

# AllType™ NGS Assay

## On the Illumina® MiSeq®, MiniSeq® and iSeq® Systems

### Application Note

For Research Use Only.  
Not For Use in Diagnostic Procedures.





## Purpose of this Guide

This application note provides reference information for using AllType NGS Amplification Kits for amplification of targeted genes in DNA samples, and subsequent library preparation, for downstream sequencing on the Illumina MiSeq®, MiniSeq® and iSeq® Systems.

This Application Note is only applicable to the use of the AllType NGS Amplification Kits and AllType NGS Index Flex Kit contained within this document.

## Limitations of the Procedure

1. For Research Use Only. This product is not intended to provide information for the diagnosis, prevention or treatment of disease or to aid in the clinical decision-making process. This product is not cleared or approved for clinical use by the FDA or approved in the EU as an in vitro diagnostic assay, nor is it CE marked.
2. Sub-optimal sample or library quality and/or quantity may cause test failures. The causes of such failures may include low sample quantity and quality, contamination, presence of inhibitors, random enzymatic reaction failures, uncalibrated and malfunctioning instruments, use of expired reagents or third-party reagents, incorrect reagent maintenance, protocol modification, and incorrect quantification or calculation.
3. Sample DNA should be quantified with a fluorometer and be free any known PCR inhibitor. PCR inhibitors may be introduced from the original sample source or from various DNA extraction methods. Routine samples should be validated for amplification using the AllType reagents.
4. The PCR and library preparation workflow described in this protocol requires highly controlled conditions. Please follow standard laboratory practices and PCR guidelines to minimize contaminations.
5. The AllType NGS assay has been verified for use with the Applied Biosystems Veriti™ 96-Well Thermal Cycler (Cat. No. 4375786). Other thermal cyclers considered for use are not supported by this application and require end-user evaluation and validation.
6. The instruction provided in this protocol has been verified to produce a final sequence-compatible, barcoded DNA fragment library using amplified DNA from AllType NGS amplification kits (Cat. No. ALL-11LX, ALL-9LX, and ALL-8LX) for generating 4nM final library pools for the Illumina MiSeq Standard, Micro and Nano Reagent Kits v2 with 2x150bp (300 cycle) output, 1nM final library pools for the Illumina [MiniSeq](#) Mid Output and High Output kits, and 100pM final library pools for the Illumina iSeq 100 reagent.
7. The AllType NGS 11 Loci (Cat. No. ALL-11LX), AllType NGS 9 Loci (Cat. No. ALL-9LX) and AllType NGS 8 Loci (Cat. No. ALL-8LX) Amplification Kits have been verified on the Illumina MiSeq, MiniSeq and iSeq 100 sequencers. For a list of verified throughputs per sequencer and per sequencing kit, please refer to the “No. of Samples Based on Targeted Region” table in the ‘Indexing Strategy’ section below. Alternative configurations, kits, and sequencing systems are not supported by this application and must be determined and validated by the user.
8. Genotyping at high resolution using the NGS technology is a complex process that requires qualified personnel to review data and make final allele assignments.
9. Please refer to AllType NGS 11 Loci Amplification Kit Ambiguities List for a known list of lot-specific allele assignment ambiguities for polymorphisms located outside of the amplified region. Any allele listed may produce incorrect results and needs to be evaluated before assigning a result.
10. Genotype ambiguities are expected from the limitation in the primer design and heterozygous genotype phasing due to limitations in sequencing read length and associated sequence alignment.
11. The AllType primers for DRB1, DQB1 and DPB1 do not amplify exon 1. Associated ambiguities are listed in the Ambiguities List.
12. In rare cases, unknown sequence variants at the amplification primer binding sites in the untranslated regions (UTRs) may affect amplification efficiency of molecular typing reagents listed above. Homozygous typing needs be confirmed by a secondary method before assigning a result.

13. The AllType NGS primers were tested using alleles identified in the Nomenclature List in brackets in Index 4 for AllType (e.g. A\*01:01<sup>[1234]</sup>). The reactivity of alleles that were not available has been predicted from its available sequence and may produce false reactions and needs to be evaluated before assigning a result.
14. The AllType NGS primers were tested based on Field-3 (6-digits genotypes) only. There are no performance claims outside this field.
15. Failure to completely read and explicitly follow all of the instructions contained herein may result in invalid test results, damage to the product(s), injury to persons, including to users or others, and damage to other property. One Lambda, Inc. does not assume any liability arising out of the improper use of the product(s) described herein (including parts thereof or software).

## **Warning or Caution**

Refer to the Safety Data Sheet for detailed information. Individual Safety Data Sheets can be downloaded at [www.onelambda.com](http://www.onelambda.com) and/or [www.thermofisher.com](http://www.thermofisher.com).



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# Product Information

## Product Description

This application note describes how to prepare Illumina MiSeq System compatible libraries from amplicons generated using AllType NGS Amplification Kits. Following the protocol in this application note, the following PCR amplicons, spanning highly variable regions of the genes, are generated:

### AllType 11-Loci Amplification Kit:

Locus	Targeted Region	Locus	Targeted Region
A	Full gene	DQB1	Exon 2 thru 3' UTR <sup>[1]</sup>
B	Full gene	DPB1	Exon 2 thru 3' UTR <sup>[1]</sup>
C	Full gene	DQA1	Full gene
DRB1	Exon 2 thru 3' UTR <sup>[1]</sup>	DPA1	Full gene
DRB3/4/5	Exon 2 thru 3' UTR <sup>[1]</sup>		

<sup>[1]</sup> Includes intronic sequence

### AllType 9 Loci Amplification Kit:

Locus	Targeted Region	Locus	Targeted Region
A	Full gene	DQB1	Exon 2 thru 3' UTR <sup>[1]</sup>
B	Full gene	DPB1	Exon 2 thru 3' UTR <sup>[1]</sup>
C	Full gene		
DRB1	Exon 2 thru 3' UTR <sup>[1]</sup>		
DRB3/4/5	Exon 2 thru 3' UTR <sup>[1]</sup>		

<sup>[1]</sup> Includes intronic sequence

### AllType 8 Loci Amplification Kit:

Locus	Targeted Region	Locus	Targeted Region
A	Full gene	DQB1	Exon 2 thru 3' UTR <sup>[1]</sup>
B	Full gene		
C	Full gene		
DRB1	Exon 2 thru 3' UTR <sup>[1]</sup>		
DRB3/4/5	Exon 2 thru 3' UTR <sup>[1]</sup>		

<sup>[1]</sup> Includes intronic sequence

After amplification, AllType amplicons are introduced into the remaining steps of the workflow and further manipulated using various library preparation reagent kits.

## AllType NGS Amplification Kits

The AllType NGS 11-Loci Amplification Kit (Cat. No. ALL-11LX) contains the following components:

AllType NGS 11-Loci Amplification Kit			
Component	Quantity	Amount	Storage
AllType 11-Loci Primer Mix	1 vial	500 µL	-20°C
AllType Buffer	1 vial	500 µL	
AllType dNTPs	1 vial	200 µL	
AllType Polymerase	1 vial	100 µL	

The AllType NGS 9 Loci Amplification Kit (Cat. No. ALL-9LX) contains the following components:

AllType NGS 9 Loci Amplification Kit			
Component	Quantity	Amount	Storage
AllType 9 Loci Primer Mix	1 vial	500 µL	-20°C
AllType Buffer	1 vial	500 µL	
AllType dNTPs	1 vial	200 µL	
AllType Polymerase	1 vial	100 µL	

The AllType NGS 8 Loci Amplification Kit (Cat. No. ALL-8LX) contains the following components:

AllType NGS 8 Loci Amplification Kit			
Component	Quantity	Amount	Storage
AllType 8 Loci Primer Mix	1 vial	500 µL	-20°C
AllType Buffer	1 vial	500 µL	
AllType dNTPs	1 vial	200 µL	
AllType Polymerase	1 vial	100 µL	

## AllType NGS Library Prep Kit

The AllType NGS Library Prep Kit (Cat. No. ALL-LIBX) contains the following components:

AllType NGS Library Prep Kit			
Component	Quantity	Amount	Storage
Fragmentase	1 vial	900 µL	-15°C to -25°C
Fragmentation Buffer	1 vial	480 µL	
Ligase	1 vial	200 µL	
Ligase Buffer	1 vial	1 mL	
Nick Repair Polymerase	1 vial	800 µL	
dNTP Mix	1 vial	200 µL	
Library Amp Master Mix	4 vials	1.8 mL	
(Low) TE Buffer	1 bottle	30 mL	

## AllType NGS Index Flex Kit

The AllType NGS Index Flex Kit (Cat. No. ALL-FLEXX) contains the following components:

AllType NGS Index Flex Kit			
Component	Quantity	Amount	Storage
AllType Barcode Adapter Plate	1 plate	6 µL/well	-20°C
AllType NGS Library Amp Primer	1 vial	400 µL	

## Required materials and equipment not supplied

General Supplies and Consumables		
Description	Supplier	Cat. No.
96-Well, 0.2mL, PCR Plates	MLS <sup>[1]</sup>	---
96 Well Plastic/Foil Plate Seals	MLS <sup>[1]</sup>	---
Thermo Scientific Nunc™ 96-Well Polypropylene MicroWell™ Plates	Fisher Scientific	12-565-369
PCR Plate & 0.2mL Tube Cooler	MLS <sup>[1]</sup>	---
Eppendorf DNA LoBind Tubes, 5.0mL	Eppendorf	0030108310
Eppendorf DNA LoBind Tubes, 1.5mL	Eppendorf	022431021
50mL Conical Tubes Nuclease free	MLS <sup>[1]</sup>	---
15mL Conical Tubes Nuclease free	MLS <sup>[1]</sup>	---
Serological Pipettes, 10 or 25mL	MLS <sup>[1]</sup>	---
Pipette Controller	MLS <sup>[1]</sup>	---
Full array of manual single channel pipettes (20µL, 200µL, 1mL)	MLS <sup>[1]</sup>	---
20µL and 200µL manual multichannel pipettes	MLS <sup>[1]</sup>	---
Full array of filtered, pre-sterilized, pipette tips	MLS <sup>[1]</sup>	---
Sterile Reagent Reservoirs	MLS <sup>[1]</sup>	---
Thermal cycler PCR Pressure Pad	MLS <sup>[1]</sup>	---

<sup>[1]</sup> Major Laboratory Supplier

## Required materials and equipment not supplied

Standalone Kits and Reagents		
Description	Supplier	Cat. No.
PhiX Control Kit	Illumina	FC-110-3001
MiSeq Reagent Kit v2 (300 cycle)	Illumina	MS-102-2002
MiSeq Reagent Nano Kit v2 (300 cycle)	Illumina	MS-103-1001
MiSeq Reagent Micro Kit v2 (300 cycle)	Illumina	MS-103-1002
MiniSeq Mid Output Kit (300 cycle)	Illumina	FC-420-1004
MiniSeq High Output Reagent Kit (300 cycle)	Illumina	FC-420-1003
iSeq 100 i1 Reagent	Illumina	20021533
Agencourt® AMPure® XP Beads, 60mL	Beckman Coulter	A63881
Nuclease Free Water	MLS <sup>[1]</sup>	---
(Low) TE Buffer	Life Technologies	12090-015
NaOH Solution ≥1.0M, Molecular Biology Grade	MLS <sup>[1]</sup>	---
200 Proof Ethanol, Molecular Biology Grade	MLS <sup>[1]</sup>	---

