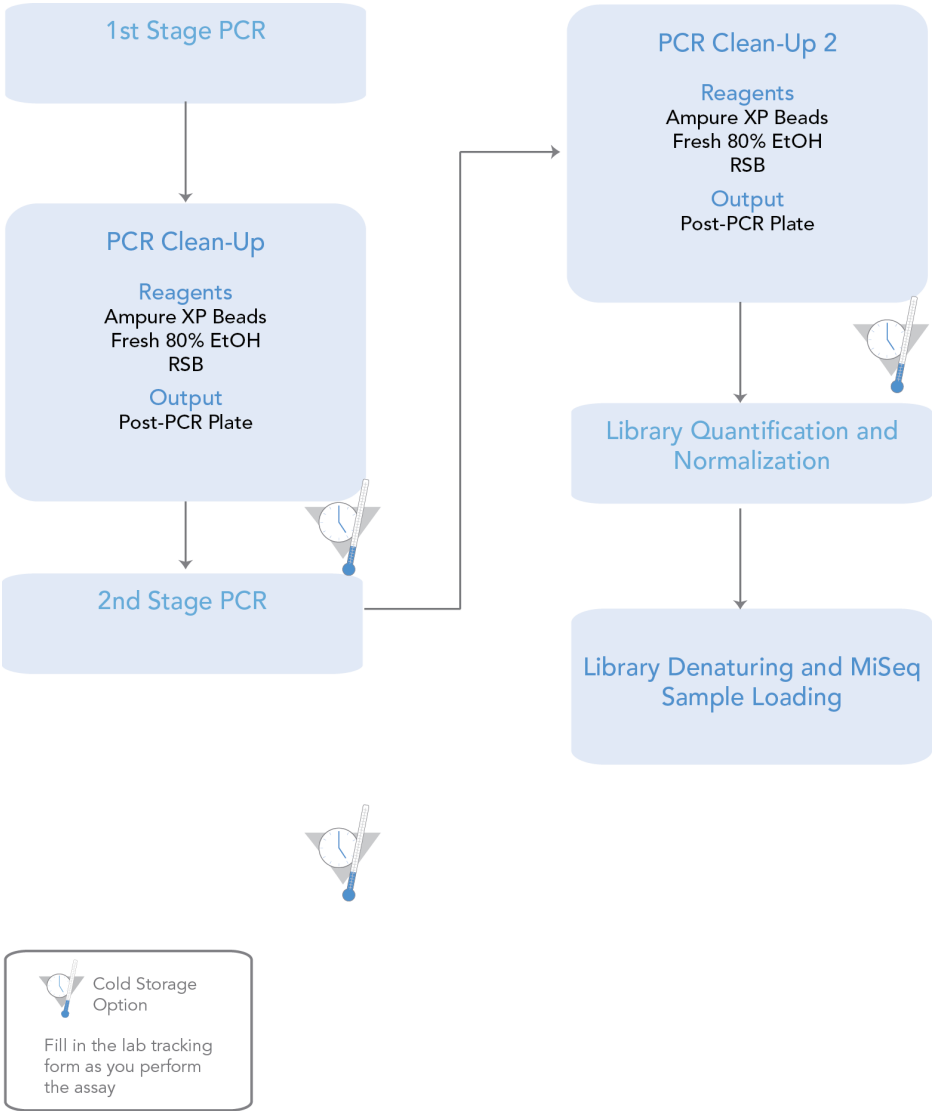
Avian 12S Library Prep & Sequencing with Illumina MiSeq for Water Sample Analysis

Linda Rutledge, January 22, 2020

1 INTRODUCTION

Before proceeding with the library prep be sure to review Appendix B: Preventing Contamination. Figure 1.1 shows the general workflow for this library preparation.

1.1 FLOWCHART OF GENERAL WORKFLOW FOR LIBRARY PREP (FROM ILLUMINA 16S PROTOCOL).



2 LIBRARY PREPARATION DETAILS

2.1 1ST STAGE PCR (AMPLICON PCR)

2.1.1 Day 1 – Plate DNA

- ☐ Prepare the plate of DNA the day before the PCR by adding 4 uL of template into each well.
- ☐ Thaw, mix and spin DNA prior to plating.
- ☐ **4 uL of each sample** should be amplified and a **PCR negative** and **PCR positive** should be included.
- ☐ With the MiSeq Nano kit v2 we can pool at least 48 samples (exclude negatives assuming they do not amplify anything).
- ☐ With the MiSeq Standard Kit v2 we can pool >384 samples (exclude negatives assuming they do not amplify anything).
- ☐ Label Plate in the following way: Avian_eDNA_year_SampleLocation_PlateNumber_Date

2.1.2 Day 2 - 1st Stage PCR (Amplicon PCR)

Use 4 uL of DNA for this reaction.
Total reaction volume per sample is 25uL.

