA should be in the range of 5kB and above. If it is below that, the DNA is heavily degraded and this might affect analysis.

Sample DNA concentration

1. Measure DNA concentration using Quant-iT 1X dsDNA BR assay kit (manual # MAN0019618).
    a. Follow the protocol in step 2.
    b. Use 1 ul of sample.
    c. Perform single measurement for each sample. If all of the samples do not fit onto a single 96-well plate, then add a standard extracted DNA to one well of each plate; if the measurements for the standard sample is highly similar across the plates, then you probably have little to no plate-to-plate measurement discrepencies.
2. Quant-iT BR procedure:
    a. Equilibrate the Quant-iT BR 1X assay bottle and standard lambda solutions to room temperature.
    b. Load **195 uL** of Quant-iT BR 1X solution in each microplate well **of column 1** with a multichannel pipetter. Load **199 uL** of Quant-iT BR 1X to the **sample wells and suggested control wells** (e.g. columns 2-12).
    c. Add 5 uL of each of the Quant-iT dsDNA BR standards to separate wells down one column (1A-1H) (Refer to tables below). Pipette 4 times with the same tip to wash out the DNA.
    d. Add 1 uL of each DNA sample to separate wells for columns 2-11. Pipette 4 times with the same tip to wash out the DNA.
    e. Add 1 uL of at least one of the positive control DNA, and leave 1 well empty as negative control.
    f. Mix the 96-well plate using a plate mixer or using the plate reader ("figure 8" shaking mode if possible) for 10 seconds.
    g. Let the plate incubate at room temperature for 2 minutes.
    h. Measure the fluorescence using a microplate reader (Excitation = 506 nm; Emission = 523 nm).
    i. Plot the lambda DNA standards (x-axis = ng; y-axis = relative fluorescence (RFU)). Calculate the regression line equation (y=ax+b) and convert it to calculate ng/sample: x=(y-b)/a
    j. Make sure that the positive controls concentrations are consistent across 96-well plates and the negative control well with no DNA reads as close as possible to 0 ng.
    k. Convert the ng/sample to ng/uL. Remember that 1 uL was added to each well during quantification so the ng amount you measured is the ng/uL of the original sample.
    l. For i-k, you can use the template Quant-it calculator Fluorescence_calculation_template.xlsx

Which lambda standards to add (5 uL / well), and reminder to add appropriate positive control(s) and a negative control. Sample wells are denoted as S:

	1	2	3	4	5	6	7	8	9	10	11	12
A	λ 0 ng/uL	S	S	S	S	S	S	S	S	S	S	+ soil
B	λ 5 ng/uL	S	S	S	S	S	S	S	S	S	S	+ rhizosphere
C	λ 10 ng/uL	S	S	S	S	S	S	S	S	S	S	+ rhizoplane
D	λ 20 ng/uL	S	S	S	S	S	S	S	S	S	S	+ root endosphere
E	λ 40 ng/uL	S	S	S	S	S	S	S	S	S	S	+ phyloplane
F	λ 60 ng/uL	S	S	S	S	S	S	S	S	S	S	+ plhylosphere
G	λ 80 ng/uL	S	S	S	S	S	S	S	S	S	S	+ leaf endosphere
H	λ 100 ng/uL	S	S	S	S	S	S	S	S	S	S	water

Amount of DNA (ng) in each standard well:

	1	2	3	4	5	6	7	8	9	10	11	12
A	λ 0 ng											
B	λ 25 ng											
C	λ 50 ng											
D	λ 100 ng											
E	λ 400 ng											
F	λ 300 ng											
G	λ 400 ng											
H	λ 500 ng											

Sample dilution

1. Based on the Qubit measurements, dilute the extracted genomic DNA to 5 ng/ul with nuclease free water. Do this by transferring appropriate amount of gDNA to a sterile PCR plate and adding UV-irradiated nuclease-free water. There are a couple different ways to do this, choose one of the methods.
    a. General: transfer systematically a set volume 5 ul of gDNA to a PCR plate. Then add appropriate volume of water to dilute the DNA to 5 ng/ul. If you have very concentrated gDNA, then adjust the input DNA volume to < 5 ul to avoid sample overflow from the wells.
    b. For expensive gDNA: Add appropriate volume of water for 1ul of each gDNA sample to the PCR plate, then add 1 ul of gDNA and pipet and stir the solution well.