<sup>[1]</sup> Major Laboratory Supplier

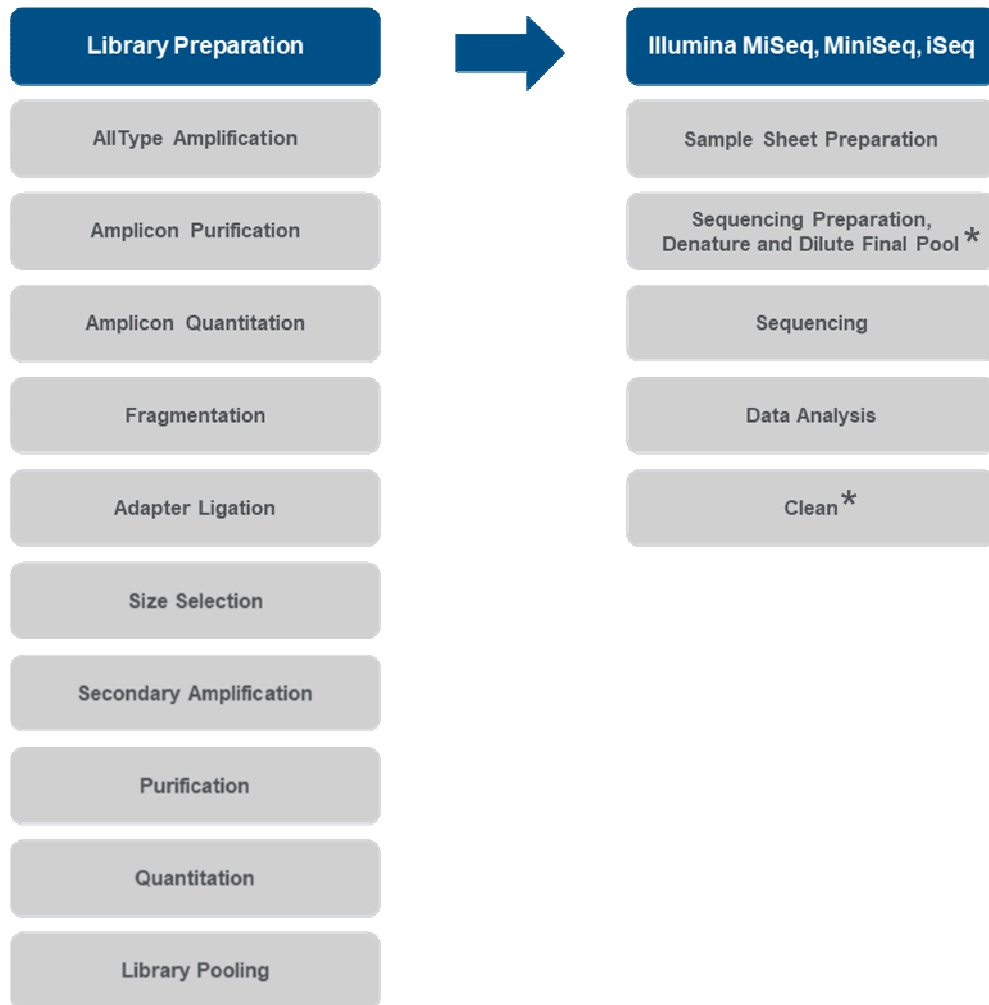
Standalone Kits and Reagents Distributed by One Lambda	
Description	Cat. No.
AllType NGS 11 Loci Amplification Kit	ALL-11LX
AllType NGS 9 Loci Amplification Kit	ALL-9LX
AllType NGS 8 Loci Amplification Kit	ALL-8LX
Ion Xpress™ Plus Fragment Library Kit (includes Ion Shear™ Plus Reagents Kit); or AllType NGS Library Prep Kit	ION-XLIB; or ALL-LIBX
AllType NGS Index Flex Kit	ALL-FLEXX

## Required materials and equipment not supplied

Equipment and Associated Reagents & Consumables		
Description	Supplier	Cat. No.
Magnetic-Ring Stand (96 well)	Life Technologies	AM10050
2.0mL Tube Magnet Stand	MLS <sup>(1)</sup>	---
Veriti 96-Well Thermal Cycler; Or other thermal cycler capable of specific temperature settings optimized for the PCR reaction = 0.8°C/sec heating and 1.6°C/sec cooling rate and 100µL Reaction Volumes	Life Technologies	4375786
Qubit <sup>®</sup> Fluorometer	Life Technologies	Q33216 or Q33226
Qubit dsDNA HS Assay Kit	Life Technologies	Q32854
Qubit Assay Tubes	Life Technologies	Q32856
Plate Centrifuge capable of 1,500 x g	MLS <sup>(1)</sup>	---
Mini/Micro Centrifuge	MLS <sup>(1)</sup>	---
Vortex	MLS <sup>(1)</sup>	---

<sup>(1)</sup> Major Laboratory Supplier

## Workflow Overview



\* The Illumina iSeq 100 sequencer does not require Denature or Clean steps.



## General Workflow Guidelines

- Wear gloves and any other appropriate PPE and follow clean bench work techniques at all times.
- Thoroughly clean the workbench with a DNA-removing agent (e.g.: DNA Away™, Termi-DNA-Tor, 10% bleach followed by 70% alcohol, or equivalent) to reduce the risk of sample contamination.
- While working at the bench, wipe gloves frequently with a DNA-removing agent to reduce the risk of sample and reagent cross-contamination. Alternatively, change gloves frequently.
- Separate pre- and post-PCR areas. Use of dedicated equipment in both the pre- and post-PCR environment is advised.
- All instruments and pipettes should be calibrated and maintained per the manufacturer's guidelines.
- Before beginning, gather the following items and reagents so that they are accessible throughout the workflow: a full range of filtered pipette tips, single and multi-channel pipettes, 96-Well 0.2mL PCR Plates, Eppendorf LoBind® tubes, nuclease-free water, ice, PCR plate cooler, tube-labeling supplies, Low TE, 200-proof Ethanol, and sterile reagent reservoirs.
- Use of manual multi-channel pipettes is recommended for Purification steps. Alternatively, use of a multi-dispensing pipette such as an Eppendorf Multipipette® Xstream may be used. Single channel pipettes are only recommended for workflows containing a small quantity of samples.
- ***Master mix preparation and dispensation should be done on ice and at a reasonably fast pace to avoid unintended results.***

## Indexing Strategy

An indexed library represents one biological sample. The number of indexed libraries that can be accommodated in a single sequencing run depends on the size of the flow cell, the size of the target region(s) of interest, and the coverage required. For a given flow cell and coverage depth, as the size of the target region to be sequenced decreases, the number of indexed libraries that can be accommodated per sequencing run increases.

Instrument / Kit	No. of Samples Based on Targeted Region		
	ALL-11LX	ALL-9LX	ALL-8LX
MiSeq Standard v2	Up to 96	Up to 96	Up to 96
MiSeq Micro v2	Up to 24	Up to 24	Up to 24
MiSeq Nano v2	Up to 8	Up to 8	Up to 8
MiniSeq High Output	Up to 96	Up to 96	Up to 96
MiniSeq Mid Output	Up to 72	Up to 72	Up to 72
iSeq 100 i1	Up to 24	Up to 24	Up to 24

## Specimen Collection and Preparation

### Sample Type

- Sample specimens such as anticoagulated peripheral blood, cultured lymphocytes, and buccal epithelial cells have been verified for use with AllType NGS amplification kits.

### Sample Storage (prior to extraction)

- Samples from whole blood or buffy coat can be extracted immediately after draw or stored up to 14 days under the following conditions: whole blood should be collected in ACD or EDTA anticoagulants and stored at 4°C.
- Buccal swab samples should be collected using swabs that are validated for epithelial cell collection and can be stored at ambient temperature for up to 14 days in a sealed container to avoid excessive drying.

### DNA Extraction Methods

- The AllType NGS 11 Loci Amplification Kit has been validated with manual QIAamp DNA Blood Mini Kit (QIAGEN, Cat No./ID: 51104) and automated MagMAX™ DNA Multi-Sample Ultra Kit (Thermo Fisher Scientific, Cat. No. A25597) and KingFisher Flex Purification System (Thermo Fisher Scientific, Cat. No. 5400630, 5400610). Alternatively, DNA can be extracted using other methods and equipment that are user-validated to isolate PCR amplifiable DNA for a multiplex PCR.
- Samples should be free from any inhibitors of DNA polymerase and should not be re-suspended in solutions containing chelating agents, such as EDTA, above 0.1 mM in concentration.
- The DNA sample to be used for PCR should be re-suspended in sterile water or in 10 mM Tris-HCl, pH 8.0 to 9.0 at an optimal concentration of 25 ng/μl with a fluorometric method such as Qubit™ Fluorometer and Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific).
- Other specifications used should be validated by the laboratory.

### Extracted Sample Storage

- DNA samples may be used immediately after isolation or stored at -20°C or below.

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## Methods

### PCR and amplicon purification protocol

The following procedure describes how to use AllType NGS Amplification Kits to generate amplicons in a multiplex PCR, followed by purification using Agencourt AMPure XP Reagent.

#### Required materials and equipment

- AllType 11-Loci, 9-Loci, or 8-Loci Primer Mix
- AllType Buffer, dNTPs, and Polymerase
- 96-Well 0.2mL PCR Plate and Plate Seals
- 1.5-mL Eppendorf LoBind® Tubes
- PCR Plate Cooler and Ice
- Nuclease-Free Water
- Agencourt AMPure XP Reagent
- 96-well Magnetic-Ring Stand
- 200-Proof Ethanol, Molecular Biology Grade
- Thermo Scientific Nunc 96-Well Polypropylene MicroWell Plates
- Reagent Reservoirs
- PCR Pressure Pad
- Low TE Buffer (included in AllType NGS Library Prep Kit)
- Vortex
- Mini/Micro Centrifuge

## Sample Amplification

**Note: Sample Amplification should be carried out in a pre-PCR environment. Prior to start of setup, clean work area as indicated in the General Workflow Guidelines section of this application note.**

**IMPORTANT: Illumina MiSeq and MiniSeq users will use a different amplification program than Illumina iSeq users. Please refer to amplification programs listed below. Using an incorrect amplification program may cause drops in sequencing coverage depth.**

1. Before starting, turn on a thermal cycler to allow the heated lid to reach temperature.
2. Thaw DNA, AllType Primer Mix, AllType dNTPs, and AllType Buffer at room temperature.  
If precipitate is present in the AllType Buffer warm it at 37°C and vortex until clear. Place the AllType Polymerase on ice when not in use.
3. Once thawed, briefly vortex the AllType dNTPs, Buffer, and Primer Mixes. **Do not vortex the AllType Polymerase.** Pulse-spin all reagents and place on ice when not in use.
4. Prepare a 96-well 0.2mL PCR plate so that it will accommodate the number of samples that are to be amplified.

**Note: The combination of tests carried out is determined by the user.**

5. Pipet 2µl of DNA sample (at 25ng/µl) into the bottom of the appropriate wells on the 96-well 0.2mL PCR plate. When finished, cover and store the plate on a PCR plate cooler or ice to prevent evaporation and contamination.

**Note: Quantification of genomic DNA samples using a fluorometric method such as Qubit is recommended. Quantification by UV absorption will lead to over estimation of concentration.**

6. Create an AllType Master Mix tube using 1.5-mL Eppendorf LoBind® Tubes. To determine the total volume of each reagent, multiply the volume of each reagent listed below by the number of samples to be tested and add an additional 15% as overage. Add the reagents to the 1.5-mL Eppendorf LoBind® Tubes exactly in this order:

**Note: Amplification reaction volume should not be altered. AllType reagents are optimized for use in a 20µL reaction volume. Sub-optimal results will occur if the volumes are altered.**

Order	Component	Volume (per reaction)
1	Nuclease-free water	6.6µl
2	AllType Buffer	4µl
3	AllType dNTPs	1.6µl
4	AllType Primer Mix	5µl
	<b>Total</b>	<b>17.2µl</b>

7. Vortex each Amplification Master Mix for 10 seconds, pulse-spin, and place on ice until ready to proceed.

8. When ready to proceed add AllType Polymerase to the AllType Master Mix tube at a volume of 0.8µl per reaction. Mix well by gently pipetting up and down 15-20 times with a pipette set to approximately half the mix volume or 1 mL if the mix volume is larger than 2 mL. Do not vortex the finished AllType Master Mix. Do not vortex the finished AllType Master Mix tube.

**Note: Remember to include a 15% overage when calculating the volume of polymerase to use.**

9. Retrieve the 96-well 0.2mL PCR plate containing DNA and aliquot 18µl of the AllType Master Mix to each reaction taking special care to not cross-contaminate reactions.
10. Seal the plate with a tray seal and spin the plate down.
11. Load the 96-well 0.2mL PCR plate into a thermal cycler and cover the plate with a PCR pressure pad appropriate for your machine. Run the following PCR Program to amplify the genomic DNA targets. Set reaction volume to 20µL.

### PCR Programs:

MiSeq and MiniSeq Amplification PCR Program			
Step	Temperature	Time	# of Cycles
Step 1:	94°C	2 minutes	1
Step 2:	98°C	10 seconds	30
	69°C	3 minutes	
Step 3:	4°C	∞	1

iSeq Amplification PCR Program			
Step	Temperature	Time	# of Cycles
Step 1:	94°C	2 minutes	1
Step 2:	98°C	10 seconds	24
	69°C	3 minutes	
Step 3:	98°C	10 seconds	6
	60°C	3 minutes	
Step 4:	4°C	∞	1

Store the amplicons at –30°C to –10°C for up to one month, or at -80°C for up to six months.

