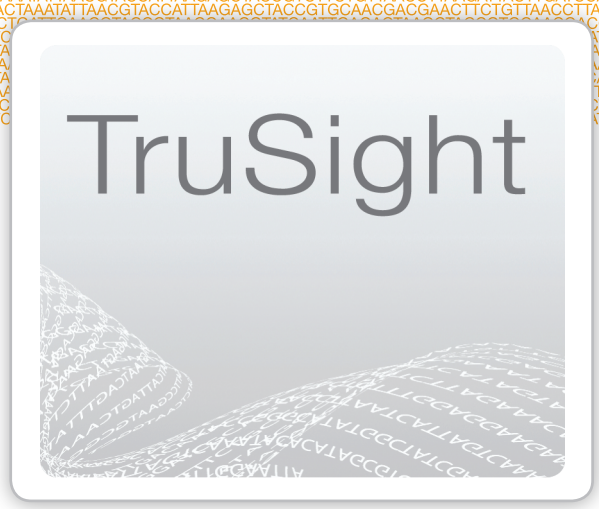
illumina®

TruSight® HLA Sequencing Panel Reference Guide



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

ILLUMINA PROPRIETARY
Part # 15056536 Rev. C
April 2015

Catalog # FC-142-9003DOC

Revision History

Part #	Revision	Date	Description of Change
15056536	C	April 2015	• Corrected duration of 72°C cycle during IndexAmp thermal cycler program in the Amplify PCR preparation steps, from 5 seconds to 5 minutes per cycle
15056536	B	March 2015	• Corrected the overfill volumes for 24 samples in step 4 of the Generate HLA PCR Amplicons procedure
15056536	A	March 2015	• Initial Release

Table of Contents

Overview

Chapter 1

Introduction

This protocol explains how to prepare indexed libraries from input DNA for subsequent cluster generation and DNA sequencing using the reagents provided in the Illumina TruSight HLA Sequencing Panel. The goal of this protocol is to isolate and amplify HLA loci with long-range polymerase chain reaction (PCR). After amplification, Nextera® tagmentation fragments the DNA and adds sequence adapters and indexing primers to generate DNA libraries ready for sequencing on the Illumina MiSeq systems.

The TruSight HLA Sequencing Panel Library Preparation protocol provides:

- A single assay and workflow to obtain ultra-high-resolution sequencing of 8 HLA Loci (Class I HLA-A, -B, -C; Class II HLA-DRB1/3/4/5, -DQA1, -DQB1, -DPA1, -DPB1)
- Unambiguous, phase-resolved HLA typing
- Sample-to-report includes assay-optimized HLA typing analysis and reporting software
- Multiplexing capabilities of up to 24 samples per MiSeq run using MiSeq v2 chemistry.
- Gene coverage including exons and introns allows for phase resolution across the entire gene, dramatically reducing the need to resolve phase ambiguities with follow-up assays

DNA Input Recommendations

- Quantify DNA with a fluorometric method specific for double-stranded DNA (dsDNA), such as the following:
  - Qubit BR assay
  - Quant-iT™ PicoGreen
- Avoid methods that measure total nucleic acid content, such as NanoDrop or other UV absorbance methods.
- Resuspend samples in water or Tris-HCl 10 mM. Normalize samples to 10 ng/µl with at least 400 ng per sample.
- The TruSight HLA Sequencing Panel Library Preparation protocol is optimized for 400 ng of purified input DNA. Illumina recommends that the DNA is intact and not fragmented; at least 50% of the DNA must be greater than 10 kb. Fragmented DNA affects the long-range PCR performance.
- If purity of the input samples is in question, Illumina recommends *Purification of DNA Samples* on page 29. Make sure that the final yield after purification is at least 40 µl at 10 ng/µl.
- Use a positive control when running the library preparation for the first time. Illumina recommends the reference DNA control sample IHW09263 from the International Histocompatibility Working Group.

Additional Resources

The following documentation is available for download from the Illumina website.

Resource	Description
TruSight HLA Sequencing Panel Protocol Guide (part # 15069450)	Provides only protocol instructions. The protocol guide is intended for experienced users. For new or less experienced users, see the TruSight HLA Sequencing Panel Library Preparation Reference Guide.
TruSight HLA Sequencing Panel Checklist (part # 15069451)	Provides a checklist of the protocol steps. The checklist is intended for experienced users. For new or less experienced users, see the TruSight HLA Sequencing Panel Library Preparation Reference Guide.
Illumina Experiment Manager Guide (part # 15031335) and *IEM TruSight HLA Quick Reference Card (part # 15069713)*	Provide information about creating and editing appropriate sample sheets for Illumina sequencing systems and analysis software and record parameters for your sample plate.
Conexio Assign™ v1.0 TruSight HLA Analysis Software (part # 15059520)	Provides information about the Conexio Assign™ v1.0 TruSight HLA Analysis Software (part # 15059520) sequencing data analysis tool. Enables you to import sequence data, perform base calling, edit sequences, and compare a consensus sequence with a library of sequences of HLA alleles.

