
ALLTYPE™ FASTPLEX™ NGS ASSAY
on
ILLUMINA SEQUENCING SYSTEMS

Application Note

ALLTYPE™ FASTPLEX™ NGS 11 LOCI FLEX KIT – 96

Product: AllType™ FASTplex™ NGS 11 Loci Flex Kit – 96



Catalog ID: ALL-FAST11LFX

For Research Use Only. Not for use in diagnostic procedures.



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INTRODUCTION

This Application Note describes how to prepare compatible libraries from amplicons generated using the AllType FASTplex NGS 11 Loci Flex Kit for the Illumina® Sequencing Systems.

The Application Note contains the steps required to generate the following PCR amplicons:

Locus	Targeted Region
• HLA-A	Full gene
• HLA-B	Full gene
• HLA-C	Full gene
• HLA-DRB1	Exon 1 & Exon 2 thru 3' UTR *
• HLA-DRB3/4/5	Exon 2 thru 3' UTR *
• HLA-DQB1	Exon 1 & Exon 2 thru 3' UTR *
• HLA-DPB1	Exon 1 & Exon 2 thru 3' UTR *
• HLA-DQA1	Full gene
• HLA-DPA1	Full gene

* Includes intronic sequence

After amplification, the remainder of the Application Note covers the steps for sample barcoding, pool barcoding, and library generation.

To prevent injury and for optimal use of the AllType FASTplex NGS 11 Loci Flex Kit, read through the entire Application Note.

PRODUCT DESCRIPTION

Official Product Name

AllType™ FASTplex™ NGS 11 Loci Flex Kit – 96

Catalog Information

REF	Catalog ID
	• ALL-FAST11LFX

Product Overview

AllType FASTplex NGS HLA 11 Loci Flex Kit include reagents to enrich the 11-loci HLA genes for HLA-A, B, C, DRB1, DRB345, DQB1, DPB1, DPA1, and DQA1 by a long-range multiplex polymerase chain reaction (PCR) method, purify the amplification products, and construct libraries containing up to 96 samples for sequencing on the Illumina systems.

AllType FASTplex NGS HLA 11 Loci Flex products consist of pre-optimized HLA gene amplification primer solution in aqueous liquid format, PCR enzyme, 5X reaction buffer, and deoxyribonucleotides (dNTP) provided at recommended storage temperature at -20°C. The amplification primer design is based on the known DNA sequences reported in public DNA sequence databases such as Genbank (<http://www.ncbi.nlm.nih.gov/genbank/>) and IMGT (<http://www.ebi.ac.uk/ipd/imgt/hla/>).

AllType FASTplex NGS HLA 11 Loci Flex products provide a 96-well plate containing unique Sample Barcoding reagents in each well and a single Universal Barcoding reagent (see Product Identification).

When combined, a user can prepare a library for 8 to 96 samples with unique i7 barcodes and a common i5 sequence for subsequent library amplification. The library amplification primer mix included in the kit identifies the P7 and P5 common sequences at the ends of DNA fragments and amplifies all properly barcoded library fragments. Additional reagents needed throughout library preparation, including barcoding buffer, stop solution, and paramagnetic beads are also provided in the kit.

Throughput for AllType FASTplex NGS HLA 11 Loci Flex Products with Illumina Sequencers

Illumina Sequencer	Illumina Reagent Kit	Number of Samples Maximum Throughput
MiSeq	MiSeq Reagent Kit v2 (300 cycle)	96
	MiSeq Reagent Micro Kit v2 (300 cycle)	24
	MiSeq Reagent Nano Kit v2 (300 cycle)	8
MiniSeq	MiniSeq High Output Kit (300 cycles)	96
	MiniSeq Mid Output Kit (300 cycles)	72
iSeq	iSeq 100 i1 Reagent v2 (300 cycles)	24

Principles of the Method

The AllType FASTplex NGS product generates target-specific amplification products for multiple HLA genes in a single multiplex polymerase chain reaction (PCR). DNA sequencing data are obtained by processing the amplified product with a fragmentation library workflow to prepare a library that can be sequenced using Illumina sequencer platforms. The AllType FASTplex NGS 11 Loci Flex Kit has been designed and optimized to produce nearly equimolar quantity of each target to provide adequate sequence coverage. Resulting data are analyzed using TypeStream™ Visual NGS Analysis Software to generate high resolution HLA allele assignments.

WARNINGS



FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.

WARNING: 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.

MATERIALS PROVIDED

Package Contents and Storage



The AllType FASTplex NGS 11 Loci Flex Kit is comprised of two separate boxes. The AllType FASTplex NGS 11 Loci Flex Kit – 96 (Box 1 of 2) can be safely stored at -20°C and the AllType FASTplex NGS Kit (Box 2 of 2) can be safely stored at +2 to 8°C. The recommended storage condition for the FAST-STOP and FAST-BBUF is +20 to 25°C. The -20°C product may be freeze/thawed up to 12 times.



CAUTION: Upon receipt, keep each box intact and stored until ready to use. Avoid unnecessary handling.

A full list of the provided materials and their appropriate storage conditions can be found in the [Components Provided in the AllType FASTplex NGS 11 Loci Flex Kit](#) section. The product label also contains individual component storage conditions.

Components Provided in the AllType FASTplex NGS 11 Loci Flex Kit

Box 1 of 2

Component	Catalog ID	Quantity	Amount (µL)	Storage
AllType FASTplex 11 Loci Primer Flex Mix	ALF-P11F	1 vial	600	-20°C
AllType FASTplex 11 Loci Exon 1 Primer Flex Mix	ALF-P11FE1	1 vial	240	
AllType dNTPs	ALL-NTP	1 vial	200	
AllType Buffer	ALL-BUF	1 vial	500	
AllType LR Polymerase	ALL-LRPOL	1 vial	100	
FASTplex Sample Flex Plate 96	FAST-SFP96	1 vial	6 per well	
FASTplex Univ Barcode Flex A	FAST-UBFA	1 vial	48	
FASTplex Library Primer Flex Mix	FAST-LPFM	1 vial	100	
FASTplex Library Amp Mix	FAST-LAM	1 vial	900	

Box 2 of 2

Component	Catalog ID	Quantity	Amount (µL)	Storage
FASTplex Barcoding Buffer	FAST-BBUF	1 vial	1500	+2 to 25°C
FASTplex Stop Solution	FAST-STOP	2 vials	1200 per vial	
FASTplex Paramagnetic Beads	FAST-BEAD	1 vial	7,700	+2 to 8°C
FASTplex DNA Suspension Buffer	FAST-SUSP	1 vial	8,000	

Instability Indications



WARNING: Do not use if the boxes are damaged, product is not received at required storage temperature, or the product has leaked. Contact One Lambda Technical Support at 1lambda-TechSupport@thermofisher.com.

MATERIALS – REQUIRED but not PROVIDED

Supplies and Consumables

Description	Supplier	Catalog No.
96-Well, 0.2 mL, PCR Plates	MLS*	---
96 Well Plastic/Foil Plate Seals	MLS	---
Thermo Scientific Nunc™ 96-Well Polypropylene MicroWell™ Plates	Fisher Scientific	12-565-369
PCR Plate & 0.2 mL Tube Cooler	MLS	---
Eppendorf DNA LoBind Tubes, 5.0 mL	Eppendorf	0030108310
Eppendorf DNA LoBind Tubes, 2.0 mL	Eppendorf	022431048
PCR tube 0.2 mL	MLS	---
50mL Conical Tubes Nuclease free	MLS	---
15mL Conical Tubes Nuclease free	MLS	---
Serological Pipettes, 10 or 25 mL	MLS	---
Pipette Controller	MLS	---
Full array of manual single channel pipettes (20 µL, 200 µL, 1 mL)	MLS	---
20 µL and 200 µL manual multi-channel pipettes	MLS	---
Full array of filtered, pre-sterilized, pipette tips	MLS	---
Sterile Reagent Reservoirs	MLS	---
Strip Tubes / Caps	MLS	---
Thermal Cycler PCR Pressure Pad	MLS	---
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
iSeq 100 i1 Reagent v2 (300 cycles)	Illumina	20031371
MiniSeq High Output kit (300 cycles)	Illumina	FC-420-1003
MiniSeq Mid Output kit (300 cycles)	Illumina	FC-420-1004

*MLS: Major Laboratory Supplier

EQUIPMENT – REQUIRED but not PROVIDED

Equipment

Description	Supplier	Catalog No.
TypeStream™ Visual NGS Analysis Software (Version 2.0.1 or higher)	One Lambda, Inc.	TSVPGR
iSeq® 100 System	Illumina, Inc	FS10000717
MiniSeq® System	Illumina, Inc	MN01037
MiSeq® System	Illumina, Inc	M01817
Magnetic-Ring Stand (96 well)	Thermo Fisher Scientific	AM10027 or AM10050
2.0 mL Tube Magnet Stand	Thermo Fisher Scientific	12321D
Veriti 96-Well Thermal Cycler*	Thermo Fisher Scientific	4375786
Qubit® Fluorometer or equivalent	Thermo Fisher Scientific	Q33216 or Q33226
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32854
Qubit Assay Tubes	Thermo Fisher Scientific	Q32856
Plate Centrifuge capable of 1,500 x g	MLS**	---
Mini/Micro Centrifuge	MLS	---
Vortex	MLS	---

**The Veriti 96-Well Thermal Cycler may be substituted with a thermal cycler capable of temperature settings optimized for the PCR reaction – 0.8 °C per second heating rate and 1.6°C per second cooling rate – and reaction volumes of 100 µL.*

***MLS: Major Laboratory Supplier*

PRECAUTIONS



SAFETY PRECAUTION: Wear gloves and appropriate PPE.

CAUTION: Follow clean bench work techniques to reduce the risk of contamination.

Clean the workbench thoroughly 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.

IMPORTANT GUIDELINES

General Laboratory Practice

- Use of manual multi-channel pipettes is recommended. Single channel pipettes are only recommended for workflows containing a small quantity of samples.
- All instruments and pipettes should be calibrated and maintained per the manufacturer's guidelines.


Technical and Equipment

- Use of dedicated equipment in both the pre- and post-PCR environment is advised.
- For convenience, set up all the thermal cycler programs before starting.
- Use the Veriti 96-Well Thermal Cycler or a model capable of 9600 emulation speed or +0.8°C/sec heating and -1.6°C/sec cooling and a heated cover at 105°C or equivalent for all programs.
 - If a thermal cycler other than the Veriti-96 will be used, validate the thermal cycler.
- If a quantification method other than a Qubit™ Fluorometer will be used, validate the quantification method.

Reagents

- The items in Box 1 of 2 of the kit should be kept on ice during use and returned to -20°C immediately after use.
- Store pre-amplification (Box 1 of the AllType FASTplex NGS 11 Loci Flex Kit) and post-amplification reagents separately.
- Master mix preparation and dispensation should be done on ice and at a reasonably fast pace to avoid unintended results.
- Use FASTplex DNA Suspension Buffer for all gDNA and amplicon dilution and library elution.
- Do not use EDTA-containing solutions (e.g., TE buffer or Low TE buffer) post amplification, as it may inhibit the reactions in this assay. See below, genomic DNA may be diluted in Low (0.1mM or below) EDTA DNA Suspension Buffer.