**Note: Thermal cycler in use should be capable of specific temperature settings that are optimized for the PCR reaction and capable of achieving standard ramp rates of +0.8°C/sec heating and -1.6°C/sec cooling (Applied Biosystems refers to this ramp speed as “9600 Emulation Mode”). For other systems, users should contact their thermal cycler manufacturer for guidance on how to program the equivalent ramp rates of +0.8°C/sec heating and -1.6°C/sec cooling. Use of a significantly different ramp speed will affect amplification efficiency.**

## Purify the amplicons

1. Remove the Agencourt AMPure XP Reagent from 2°C - 8°C storage and allow the mixture to come to room temperature (~30 minutes).
2. Prepare fresh 75% ethanol solution sufficient for 200µL per reaction, plus a 15% overage. Ethanol solution should be prepared from 200 proof, molecular biology grade, and ethanol in nuclease free water. 75% ethanol should be prepared fresh daily.
3. Obtain a new 96-well 0.2mL PCR plate that will contain the purified product and label accordingly.
4. Once the Agencourt AMPure XP Reagent has reached room temperature, vortex at medium speed for 30 seconds, or until the beads are fully re-suspended.
5. Pour a volume of Agencourt AMPure XP Reagent into a reagent reservoir sufficient for 12µL per reaction, plus a 15% overage.
6. Retrieve a Nunc 96-Well Polypropylene MicroWell Plate and, using a multi-channel pipette, transfer 12µL of Agencourt AMPure XP Reagent into the same number of wells that currently occupy amplicons in the 96-well 0.2mL PCR plate from the amplification step.
7. Pipette all of the amplified product (~20µL) into corresponding wells of the Nunc 96-Well Polypropylene MicroWell Plate containing the AMPure XP Reagent. Mix the samples with the AMPure XP Reagent by pipetting up and down 10 times. Change tips between samples. Avoid producing bubbles.
8. Incubate the amplified product and AMPure XP Reagent beads mixture at room temperature for 5 minutes.
9. When the incubation is complete place the plate on the 96-well Magnetic-Ring Stand for ~3 minutes or until the solution clears.
10. Using a multichannel pipette set to 28µL, slowly remove and discard the supernatant from each well without disturbing the ring of beads that has formed around the magnet.  
**Note: Beads hold amplicons - loss of beads should be avoided.**
11. Without removing the plate from the magnet, dispense 100µL of freshly prepared 75% ethanol into each well. Shift the plate in between the wells of the magnetic-ring stand, placing the plate on top instead of inside of the wells. When done correctly the beads will collapse towards the center of the wells. Shift the plate a total of 5 times to rinse the beads. When finished, allow the plate to sit until the ring of beads re-forms and the solution clears. Incubate the samples at room temperature for 30 seconds.
12. After the solution clears, using a multichannel pipette set to 110µL, remove and discard the supernatant from each well without disturbing the ring of beads that has formed around the magnet.
13. Repeat steps 11 & 12 for a second ethanol wash.
14. To remove residual ethanol, keep the plate on the magnet and carefully remove any remaining supernatant with a 20µL multi-channel pipette without disturbing the ring of beads.
15. Keeping the plate on the magnet, air-dry the beads at room temperature for up to 5-10 minutes. Do not over dry the beads, as this makes bead re-suspension difficult and decreases sample recovery.

16. Keeping the plate on the magnet, add 27 $\mu$ L of Low TE to each well. Remove the plate from the magnet and pipet the mixture up and down to mix thoroughly. Incubate at room temperature for 1 minute. Alternatively, seal the plate, vortex gently until the beads are re-suspended, and quick spin the plate.
17. Place the plate back on a magnet for at least 1 minute. After the solution clears, set a multi-channel pipette to 25 $\mu$ L and transfer the supernatant containing the purified amplicons to the labeled 96-well 0.2mL PCR plate retrieved at the beginning of the purification without disturbing the pellet.

**STOPPING POINT (Optional)** Store the purified amplicons at  $-30^{\circ}\text{C}$  to  $-10^{\circ}\text{C}$  for up to one month, or at  $-80^{\circ}\text{C}$  for up to six months.

## Amplicon quantification and dilution

The following procedure describes how to quantify the purified amplicons from the previous step using the Qubit Fluorometer. Once quantification is complete, this procedure will describe the process of amplicon dilution to 100ng.

### Required materials and equipment

- Qubit Fluorometer
- Qubit dsDNA HS Assay Kit
- Qubit Assay Tubes
- 50mL conical tubes, Nuclease-free
- Serological pipettes and pipette controller
- Vortex
- 96-Well 0.2mL PCR Plate and Plate Seals
- Nuclease-free Water



## Amplicon quantification

1. Retrieve Qubit Assay Tubes and label one tube for every amplicon. Label two additional tubes, one for Qubit Standard #1 and one for Qubit Standard #2.
2. Retrieve a Qubit dsDNA HS Assay Kit and create a Working Qubit Solution in a 50mL conical tube by mixing the following:
  - 199 $\mu$ L of Qubit dsDNA HS Buffer per amplicon, plus a 15% overage
  - 1 $\mu$ L of Qubit dsDNA HS Reagent Concentrate per amplicon, plus a 15% overage. Vortex prior to use
3. Vortex the Working Qubit Solution and cover with foil to protect from light. The solution must be used within 2 hours.
4. Add 199 $\mu$ L of Working Solution to each Qubit Assay Tube that will contain amplicon. Add 190 $\mu$ L of Working Solution to the two tubes set aside for Standard #1 and Standard #2.
5. To the assay tubes containing 199 $\mu$ L of working solution, add 1 $\mu$ L of the appropriate amplicon.
6. To the two assay tubes containing the 190 $\mu$ L of working solution, add 10 $\mu$ L of the designated standard to the appropriate tube.
7. Briefly vortex all tubes, spin, incubate at room temperature for 2 minutes, while keeping out of light.
8. Turn on the Qubit Fluorometer, select **DNA** from the home screen, and then select **dsDNA High Sensitivity**.
9. Press the appropriate button to begin reading the Qubit standards. As directed by the Qubit Fluorometer, insert and measure previously prepared Standard #1 and Standard #2 to complete the calibration.
10. Begin the reading of your assay tubes containing amplicon. When prompted, change the volume of sample used to **1 $\mu$ L** and the units to **ng/ $\mu$ L**. Manually record the concentration for the sample.
11. Repeat step 10 for all assay tubes containing amplicon.
12. For all samples that exceed the Qubit Fluorometer's upper detection limit of 600ng/mL, repeat preparation of the amplicon in the Working Qubit Solution by using a twofold dilution of the sample. When prepared, re-read the sample.

**Note: When recording the final concentration of amplicons requiring two-fold dilution, multiply the Qubit reading by two.**

For samples that fall below the lower detection limit of < 0.5ng/mL, re-amplify the sample, purify, and quantitate according to the previous instructions in this application note.
13. Insert the Qubit USB drive into the USB port of the Qubit Fluorometer to export the data.

## Amplicon dilution

1. Open the **NGS Library Pooling Calculator** Excel file, which can be obtained from the One Lambda website.
2. Using data exported from the Qubit Fluorometer, in the **AllType Amplicon Dilution** tab of the Excel file, input the sample names in the **DNA** column and the corresponding sample concentration (in ng/ $\mu$ L) in the **Conc. (ng/ $\mu$ L)** column.
3. Acquire a new 96-well 0.2mL PCR plate that will be used to create the 100ng amplicon dilutions.
4. Add 35 $\mu$ L of nuclease free water to each well that will contain diluted amplicon.
5. Using the values in the column labeled **Variable Volume ( $\mu$ L)**, dilute each amplicon individually by first removing a volume of water from the 35 $\mu$ L that is equal to the value in this column of the spreadsheet and discard. Change tips and add the same volume of amplicon to the remaining volume of water. The total volume for each diluted sample will be 35 $\mu$ L.
6. Proceed to Amplicon Fragmentation with the 100ng dilutions.

**STOPPING POINT (Optional)** Store the remaining amplicon and/or amplicon pools at  $-30^{\circ}\text{C}$  to  $-10^{\circ}\text{C}$  for up to one month, or at  $-80^{\circ}\text{C}$  for up to six months.

# Amplicon fragmentation

The following procedure describes the process to fragment the amplicon.

## Required materials and equipment

- Ion Shear Plus Reagents Kit(s) (part of Ion Xpress Plus Fragment Library Kit), or AllType NGS Library Prep Kit
- PCR Plate Cooler and Ice
- 1.5-mL Eppendorf LoBind® Tubes
- Vortex
- Mini/Micro Centrifuge
- Plate Centrifuge

## Amplicon Fragmentation

1. Before starting, turn on a thermal cycler to allow the heated lid to reach temperature.
2. Obtain enough Ion Shear Plus Reagent Kits suitable for the number of samples, based on the volumes indicated in the following table. Only one AllType NGS Library Prep Kit is required to prepare up to 96 fragmented amplicons.

Component Label if using Ion Shear Plus	Component Label if using AllType NGS Library Prep	Volume (per amplicon pool/sample)
Enzyme II Mix	Fragmentase	9µL
10X Reaction Buffer	Fragmentation Buffer	5µL

3. The Enzyme Mix II (or Fragmentase) should be pulse spun and immediately placed in a cold block or on ice. The 10X Reaction Buffer should be thawed at room temperature. Once thawed, vortex, and place the reagent in a cold block or on ice.
4. Create a Fragmentation Master Mix for all samples in a 1.5mL LoBind tube by combining Enzyme II Mix (or Fragmentase) and 10X Reaction Buffer (or Fragmentation Buffer) in the volumes listed in Step 2, plus a 15% overage. Mix well by gently pipetting up and down 15-20 times with a pipette set to approximately half the mix volume or 1 mL if the mix volume is larger than 2 mL. Do not vortex the finished Fragmentation Master Mix. Place the Fragmentation Master Mix on ice until use.
5. Obtain the plate(s) of 100ng diluted amplicon. Place on ice.
6. Keeping the plate on ice, to each sample, add 14µL of Fragmentation Master Mix to the top side of the well, making sure not to cross-contaminate reactions, so as to prevent the master mix from falling prematurely into the reaction. Do not plunge the tip into the DNA sample as simultaneous mixing is later required to achieve uniform fragmentation.
7. When finished, place a new seal firmly over the plate. Pulse-vortex the plate for 5 times and pulse-spin for 5 seconds at 1500g.

**Note: Steps 6 & 7 should be performed quickly to ensure the targeted level of fragmentation. In certain cases, adjustment of fragmentation time may be required.**

8. Immediately place the plate in the thermal cycler with pre-heated lid and run the following program.

Set reaction volume to 49µL.

Fragmentation PCR Program			
Step	Temperature	Time	# of Cycles
Step 1:	37°C	6 minutes	1
Step 2:	70°C	10 minutes	1
Step 3:	4°C	∞	1

9. At the conclusion of the incubation, remove the tray from the thermal cycler, pulse-spin, and immediately place on ice.
10. Proceed to index ligation.

## Index ligation, and nick repair

The following procedure describes the process to ligate indexes to the fragmented amplicons and to simultaneously carry out the nick-repair process.

**Materials required from the Ion Plus Fragment Library Kit** (may be substituted by AllType NGS Library Prep Kit)

- 10X Ligase Buffer
- dNTP Mix
- DNA Ligase
- Nick Repair Polymerase

**Materials required from the AllType NGS Library Prep Kit** (may be substituted by Ion Plus Fragment Library Kit)

- Ligase Buffer
- dNTP Mix
- Ligase
- Nick Repair Polymerase

**Materials required from the AllType NGS Index Flex Kit**

- AllType NGS Index Adapter Plate (1 index per sample)

**Other materials and equipment**

- PCR Plate Cooler and Ice
- Nuclease-Free Water
- 1.5mL Eppendorf LoBind® Tubes or 15mL conical tubes
- Vortex
- Mini/Micro Centrifuge
- Plate Centrifuge

## Index ligation and nick repair

1. Before beginning, turn on a thermal cycler to allow the heated lid to reach temperature.
2. Obtain enough Ion Plus Fragment Library Kits suitable for the number of fragmented amplicons based on the volumes indicated in the table in Step 6. Only one AllType NGS Library Prep Kit is required to prepare up to 96 fragmented amplicons.
3. Obtain the DNA Ligase (or Ligase) and Nick Repair Polymerase. Pulse-spin and immediately place on ice.
4. Thaw all other reagents at room temperature. Once thawed, vortex, pulse-spin, and then place on ice until use. Precipitate may be visible in the 10X Ligase Buffer (or Ligase Buffer) but when added to the Ligation Master Mix, it will have no effect on the reaction.
5. Thaw the AllType Index Adapter Plate at room temperature. Once thawed, vortex, pulse-spin, and then place on ice until use.