Visit the TruSight HLA Sequencing Panel Library Preparation support pages on the Illumina website for requirements and compatibility, additional documentation, software downloads, online training, frequently asked questions, and best practices.

Protocol

Introduction

This chapter describes the TruSight HLA Sequencing Panel Library Preparation protocol.

- Review Best Practices before proceeding. See *Additional Resources* on page 4 for information on how to access TruSight HLA Sequencing Panel Library Preparation Best Practices on the Illumina website.
- Review Appendix A Supporting Information to confirm your kit contents and make sure that you have obtained all of the requisite equipment and consumables.
- Follow the protocols in the order shown, using the specified volumes and incubation parameters.
- If you are pooling samples, record information about your samples in a sample sheet before beginning library prep.
  - Use IEM to create and edit sample sheets for Illumina sequencing systems and analysis software. See *Additional Resources* on page 4 for information on how to download IEM software and documentation from the Illumina website.

Tips and Techniques

Unless a safe stopping point is specified in the protocol, proceed immediately to the next step.

Avoiding Cross-Contamination

- When adding or transferring samples, change tips between ***each sample***.
- When adding adapters or primers, change tips between ***each row*** and ***each column***.
- Remove unused index adapter tubes from the working area.

Sealing the Plate

- Always seal the 96-well plate before the following steps in the protocol:
  - Shaking steps
  - Centrifuge steps
  - Thermal cycling steps
- Apply the adhesive seal to cover the plate and seal with a rubber roller.
- Microseal 'B' adhesive seals are effective at -40°C to 110°C, and suitable for skirted or semi-skirted PCR plates.
- Foil seals are effective at -70°C to 105°C, and suitable for skirted or semi-skirted plates.
- Microseal 'A' adhesive film is effective for thermal cycling and easy to cut when using fewer than 96 wells.

Plate Transfers

- When transferring volumes between plates, transfer the specified volume from each well of a plate to the corresponding well of the other plate.
- When multiple plates are used in a step, such as a plate 1 and a plate 2, transfer volumes from the existing plate 1 to the new plate 1. Transfer volumes from the existing plate 2 to the new plate 2.

TruSight HLA Sequencing Panel Library Preparation Workflow

The following diagram illustrates the workflow using the TruSight HLA Sequencing Panel Library Preparation. Safe stopping points are indicated as cold storage options.

Figure 1 Workflow Diagram

Generate HLA PCR Amplicons

This PCR step generates 8 HLA gene amplicons from each sample using 3 PCR plates for 24 samples.

NOTE
Illumina recommends performing this PCR step overnight due to the length of the PCR program.

Consumables

- HLA Locus-Specific Primers: HLA-A, HLA-B, HLA-C, DPA1, DPB1, DQA1, DRB1, DQB1
- HPM (HLA PCR Mix)
- MasterAmp Extra-Long DNA Polymerase
- Foil adhesive seals
- PCR 8-tube strip
- PCR-grade water
- Purified DNA, 10 ng/µl (40 µl per sample)
- 96-well PCR plate, semi-skirted (3)
- 96-well midi plates (2)
- 15 ml conical tube

Preparation

1. Review *DNA Input Recommendations* on page 3.
2. Prepare a sample sheet as described in the *TruSight HLA Sample Sheet Preparation Guide*.
3. Label 3 new semi-skirted 96-well PCR plates LRP1, LRP2, and LRP3.
4. Save the following programs as PCR1 and PCR2 on a thermal cycler with a heated lid (95°C to 101°C).

PCR1: LRP1 and LRP2 Plates	PCR2: LRP3 Plate
• 94°C for 3 minutes • 35 cycles of: — 94°C for 30 seconds — 60°C for 2 minutes — 68°C for 15 minutes • 68°C for 10 minutes • Hold at 10°C	• 94°C for 3 minutes • 10 cycles of: — 94°C for 30 seconds — 55°C for 2 minutes — 72°C for 15 minutes • 25 cycles of: — 94°C for 30 seconds — 60°C for 2 minutes — 72°C for 15 minutes • 72°C for 10 minutes • Hold at 10°C

Procedure

NOTE
This procedure is time sensitive. For best results, complete the steps within 30 minutes.