Assemble the following reagents:

√	Item	Quantity (uL) per sample	Quantity (uL) for 30 (24) Samples	Quantity (uL) for 55 (48) Samples	Quantity (uL) for 110 (96) Samples
	NEB Q5 Mastermix (2x)	12.5	375	687.5	1375
	10 uM Primers with Illumina Overhang: (MiBird Illumina U-**F**)*	0.5	15	27.5	55
	10 uM Primers with Illumina Overhang: (MiBird Illumina U-**R**)*	0.5	15	27.5	55
	DNAase/RNAase Free Molecular Grade Water	7.5	225	412.5	825
	DNA Sample	4	-	-	-

***Primer Sequences (**Ushio et al 2018. Demonstration of the potential of environmental DNA as a tool for the detection of avian species. Scientific Reports. DOI:10.1038/s41598-018-22817-5). Amplifies 171bp insert of mtDNA 12S region.

MiBird Illumina U-**F:**
5'- **TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GGGTTGGTAAATCTTGTGCCAGC - 3'

MiBird Illumina U-**R:**
5'-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CATAGTGGGGTATCTAATCCCAGTTTG**-** 3'

☐ Prepare the PCR cocktail in the NON-DNA Paleo Lab according to the following guidelines:

Reagent	C1	V1 (uL)	C2	V2 (uL)	30 (24) Samples	√	55 (48) Samples	√	110 (96) samples	√	Other Amount
NEB Q5 Mastermix (2x)	2	12.5	1	25	375		687.5		1375		
10 uM Primers with Illumina Overhang: (MiBird Illumina U-**F**)*	10	0.5	0.2	25	15		27.5		55		
10 uM Primers with Illumina Overhang: (MiBird Illumina U-**R**)*	10	0.5	0.2	25	15		27.5		55		
Water (uL)		7.5		25	225		412.5		825		
DNA (uL)		4									

☐ Move the PCR cocktail into the Paleo Lab DNA room. Remove previously aliquoted DNA from freezer. Thaw and quick spin.
☐ Add 21 uL of cocktail to each well.
☐ Cover loosely with a seal for heat sealing outside the paleo lab.
☐ Add 4 uL of control DNA to well H12 (standardized to 0.25 ng/uL)
☐ Add 4 uL of dH2O (PCR Negative control) to well H11.
☐ Heat seal the plate and quickly spin it down.
☐ Place in Thermal Cycler (MiBird1) and run with the following conditions.

Step	Temperature (°C)	Duration	Cycles
Initial Denaturation	98	30 sec	1
Denaturation	98	10 sec	35
Annealing	54	30 sec	
Extension	72	25 sec	
Final Extension	72	2 min	1
hold	4	HOLD	

2.2 AMPLICON PCR BEAD CLEAN-UP

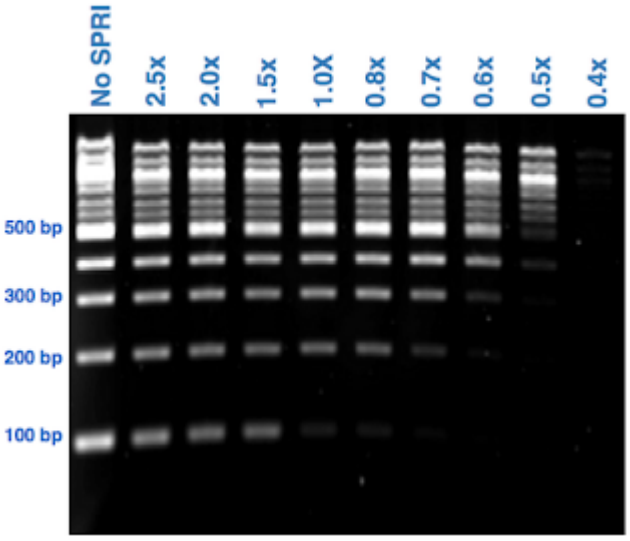
Use AMPure XP beads (Beckman Coulter) to purify the amplicon away from the free primers and primer dimer sequences.

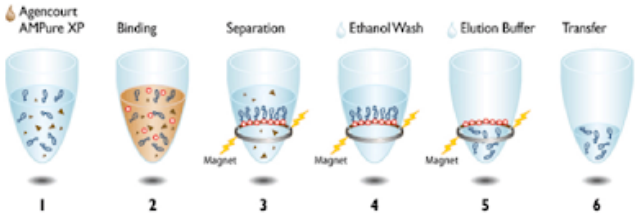
> **Safety note:** AMPure beads are in a sodium azide solution Sodium azide forms explosive compounds with heavy metals. This product contains concentrations of azide <0.1% (w/w) which with repeated contact with lead and copper commonly found in plumbin drains may result in the build-up of shock sensitive compounds. Supernatant and used beads must be disposed of in hazardous waste.