Safe Stopping Point

- For optimal results, perform all the library preparation steps in one day.
 - But if more time is needed, look for the  **SAFE STOPPING POINT** to store an intermediate material at -20°C.
- Due to an unknown amount of left-over enzyme activities, do not to keep the materials for more than 72 hours at -20°C; longer storage time would require validation.

SAMPLE COLLECTION and SAMPLE PREPARATION

Sample Type

Specimens, including anticoagulated peripheral blood, cultured lymphocytes, and buccal epithelial cells, have been tested and verified for use using the AllType FASTplex NGS 11 Loci Flex Kit.

DNA can be isolated from samples as long as 2 weeks after the initial blood draw or buccal epithelial cell collection, although it is recommended that samples be processed within 2 to 3 days of collection (4, 5). DNA may be kept the storage condition and length as validated by the individual laboratory.

Sample Storage

Whole blood must be collected in ACD or EDTA anticoagulants and stored at 4°C. Samples containing heparin are not suitable for use.

Buccal swab must be collected using swabs that are validated for epithelial cell collection and should be stored at ambient temperature in a sealed container to avoid excessive drying.

DNA Extraction Methods

The following extraction methods have been tested with the AllType FASTplex NGS 11 Loci Flex Kit:

- Maxwell 16 Blood DNA Purification Kit (Promega Corporation, Cat No. AS1010)
- QIAamp DNA Blood Mini Kit (QIAGEN, Cat No./ID: 51104)
- Automated MagMAX™ DNA Multi-Sample Ultra Kit (Thermo Fisher Scientific, Cat. No. A25597) using KingFisher Flex Purification System (Thermo Fisher Scientific, Cat. No. 5400630, 5400610).

Other DNA extraction methods can be used, but the methods and equipment must be validated by the end-user.

Make sure the samples are of acceptable integrity, purity and free from any inhibitors of DNA polymerase.

DNA integrity may be determined by 0.8% agarose gel electrophoresis to verify that the DNA for each sample is of high molecular weight and double stranded. A single band above the location of the 20 KB band Ladder indicates good integrity. The DNA should also have a single band of high molecular weight. If a high molecular weight band is not present and a significant amount of DNA is smeared below the 5 KB band (from DNA ladder, size marker), the sample is degraded and will not generate good results when used for creating NGS libraries.

Following DNA extraction, samples should be measured for purity using the A260/280 ratio for which a ratio of ~1.8 is generally accepted as “pure” DNA. Abnormal 260/280 ratios usually indicate that a sample is contaminated by residual phenol, guanidine, or other reagent used in the extraction protocol, in which case the ratio is normally low. Inaccurate ratios may also be encountered at very low concentrations (< 10 ng/μl) of nucleic acids. High 260/280 purity ratios are not indicative of an issue. An A260/A230 ratio higher than 2.2 may indicate the presence of unwanted organic compounds such as Trizol, phenol, Guanidine HCL and guanidine thiocyanate that may result in PCR inhibition.

It is not required but recommended as a best practice to investigate the quality of the amplification products by gel electrophoresis. Such methods are useful in determining the presence of PCR products based on molecular weight and may provide valuable information regarding the integrity of a DNA amplicon, including the potential for a fragmented template. The use of an automated gel electrophoresis is preferred over traditional methods to assess for amplicon quality (e.g. Agilent BioAnalyzer or

TapeStation). Below is an example of a poor amplification product (left) and high-quality amplification product (right). The product on the left would not provide sequencing results.

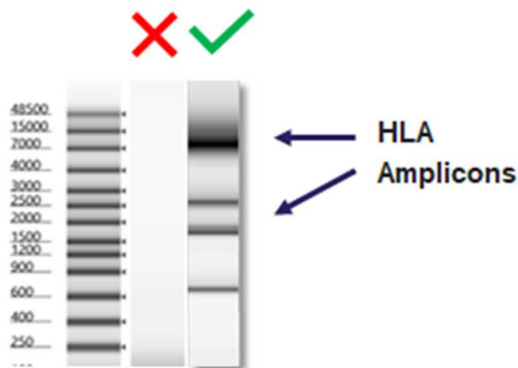


Figure 1. Gel images of a poor amplification product, which is the gel image on the left under the **X**, and a high-quality amplification product, which is the gel image on the right under the **✓**.

Do not resuspend the samples in solutions containing chelating agents, such as EDTA, above 0.1 mM in concentration.

Resuspend the DNA samples to be used for PCR in Low (below 0.1mM) EDTA DNA Suspension buffer, 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 can be used but must be validated by the laboratory.

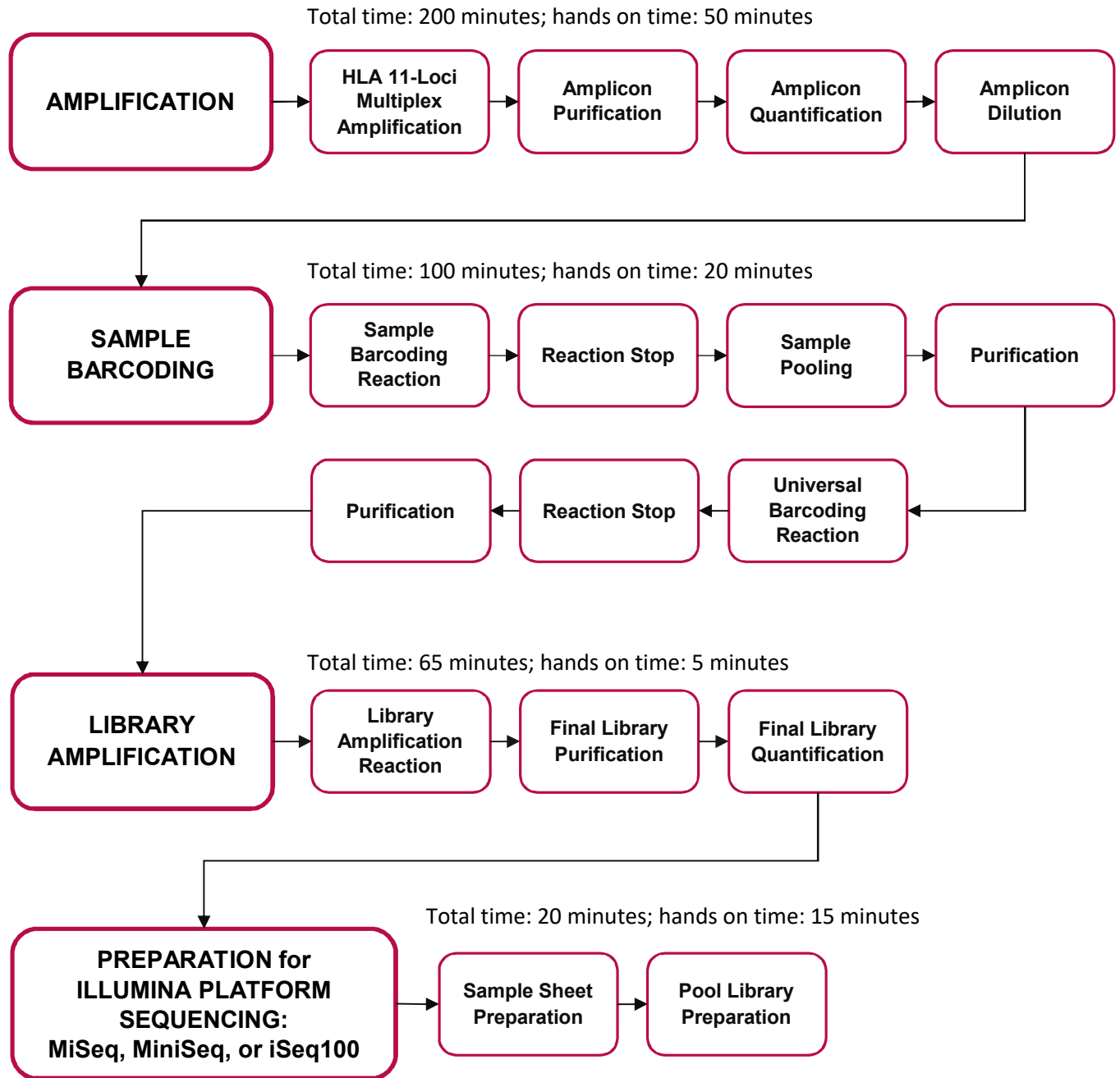
IMPORTANT: Only in the dilution of the genomic DNA can the above reagents be used. In all subsequent steps, when DNA Suspension Buffer is listed, only the FASTplex DNA Suspension Buffer can be used.

DNA Storage

- Use DNA samples immediately after isolation or store DNA at -20°C or below.
- Ship DNA samples at 4°C or below to preserve their integrity during transport.

Extracted DNA has been tested that has been stored up to 14 days using the AllType FASTplex NGS 11 Loci Flex Kit. Additional DNA storage may be validated by the individual laboratory.

WORKFLOW for the ASSAYS



GENERAL PREPARATION for the ASSAYS

1. **Separate** pre- and post-PCR areas.
2. **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 0.2 mL PCR Plates
 - Eppendorf LoBind® tubes
 - 0.2ml PCR tubes
 - Magnetic racks for a 96-well plate and a 2.0 mL tube
 - Nuclease-free water
 - Ice
 - PCR plate cooler
 - Tube-labeling supplies
 - FASTplex DNA Suspension Buffer
 - 200-proof Ethanol
 - Sterile reagent reservoirs
3. **Resuspend** DNA samples in FASTplex DNA Suspension Buffer, Low (below 0.1mM) EDTA DNA Suspension buffer, sterile water, or in 10 mM Tris-HCl, pH 8.0 to 9.0 at an optimal concentration of 25 ng/μL using a Qubit dsDNA HS Assay Kit and Qubit Fluorometer for measurement.

An acceptable range of genomic DNA concentrations is 3.00 ng/μL to 50 ng/μL.

Optical density (OD) methods should not be used to measure DNA concentration; the readings could be overestimated. If optical density (OD) method is used to measure DNA quality, the expected A260/A280 ratio is 1.80.

4. **Record** each sample location, the volume of the FASTplex Sample Flex Plate 96 (Refer to [Appendix 2](#) for a diagram of the plate.), and the FASTplex Univ Barcode Flex A (UB) reagent to be used for library prep.
5. **Before starting the procedure, label** the PCR 8-tube strip(s) or 96-well plate clearly.
6. **Keep track** of which wells from the FASTplex Sample Flex Plate 96 and how much UB reagent has been consumed in the library preparation. Users may refer to the plate grid at the end of this document.
7. **Do not discard** reagents in the kit until empty since they are intended for multiple uses.
8. **Avoid** contamination of all kit reagents/components.
9. **Return** the unused portions to storage at the temperature indicated on the label.
10. **Take** precautions to avoid well-to-well cross-contaminations.

