Preparing multiplexed 16S/18S/ITS amplicons for the Illumina MiSeq

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We use this protocol and it's working

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ABSTRACT

The following detailed protocol is for the generation of paired-end sequencing reads of 16S/18S/ITS PCR amplicons with dual barcodes (i.e.: “indices”) on the *Illumina MiSeq* machine of length ≈450 bp (ie: 300+300 bp sequencing with ~150 bp overlap) using v3 600 cycle chemistry. It assumes an input of 380 individual samples (+4 controls = 384) in four 96-well plates.

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MATERIALS

The following materials list contains those consumables+quantities used specifically at the IMR to complete the present protocol for 380 amplicons (4 plates) on one MiSeq run.

16S/18S/ITS PCR Supplies

Phusion Plus XL pack (2000 U = 4000 rxns)
Thermo dNTP mix (10 mM x 10 mL = 20000 rxns)
Separate Illumina fusion primer sets (4000 uL/ea. @ 1 uM = 1000 rxns)
…or Combined UDI F+R Illumina fusion primer sets (4000 uL/pair @ 1 uM = 1000 rxns/pair)
PCR microplates 96-well Bio-Rad
PCR microplates sealing film Bio-Rad
Tips ClipTip 20
Tips ClipTip 200
UltraPure water

Gel Verification

PCR microplates 96-well Bio-Rad
PCR microplates sealing film Bio-Rad
E-gels **or** Coastal Genomics analytical gels

Tips ClipTip 20
Tips ClipTip 200
Tips ClipTip 300

Initial Clean-up and Normalization
PCR microplates sealing film Bio-Rad
Charm Normalization 96-well kit
Ethanol
Tips ClipTip 200
Tips ClipTip 300
Hamilton tips 300
Reservoir

Final Pooling and Clean-up
Illumina SPB (or AMPure or Pronex beads)
PCR microplates 96-well Bio-Rad
1.5 mL Eppendorf tubes
Ethanol
Tips ClipTip 20
Manual p100 tips
Manual p1000 tips
UltraPure water

Loading and Sequencing
PhiX Control Kit v3
MiSeq Reagent Kit v3 600 cycle
Quant-iT 1X HS dsDNA Kit (1000 samples)
Qubit Tubes
1.5 mL Eppendorf tubes
1 N NaOH
200 mM Tris-HCl pH 7
Various manual pipette tips

Barcoded PCRs

1 Prepare the following PCR master-mix for **PCR Plate 1 (2 and 0.2 µL)** in two 1.5 mL Eppendorf tubes (one per duplicate plate; adjust if not using *Phusion Plus*), using either of the primer formats prepared in the IMR protocols **Preparing Indexed Primer Plates (IDT Ultramers) for the Illumina MiSeq - Nextera Dual Indices** or **Preparing Combined Indexed Primer Plates (IDT Ultramers) for the Illumina MiSeq - IDT UDIs**:

		16S/18S/ITS standard		**18S with blocking primer**	
	Reagent	per 20 µL rxn	x100 rxns	per 20 µL rxn	x100 rxns

	Reagent	per 20 µL rxn	x100 rxns	per 20 µL rxn	x100 rxns
	5x Phusion Plus Buffer	4 µL	400 µL	4 µL	400 µL
	dNTPs (40 mM)	0.4 µL	40 µL	0.4 µL	40 µL
	F primer (1 µM)	4 µL	added after	4 µL	added after
	R primer (1 µM)	4 µL	added after	4 µL	added after
	Blocking primer (10 µM)	-	-	3.2 µL	320 µL
	Phusion Plus (2 U/µl)	0.2 µL	20 µL	0.2 µL	20 µL
	PCR-grade Water	5.4 µL	540 µL	2.2 µL	220 µL
	Template	2 µL	added after	2 µL	added after

Note

We prepare two aliquots of "100 rxn" master-mix here above since we tend to prepare both dilution plates of the one PCR plate together. Below we fill the first PCR plate with double the final volumes, since it will be split into two plates right before adding the templates.

2 Dispense 120 µL of the master-mix into the 8 wells of 1 column of a 96-well plate (remaining wells to be used in subsequent PCR preps) – this plate now becomes the **Master-Mix Plate** and is used to transfer the master-mix into the PCR plate using a multichannel pipette (MCP).

