AllType™ and NXType™ NGS Reagents

On Ion Chef™ and Ion S5™ and Ion GeneStudio™ S5 Series Systems

Application Note

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

ONE LAMBDA
A Thermo Fisher Scientific Brand

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About this Application Note

Purpose of this Guide

This application note provides reference information for using AllType and NXType NGS Reagents for amplification of targeted genes in DNA samples, and subsequent library preparation, followed by template preparation on the Ion Chef and downstream sequencing on the Ion S5 Sequencer.

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 100pM final library pools for the Ion 530™ Chip.
7. The AllType NGS amplification kits (Cat. No. ALL-11LX, ALL-9LX, and ALL-8LX) have been verified using 48 samples per sequencing run on the Ion S5 (IONS5), Ion S5 XL (IONS5XL), Ion GeneStudio S5 (IONGSS5) and Ion GeneStudio S5 Plus (IONGSS5PL) Systems using the Ion 520 & 530 ExT Kit - Chef (Cat. No. IONCHEF-EXT) and Ion 530 Chip (Cat. No. IONS5-530C4). 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$^{[1234]}$). 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 and/or www.thermofisher.com.

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Product Information

Product Description

This application note describes how to prepare Ion S5 System or Ion GeneStudio S5 System compatible libraries from amplicons generated using the AllType NGS 11-Loci Amplification and NXType NGS Genotyping 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 [1]
B	Full gene	DPB1	Exon 2 thru 3' UTR [1]
C	Full gene	DQA1	Full gene
DRB1	Exon 2 thru 3' UTR [1]	DPA1	Full gene
DRB3/4/5	Exon 2 thru 3' UTR [1]		

[1] Includes intronic sequence

AllType 9 Loci Amplification Kit:

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

[1] Includes intronic sequence

AllType 8 Loci Amplification Kit:

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

[1] Includes intronic sequence

NXType NGS Reagents:

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

[1] Includes intronic sequence

After amplification, AllType and NXType amplicons are introduced into the remaining steps of the workflow and further manipulated with various Ion Torrent reagent kits.

AllType NGS Amplification Kits

AllType NGS Amplification kits have been validated for use on the Ion S5, Ion S5 XL, Ion GeneStudio S5 and Ion GeneStudio S5 Plus Systems.

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 (each)**	**Storage**
Fragmentase	1 vial	900 µL	–15˚ 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 Library Prep Kit (Cat. No. ALL-LIBX) components contain solutions that are identical to those contained in the Ion Xpress Plus Fragment Library and Ion Shear Kits (Cat. No. ION-XLIB).

AllType NGS Index Kit

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

AllType NGS Library Prep Kit			
Component	**Quantity**	**Amount (each)**	**Storage**
Ion Barcode Adapter Plate	1 plate	8 µL/well	–30˚ to –10˚C
Ion Library Amp Primers	1 vial	400 µL	

* AllType NGS Library Prep Kit (Cat. No. ALL-LIBX) components contain solutions that are identical to those contained in the Ion Xpress Barcodes Kits (Cat. No. ION-XBA1, ION-XBA2, ION-XBA3, ION-XBA4, ION-XBA5, and ION-XBA6).

NXType NGS Reagents

NXType NGS Amplification kits have been validated for use on the Ion S5 and Ion S5 XL Systems only.

The Class I (A, B, and C Locus) NXType NGS Genotyping Kit (Cat. No. NXT1ABCX) contains the following components:

Class I NXType NGS Typing Kit			
Component	**Quantity**	**Amount**	**Storage**
NXType Class I Primer Mix	1 vial	500 µL	–20˚C
NXType Buffer	1 vial	500 µL	
NXType dNTPs	1 vial	200 µL	
NXType Polymerase	1 vial	100 µL	

The Class II (DRB1, DRB3/4/5, DQB1, and DPB1 loci) NXType NGS Genotyping Kit (Cat. No. NXT2RQP1X) contains the following components:

Class II NXType NGS Genotyping Kit			
Component	**Quantity**	**Amount**	**Storage**
NXType Class II Primer Mix	1 vial	500 µL	–20˚C
NXType Buffer	1 vial	500 µL	
NXType dNTPs	1 vial	200 µL	
NXType Polymerase	1 vial	100 µL	

The DQA1 NXType NGS Genotyping Kit (Cat. No. NXT2QA1X) contains the following components:

DQA1 NXType NGS Genotyping Kit			
Component	**Quantity**	**Amount**	**Storage**
NXType DQA1 Primer Mix	1 vial	125 µL	–20˚C
NXType Buffer	1 vial	125 µL	
NXType dNTPs	1 vial	50 µL	
NXType Polymerase	1 vial	28 µL	

The DPA1 NXType NGS Genotyping Kit (Cat. No. NXT2PA1X) contains the following components:

DPA1 NXType NGS Genotyping Kit			
Component	**Quantity**	**Amount**	**Storage**
NXType DPA1 Primer Mix	1 vial	125 µL	–20˚C
NXType Buffer	1 vial	125 µL	
NXType dNTPs	1 vial	50 µL	
NXType Polymerase	1 vial	28 µL	

Required materials and equipment not supplied

General Supplies and Consumables		
Description	**Supplier**	**Cat. No.**
96-Well, 0.2mL, PCR Plates	MLS[1]	---
96 Well Plastic/Foil Plate Seals	MLS[1]	---
Thermo Scientific Nunc™ 96-Well Polypropylene MicroWell™ Plates	Fisher Scientific	12-565-369
0.2mL 8-Tube PCR Strips with Caps	MLS[1]	---
PCR Plate & 0.2mL Tube Cooler	MLS[1]	---
Eppendorf DNA LoBind Tubes, 5.0mL	Eppendorf	0030108310
Eppendorf DNA LoBind Tubes, 2.0mL	Eppendorf	022431048
Eppendorf DNA LoBind Tubes, 1.5mL	Eppendorf	022431021
50mL Conical Tubes Nuclease free	MLS[1]	---
15mL Conical Tubes Nuclease free	MLS[1]	---
Serological Pipettes, 10 or 25mL	MLS[1]	---
Pipette Controller	MLS[1]	-
Full array of manual single channel pipettes	MLS[1]	-
20µL and 200µL manual multichannel pipettes	MLS[1]	---
Full array of filtered, pre-sterilized, pipette tips	MLS[1]	---
Sterile Reagent Reservoirs	MLS[1]	---
0.2mL Centrifuge Adapter	MLS[1]	---
Thermal cycler PCR Pressure Pad	MLS[1]	---

[1] **Major Laboratory Supplier**

Required materials and equipment not supplied

Standalone Kits and Reagents		
Description	**Supplier**	**Cat. No.**
Agencourt® AMPure® XP Beads, 60mL	Beckman Coulter	A63881
Nuclease Free Water	MLS[1]	---
(Low) TE Buffer; or (Low) TE Buffer (part of ALL-LIBX)	Life Technologies; or One Lambda	12090-015; or part of ALL-LIBX
200 Proof Ethanol, Molecular Biology Grade	MLS[1]	---