The FASTplex Sample Flex Plate supplied in the kit contains enough volume to prepare a 96-sample library once or up to twelve 8-sample libraries. The FASTplex kit is also compatible with any library size between 8 and 96 samples. The plate is designed to allow for columns of barcodes (wells) to be

separated; the remaining contents of the FASTplex Sample Plate should be stored at -20°C until the next use. Care must be taken to avoid well-to-well cross-contaminations.

11. **Program** the thermal cyclers(s).
12. **Set up** all the thermal cyclers programs before starting.
13. **Use** the following programs:

- **HLA 11-Loci Amplification Illumina Program:**

Temperature	Time	Cycle
94°C	2 min	1
98°C	10 sec	30
69°C	3 min	
4°C	HOLD	1

9600 emulation speed and heated cover on

- **TAG Program:** 55°C for 15 min., 25°C hold, heated cover on
- **STOP Program:** 68°C for 10 min., 25°C hold, heated cover on
- **FASTplex Illumina Library Amplification Program:**

Temperature	Time	Cycle
72°C	10 min	1
98°C	3 min	1
98°C	15 sec	12
64°C	30 sec	
72°C	1 min	
72°C	5 min	1
4°C	HOLD	1

9600 emulation speed and heated cover on

IMPORTANT: At the completion of each PCR step, select the next thermal cycler program to be used, so the thermal lid is pre-warmed. The thermal cycler lid must be at the appropriate target temperature before placing the reaction. Avoid placing reactions onto a cold thermal cycler.

14. **Pulse-spin** the kit components in a suitable centrifuge to gather the reagents at the bottom of the well or tube before every use of the FASTplex Sample Plate and before dispensing from the reagent tubes, since liquids can condense or shift locations inside containers during shipment or storage.
15. **If the Room Temperature kit components freeze during shipment or storage, perform** the following steps before use:
 - a) Thaw the kit components.
 - b) Mix the kit components.
 - c) Pulse-spin the kit components.
16. **Equilibrate** FASTplex Paramagnetic Beads to room temperature. FASTplex Paramagnetic Beads can be stored for up to 2 weeks at room temperature or for longer periods at 2 - 8°C.

- If the FASTplex Paramagnetic Beads are stored cold, perform the following steps before use:
 - a) Set the beads at room temperature for 30 minutes.
 - b) Vortex the beads thoroughly to resuspend.

IMPORTANT: To transfer volumes accurately, do not pre-wet pipette tips and pipette slowly.

17. Check the Stop Solution for precipitate before use.

- If a precipitate is visible, perform the following steps:
 - a) Incubate the tube at 37°C for 5 minutes.
 - b) Mix gently by inversion until the precipitate dissolves.

IMPORTANT: Do not vortex. Stop Solution contains SDS and will precipitate if stored below room temperature. Overly vigorous mixing will cause foaming.

18. Store the FASTplex Barcoding Buffer at room temperature when in use because it is viscous.

IMPORTANT: To transfer Barcoding Buffer accurately, do not pre-wet pipette tips and pipette slowly. While adding Barcoding Buffer to reactions, mix in the Barcoding Buffer completely by pipetting up and down several times with the same pipette tip(s) that was used for addition.

19. Prepare 80% ethanol daily.

20. Plan sample barcoding before starting the assay.

21. When planning for sample barcoding, keep the following in mind:

- The kit contains a total of 96 unique Barcodes as Sample Barcodes (SB) and a unique FASTplex Universal Barcode (UB) to prepare up to a 96-sample library.
- The AllType FASTplex NGS 11 Loci Flex Kit (Cat. No. ALL-FAST11LFX) has been tested using Illumina MiSeq v2 Standard (up to 96 samples), MiSeq v2 Micro (up to 24 samples), MiSeq v2 Nano (up to 8 samples), MiniSeq High Output (up to 96 samples), MiniSeq Mid Output (up to 72 samples), and iSeq v2 (up to 24 samples).
- A library can contain between 8 to 96 samples and is barcoded with one unique UB.
- Each well contains enough barcoding reagents for one reaction.
- Figure 2 provides the layout for a 96-sample library. Illumina barcode IDs and their positions are shown.

	1	2	3	4	5	6	7	8	9	10	11	12
A	X101	X102	X103	X104	X105	X106	X107	X108	X109	X110	X111	X112
B	X113	X114	X115	X116	X117	X118	X119	X120	X121	X122	X123	X124
C	X125	X126	X127	X128	X129	X130	X131	X132	X133	X134	X135	X136
D	X137	X138	X139	X140	X141	X142	X143	X144	X145	X146	X147	X148
E	X149	X150	X151	X152	X153	X154	X155	X156	X157	X158	X159	X160
F	X161	X162	X163	X164	X165	X166	X167	X168	X169	X170	X171	X172
G	X173	X174	X175	X176	X177	X178	X179	X180	X181	X182	X183	X184
H	X185	X186	X187	X188	X189	X190	X191	X192	X193	X194	X195	X196

Figure 2. Barcode sample plate layout

- The TypeStream Visual (TSV) NGS Calculator has a tab for the sample sheet that reflects this barcode order.

IMPORTANT: Alternative configurations, kits, and sequencing systems are not supported by this application and must be determined and validated by the user.

22. If working with various sample sizes, keep the following in mind for dealing with reagent volumes:

- When processing different sample sizes, different volumes of the pooled SB reaction and of the FASTplex Paramagnetic Beads will be used after Sample Barcoding step.
- The TSV NGS Calculator displays the required reagent volumes. In the “SB UB Lib Amp” tab, the total number of samples will automatically update based on the number of samples added in the “Pool Configuration” tab. In the “SB – UB-Lib. Amp” tab, the required reagent volumes are displayed.
- To ensure sample IDs are properly populated throughout, please refer to the TypeStream Visual NGS Analysis Software User Manual.
- The kit contains enough barcoding reagents, barcoding buffer and stop solution to process 12 runs of 8 samples

AMPLIFICATION

Materials and Equipment

- Diluted genomic DNA (25ng/μL)
- Eppendorf DNA LoBind Tubes, 2.0 mL
- 96-Well, 0.2 mL, PCR Plates
- AllType FASTplex 11 Loci Primer Flex Mix (Cat. ID ALF-P11F)
- AllType FASTplex 11 Loci Exon 1 Primer Flex Mix (Cat. ID ALF-P11FE1)
- AllType Buffer (Cat. ID ALL-BUF)
- AllType dNTPs (Cat. ID ALL-NTP)
- AllType LR Polymerase (Cat. ID ALL-LRPOL)
- Nuclease Free Water
- FASTplex Paramagnetic Beads (Cat. ID FAST-BEAD)
- FASTplex DNA Suspension Buffer (Cat. ID FAST-SUSP)
- 200 Proof Ethanol, Molecular Biology Grade
- Nunc 96-Well Polypropylene MicroWell Plates
- Magnetic Plate Stand
- 50 mL Polystyrene Reagent Reservoirs
- Qubit dsDNA High Sensitivity Assay Kit
- Qubit Fluorometer
- Full array of manual single channel pipettes (20 μL, 200 μL, 1 mL)
- Full array of filtered, pre-sterilized, pipette tips
- Serological Pipettes, 10 or 25 mL
- PCR Plate Centrifuge
- Mini Centrifuge
- Vortex
- Ice or Cold Block
- Thermal cycler PCR Pressure Pad
- [Veriti 96-Well Thermal Cycler or equivalent](#)
- TSV NGS Calculator

Part 1: Sample Amplification

1. **Turn on** a thermal cycler.
2. **Program** the thermal cycler to run the following HLA 11-loci Amplification Illumina Program:

HLA 11-Loci Amplification Illumina Program

Temperature	Time	Cycle
94°C	2 min	1
98°C	10 sec	30
69°C	3 min	
4°C	HOLD	1

3. **Use** 9600 emulation speed or +0.8°C/sec heating and -1.6°C/sec cooling with the heated cover set to 105°C

IMPORTANT: Check the thermal cycler lid temperature before adding the reaction. Make sure the thermal cycler lid is at the appropriate target temperature. Do not place the reaction onto a cold thermal cycler.

4. **Use** the “gDNA Dilution” tab in the TSV NGS Calculator to calculate the original sample volumes and DNA Suspension Buffer volumes to prepare 25 ng/μL dilutions.



Note: To ensure sample IDs are properly populated throughout, please refer to the TypeStream Visual NGS Analysis Software User Manual .

5. **Thaw** AllType FASTplex 11 Loci Primer Flex Mix, AllType FASTplex 11 Loci Exon 1 Primer Flex Mix, AllType dNTP, and AllType Buffer at room temperature.
6. **Keep** the AllType LR Polymerase tube on ice.
7. **Briefly** vortex and spin down all reagents EXCEPT polymerase.
8. **Keep** them on ice until ready for use.
9. **In a 96-Well 0.2 mL PCR Plate, aliquot** 4.0 μL of genomic DNA with the concentration adjusted to 25 ng/μL.
10. **Go to** the “Amp-Clean Up” tab of the TSV NGS Calculator, which will automatically calculate the volumes of each amplification reagent based on the number of samples input into the “gDNA Dilution” tab.
11. **Check** the “Exon 1” box if using the Exon 1 primer mix.
12. **In a 2.0 mL LoBind Tube, prepare** an Amplification Master Mix based on the reagent volumes shown in the “Amp Clean Up” tab of the TSV NGS Calculator.
13. **Prepare** reagents in the order shown in the “Amplification” table of the “Amp-Clean Up” tab in the TSV NGS Calculator.

IMPORTANT: At this stage, only prepare the Amplification Mix inclusive of the first five components. Do not add the Polymerase at this stage.
14. **Vortex** and pulse-spin the mix for 10 seconds.
15. **Keep** the mix on ice.
16. **Quick spin** the Polymerase.
17. **Add** the Polymerase to the Master Mix according to the “Amplification” table in the TSV NGS Calculator.
18. **Mix well** by pipetting 15-20 times with the pipet volume set to half the total volume of the Master Mix.
19. **Aliquot** 16 μL of the Master Mix to each well with DNA.
20. **Seal** the plate with a tray seal.
21. **Pulse-spin** the plate.
22. **Load** the plate into a pre-warmed thermal cycler.

23. Cover the plate with a pressure pad.

24. Run the HLA 11-loci Amplification program.

IMPORTANT: Check the thermal cycler lid temperature before adding the reaction. Make sure the thermal cycler lid is at the appropriate target temperature. Do not place the reaction onto a cold thermal cycler.



SAFE-STOPPING POINT: Proceed immediately to the next step or store the amplicon at -20°C.

Part 2: Amplicon Purification

- 1. Equilibrate** the FASTplex Paramagnetic Beads to room temperature for 30 minutes.
- 2. Prepare** fresh 80% ethanol solution by measuring ethanol and nuclease-free water separately and mixing. Refer to the TSV NGS Calculator and see the “Amp Clean Up” tab in the Amplification Purification section.
- 3. Vortex** the FASTplex Paramagnetic Beads at medium speed for 30 seconds to fully resuspend the beads.
- 4. In a Nunc LoBind 96-well plate, aliquot** 12 µL of the beads to each well. Use a multi-channel pipette and reagent reservoir for convenience.
- 5. Transfer** all amplified product (~20 µL) to the corresponding wells.
- 6. Mix** by pipetting up and down 10 times.