1. Quantify DNA according to the *DNA Input Recommendations* on page 3.

2 Using the Illumina Index Plate Fixture, add 5 µl of each HLA primer to the LRP plates, as follows.

- HLA-A—LRP1 and LRP2 row A
- HLA-B—LRP1 and LRP2 row B
- HLA-C—LRP1 and LRP2 row C
- DPA1—LRP1 and LRP2 row D
- DPB1—LRP1 and LRP2 row E
- DQA1—LRP1 and LRP2 row F
- DRB1—LRP1 and LRP2 row G
- DQB1—LRP3 rows A and B

Figure 2 LRP1 and LRP2 Plates

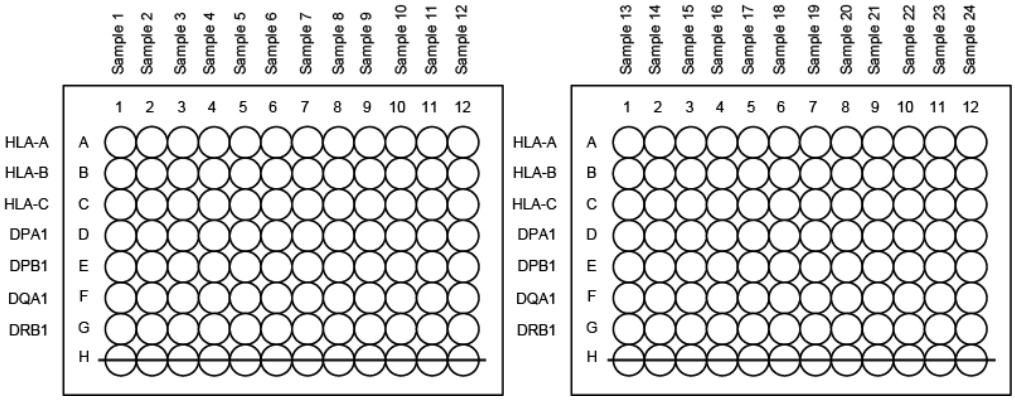
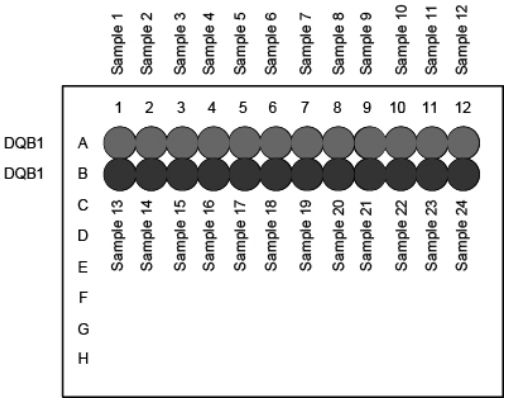


Figure 3 LPR3 Plate



3 Add 5 µl of 10 ng/µl template DNA to the LRP plates, as follows.

- Samples 1–12—LRP1 rows A–G
- Samples 1–12—LRP3 row A
- Samples 13–24—LRP2 rows A–G
- Samples 13–24—LRP3 row B

4 Combine the following reagents in a 15 ml conical tube to create PCR master mix. Volumes listed for 24 samples include 5% overfill.

PCR Component	Per Well	Per 24 Samples
HPM (HLA PCR Mix)	25 µl	5040 µl
MasterAmp Extra-Long DNA Polymerase	2 µl	403.2 µl
PCR-grade water	13 µl	2620.8 µl

5 Add 40 µl PCR master mix to each well that contains sample. Pipette to mix.

6 Centrifuge at 280 × g for 2 minutes.

7 Place the LRP1 and LRP2 plates on a thermal cycler and run the PCR1 program.

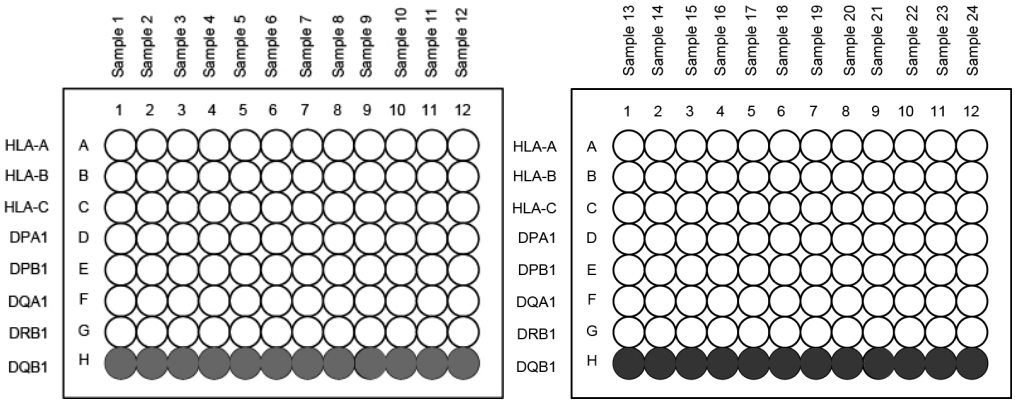
8 Place the LRP3 plate on a thermal cycler and run the PCR2 program.