Preparation for Amplicon PCR

1. Clean the laminar flow hood working space with 70% EtOH or chlorite solution.
2. UV irradiate the flow hood while collecting all the needed materials (~5-10 min). Turn off UV in the flow hood.
3. Put all of the required equipment in the laminar flow hood (pipetters with the tip side facing the UV lamp, tips, dispensing tubs, water, tubes, PCR plates, PCR caps OR PCR silicon seals, aluminum plate seal, fresh emptied trash container). Make sure that the bottle of nuclease-free water is capped off and facing the UV lamp. UV irradiate for 15-20 minutes. Do no place enzymes and DNA in under UV.
4. Once UV period is over, recap the nuclease-free water. Put the PCR caps or silicon seals to the back corner of the hood where the sterile air is flowing over them without contaminating obstacles. The strip caps/seals are the last things to be used so they need to stay sterile throughout the setup procedure.

Amplicon PCR

The amplicon PCR reaction is run in duplicate for each sample to reduce the impact of PCR drift. The reagents/template for the duplicates are mixed together in the same PCR tube and afterwards divided into two duplicate reactions. Make sure to reserve TWO identical well calibrated thermocyclers for each 96

samples run. When a Tapestation machine is not available DNA quality spot-checking, select ~8 samples which you will run 2 sets of reactions on the same PCR plate. The duplicated product will run on a 2% agarose gel AFTER PCR product cleanup.

1. Thaw the PCRBIO 2x Ultra Mix and primers and put on ice.
2. Remember negative control (Nuclease free water) and positive control (known environmental sample of the same type that used the same extraction method and contains similar known inhibitors of PCR) for the PCR.
3. Always prepare 5% more master mix than needed.
4. Prepare master mix:

Bacteria V4			
Reagents	1x duplicate reaction (µl)	N x duplicate reactions (µl)	Final concentration
Nuclease free water	0		-
PCRBIO 2x Ultra Mix	25		1x
Tailed primer F (5µM)	4		400 nM
Tailed primer R (5µM)	4		400 nM
mPNA (5µM) (for bacterial V4)	6.5		600 nM
pPNA (5µM) (for bacterial V4)	6.5		600 nM
Total volume	46		

Bacteria V5-7 / Archaea V4			
Reagents	1x duplicate reaction (µl)	N x duplicate reactions (µl)	Final concentration
Nuclease free water	13		-
PCRBIO 2x Ultra Mix	25		1x
Tailed primer F (5µM)	4		400 nM
Tailed primer R (5µM)	4		400 nM
Total volume	46		

Fungal ITS1			
Reagents	1x duplicate reaction (µl)	N x duplicate reactions (µl)	Final concentration
Nuclease free water	13		-
PCRBIO 2x Ultra Mix	25		1x
Tailed primer ITS1-f (5µM)	4		400 nM
Tailed primer ITS2 (5µM)	4		400 nM
Total volume	46		

Oomycetes ITS1			
Reagents	1x duplicate reaction (µl)	N x duplicate reactions (µl)	Final concentration
Nuclease free water	13		-
PCRBIO 2x Ultra Mix	25		1x
Tailed primer ITS1-O (5µM)	4		400 nM
Tailed primer 5.8s-O-Rev (5µM)	4		400 nM
Total volume	46		

List of duplicated samples for agarose gel checking later:

Sample	Type	Sample	Type

5. Transfer 46 ul of mastermix to the wells of a 96-well plate.
6. Add 4 ul of template DNA (ca. 20 ng DNA for bacteria and fungi)