IMPORTANT: Change tips for each sample and avoid producing bubbles.
- 7. Incubate** at room temperature for 5 minutes.
- 8. Place** the plate on the magnetic stand. Adjust the plate as needed so the beads form a pellet on one side of each well. Let stand for ~3 minutes or until clear.
- 9. Using a pipette set to 28 µL, carefully remove and discard** the supernatant from each well without disturbing the bead pellet.
- 10. With the plate on the magnet, add** 100 µL of 80% ethanol to each well.
- 11. Incubate** at room temperature for 30 seconds or longer until the solution clears.
- 12. With a pipette set to 110 µL, remove and discard** the supernatant.

IMPORTANT: Be careful not to disturb the bead pellet.
- 13. Repeat** steps 10 through 12 above for a second ethanol wash.
- 14. With the plate on the magnet, remove** visible ethanol residue with a P-20 pipette.
- 15. Air-dry** the beads at room temperature for up to 3 minutes on the magnet.

IMPORTANT: Avoid over-drying.
- 16. With the plate on magnet, add** 27 µL of FASTplex DNA Suspension Buffer to each well.

17. **Remove** the plate from the magnet.
18. **Mix** each sample by pipetting up and down 10 times avoiding making any bubbles.
19. **Incubate** at room temperature for 5 minutes.
20. **Place** the plate back on magnet for ~3 minutes or until clear.
21. **With a pipette volume set to 25 μ L, transfer** the supernatant to a new 0.2 mL 96-well PCR plate without disturbing the beads.



SAFE-STOPPING POINT: Proceed immediately to the next step or store the purified products at -20°C.

Part 3: Amplicon Quantification

1. **Label** Qubit Assay Tubes for each sample and two additional tubes for Qubit Standard #1 and #2.
2. **Prepare** a Working Qubit Solution using the volumes per sample shown below plus 15% overage by mixing the equivalent of:
 - a) 199 μ L of Qubit dsDNA HS Buffer (Component B) per sample.
 - b) 1 μ L of Qubit dsDNA HS Reagent (Component A) per sample. Vortex prior to use.
3. **Vortex** the Working Qubit Solution and protect from light. Use within 2 hours.
4. **Add** 198 μ L of Working Solution to each labeled Qubit Assay Tube.
5. **Add** 190 μ L of Working Solution to the two tubes for the Standards.
6. **Add** 2 μ L of the corresponding purified amplicon to each labeled tube.
7. **Add** 10 μ L of the corresponding Standard to the appropriate, labeled tube.
8. **Briefly** vortex and spin all tubes.
9. **Protect** all tubes from light.
10. **Incubate** at room temperature for 2 minutes.
11. **Turn on** the Qubit Fluorometer.
12. **Select** DNA from the home screen.
13. **Select** dsDNA High Sensitivity.
14. **Press** the appropriate button to begin reading the standards.
15. **Measure** Standards 1 and 2 to complete the calibration.
16. **Begin reading** the amplicon tubes.
17. **When prompted, set** the volume of sample used to 2 μ L and the units to ng/ μ L.
18. **Record** the concentration of sample.
19. **Repeat** steps 16 through 18 for all samples.
20. **If any sample exceeds the upper detection, perform** the following steps:
 - a) Dilute the samples.
 - b) Remeasure the diluted samples.
 - c) Multiply the reading by the dilution factor. See the Qubit protocol for additional clarification.

Part 4: Amplicon Dilution

Lower concentration samples may not contain an adequate quantity of the amplicons. The following steps in this section will help calculate a volume of FASTplex DNA Suspension Buffer to dilute amplicons.

1. **Use** the “Amplicon Dilution” and “Plate Layout” tabs of the TSV NGS Calculator for amplicon dilution calculation.

Functions of the “Amplicon Dilution” and “Plate Layout” Tabs

Calculation Tool Tab ID	Functions
Amplicon Dilution	Calculation of 96 (max) amplicon dilution factors and diluent volumes to prepare 3-30ng input DNA for Sample Barcoding reaction
Plate Layout	View the result of input DNA and FASTplex DNA Suspension Buffer volumes in an 8 x 12 plate format

2. **Open** the TSV NGS Calculator.
 Sample IDs will be automatically populated from the sample IDs input into the “gDNA Dilutions” tab.
3. **In the “Amplicon Dilution” tab, enter** the amplicon concentrations in the column labeled “Amplicon Conc. (ng/μL).”
 - The table will display a volume of FASTplex DNA Suspension Buffer to add to 2 μL of each amplicon [“Sample (μL)” column].
 - Samples whose amplicon concentrations, after dilution, fall outside of the desired 3-30ng input into Library Preparation will be highlighted in orange (“High”) or red (“Low”).
 - The “Range” column will show either “High” or “Low” for the respective sample, and volume of sample and buffer to use.
 - The stock sample volume cell in the calculator is adjustable if more volume is needed.
4. **In a clean 96-well plate, add** the indicated volume of FASTplex DNA Suspension Buffer and 2 μL of the stock amplicon.
 - **For any outlier samples, add** volumes of the FASTplex DNA Suspension Buffer and stock amplicon that are indicated in the Sample (μL) and FASTplex DNA Suspension Buffer (μL) columns for each sample. The optimal input amount is 10 ng in a 4 μL volume at 2.5 ng/μL.
 - **Use** the “Plate Layout” tab to locate positions of outlier samples in the plate

IMPORTANT: For any samples that fall outside the normal range of concentrations, it is important to add the indicated volume.
5. **Carefully mix** the diluted amplicon well by pipetting. Avoid cross-contamination.
6. **Seal** the plate
7. **Pulse-spin** the plate.
8. **Store** on ice for immediate use or store at -20°C.



SAFE-STOPPING POINT: Proceed immediately to the next step or store the purified products at -20°C.

SAMPLE BARCODING

Materials and Equipment

- Diluted amplicons
- Eppendorf DNA LoBind Tubes, 2.0 mL
- PCR tube 0.2 mL
- 96-Well, 0.2 mL, PCR Plates
- 2.0 mL Tube Magnet Stand
- Serological Pipettes, 10 or 25 mL
- FASTplex Sample Flex Plate 96 (Cat. ID FAST-SFP96)
- FASTplex Univ Barcode Flex A (Cat. ID FAST-UBFA)
- FASTplex Barcoding Buffer (Cat. ID FAST-BB)
- FASTplex Stop Solution (Cat. ID FAST-STOP)
- FASTplex Paramagnetic Beads (Equilibrated to room temperature) (Cat. ID FAST-BEAD)
- FASTplex DNA Suspension Buffer (Cat. ID FAST-SUSP)
- 200 Proof Ethanol, Molecular Biology Grade
- Full array of manual single channel pipettes (20 µL, 200 µL, 1 mL)
- Full array of filtered, pre-sterilized, pipette tips
- 8 tube strips
- Thermal cycler PCR Pressure Pad
- [Veriti 96-Well Thermal Cycler or equivalent](#)
- TSV NGS Calculator

Part 1: Sample Barcoding (SB) Reaction, Reaction Stop, Sample Pooling, and Purification

IMPORTANT: Before starting this step, make sure the thermal cycler lid is at the appropriate target temperature. Do not place the reaction onto a cold thermal cycler.

IMPORTANT: Before starting this step, ensure the “SB UB Lib Amp” tab of the TSV NGS Calculator has been populated.



Useful Tip: In the following Sample Barcoding section below through step 20 the individual sample must be treated separately. An 8-channel pipette and use of an 8-tube strip can make this step faster and less error-prone. Common reagents, Barcoding Buffer and Stop Solution, can be aliquoted into an 8-tube strip or equivalent for use with the 8-channel pipette. The volume of reagents to aliquot using an 8-tube strip are summarized below.

Barcoding and Stop Buffer Volume (µL) per Well in 8-Tube Strip (W/Overage)

Reagent	8 Samples	16 Samples	24 Samples	48 Samples	96 Samples
Barcoding Buffer	7.0	14.0	21.0	42.0	84.0
Stop Solution	10.0	20.0	30.0	60.0	120.0

1. **Pulse-spin** the FASTplex Sample Flex Plate 96 in a centrifuge.
2. **After centrifugation, carefully remove** the plate seal from the FASTplex Sample Flex Plate.

3. **Discard** the plate seal.

IMPORTANT: Do not reuse the plate seal.

4. **Place** the FASTplex Sample Flex Plate on ice.
5. **Equilibrate** the FASTplex Paramagnetic Beads at room temperature.
6. **Set up** the Sample Barcoding reaction at ambient temperature.
7. **Transfer** 6 µL of the reagent from the FASTplex Sample Flex Plate 96 to a pre-labeled PCR plate or an 8-tube PCR strip(s) using an accurate pipette.

IMPORTANT: Use clean tips for each transfer.

8. **Visually confirm** that the volume of reagents in the plate appears equal.
9. **Return** the plate to the freezer (-20°C storage) if not all 96 samples are used.
10. **Transfer** 4 µL of diluted input DNA (2.5 ng/µL) to each well (one sample per well) or tube using an accurate pipette.
11. **Mix** the DNA thoroughly with the sample barcode reagent in each tube by pipetting up and down ten times at 4µL.

IMPORTANT: Be careful not to introduce excessive foams.

12. **Use** clean tips for each sample.
13. **Carefully pipette** 5 µL of Barcoding Buffer to each well or tube using new pipette tips for each transfer.
14. **Mix thoroughly but slowly by pipetting up and down** ten times at 5µL.

IMPORTANT: Be careful not to introduce excessive foams. Barcoding Buffer is very viscous and is subject to foaming.

15. **Carefully and completely seal or cap** the SB reactions to prevent evaporation.
16. **Pulse-spin** the plate.
17. **Transfer** to a thermal cycler.
18. **Place** a PCR pressure pad over the plate.

19. **Run** the following program (TAG) with heated cover on (105°C):

TAG program: 55°C for 15 min., 25°C hold, Reaction volume: **15 µL**

IMPORTANT: Check the thermal cycler lid temperature before adding the reaction. Make sure the thermal cycler lid is at the appropriate target temperature. Do not place the reaction onto a cold thermal cycler.

20. **Immediately take** the plate out of the thermal cycler, pulse-spin.
21. **Add** 7.5 µL of FASTplex Stop Solution to each SB reaction.
22. **Slowly pipette** up and down 5 times to mix, being careful not to introduce excessive bubbles.

IMPORTANT: Use clean tips for each transfer.

23. After mixing with the Stop Solution, securely re-seal the PCR plate.

24. Pulse-spin the PCR plate.

25. Transfer the PCR plate to a thermal cycler

26. Run the following STOP program with the heated cover on:

STOP program: 68°C for 10 min., 25°C hold, Reaction volume: **22.5 µL**

IMPORTANT: Check the thermal cycler lid temperature before adding the reaction. Make sure the thermal cycler lid is at the appropriate target temperature. Do not place the reaction onto a cold thermal cycler.