**Note: The AllType Index Flex adapter plate features a segmented design which allows it to be cut into 24 and 48 well sections at a time. Users are encouraged to cut the adapter plate into sections, as required, to minimize the number of freeze-thaw cycles. Unintended freeze-thaw cycles of unused barcodes may degrade performance and result in low secondary amplification yields.**

6. Depending on sample number, combine the following components in either a 1.5mL Eppendorf LoBind® tube, or 15mL conical tube to create a Ligation Master Mix based on the number of samples in-process. Include a 15% overage. Pulse-vortex and keep on ice until ready to use.

**Note: Do not add the DNA Ligase and Nick Repair Polymerase until immediately prior to dispensing master mix over sample.**

Ligation Master Mix Component	Volume (per fragmented
Nuclease-Free Water	25µL
10X Ligase Buffer (or Ligase Buffer)	10µL
dNTP Mix	2µL
DNA Ligase (or Ligase)	2µL
Nick Repair Polymerase	8µL

7. Obtain the PCR plate containing the fragmented amplicon.
8. Add 4ul of each AllType Index into the desired wells using a multichannel pipette. Change tips between samples. Only one index should be used per well.

**Note: Based on the index layout below, keep track of the index ID (D###'s) per each sample so correct information will be transferred to a sample sheet for MiSeq, MiniSeq or iSeq input.**

**Important: Take care to avoid contamination. Additionally, wipe gloves routinely with DNA Away or 70% ethanol while working with indexes.**

**AllType NGS Index Flex Kit plate layout:**

	1	2	3	4	5	6	7	8	9	10	11	12
<b>A</b>	D501 D701	D501 D702	D501 D703	D501 D704	D501 D705	D501 D706	D501 D707	D501 D708	D501 D709	D501 D710	D501 D711	D501 D712
<b>B</b>	D502 D701	D502 D702	D502 D703	D502 D704	D502 D705	D502 D706	D502 D707	D502 D708	D502 D709	D502 D710	D502 D711	D502 D712
<b>C</b>	D503 D701	D503 D702	D503 D703	D503 D704	D503 D705	D503 D706	D503 D707	D503 D708	D503 D709	D503 D710	D503 D711	D503 D712
<b>D</b>	D504 D701	D504 D702	D504 D703	D504 D704	D504 D705	D504 D706	D504 D707	D504 D708	D504 D709	D504 D710	D504 D711	D504 D712
<b>E</b>	D505 D701	D505 D702	D505 D703	D505 D704	D505 D705	D505 D706	D505 D707	D505 D708	D505 D709	D505 D710	D505 D711	D505 D712
<b>F</b>	D506 D701	D506 D702	D506 D703	D506 D704	D506 D705	D506 D706	D506 D707	D506 D708	D506 D709	D506 D710	D506 D711	D506 D712
<b>G</b>	D507 D701	D507 D702	D507 D703	D507 D704	D507 D705	D507 D706	D507 D707	D507 D708	D507 D709	D507 D710	D507 D711	D507 D712
<b>H</b>	D508 D701	D508 D702	D508 D703	D508 D704	D508 D705	D508 D706	D508 D707	D508 D708	D508 D709	D508 D710	D508 D711	D508 D712

9. When ready, add the calculated volumes of DNA Ligase (or Ligase) and Nick Repair Polymerase to the Ligation Master Mix by using the table above. Remember to include the 15% overage. Mix well by gently pipetting up and down 15-20 times with a pipette set to approximately half the mix volume or 1 mL if the mix volume is larger than 2 mL. Do not vortex the finished Ligation Master Mix. Place completed master mix on ice.
10. Dispense 47µL of Ligation Master Mix into each well of the PCR plate containing fragmented product and index, taking special care not to cross-contaminate reactions.
11. Seal the plate, spin down, and place into a thermal cycler using the following program. Set reaction volume to 100µL.

Ligation PCR Program *			
Step	Temperature	Time	# of Cycles
<b>Step 1:</b>	25°C	15 min.	1
<b>Step 2:</b>	72°C	5 min.	1
<b>Step 3:</b>	4°C	∞	1

*\* Thermal cycler should be programmed to simulate the GeneAmp® 9600 Emulation Mode. For other systems, consult the manufacturer's documentation to adjust ramp speed to simulate the GeneAmp® 9600 program.*

12. At the conclusion of the incubation, pulse-spin the plate, place on ice, and proceed to size- selection.

## Size-selection, secondary amplification, and purification

The following procedure describes the process to perform a size-selection of the index-ligated product using Agencourt AMPure XP Reagent. At the completion of size-selection, a secondary amplification will be performed on the size-selected product. Finally, purification of the amplified libraries will be performed using Agencourt AMPure XP Reagent.

### Required materials and equipment

- Low TE Buffer (included in AllType NGS Library Prep Kit)
- 96-Well 0.2mL PCR Plate and Plate Seals
- PCR Plate Cooler and Ice
- Agencourt AMPure XP Reagent
- 96-well Magnetic-Ring Stand
- 200-Proof Ethanol, Molecular Biology Grade
- Thermo Scientific Nunc 96-Well Polypropylene MicroWell Plates
- Reagent Reservoirs
- 1.5mL Eppendorf LoBind® Tubes or 15mL Conical
- Nuclease-free Water
- Vortex
- Mini/Micro Centrifuge
- Plate Centrifuge

**Materials required from the Ion Plus Fragment Library Kit** (may be substituted by AllType NGS Library Prep Kit)

- Platinum PCR SuperMix High Fidelity

**Materials required from the AllType NGS Library Prep Kit** (may be substituted by Ion Plus Fragment Library Kit)

- Library Amp Master Mix

**Materials required from the AllType NGS Index Flex Kit**

- AllType NGS Library Amplification Primer (used for secondary amplification)



## Size-Selection

1. Remove the Agencourt AMPure XP Reagent from 2°C - 8°C storage and allow the mixture to come to room temperature (~30 minutes).
2. Prepare fresh 75% ethanol solution sufficient for 200µL per reaction, plus a 15% overage. Ethanol solution should be prepared from 200 proof, molecular biology grade, and ethanol in nuclease free water. 75% ethanol should be prepared fresh daily.
3. Obtain a new 96-well 0.2mL PCR plate that will contain the size selected product and label accordingly.
4. Once the Agencourt AMPure XP Reagent has reached room temperature, vortex at medium speed for 30 seconds, or until the beads are fully re-suspended.
5. Retrieve two Nunc 96-Well Polypropylene MicroWell Plates.
6. Pour a volume of Agencourt AMPure XP Reagent into a reagent reservoir sufficient for 63.1µL per reaction, plus a 15% overage.
7. In the first Nunc 96-Well Polypropylene MicroWell Plate, using a multi-channel pipette, transfer 48.5µL of Agencourt AMPure XP Reagent into each well that will contain sample.
8. Pipette 97µL of the adapter-ligated product into corresponding wells of the first Nunc 96-Well Polypropylene MicroWell Plate. Mix the sample with the AMPure XP Reagent by pipetting up and down 10 times. Change tips between samples. Avoid producing bubbles.
9. Incubate the adaptor-ligated product and AMPure XP Reagent beads mixture at room temperature for 5 minutes.
10. During the 5 minute incubation, using a multi-channel pipette, transfer 14.6µL of Agencourt AMPure XP Reagent to the second Nunc 96-Well Polypropylene MicroWell Plate, into each well that will contain sample.
11. When the 5 minute incubation of the first plate is complete, place the plate on the 96-well Magnetic- Ring Stand for ~3 minutes or until the solution clears.
12. Using a multichannel pipette set to 130µL , collect the supernatant and transfer to the second Nunc 96-Well Polypropylene MicroWell Plate containing 14.6µL of AMPure XP Reagent per well. Mix the supernatant with the AMPure XP Reagent by pipetting up and down 10 times. Change tips between samples. Avoid producing bubbles. **The supernatant contains the amplicons - loss of supernatant should be avoided.**
13. Incubate the supernatant and AMPure XP Reagent beads mixture in the second plate at room temperature for 5 minutes.
14. When the 5-minute incubation of the second plate is complete, place the plate on the 96-well Magnetic-Ring Stand for ~3 minutes or until the solution clears.
15. Using a multichannel pipette set to 140µL, remove and discard the supernatant from each well without disturbing the ring of beads that has formed around the magnet. **Beads hold amplicons - loss of beads should be avoided.**
16. Without removing the plate from the magnet, dispense 100µL of freshly prepared 75% ethanol into each well. Shift the plate in between the wells of the magnetic ring stand, placing the plate on top instead of inside of the wells. When done correctly the beads will collapse towards the center of the wells. Shift the plate a total of 5 times to rinse the beads. When finished, allow the plate to sit until the ring of beads re-forms and the solution clears. Incubate the samples at room temperature for 30 seconds.
17. After the solution clears, using a multichannel pipette set to 110µL, remove and discard the supernatant from each well without disturbing the ring of beads that has formed around the magnet

18. Repeat steps 16 & 17 for a second ethanol wash.
19. To remove residual ethanol, keep the plate on the magnet and carefully remove any remaining supernatant with a 20- $\mu$ L multi-channel pipette without disturbing the ring of beads.
20. Keeping the plate on the magnet, air-dry the beads at room temperature for 5-10 minutes. Do not over dry the beads, as this makes bead re-suspension difficult and decreases sample recovery.
21. Keeping the plate on the magnet, add 27 $\mu$ L of Low TE to each well. Remove the plate from the magnet, seal the plate, vortex gently until the beads are re-suspended, and quick spin the plate.
22. Place the plate back on a magnet for approximately 1 minute. After the solution clears, set a multi-channel pipette to 25 $\mu$ L and transfer the supernatant containing the size-selected product to the labeled 96-well 0.2mL PCR plate retrieved at the beginning of size-selection without disturbing the pellet.

**STOPPING POINT (Optional)** Store the purified amplicons at  $-30^{\circ}\text{C}$  to  $-10^{\circ}\text{C}$  for up to two weeks. Before use, thaw on ice.

## Secondary Amplification

1. Before beginning, turn on a thermal cycler to allow the heated lid to reach temperature.
2. Obtain the Platinum PCR SuperMix High Fidelity (or Library Amp Master Mix) reagent and the AllType Library Amplification Primer from the AllType NGS Index Flex Kit and thaw at room temperature. When thawed, vortex, pulse-spin, and then place on ice until use.
3. In a 1.5mL Eppendorf LoBind tube or 15mL conical tube, create a Secondary Amp Master Mix for the desired number of size-selected products using the table below. Include a 15% overage.

Component Required for Secondary Amp Master Mix	Volume (per size-selected product)
Platinum PCR SuperMix High Fidelity; or Library Amp Master Mix	71.4 $\mu$ L
AllType Library Amplification Primer	3.6 $\mu$ L

4. Mix well by gently pipetting up and down 15-20 times with a pipette set to approximately half the mix volume or 1 mL if the mix volume is larger than 2 mL. Add 75 $\mu$ L of the Master Mix to each size-selected product.

- Seal the plate, spin down, and place the plate into a thermal cycler using the following program. Set reaction volume to 100µL.

Secondary Amplification PCR Program			
Step	Temperature	Time	# of Cycles
<b>Step 1:</b>	95°C	5 min.	1
<b>Step 2:</b>	95°C	15 sec.	8
	58°C	15 sec.	
<b>Step 3:</b>	4°C	∞	1

- After the program has finished, pulse spin the plate and proceed to the final purification.

## Purification

- Remove the Agencourt AMPure XP Reagent from 2°C - 8°C storage and allow the mixture to come to room temperature (~30 minutes).
- Prepare fresh 75% ethanol solution sufficient for 200µL per amplicon, plus a 15% overage. Ethanol solution should be prepared from 200 proof, molecular biology grade, and ethanol in nuclease free water. 75% ethanol should be prepared fresh daily.
- Obtain a new 96-well 0.2mL PCR plate that will ultimately contain the purified product and label accordingly.
- Once the Agencourt AMPure XP Reagent has reached room temperature, vortex at medium speed for 30 seconds, or until the beads are fully re-suspended.
- Pour a volume of Agencourt AMPure XP Reagent into a reagent reservoir sufficient for 100µL per amplicon, plus a 15% overage.
- Retrieve a Nunc 96-Well Polypropylene MicroWell Plate and, using a multi-channel pipette, transfer 100µl of Agencourt AMPure XP Reagent into the same number of wells that currently occupy ligated PCR product.
- Pipette all of the amplified product (~100µl) into corresponding wells of the Nunc 96-Well Polypropylene MicroWell Plate containing the AMPure XP Reagent. Mix the samples with the AMPure XP Reagent by pipetting up and down 10 times. Change tips between samples.
- Incubate the amplified product and AMPure XP Reagent beads mixture at room temperature for 5 minutes.
- When the incubation is complete place the plate on the 96-well Magnetic-Ring Stand for ~3 minutes or until the solution clears.
- Using a multichannel pipette set to 195µL, remove and discard all the supernatant from each well without disturbing the ring of beads that has formed around the magnet.