3 Dispense 20 µL of master-mix into each well of the **PCR Plate 1 (2 µL)**, one column (or row) at a time with the MCP.

4 If using **Combined Primer Plates**, proceed to **step 4.1** below instead.

Remove the protective film (using a scalpel) from one **column** of the **Forward Set 1 Primer Plate**, align it horizontally on the bench to the left of **PCR Plate 1 (2 µL)** and dispense 8 µL into each well, one **column** at a time using the MCP.

Note

You can use the same set of 8 tips for all.

4.1 Remove the protective film from the entire combined **F1R1 Primer Plate**, align it either horizontally or vertically on the bench to the left of or above the **PCR Plate 1 (2 µL)** and dispense 16 µL into each corresponding **column**, one **column** at a time using the MCP. Once complete, skip step 5 and proceed directly to **step 6**.

> Note
>
> You must **change tips** after every column to avoid cross-contamination (since working with different Forward+Reverse combined primers unique to each well).

5 Uncover one **row** of the **Reverse Set 1 Primer Plate**, align it vertically on the bench along the top of **PCR Plate 1 (2 µL)** and dispense 8 µL into each well, one **row** at a time using the MCP.

> Note
>
> You must now **change tips** after every row to avoid cross-contamination (since different Forward primers/indices are now in each row).

6 Transfer 18 µL (half the volume) from each column of **PCR Plate 1 (2 µL)** into a new **PCR Plate 1 (0.2 µL)**, one column at a time using the MCP.

> Note
>
> Remember to **change tips** after every column.

7 Uncover the **DNA Template Plate 1**, align it along the top of **PCR Plate 1 (2 µL)** and dispense 2 µL into each well, one **column** at a time using the MCP. Seal the **PCR Plate 1 (2 µL)** with PCR film and either keep on ice until both PCR plates are ready for the thermocyclers or place in the thermocycler right away, as per below.

Note

Remember to **change tips** after every column.

8 While the plate is still unsealed, prepare the 1/10th dilution of **DNA Template Plate 1**. Add 18 µL of PCR-grade water to each well of a new PCR plate using a reservoir (~3 mL required) and MCP (same column of tips) and label it **1:10 DNA Template Plate 1**. Align it vertically below the original DNA plate. Label a new third PCR plate **PCR Plate 1 (0.2 µL)** and align it below the 1/10th plate. Then, working one **column** at a time using the MCP, transfer 2 µL of the original template from **DNA Template Plate 1** into the new **1:10 DNA Template Plate 1** (for a total of 20 µL final volume), mix with the pipette, then transfer 2 µL of this new dilution into the corresponding column of the **PCR Plate 1 (0.2 µL)**. Seal the DNA template plates with PCR film and archive.

Note

Remember to **change tips** after every column.

9 Once PCR setup is complete, seal the plates with PCR film, place in thermocyclers and run the following program with **25 cycles** (adjust if not using *Phusion Plus*):

		without BP		**with BP**	
	A	B	C	D	E
	Initial denaturation	98°C	30 s	98°C	30 s
	Denaturation	98°C	10 s	98°C	10 s
	Blocking primer annealing	-	-	70°C	30 s
	Annealing	55°C	30 s	55°C	30 s
	Extension	72°C	30 s	72°C	30 s
	Final Extension	72°C	4:30	72°C	4:30
	Hold	4°C	forever	4°C	forever

10 Once the two PCRs for **Plate 1** are complete, repeat **steps 1-9** to prepare **PCR Plates 2 (2 µL) & (0.2 µL)** from **DNA Template Plate 2** using **Forward Set 1 Primer Plate** and **Reverse Set 2 Primer Plate** (*change to F1+R2 here*), or combined **F1R2 Primer Plate**.

11 Once the two PCRs for **Plate 2** are complete, repeat **steps 1-9** to prepare **PCR Plates 3 (2 µL) & (0.2 µL)** from **DNA Template Plate 3** using **Forward Set 2 Primer Plate** and **Reverse Set 1 Primer Plate** (*change to F2+R1 here*), or combined **F2R1 Primer Plate**..

12 Once the two PCRs for **Plate 3** are complete, repeat **steps 1-9** to prepare **PCR Plates 4 (2 µL) & (0.2 µL)** from **DNA Template Plate 4** using **Forward Set 2 Primer Plate** and **Reverse Set 2 Primer Plate** (*change to F2+R2 here*), or combined **F2R2 Primer Plate**..

Gel Verification

13 Plug in the *Mother E-Base*, unwrap a fresh *E-Gel 96* and insert it into the base.

Note

We leave these instructions for the *E-Gels* here in this section, as we are sometimes using them when our regular supplies run low, but we now are regularly using our *Coastal Genomics (Yourgene Health) Nimbus Select* robot platform which runs gel cassettes for the resolution of PCR products (and enables on-board BioAnalyzer-type analytics, as well as size-selection).