7. Mix content of each well by pipetting up and down 10 times and then transfer 25 ul of each reaction to the same wells in a new 96-well PCR plate. The final result should be duplicate 96-well PCR plates with 25 ul reaction volume in each well.
8. Seal adaptor plate wells with 8-strip caps. Check that all wells have been properly sealed.
9. Briefly centrifuge the PCR plate to bring down all of the solution to the bottom of the wells and ensure that there are no bubbles at the bottom of the wells.
10. Run variable region specific PCR program. The anneleaing temperature is the same for both the V4 and ITS1 (fungi and oomycetes) amplicons and elongation temperature is more than sufficient for these regions:

Amplicon PCR		
Temp	Time	
110 °C	Heated lid	
95 °C	2 min	
95 °C	15 sec	25-30 cycles
55 °C	15 sec	
72 °C	50 sec	
72 °C	5 min	
4 °C	Hold	

List of sample type and PCR conditions:

Primer pair	Sample type	Ta	Cycling number	Extra step
Nex-799F / Nex-1193R	Rhizoplane	55C	30x	
Nex-799F / Nex-1193R	Root	55C	30x	Gel remove 800bp band
Nex-799F / Nex-1193R	Rhizosphere, soil	55C	25x	
Nex-ITS1-F / Nex-ITS2	All	55C	30x	
Nex-ITS1-O / Nex5.8s-O-Rev	All	55C	30x	

Note: Oomycete positive control should be 4ul CAS16-2 soil + 0.5-1ul of Albugo, both of them diluted to 5ng/ul. This is because CAS soil contains little detectable oomycete DNA.

11. Pool duplicates and perform cleanup.
12. After the PCR, the samples will be referred to as “amplicons”.

Amplicon Cleanup

Note: The root samples amplified with V5-V7 bacterial primers must be gel extracted rather than bead cleaned in order to remove the 800bp mitochondria DNA that co-amplifies. Follow kit protocols for gel extraction.

1. Gently shake Agencourt AMPure XP Bottle to resuspend the beands, remove required volume and let it equilibrate to room temperature.
2. Transfer 40 ul of bead solution to each pooled library (50 ul) and mix by pipetting 10 times up and down with a 100-200 ul pipette.
3. Incubate for 2 minutes at room temperature.

4. Place the amplicon mixture on a magnetic rack until liquid is clear (>1 minute). The container should stay on the rack until stated otherwise.
5. Remove liquid with pipette and discard it.
6. Wash bead-pellet with 200 ul **freshly** prepared 80 % EtOH by gently dispensing it over the beads with a pipette. Do not touch the pellet with the pipette tip! Let the EtOH rest for 30 sec and then remove the liquid. The more EtOH you can remove, the cleaner your final eluate will be.
7. **Repeat** above step 6.
8. Ensure no excess ethanol is left after the washes. If there is, remove it with a 10 uL pipette.
9. Air dry for approximately 5 minutes to evaporate the last of the ethanol. Longer dry times will dry out the pellet and make it difficult to resuspend, resulting in product loss.
10. Remove library plate from magnetic rack. Add 25 ul of nuclease free water and mix with pipette (>10 times up and down) to resuspend the beads.
11. Place the library plate back on the magnetic rack and wait until the liquid clears.
12. Transfer 20 ul of the liquid to a new plate.