[1] **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 Kit	ALL-IONX
Ion Xpress Barcode Adapters 1-16 Kit (need is dependent on run sample size)	ION-XBA1
Ion Xpress Barcode Adapters 17-32 Kit (need is dependent on run sample size)	ION-XBA2
Ion Xpress Barcode Adapters 33-48 Kit (need is dependent on run sample size)	ION-XBA3
Ion Xpress Barcode Adapters 49-64 Kit (need is dependent on run sample size)	ION-XBA4
Ion Xpress Barcode Adapters 65-80 Kit (need is dependent on run sample size)	ION-XBA5
Ion Xpress Barcode Adapters 80-96 Kit (need is dependent on run sample size)	ION-XBA6
Ion Xpress Barcode Adapters 1-96 Kit (need is dependent on run sample size)	ION-XBA7
Ion 520 & 530 ExT Kit - Chef (4 rxn) Contains the following kits: • Ion S5 ExT Sequencing Reagents • Ion S5 ExT Sequencing Solutions • Ion S5 ExT Chef Reagents • Ion S5 ExT Chef Supplies • Ion S5 ExT Chef Solutions	IONCHEF-EXT
Ion 530 Chip	IONS5-530C4

Ion S5 ExT Sequencing Reagents	Not sold individually. Refer to IONCHEF-EXT
Ion S5 ExT Sequencing Solutions	Not sold individually. Refer to IONCHEF-EXT
Ion S5 ExT Chef Reagents	Not sold individually. Refer to IONCHEF-EXT
Ion S5 ExT Chef Supplies	Not sold individually. Refer to IONCHEF-EXT
Ion S5 ExT Chef Solutions	Not sold individually. Refer to IONCHEF-EXT

* Users preparing libraries with AllType™ NGS Library Prep Kit (Cat. No. ALL-LIBX) for use on Ion sequencers (Cat. No. IONS5, IONS5XL, IONGSS5, or IONGSS5PL) require the use of either: (a) the Library Amplification Primer Mix component from the Ion Xpress™ Plus Fragment Library Kit (Cat. No. ION-XLIB), or (b) the Ion Library Amp Primers from the AllType NGS Index Kit (Cat. No. ALL-IONX).

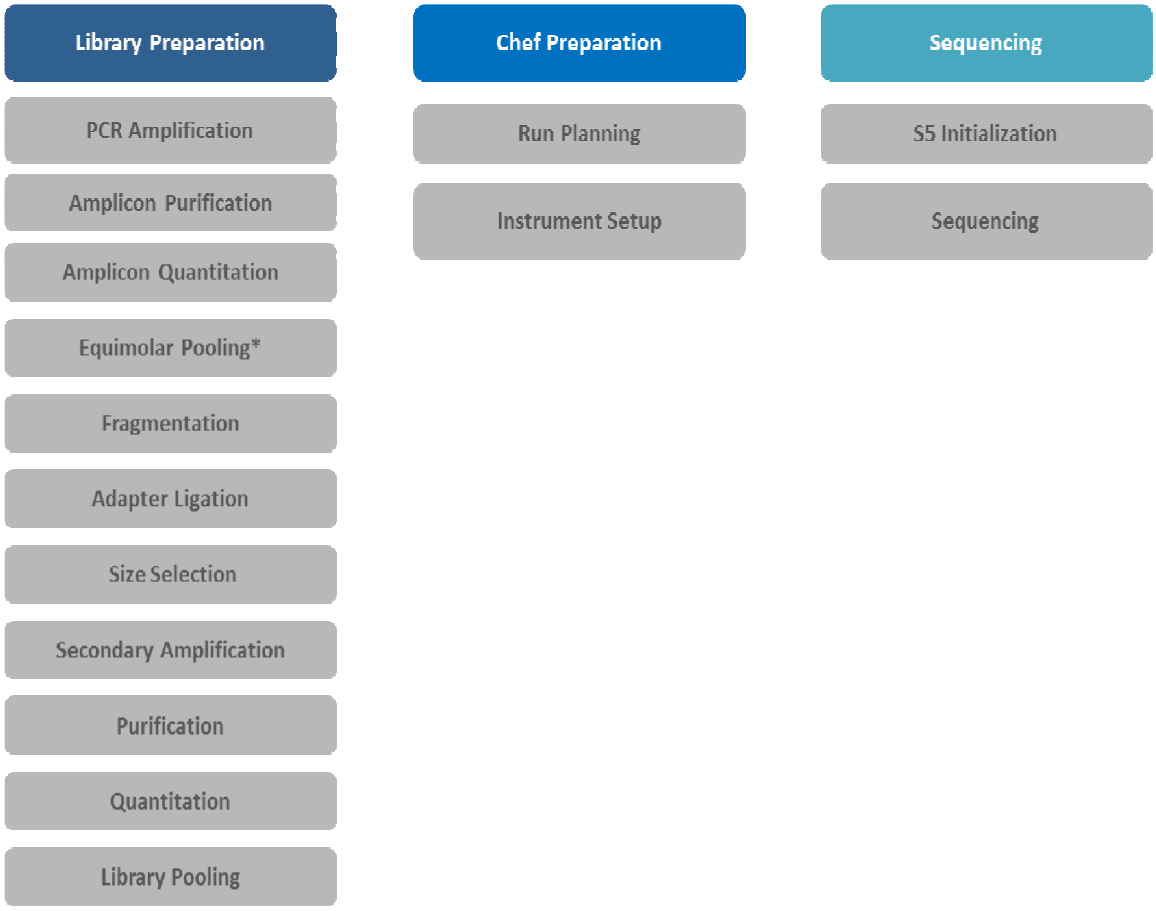
Required materials and equipment not supplied

Equipment and Associated Reagents & Consumables		
Description	**Supplier**	**Cat. No.**
Magnetic-Ring Stand (96 well)	Life Technologies	AM10050
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® Fluorometer	Life Technologies	Q33216 or Q33226
Qubit dsDNA HS Assay Kit	Life Technologies	Q32854
Qubit Assay Tubes	Life Technologies	Q32856
Microcentrifuge capable of 15,000 x g	MLS[1]	---
Plate Centrifuge capable of 1,500 x g	MLS [1]	---
Mini Centrifuge	MLS [1]	---
Vortex	MLS [1]	---

[1] **Major Laboratory Supplier**

Equipment and Associated Reagents & Consumables Distributed by One Lambda	
Description	**Cat. No.**
Ion GeneStudio S5 or Ion GeneStudio S5 Plus System	IONGSS5 or IONGSS5PL
Ion S5 or S5XL System	IONS5 or IONS5XL
Ion Chef System	IONCHEF

Workflow Overview



*** Equimolar Pooling step not required when processing AllType amplicons.**

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 are 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.
- The recommended number of samples per sequencing run on Ion S5 530 Chip is 48-96 depending on amplicon panels used to generate the library.