14 The duplicate PCR reactions of **Plate 1** are aggregated then loaded onto the gel in the same action: using the MCP and working by **rows** (the gel cannot be loaded by columns as they are staggered), pipette 20 µL out of the **PCR Plate 1 (0.2 µL)** into the corresponding wells of **PCR Plate 1 (2 µL)**, mix by pipetting, then take 20 µL of this aggregate and load it into the appropriate wells of the gel.

Note

Remember to **change tips** after every row. Discard the empty **PCR Plate 1 (0.2 µL)** when finished and relabel the **PCR Plate 1 (2 µL)** the **Aggregated PCR Plate 1**.

15 Once all rows are complete, load 20 µL of the *E-Gel Low Range Ladder* into some of the marker ("M") wells, then run the gel for the pre-set 00:12:00. 12m

16 Visualize the gel and photograph on a UV/blue transilluminator with a SYBR filter. Remember that your expected band sizes will be the normal product/insert sizes + an additional ~140 bp with the addition of the Illumina adapters+barcodes in the fusion primers (only 32 bp if doing our *PacBio* versions).

17 Repeat **steps 13-16** for **PCR Plates 2 (2 µL) & (0.2 µL)**.

18 Repeat **steps 13-16** for **PCR Plates 3 (2 µL) & (0.2 µL)**.

19 Repeat **steps 13-16** for **PCR Plates 4 (2 µL) & (0.2 µL)**.

20 If there are large numbers of samples on a given plate with failed PCRs (or spurious bands), they are re-amplified by optimizing the PCR (further template dilution to 1:100 or, conversely, increased template amount; or using BSA/other additives) to produce correct bands in order to complete the amplicon plate (either in new plates or individual strips).

Once correct bands have been obtained, amalgamate those few strips or plate columns into the appropriate wells of the respective **Aggregated PCR Plates** before continuing.

PCR Clean-up + Normalization & Final Library Pool

21 Use the remaining 20 µL of each well in the **Aggregated PCR Plate 1** to cleaned-up and normalize the amplicons using the high-throughput *Charm Biotech Just-a-Plate 96 PCR Normalization and Purification Kit*. Label this final plate **Charm Plate 1**.

Note

You have the option here forward of only using one set of tips for each step (ex: one column per step) as amplicons are now barcoded (therefore can't "contaminate" each other's reads) and will shortly be pooled anyways. This will not cause problems within the one plate, but just be aware the whole plate will have to be considered a "unit" from now onward - if coming back to the plate, you can resequence the whole plate without any problems of barcode contamination, but you could not pull out individual wells from the plate to resequence alongside new samples/PCR products from the same primer set (ex: could not mix older F1R1 samples with a new plate of F1R1, even if not using those exact well locations, because the older samples will have traces of other F1R1 combo products from other wells mixed in).

22 Once the *Charm* protocol is complete, pool the 95 samples from **Charm Plate 1** by using the MCP to transfer 5 µL of each column into one column of a new 96-well plate named the **Library Pooling Plate** (remaining columns to be used in subsequent pooling). Once complete, pipette 50 µL of each of the 8 wells into one 1.5 mL Eppendorf tube and label **Plate 1 Library Pool**.

Note

As discussed in the step above, you can use the same set of 8 tips for all columns if not needing to return to individual samples on the plate in future sequencing.

23 Repeat **steps 21-22** for **Aggregated PCR Plate 2**.

24 Repeat **steps 21-22** for **Aggregated PCR Plate 3**.

25 Repeat **steps 21-22** for **Aggregated PCR Plate 4.**

26 Once all four pools are complete, pipette 100 µL of each of the four tubes into one 1.5 mL Eppendorf tube and label **Final Library Pool** (add the run name to the tube or some other identifier to keep your various pools separate).

27 *Optional:* Proceed with a final bead cleaning of 100 µL of the **Final Library Pool** - resuspend into a final volume of 50 µL and label **Final Cleaned Library Pool**. We found that this step was not required in the initial years of our library preps for the *MiSeq* using the *Charm* kits, however we started encountering problems of primer dimer carryover into the final pools which then impacted sequencing runs. We now systematically incorporate this step into our procedure. Given the large amount of leftover NGS products we have on-hand, we use the *Illumina SPB* beads (from the "*Nextera Flex*" kit, according to that protocols instructions) as there are substantial extras available, but you can use any similar SPRI beads (such as *AMPure* or *Pronex*).