☐ **Assemble the following reagents:**

√	Item	Quantity (uL) per sample	Quantity (uL) for 30 (24) Samples	Quantity (uL) for 55 (48) Samples	Quantity (uL) for 110 (96) Samples	Other Amount
	10 mM Tris HCl (pH 8.5) (Qiagen EB)	52.5 uL per sample	1575	2887.5	5775	
	AMPure Beads (Beckman Coulter) at Room Temperature	20 uL per sample	600	1100	2200	
	Freshly Prepared 80% Molecular Grade Ethanol (EtOH)**	400 uL per sample	12,000	22,000	44,000	
	96-well 0.2 mL PCR plate	1 plate				

** Note that the purity of the ethanol is important to this step. Use only 200 proof molecular grade ethanol (e.g. Fisher BP2818500) and make it up fresh each time.





Preparation:

- Bring the AMPure XP beads to room temperature by mixing them on a spinner for 30 minutes prior to bead clean-up.

Procedure - Amplicon PCR Bead Clean Up

- ☐ Centrifuge the Amplicon PCR plate at 1,000 x g at 20ºC for 1 minute to collect condensation. Carefully remove seal.
- ☐ Vortex the AMPure XP bead for 30 seconds to make sure that the beads are evenly dispersed. Add an appropriate volume of beads to a trough or to a strip of PCR plate wells depending on the number of samples processing.
- ☐ Using a multichannel pipette, add 20 uL of AMPure XP beads (ratio of beads to DNA is 0.8x) to each well of the Amplicon PCR plate and gently pipette up and down 10 times. Change tips between columns. *Ratio of beads may change depending on size fragment
- ☐ Incubate at room temperature for 5 minutes.
- ☐ Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared. The time to bind will depend on the strength of the magnet.
- ☐ With the Amplicon PCR plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant (into hazardous waste glass contained). Change tips between samples. (DO NOT DISTURB THE MAGNET PELLET).
- ☐ With the Amplicon PCR plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
    - Using a multichannel pipette, add 200 uL of freshly prepared 80% ethanol to each sample well.
    - Incubate the plate on the magnetic stand for 30 seconds.
    - Carefully remove and discard the supernatant. (You can discard this onto a Kimwipe place in a beaker and allow the ethanol to evaporate before discarding in waste).
- ☐ Repeat Step #7 (previous step).
    - With the Amplicon PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes. DO NOT OVER DRY THE BEADS.
    - Use a P10 multichannel with long fine tips to remove excess ethanol at the bottom of the wells. Be careful not to touch the beads.
- ☐ Remove the Amplicon PCR plate from the magnetic stand. Using a multichannel pipette, add 52.5 uL of 10 mM Tris pH 8.5 (EB Solution) to each well of the Amplicon PCR plate.
- ☐ Gently pipette mix up and down 10 times, changing tips after each column. Make sure that beads are fully resuspended.
- ☐ Incubate at room temperature for 2 minutes.
- ☐ Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.
- ☐ Using a multichannel pipette, carefully transfer 50 uL of supernatant from the Amplicon PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination.

Quality Control of Cleaned Amplicon PCR:
Run 5 uL of cleaned PCR product on a 1.5% agarose gel. Include a positive control to ensure amplicon is at correct fragment size.

Optional: Run 1 uL of PCR product on Agilent Tape Station with the D1000 protocol to ensure target amplified. Our target fragment will be ~300bp (target fragment (175 bp) + primers + Illumina overhang). The figure below (figure 1a) shows expectation from TapeStation trace for an amplified 12S fragment. This sample is from Ripley's Aquarium. Note that samples from natural lakes will have lower DNA yields and peak height (Figure 1b); samples from diet analysis will have higher yields. but should be the same fragment size.
Note for diet where yields are expected to be higher, you can also run 5 uL of cleaned product on an agarose gel.

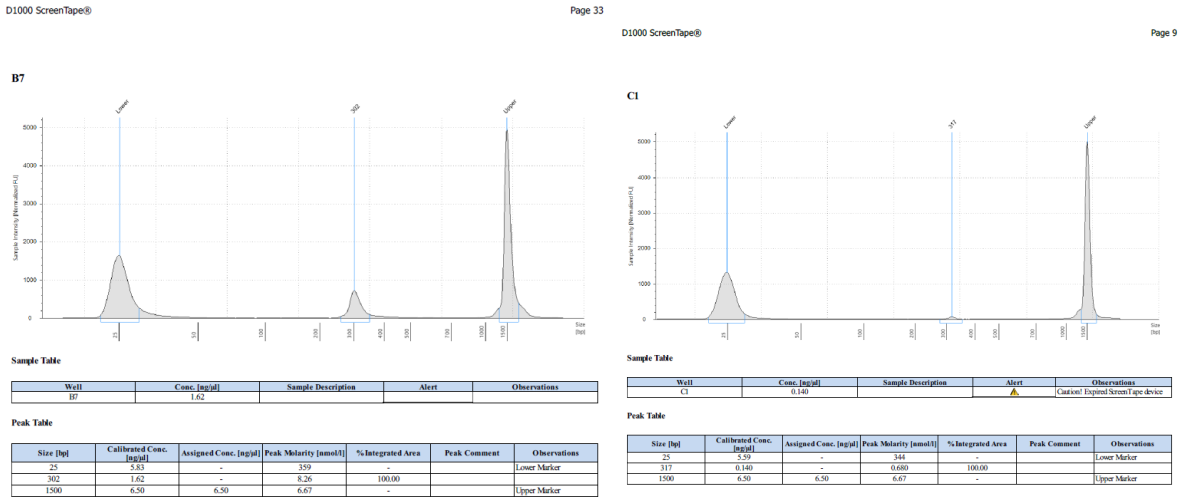


Figure 1: a) Example Amplicon amplification from Ripleys Aquarium. b) Example amplicon amplification from Lake sample.