**Beads hold amplicons - loss of beads should be avoided.**

11. Without removing the plate from the magnet, dispense 100 $\mu$ L of freshly prepared 75% ethanol into each well. Shift the plate in between the wells of the magnetic-ring stand, placing the plate on top instead of inside of the wells. When done correctly the beads will collapse towards the center of the wells. Shift the plate a total of 5 times to rinse the beads. When finished, allow the plate to sit until the ring of beads re-forms and the solution clears.
12. After the solution clears, using a multichannel pipette set to 110 $\mu$ L, remove and discard the supernatant from each well without disturbing the ring of beads that has formed around the magnet.
13. Repeat steps 11 & 12 for a second ethanol wash.
14. To remove residual ethanol, keep the plate on the magnet and carefully remove any remaining supernatant with a 20- $\mu$ L multi-channel pipette without disturbing the ring of beads.
15. Keeping the plate on the magnet, air-dry the beads at room temperature for up to 10 minutes. Do not over dry the beads, as this makes bead re-suspension difficult and decreases sample recovery.
16. Keeping the plate on the magnet, add 27 $\mu$ L of Low TE to each well. Remove the plate from the magnet and pipet the mixture up and down to mix thoroughly. Incubate at room temperature for 1 minute. Alternatively, seal the plate, vortex gently until the beads are re-suspended, and quick spin the plate.
17. Place the plate back on a magnet for at least 1 minute. After the solution clears, set a multi-channel pipette to 25 $\mu$ L and transfer the supernatant containing the purified amplicons to the labeled 96-well 0.2mL PCR plate retrieved at the beginning of the purification without disturbing the pellet.

**STOPPING POINT (Optional)** Store the purified libraries product at  $-30^{\circ}\text{C}$  to  $-10^{\circ}\text{C}$  for up to two months. Before use, thaw on ice.

## Final quantification and library pooling

The following procedure describes the process to perform the final quantification of all samples using the Qubit Fluorometer. Following quantification all individual samples will be pooled into a single reaction and purified one final time before initiating sequencing preparation.

### Required materials and equipment

- Low TE Buffer (included in AllType NGS Library Prep Kit)
- 1.5-mL Eppendorf LoBind® Tubes
- Qubit Fluorometer
- Qubit dsDNA HS Assay Kit
- Qubit Assay Tubes
- Agencourt AMPure XP Reagent
- Magnetic Tube Stand
- 200-Proof Ethanol, Molecular Biology Grade
- 15/50mL Conical Tubes
- Vortex
- Mini/Micro Centrifuge

## Final Quantification

1. Retrieve Qubit Assay Tubes and label one tube for every sample. Label two additional tubes, one for Qubit Standard #1 and one for Qubit Standard #2.
2. Retrieve a Qubit dsDNA HS Assay Kit and create a Working Qubit Solution in a 15/50mL conical tube by mixing the following:
  - 199 $\mu$ L of Qubit dsDNA HS Buffer per sample, plus a 15% overage
  - 1 $\mu$ L of Qubit dsDNA HS Reagent Concentrate per sample, plus a 15% overage.
3. Vortex the Working Qubit Solution and cover with foil to protect from light. The solution must be used within 2 hours.
4. Add 195 $\mu$ L of Working Solution to each Qubit Assay Tube that will contain sample. Add 190 $\mu$ L of Working Solution to the two tubes set aside for Standard #1 and Standard #2.
5. In a new 96 well 0.2mL PCR plate, perform 1:1 dilution using 10 $\mu$ L of purified sample with 10 $\mu$ L of Low TE. These dilutions will serve as the sample to be quantified and used for final library pooling.
6. To the assay tubes containing 195 $\mu$ L of working solution, add 5 $\mu$ L of the appropriate 1:1 diluted sample.
7. To the two assay tubes containing the 190 $\mu$ L of working solution, add 10 $\mu$ L of the designated standard to the appropriate tube.
8. Briefly vortex all tubes, spin, incubate at room temperature for 2 minutes, while keeping out of light.
9. Turn on the Qubit Fluorometer, select DNA from the home screen, and then select dsDNA High Sensitivity. Press the appropriate button to begin reading Qubit Standards as directed by the Qubit Fluorometer. Insert and measure previously prepared Standard#1 and Standard#2 to complete the calibration.
10. Begin the reading of your assay tubes containing sample. When prompted, change the volume of sample used to **5 $\mu$ L** and the units to **ng/ $\mu$ L**. Manually record the concentration for the sample.
11. Repeat step 10 for all assay tubes containing sample.
12. For samples that fall below the lower detection limit of < 0.5ng/mL, try quantifying the undiluted sample.
13. If desired, insert the Qubit USB drive into the USB port of the Qubit Fluorometer to export the data.

## Library Pooling

### A. Users of the Illumina MiSeq System

1. Open the **NGS Library Pooling Calculator** Excel file, which can be obtained from the One Lambda website.  
Navigate to the **Illumina Library Pooling - Qubit** tab once the Excel file opens.
2. For every sample, input the concentration obtained from the Qubit in the **Concentration, ng/ $\mu$ L** column.
3. The columns labeled **Lowest Concentration, ng/ $\mu$ L** and **Sample Volume,  $\mu$ L** should auto populate.
4. Check all samples to ensure that the value in the **Sample Volume,  $\mu$ L** column is at least 2 $\mu$ L (cells with volumes less than 2 $\mu$ L will turn purple). If it is not, adjust the value for all samples in the **Total Volume,  $\mu$ L** column so that at least 2 $\mu$ L of each sample is being used. Adjusting the value in this column will update the value in the **Sample Volume,  $\mu$ L** column.
5. Begin the process of creating the equimolar pool by first adding a volume of Low TE into a 1.5mL LoBind tube that equals the value from the Excel file in the cell labeled **TOTAL Low TE ( $\mu$ L)**.
6. Next, for each sample, add a volume of each sample to the LoBind tube which equals the volume indicated in the **Sample Volume,  $\mu$ L** column of the Excel file.  
**Note: Sample should be taken from the plate containing the diluted samples (1:1 dilution). Or, in the case where an un-diluted sample required quantification, use the un-diluted sample.**
7. With the pooling completed, transfer half of the pooled library to a new LoBind tube. Make note of the actual volume transferred.
8. Conduct a 0.8X AMPure cleanup in the new LoBind tube containing library. For example, if 100 $\mu$ L of library was transferred, add 80 $\mu$ L of AMPure beads.
9. Pipette the AMPure bead & library pool mixture approximately 10 times to mix. Incubate at room temperature for 5 mins.
10. When the incubation is complete, place the tube in a magnetic rack and let stand for approximately 3 mins, or until the solution clears.
11. During the 3-minute incubation, prepare 500 $\mu$ L of 75% EtOH.
12. When the 3-minute incubation is complete, remove and discard the supernatant from the LoBind tube without disturbing the bead pellet.
13. With the tube still on the magnet, add 200 $\mu$ L of freshly prepared 75% EtOH. Rotate the tube on the magnet approximately 5 times to mix the bead pellet with the EtOH. If rotating the tube does not allow for mixing, use a pipette.
14. Once the bead pellet has re-formed, remove and discard the supernatant from the LoBind tube without disturbing the bead pellet.
15. Repeat Steps 13 & 14 for a second EtOH wash.
16. With the tube open to the environment, allow the bead pellet to air dry for up to 5 minutes. Monitor the bead pellet throughout the drying process so that the pellet does not over-dry. Over-dried beads make for difficult resuspension.
17. When the bead pellet has dried, elute the library from the beads by adding 25 $\mu$ L (or 1/4<sup>th</sup> of the volume transferred in Step 7) of Low TE Buffer.

18. Remove the tube from the magnet and fully resuspend the pellet in the Low TE Buffer either via gentle vortexing or through the use of pipette mixing. Once mixed, let the solution sit for 1 minute.
19. Place the tube back on the magnet, allow the bead pellet to form, and transfer 23 $\mu$ L of eluted library to a new 1.5mL LoBind tube.
20. The library should now be at a concentration higher than 4nM, the required library concentration for the Illumina MiSeq System. Quantitate 5 $\mu$ L of the eluted library using the Qubit Fluorometer (i.e.: 5 $\mu$ L of eluted library in 195 $\mu$ L of Working Qubit Solution prepared in the previous step).
21. The exact Qubit concentration required to reach 4nM may vary depending on the size of the final sequencing library. Based on the average size of AllType libraries, we recommend diluting the library in Low TE Buffer to a concentration of 1.60ng/ $\mu$ L\* by using the calculator/table located at the bottom of the **Illumina Library Pooling – Qubit** tab. Input the current library concentration into the cell labeled **Concentration, ng/ $\mu$ L**, and the table will subsequently display the volume of sample and the volume of Low TE buffer to combine to reach 1.60ng/ $\mu$ L.
22. Once diluted, it is best practice to conduct a duplicate/triplicate quantification of the library using the Qubit Fluorometer to ensure that the library is as close to 1.60ng/ $\mu$ L as possible. (i.e.: 5 $\mu$ L of diluted library in 195 $\mu$ L of Working Qubit Solution).
23. If further dilution to 1.60ng/ $\mu$ L is required following the duplicate/triplicate Qubit reading, follow Step 21.

***\*Note: The final concentration of the sequencing library may need to be adjusted by the user depending on the size of the final library. We recommend 1.60 ng/ $\mu$ L as a starting point, but if the flow cell cluster density is outside of the normal range for the MiSeq (1000 -1200) it may be necessary to adjust the final library concentration.***

**STOPPING POINT (Optional)** Store undiluted library at  $-30^{\circ}\text{C}$  to  $-10^{\circ}\text{C}$  for up to two months. Store diluted library at  $-30^{\circ}\text{C}$  to  $-10^{\circ}\text{C}$  for up to two weeks. Before use, thaw on ice, and re-quantify if stored for more than one week.



## B. Users of the Illumina MiniSeq System

1. Open the **NGS Library Pooling Calculator** Excel file, which can be obtained from the One Lambda website. Navigate to the **Illumina Library Pooling** - Qubit tab once the Excel file opens.
2. For every sample, input the concentration obtained from the Qubit in the **Concentration, ng/ $\mu$ L** column.
3. The columns labeled **Lowest Concentration, ng/ $\mu$ L** and **Sample Volume,  $\mu$ L** should auto populate.
4. Check all samples to ensure that the value in the **Sample Volume,  $\mu$ L** column is at least 2 $\mu$ L (cells with volumes less than 2 $\mu$ L will turn purple). If it is not, adjust the value for all samples in the **Total Volume,  $\mu$ L** column so that at least 2 $\mu$ L of each sample is being used. Adjusting the value in this column will update the value in the **Sample Volume,  $\mu$ L** column.
5. Begin the process of creating the equimolar pool by first adding a volume of Low TE into a 1.5mL LoBind tube that equals the value from the Excel file in the cell labeled **TOTAL Low TE ( $\mu$ L)**.
6. Next, for each sample, add a volume of each sample to the LoBind tube which equals the volume indicated in the Sample Volume,  $\mu$ L column of the Excel file.  
**Note: Sample should be taken from the plate containing the diluted samples (1:1 dilution). Or, in the case where an un-diluted sample required quantification, use the un-diluted sample.**
7. With the pooling completed, transfer half of the pooled library to a new LoBind tube. Make note of the actual volume transferred.
8. Conduct a 0.8X AMPure cleanup in the new LoBind tube containing library. For example, if 100 $\mu$ L of library was transferred, add 80 $\mu$ L of AMPure beads.
9. Pipette the AMPure bead & library pool mixture approximately 10 times to mix. Incubate at room temperature for 5 mins.
10. When the incubation is complete, place the tube in a magnetic rack and let stand for approximately 3 mins, or until the solution clears.
11. During the 3-minute incubation, prepare 500 $\mu$ L of 75% EtOH.
12. When the 3-minute incubation is complete, remove and discard the supernatant from the LoBind tube without disturbing the bead pellet.
13. With the tube still on the magnet, add 200 $\mu$ L of freshly prepared 75% EtOH. Rotate the tube on the magnet approximately 5 times to mix the bead pellet with the EtOH. If rotating the tube does not allow for mixing, use a pipette.
14. Once the bead pellet has re-formed, remove and discard the supernatant from the LoBind tube without disturbing the bead pellet.
15. Repeat Steps 13 & 14 for a second EtOH wash.
16. With the tube open to the environment, allow the bead pellet to air dry for up to 5 minutes. Monitor the bead pellet throughout the drying process so that the pellet does not over-dry. Over-dried beads make for difficult resuspension.
17. When the bead pellet has dried, elute the library from the beads by adding 25 $\mu$ L ( or 1/4<sup>th</sup> of the volume transferred in Step 7) of Low TE Buffer.
18. Remove the tube from the magnet and fully resuspend the pellet in the Low TE Buffer either via gentle vortexing or through the use of pipette mixing. Once mixed, let the solution sit for 1 minute.
19. Place the tube back on the magnet, allow the bead pellet to form, and transfer 23 $\mu$ L of eluted

library to a new 1.5mL LoBind tube.