Barcoding Strategy

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

Chip type	No. of Samples Based on Targeted Region			
	ALL-11LX	ALL-9LX	ALL-8LX	NXT1ABCX + NXT2RQP1X
Ion 530 Chip	Up to 48	Up to 48	Up to 48	Up to 96

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Methods

PCR and amplicon purification protocol

The following procedure describes how to use AllType and NXType 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
- NXType Class I, Class II, DQA1, and/or DPA1 Primer Mixes
- NXType Buffer, dNTPs, and Polymerase
- 96-Well 0.2mL PCR Plate and Plate Seals
- 1.5-mL or 2.0mL 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)

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.

1. Before starting, turn on a thermal cycler to allow the heated lid to reach temperature.
2. Thaw DNA and AllType/ NXType Primer Mixes, dNTPs, and Buffer at room temperature. If precipitate is present in the AllType/ NXType Buffer warm it at 37°C and vortex until clear. Place the AllType/ NXType Polymerase on ice when not in use.
3. Once thawed, briefly vortex the AllType/ NXType dNTPs, Buffer, and Primer Mixes. **Do not vortex the AllType/ NXType Polymerase**. Pulse-spin all reagents and place on ice when not in use.
4. Prepare a 96-well 0.2mL PCR plate to accommodate for the amplification parameters specific for each PCR reaction. See PCR Programs A & B below.
   Note: The combination of tests carried out is determined by the user.
5. Pipet the correct volume of DNA sample (at 25ng/µl) into the bottom of the appropriate wells on the 96-well 0.2mL PCR plate:
   - 2µl of DNA for AllType 11-Loci reactions
   - 2µl of DNA for NXType Class I and Class II reactions
   - 4µl of DNA for NXType DQA1 and DPA1 reactions
   - When finished, cover and store the plate on a PCR plate cooler or ice to prevent evaporation and contamination
6. Using 1.5-mL Eppendorf LoBind® Tubes, create a separate Amplification Master Mix for each primer mix utilized. 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 from the volumes directed in steps 6 & 8 below. AllType and NXType reagents are optimized for use in a 20µL reaction volume. Sub-optimal results will occur if the volumes are altered.

Order	Component	AllType 11-Loci Volume (per reaction)	NXType Class I and Class II Volume (per reaction)	NXType DQA1 and DPA1 Volume (per reaction)
1	Nuclease-free water	6.6µl	6.6µl	4.6µl
2	AllType/ NXType Buffer	4µl	4µl	4µl
3	AllType/ NXType dNTPs	1.6µl	1.6µl	1.6µl
4	AllType/ NXType Primer Mixes	5µl	5µl	5µl
	Total	**17.2µl**	**17.2µl**	**15.2µl**

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 the AllType/ NXType Polymerase to the Amplification Master Mix at a volume of 0.8µl per reaction. Mix well by pipetting up and down 15-20 times with a pipette set to approximately half the mix volume. Do not vortex the finished Amplification Master Mix.

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 place on ice or cold block. Aliquot the appropriate Amplification Master Mix to each reaction – 18µl for AllType 11-Loci and NXType Class I and Class II or 16µl for NXType DQA1 and DPA1 – taking special care so as not to cross-contaminate reactions.
10. Seal the plate with a tray seal and spin down the plate.
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 program(s) to amplify the genomic DNA targets.

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.*

PCR Program "A" for AllType 8-Loci, 9-Loci and 11-Loci, and NXType Class I (Lots 004 and higher) primer mixes:

Amplification PCR Program			
Step	**Temperature**	**Time**	**# of Cycles**
Step 1:	94°C	2 minutes	1
Step 2:	98°C 69°C	10 seconds 3 minutes	22
Step 3:	98°C 60°C	10 seconds 3 minutes	8
Step 4:	4°C	∞	1

PCR Program "B" for NXType Class I (Lots 001-003 only), Class II, DQA1, and DPA1 primer mixes:

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

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

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 27µL 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 25µL 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°C to –10°C for up to one month, or at -80°C for up to six months.

Amplicon quantification and equimolar pooling

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 pooling and dilution.

Required materials and equipment

- Qubit Fluorometer
- Qubit dsDNA HS Assay Kit
- Qubit Assay Tubes
- 50mL conical tubes, Nuclease-free
- 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:
    - 199µL of Qubit dsDNA HS Buffer per amplicon, plus a 15% overage
    - 1µL 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 199µL of Working Solution to each Qubit Assay Tube that will contain amplicon. Add 190µL of Working Solution to the two tubes set aside for Standard #1 and Standard #2.
5. To the assay tubes containing 199µL of working solution, add 1µL of the appropriate amplicon.
6. To the two assay tubes containing the 190µL of working solution, add 10µL 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 **1µL** and the units to **ng/µL**. 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 – AllType NGS Reagents

Note: If using NXType NGS Reagents, skip this section and proceed to the section titled "Amplicon pooling and dilution – NXType NGS Reagents"

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/µL) in the **Conc. (ng/µL)** column.
3. Acquire a new 96-well 0.2mL PCR plate that will be used to create the 100ng amplicon dilutions.
4. Add 35µL of nuclease free water to each well that will contain diluted amplicon.
5. Using the values in the column labeled **Variable Volume (µL)**, dilute each amplicon individually by first removing a volume of water from the 35µL 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 35µL.
6. Proceed to Amplicon Fragmentation with the 100ng dilutions.

STOPPING POINT (Optional) Store the remaining amplicon and/or amplicon pools at –30°C to –10°C for up to one month, or at -80°C for up to six months.

Amplicon pooling and dilution – NXType NGS Reagents

Note: If using AllType NGS Reagents, skip this section and reference the section above titled "Amplicon dilution – AllType NGS Reagents".

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 **"NXT CI-CII Amp Pooling"** tab of the Excel file, input the sample names in the **Sample** column and the corresponding sample concentration (in ng/µL) in the **Conc. (ng/µL)** column.

   Note: Depending on the combination of NXType NGS Reagent kits used, additional tabs are present for amplicon pooling when using all four amplification kits, as well as for users utilizing only the DPA and DQA amplification kits.
3. In cell F1 labeled **Primer Lot Specific Ratio**, enter the appropriate CI/CII pooling ratio as specified by the vendor.
4. If the value in the **Variable Volume** or **Combined Volume to make 100ng** columns turns red to indicate that they are outside the ideal range of 2-16µL, adjust the value in the **Constant Volume** column accordingly. Once an adjustment has been made to this value, adjust all remaining samples in the same manner. Adjusting all samples will allow for the use of a multichannel pipette during the pooling process.
5. Based on the calculations performed in the previous step, using a new 96-well 0.2mL PCR plate, and multichannel pipette, add a volume of Class II (or DQA1) amplicon to the plate that is equivalent to the value in the **Constant Volume** column. Next, using a single channel pipette, add a volume
   of Class I (or DPA1) amplicon to the corresponding well on the plate that is equivalent to the value in the **Variable Volume** column. At the conclusion of this addition you will have a Class I + Class II and/or DPA1 + DQA1 equimolar pool for each sample.
6. You should now have a number of Class I + Class II and/or DPA1 + DQA1 equimolar amplicon pools that equals the total number of samples included in your run.