27. Vortex (or vigorously pipette) room temperature FASTplex Paramagnetic Beads to ensure that the beads are fully resuspended.

28. After thermal cycling, pulse-spin the PCR plate containing the stopped SB reactions.

29. Using a 10-20 µL pipette, transfer 15 µL of each SB reaction into a single 2 mL LoBind tube, label this tube as “Tube A” and gently mix by pipetting up and down slowly.

The final volume of the combined SB reaction pool is determined by the batch size.

30. Once pooling is complete, refer to the “Sample and Universal Barcoding” section of the “SB UB Lib Amp” tab of the TSV NGS Calculator for the volume of FASTplex Paramagnetic Beads to be added to the pooled SB reaction.

31. In a new 2.0mL LoBind tube, transfer the volume specified in the calculator cell “Volume of stopped, pooled SB reaction” from “Tube A” over to “Tube B”. Not all of Tube A volume will be used.

32. Label this tube as “Tube B.”

33. Proceed to the next step with “Tube B” and its contents

Volumes of the pool and paramagnetic bead for common sample sizes are listed in the following SB Reaction Pool and FASTplex Paramagnetic Bead Volumes table. The volumes for different sample sizes can be displayed using the TSV NGS Calculator.

SB Reaction Pool and Paramagnetic Bead Volumes

Batch Size (Samples per Pool)	8 Samples	16 Samples	24 Samples	48 Samples	96 Samples
Volume of stopped SB reaction pool	80 µL	160 µL	240 µL	480 µL	960 µL
Add FASTplex Paramagnetic Beads	72 µL	144 µL	216 µL	432 µL	864 µL

34. Go to the “SB UB Lib Amp” tab to find the volumes of the pool, paramagnetic bead, and the reagents for the rest of workflow.

35. Add the appropriate volume of FASTplex Paramagnetic Beads to the stopped SB reaction pool in “Tube B”.

36. Mix thoroughly by pipetting up and down 10x.

37. Incubate in a tube rack (non-magnetic) on the bench for 5 minutes to allow the DNA to bind.

38. Transfer the tube to a magnetic stand for ~ 5 minutes or until clear. A bead pellet will form.

39. Slowly remove the supernatant with a pipette and discard.

IMPORTANT: Be careful not to disturb the bead pellet.

40. With the tube in the magnetic stand, add 2.0 mL of 80% ethanol to completely immerse the bead pellet without disturbing the beads.

IMPORTANT: Be careful not to disturb the beads.

41. After 30 seconds, slowly remove and discard the supernatant.

IMPORTANT: Be careful not to disturb the bead pellet.

42. Repeat steps 40 and 41 for a total of two washes with 80% ethanol. Use a small pipette (e.g., P20) to remove the residual ethanol after the second wash.

43. Air-dry beads by leaving the tube uncapped on the magnetic stand for 2 minutes.

44. Check to ensure that there are no visible ethanol droplets in the tubes at end of 2 minutes.

- **If ethanol droplets are still visible, air-dry** the beads longer.



CAUTION: Do not dry the pellet longer than 3 minutes in total or the DNA recovery will be compromised.

45. Remove the tube from the magnetic stand.

46. Pipette the volume of FASTplex DNA Suspension Buffer provided in the “SB UB Lib Amp” tab of the TSV NGS Calculator. This volume is directly dependent on the number of samples and will vary accordingly.

Volumes of buffer for common sample sizes are listed in the following SB Reaction Pool and Paramagnetic Bead Volumes table.

SB Reaction Pool and Paramagnetic Bead Volumes

Batch Size (Samples per Pool)	8 Samples	16 Samples	24 Samples	48 Samples	96 Samples
Volume of FASTplex DNA Suspension Buffer to elute	50 µL	96 µL	144 µL	288 µL	576 µL

47. Pipette the buffer-bead solution along the inner wall of the tube multiple times to thoroughly mix and resuspend the bead pellet.

48. Incubate the tube in a tube rack (non-magnetic) on the bench for at least 5 minutes to elute the purified SB reaction pool from the beads.

49. Return the tube to a magnetic stand and allow a bead pellet to reform on the inner wall of the tube ~ 2 minutes or until clear.

50. When the supernatant has cleared completely, carefully transfer the full library eluate to a PCR tube. The transferred eluate contains the purified SB reaction pool.



SAFE-STOPPING POINT: Proceed immediately to the next step or store the purified products at -20°C.

Part 2: Universal Barcoding (UB) Reaction, Reaction Stop, and Purification

IMPORTANT: Before starting this step, make sure the thermal cycler lid is at the appropriate target temperature. Do not place the reaction onto a cold thermal cycler.

1. **Set up** a UB reaction in a 0.2 ml PCR tube using 48 µL of the purified SB reaction pool from the last step. For all library pools > 8 samples, there will be excess purified SB reaction pool.
2. **Add** the UB Reagent and Barcoding Buffer by using the TSV NGS Calculator or according to the amounts in the following table:

UB Reagent and Barcoding Buffer Volumes

UB Reagent and Barcoding Buffer	Volume
Purified SB reaction	48.0 µL
FASTplex Univ. Barcode Flex A	4.0 µL
FASTplex Barcoding Buffer	26.0 µL
Total Reaction Volume	78.0 µL

3. **Using a pipette set at 50 µL, mix** the UB reaction thoroughly by pipetting up and down 10 times.
4. **Cap** the PCR tube containing the UB reaction.
5. **Pulse-spin** the PCR tube.
6. **Transfer** the tube to a thermal cycler.
7. **Use** a PCR pressure pad.
8. **Run** the following program (TAG) with appropriate reaction volume and lid-heating on (105°C):

TAG program: 55°C for 15 min., 25°C hold, **Reaction Volume:** 78 µL for all library sizes.

IMPORTANT: Check the thermal cycler lid temperature before adding the reaction. Make sure the thermal cycler lid is at the appropriate target temperature. Do not place the reaction onto a cold thermal cycler.

9. **After the TAG program, remove** the tube from thermal cycler.
10. **Add** 39 µL Stop Solution.
11. **With the pipette set at 50 µL, mix** thoroughly by pipetting up and down 10 times.

IMPORTANT: Be careful not to produce excessive foaming.

12. **Re-cap** the PCR tube containing the UB reaction.
13. **Pulse-spin** the PCR tube.
14. **Transfer** the tube to a thermal cycler.
15. **Run** the following program (STOP) with appropriate reaction volume and lid-heating on:

STOP program: 68°C for 10 min., 25°C hold. Use 100 µL volume in the PCR program.

IMPORTANT: Check the thermal cycler lid temperature before adding the reaction. Make sure the thermal cycler lid is at the appropriate target temperature. Do not place the reaction onto a cold thermal cycler.

16. **Transfer** the entire volume of the stopped UB reaction to a single 2.0 ml LoBind tube.
17. **Vortex** (or vigorously pipette) room temperature Paramagnetic Beads to ensure they are completely resuspended. The UB reaction volume should be 117 μ L, but evaporation during the reaction may reduce its volume.
18. **Measure** the volume of UB reaction using a pipette.
19. **Add** an equal volume as measured in step 18 of paramagnetic beads in the 2.0 ml LoBind tube
20. **Mix** thoroughly by pipetting.
21. **Incubate** at room temperature in a tube rack (non-magnetic) on the bench for 5 minutes to allow the DNA to bind.
22. **Transfer** the 2.0 ml LoBind tube to a magnetic stand.
23. **Let** the beads separate completely for 3 minutes. A bead pellet will form along one side of the tube and the supernatant should appear completely clear after ~3 minutes.
24. **Slowly remove** the supernatant with a pipette and discard.
IMPORTANT: Be careful not to disturb the bead pellet.
25. **With the tube in the magnetic stand, add** 500 μ L of 80% ethanol, ensuring the bead pellet is submerged.
IMPORTANT: Be careful not to disturb the pellet.
26. **Slowly remove and discard** the supernatant after 30 seconds or until clear.
IMPORTANT: Be careful not to disturb the bead pellet.
27. **Repeat** steps 25 and 26 for a total of 2 washes with 80% ethanol.
28. **Use** a large pipette to remove most of the ethanol waste.
29. **Use** a smaller pipette (e.g., P20) to remove the residual ethanol that collects at the bottom of the tube.
30. **Air-dry** beads by leaving the tube uncapped on the magnetic stand for 2 minutes.
31. **Check** to ensure that there are no visible ethanol droplets in the tube at end of 2 minutes.
 - **If ethanol droplets are still visible, air-dry** the beads longer.



CAUTION: Do not dry the pellet longer than 3 minutes in total or the DNA recovery will be compromised.

32. **Remove** the tube from the magnetic stand.
33. **Add** 20 μ L of FASTplex DNA Suspension Buffer.
 - **Pipette** the liquid along the inside of the tube several times to thoroughly resuspend the bead pellet.
34. **Incubate** in a tube rack (non-magnetic) on the bench for at least 5 minutes to elute the purified DNA from the beads.
35. **Return** the tube to the magnetic stand.

- 36. Allow** the bead pellet to form on the inner wall of the tube for ~2 minutes or until clear.
- 37. When the supernatant has cleared completely, carefully transfer** 17 µL of DNA eluate to a clean 0.2 mL PCR tube. The transferred eluate contains the DNA purified from the UB reaction and is now ready for library amplification.



SAFE-STOPPING POINT: Proceed immediately to the next step or store the purified products at -20°C.

LIBRARY AMPLIFICATION

Materials and Equipment

- UB Barcoded library
- 0.2 mL PCR tube
- FASTplex Library Amp Mix (Cat. ID FAST-LAM)
- FASTplex Library Primer Flex Mix (Cat. ID FAST-LPFM)
- FASTplex Paramagnetic Beads (Cat. ID FAST-BEAD)
- FASTplex DNA Suspension Buffer (Cat. ID FAST-SUSP)
- Manual single channel pipettes (20 µL, 200 µL, 1 mL)
- Filtered, pre-sterilized, pipette tips
- 200 Proof Ethanol, Molecular Biology Grade
- [Veriti 96-Well Thermal Cycler or equivalent](#)
- 2.0 mL Tube Magnet Stand
- Nuclease Free Water
- Eppendorf DNA LoBind Tubes, 2.0 mL
- Qubit® Fluorometer or equivalent
- Qubit dsDNA HS Assay Kit
- Qubit Assay Tubes
- Calculation Tool

Part 1: Library Amplification Reaction

IMPORTANT: Before starting this step, make sure the thermal cycler lid is at the appropriate target temperature. Do not place the reaction onto a cold thermal cycler.

