10.March.2016\_Version10

**Dunfield Lab Illumina Amplicon Protocol**

Based on: 16S Metagenomic Sequencing Library Preparation Part # 15044223 Rev. B

The following will be performed individually, with each lab member processing their own samples:

1. DNA extraction

-Use your preferred method

-Elute your DNA in 10 mM Tris pH 8.5 (Qiagen Buffer EB)

2. PCR Amplify your samples

-Input quantity is flexible, but ***it must be the same for each sample within a single experiment***. The original protocol calls for 5.0ng/uL of input DNA, but input concentrations as low as have 1.25 ng/μL amplified in the past.

-You must use filter tips.

|  |  |
| --- | --- |
| **Reagent** | **Volume** |
| Microbial DNA | 2.5 μL |
| Forward Primer 1 μM | 5.0 μL |
| Reverse Primer 1 μM | 5.0 μL |
| 2x KAPA HiFi HotStart ReadyMix | 12.5 μL |
| **Total** | **25 μL** |

2.1 Set up the following PCR reaction:

2.2 If using a PCR plate, seal the PCR plate with a MicroAmp seal; use the rubber plate-sealing tool to get an airtight seal. Make sure each well is individually sealed. We have two types of seals in the lab; you must use the ones that are wrapped in aluminum foil. Please put foil back on the unused seals; the adhesive is light sensitive.

2.3 Run the following PCR program:

95°C, 3 min

95°C, 30 sec

25 cycles 55°C, 30 sec

72°C, 30 sec

72°C, 5 min

This program is saved on PCR machine #3, User: illumi, Method: pcr one

3. Run each PCR reaction on a gel.

-Centrifuge the plate using the centrifuge in room 107

-Run out exactly 5 μL of unpurified PCR product.

-If no product is visible, re-do the PCR. Multiple PCR reactions can be pooled together.

-If the desired product is present, along with extraneous bands, gel-purify your sample using the Qiagen Gel Purification kit. Note: very faint extraneous bands are acceptable

-If the anticipated product is visible on the gel, proceed to Step 4

4. Purify the PCR reactions using AMPure XP beads.

-Use your own personal aliquot of the beads

-Bring the beads to RT before use (20-30 minutes)

1. Resuspend the beads by hand, until no more dark patches are visible

2. Add 16.0 μL (0.8X PCR volume) of AMPure XP beads to each PCR reaction well

3. Gently pipette the entire volume (36 μL) up and down 10 times. Avoid inducing excessive frothing of the sample.

4. Incubate at room temperature, 5 minutes

5. Place plate on the magnet, 2 minutes (or more, if supernatant is still not clear). If using PCR tubes, open the caps *before* placing them on the magnet, as this action may jostle the beads if done after the incubation.

6. With the plate still on the magnet, aspirate and discard the supernatant.

7. With the plate still on the magnet, add 200 μL of ***freshly prepared*** 80% ethanol. Incubate for 30 seconds. Remove and discard the ethanol.

8. With the plate still on the magnet, add 200 μL of freshly prepared 80% ethanol. Incubate for 30 seconds. Remove and discard the ethanol. Use a P10 pipette to remove residual ethanol.

9. With the plate still on the magnet, allow the beads to air-dry for 10 minutes.

10. Remove the plate from the magnet. Add 52.5 μL of Qiagen Buffer EB (10 mM Tris pH 8.5). Pipette mix up and down 10 times.

11. Incubate at room temperature, 2 minutes.

12. Place plate on the magnet, 2 minutes (or more, if supernatant is still not clear).

13. With the plate still on the magnet, transfer 50 μL of the supernatant to a new PCR plate.

5. Refer to the excel sample spreadsheet for the PCR2 primers you have been assigned (located in: All\_Dunfield\_NGS/All\_Illumina\_DunfieldLab/ LaneX\_201X\_

SampleList.xlsx).

6. PCR\_2: Barcoding the samples

1-1. Make 1uM dilutions of the barcoded PCR primers from the 10uM stock in ultrapure water.