Amplicon QC screen

1. Measure the DNA concentration of each individual library using Quant-iT 1X dsDNA HS assay kit.
    a. Follow the protocol in step 2.
    b. Use 2 ul of sample.
    c. Perform single measurement for each sample. If all of the samples do not fit onto a single 96-well plate, then add the standard PCR sample to one well of each plate; if the measurements for the standard are highly similar across the plates, then you probably have little to no plate-to-plate measurement discrepencies.
2. Quant-iT 1X dsDNA HS procedure:
    a. Equilibrate the Quant-iT HS 1X assay bottle and standard lambda solutions to room temperature.
    b. Load **190 uL** of Quant-iT HS 1X solution to each well of **column 1** and **198 uL** to the **sample wells and control wells** with a multichannel pipetter. (For half a plate of samples, that's about 10ml HS 1X in a polypropylene tip tub)
    c. Add 10 uL of each of the Quant-iT dsDNA HS standards to separate wells down one column (A-H) (Refer to tables below). Pipette 4 times with the same tip to wash out the DNA.
    d. Add 2 uL of each DNA sample to separate wells for columns 2-11. Pipette 4 times with the same tip to wash out the DNA.
    e. Add 2 uL of at least one of the positive control AMPure bead cleaned PCR product DNA, and leave 1 well empty as negative control.
    f. Mix the 96-well plate using a plate mixer or using the plate reader ("figure 8" shaking mode if possible) for 10 seconds.
    g. Let the plate incubate at room temprature 2 minutes.
    h. Measure the fluorescence using a microplate reader (Excitation = 502; Emission = 523 nm).
    i. Plot the lambda DNA standards (x=axis = ng; y=axis = relative fluorescence RFU).
    j. Calculate the regression line equation (y=ax+b) and convert it to calculate ng/sample: x=(y-b)/a
    k. Make sure that the positive controls concentrations are consistent across 96-well plates and the negative control well with no DNA reads as close as possible to 0 ng.

l. Convert the ng/sample to ng/uL. Remember that 2 uL were added to each well during quantification so simply divide the ng/sample by 2 and you have the ng/ul of the original sample.
m. You can use the template concentration calculator file to do steps j-l. Fluorescence_calculation_template.xlsx

Which lambda standards to add (10 uL / well), and reminder to add appropriate positive control(s) and a negative control. Sample wells are denoted as S:

	1	2	3	4	5	6	7	8	9	10	11	12
A	λ 0 ng/uL	S	S	S	S	S	S	S	S	S	S	+ soil
B	λ 0.5 ng/uL	S	S	S	S	S	S	S	S	S	S	+ rhizosphere
C	λ 1.0 ng/uL	S	S	S	S	S	S	S	S	S	S	+ rhizoplane
D	λ 2.0 ng/uL	S	S	S	S	S	S	S	S	S	S	+ root endosphere
E	λ 4.0 ng/uL	S	S	S	S	S	S	S	S	S	S	+ phyloplane
F	λ 6.0 ng/uL	S	S	S	S	S	S	S	S	S	S	+ plhylosphere
G	λ 8.0 ng/uL	S	S	S	S	S	S	S	S	S	S	+ leaf endosphere
H	λ 10.0 ng/uL	S	S	S	S	S	S	S	S	S	S	water

Amount of DNA (ng) in each standard well:

	1	2	3	4	5	6	7	8	9	10	11	12
A	λ 0 ng											
B	λ 5 ng											
C	λ 10 ng											
D	λ 20 ng											
E	λ 40 ng											
F	λ 60 ng											
G	λ 80 ng											
H	λ 100 ng											

3. Tapestation Gel electrophoresis / Bioanalyzer / agarose gel
    a. Based on the Qubit measured DNA concentration, pick samples with low DNA concentrations (potentially failed), as well as 2 good samples and the negative and positive control.
    b. Run the Tapestation 220 with D1000 Screentapes, no replicates. Alternatively, run either bioanalyzer or gel to assess the quality of the DNA.
    c. Ensure that the target amplicon is present (see size in introduction) and that no unspecific products are present. Unspecific products are most likely primer dimers. These need to be completely removed because they have a negative impact on sequencing.
4. If the concentration is higher than 5 ng/uL dilute to 5 ng/uL. To do this transfer 5 ul DNA to a fresh PCR plate and add appropriate volume of nuclease-free water to each well.

Preparation for Library PCR

1. Clean the laminar flow hood working space with 70% EtOH or chlorite solution.
2. UV irradiate the flow hood while collecting all the needed materials (~5-10 min). Turn off UV in the flow hood.
3. Put all of the required equipment in the laminar flow hood (pipetters with the tip side facing the UV lamp, tips, dispensing tubs, water, tubes, PCR plates, PCR caps OR PCR silicon seals, aluminum plate seal, fresh emptied trash container). Make sure that the bottle of nuclease-free water is capped off and facing the UV lamp. UV irradiate for 15-20 minutes. Do no place enzymes and DNA in under UV.
4. Once UV period is over, recap the nuclease-free water. Put the PCR caps or silicon seals to the back corner of the hood where the sterile air is flowing over them without contaminating obstacles. The strip caps/seals are the last things to be used so they need to stay sterile throughout the setup procedure.