Reagent Volumes for the Steps Below

Reagent	Volume
FASTplex Library Primer Flex Mix	8 µL
Barcoded Library from step 37 in Part 2 of the Sample Barcoding section	17 µL
FASTplex Library Amp Mix	75 µL

1. **As described in the table above, add 8 µL of Library Primer Mix to the 17 µL Barcoded Library eluate in the 0.2 mL PCR tube from [step 37](#) in Part 2 of the Sample Barcoding process.**
2. **Add 75 µL of Library Amp Mix.**
3. **Mix well by pipetting.**
4. **Cap the PCR tube and pulse spin.**
5. **Use an appropriate PCR pressure pad.**
6. **Run the following FASTplex Illumina Library Amplification PCR program with a heated cover on and 9600 emulation mode or +0.8°C/sec heating and -1.6°C/sec cooling with the heated cover set to 105°C. The total reaction volume is 100 µL.**

- **FASTplex Illumina Library Amplification Program:**

Temperature	Time	Cycle
72°C	10 min	1
98°C	3 min	1
98°C	15 sec	12]
64°C	30 sec	
72°C	1 min	
72°C	5 min	1
4°C	HOLD	1

9600 emulation speed and heated cover on

IMPORTANT: Check the thermal cycler lid temperature before adding the reaction. Make sure the thermal cycler lid is at the appropriate target temperature. Do not place the reaction onto a cold thermal cycler.

7. **After PCR, pulse-spin** the library amplification reaction.
8. **Transfer** 95 µL of the library amplification reaction to a 2.0 mL LoBind tube.
 - **If it's less than 95 µL, note** the volume for PART 2: Purification of the Amplified Library, [step 1](#).

IMPORTANT: The volume will normally decrease due to evaporation during thermal cycling, so it is important to measure the volume prior to the purification steps.



SAFE-STOPPING POINT: Proceed immediately to the next step or store the purified products at -20°C.

Part 2: Purification of the Amplified Library

1. **In a new 2.0 mL LoBind tube, dilute** the library amplification reaction to a final volume of 200 µL by adding 105 µL (or more in case of evaporation) of FASTplex DNA Suspension Buffer to the amplified product.
2. **Vortex** room temperature FASTplex Paramagnetic Beads to ensure they are completely resuspended.
3. **Add** 160 µL of FASTplex Paramagnetic Beads (0.8x volume equivalents) to the diluted multiplexed library.
4. **Mix** thoroughly by pipetting up and down.
5. **Incubate** in a tube rack (non-magnetic) on the bench for 5 minutes to allow the DNA to bind.
6. **Transfer** the 2.0 ml LoBind tube to a magnetic stand.
7. **Let** the beads settle completely for approximately 3 minutes. A bead pellet will form along one side of the tube and the supernatant should appear completely clear blue after ~3 minutes.
8. **Slowly remove** the supernatant with a pipette and discard.

IMPORTANT: Be careful not to disturb the bead pellet.

9. **With the tube in the magnetic rack, add** 500 µL of 80% Ethanol.

IMPORTANT: Be careful not to disturb the beads.

10. **Incubate** for 30 seconds, until the solution clears.
11. **Keep** the tube on the magnetic rack.
12. **Carefully remove and discard** the supernatant.
13. **Repeat** steps 9 through 12 for a total of two washes with 80% ethanol.
14. **Use** a small pipette (e.g., P20) to remove the residual ethanol after the second wash.
15. **Air-dry** the beads by leaving the tube uncapped on the magnetic stand for 2 minutes.
16. **Check** to ensure that there are no visible ethanol droplets in the tubes after 1 minute.
 - **If ethanol droplets are still visible, air-dry** the beads longer.



CAUTION: Do not dry the pellet longer than 3 minutes in total or the DNA recovery will be compromised.

17. **Remove** the tube from the magnetic stand.
18. **Add** 35 µL of FASTplex DNA Suspension Buffer.
19. **Pipette** the liquid along the inside of the tube several times to thoroughly disperse the beads.
20. **Incubate** for 5 minutes in a non-magnetic tube rack on the bench to elute the multiplexed library from the magnetic beads.
21. **Return** the tubes to magnetic stand.
22. **Allow** the bead pellet to form on the inner wall of the tube for ~2 minutes or until clear.
23. **When the supernatant has cleared completely, carefully transfer** 33 µL of DNA eluate to a new 2.0 ml LoBind tube. The transferred eluate contains the final library.



SAFE-STOPPING POINT: Proceed immediately to the next step or store the purified products at -20°C.

Part 3: Quantification of the Final Library

1. **Label** five Qubit Assay Tubes – three for triplicate quantification of the library and two additional tubes for Qubit Standard #1 and #2.
2. **Prepare** a Working Qubit Solution in a 5 ml tube for 10 samples (to account for the measurement in [step 16](#) and [step 23](#) for pre-dilution and post-dilution samples) by mixing the equivalent of:
 - a) 199 µL of Qubit dsDNA HS Buffer (Component B) per sample.
 - b) 1 µL of Qubit dsDNA HS Reagent (Component A) per sample. Vortex before use.
3. **Vortex** the Working Qubit Solution.
4. **Cover** with foil.

IMPORTANT: Use within 2 hours.
5. **Add** 198 µL of Working Solution to the three Qubit Assay Tubes used for triplicate quantification of the library.
6. **Add** 190 µL of Working Solution to the two tubes used for the Qubit Standards.

7. **Add** 2 μL of the purified library to the three Qubit Assay Tubes used for triplicate quantification of the library.
8. **Add** 10 μL of the corresponding Qubit Standard to the two tubes used for the Qubit Standards.
9. **Briefly vortex** and spin all tubes.
10. **Cover** the tubes with foil.
11. **Incubate** the tubes at room temperature for 2 minutes.
12. **Turn on** the Qubit Fluorometer,
13. **Select** DNA from the home screen.
14. **Select** dsDNA High Sensitivity.
15. **Press** the appropriate button to begin reading the standards.
16. **Measure** Standards 1 and 2 to complete the calibration. This calibration can be used for quantification of the final library in [step 23](#) of this section, if the final quantification is performed within 2 hours.
17. **Begin reading** the three library tubes.
18. **When prompted, change** the volume of sample used to 2 μL and the units to ng/ μL .
19. **Record** the concentration of the library in triplicate.
20. **In the TSV NGS Calculator, open** the tab named “Final Quant”
21. **Choose** the appropriate Instrument from the drop-down menu in the upper left-hand corner of the tab.

Different sequencing systems require different final library concentrations. The following table lists the concentrations for the MiSeq, MiniSeq, and iSeq sequencing systems.

Final Library Concentrations

Sequencing System	Library Concentration
MiSeq	2.1 ng/ μL
MiniSeq	0.53 ng/ μL
iSeq	0.056 ng/ μL

22. **Input** the average concentration of the three triplicate readings that was determined by Qubit into the TSV NGS Calculator “Pool Quant Values (ng/ μL).”

The calculator will calculate the amount of FASTplex DNA Suspension Buffer and sample pool volume that should be combined in a new 2.0 mL LoBind tube to achieve the proper library concentration.

The “Diluted Pool Volume (μL)” can be adjusted as needed, but the final desired target concentration cannot be edited in the calculator. It may be helpful to make an intermediate dilution, especially for libraries that are to be sequenced on the iSeq as the final library concentration is low.

23. **Quantify** the diluted library in triplicate using the Qubit Working Solution in order to verify that it is at the appropriate concentration.

Qubit Working Solution Volumes per Sequencing System

Sequencing System	Qubit Working Solution Volume	Library Volume
MiSeq	198 µL	2 µL
MiniSeq	198 µL	2 µL
iSeq	195 µL	5 µL

PREPARING for ILLUMINA PLATFORM SEQUENCING

Materials and Equipment

- Final library with the concentration adjusted for appropriate Illumina sequencing kit
- Illumina Sequencing Kit(s) of choice (see Required Materials and Equipment Not Provided above for compatible kits)
- PhiX Control Kit
- NaOH Solution $\geq 1.0M$, Molecular Biology Grade (for MiSeq and MiniSeq only)

Part 1: Preparing PhiX Control

1. **In a new 2.0 mL LoBind tube, combine** 2 μ L of 10nM PhiX stock to 3 μ L of FASTplex DNA Suspension Buffer to dilute the PhiX to 4nM.
2. **Vortex** and pulse-spin.
3. **To the same tube containing 4 nM PhiX, add** 5 μ L of freshly prepared 0.2M NaOH solution
4. **Vortex** and pulse-spin.
5. **Incubate** at room temperature for 5 mins.
6. **When the 5-minute incubation is complete, add** 990 μ L of HT1/Hybridization buffer to the denatured PhiX to bring the concentration down to 20 pM.
7. **Vortex** and pulse-spin the 20 pM PhiX.

20 pM PhiX can be used immediately in the following library preparation or stored at -20°C for up to 2 weeks.

Part 2: MiSeq Sequencing – Preparing the Sample Sheet

1. **Open** the “Sample Sheet” tab in the TSV NGS Calculator.
2. **If using MiSeq with Windows 7 Operating System, choose** “MiSeq” from the drop-down menu in the top left corner of the calculator.

The Sample Sheet “Sample_Name” and “Sample_ID” columns will automatically populate.
3. **If using MiSeq with Windows 10 Operating System, choose** “MiSeqV2” from the drop-down menu in the top left corner of the calculator.

The Sample Sheet “Sample_Name” and “Sample_ID” columns will automatically populate.
4. **Fill in** Experiment Name in the highlighted cell (yellow). The Date will automatically populate, but it can be edited.
5. **Export** the completed sample sheet using the “Export” button
6. **Name** the sample sheet.
7. **Copy** the file on a USB memory drive.

Part 3: MiSeq Sequencing – Preparing the Pool Library

IMPORTANT: Prior to beginning the next series of steps, fully thaw the MiSeq Reagent Cartridge by placing it in room temperature water up to the water line for at least an hour. Once fully thawed, the cartridge can be stored at 4°C until ready for sequencing.

1. **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.
2. **In a new 1.5 mL LoBind tube, combine** 5 µL of the 2.1 ng/µL (or other value used) library pool with 5 µL of freshly prepared 0.2 M NaOH solution.
3. **Vortex** and pulse-spin.
4. **Incubate** at room temperature for 5 mins.
5. **When the 5-minute incubation is complete, add** 990 µL of HT1/Hybridization buffer to the denatured library to bring the concentration down to 20 pM.
6. **Vortex** and pulse-spin.
7. **Add** 25 µL of 20 pM PhiX to the 1 mL denatured and diluted library. Vortex, and pulse-spin.
8. **Obtain** the thawed MiSeq Reagent Cartridge.
9. **Invert** the MiSeq Reagent Cartridge 10 times to mix.
10. **Gently tap** the cartridge in the upright position on a countertop.
11. **Wipe off** any liquid or condensation from the cartridge using a lab wipe.
12. **Pierce** well 17 of the MiSeq Reagent Cartridge using the 1mL pipette tip.
13. **Discard** the pipette tip once the MiSeq Reagent Cartridge is pierced.
14. **Transfer** 600µL of the combined library and PhiX solution to well 17 using a pipette.
15. **Make sure** that the solution is dispensed to the bottom of the well.
16. **Press** the Sequence button on the instrument interface to walk through the remainder of the sequencing run setup.
17. **When prompted to insert the flow cell, remove** the flow cell from its container.
18. **Clean** the flow cell by thoroughly rinsing it with lab grade water.
19. **Once rinsed, thoroughly dry** the flow cell surface by blotting gently with a lab wipe.
20. **Ensure** that all visible smudges, dust, and lab wipe lint are removed from the flow cell surface.
21. **Insert** the flow cell into the MiSeq flow cell compartment and close the latch.
22. **Proceed** with initiation of the sequencing run on MiSeq sequencer screen.
23. **After loading the sequencing cartridge and Incorporation Buffer, load** the sample sheet made in the previous section, [Part 2: MiSeq Sequencing – Preparing the Sample Sheet](#), as a sample sheet from the USB memory drive by using the browse button.
24. **Press** the Restart/Check button to proceed with the sequencing run setup.