SAFE STOPPING POINT: If you do not immediately proceed to *Index PCR,* seal plate adhesive seal and store it at -20C for up to a week.

2.2.1 Index PCR

Day 3 – 2nd Stage Index PCR
This step attaches dual indices and Illumina sequencing adapters using the Nextera XT Index Kit. (Refer to Nextera Low Plex Pooling Guidelines for acceptable Index combinations https://www.illumina.com/documents/products/technotes/technote_nextera_low_plex_pooling_guidelines.pdf)

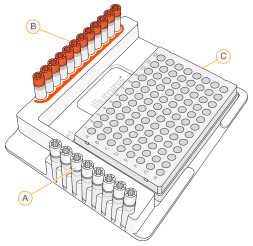
- ☐ **Assemble the following reagents:**

√	Item	Quantity (uL) per sample	Quantity (uL) for 30 (24) Samples	Quantity (uL) for 55 (48) Samples	Quantity (uL) for 110 (96) Samples	Other quantity
	Amplicon PCR product	5				
	NEB Q5 Mastermix	25	750	1375	2750	
	Nextera XT Index 1 Primers (N7XX) from the Nextera XT Index kit (FC-131-1001 or FC-131-1002)	5	150	275	550	
	Nextera XT Index 2 Primers (S5XX) from the Nextera XT Index kit (FC-131-1001 or FC-131-1002)	5	150	275	550	
	PCR Grade Water	10	300	550	1100	
	TruSeq Index Plate Fixture (FC-130-1005)	1				
	96-well 0.2 ml PCR plate	1				
	Plate Seal	1				

Procedure – Index PCR

1. Using a multichannel pipette, transfer 5 µl from each well to a new 96-well plate. The remaining 1st stage PCR product is not used in the protocol and can be stored in the freezer for other uses.
2. Arrange the Index 1 and 2 primers in a rack (i.e. the TruSeq Index Plate Fixture), on ice, using the following arrangements as needed:
   i) Arrange Index 2 primer tubes (white caps, clear solution) vertically, aligned with rows A through H.
   ii) Arrange Index 1 primer tubes (orange caps, yellow solution) horizontally, aligned with columns 1 through 12.

Figure 4 TruSeq Index Plate Fixture
A Index 2 primers (white caps)
B Index 1 primers (orange caps)
C 96-well plate
Place the 96-well PCR plate with the 5 µl of resuspended PCR product DNA in the TruSeq Index Plate Fixture.



3. Set up the following reaction of DNA, Index 1 and 2 primers, 2x NEB mastermix, and PCR Grade water: total 50 uL reaction
   a. Add 5 uL of each index to each 5 uL DNA well. Replace all index caps with new, clean caps after use.
   b. Then make a cocktail by combining Mastermix (25 uL per sample) and water (10 uL per sample) according to the following guidelines.

√	Item	C1	V1	C2	V2	30 (24) Samples	55 (48) Samples	110 (96) Samples
	NEB Q5 mastermix	2	25	1	50	750	1375	2750
	PCR Grade Water		10			300	550	1100

   c. Add 35 uL of the Mastermix/water cocktail to each well to make a 50 uL reaction.

4. Gently pipette up and down 10 times to mix.
5. Cover the plate with a foil heat seal.
6. Centrifuge the plate at 1,000 × g at 20°C for 1 minute.
7. Perform PCR on a thermal cycler using the following program (*Note NEB says 65C when using their indices, but Isabel at NEB tech support confirmed our Illumina indices anneal at 67C)

Step	Temperature (°C)	Duration	Cycles
Initial Denaturation	98	30 sec	1
Denaturation	98	10 sec	8
Annealing/Extension	67*	75 sec	
Final Extension	67*	5 min	1
Hold	4	HOLD	

2.2.2 Index PCR Bead Clean Up

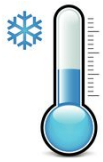
This step uses AMPure XP beads to clean up the index PCR before quantification.