Library PCR

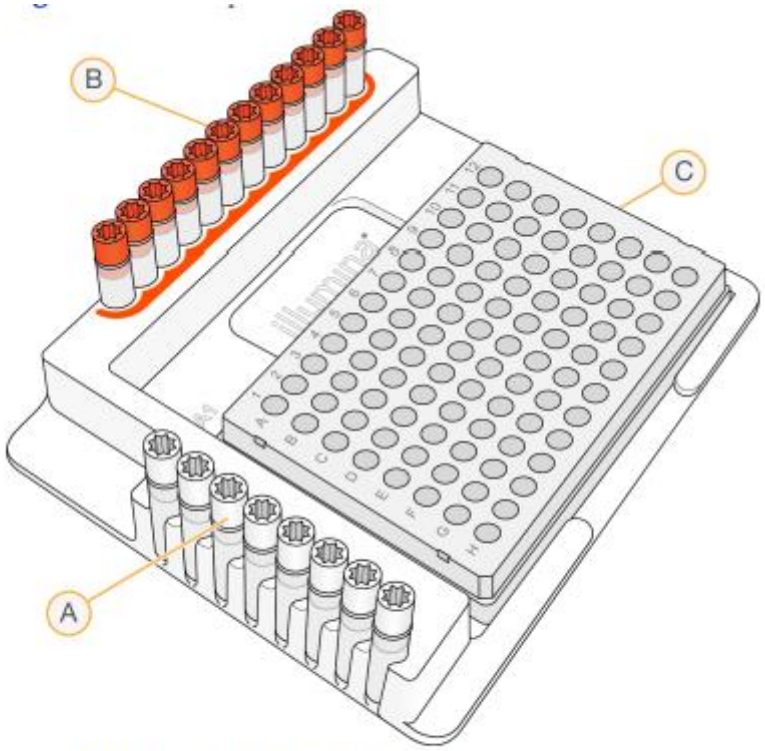
The library PCR adds Illumina sequencing adapters necessary for sequencing to the amplicons from the first PCR. A single reaction is run for each sample. The Nextera adapters are placed in 96-well plates**. Refer to appendix for barcode plate preparation**. Do this first before starting the procedure. Multiple barcode plates can be prepared and stored in the freezer.

1. Thaw PCRBIO Reaction buffer and put on ice together with the polymerase.
2. Remove the Nextera adapter mixed plates, thaw them, spin them down. Store on ice until use.
3. Calculate amount of reagent needed for the master mix. Always prepare 5% more master mix than needed.
4. Prepare master mix:

Reagents	1x reaction (uL)	N x reactions (uL)	Final conc.
Nuclease free water	7.75		-
5x PCRBIO Reaction buffer (includes Mg2+ and dNTPs)	5		1 x
PCRBIO Hifi Polymerase (2 U/UL)	0.25		1 U
Total volume	13		

5. Transfer 13 uL of master mix to the wells of a 96-well PCR plate containing the 10 uL of Nextera adapter mixes (1 uM). Final adapter concentration will be 400 nM.
6. **IF YOU ARE NOT USING THE PREMIXED NEXTERA ADAPTERS:** Arrange the barcode primers (2uM each primer) so that
    a. the Index 1 primers are along the top of the PCR plate and
    b. Index 2 are along the side of the PCR plate.

    This avoids errors when adding the unique-indexed primers. Add 5 uL of each Nextera adapter (2 uM) down each column or across each row, respectively. (Total of 10 uL of adapter mix per well.)