1-2. Make KAPA/Ultrapure H2O master mix

-Place a clean boat onto the boat ice block

-Do this directly in the *small section* of a boat, to minimize waste

-Add 25uL KAPA x # of reactions +2

-Add 10uL Ultrapure H2O

-Pipette up and down to mix

1-3. Set up the PCR reactions

-To a clean PCR plate, add the following:

5uL bead-purified PCR\_1 product

5uL Forward barcoded primer (1uM)

5uL Reverse barcoded primer (1uM)

35uL KAPA/H2O master mix

-After adding the master mix, pipette up and down to mix

1-4. Seal the PCR plate with a Microseal A film using the blue plate sealer tool. Please remember to rewrap unused films in aluminum foil.

1-5. Run the following PCR reaction:

95°C, 3 min

95°C, 30 sec

8 cycles 55°C, 30 sec

72°C, 30 sec

72°C, 5 min

This program is saved on PCR machine #3, User: illumi, Method: pcr two

7. Run the PCR reactions on a 1% agarose gel

Load 5uL of each sample

8. Purify the PCR reactions using AMPure XP beads.

-Use your own personal aliquot of the beads

-Bring the beads to RT before use (20-30 minutes)

1. Resuspend the beads by hand, until no more dark patches are visible

2. Add 50.4μL of AMPure XP beads to each PCR reaction well

3. Gently pipette the entire volume (95.4μL) up and down 10 times. Avoid inducing excessive frothing of the sample.

4. Incubate at room temperature, 5 minutes

5. Place plate on the magnet, 2 minutes (or more, if supernatant is still not clear). If using PCR tubes, open the caps *before* placing them on the magnet, as this action may jostle the beads if done after the incubation.

6. With the plate still on the magnet, aspirate and discard the supernatant.

7. With the plate still on the magnet, add 200 μL of ***freshly prepared*** 80% ethanol. Incubate for 30 seconds. Remove and discard the ethanol.

8. With the plate still on the magnet, add 200 μL of freshly prepared 80% ethanol. Incubate for 30 seconds. Remove and discard the ethanol. Use a P10 pipette to remove residual ethanol.

9. With the plate still on the magnet, allow the beads to air-dry for 10 minutes.

10. Remove the plate from the magnet. Add 27.5 μL of Qiagen Buffer EB (10 mM Tris pH 8.5). Pipette mix up and down 10 times.

11. Incubate at room temperature, 2 minutes.

12. Place plate on the magnet, 2 minutes (or more, if supernatant is still not clear).

13. With the plate still on the magnet, transfer 25 μL of the supernatant to a new PCR plate.

9. Provide the person performing the rest of the library with your amplicons:

9-1. Place your samples into the designated 96-well-PCR-plate-rack in the exact well positions you have been assigned in the excel sample spreadsheet (located in: All\_Dunfield\_NGS/All\_Illumina\_DunfieldLab/ LaneX\_201X\_SampleList.xlsx). Please orient the tubes so that the hinge faces Column 12.

9-2. Make sure you have your gel image from Step 7 archived, as this may be required later.

The following will be performed by a single individual:

1. Quantify the bead-purified libraries

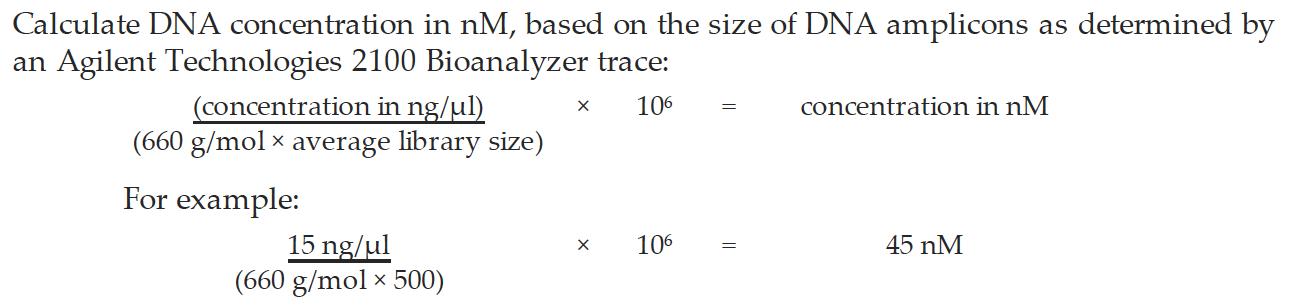
-Use Qubit BR kit

-It is extremely important to quantify all of the samples using *the same master mix and standards.* Make enough master mix for all of the samples (this is usually the number of samples you have plus 4- 2 for the standards plus another 2 for pipette error).