   Example: *If 48 samples are setup in a given run, a total of 48 Class I + Class II amplicon pools should have been created in the previous step.*
7. Using a new 96-well 0.2mL PCR plate, add 35µL of nuclease free water to each well that will contain diluted amplicon pool. Set a pipette to a specific volume for amplicon pool transfer based on the information in the **Combined Volume for 100ng (µL)** column in the Excel file. Remove this volume of water from the corresponding well, discard, and then add the same volume of amplicon pool to the well.
8. Proceed to Fragmentation with the 100ng amplicon pools.

STOPPING POINT (Optional) Store the remaining amplicon and/or amplicon pools at –30°C to –10°C for up to one month, or at -80°C for up to six months.

Amplicon fragmentation

The following procedure describes the process to fragment the amplicon present in the amplicon pools.

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 or 2.0mL Eppendorf LoBind® Tubes

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 amplicon pools 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 Reagent Kit	Component Label if using AllType NGS Library Prep Kit	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 amplicon pools/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 gently by 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 more than 2 mL. Place the Fragmentation Master Mix on ice until use.
5. Obtain the plate(s) of 100ng amplicon pools. Place on ice.
6. Keeping the plate on ice, to each amplicon pool/sample, add 14µL of Fragmentation Master Mix to the top side of the wells that hold amplicon, 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 and cover the plate with a PCR pressure pad appropriate for your machine. Use 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. Immediately proceed to barcode ligation.

Adaptor ligation, and nick repair

The following procedure describes the process to ligate adaptors 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 Ion Xpress Barcode Adapters Kits

- Ion Xpress P1 Adapter
- Ion Xpress Barcodes (1 barcode adapter per sample)

Materials required from the AllType NGS Index Kit (may be substituted by Ion Xpress Barcode Adapters Kits)

- Ion Barcode Adapter Plate

Other materials and equipment

- PCR Plate Cooler and Ice
- Nuclease-Free Water
- 2.0mL or 5.0mL Eppendorf LoBind® Tubes or 15mL conical tubes
- 0.2mL 8-Tube PCR Strips with Caps

Adaptor ligation and nick repair

Note: If using IonXpress Barcodes, it is recommended that users pre-aliquot the IonXpress Barcode Adapters into 0.2mL 8-Tube PCR Strips with Caps prior to beginning the Adaptor ligation and nick repair. Prior to aliquoting, label the PCR Strip tubes with the appropriate barcode number, thaw, vortex, and subsequently aliquot the Ion Xpress Barcode Adapters. Store aliquoted barcodes at -20˚C.

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 7. 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. Obtain enough pre-aliquoted Ion Xpress Barcode Adapters suitable for the number of samples present in your run (1 distinct barcode adapter per sample).
6. Thaw the Ion Xpress Barcodes and Ion Xpress P1 Adapter at room temperature. Once thawed, vortex, pulse-spin, and then place on ice until use.
7. If using Ion Xpress Barcode adapters, combine the following components in either a 1.5 mL Eppendorf LoBind tube or 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 (or Ligase) and Nick Repair Polymerase until immediately prior to dispensing master mix over sample.

Ligation Master Mix Component	Volume (per fragmented amplicon/sample)
10X Ligase Buffer (or Ligase Buffer)	10µL
dNTP Mix	2µL
Ion Xpress P1 Adapter	2µL
Nuclease-Free Water	25µL
DNA Ligase (or Ligase)	2µL
Nick Repair Polymerase	8µL

If using the Ion Barcode Adapter Plate, combine the following components in either a 1.5 mL Eppendorf LoBind tube or 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 (or Ligase) and Nick Repair Polymerase until immediately prior to dispensing master mix over sample.

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

8. Obtain the PCR plate containing the fragmented product.
9. If using **Ion Xpress Barcode adapters**, add 2µL of the appropriate Ion Xpress Barcode into the respective well containing fragmented product. Only one barcode should be used per DNA sample.

   Important: Take care to avoid contamination. Work with only one strip of barcodes at a time. Additionally, wipe gloves routinely with DNA Away or 70% ethanol while working with barcodes.

   If using the **Ion Barcode Adapter Plate**, add 4µL of each Ion Barcode adapter into the respective well containing fragmented product. Only one index should be used per DNA sample.

   Note: Based on the index layout below, keep track of the index IDs used with each sample so correct information will be transferred to a Sample Sheet.

   Important: Take care to avoid contamination. Work with only one strip of barcodes at a time. Additionally, wipe gloves routinely with DNA Away or 70% ethanol while working with barcodes.

	1	2	3	4	5	6	7	8	9	10	11	12
A	001	009	017	025	033	041	049	057	065	073	081	089
B	002	010	018	026	034	042	050	058	066	074	082	090
C	003	011	019	027	035	043	051	059	067	075	083	091
D	004	012	020	028	036	044	052	060	068	076	084	092
E	005	013	021	029	037	045	053	061	069	077	085	093
F	006	014	022	030	038	046	054	062	070	078	086	094
G	007	015	023	031	039	047	055	063	071	079	087	095
H	008	016	024	032	040	048	056	064	072	080	088	096

10. 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.

11. If using the **Ion Xpress Barcode adapters,** dispense 49µL of Ligation Master Mix into each well of the PCR plate containing fragmented product with barcodes, taking special care not to cross-contaminate reactions.

    If using the **Ion Barcode Adapter Plate,** use 47µL of Ligation Master Mix into each well of the PCR plate containing fragmented product with barcodes, taking special care not to cross-contaminate reactions.

12. 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**
Step 1:	25°C	15 min.	1
Step 2:	72°C	5 min.	1
Step 3:	4°C	∞	1

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

Size-selection, secondary amplification, and final purification

The following procedure describes the process to perform a size-selection of the adaptor-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
- 5.0mL 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
- Library Amplification Primer Mix

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

- Ion Library Amp Primers

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

- Library Amp Master Mix

* Users preparing libraries with AllType™ NGS Library Prep Kit (Cat. No. ALL-LIBX) for use on Ion sequencers (Cat. No. IONS5, IONS5XL, IONGSS5, or IONGSS5PL) require the use of either: (a) the Library Amplification Primer Mix component from the Ion Xpress™ Plus Fragment Library Kit (Cat. No. ION-XLIB), or (b) the Ion Library Amp Primers from the AllType NGS Index Kit (Cat. No. ALL-IONX).

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-µL 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 27µL 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 25µL 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°C to –10°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 enough Secondary Amplification reagents from the Ion Plus Fragment Library Kits suitable for the number size-selected products 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 Required for Secondary Amp Master Mix	Volume (per size-selected product)
Platinum PCR SuperMix High Fidelity; or Library Amp Master Mix	71.4µL
Library Amplification Primer Mix * or Ion Library Amp Primers	3.6µL

* Users preparing libraries with AllType™ NGS Library Prep Kit (Cat. No. ALL-LIBX) for use on Ion sequencers (Cat. No. IONS5, IONS5XL, IONGSS5, or IONGSS5PL) require the use of either: (a) the Library Amplification Primer Mix component from the Ion Xpress™ Plus Fragment Library Kit (Cat. No. ION-XLIB), or (b) the Ion Library Amp Primers from the AllType NGS Index Kit (Cat. No. ALL-IONX).