9 Centrifuge the plates at 280 × g for 2 minutes.

10 Label 2 new midi plates LRC1 and LRC2.

11 Transfer samples from 3 LRP plates to 2 LRC plates, as follows.
- LRP1 rows A–G to LRC1 rows A–G
- LRP3 row A to LRC1 row H
- LRP2 rows A–G to LRC2 rows A–G
- LRP3 row B to LRC2 row H

Figure 4 LRC1 and LRC2 Plates



SAFE STOPPING POINT

If you are stopping, seal the plates and store at -25°C to -15°C for up to 7 days.

Clean Up HLA PCR Amplicons

This step uses SPB (Sample Purification Beads) to purify the HLA PCR amplicons from other reaction components. The cleanup step is performed with large volumes in midi plates to maximize yield and minimize cross-contamination.

NOTE
This procedure is time sensitive. There is sufficient time to process the first plate and then repeat the steps to process the second plate. However, if 2 sets of equipment are available, process both plates in parallel.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- 96-well midi plates
- Microseal 'B' adhesive seals

Preparation

1 Prepare the following consumables.

Reagent	Storage	Instructions
RSB	-25°C to -15°C	Thaw at room temperature. Let stand for 30 minutes to bring to room temperature.
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Prepare fresh 80% EtOH.

3 Label 2 new midi plates LRB1 and LRB2.

Procedure

Binding

1 Add 45 µl SPB to each well of the LRC1 and LRC2 plates.

2 Shake at 1800 rpm for 2 minutes.

3 Incubate at room temperature for 5 minutes.

Washing

1 Place on the magnetic stand and wait until the liquid is clear (~2 minutes).

2 Remove and discard all supernatant.

3 Wash 2 times, as follows.
   a Add 200 µl freshly prepared 80% EtOH to each sample well.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well.

4 Use a 20 µl pipette to remove residual EtOH from each well.

5 Air-dry on the magnetic stand for 5 minutes.

Elution

1 Add 30 µl RSB to each well.

2 Shake at 1800 rpm for 2 minutes.

3 Incubate at room temperature for 2 minutes.

4 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

5 Transfer 20 µl supernatant from each well of the LRC plates to the corresponding wells of the LRB plates.

6 [Optional] Check amplicon size using the following options:

- Run 5 µl resulting HLA PCR amplicon remaining in the LRC plate on a 0.8% agarose gel at 100 volts. Use a 1 kB ladder for 30 minutes or until the ladder is resolved to verify the size.
- Run 1 µl resulting HLA PCR amplicon remaining in the LRC plate on a bioanalyzer using the Agilent DNA 12000 Kit.

Class	Gene	Size (kb)
I	HLA-A	4.1
	HLA-B	2.6
	HLA-C	4.2
II	DPA1	10.3
	DPB1	9.7
	DQA1	7.3
	DRB	4.1
	DQB1	7.1

Figure 5 Resulting HLA PCR Amplicon Sizes Using Agarose Gel

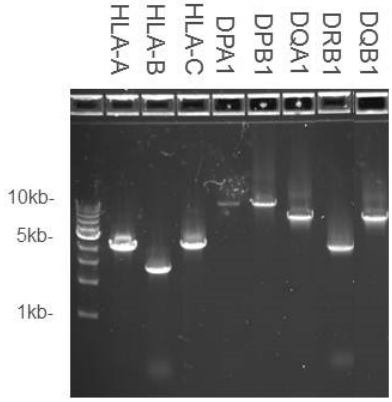
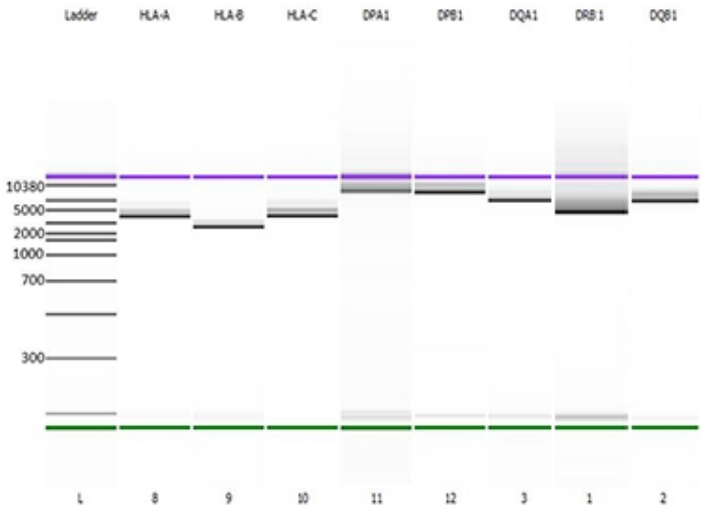


Figure 6 Example HLA PCR Amplicon Sizes Using Bioanalyzer Gel



SAFE STOPPING POINT

If you are stopping, seal the plates and store at -25°C to -15°C for up to 7 days.