2. Normalize the libraries

2-1. Calculate each sample’s molarity (in nM):

From 16S Metagenomic Sequencing Library Preparation Part # 15044223 Rev. B:



Note, you will need to know which primers were used to amplify each sample during PCR-1

Expected library sizes after PCR-2:

341/785: 581bp

926/1392: 600bp

pmo: 608bp

2-2. Calculate total volume needed to dilute each library to 4nM, using the equation V1\*C1=V2\*C2

-Personally, I (Ilona) do not work with volume below 2.0uL, so I plug

the following number to the above equation:

(2uL lib \* current conc. in nM) = (x total volume \* 4nM). Plug the values calculated in 5-1 into the “current conc. in nM” spot and solve for “x”

-*The minimum total volume calculated needs to be at least 7uL* to ensure enough volume for subsequent pooling.

2-3. Subtract 2uL from the total volume to calculate the amount of Buffer EB to add

2-4. Add the calculated amount of Buffer EB to a clean PCR plate.

2-5. Using a multichannel pipette, add 2uL of each sample to the PCR plate with EB Buffer. Take the time to visually inspect that each channel aspirated the appropriate amount of sample.

3. Pooling the libraries, Part 1

6-1. Create a pooled sample for each primer type: Combine 5uL of each sample that uses the same PCR-1 primer into one 1.5mL eppendorf tube.

4. BioAnalyze the pools and the metagenomes

7-1. Using a High Sensitivity BioAnalyzer chip (available for purchase from the Stous lab in EEEL), analyze the amplicon pools and the metagenomes going on the same lane.

7-2. Provide the BioAnalyzer data on the average size of each metagenome to the person who prepared them.

-That person can then use this value to calculate the molarity of their metagenome and thereby dilute their sample to 4nM

5. Pooling the libraries, Part 2

Now that the metagenomes have been diluted to 4nM, all of the samples can be pooled.

8-1 Combine the entire volume of the amplicon pools into 1 tube.

8-2 Add the metagenomes in whatever ratio has been decided prior to running on the lane.

**Running the MiSeq**

Based on: Preparing Libraries for Sequencing on the MiSeq Part # 15039740 Rev. D

The MiSeq is run in the Harrison Lab. Bring with you:

pipettes and pipette tips (P10, P1000)

microcentrifuge tubes

all reagents (tween, NaOH. buffer EB)

gloves

1. Check to make sure that there is at least 100GB of free space on Drive D:

-If there is less than 100GB of free space:

1. Empty the recycling bin

2. CUT folders from/Data/Illumina/MiSeqAnalysis to the external hard drive

2. Thaw the MiSeq reagent cartridge at 4°C, overnight

3. It *may* be necessary to perform a Maintenance Wash on the MiSeq before you -begin your run. A maintenance wash needs to be run once per week; if it has been longer than 1 week (as indicated by a yellow exclamation point pop up on the MiSeq welcome screen) then you need to perform this wash before you begin. If you see a green check mark, you do not need to perform a wash.

-A maintenance wash takes approximately an hour to complete.

-There is a stock solution of 1% Tween20 in the Harrison Lab. The Harrison Lab is NOT responsible for keeping this stock bottle full; it is refilled by all the MiSeq users in the department. The last person who fills it leaves their name and a date on the bottle.

-Dilute the stock solution to 0.5% Tween20 with MilliQ water (750mL of 1% Tween and 750mL of MilliQ water, for a total of 1.5L 0.5% Tween).

-Follow pages 83-85 of the MiSeq System User Guide (The guide is in the “Illumina Documents” drawer opposite the MiSeq in the Harrison lab)

4. Power cycle the MiSeq: Shut down the MiSeq computer. Turn off the machine. Wait 15 seconds. Restart the machine.

5. Set up the MCS Software (Preparing the Sample Sheet File)

-Windows desktop

-Illumina Experiment Manager

-Edit Sample Sheet

-Select MS3860243\_Dunfield\_Lane4 as your template

-Open the MiSeq reagent Cartridge and get its number (example number: MS2865376)

-NexteraXT

-Index Reads 2

-Year\_Dunfield\_Lane#

-Your initials

-For the description you can put anything, suggestions: names of metagenomes and number of amplicons

-Paired end

-301 cycles

-301 cycles

-Make sure “Use Adapter trimming” is selected

-Next

-The Dunfield Lane 4 Your Sample Sheet template opens. The template is written for 96 samples. If you have less than 96 samples, delete the wells not being run.