3. The Platinum PCR SuperMix High Fidelity (or Library Amp Master Mix) reagent and the Library Amplification Primer Mix should be thawed at room temperature. When thawed, vortex, pulse-spin, and then place on ice until use.
4. In a 5.0mL Eppendorf LoBind tube or 15mL conical, create a Secondary Amp Master Mix for the desired number of size-selected products using the table above. Include a 15% overage.
5. 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 uL of the Master Mix to each size-selected product.

6. 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**
Step 1:	95°C	5 min.	1
Step 2:	95°C 58°C 70°C	15 sec. 15 sec. 1 min.	8
Step 3:	4°C	∞	1

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

Final Purification

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 amplicon, plus a 15% overage. Ethanol solution should be prepared from 200 proof, molecular biology grade, ethanol in nuclease free water. 75% ethanol should be prepared fresh daily.
3. Obtain a new 96-well 0.2mL PCR plate that will ultimately 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 100µL per amplicon, plus a 15% overage.
6. 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.
7. 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.
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 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 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.
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 20-µL 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 27µL 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 25µL 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°C to –10°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 prior to the start of Template 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, Nuclease Free
- Vortex
- Mini/Micro Centrifuge

Final Quantification – Qubit

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:
    - 199µL of Qubit dsDNA HS Buffer per amplicon, plus a 15% overage
    - 1µL 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 195µL of Working Solution to each Qubit Assay Tube that will contain amplicon. Add 190µL 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 1:5 dilution using 4µL of purified sample from Final Purification with 16µL 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 195µL of working solution, add 5µL of the appropriate amplicon.
7. To the two assay tubes containing the 190µL of working solution, add 10µL 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 amplicon. When prompted, change the volume of sample used to **5µL** and the units to **ng/µL**. 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.

Library Pooling - Qubit

1. Open the **NGS Library Pooling Calculator** Excel file which can be downloaded from the One Lambda website.
   Navigate to the **S5 Library Pooling - Qubit** tab once the Excel file opens.
2. For every sample, input the concentration obtained from the Qubit in the **Concentration, ng/µL** column.
3. The columns labeled **Lowest Concentration** and **Sample Volume** should auto populate.
4. Check all samples to ensure that the value in the **Sample Volume** column is at least 2µL (cells with volumes less than 2µL will turn purple). If it is not, adjust the value for all samples in the **Total Volume (µL)** column so that at least 2µL of each sample is being used. Adjusting the value in this column will update the value in the **Sample Volume** column.
5. Begin the process of creating the equimolar pool by first adding a volume of Low TE into a LoBind tube labeled "Library Pool" that equals the value from the Excel file in the cell labeled **TOTAL Low TE (µL).**
6. Next, for each sample, add a volume of each sample to the LoBind tube, labeled "Library Pool", which equals the volume indicated in the **Sample Volume** column of the Excel file.

Note: Sample should be taken from the plate containing the diluted samples (1:5 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 100µL of library was transferred, add 80µL of AMPure beads.
9. Pipette the AMPure bead & library pool mixture approximately 10 times to mix. Incubate at room temperature for 5 minutes.
10. When the incubation is complete, place the tube in a magnetic rack and let stand for approximately 3 minutes or until the solution clears.
11. During the 3 minute incubation, prepare 500µL 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 200µL 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 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.
17. When the bead pellet has dried, elute the library from the beads by adding 27µL 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 mxing. 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 25µL of eluted

library to a new 1.5mL LoBind tube.

20. Quantitate 5µL of the eluted library using the Qubit Fluorometer (i.e. 5µL of eluted library in 195µL of working Qubit solution).

21. The library should now be at a concentration higher than 0.045ng/µL. Dilute the concentrated library down to 0.045 ng/µL in Low TE buffer using the calculator/ table located at the bottom of the **S5 Library Pooling – Qubit** tab. Input the current library concentration into the cell labeled **Concentration, ng/µL** and set the cell labeled **Total Volume, µL** to 100. 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.
    If further dilution down to 0.045ng/µL is required following the duplicate/triplicate Qubit reading, follow Step 21.

23. **If the lowest molarity for a sample is less than 0.045ng/µL:** No further dilution is necessary. The pool in its current state is the final pool that will be used as the input into the Isothermal Amplification reaction. Proceed to Template Preparation.

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.

Ion Chef ExT preparation – Template and Sequencing Preparation

The following procedure describes the how to prepare a run on the Ion Chef, which will automate the Template Preparation and Sequencing Preparation process.

Template Preparation is the process in which library fragments present in the equimolar library pool from the previous step are individually amplified across the surface of Ion Sphere™ Particles (ISPs) through a process called Isothermal Amplification (IA). At the conclusion of IA, each ISP should contain a unique library fragment clonally amplified across its surface.

The instrument will then perform Enrichment where non-templated ISP's are removed from the reaction.

Finally, the instrument will add a sequencing primer and polymerase to the enriched ISP's. The procedure will conclude with loading the reaction onto the Ion 530 Chip.

Materials and Kits required

- Ion S5 ExT Chef Solutions Kit*
- Ion S5 ExT Chef Reagents Kit*
- Ion S5 ExT Chef Supplies Kit*
- Ion 530 Chip Kit

**Included with the ION 520™ & 530 Ext Kit - Chef (Cat. No. IONCHEF-EXT)*

Plan a Run on the Torrent Browser

1. Log into the Torrent Browser for the Torrent Server that is connected to the Ion S5 System or Ion GeneStudio S5 System and Ion Chef Instrument.
2. Select the **Plan** tab located at the top left corner of the screen, and then select the **Plan Template Run** button on the subsequent screen.
3. From the left-hand panel, select **Generic Sequencing**.
4. Towards the right-top corner of the screen select **Plan New Run**.
5. Proceed with the default options in the **Ion Report** and **Application** tabs by hitting the **Next** button.
6. In the **Kits** tab click the "Details +" button to bring up additional options. **Ensure that the following parameters are selected:**

Note: All of the following selections will need to be made in TSS 5.2.1 or higher. If using a previous version of the software some fields may not be present.