Normalize HLA PCR Amplicons

This process normalizes the HLA PCR amplicons, which eliminates the need for manual quantitation and normalization.

NOTE
This procedure is time sensitive. If possible, process both plates in parallel. Otherwise, process the first plate up to the Amplify PCR step and then repeat the process for the second plate.

Consumables

- HTB (HLA Tagmentation Buffer)
- LNA1 (Library Normalization Additives 1)
- LNB1 (Library Normalization Beads 1)
- RSB (Resuspension Buffer)
- 15 ml conical tube
- Microseal 'B' adhesive seals

WARNING
This set of reagents contains formamide, an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region.
For more information, see the SDS for this kit, at support.illumina.com/sds.ilmn.

About Reagents

- Do not use a P200 pipette to handle LNB1.
- Mix only the amounts of LNA1 and LNB1 required for the current experiment.
- Store remaining LNA1 and LNB1 separately at their respective temperatures.
- Make sure that LNB1 is resuspended before use. Homogeneous resuspension is essential for consistent cluster density on the flow cell.

Preparation

1 Prepare the following consumables.

Reagent	Storage	Instructions
LNA1	-25°C to -15°C	Thaw at room temperature. Let stand for 30 minutes to bring to room temperature. Vortex vigorously, and then inspect in front of a light to make sure that all precipitate has dissolved.
LNB1	2°C to 8°C	Let stand for 30 minutes to bring to room temperature. Vigorously vortex at least 1 minute, and then invert to resuspend. Make sure that no pellet is present at the bottom of the tube.
HTB	-25°C to -15°C	Thaw at room temperature.

Procedure

1 Add 8.8 ml LNA1 to a new 15 ml conical tube.

NOTE
A volume of 4.4 ml is required for each plate.

2 Pipette to further resuspend LNB1.

3 Transfer 1.6 ml LNB1 to the 15 ml conical tube that contains LNA1.

NOTE
A volume of 800 µl is required for each plate.

4 Invert the tube to mix.

Binding

1 If the LRB plates were stored, centrifuge the plate at 280 × g for 2 minutes.
2 Add 45 µl LNB1/LNA1 mixture to each well of the LRB plates.
3 Shake at 1800 rpm for 30 minutes.

Washing

1 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
2 Remove and discard all supernatant.
3 Add 45 µl RSB to each well.
4 Shake at 1800 rpm for 5 minutes.
5 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
6 Remove and discard all supernatant.
7 Add 40 µl HTB to each well.
8 Shake at 1800 rpm for 5 minutes.

NOTE
Proceed ***immediately*** to the next step.

Tagment HLA PCR Amplicons

During this step, the Long Range HLA PCR amplicon DNA is tagmented (tagged and fragmented) by the TruSight HLA Sequencing Panel transposome. The TruSight HLA Sequencing Panel transposome simultaneously fragments the amplicon DNA and adds adapter sequences to the ends, which allows amplification by Nextera PCR in subsequent steps.

NOTE
Perform tagmentation using a thermal cycler (Option 1) or a TruTemp microheating system (Option 2).
Do not use a foil seal for the tagmentation step when using Option 2 on a TruTemp microheating system. For this step, use Microseal 'B' adhesive seals.

Consumables

- HTM (HLA Tagmentation Mix)
- PCR 8-tube strip
- [Optional] PCR plates
- Microseal 'B' adhesive seals

Preparation

1. Thaw HTM at room temperature. Gently invert to mix, and then centrifuge briefly.
2. [Option 1] Preheat a thermal cycler to 58°C.
3. [Option 1] Label 2 new 96-well PCR plates TAG1 and TAG2.
4. [Option 2] Preheat a TruTemp microheating system to 58°C.
5. Label 2 new midi plates NTC1 and NTC2.

Procedure

1. Calculate the total volume of HTM for all reactions, including 10% extra. Divide the volume equally in each well of a PCR 8-tube strip.
2. **[Option 1]** Using a thermal cycler:
   a. Transfer 40 µl from each well of the LRB plates to the corresponding wells of the TAG plates.
   b. Add 10 µl HTM to each well of the TAG plates, and then pipette to mix.
   c. Place on a thermal cycler (58°C) for 12 minutes.
   d. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
3. **[Option 2]** Using a TruTemp microheating system:
   a. Add 10 µl HTM to each well of the LRB plates.
   b. Shake for 1 minute at 1600 rpm.
   c. Place on a TruTemp microheating system set to 58°C for 12 minutes.
   d. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
4. Transfer all supernatant from each tagmentation reaction to the corresponding wells of the NTC plates.