Assemble the following reagents:

√	Item	Quantity	Quantity (uL) for 30 (24) Samples	Quantity for 55 (48) Samples	Quantity for 110 (96) Samples	Other Amount
	10 mM Tris pH 8.5 (Qiagen Buffer EB)	27.5 uL per sample	825	1512.5	3025	
	AMPure XP beads (Beckman Coulter)	56 uL per sample	1680	3080	6160	
	Freshly Prepared 80% Ethanol	400 uL per sample	12,000	22,000	44,000	
	96-well 0.2 mL PCR plate	1 plate				

Procedure – Index PCR Bead Clean Up

- ☐ Centrifuge the Index PCR plate at 280 × g at 20°C for 1 minute to collect condensation.
- ☐ Vortex the AMPure XP beads for 30 seconds to make sure that the beads are evenly dispersed. Add an appropriate volume of beads to a trough.
- ☐ Using a multichannel pipette, add 56 µl of AMPure XP beads to each well of the Index PCR plate.
- ☐ Gently pipette mix up and down 10 times if using a 96-well PCR plate.
- ☐ Incubate at room temperature without shaking for 5 minutes.
- ☐ Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared.
- ☐ With the Index PCR plate on the magnetic stand, use a multichannel pipette to remove and discard the supernatant. Change tips between samples.
- ☐ With the Index PCR plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:
    - Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
    - Incubate the plate on the magnetic stand for 30 seconds.
    - Carefully remove and discard the supernatant.
- ☐ With the Index PCR plate on the magnetic stand, perform a second ethanol wash as follows:
    - Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.
    - Incubate the plate on the magnetic stand for 30 seconds.
    - Carefully remove and discard the supernatant.
    - With the Index PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.
    - Use a P20 multichannel pipette with fine pipette tips to remove excess ethanol.
- ☐ Remove the Index PCR plate from the magnetic stand. Using a multichannel pipette, add 27.5 µl of 10 mM Tris pH 8.5 (EB solution) to each well of the Index PCR plate.
- ☐ Gently pipette mix up and down 10 times until beads are fully resuspended, changing tips after each column
- ☐ Incubate at room temperature for 2 minutes.
- ☐ Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.
- ☐ Using a multichannel pipette, carefully transfer 25 µl of the supernatant from the Index PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross contamination.



SAFE STOPPING POINT

If you do not plan to proceed to *Library Quantification, Normalization, and Pooling,* seal the plate with an adhesive seal. Store the plate at -15° to -25°C for up to a week.

2.2.3 Library Quantification, Normalization, and Pooling

Illumina recommends quantifying your libraries using a fluorometric quantification method that uses dsDNA binding dyes.

1. Calculate DNA concentration in nM, based on the size of DNA amplicons as determined by an Agilent Technologies 2100 Bioanalyzer trace or Tape Station trace:

$$\frac{(concentration\ in\ \frac{ng}{uL})}{(660\frac{g}{mol}\ x\ average\ library\ size)} \times 10^6 = concentration\ in\ nM$$

Example for our libraries from lake water samples (after indexing the Qubit HS quantification is about 15 ng/uL (Note that Ripleys samples were up to about 100 ng/uL):

$$\frac{15\ ng/uL}{(660\frac{g}{mol}x\ 380\ bp)} \times\ 10^6 = 59.8\ nM$$

2. Dilute each final index PCR product library with Resuspension Buffer (RSB) or 10 mM Tris pH 8.5 (Qiagen Buffer EB) to 4nM with the following formula as an example based on the above concentration calculation:

$$C1 \times V1\ = C2 \times V2$$
$$59.8\ nM\ \times V1 = 4\ nM\ \times 50\ uL$$
$$V1\ = (4\ nM\ \times 50\ uL) \div 59.8\ nM$$
$$V1\ = 200\ \div 59.8$$
$$V1\ = 3.3\ uL$$

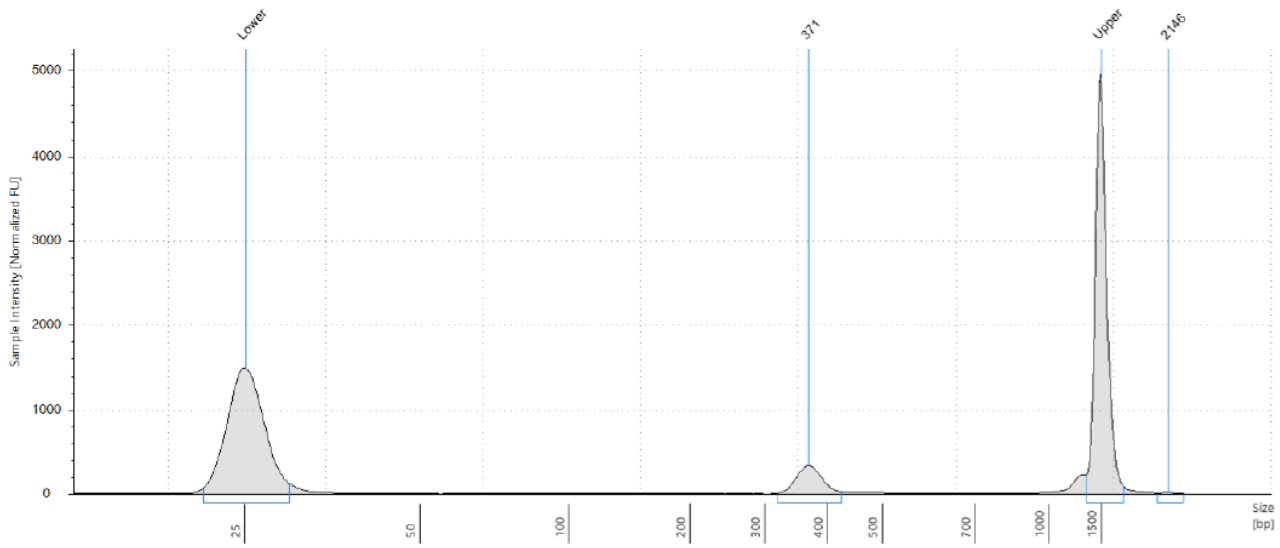
   a. So to make a 4nM library from a 56.8 nM library, combine 3.3 uL of the index PCR product with 46.7 uL (50 – 3.3 = 46.7 uL) of RSB or Buffer EB
3. Aliquot 5 µL of each standardized 4 nM DNA index PCR product library into a low-binding 1.5 mL tube.
4. For our library, we can combine 24 samples for a MiSeq Micro Kit or 96 samples with a MiSeq Standard Kit.