25. **When the run is complete, clean** the instrument in a timely fashion per the manufacturer's recommendations.

Part 4: MiniSeq Sequencing – Preparing the Sample Sheet

1. **Open** the “Sample Sheet” tab in the TSV NGS Calculator.
2. **Users of the MiniSeq, choose** “MiniSeq” from the drop-down menu in the top left corner of the calculator.

The Sample Sheet “Sample_Name” and “Sample_ID” columns will automatically populate.

3. **Fill in** the Experiment Name in the highlighted cell (yellow).
The Date will automatically populate, but the date can be edited.
4. **Export** the completed sample sheet clicking the “Export” button.
5. **Name** the sample sheet.
6. **Copy** the file on a USB memory drive.
7. **Log into** Local Run Manager.
8. **Click** the blue “Create Run” button on the top right of the page.
9. **Choose** “GenerateFASTQ”.
10. **Click** the “Import Sample Sheet” button on the top left side of the page.
11. **Navigate** to the location of the sample sheet.
12. **Open** the sample sheet.

The Run Name should automatically update with what was entered in cell B3 of the Sample Sheet.

The Library Prep kit should automatically update to “Custom.”

13. **Scroll** to the bottom of the page.
14. **Click** the “Save Run” button.

Part 5: MiniSeq Sequencing – Preparing the Pool Library

IMPORTANT: Prior to beginning the next series of steps, fully thaw the MiniSeq Reagent Cartridge by putting it in room temperature water up to the bottom of the handle for 3+ hours. Once fully thawed, the cartridge can be stored at 4°C until ready for sequencing.

Equilibrate the flow cell, which is stored separately at 4°C, to Room Temperature for 30 minutes before sequencing.

1. **In addition to thawing the reagent cartridge, thaw** the HT1/Hybridization Buffer that is packaged separately from the cartridge.
2. **Once thawed, keep** the HT1/Hybridization Buffer on ice.
3. **In a new 1.5 mL LoBind tube, combine** 5 µL of the 0.53 ng/µL library pool with 5µL of freshly prepared 0.1M NaOH solution.

IMPORTANT: MiniSeq denaturation uses a lower concentration of NaOH than MiSeq.

4. **Vortex** and pulse-spin.
5. **Incubate** at room temperature for 5 mins.
6. **When the 5-minute incubation is complete, add 5 µL** of FASTplex DNA Suspension Buffer to the denatured library to bring the concentration down to 333 pM.
7. **Vortex** and pulse-spin.
8. **Add 985 µL** of HT1/Hybridization Buffer to the denatured library to bring it down to 5 pM.
9. **Vortex** and pulse-spin.
10. **Add 2.5 µL** of prepared 20 pM PhiX to the denatured and diluted library.
11. **Add 180 µL** of the denatured and diluted library to 320 µL of HT1/Hybridization Buffer to bring it down to the final loading concentration of 1.8 pM.
12. **Vortex** and pulse-spin.
13. **Obtain** the thawed MiniSeq Reagent Cartridge.
14. **Invert** 10 times to mix.
15. **Gently tap** the cartridge in the upright position on a countertop.
16. **Wipe off** any liquid or condensation from the cartridge using a lab wipe.
17. **Pierce** well 16 of the MiniSeq Reagent Cartridge using a 1mL pipette tip. Discard the tip once pierced.
18. **Transfer** 500µL of the combined library and PhiX solution to well 16 using a pipette.
19. **Make sure** the solution is dispensed to the bottom of the well.
20. **Press** the Sequence button on the instrument interface to walk through the remainder of the sequencing run setup.

The interface will prompt the user to insert the flow cell.
21. **Inspect** for the flow cell carefully for any dust or marks.
22. **Ensure** that all visible smudges, dust, and lab wipe lint is removed from the flow cell surface.
23. **Insert** the flow cell into the receptacle, secure it, and close the lid.
24. **Proceed** with initiation of the sequencing run on MiniSeq sequencer screen.
25. **Load** the MiniSeq Reagent Cartridge containing the prepared sequencing library.



Note: When the run is complete, the MiniSeq is capable of auto-cleaning if the option is chosen.

Part 6: iSeq 100 Sequencing – Preparing the Sample Sheet

1. **Open** the “Sample Sheet” tab in the TSV NGS Calculator.
2. **If using iSeq, choose** “iSeq” from the drop-down menu in the top left corner of the calculator.
The Sample Sheet “Sample_Name” and “Sample_ID” columns will automatically populate.
3. **Fill in** the Experimental Name in the highlighted cell (yellow).
The date will automatically populate, but the date can be edited.
4. **Export** the completed sample sheet using the “Export” button and name the sample sheet. Copy the file on a USB memory drive.
5. **Log into** Local Run Manager and click the blue “Create Run” button on the top right of the page and choose “GenerateFASTQ”.
6. **Click** the “Import Sample Sheet” button on the top left side of the page, navigate to the location of your sample sheet, and open it.
The Run Name should automatically update with what was entered in cell B3 of the Sample Sheet.
The Library Prep kit should automatically update to “Custom”.
7. **Scroll** to the bottom of the page.
8. **Click** the “Save Run” button.

Part 7: iSeq 100 Sequencing – Preparing the Pool Library

IMPORTANT: Prior to beginning the next series of steps, fully thaw the iSeq Reagent v2 Cartridge thawed by submerging it in room temperature water for at least 6 hours. Alternatively, it can be thawed overnight at 4°C and brought to room temperature for ~ 1 hour before use.

1. **In addition to thawing the reagent cartridge, take** the flow cell cartridge from the 4°C storage and allow it to equilibrate at room temperature for at least 15 minutes.
2. **Add** 20 µL of 20 pM PhiX to 100 µL of the 0.056 ng/µL library pool.
3. **Vortex** and spin-down.
4. **Invert** the bagged cartridge 5 times to mix reagents.
5. **Tap** the cartridge with the label facing upwards several times to bring reagents down from the wall of the wells.
6. **Remove** the cartridge from the bag.
7. **Open** the flow cell package.
8. **Remove** the flow cell package from the plastic tray.
9. **Inspect** the flow cell for any damage.
10. **Insert** the flow cell into the cartridge so that the label is visible from outside of the cartridge and the glass portion of the flow cell is visible through the opening in the cartridge. There will be an audible click when the flow cell is properly inserted.

11. **Pierce** the foil in the orange box marked “Library.”
12. **Add** 20 µL of your library plus PhiX to the bottom of the Library reservoir.
13. **Proceed** with initiation of the sequencing run on the iSeq sequencer screen.
14. **Follow** the instructions on the screen to proceed.
15. **When the run options screen is presented, choose “1”** under the “Read Index” option for single index sequencing.
16. **Proceed** with sequencing.

RESULTS

Data Acquisition

Upon completion of sequencing, the sequencer system software demultiplexes all sequence reads using the barcodes and generates a read 1 and 2 file per sample. Refer to user manual of the sequencer system software to download sequence data in its default or specified format such as a FASTQ file for each sample. HLA TypeStream Visual NGS Analysis Software imports a FASTQ file from the sequencer for analysis.

Sample ID can be embedded in the data file from the sequencer software or can be added at the time of data acquisition through TypeStream Visual NGS Analysis Software.

Data Calculation

Please see TypeStream Visual NGS Analysis Software User Manual for details. The HLA TypeStream Visual NGS Analysis Software provides all the necessary quality metrics depth of coverage, quality score for the base reads, read alignment and variant call, and the laboratory should determine the acceptable values for each quality metric in order to assure a successful run.

Data Analysis

HLA TypeStream Visual NGS Analysis Software sorts imported sequence reads using the locus-specific reference sequences from the IPD-IMGT/HLA Database which provides a specific database for sequences of the human major histocompatibility complex, identifies reference alleles that best match the sample reads, quality-trims, maps and assembles contiguous sequences from the mapped reads, and assigns alleles per locus based on the assembled consensus contiguous sequences. The use of other analysis software may lead to incorrect results and is not supported.

LIMITATIONS of the PROCEDURE

- A. 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.
- B. Sample DNA should be quantified with a fluorometer and be free from any known PCR inhibitor. PCR inhibitors may be introduced from the original sample source or from various DNA extraction methods. Reference samples should be validated for amplification using the AllType FASTplex NGS 11 Loci Flex Kit reagents.
- C. The PCR and library preparation workflow described in this protocol requires highly controlled conditions. Follow the standard PCR guidelines listed in the section above, [GENERAL PREPARATION for the ASSAYS](#) to minimize contaminations.
- D. The AllType FASTplex NGS 11 Loci Flex Kit - 96 has been tested for use with the Applied Biosystems Veriti 96-Well Thermal Cycler (Cat. No. 4375786), a model capable of 9600 emulation speed or +0.8°C/sec heating and -1.6°C/sec cooling and a heated cover at 105°C or equivalent for all programs. Other thermal cyclers considered for use require end-user evaluation and validation.
- E. The AllType FASTplex NGS 11 Loci Flex Kit - 96 (Cat. No. ALL-FAST11LFX) has been tested using Illumina the MiSeq Sequencing System (300 cycles) using the MiSeq Reagent Kit v2 (≤96 samples), Micro Kit v2 (≤24 samples) kit, Nano Kit v2 (≤8 samples), MiniSeq Sequencing System using the High (≤96 samples) and Mid Output Kit (≤72 samples), and iSeq Sequencing System using the 100 i1 Reagent v2 Kit (≤24 samples). Alternative configurations, kits, and sequencing systems are not supported by this application and must be determined and validated by the user.
- F. The minimum sample size per run is eight.
- G. The AllType FASTplex NGS 11 Loci Flex Kit - 96 (Cat. No. ALL-FAST11LFX) has not been tested using any protocol that deviates from the one described above and may lead to erroneous results.
- H. HLA typing at high resolution using the NGS technology is a complex process that requires qualified personnel to review data and final HLA allele assignments.
- I. This test must not be used for making a clinical decision
- J. Please refer to the AllType FASTplex NGS 11 Loci Flex Kit - 96 Resolution Limitations – 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.
- K. AllType FASTplex NGS 11 Loci Flex Kit - 96 can detect DRB4*03:01N. The kit can eliminate DQB1*03:276N when the supplemental Class II Exon 1 primer mix is used and the test sample is heterozygous in exon 1, using TypeStream Visual 2.1 or later.
- L. 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.
- M. The AllType FASTplex 11 Loci Primer Flex Mix for HLA-DRB1, -DQB1 and -DPB1 primers do not amplify exon 1. The AllType FASTplex Exon 1 Primer Flex Mix must be used to resolve Exon 1 ambiguities for these genes. When using the AllType FASTplex 11 Loci Exon 1 Primer Flex Mix with low quality or highly fragmented DNA, users may experience low uniformity across sequence reads.
- N. 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 results needs be confirmed by a secondary method before assigning a result.