-Double check Sample Sheet

-Click Finish

-Name your file MSxxxxxxx\_Dunfield\_LaneX

6. Prepare 10mM TrisHCl (=Qiagen Buffer EB) with 0.1% Tween20

-This solution will be used later to dilute the PhiX control

|  |
| --- |
| 18uL Qiagen Buffer EB (=10mM TrisHCl)  2uL 100% Tween20 |
| 20uL 10% Tween20 in EB |

|  |
| --- |
| 198uL Qiagen Buffer EB  2uL 10%Tween20 prepared above |
| 200uL of 0.1% Tween20 |

7. Prepare 1mL of 0.2N NaOH

-This solution will be used to denature both the library and the PhiX control

-Our lab has a stock solution of 1N NaOH

|  |
| --- |
| 800uL Laboratory-grade water  200uL 1.0N NaOH |
| 1000uL 0.2N NaOH |

8. Get ice

9. Dilute a PhiX library. The PhiX library can be found in the Harrison -20.

|  |
| --- |
| 2uL of 10nM PhiX  3uL of EB with 0.1% Tween20 |
| 5uL of 4nM PhiX library |

10. Working on ice, denature your library and the PhiX library:

-Denaturing your Library: Denaturing PhiX (in Harrison -20)

|  |  |
| --- | --- |
| 5uL of 4nM library  5uL of 0.2N NaOH | 5uL of 4nM PhiX library  5uL of 0.2N NaOH |

-Discard remaining 0.2N NaOH

-Vortex briefly and spin down at 1700rpm (280g) for 1 minute

-Incubate for 5 minutes, at room temperature (this is when the library is denatured into single strands)

-Add HT1 (“Hyb buffer”) to the denatured library and Phi X. HT1 can be found the reagent cartridge and will be thawed overnight with the cartridge.

|  |  |
| --- | --- |
| 10uL denatured, diluted library  990uL HT1 | 10uL denatured, diluted PhiX  990uL HT1 |
| 1000uL 20pM library | 1000uL 20pM PhiX |

-For V3 chemistry kits, both the library and the PhiX control are used at the 20pM concentration

11. Combine the library and the PhiX control:

-Since we always combine metagenomes with our amplicons, our libraries are considered to be high diversity; therefore, combine:

|  |
| --- |
| 693uL denatured, diluted library  7uL denatured, diluted PhiX control |
| 700uL ready to load library |

12. Place library on ice until you are ready to load the cartridge

13. Open the MiSeq software

-Windows desktop

-Double click MiSeq Control Software

-Select “Sequence” and press “Next”

14. Follow pages 66-67 in the MiSeq System User Guide (The guide is in the “Illumina Documents” drawer opposite the MiSeq in the Harrison lab).

-Load the flowcell in the Harrison cold room

15. Follow pages 69-72 in the MiSeq System User Guide

-Make sure you are using MilliQ water to rinse the flowcell

-Lens paper is in the drawer near the gel doc.

-Alcohol wipes are located in the first drawer near the MiSeq

16. Follow pages 73-75 in the MiSeq System User Guide

-When loading the PR2 bottle and the reagent cartridge, remove the wash bottle and wash tray, empty them in the sink, and leave them near the MiSeq

-Click “Browse” to select your Sample Sheet File, prepared in Step 3.

-Click “Restart check” and review the run set up. Press “Next”

17. Tape up the “MiSeq run in progress” signs near the MiSeq, PCR machines, and gel doc.

18. A MiSeq V3-600 cycle run takes ~50 hours to complete

**After the Run**

1. REMOVE your data from the MiSeq (transfer it to your personal USB and then either delete it off the MiSeq or CUT it to the Harrison external hard drive).

Computer/Data/Illumina/MiSeq Output

2. Perform a Post Run Wash

-Follow pages 81-82 in the MiSeq System User Guide

3. Waste disposal:

-flowcell: stays inside

-pink waste: pour into “MiSeq Waste” container in the Harrison hood

-PR2 bottle: pour contents down the sink; discard the bottle in the regular

garbage

-reagent cartridge: take cartridge to the hood and using a glass pipette, siphon out the blue liquid in the position 1 well, disposing of it in the “MiSeq Waste” container; discard of the glass pipette in the yellow bucket; discard the rest of the reagent cartridge in the regular garbage.