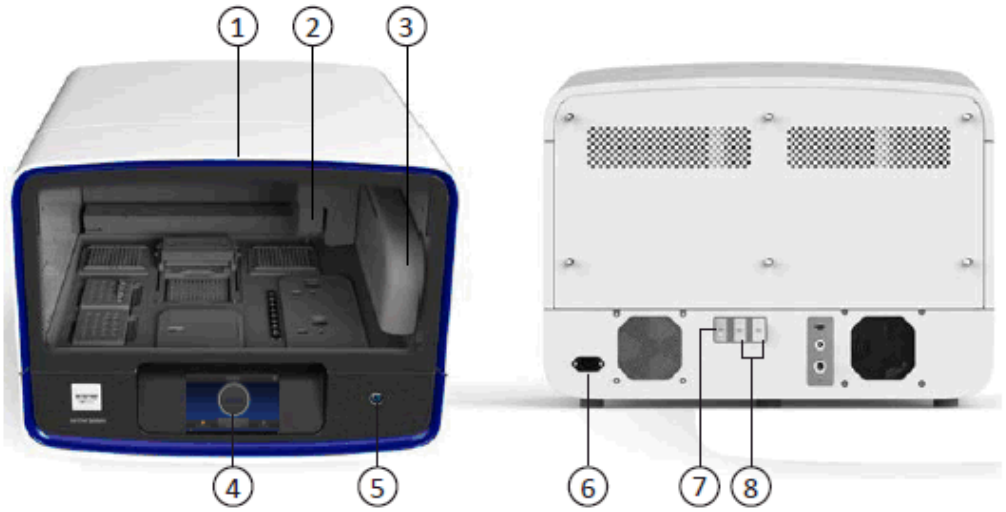
Parameter	Value
Instrument Type:	Ion S5 System or Ion GeneStudio S5 System
Chip Type:	Ion 530 Chip
Library Kit Type:	Ion Xpress Plus Fragment Library Kit
Forward Library Key:	Ion TCAG
Test Fragment Key:	ATCG
Barcode Set:	IonXpress
Forward 3' Adapter:	Ion P1B
FlowOrder	Ion samba contradanza flow
Template Kit:	Ion 520 and 530 ExT Kit – Chef
Sequencing Kit:	Ion S5 ExT Sequencing Kit
Base Calibration Mode:	No Calibration
Flows:	1350

7. In the **Projects** tab, users have the option to select a Project that the completed run will be grouped into. This step is optional. Click **Next.**
8. In the **Plan** tab, assign a run name. Additionally, use this screen to input the samples involved in the run. Enter the number of samples to be run in the **Number of Barcodes** box. Click the check box next to it. Insert the **Sample Name** that corresponds to each barcode. When complete, click **Plan Run.**
9. This run will now later appear as an option when starting the Ion Chef and the Ion S5 System or Ion GeneStudio S5 System.

Note: Users may alternatively choose to plan their run by creating Sample List imports or Run Templates. Reference the Torrent Suite™ Software User Guide, available from the Ion Community, or contact One Lambda Technical Support for detail on these alternate processes.

Ion Chef System Components

Figure 2



① **Door** – Provides access to the interior of the instrument. The door is locked in the closed position during operation.

② **Micropipettor** – A mechanical positive-displacement pipettor that performs all fluid transfers during sample and chip preparation.

③ **Robotic arm** – Performs fluid transfer during chip loading. The arm also contains an optical sensor that reads the barcodes of instrument reagents and consumables.

④ **Touchscreen** – Provides access to all instrument functions for operation, maintenance, and troubleshooting.

⑤ **Power button** – Power switch for the Ion Chef™ Instrument, where the states are on (illuminated) and off.

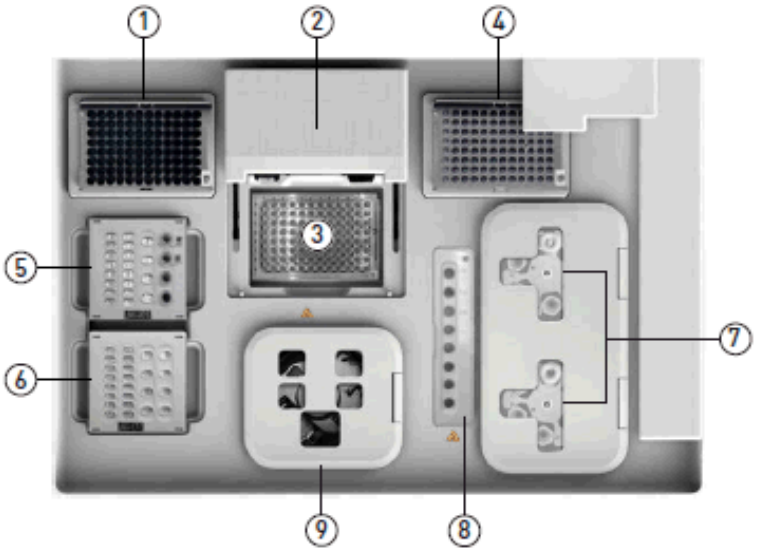
⑥ **Power port** – A 100–240 VAC port that provides power to the Ion Chef™ Instrument.

⑦ **Ethernet port** – An RJ45 port that provides Ethernet (100Mbit) communication with the Ion Chef™ Instrument.

⑧ **USB port** – Provides USB communication with the Ion Chef™ Instrument. Used to update the instrument firmware and to transfer data during service or maintenance.

Ion Chef System Interior Hardware and Consumables

Figure 3



① **New pipette tips** – The position of the rack containing unused pipette tips.

② **Automated heated cover** – Transfers the plate cover to the PCR reaction plate within the sample block. During thermal cycling, the heated cover applies compression to seal the reaction plate and heats the cover to prevent condensation.

③ **Thermal cycler sample block** – Performs thermal cycling of the sequencing reactions on a 96-well PCR reaction plate.

④ **Waste pipette tip rack** – The position of the rack containing waste (used) pipette tips.

⑤ **Reagents station** – The position on the instrument deck of the diluted libraries, NaOH, and the Ion 520™ & 530™ Chef Reagents Cartridge.

⑥ **Solutions station** – The position on the instrument deck of the Ion S5™ Chef Solutions Cartridge, which is maintained at room temperature.

⑦ **Recovery Centrifuges** – Twin stations that perform centrifugation of the ISPs during the recovery phase of template preparation.

⑧ **Enrichment station** – The position of the rack containing consumables for the enrichment of the template-positive ISPs.

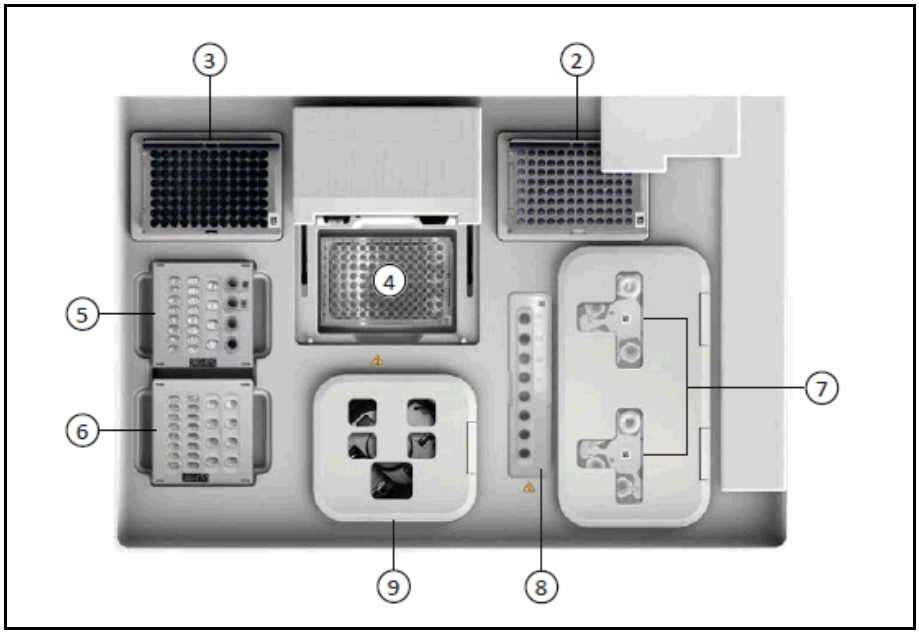
⑨ **Chip-loading Centrifuge** – Performs centrifugation of sequencing chips that have been mounted to chip-loading adapters and loaded with template-positive ISPs.