- O. The AllType FASTplex primer mixes 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.
- P. The AllType FASTplex primer mixes were tested based on Field-3 (6-digits HLA types) only. There are no performance claims outside this field.
- Q. Software analysis for this kit is only supported with the TypeStream Visual NGS Analysis Software 2.1 or above using AllType FASTplex NGS 11 Loci Flex Kit catalog file. The use of other software is not supported.
- R. Failure to completely read and explicitly follow all 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).

EXPECTED VALUES

Sample Amplification

The AllType FASTplex NGS 11 Loci Flex Kit is expected to amplify and produce HLA locus-specific amplification products consisting of ~5000 base pairs in averaged length (subject to change per lot). Physical separation and monitoring of contamination in a pre-amplification laboratory and equipment is highly recommended. Exon 1 amplicons will range from 1.6-2.2 kb.

Library Preparation

The instruction provided in this Application Note has been tested to produce a final sequence-compatible barcoded library using amplified DNA from the AllType FASTplex NGS 11 Loci Flex Kit for the final concentration of 2.1 ng/μL, 0.53 ng/μL, and 0.056 ng/μL for Illumina MiSeq, MiniSeq, and iSeq sequencing systems, respectively. The expected final library concentration is 10 ng/μL or above, as measured in [LIBRARY AMPLIFICATION, Part 3, step 19](#). A lower concentration library may give incorrect or ambiguous results due to lower reads.

DNA Sequencing

The AllType FASTplex NGS 11 Loci Flex Kit has been tested using up to 96 samples per sequencing run to ensure a minimum average read depth of 50 reads or greater per allele when using Illumina MiSeq Reagent Kit V2 with 2x150bp (300 cycle) output, 24 samples with V2 Micro Reagent Kit, and 8 samples with V2 Nano Reagent Kit. Ninety-six (96) samples have been tested for the MiniSeq High Output kit and 72 samples for the MiniSeq Mid Output Kit. Twenty-four samples have been tested for the iSeq 100 i1 Reagent V2 kit. Alternative sample-per-run throughputs are not supported by this application and must be determined by the user.

The following sequencing metrics in the Illumina BaseSpace Sequencing Hub report have been tested to produce reliable results for the 96-sample run. %Q30 Reads is the number of reads that meets the sequencer QC filters and is a good metric to assess if the run produced enough reads. Mean read length indicates if the sequenced reads meet the minimum length needed. %CV of % Reads Identified (PF) Per Index indicates if the library contains an even number of reads for each sample. A high %CV with samples with significantly lower reads indicates poor sample input or normalization that may lead to sample-specific issues. A deviation of a single metric does not necessarily indicate a sequencing error. A combination of low total reads and high barcode read balance %CV due to multiple low reads samples, or low read length may lead to ambiguous or incorrect results due to insufficient reads.

QC Metrics for Sequencing:

- %Q30 Reads: ≥70% for MiSeq and MiniSeq, >60% for iSeq
- Mean Read Length: ≥130 bases
- %CV of % Reads Identified (PF) Per Index: ≤30% without excessively low reads sample(s); ≤30% without excessively low reads sample(s)

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APPENDICES

Appendix 1: PCR Program Reference Guide

The following is a list of PCR programs used for the assays:

A. HLA 11-Loci Amplification Illumina Program:

Temperature	Time	Cycle
94°C	2 min	1
98°C	10 sec	30
69°C	3 min	
4°C	HOLD	1

9600 emulation speed and heated cover on

B. TAG Program: 55°C for 15 min., 25°C hold, heated cover on

C. STOP Program: 68°C for 10 min., 25°C hold, heated cover on

D. FASTplex Illumina Library Amplification Program:

Temperature	Time	Cycle
72°C	10 min	1
98°C	3 min	1
98°C	15 sec	12
64°C	30 sec	
72°C	1 min	
72°C	5 min	1
4°C	HOLD	1

9600 emulation speed and heated cover on

Appendix 2: FASTplex Sample Flex Plate 96 Worksheet

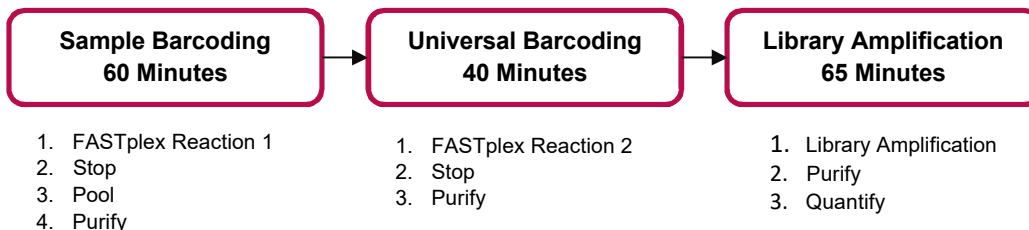
I	G	F	E	D	C	B	A	
								1
								2
								3
								4
								5
								6
								7
								8
								9
								10
								11
								12

Figure 3. FASTplex Sample Flex Plate 96

Appendix 3: Quick Guide (Refer to the Application Note for more detail.)

Illumina – AllType FASTplex NGS Assay Quick Guide

- After amplification of DNA sample, purify with magnetic beads, and measure concentration with standard Qubit protocol.
- Enter the Qubit values into the TSV NGS Calculator. Dilute the amplicons with the FASTplex DNA Suspension Buffer from kit as indicated.
- Diluted amplicons are ready for library preparation



Note: Before beginning, thaw reagents and keep on ice. Place FASTplex Paramagnetic Beads at room temp for at least 30 minutes. Ensure that Buffers contain NO crystals

IMPORTANT: Only use FASTplex DNA Suspension Buffer

Sample Barcoding Steps:

- Centrifuge FASTplex Sample Plate and transfer **6 µL** barcoding reagent into a fresh plate
- Add **4 µL** diluted amplicon and pipette mix 10x
- Add **5 µL** FASTplex Barcoding Buffer and pipette mix 10x
- Seal Plate and pulse centrifuge. Run FASTplex **TAG program**.
- Add **7.5 µL** FASTplex Stop Solution and pipette mix 5x.
- Seal plate and pulse centrifuge. Run FASTplex **STOP program**.
- Pool **10 µL** from each sample barcoding reaction well.
- Complete Sample Barcode (SB) purification following TSV NGS Calculator
- Elute in volume of DNA suspension Buffer **according** to the TSV NGS Calculator.
- Transfer **48 µL** Purified SB pool to 0.2 ml PCR tube

Universal Barcoding Steps:

- Add **4 µL** FASTplex Univ Barcode Flex A reagent to 48µL Purified SB Pool
- Add **26 µL** FASTplex Barcoding Buffer, pipette mix and pulse centrifuge. Run FASTplex **TAG program**.
- Add **39 µL** FASTplex Stop Solution, pipette mix, and pulse centrifuge. Run FASTplex **STOP program**.
- Complete Univ Barcode (UB) Purification (1 volume equivalent)
- Elute in **20 µL** of FASTplex DNA Suspension Buffer
- Transfer **17 µL** Purified UB pool to 0.2 ml PCR tube

Library Amplification Steps:

- Add **8 µL** FASTplex Library Primer Flex Mix to 17ul purified UB pool,
- Add **75 µL** FASTplex Library Amp Mix and pipette to mix
- Run **FASTplex Illumina Library Amplification Program**
- Dilute Library – **95 µL** Library in **105 µl** FASTplex DNA Suspension Buffer
- Complete Library Purification with **160 µl** of FASTplex Paramagnetic Beads (0.8x volume equivalent)
- Elute in **35 µl** of FASTplex DNA Suspension Buffer
- Transfer **33 µL** of final library to a 2ml LoBind Tube
- Dilute final library for sequencer loading: Miseq 2.1 ng/µL, MiniSeq 0.53 ng/µL and iSeq 0.056 ng/µL

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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

EXPLANATION of SYMBOLS




Reference EN ISO 15223-1: Medical Devices – symbols to be used with medical device labels, labeling and information to be supplied.

Symbol	Description	Symbol	Description
 ISO 7000 Reg No. 1641	Consult Application Note. An eIFU indicator can be a manufacturer's website URL or some other appropriate indication that the Application Note is available in an electronic format.	 ISO 7000 Reg No. 0518	Contains sufficient for <n> tests
 ISO 7000 Reg No. 2493	Catalog number	 ISO 7000 Reg No. 3082	Manufacturer
 ISO 7000 Reg No. 0434A	Caution	 ISO 7000 Reg No. 2497	Date of Manufacture
 ISO 7000 Reg No. 0632	Temperature limitation	 ISO 7000 Reg No. 2607	Use by Date
 ISO 7000 Reg No. 0633	Upper limit of temperature	 ISO 7000 Reg No. 2492	Batch code
Other Symbols			
	Irritant (Skin, Eyes)		Carcinogen
Symbol	Description	Symbol	Description
 ISO 7000 Reg No. 1641	Consult Application Note. An eIFU indicator can be a manufacturer's website URL or some other appropriate indication that the Application Note is available in an electronic format.	 ISO 7000 Reg No. 0518	Contains sufficient for <n> tests
 ISO 7000 Reg No. 2493	Catalog number	 ISO 7000 Reg No. 3082	Manufacturer
	In vitro diagnostic medical device		Authorized representative in the European Community

Symbol	Description	Symbol	Description
 <i>ISO 7000 Reg No. 0434A</i>	Caution	 <i>ISO 7000 Reg No 2497</i>	Date of Manufacture
 <i>ISO 7000 Reg No. 0632</i>	Temperature limitation	 <i>ISO 7000 Reg No 2607</i>	Use by Date
 <i>ISO 7000 Reg No. 0633</i>	Upper limit of Temperature	 <i>ISO 7000 Reg No. 2492</i>	Batch Code
Other Symbols			
	Irritant (Skin, Eyes)		Carcinogen

The batch field on the label is for traceability of a manufacturing event.

EXPLANATION of HELPFUL SYMBOLS

Icon	Description
	Safe Stopping Point
	Note
	Useful tip

REVISION HISTORY

Revision	Date of Issue	Revision Description
06	19 Aug 2021	<ul style="list-style-type: none"> Application Note converted to new template layout. Protocol updated for new product configuration (Lot 003 and greater). See sections for Amplification (Part 1 & 2) and Sample Barcoding (Part 1 & 2).
07	14 Sep 2021	<ul style="list-style-type: none"> Added best practice recommendations under section: DNA Extraction Methods
08	Current	<ul style="list-style-type: none"> Corrected SB Reaction Pool and Paramagnetic Bead Volumes For 8 samples to 50uL as per the validated protocol Corrected the Typo in Part 5 Miniseq Sequencing – Preparing the Pool Library #6. From 500pM to 333pM.