NOTE
Proceed ***immediately*** to the next step.

Clean Up Tagmentation Reaction

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- 96-well PCR plate, semi-skirted
- Freshly prepared 80% ethanol (EtOH)
- Microseal 'B' adhesive seals

Preparation

1. Prepare the following consumables.

Reagent	Storage	Instructions
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2. Prepare fresh 80% EtOH.
3. Label 2 new semi-skirted PCR plates NPP1 and NPP2.

Procedure

Binding

1. Add 25 µl SPB to each well of the NTC plates.
2. Shake at 1800 rpm for 2 minutes.
3. Incubate at room temperature for 5 minutes.

Washing

1. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
2. Remove and discard all supernatant.
3. Wash 2 times with 200 µl 80% EtOH, as follows.
   a. Add 200 µl freshly prepared 80% EtOH to each sample well.
   b. Incubate on the magnetic stand for 30 seconds.
   c. Remove and discard all supernatant from each well.
4. Use a 20 µl pipette to remove residual EtOH from each well.
5. Air-dry on the magnetic stand for 5 minutes.

Elution

1. Add 22.5 µl RSB to each well.
2. Shake at 1800 rpm for 2 minutes.
3. Incubate at room temperature for 2 minutes.
4. Centrifuge at 280 × g for 2 minutes.
5. Place on a magnetic stand and wait for the liquid to clear (~2 minutes).

6 Transfer 20 µl supernatant to the corresponding wells of the NPP plates.

SAFE STOPPING POINT

If you are stopping, seal the plates and store at -25°C to -15°C for up to 1 day.

Amplify PCR

In this step, the tagmented DNA is amplified and sequencing adapters are added using a limited-cycle PCR program. The PCR step adds index 1 (i7) adapters, index 2 (i5) adapters, and sequences required for cluster formation. To make sure that libraries produce quality sequencing results, use the recommended amount of input DNA instead of adding extra PCR cycles. Use the index combinations selected during preparation of the sample sheet.

Consumables

- Nextera XT Index Kit v2 Set A-D (2 different index kits per 24 samples)
- NLM Library Amp Mix (Nextera Library Amplification Mix)
- PCR 8-tube strips
- TruSeq Index Plate Fixture
- Foil adhesive seals

Preparation

1 Prepare the following consumables.

Reagent	Storage	Instructions
NLM	-25°C to -15°C	Thaw at room temperature for 20 minutes. Invert each tube to mix.
Index adapters (i5 and i7)	-25°C to -15°C	Thaw at room temperature for 20 minutes. Invert each tube to mix. Centrifuge briefly using a 1.7 ml Eppendorf tube.

2 Save the following program as IndexAmp on a thermal cycler with a heated lid.

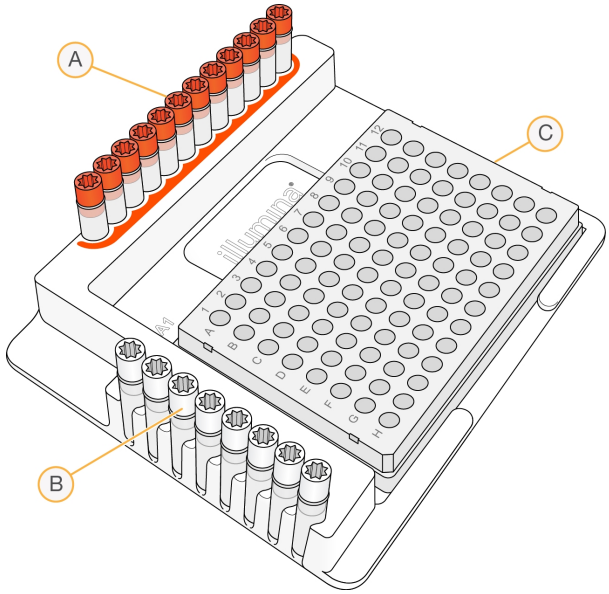
- 72°C for 3 minutes
- 98°C for 30 seconds
- 12 cycles of:
  - 98°C for 10 seconds
  - 60°C for 30 seconds
  - 72°C for 5 minutes
- 72°C for 5 minutes
- Hold at 10°C (overnight maximum)

Procedure

1 Arrange the first Nextera XT Index Kit in the TruSeq Index Plate Fixture, as follows.

- Index 1 (i7) adapters in columns 1–12.
- Index 2 (i5) adapters in rows A–H.

Figure 7 TruSeq Index Plate Fixture



A Columns 1–12: Index 1 (i7) adapters (orange caps)
B Rows A–H: Index 2 (i5) adapters (white caps)
C NPP1 plate or NPP2 plate

2 Place the NPP1 plate on a TruSeq Index Plate Fixture.

3 Using a multichannel pipette, add 5 µl of each Index 1 (i7) adapter to each row. Replace the cap on each i7 adapter tube with a new orange cap.