20. The library should now be at a concentration higher than 1nM, the required library concentration for the Illumina MiniSeq System. Quantitate 5µL of the eluted library using the Qubit Fluorometer (i.e.: 5µL of eluted library in 195µL of Working Qubit Solution prepared in the previous step).
21. The exact Qubit concentration required to reach 1nM may vary depending on the size of the final sequencing library. Based on the average size of AllType libraries, we recommend diluting the library in Low TE Buffer to a concentration of 0.40ng/µL\* by using the calculator/table located at the bottom of the **Illumina Library Pooling – Qubit** tab. Input the current library concentration into the cell labeled **Concentration, ng/µL**, and the table will subsequently display the volume of sample and the volume of Low TE buffer to combine to reach 0.40ng/µL.
22. Once diluted, it is best practice to conduct a duplicate/triplicate quantification of the library using the Qubit Fluorometer to ensure that the library is as close to 0.40ng/µL as possible. (i.e.: 5µL of diluted library in 195µL of Working Qubit Solution).
23. If further dilution down to 0.40ng/µL is required following the duplicate/triplicate Qubit reading, follow Step 21.

***\*Note: The final concentration of the sequencing library may need to be adjusted by the user depending on the size of the final library. We recommend 0.40ng/µL as a starting point, but if the flow cell cluster density is outside of the normal range for the MiniSeq (170 -220) it may be necessary to adjust the final library concentration.***

**STOPPING POINT (Optional)** Store undiluted library at –30°C to –10°C for up to two months. Store diluted library at –30°C to –10°C for up to two weeks. Before use, thaw on ice, and re-quantify if stored for more than one week.

### C. Users of the Illumina iSeq System

1. Open the **NGS Library Pooling Calculator** Excel file, which can be obtained from the One Lambda website. Navigate to the **Illumina Library Pooling - Qubit** tab once the Excel file opens.
2. For every sample, input the concentration obtained from the Qubit in the **Concentration, ng/ $\mu$ L** column.
3. The columns labeled **Lowest Concentration, ng/ $\mu$ L** and **Sample Volume,  $\mu$ L** should auto populate.
4. Check all samples to ensure that the value in the **Sample Volume,  $\mu$ L** column is at least 2 $\mu$ L (cells with volumes less than 2 $\mu$ L will turn purple). If it is not, adjust the value for all samples in the **Total Volume,  $\mu$ L** column so that at least 2 $\mu$ L of each sample is being used. Adjusting the value in this column will update the value in the **Sample Volume,  $\mu$ L** column.
5. Begin the process of creating the equimolar pool by first adding a volume of Low TE into a 1.5mL LoBind tube that equals the value from the Excel file in the cell labeled **TOTAL Low TE ( $\mu$ L)**.
6. Next, for each sample, add a volume of each sample to the LoBind tube which equals the volume indicated in the **Sample Volume,  $\mu$ L** column of the Excel file.

**Note: Sample should be taken from the plate containing the diluted samples (1:1 dilution). Or, in the case where an un-diluted sample required quantification, use the un-diluted sample.**

7. With the pooling completed, transfer half of the pooled library to a new LoBind tube. Make note of the actual volume transferred.
8. Conduct a 0.8X AMPure cleanup in the new LoBind tube containing library. For example, if 100 $\mu$ L of library was transferred, add 80 $\mu$ L of AMPure beads.
9. Pipette the AMPure bead & library pool mixture approximately 10 times to mix. Incubate at room temperature for 5 mins.
10. When the incubation is complete, place the tube in a magnetic rack and let stand for approximately 3 mins, or until the solution clears.
11. During the 3-minute incubation, prepare 500 $\mu$ L of 75% EtOH.
12. When the 3-minute incubation is complete, remove and discard the supernatant from the LoBind tube without disturbing the bead pellet.
13. With the tube still on the magnet, add 200 $\mu$ L of freshly prepared 75% EtOH. Rotate the tube on the magnet approximately 5 times to mix the bead pellet with the EtOH. If rotating the tube does not allow for mixing, use a pipette.
14. Once the bead pellet has re-formed, remove and discard the supernatant from the LoBind tube without disturbing the bead pellet.
15. Repeat Steps 13 & 14 for a second EtOH wash.
16. With the tube open to the environment, allow the bead pellet to air dry for up to 5 minutes. Monitor the bead pellet throughout the drying process so that the pellet does not over-dry. Over-dried beads make for difficult resuspension.
17. When the bead pellet has dried, elute the library from the beads by adding an equal volume of Low TE Buffer to the amount of library transferred in Step 7 (elute with 100 $\mu$ L if 100 $\mu$ L was initially transferred).
18. Remove the tube from the magnet and fully resuspend the pellet in the Low TE Buffer either via gentle vortexing or through the use of pipette mixing. Once mixed, let the solution sit for 1

minute.

19. Place the tube back on the magnet, allow the bead pellet to form, and transfer 95µL of eluted library to a new 1.5mL LoBind tube.
20. The library should now be at a concentration higher than 100pM, the required library concentration for the Illumina iSeq System. Quantitate 5µL of the eluted library using the Qubit Fluorometer (i.e.: 5µL of eluted library in 195µL of Working Qubit Solution prepared in the previous step).
21. The exact Qubit concentration required to reach 100pM may vary depending on the size of the final sequencing library. Based on the average size of AllType libraries, we recommend diluting the library in Low TE Buffer to a concentration of 0.045\*ng/µL by using the calculator/table located at the bottom of the **MiSeq Illumina Library Pooling – Qubit tab**. Input the current library concentration into the cell labeled **Concentration, ng/µL**, and the table will subsequently display the volume of sample and the volume of Low TE buffer to combine to reach 0.045ng/µL.
22. Once diluted, it is best practice to conduct a duplicate/triplicate quantification of the library using the Qubit Fluorometer to ensure that the library is as close to 0.045ng/µL as possible. (i.e.: 5µL of diluted library in 195µL of Working Qubit Solution).
23. If further dilution down to 0.045ng/µL is required following the duplicate/triplicate Qubit reading, follow Step 21.

***\*Note: The final concentration of the sequencing library may need to be adjusted by the user depending on the size of the final library. We recommend 0.045ng/µL as a starting point, but if the flow cell Occupancy is outside of the normal range for the iSeq (75 - 95%) it may be necessary to adjust the final library concentration.***

**STOPPING POINT (Optional)** Store undiluted library at –30°C to –10°C for up to two months. Store diluted library at -30°C to –10°C for up to two weeks. Before use, thaw on ice, and re-quantify if stored for more than one week.

# Illumina MiSeq Sample Plate Creation, Sample Sheet Creation, and Sequencing Preparation

The following process describes how to use the Illumina Experiment Manager software to create a Sample Plate and a Sample Sheet for an AllType NGS Assay Sequencing Run using the MiSeq sequencing system.

## Sample Plate Creation

1. Open the Illumina Experiment Manager software.
2. Select the **Create Sample Plate** button.
3. When prompted to select a Library Prep Kit, select **TruSeq DNA CD Indexes (96 Indexes)** and click Next.
4. In the Plate Parameters screen, assign the Plate a name in the open field, and select **2 (Dual)** for the Index Reads radio button selection.
5. In the Plate Samples screen, populate the Sample ID for each sample in your library, along with the appropriate Index 1 and Index 2 applied to each sample. Use the **AllType NGS Index Flex Kit plate layout** provided in the **Index ligation and nick repair** section of this document as a guide.
6. When complete, click the Finish button and save the Sample Plate.

## Sample Sheet Creation

1. When finished saving the Sample Plate, the Illumina Experiment Manager takes you back to the home screen.
2. Select the **Create Sample Sheet** button.
3. In the Instrument Selection screen, select the **MiSeq** option and then click Next.
4. In the MiSeq Application Selection screen, select the **Other** option.
5. Within the same screen, select the **FASTQ Only** option and click Next.
6. In the Workflow Parameters screen, populate the fields as seen in the following screenshot:

## Sample Sheet Wizard - Workflow Parameters

FASTQ Only Run Settings	FASTQ Only Workflow-Specific Settings
Reagent Cartridge Barcode*	<input type="checkbox"/> Custom Primer for Read 1
Library Prep Workflow TruSeq DNA PCR-Free	<input type="checkbox"/> Custom Primer for Index
Index Adapters TruSeq DNA CD Indexes (96 Indexes)	<input type="checkbox"/> Custom Primer for Read 2
Index Reads <input type="radio"/> 0 (None) <input type="radio"/> 1 (Single) <input checked="" type="radio"/> 2 (Dual)	<input type="checkbox"/> Reverse Complement
Experiment Name	<input checked="" type="checkbox"/> Use Adapter Trimming
Investigator Name	<input checked="" type="checkbox"/> Use Adapter Trimming Read 2
Description	
Date 7/ 2/2017	
Read Type <input checked="" type="radio"/> Paired End <input type="radio"/> Single Read	
Cycles Read 1 151	
Cycles Read 2 151	
* - required field	

Populate any additional required and/or non-required fields based on user preference. Click Next.

7. In the Sample Selection screen, load the Sample Plate created in the previous section. With the sample information loaded, transfer the applicable samples for the upcoming run to the right of the screen. Once completed, click Finish and save the Sample Sheet as a .csv file for later transfer to the Illumina MiSeq instrument.

## Sequence Preparation and Setup

***The following procedure describes to dilute and denature the library pool. Following dilution and denaturation of the library pool, a PhiX control is prepared. The prepared library and prepared PhiX control are then combined and loaded onto the MiSeq Reagent Cartridge for sequencing.***

### Required materials and equipment

- MiSeq Reagent Kit v2 (300 cycle)
- PhiX Control Kit
- NaOH Solution
- Nuclease Free Water
- Low TE Buffer (included in AllType NGS Library Prep Kit)
- 1.5mL Eppendorf LoBind Tubes
- Vortex
- Mini/Micro Centrifuge

## Preparing the Pooled Library for Sequencing

***Note: Prior to beginning the next series of steps, thaw the MiSeq Reagent Cartridge for at least one hour by leaving the cartridge at room temperature, or by submerging in room temperature water up to the noted fill line. Once thawed, the cartridge can be stored at 4°C until ready for sequencing.***

***In addition to thawing the Reagent Cartridge, thaw the HT1/Hybridization Buffer that is included with the MiSeq Reagent Kit. Once thawed, keep the HT1/Hybridization Buffer on ice.***

1. In a new 1.5mL LoBind tube, combine 5µL of the 1.60ng/µL (4nM) library pool with 5µL of freshly prepared 0.2M NaOH solution. Vortex, pulse-spin, and incubate at room temperature for 5 mins.
2. When the 5 minute incubation is complete, add 990µL of HT1/Hybridization buffer to the denatured library to bring the concentration down to 20pM. Vortex and pulse-spin.

## Preparing the PhiX Control for Sequencing

1. In a new 1.5mL LoBind tube, combine 2µL of 10nM PhiX stock to 3µL of Low TE buffer to dilute the PhiX to 4nM. Vortex and pulse-spin.
2. To the same tube containing 4nM PhiX, add 5µL of freshly prepared 0.2M NaOH solution. Vortex, pulse-spin, and incubate at room temperature for 5 mins.
3. When the 5-minute incubation is complete, add 990µL of HT1/Hybridization buffer to the denatured PhiX to bring the concentration down to 20pM. Vortex and pulse-spin.  
20pM PhiX can be stored at -20°C for up to 2 weeks.
4. In a new 1.5mL LoBind tube, combine 37.5µL of 20pM PhiX to 22.5µL of HT1/Hybridization buffer to bring the concentration down to 12.5pM. This dilution will serve as the control for the sequencing run.