Ion Chef System Procedural Guidelines

- The Isothermal Amplification (IA) reaction is sensitive to contamination from spurious DNA fragments from previous sample processing steps. Do not introduce amplified DNA into the library preparation work area.
- All Ion Chef Instrument components are single-use only.
- Store all Ion Chef System consumables and cartridges under the recommended conditions and in an upright position.
- Hold sequencing chips by gently gripping them by their edges.
- When the Ion Chef System is not in use, remove all consumables and reagents from the deck and close the instrument door.
- Before use confirm that the Ion Chef has been cleaned following the last use.
- Ensure that the Ion S5 ExT Chef Reagents are fully thawed by keeping them at room temperature for at least an hour. If reagents are fully thawed and will not be used immediately, place the cartridge in 4°C storage until ready for use.
- Confirm that all components are clean and dry before loading them onto the Ion Chef Instrument.
- Confirm that the Reagents and Solutions station compartments are dry and free of condensate before loading components.
- Except for the Pipette Tip Cartridge V2, do not reuse any of the Ion Chef System consumables or reagents.
- Use only Ion Torrent kits and supplies with the Ion Chef Instrument. The use of third-party reagents and supplies can adversely affect the performance of the Ion Chef Instrument and chips prepared.
- The Ion Chef chip-loading centrifuge is rated to operate at the listed rotational frequencies with the chip buckets, chips, and adapters. The centrifuge must be load balanced.
- Remove and sequence chip within 1 hour after the Ion Chef System finishes loading them. If you cannot sequence a loaded chip immediately, store it within a chip storage container at 4°C until you are ready to run it (8 hours maximum).
- If you choose to store a loaded chip, remove the chip from 4°C storage for at least 20 minutes prior to running it. Keep the chip in the storage container while allowing it to reach room temperature.

Ion Chef Instrument Setup and Run

Figure 4



1. From the home screen click the eject button in the top right corner. This will unlock the instrument's door.
2. Slide the door up the tracks until the latch mechanism engages.
   For the following instructions refer to Figure 4 above.
3. Place a new tip rack into Position **3**. An empty tip rack from a previous run should be placed in position **2**. To insert tip racks pull the catch forward, and the locking bracket upwards. While the locking bracket is pulled up slid a new rack into position, pull down the locking bracket, and push the catch backwards.
4. Place the semi-skirted 96 well plate onto the thermal cycler sample block, position **4**. Slide a plate seal underneath the cover that is homed behind the 96 well plate. The notches should be facing out and up.
5. Uncap the 4 tubes on the Reagents Cartridge. Add 50µL of 100pM prepared library into the Ion S5 ExT Chef Library Sample Tube (barcoded tube on Reagents Cartridge). Place the Ion S5 ExT Chef Reagents Cartridge into Position **5**.
6. Place the Ion S5 ExT Chef Solutions Cartridge into Position **6**.
7. Place a new Ion 530 chip into a chip adaptor, place the adaptor/chip into a chip bucket, and place the bucket with the adapter/chip into Location A of the Chip Centrifuge, Position **9**. Location A is located 90° clockwise from the Location A marker hole, refer to *Figure 5* on the following page.
8. Repeat Step 7 using an Ion Chef S5 Series Chip Balance with the other adaptor and other chip bucket. Place the chip bucket with the chip balance into Location B. Location B is located 90° clockwise from the Location B marker holes, refer to *Figure 5* on the following page.
   Note: The chip can only fit into the bucket in one orientation. The notch of the chip and the bucket should be aligned. Also, the bucket can only fit into the instrument in one orientation. The adaptor should be slid into the notch and the tab pressed back to fit in the other notch, to firmly seat the adaptor on the chip bucket. Refer to *Figure 6* on the

following page.

Figure 5

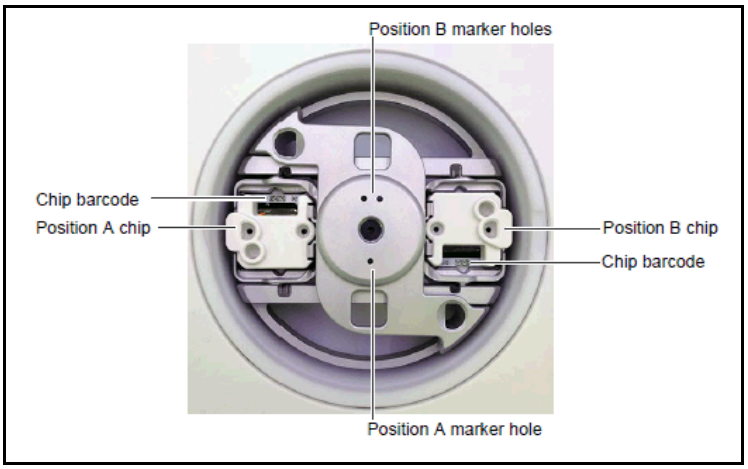
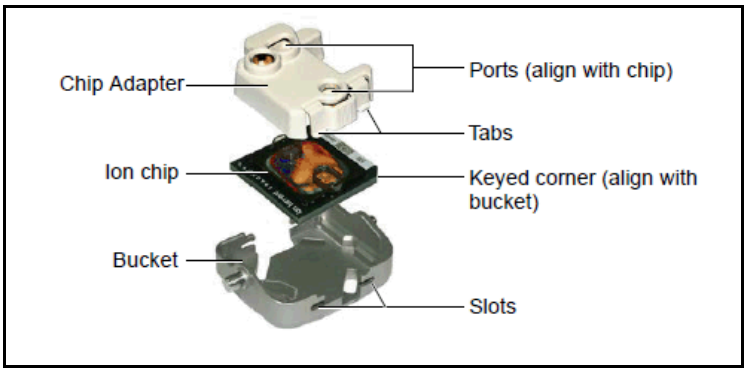


Figure 6



9. Close the lid to the chip centrifuge.
10. Place the Enrichment Cartridge into Position **8**.
11. Load six Recovery Tubes into each Recovery Centrifuge, Position **7**. Then place Recovery Station Disposable Lids over each centrifuge. The lids will only fit on the centrifuges in one position. The barcode should be facing up.
12. Closed the hinged lid of the Recovery Centrifuges. Confirm that the port of each lid is oriented towards the back of the instrument.
13. Before continuing, verify that the new tip rack is locked in place (bracket is down and catch is back).