4 Using a multichannel pipette, add 5 µl of each Index 2 (i5) adapter to each column. Replace the cap on each i5 adapter tube with a new white cap.

5 Add 20 µl NLM to each well. Pipette to mix.

6 Centrifuge at 280 × g at 20°C for 1 minute.

7 Repeat steps 1–6 for the NPP2 plate using a different Nextera XT Index Kit.

8 Place both plates on the thermal cycler and run the IndexAmp program.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at 2°C to 8°C for up to 2 days. Alternatively, leave on the thermal cycler overnight.

Clean Up PCR

This step uses SPB (Sample Purification Beads) to purify the library DNA, and provides a size-selection step that removes small fragments from the library.

NOTE
This procedure is time sensitive. There is sufficient time to process the first plate and then repeat the steps to process the second plate. However, if 2 sets of equipment are available, process both plates in parallel.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- 96-well midi plates
- 96-well PCR plates
- Microseal 'B' adhesive seals

Preparation

1 Prepare the following consumables.

Reagent	Storage	Instructions
SPB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.
RSB	2°C to 8°C	Let stand for 30 minutes to bring to room temperature.

2 Prepare fresh 80% EtOH.

3 Label 2 new midi plates NPC1 and NPC2.

4 Label 2 new PCR plates HLP1 and HLP2.

Procedure

Binding

1 Transfer the PCR reactions from each well of the NPP plates to the corresponding wells of the NPC plates.

2 Add 25 µl SPB to each well.

NOTE
A 2:1 ratio of PCR reaction to SPB is critical.

3 Shake at 1800 rpm for 2 minutes.

4 Incubate at room temperature for 5 minutes.

Washing

1 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).

2 Remove and discard all supernatant.

3 Wash 2 times, as follows.
   a Add 200 µl freshly prepared 80% EtOH to each sample well.
   b Incubate on the magnetic stand for 30 seconds.
   c Remove and discard all supernatant from each well.
4 Use a 20 µl pipette to remove residual EtOH from each well.
5 Air-dry on the magnetic stand for 5 minutes.

Elution

1 Add 32.5 µl RSB to each well.
2 Shake at 1800 rpm for 2 minutes.
3 Incubate at room temperature for 2 minutes.
4 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
5 Transfer 30 µl supernatant from each well of the NPC plates to the corresponding well of the HLP plates.

SAFE STOPPING POINT

If you are stopping, seal the plates and store at 2°C to 8°C for up to 7 days.

Library Pooling and MiSeq Sequencing

In preparation for cluster generation and sequencing, equal volumes of library are combined, denatured in NaOH, and diluted in Hybridization Buffer before MiSeq sequencing. This procedure is a guideline for dilution for optimal cluster density. Adjust final dilution on a sample-to-sample basis.

Illumina recommends the following pooling guidelines:

- For a standard MiSeq v2 flow cell—Pool 24 samples, 192 libraries
- For a nano MiSeq v2 flow cell—Pool 6 samples, 48 libraries

Consumables

- HT1 (Hybridization Buffer) provided in the MiSeq Reagent Kit
- MiSeq reagent cartridge v2 (500 cycles), standard or nano
- 0.1 N NaOH (prepared fresh)
- 1.5 ml Eppendorf Tube
- Deionized water
- Qubit dsDNA BR Assay Kit

Preparation

1 Prepare the following consumables.

Reagent	Reagent	Instructions
MiSeq reagent cartridge	-25°C to -15°C	Thaw in a room temperature water bath for 1 hour, or overnight at 2°C to 8°C.
HT1	-25°C to -15°C	Thaw at room temperature. Set aside at 2°C to 8°C.

2 Prepare a fresh dilution of 0.1 N NaOH from 1 N NaOH.

3 Label a new 1.5 ml Eppendorf tube PHL.

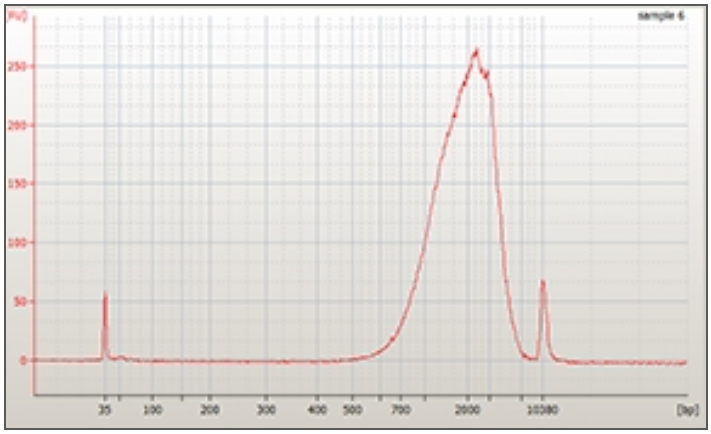
4 Label a new 1.5 ml Eppendorf tube DHL.