Validate Library

Run 1 µl of the final pooled library on the Agilent 4200 Tapestation (In Aaron Shafer's lab) with the D1000 protocol to verify the size. With the MiFish primer pairs in the protocol, the expected size on a Bioanalyzer trace of the final library is about 380 bp.

Figure: Example Agilent 4200 Tapestation Trace of pooled Library (with 12S target).

B1: CommEdnaTest3Take3



Sample Table

Well	Conc. [ng/µl]	Sample Description	Alert	Observations
B1	1.03	CommEdnaTest3Take3		

2.2.4 Library Denaturing and MiSeq Sample Loading

In preparation for cluster generation and sequencing, pooled libraries are denatured with NaOH, diluted with hybridization buffer, and then heat denatured before MiSeq sequencing. Each run for our purpose should include 30% PhiX to serve as an internal control for these low diversity libraries. Illumina recommends using MiSeq v3 reagent kits for improved run metrics. But we are using the v2 kits to get 300 cycles.

Assemble the following items:

√	Item	Quantity	Storage
	10 mM Tris pH 8.5 or RSB (Resuspension Buffer)	6 uL	-15° to -25°C
	HT1 (Hybridization Buffer) 1540 µl	1540 µl	-15° to -25°C
	0.2 N NaOH (less than a week old)	10 µl	-15° to -25°C
	PhiX Control Kit v3 (FC-110-3001)	4 µl	-15° to -25°C
	MiSeq reagent cartridge	1 cartridge	-15° to -25°C
	1.7 ml microcentrifuge tubes (screw cap recommended)	3 tubes	
	2.5 L ice bucket		

Preparation

1. Set a heat block suitable for 1.7 ml microcentrifuge tubes to 96°C
2. Remove a MiSeq reagent cartridge from -15°C to -25°C storage and thaw at room temperature.
3. In an ice bucket, prepare an ice-water bath by combining 3 parts ice and 1 part water.

Denature DNA

1. Combine the following volumes of pooled final DNA library and freshly diluted 0.2 N NaOH in a microcentrifuge tube:
    a. 4 nM pooled library (5 µl)
    b. 0.2 N NaOH (5 µl)

2. Set aside the remaining dilution of 0.2 N NaOH to prepare a PhiX control within the next 12 hours.
3. Vortex briefly to mix the sample solution, and then centrifuge the sample solution at 280 × g at 20°C for 1 minute.
4. Incubate for 5 minutes at room temperature to denature the DNA into single strands.
5. Add the following volume of pre-chilled HT1 to the tube containing denatured DNA:
    a. Denatured DNA (10 µl)
    b. Pre-chilled HT1 (990 µl)
    Adding the HT1 results in a 20 pM denatured library in 1 mM NaOH.
6. Place the denatured DNA on ice until you are ready to proceed to final dilution.

Dilute Denatured DNA

1. Dilute the denatured DNA to the desired concentration using the following example:

NOTE
Illumina recommends targeting 800–1000 K/mm² raw cluster densities (MiSeq v 3 reagents) and 600 – 800 K/mm² for MiSeq v2 reagents (600-800 with v2). We are using v2 so we expect about 700 K/mm². After some initial runs, we decided to run a 6 pM loading concentration.

Final Concentration	2 pM	4 pM	6 pM	8 pM	10 pM
20 pM denatured library	60 uL	120 uL	180 uL	240 uL	300 uL
Pre-chilled HT1	540 uL	480 uL	420 uL	360 uL	300 uL

2. Invert several times to mix and then pulse centrifuge the DNA solution.
3. Place the denatured and diluted DNA on ice.

Denature and Dilution of PhiX Control

Use the following instructions to denature and dilute the 10 nM PhiX library to the same loading concentration as the Amplicon library. For our purposes, the final library mixture should contain 30% PhiX.