A Index 2 primers (white caps)
B Index 1 primers (orange caps)
C 96-well plate

7. Add 2 uL of purified amplicon PCR product (ca. 5 ng/uL) to each reaction.
8. Mix contents of each well by pipetting up and down with P10 pipetter set to 10 uL or P20 set to 20 uL.
9. Seal PCR plate with 8-strip caps. Check that all wells have been properly sealed.
10. Briefly centrifuge the PCR plate to bring down all of the solution to the bottom of the wells and ensure that there are no bubbles at the bottom of the wells.
11. Run PCR program

Amplicon PCR		
Temp	Time	
110 °C	Heated lid	
95 °C	2 min	
95 °C	20 sec	8 cycles
55 °C	30 sec	
72 °C	60 sec	
72 °C	5 min	
4 °C	Hold	

12. After the PCR, samples are referred to as “libraries”.

Library Cleanup

1. Gently shake Agencourt AMPure XP Bottle to resuspend the beands, remove required volume and let it equilibrate to room temperature.
2. Transfer 20 ul of bead solution to each pooled library (25 ul) and mix by pipetting 10 times up and down.
3. Incubate for 2 minutes at room temperature.
4. Place the amplicon mixture on a magnetic rack until liquid is clear (>1 minute). The container should stay on the rack until stated otherwise.
5. Remove liquid with pipette and discard it.
6. Wash bead-pellet with 200 ul **freshly** prepared 80 % EtOH by gently dispensing it over the beads with a pipette. Do not touch the pellet with the pipette tip! Let the EtOH rest for 30 sec and then remove the liquid. The more EtOH you can remove, the cleaner your final eluate will be.
7. **Repeat** above step 6.
8. Ensure no excess ethanol is left after the washes. If there is, remove it with a 10 uL pipette.
9. Air dry for approximately 5 minutes to evaporate the last of the ethanol. Longer dry times will dry out the pellet and make it difficult to resuspend, resulting in product loss.
10. Remove library plate from magnetic rack. Add 25 ul of nuclease free water and mix with pipette (>10 times up and down) to resuspend the beads.
11. Place the library plate back on the magnetic rack and wait until the liquid clears.
12. Transfer 20 ul of the liquid to a new plate.

Library QC

5. Measure the DNA concentration of each individual library using Qubit dsDNA HS assay kit.
    a. Follow the Quant-iT 1X dsDNA HS procedure in the Amplicon QC section.
    b. Use 2 ul of sample.
    c. Perform single measurement for each sample. If all of the samples do not fit onto a single 96-well plate, then add a few samples (e.g. 1-5 samples) from one plate to the next plate; if the measurements for these ~5 samples are highly similar across the plates, then you probably have little to no plate-to-plate measurement discrepencies.
6. Tapestation Gel electrophoresis / Bioanalyzer / agarose gel
    a. Based on the Qubit measured DNA concentration, pick samples with low DNA concentrations (potentially failed), as well as 2 good samples and the negative and positive control.
    b. Run the Tapestation 220 with D1000 Screentapes, no replicates. Alternatively, run either bioanalyzer or gel to assess the quality of the DNA.
    c. Ensure that the target amplicon is present (see size in introduction) and that no unspecific products are present. Unspecific products are most likely primer dimers. These need to be completely removed because they have a negative impact on sequencing.

Library Pooling

1. Calculate the required volume of each sample.
    a. Libraries with a concentration of <1 ng/uL should be excluded (either leave out or rerun PCR).
    b. Detect the sample with the lowest concentration and multiple this concentration with 15 uL (e.g. 1 ng/uL x 15 uL = 15 ng). This is the amount of library wanted from each library.
    c. Calculate the volumes required to obtain the same amount of library for each of the other libraries. Use a spreadsheet to avoid making mistakes.
    d. If volumes less than 1 uL are required for some libraries, dilute the libraries and recalculate the required volumes. This avoids errors from pipetting very small volumes.
2. Pool libraries
    a. Only pool samples from the same primer set together. Do not mix samples prepared with different primers sets into the same pool prior to submitting to the sequencing facility.
    b. Use a new microcentrifuge tube (1.5 mL).
    c. Transfer the calculated volume of each sample to the tube.
    d. Mix well after all the samples have been added.
    e. If the negative control libraries had undetectable ng of DNA, add 15 uL of the Ampure purified library.