## Preparing the Sequencing Run

1. In a new 1.5mL LoBind tube, combine 570 $\mu$ L of 20pM library that was prepared in the previous steps, along with 30 $\mu$ L of 12.5pM PhiX control. Vortex, and pulse-spin.
2. Obtain the thawed MiSeq Reagent Cartridge and invert 10 times to mix, followed by gentle tapping of the cartridge in the upright position on a countertop. Wipe off any liquid or condensation from the cartridge using a lab wipe.
3. Pierce well 17 of the MiSeq Reagent Cartridge using the 1mL pipette tip. Discard the tip once pierced.
4. Transfer 600 $\mu$ L of the combined library and PhiX solution to well 17 using a pipette. Make sure that the solution is dispensed to the bottom of the well.
5. If applicable, transfer the Sample Sheet created in previous sections to the appropriate location on the MiSeq instrument.
6. Press the Sequence button on the instrument interface to walk through the remainder of the sequencing run setup.
7. When prompted to insert the flow cell, clean the flow cell after careful removal from its container by thorough rinsing with lab grade water. Once rinsed, thoroughly dry the flow cell surface with a lab wipe. Ensure that all visible smudges, dust, and lab wipe lint is removed from the flow cell surface.
8. Proceed with initiation of the sequencing run.
9. When the run is complete, clean the instrument in a timely fashion per Illumina's recommendations.

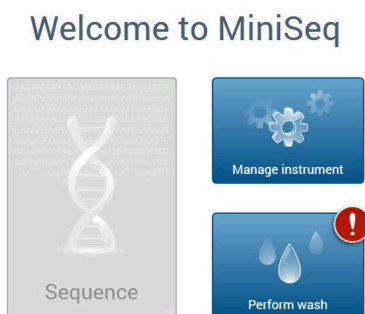


# Illumina MiniSeq Sample Sheet Creation, Template Preparation, and Sequencing

The following process describes how to use the Illumina Experiment Manager software to create a Sample Plate and a Sample Sheet for an AllType NGS Assay Sequencing Run using the MiniSeq sequencing system.

## Illumina MiniSeq Sample Sheet and Sequencing Run setup

1. Set up MiniSeq for on-instrument analysis through Local Run Manager by choosing “**Manage Instrument**” on the Home screen.



2. Choose System Configuration>Analysis Configuration>**Local Run Manager**

- On the Local Run Manager tab, fill out the fields with the username and password and optionally, the BaseSpace login credentials can be filled out to enable run monitoring. Also, an output folder must be designated.
- Install Chromium, if not already installed. Then enter “localhost” into the URL field to go to Local Run Manager and log in.
- Click on Create Run>**GenerateFASTQ** to be taken the “Create Run” page.

The screenshot shows the 'Local Run Manager' interface. At the top, there are navigation icons for search, settings, and a user profile labeled 'Admin User'. Below this is a summary section with five circular indicators: 'Ready' (1), 'In Progress' (0), 'Stopped or Unsuccessful' (0), 'Complete' (2), and 'Total' (3). A 'Create Run' button is visible, with a dropdown menu showing 'GenerateFASTQ'. Below the summary is a table with the following data:

RUN NAME / ID	MODULE	STATUS	LAST MODIFIED
11L-196 QC Panel 1 HL Mini... 190111_MN01037_0003_A000H2MM2J	GENERATEFASTQ	Analysis Completed	2019-01-12 17:29
MiniSeq Test Run HT 181_1... 190108_MN01037_0002_A000H2MLNY	GENERATEFASTQ	Analysis Completed	2019-01-09 14:53
OneLamda_MNSQ_PhiX_Ve... --	GENERATEFASTQ	Ready for Sequencing	2018-12-21 14:05

At the bottom of the table, there is a pagination control showing 'Showing 1-3 of 3' and a dropdown menu for 'Active Runs'. The footer of the interface indicates 'Instrument: MiniSeq MINISEQ-MN01037' and 'Local Run Manager Version: 1.3.1'.

- Fill out the Run Name field with the appropriate information. Fill out the Run Description with any other relevant info. For Library Prep Kit, choose “**TruSeq HT.**” The Template button can be clicked to download a MiniSeq barcode template.
- Fill the template “**GenerateFastQ-samples.csv**” out with the sample name associated with the appropriate barcode combinations. Then click the blue “Import Samples” button to upload the sample list.

## Create Run GENERATEFASTQ

Run Name\*  Run Description

### Run Settings

Library Prep Kit\*

Index Reads\*  0  1  2

Read Type\*  Single Read  Paired End

READ 1	INDEX 1	INDEX 2	READ 2
<input type="text" value="151"/>	<input type="text" value="8"/>	<input type="text" value="8"/>	<input type="text" value="151"/>

Custom Primers  Read 1  Index 1  Index 2  Read 2

### Module-Specific Settings

**i** No module-specific settings available for the selected Library Prep Kit.

[Show advanced module settings...](#)

### Import Samples

[Template](#) [Export](#)

	SAMPLE ID*	SAMPLE DESCRIPTION	INDEX 1 (I7)*	INDEX 2 (I5)*	
1	LITTLE-1	11L_196_mmm09ann_QC_P1_HL	D707	D501	x
2	TER254-2	11L_196_mmm09ann_QC_P1_HL	D707	D502	x
3	BCK-3	11L_196_mmm09ann_QC_P1_HL	D707	D503	x
4	NIE-4	11L_196_mmm09ann_QC_P1_HL	D707	D504	x
5	TER262-5	11L_196_mmm09ann_QC_P1_HL	D707	D505	x
6	TER204-6	11L_196_mmm09ann_QC_P1_HL	D707	D506	x
7	TER238-7	11L_196_mmm09ann_QC_P1_HL	D707	D507	x
8	TER250-8	11L_196_mmm09ann_QC_P1_HL	D707	D508	x
9	TER150-9	11L_196_mmm09ann_QC_P1_HL	D708	D501	x
10	TER221-10	11L_196_mmm09ann_QC_P1_HL	D708	D502	x
...					

## 8. Example of MiniSeq Sample Sheet

	A	B	C	D	E
1	Sample_ID	Descriptive	I7_Index_ID	I5_Index_ID	
2	Sample 1		D701	D501	
3	Sample 2		D701	D502	
4	Sample 3		D701	D503	
5	Sample 4		D701	D504	
6	Sample 5		D701	D505	
7	Sample 6		D701	D506	
8	Sample 7		D701	D507	
9	Sample 8		D701	D508	
10	Sample 9		D702	D501	
11	Sample 10		D702	D502	
12	Sample 11		D702	D503	
13	Sample 12		D702	D504	
14	Sample 13		D702	D505	
15	Sample 14		D702	D506	
16	Sample 15		D702	D507	

## Illumina MiniSeq Sample Preparation

The following procedure describes how to dilute and denature the library pool and PhiX control. Following dilution and denaturation of the library pool, the prepared library and prepared PhiX control are combined and loaded onto the MiniSeq Reagent Cartridge for sequencing.

### Required Materials:

- **PhiX**
- *0.1 M NaOH (freshly prepared)*
- *DNA Suspension Buffer*
- *Hybridization Buffer (supplied with MiniSeq kit)*

**Note:** Thaw reagent cartridge for 2 – 3 hours in a RT water bath. Reagent will be stable for up to 24 hours after thawing. **Invert cartridge 5 times to mix then tap gently after checking that cartridge is fully thawed to reduce air bubbles just before loading.** The MiniSeq flow cell should be removed from 4°C storage and equilibrated to RT 30 minutes prior to sequencing. The MiniSeq flow cell is packaged dry, so dab flow cell gently with a KimWipe if needed.

**Note:** The Hybridization Buffer is included with the MiniSeq Reagent Kit and stored separately at -20°C. Prior to PhiX and Sample preparation, remove Hybridization Buffer from -20°C and once thawed, keep the Hybridization Buffer on ice.

## Preparing the Pooled Library for Sequencing

1. In a new 1.5mL LoBind tube, combine 5 µl of the 1nM (0.40 ng/µl) library with 5 µl of 0.1M NaOH. Vortex and spin down. Then incubate for 5 minutes.
2. Add 5 µl of 200 mM **DNA Suspension Buffer**. Vortex and spin down.
3. Add 985 µl of **Hybridization Buffer** to denatured **Library** immediately. The concentration is now 20 pM.

## Preparing the PhiX Control for Sequencing

1. In a new 1.5mL LoBind tube, combine 10 µl of 10 nM **PhiX** with 15 µl of **DNA Suspension Buffer** to make the concentration 4 nM.

**Note:** The 4 nM **PhiX** can be stored at -20°C for up to three months.

2. In a new 1.5mL LoBind tube, combine 5 µl of the 4 nM **PhiX** with 5 µl of 0.1M **NaOH**. Vortex and spin down. Then incubate at room temperature for 5 minutes.
3. Add 5 µl of 200 mM **DNA Suspension Buffer**. Vortex and spin down.
4. Add 985 µl of **Hybridization Buffer** to denatured **PhiX** immediately. The concentration is now 20 pM.

**Note:** The 20 pM **PhiX** can be stored at -20°C for up to two weeks.

## Preparing the Sequencing Run

1. Add 5  $\mu\text{l}$  of the prepared 20pM PhiX to the denatured and diluted library.
2. Transfer 180  $\mu\text{l}$  of the combined library to a new 1.5mL LoBind tube. Add 320  $\mu\text{l}$  of **Hybridization Buffer**. The final concentration of the diluted library should now be 1.8pM.
3. Add 500  $\mu\text{l}$  of the final library to reservoir **#16** and load MiniSeq per instructions on the screen.

# Illumina iSeq Sample Sheet Creation, Template Preparation, and Sequencing

The following process describes how to use the Illumina Experiment Manager software to create a Sample Sheet for an AllType NGS Assay Sequencing Run using the iSeq sequencing system.

**Note:** The iSeq reagent cartridge, stored at -25°C to -15°C, requires a minimum of 6 hours to thaw in a RT water bath, not exceeding 18 hours. Alternatively, the cartridge can be thawed overnight at RT at a minimum of 9 hours, not exceeding 18 hours. The flow cell, which is stored at 4°C, should be left at RT for 30 minutes before use.

**Important:** The cartridge should be thawed outside of its box but with the white foil bag intact. Thaw the cartridge with the label facing upwards.

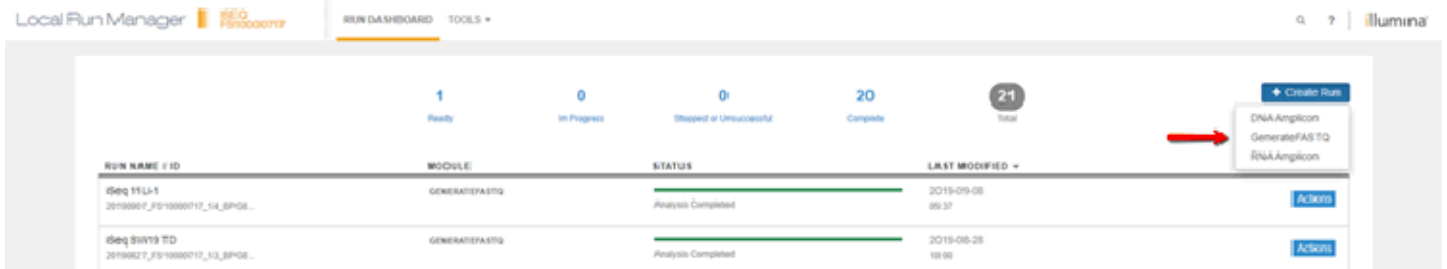
## Illumina iSeq Sample Sheet Creation

1. Open the iSeq Sample Sheet Template.csv file. Fill out the sheet with the **Experiment name** in cell B3 and the **Date** in cell B4. Populate cells B20 to B115 with the respective DNA sample name/ID following the plate positions in below cell C19. Delete rows that correspond to unused positions.
2. Save the .csv file and transfer to a thumb drive. Below is an example of the sample sheet.