1. Combine the following volumes to dilute the PhiX library to 4 nM:
    a. 10 nM PhiX library (2 µl)
    b. 10 mM Tris pH 8.5 (3 µl)
2. Combine the following volumes of 4 nM PhiX and 0.2 N NaOH in a microcentrifuge tube:
    a. 4 nM PhiX library (5 µl)
    b. 0.2 N NaOH (5 µl)
3. Vortex briefly to mix the 2 nM PhiX library solution.
4. Incubate for 5 minutes at room temperature to denature the PhiX library into single strands.
5. Add the following volumes of pre-chilled HT1 to the tube containing denatured PhiX library to result in a 20 pM PhiX library:
    a. Denatured PhiX library (10 µl)
    b. Pre-chilled HT1 (990 µl)
6. Dilute the denatured 20 pM PhiX library to the same loading concentration as the Amplicon library as follows:

Final Concentration	2 pM	4 pM	6 pM	8 pM	10 pM
20 pM denatured library	60 uL	120 uL	180 uL	240 uL	300 uL
Pre-chilled HT1	540 uL	480 uL	420 uL	360 uL	300 uL

7. Invert several times to mix and then pulse centrifuge the DNA solution.
8. Place the denatured and diluted PhiX on ice.

Combine Amplicon Library and PhiX Control

1. For a 30% Phix, combine the following volumes of denatured PhiX control library and your denatured amplicon library in a microcentrifuge tube:
    a. Denatured and diluted PhiX control (180 µl)
    b. Denatured and diluted index PCR pooled library (420 µl)
2. Set the combined sample library and PhiX control aside on ice until you are ready to heat denature the mixture immediately before loading it onto the MiSeq v3 reagent cartridge.
3. Using a heat block, incubate the combined library and PhiX control tube at 96°C for 2 minutes.
4. After the incubation, invert the tube 1–2 times to mix and immediately place in the icewater bath.
5. Keep the tube in the ice-water bath for 5 minutes.

NOTE
Perform the heat denaturation step immediately before loading the library into the MiSeq reagent cartridge to ensure efficient template loading on the MiSeq flow cell.

3 APPENDIX B: PREVENT PCR CONTAMINATION

This section is taken from the Illumina 16SMetagenomic Sequencing Library Preparation protocol.

Prevent PCR Product Contamination

The PCR process is commonly used in the laboratory to amplify specific DNA sequences. Unless proper laboratory hygiene is used, PCR products can contaminate reagents, instrumentation, and genomic DNA samples, causing inaccurate and unreliable results. PCR product contamination can shut down lab processes and significantly delay normal operations.

Make sure that the lab is set up appropriately to reduce the risk of PCR product contamination:

1. **Physically Separate Pre-PCR and Post-PCR Areas**

- Physically separate laboratory space where pre-PCR processes are performed (DNA
- extraction, quantification, and normalization) from the laboratory space where PCR
- products are made and processed (post-PCR processes).
- Never use the same sink to wash pre-PCR and post-PCR troughs.
- Never share water purification systems for pre-PCR and post-PCR processes.
- Store all supplies used in the protocols in the pre-PCR area, and transfer to the post-PCR area as needed.

2. **Use Dedicated Equipment and Supplies**

- Dedicate separate full sets of equipment and supplies (pipettes, centrifuges, oven, heat block, etc.) to pre-PCR and post-PCR lab processes, and never share between processes.
- Dedicate separate storage areas (freezers and refrigerators) to pre-PCR and post-PCR consumables.

Because the pre- and post-amplification reagents are shipped together, it is important to unpack the reagents in the pre-PCR lab area. After unpacking the reagents, move the post-amplification reagents to the proper post-PCR storage area.

Pre-PCR and Post-PCR Lab Procedures

To prevent PCR product contamination, it is important to establish lab procedures and follow best practices. Illumina recommends daily and weekly cleaning of lab areas using 0.5% Sodium Hypochlorite (10% Bleach).

! CAUTION

To prevent sample or reagent degradation, make sure that all vapors from the cleaning solution have fully dissipated before beginning any processes.

Daily Cleaning of Pre-PCR Area

A daily cleaning of the pre-PCR area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps to eliminate PCR product that has entered the pre-PCR area.
Identify pre-PCR areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution before beginning any pre-PCR processes. High-risk areas might include, but are not limited to, the following items:
- Benchtops
- Door handles
- Refrigerator/freezer door handles
- Computer mouse
- Keyboards

Daily Cleaning of Post-PCR Area

Reducing the amount of PCR product in the post-PCR area helps reduce the risk of contamination in the pre-PCR area. Daily cleaning of the post-PCR area using a 0.5% Sodium Hypochlorite (10% Bleach) solution helps reduce the risk of contamination.
Identify post-PCR areas that pose the highest risk of contamination, and clean these areas with a 0.5% Sodium Hypochlorite (10% Bleach) solution daily. High-risk areas might include, but are not limited to, the following items:
- Thermal cyclers
- Bench space used to process amplified DNA
- Door handles
- Refrigerator/freezer door handles
- Computer mouse
- Keyboards

Weekly Cleaning of All Lab Areas

One time a week, perform a thorough cleaning of the pre-PCR and post-PCR areas using 0.5% Sodium Hypochlorite (10% Bleach).
- Clean all benchtops and laboratory surfaces.
- Clean all instruments that are not cleaned daily.
- Thoroughly mop lab floors.
- Make sure that personnel responsible for weekly cleaning are properly trained on prevention of PCR product contamination.