Library Pool QC

1. Measure the DNA concentration of the library pool with Qubit dsDNA HS assay kit.
    a. Follow vendor recommended protocol.
    b. Measure in triplicate and calculate the mean concentration.
    c. Use 2 uL sample for each measurement.
2. Tapestation gel electrophoresis / Bioanalyzer
    a. Tapestation: Run Tapestation 220 with D1000 Screentapes. Follow standard protocol. No replicates.
    b. Only the target amplicon should be present on the gel. Note that the target amplicon size is now amplicon + adapters.
3. Calculate the library pool concentration in nanomolar (nM):

$$nM = \frac{c}{bp \cdot 650} \cdot 1000\,000$$

4. c = concentration in ng/µL
5. bp = fragmentsize in basepairs

Submit to sequencing

1. Make sure to follow the instructions of the sequencing facility to which the samples are being submitted to. This includes the following:
    a. Metadata sheet
    b. Sample preparation
    c. Shipping instructions

Appendix

Preparation of Nextera oligos.

Consumables and reagents needed:

- Nuclease-free water
- Nextera primers (100 uM)
- 96-well PCR plates
- Sterile aluminum plate seals
- Plate seal rollers or thick knitted fabric
- Multichannel and Single pipettes and tips
- 1x 96-well plate rack
- 2x 1.8 ul tube rack (to organize the Nextera primer stocks alongside the PCR master mix plate)

The Nextera barcoded oligos are ordered from IDT as desalted ultramers and are ordered in 100 uM stocks. If a large sequencing project is planned, it is advised to reduce the amount of hands-on handling by preparing a large number of 96-well plates with premixed adapter mixes. This will also avoid making mistakes and misbarcoding samples. Refer to the Barcodes spreadsheet for the barcode pairs that should be in each well.

Below is the recipe to prepare a small-scale set of barcode mix plates, a medium-scale, and a large-scale recipe. Remember that there are a total of 40 Nextera barcoded primers (16 S5xx and 24 N7xx), so a total of 384 unique barcode pairs can be prepared.

1. For small-scale sequencing project (5 plates of each barcode pair), dilute an aliquot of the 100 uM Nextera barcodes to 10 uM stocks. You will need 144 uL of each S5xx primer and 96 uL of each N7xx primer. Below is the recipe that accounts for pipetting error.

Reagent	**Small-scale (5x)**	**Medium-scale (20x)**	**Large-scale (100x)**
Nuclease-free water	144 uL		
Nextera **S5xx** (100 uM)	16 uL	-	-
		-	-
Total	160 uL (10 uM)		

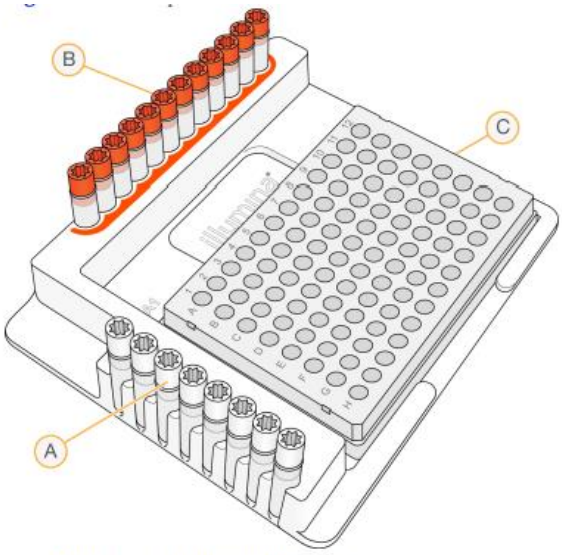
Reagent	**Small-scale (5x)**	**Medium-scale (20x)**	**Large-scale (100x)**
Nuclease-free water	99 uL		
Nextera **N7xx** (100 uM)	11 uL	-	-
		-	-
Total	110 uL (10 uM)		

2. The configuration of the barcodes will be as follows, shown in the table below. The numbers in each cell denotes the code for each barcode pair. **Process one plate at a time** in order to avoid confusion.