28 Quantify the **Final (Cleaned) Library Pool** using the Invitrogen Qubit dsDNA HS assay (or similar fluorescence-based alternative; 5 µL of pool to be assayed) and calculate the molar concentration using the following formula, knowing that 1 ng/µL of a 500 bp amplicon = 3.29 nM:

(500 bp/size in bp of amplicon) x (concentration in ng/µl) x (3.29)

For the 16S amplicon generated using our V6-V8 primers (= 574 bp, including target region + adapters + indices) at a concentration of 3.0 ng/µL, for example: (500 bp / 574 bp) x 3.0 x 3.29 = 8.6 nM.

Note

We have found that the anticipated >1 ng/µL output from the *Charm* plate is near impossible to achieve; we typically see concentrations in the range of 0.3-0.9 ng/µL.

Illumina MiSeq Sequencing

5m

29 This section is based upon the following *Illumina* documents, with some small procedural changes (including using the *NextSeq* variant for sample denaturation), and the inclusion of instructions to be able to load >96 samples (i.e.: 384 combinations of indices) which are not written out by *Illumina* – familiarize yourself with these documents / have them on-hand:

- *MiSeq Reagent Kit v3 – Reagent Preparation Guide*
- *Preparing Libraries for Sequencing on the MiSeq*
- *Denaturing and Diluting Libraries for the NextSeq 500*
- *MiSeq System User Guide*

30 Begin thawing the **v3 Reagent Cartridge** and tube of **HT1** as instructed. Put at 4 °C when complete.

Note

Optional: Take out the day before and thaw the reagents overnight at 4 °C .

31 While waiting, prepare the **Sample Plate** and **Sample Sheet** files that will be used to run the *MiSeq* by opening the *Illumina Experiment Manager (iEM)* software.

Note

The info contained in this section was originally written for the previous versions of the *MiSeq Control Software (MCS)* which has since been replaced by *MiSeq Local Run Manager (LRM)*. *Illumina* has stated that *LRM* is no longer compatible with sample sheets generated in *iEM*, however we have found that to not be the case. We continue to create our sheets in *iEM*, due to the better ease-of-use for making the **Sample Plates** and the flexibility of the custom index system files we created (for our IDT UDI primer sets). It is true that you can import a sheet using a template into *LRM* (which we will exploit to bring the *iEM* sheet into it), but it is more difficult to create that template in the format they want it in (reformatting all your sample names) than to simply copy-and-paste from our Excel run designs into *iEM*. All that needs to be changed to make the final *iEM* **Sample Sheet** compatible for an import into *LRM* is to slightly change the old header for a new *LRM* version (in **step 37** below).

32 Create the **Sample Plates** first – in order to run all 384 combinations of indices, four separate **Sample Plates** (one per plate from our protocol above) will be required. For the samples that were in **DNA Template Plate 1**:

- Choose *Nextera XT v2 (Set A)* in the *iEM* wizard.
- Give the plate a unique name (we usually use our run number and append an "**A**" to the end; ex.: *15A*).
- Copy-and-paste the 96 sample names from your Excel sheet (after having brought the file over to the *MiSeq* via USB, *Dropbox* or email) into the *Plate* tab, then press *Apply Default Index Layout*. You will sometimes not see the index names show up on the top row and left-hand column of this tab, but if you switch to the *Plate Graphic* or *Table* views, they will be there correctly.
- Click on *Finish* and save the **A.nexxt28.plt* file in the directory of your choice.

33 For the samples that were in **DNA Template Plate 2**, repeat **step 32** except change to *Nextera XT v2 (Set B)* and append a "**B**" to the filename.

34 For the samples that were in **DNA Template Plate 3**, repeat **step 32** except change to *Nextera XT v2 (Set C)* and append a "**C**" to the filename.

35 For the samples that were in **DNA Template Plate 4**, repeat **step 32** except change to *Nextera XT v2 (Set D)* and append a "**D**" to the filename.