Start Chef ExT Run

1. From the home screen select “Set Up Run”.
2. Select “Quick Start”. Alternatively, “Step by Step” can be selected. This will take the user through the same set of instructions as shown above, accompanied by on-screen prompts.
3. Press “Next” to start the tip verification process.
4. When prompted close the door of the instrument.
5. Press “Start Check”. The instrument will scan the deck to ensure proper setup.
6. When prompted choose the run plan from the Data Destination screen.
7. The Run Options screen allows the user to select when the run will complete through chip loading (~6.5 hours). The time initially shown to the right of the “Timer” is if the instrument runs without a pause. To change when the run will end, click the “Timer” and enter a new time. When finished click “Start Run”.
8. When the run is complete remove the chip from the adaptor. Draw off any excess liquid with a 200µL pipette. Proceed to sequencing on the Ion S5 System or Ion GeneStudio S5 System.

NOTE: For additional support with the Ion Chef Instrument, refer to the manufacturer’s user documentation.

Sequencer cleaning and maintenance

The following procedure describes the process for manually cleaning the Ion S5 System or Ion GeneStudio S5 System. This cleaning is required prior to instrument initialization. Cleaning is normally done automatically after the completion of a run. If a run is canceled for whatever reason (power failure, user-aborted, etc.) then the instrument will not allow a subsequent initialization to proceed until a cleaning has been completed.

Required materials and equipment

- Ion S5 ExT Cleaning Solution Bottle
- Used Ion S5 ExT Wash Solution Bottle
- Used Ion S5 Sequencing Reagents Cartridge
- Used Ion Chip™

Manual Sequencer Cleaning

1. From the main menu of the instrument select “Clean”. The instrument door should unlock, allowing access to the consumables.
2. Remove the Ion S5 ExT Wash Solution Bottle and waste reservoir, and empty the waste reservoir.
3. Re-install the empty waste reservoir and the used Ion S5 ExT Wash Solution Bottle.
4. Install a used Ion S5 ExT Sequencing Reagents Cartridge and Ion S5 ExT Cleaning Solution Bottle.
5. Place a used chip in the chip clamp and push the chip clamp all the way in to engage.
6. Close the instrument door and press “Next” The cleaning takes approximately 35 minutes to complete. Upon completion, the instrument door unlocks and the chip and cartridge clamps disengage.

Disposing of Reagent Consumables

Following the conclusion of a sequencing run:

1. Carefully invert the Ion S5 ExT Sequencing Reagents Cartridge over an appropriate receptacle to drain all residual liquid.
2. Wearing gloves, insert the S5 ExT Cartridge Tool firmly into the CO_2 scrubber until the flange stops on top of the cartridge.
3. Pull straight up on the tool while holding the nucleotide reagent cartridge down.
4. Remove the scrubber from the cartridge tool, then dispose of the scrubber.
5. Open the expended S5 ExT Wash Solution Bottle by unscrewing the cap.
6. Remove the cap, sipper, and filter, and then pour any residual liquid into an appropriate receptacle.
7. Rinse out the used bottle with water.
8. Recycle or dispose of the bottle, cap, sipper, and filter.

Maintain the Sequencer

In the event spill or leak on or inside the instrument, perform the following steps.

1. Remove the Ion S5 ExT Wash Solution Bottle and Waste Reservoir, and empty the Waste Reservoir.
2. Remove the Ion S5 ExT Sequencing Reagent Cartridge.
3. Inspect the waste and nucleotide reagent bays for liquid.
4. Using absorbent paper soak up as much liquid as possible. Wash the affected area with 10% bleach solution.
5. Wipe the affected surfaces with 70% Isopropanol. Allow to air-dry.

NOTE: For additional support with the Ion S5 System or Ion GeneStudio S5 System, refer to the manufacturer’s user documentation.

Sequencer and Run Initialization

The following procedure describes the process to prepare the Ion S5 System or Ion GeneStudio S5 System for a sequencing run after the instrument has been through the cleaning process. Instrument initialization should be carried out the same day that the chip is to be loaded onto the instrument, and at least one hour prior to the conclusion of the Ion Chef run.

Materials required from the Ion S5 ExT Sequencing Reagents Kit

- Ion S5 ExT Sequencing Reagents cartridge

Materials required from the Ion S5 ExT Sequencing Solutions Kit

- Ions S5 ExT Wash Solution
- Ion S5 ExT Cleaning Solution

Other materials and equipment

- Used Ion Chip

Sequencer Initialization

Note: *It is recommended that sequencing be started as soon as possible after chip loading and instrument initialization are complete.*

Note: *The Ion S5 ExT Sequencing Reagents Cartridge and Ion S5 ExT Sequencing Solutions are different than the Ion S5 Reagents Cartridge and Ion S5 Sequencing Solutions used for Manual Template Preparation, respectively.*

1. Allow an Ion S5 ExT Sequencing Reagents Cartridge to equilibrate to room temperature prior to initialization. It is recommended to thaw the cartridge at least 1 hour prior to initialization to allow the nucleotides to fully thaw. If reagents are fully thawed and will not be used immediately, place the cartridge in 4°C storage until ready for use.
2. From the main menu of the instrument select “Initialize”. The door, chip, and reagent cartridge clamps will all unlock.
3. When prompted remove the Ion S5 ExT Sequencing Solution Bottle and Waste Reservoir, and empty the Waste Reservoir.
4. Re-install the empty Waste Reservoir.
5. Replace the expended Ion S5 ExT Sequencing Reagents Cartridge with a new cartridge.
6. Invert the Ion S5 ExT Sequencing Solution Bottle 5 times, remove the red cap, and then install.
7. Ensure that a used sequencing chip from a previous run is properly seated in the chip clamp, and the chip clamp is pushed in all the way.
8. If necessary install a new Ion S5 ExT Cleaning Solution Bottle. The Ion S5 ExT Cleaning Solution Bottle can be used up to 4 times.
9. Close the door and select “Next”. The instrument will confirm that the consumables and chip are properly installed and that the Ion S5 ExT Cleaning Solution contains enough reagents to perform the post-run cleaning.
10. Follow all on-screen recommendations to ensure proper installation of required consumables.
11. When initialization is complete (approximately 40 minutes), select “Next” to return to the main menu. The instrument is now ready to initiate a sequencing run.

Start the Sequencing Run

1. From the main menu of the instrument select “Run”. The door and chip clamp will unlock.
2. Remove the used sequencing chip. Then secure the chip loaded with templated ISPs in the chip clamps, with the chip notch facing downwards.
3. Push the chip clamp all the way in to engage. Then close the instrument door and press “Next”.
4. From the drop-down list, select the Planned Run that was created in the Torrent Suite Software, then press “Next”.
5. Confirm the pre-populated settings are correct, or make changes using the buttons and drop-down lists if necessary.
6. Confirm that the instrument door is closed, then press “Next”.
7. Press “Calibrate” to begin the sequencing run.

Explanation of Symbols

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

Revision History

Revision	Date	Description of Change
02	07/02/2019	• 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).
03	09/20/2019	• Updated contact information and address to reflect change in legal manufacture site.
04	Current	• Addition of AllType™ NGS Index Kit (Cat. No. ALL-IONX) as an equivalent substitution for the Ion Xpress Barcode Kits (Cat. No. ION-XBA1, ION-XBA2, ION-XBA3, ION-XBA4, ION-XBA5 and ION-XBA6).

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