			Index 1																								
			N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715	N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729	
	Index 2		1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9	10	11	12	
Plate 1	S502	A	1	9	17	25	33	41	49	57	65	73	81	89	97	105	113	121	129	137	145	153	161	169	177	185	Plate 2
	S503	B	2	10	18	26	34	42	50	58	66	74	82	90	98	106	114	122	130	138	146	154	162	170	178	186	
	S505	C	3	11	19	27	35	43	51	59	67	75	83	91	99	107	115	123	131	139	147	155	163	171	179	187	
	S506	D	4	12	20	28	36	44	52	60	68	76	84	92	100	108	116	124	132	140	148	156	164	172	180	188	
	S507	E	5	13	21	29	37	45	53	61	69	77	85	93	101	109	117	125	133	141	149	157	165	173	181	189	
	S508	F	6	14	22	30	38	46	54	62	70	78	86	94	102	110	118	126	134	142	150	158	166	174	182	190	
	S510	G	7	15	23	31	39	47	55	63	71	79	87	95	103	111	119	127	135	143	151	159	167	175	183	191	
	S511	H	8	16	24	32	40	48	56	64	72	80	88	96	104	112	120	128	136	144	152	160	168	176	184	192	
Plate 3	S513	A	193	201	209	217	225	233	241	249	257	265	273	281	289	297	305	313	321	329	337	345	353	361	369	377	Plate 4
	S515	B	194	202	210	218	226	234	242	250	258	266	274	282	290	298	306	314	322	330	338	346	354	362	370	378	
	S516	C	195	203	211	219	227	235	243	251	259	267	275	283	291	299	307	315	323	331	339	347	355	363	371	379	
	S517	D	196	204	212	220	228	236	244	252	260	268	276	284	292	300	308	316	324	332	340	348	356	364	372	380	
	S518	E	197	205	213	221	229	237	245	253	261	269	277	285	293	301	309	317	325	333	341	349	357	365	373	381	
	S520	F	198	206	214	222	230	238	246	254	262	270	278	286	294	302	310	318	326	334	342	350	358	366	374	382	
	S521	G	199	207	215	223	231	239	247	255	263	271	279	287	295	303	311	319	327	335	343	351	359	367	375	383	
	S522	H	200	208	216	224	232	240	248	256	264	272	280	288	296	304	312	320	328	336	344	352	360	368	376	384	

3. Prepare the master primer mix plate. Arrange the barcode primers so that
    a. the Index 1 primers (N7xx) are along the top of the PCR plate and
    b. Index 2 (S5xx) are along the side of the PCR plate.

    This avoids errors when adding the unique-indexed primers. To each well, add the corresponding amount of water and each Nextera adapter down each column or across each row, respectively. Add first the water, then the primers. **Change tips after each well!**



A Index 2 primers (white caps)
B Index 1 primers (orange caps)
C 96-well plate

Reagent in each well	Small-scale (5x)	Medium-scale (19x)	Large-scale (99x)
Nuclease free water	48 uL	196 uL	980 uL
Index 1 (S7xx)	6 uL (10 uM)	2 uL (100 uM)	10 uL (100 uM)
Index 2 (S5xx)	6 uL (10 uM)	2 uL (100 uM)	10 uL (100 uM)
Total	60 uL	200 uL	1000 uL

4. Using a multichannel pipette, aliquot 10 uL of the Nextera primer mix into 96 well PCR plates. The number of plates to transfer to is indicated between parentheses in the table above.

5. Discard the master plate of primer mix because the amount of primer mix left is inexact after pipetting errors.
6. Label the primer mix plates with “Nextera stock plate [Plate Number], [your initials], [date]”
7. Seal the plates VERY WELL with sterile aluminum seal.
8. Store the primer mix plates at -20°C.

Quantification of DNA using the ClarioStar Plus plate reader

1. Switch on the ClarioStar machine.
2. Open the ClarioStar controller software on the computer
3. Right click on the Quant-it protocol and select edit protocol.
4. In the layout, select the wells that are standard and sample.
5. Click the Measurement icon at the bottom of the pop-up window.
6. Click Measure in the new pop-up window.