36 Now create the **Sample Sheet** by bringing in the four **Sample Plates** that belong to it:

- Select *MiSeq* in the *iEM* wizard, then *Other --> FASTQ Only*.
- Input your Reagent Cartridge barcode, select *Nextera XT v2* for the Sample Prep Kit, input your Experiment Name (we usually use our complete run name now; ex: *IMR-Run15*) and change the cycles to **301** for both reads.
- On the next screen, uncheck the *Maximize* box, choose *Select Plate* at left and navigate to and select your **A.nexxt28.plt* file (*Plate A*).
- Once the samples are displayed, choose *Select All + Add Selected Samples*.
- Repeat the above two steps for the remaining plate files (**Plates B/C/D**).
- The **Sample Sheet** status should show as **Valid** and, if so, click *Finish* to save the file, appending the run name to the end of the filename (for the above ex.: *MSxxxxxxx-600V3-Run15.csv*). We find it helpful having the run name/# when returning to the files, otherwise they are only labelled with the less-informative cartridge barcode by default. If your status is showing as **Invalid**, then it is often due to identical sample names being used in error. Once corrected, the status will update.
- It is a good idea to then simply verify that the CSV file is all correct by opening the file in *WordPad* and checking that the header information is correct (date, run name, FASTQ generation, etc.) and that you see the four sets of samples below in the [Data] section (*Plate A* samples, followed by *Plate B*, etc.).

37 Due to the above-stated change in compatibility between *iEM* and the new *MiSeq LRM* software, you need to replace the existing *iEM*-generated header in the **Sample Sheet** with this new header below (you can do so while performing the above check of the CSV file in *WordPad*):

[Header]
Local Run Manager Analysis Id,12345
Experiment Name,RunXXX
Date,YYYY-MM-DD
Module,GenerateFASTQ - 3.0.1

Workflow,GenerateFASTQ
Library Prep Kit,Custom
Index Kit,Custom
Chemistry,Amplicon

[Reads]
301
301

[Settings]
Adapter,CTGTCTCTTATACACATCT

[Data]
...

Replace all sections above the **[Data]** section with the above header info, taking care to replace with your run name and date.

38 Prepare **0.2 N NaOH** as instructed, except make 10-fold less (20 µL of 1 N NaOH + 80 µL of water).

39 Use the nanomolar concentration from **step 28** to determine whether the final amplicon library must be diluted or concentrated prior to continuing. A fixed concentration of [M] 4 nanomolar (nM) is the standard requirement for the *MiSeq*, however, using the *NextSeq* loading protocol, a library between **0.4-4 nM** can be accommodated by simply using a larger volume of a more dilute library. Use the following guidelines:

Library	Volume to denature	0.2 N NaOH	Tris-HCl	HT1
>4 nM	dilute to 4 nM using PCR-grade water or buffer			
4 nM	5 µl	5 µl	5 µl	985 µl
2 nM	10 µl	10 µl	10 µl	970 µl
1 nM	20 µl	20 µl	20 µl	940 µl
0.5 nM	40 µl	40 µl	40 µl	880 µl
0.4 nM	50 µl	50 µl	50 µl	850 µl
<0.4 nM	concentrate using standard method/kit and repeat quantification			

40 Denature the library, using the indicated volume of **0.2 N NaOH** in the table above, for 5m

00:05:00 at room temperature.

41 Neutralize the reaction by adding the equivalent volume of **200 mM Tris-HCl** as indicated.

42 Dilute out the library to [M] 20 picomolar (pM) using the indicated amount of chilled **HT1** and place on ice.

> Note
>
> We began loading at **20 pM** many years ago, but have experienced variability in *MiSeq* kit and library performance over the years, including after the switch to the optional final bead purification after *Charm* noted above, and so are now systematically loading at **14 pM** in order to achieve a target CD of around **800 K/mm2**, which maximizes read output without compromising overal Q30 values.

43 Combine 540 µL of the library with 60 µL (=**10%**) of the already diluted and denatured **PhiX Control Library**. We always use **10%** with amplicon libraries, regardless of anticipated diversity.

> Note
>
> We began only using **5% PhiX** many years ago, which worked very well for a long time, but have experienced quality drops in our *MiSeq* run since the fall of 2022, and so are now systematically adding **10%** in order to maintain acceptable Q30 values.

44 Proceed with loading the 600 µL sample in the **v3 Reagent Cartridge** and continue the *MiSeq* run start procedure, as instructed – the only slight change in *MCS* is that the default filename (just the cartridge barcode) of the **Sample Sheet** will not be found since we appended the run name to the end of the CSV file (for the above ex.: *MSxxxxxxx-600V3-Run15.csv*); browse to and select the correct file. For *LRM* you select *Create Run*, *Generate FASTQ*, then import the **Sample Sheet** and save the run. In both software instances, confirm that the displayed run info is correct (ex: number of cycles should be **301 | 8 | 8 | 301**) before saving the run/starting the sequencing.