Items Fallen to the Floor

The floor is contaminated with PCR product transferred on the shoes of individuals coming from the post-PCR area; therefore, anything falling to the floor must be treated as contaminated.
- Disposable items that have fallen to the floor, such as empty tubes, pipette tips, gloves, lab coat hangers, must be discarded.

• Non-disposable items that have fallen to the floor, such as a pipette or an important sample container, must be immediately and thoroughly cleaned. Use a 0.5% Sodium Hypochlorite (10% Bleach) solution to remove PCR product contamination.
• Clean any lab surface that has come in contact with the contaminated item. Individuals handling anything that has fallen to the floor, disposable or non-disposable, must discard their lab gloves and put on a new pair.

Best Practices

When preparing libraries for sequencing, always adhere to good molecular biology practices. Read through the entire protocol before starting to make sure that all of the required materials are available and your equipment is programmed and ready to use.

Handling Liquids

Good liquid handling measures are essential, particularly when quantifying libraries or diluting concentrated libraries for making clusters.

- Small differences in volumes (±0.5 µl) can sometimes cause large differences in cluster numbers (~100,000).
- Small volume pipetting can be a source of potential error in protocols requiring the generation of standard curves, such as qPCR, or small but precise volumes, such as the Agilent Bioanalyzer.
- If small volumes are unavoidable, use due diligence to make sure that pipettes are correctly calibrated.
- Make sure that pipettes are not used at the volume extremes of their performance specifications.
- Prepare the reagents for multiple samples simultaneously, to minimize pipetting errors, especially with small volume enzyme additions. As a result, pipette one time from the reagent with a larger volume, rather than many times with small volumes. Aliquot to individual samples in a single pipetting movement to allow for standardization across multiple samples.

Handling Magnetic Beads

! NOTE: Cleanup procedures have only been validated using the 96-well plates and the magnetic stand specified in the *Consumables and Equipment* list. Comparable performance is not guaranteed when using a microcentrifuge tube or other formats, or other magnets.

- Before use, allow the beads to come to room temperature.
- Do not reuse beads. Always add fresh beads when performing these procedures.
- Immediately before use, vortex the beads until they are well dispersed and the color of the liquid is homogeneous.
- When pipetting the beads, pipette slowly and dispense slowly due to the viscosity of the solution.
- Take care to minimize bead loss, which can affect final yields.
- Change the tips for each sample, unless specified otherwise.

- Let the mixed samples incubate at room temperature for the time indicated in the protocol for maximum recovery.
- When removing and discarding supernatant from the wells, use a single channel or multichannel pipette and take care not to disturb the beads.
- When aspirating the cleared solution from the reaction plate and wash step, it is important to keep the plate on the magnetic stand and not disturb the separated magnetic beads. Aspirate slowly to prevent the beads from sliding down the sides of the wells and into the pipette tips.
- To prevent the carryover of beads after elution, approximately 2.5 µl of supernatant is left when the eluates are removed from the bead pellet.
- Be sure to remove all of the ethanol from the bottom of the wells, as it can contain residual contaminants.
- Keep the reaction plate on the magnetic stand and let it air-dry at room temperature to prevent potential bead loss due to electrostatic forces. Allow for the complete evaporation of residual ethanol, because the presence of ethanol affects the performance of the subsequent reactions. Illumina recommends at least minutes drying time, but a longer drying time can be required. Remaining ethanol can be removed with a 10 µl pipette.
- Avoid over drying the beads, which can impact final yields.
- Do not scrape the beads from the edge of the well using the pipette tip.
- To maximize sample recovery during elution, incubate the sample/bead mix for 2 minutes at room temperature before placing the samples onto the magnet.

Avoiding Cross-Contamination

Practice the following to avoid cross-contamination:

- Open only one adapter tube at a time.
- Change the tips for each sample, unless specified otherwise.
- Pipette carefully to avoid spillage.
- Clean pipettes and change gloves between handling different adapter stocks.
- Clean work surfaces thoroughly before and after the procedure.

Potential DNA Contaminants

When handling and processing samples using this protocol, use best practices to avoid PCR contamination, as you would when preparing PCR amplicons.

Temperature Considerations

Temperature is an important consideration for making libraries:

- Keep libraries at temperatures ≤37°C, except where specifically noted.
- Place reagents on ice after thawing at room temperature.

Equipment

- Review the programming instructions for your thermal cycler user guide to make sure that it is programmed appropriately using the heated lid function.
- It is acceptable to use the thermal cycler tracked heating lid function.