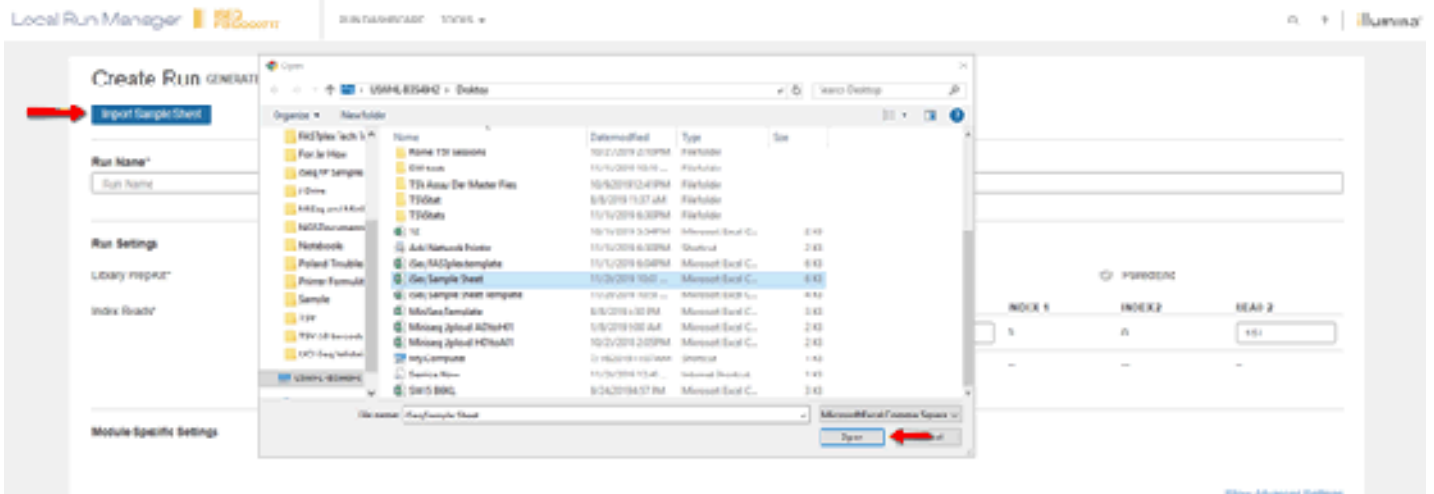
	A	B	C	D	E	F	G	H	I	J
1	[Header]									
2	Local Run	1003								
3	Experiment	EXPERIMENT NAME HERE								
4	Date	MM/DD/YYYY								
5	Module	GenerateFASTQ - 2.0.0								
6	Workflow	GenerateFASTQ								
7	Library Pri	Custom								
8	Chemistry	Amplicon								
9										
10	[Reads]									
11		151								
12		151								
13										
14	[Settings]									
15	Adapter	AGATCGGAAGAGCACACGTCTGAACTCCAGTCA								
16	AdapterR	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT								
17										
18	[Data]									
19	Sample_ID	Sample_Name	Descriptio	index	I7_Index	index2	I5_Index	Sample_Project		
20	1		A1	ATTACTCC	D701	TATAGCC1	D501			
21	2		B1	ATTACTCC	D701	ATAGAGG	D502			
22	3		C1	ATTACTCC	D701	CCTATCCT	D503			
23	4		D1	ATTACTCC	D701	GGCTCTG	D504			
24	5		E1	ATTACTCC	D701	AGGCGAA	D505			
25	6		F1	ATTACTCC	D701	TAATCTTA	D506			
26	7		G1	ATTACTCC	D701	CAGGACG	D507			
27	8		H1	ATTACTCC	D701	GTA	D508			
28	9		A2	TCCGGAG,	D702	TATAGCC1	D501			
29	10		B2	TCCGGAG,	D702	ATAGAGG	D502			
30	11		C2	TCCGGAG,	D702	CCTATCCT	D503			
31	12		D2	TCCGGAG,	D702	GGCTCTG/	D504			
32	13		E2	TCCGGAG,	D702	AGGCGAA	D505			
33	14		F2	TCCGGAG,	D702	TAATCTTA	D506			
34	15		G2	TCCGGAG,	D702	CAGGACG	D507			
35	16		H2	TCCGGAG,	D702	GTA	D508			

## Planning the Illumina iSeq Sequencing Run

1. Open Local Run Manager by clicking on the hamburger button (not sure if this is the most professional thing...but it's what it is!) and selecting Local Run Manager. A window will pop up with a blue "Open Local Run Manager" button.
2. The control software screen will minimize, and a browser window will open up with Local Run Manager.
3. Click the blue "Create Run" button and select the "GenerateFASTQ" option.



4. The "Create Run" page will open next. Click the blue "Import Sample Sheet" to the top left of the screen. Navigate to the location of your prepared sample sheet and select it.



- The Run Name section and your run sample information will populate.

**Create Run** GENERAL/ALSTG

[Import Sample Sheet](#)

**Run Name\***  **Run Description**

**Run Settings**

Library Prep Kit\*

Index Reads\*  0  1  2

Read Type\*  Single Read  Paired End

Read Length\*

Custom Primers

**Module-Specific Settings**

[Show Index Sequence](#)

	SAMPLE ID*	SAMPLE NAME	SAMPLE DESCRIPTION	INDEX 1 (51%)	INDEX 2 (51%)	SAMPLE PROJECT
1	DNA-1		TTGGAATG	CAACTGAC		X
2	DNA-1		TTAATGGG	CAACTGAC		X
3	DNA-1		AGCTACGT	CAACTGAC		X
4	DNA-1		GGCTCTGT	CAACTGAC		X
5	DNA-1		GGGACAA	CAACTGAC		X
6	DNA-1		TATCCGAC	CAACTGAC		X

- Verify that the run information, including cycle numbers and sample names, are correct. The read type should have the “Library Prep Kit” set as Custom, the “Paired End” selection chosen, and the “Index Reads” field should have 2 selected.

**Create Run** GENERAL/ALSTG

[Import Sample Sheet](#)

**Run Name\***  **Run Description**

**Run Settings**

Library Prep Kit\*

Index Reads\*  0  1  2

Read Type\*  Single Read  Paired End

Read Length\*

Custom Primers

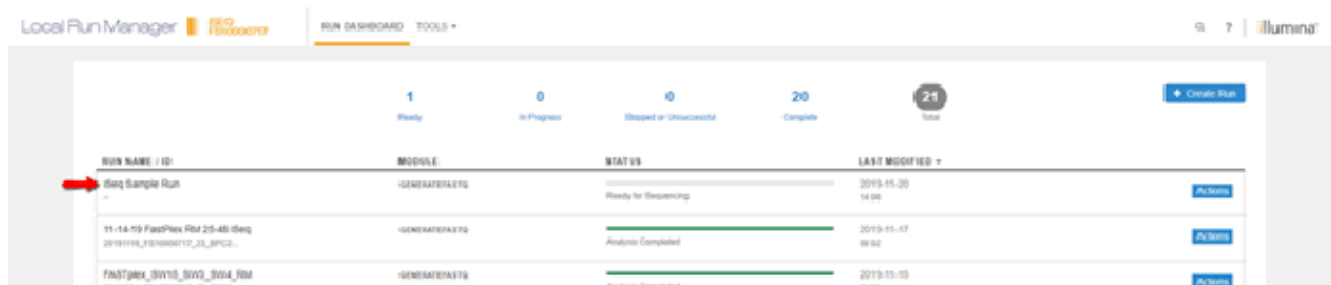
**Module-Specific Settings**

[Show Index Sequence](#)

	SAMPLE ID*	SAMPLE NAME	SAMPLE DESCRIPTION	INDEX 1 (51%)	INDEX 2 (51%)	SAMPLE PROJECT
1	DNA-1		TTGGAATG	CAACTGAC		X
2	DNA-1		TTAATGGG	CAACTGAC		X
3	DNA-1		AGCTACGT	CAACTGAC		X
4	DNA-1		GGCTCTGT	CAACTGAC		X
5	DNA-1		GGGACAA	CAACTGAC		X
6	DNA-1		TATCCGAC	CAACTGAC		X
7	DNA-1		TTGGAATG	CAACTGAC		X
8	DNA-1		TTAATGGG	CAACTGAC		X
9	DNA-1		AGCTACGT	CAACTGAC		X
10	DNA-1		GGCTCTGT	CAACTGAC		X
11	DNA-1		GGGACAA	CAACTGAC		X
12	DNA-1		TATCCGAC	CAACTGAC		X
13	DNA-1		TTGGAATG	CAACTGAC		X
14	DNA-1		TTAATGGG	CAACTGAC		X
15	DNA-1		AGCTACGT	CAACTGAC		X
16	DNA-1		GGCTCTGT	CAACTGAC		X
17	DNA-1		GGGACAA	CAACTGAC		X
18	DNA-1		TATCCGAC	CAACTGAC		X



- Click the blue “Save Run” button and you will be taken back to the home screen for Local Run Manager with your run saved.



## Illumina iSeq Sample Preparation

The following procedure describes how to dilute and denature the library pool and PhiX control. Following dilution and denaturation of the library pool, the prepared library and prepared PhiX control are combined and loaded onto the iSeq for sequencing.

### Required Materials:

- PhiX Control v3
- DNA Suspension Buffer or Low TE

## Preparing the Pooled Library for Sequencing

- The pooled library should be diluted to 100 pM (0.045 ng/μl) using the Pooling Calculator for iSeq.
- Add 5 μL of diluted 100 pM PhiX to 100 μL of the 100 pM library. Vortex briefly and spin down.
- Invert the cartridge 5 times to mix the reagents.
- Tap the cartridge (label facing upwards) on the bench to ensure reagent aspiration and then remove the thawed cartridge from the bag.
- Using a pipette tip, pierce the foil in the orange square marked “Library” and push aside the foil edges to enlarge the hole. Discard pipette tip.
- Add 20 μL of the Library to the bottom of the reservoir.
- Remove the equilibrated flow cell from its packaging and insert with the label side facing you into the slot on the front of the cartridge until an audible click is heard and felt.

**Note: Avoid touching the electrical interface, CMOS sensor, glass and gaskets.**

## Preparing the PhiX Control for Sequencing

1. To prepare 1 nM PhiX, combine 5 µl of **10nM PhiX** with 45 µl of **DNA Suspension Buffer** in a low bind microcentrifuge tube. Vortex briefly and spin down.
2. To prepare 100 pM PhiX (working concentration), combine 10 µl of **1nM PhiX** with 90 µl of **DNA Suspension Buffer** in a low bind microcentrifuge tube. Vortex briefly and spin down.






*Note: 1 nM PhiX can be stored at -25°C to -15°C for up to one month. 100 pM PhiX can be stored at -25°C to -15°C for up to two weeks.*

3. Set aside diluted PhiX on ice.

## Preparing the Sequencing Run

1. In the control software, select **Sequence** – the software opens the door at an angle, ejects the tray, and initiates a series of run setup screens. Follow the steps as described on-screen.
2. Place the thawed cartridge containing the flow cell and library onto the tray so that the access window faces up and the flow cell is inside the instrument. Do not push the cartridge or tray into the instrument, there is a groove that will secure the cartridge as it is inserted.
3. Select Close Door to retract the cartridge into the instrument.
4. Enter BaseSpace login information and **Sign In**.
5. Select **Run Setup**.
6. Login into **Local Run Manager** and select the appropriate run from the Run Name list.
7. Select the “**Start Sequencing**” button.

# Explanation of Symbols

Symbol	Description
 <p>ISO 7000 Reg No. 2493</p>	Catalog number
 <p>ISO 7000 Reg No. 1641</p>	Consult instructions for use *Please consult Application Note for Research Use Only product
 <p>ISO 7000 Reg No. 0434A</p>	Caution, consult accompanying documents
 <p>ISO 7000 Reg No. 0632</p>	Temperature limitation
 <p>ISO 7000 Reg No. 3082</p>	Manufacturer

## Revision History

Revision	Date	Description of Change
02	07/02/2019	<ul style="list-style-type: none"> <li>Addition of AllType NGS Library Prep Kit (Cat. No. ALL-LIBX) as an equivalent substitution for the Ion Xpress Plus Fragment Library Kit (Cat. No. ION-XLIB).</li> </ul>
03	09/21/2019	<ul style="list-style-type: none"> <li>Updated contact information and address to reflect change in legal manufacture site.</li> </ul>
04	Current	<ul style="list-style-type: none"> <li>Addition of Illumina MiniSeq and iSeq 100 sequencers, sequencing kits and samples throughputs to the following sections: Purpose of this Guide, Limitations Of The Procedure, Product Description, Required materials and equipment not supplied; Indexing Strategy, Methods, Library Pooling/Pooling Calculator, Illumina MiSeq Sample Plate and Sheet Creation, Sequence Preparation and Setup, Preparing the Pooled Library for Sequencing, Preparing the PhiX Control for Sequencing, and Preparing the Sequencing Run.</li> </ul>

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