Procedure

1 Transfer 5 µl from each well of the HLP plates to the PHL tube.

2 [Optional] Dilute 1 µl pooled sample 1:10 and run on High Sensitivity Bioanalyzer chip.

Figure 8 Size Distribution of HLA Library



3 Quantify the library with the Qubit BR assay or a fluorometric assay, such as PicoGreen.

4 Determine the library volume to denature using the following formula:
-0.67(X) + 11.9 = Y
- X is the library concentration (ng/µl) as determined by the fluorometric assay
- Y is the library volume (µl) to dilute and denature

5 Transfer the volume determined by Y to the DHL tube.

NOTE
If Y > 10 µl in the calculation, the library yield might be too low. Contact Illumina Technical Support at techsupport@illumina.com.

6 Dilute with deionized water to a final volume of 10 µl.

7 Add 10 µl 0.1 N NaOH.

8 Vortex and then centrifuge briefly to mix.

9 Incubate at room temperature for 5 minutes.

10 Add 980 µl HT1 for a final volume of 1000 µl, and then invert to mix.

11 Load 600 µl denatured library from the DHL tube onto the thawed reagent cartridge.

NOTE
See the *MiSeq System Guide (part # 15027617)* for information about loading consumables and setting up a run.

Supporting Information

Appendix A

Introduction

The protocols described in this guide assume that you have reviewed the contents of this appendix, confirmed your kit contents, and obtained all of the requisite consumables and equipment.

Purification of DNA Samples

If sample purity is in question, perform this step for up to 96 samples at a time. SPB beads are used to purify input DNA of interfering contaminants in the HLA PCR amplicons. If testing all 8 TruSight HLA alleles, make sure that the final DNA after purification is at least 40 µl at 10 ng/µl. For optimal yield, Illumina recommends starting sample purification with at least 50 µl at 16 ng/µl high-quality DNA.

Consumables

- RSB (Resuspension Buffer)
- SPB (Sample Purification Beads)
- Freshly prepared 80% ethanol (EtOH)
- 96-well midi plates
- 96-well PCR plate, semi-skirted
- Microseal 'B' adhesive seals

Preparation

1. Let SBP stand for 20 minutes to bring to room temperature.
2. Prepare fresh 80% EtOH.

Procedure

Binding

1. Label a new 96-well midi plate ISC.
2. Add 50 µl input sample.
3. Add 50 µl SPB.
4. Shake at 1800 rpm for 2 minutes.
5. Incubate at room temperature for 5 minutes.

Washing

1. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
2. Remove and discard 100 µl supernatant.
3. Wash 2 times with 200 µl 80% EtOH, as follows.
   a. Add 200 µl freshly prepared 80% EtOH to each sample well.
   b. Incubate on the magnetic stand for 30 seconds.
   c. Remove and discard all supernatant from each well.
4. Using a 20 µl pipette, remove residual EtOH from each well.
5. Air-dry on the magnetic stand for 5 minutes.

Elution

1. Add 52.5 µl RSB.
2. Remove from the magnetic stand.

3 Shake at 1800 rpm for 2 minutes.

4 Incubate at room temperature for 2 minutes.

5 Place on a magnetic stand and wait for the liquid to clear (~2 minutes).

6 Label a new 96-well PCR plate IHP.

7 Transfer 50 µl supernatant.

8 Quantify the sample using a fluorometric method, such as the Qubit BR assay.

9 Normalize each sample to 10 ng/µl with at least 40 µl RSB per sample.

NOTE

Do not pipette less than 2 µl during dilution. Use serial dilutions, if necessary.

SAFE STOPPING POINT

If you are stopping, seal the plate and store at -25°C to -15°C for up to 7 days.

Acronyms

Acronym	Definition
DHL	Diluted HLA Libraries
HLP	HLA Library Plate
HPM	HLA PCR Mix
HTB	HLA Tagmentation Buffer
HTM	HLA Tagmentation Mix
IHP	Input HLA Plate
ISC	Input Sample Clean Up
LNA1	Library Normalization Additives 1
LNB1	Library Normalization Beads 1
LRB	Long Range Bead Based Normalization 2
LRC	Long Range Clean Up
LRP	Long Range PCR
NLM	Nextera Library Amplification Mix
NPC	Nextera PCR Clean Up
NPP	Nextera PCR Plate
NTC	Nextera Tagmentation Clean Up
PHL	Pool HLA Libraries
RSB	Resuspension Buffer
SPB	Sample Purification Beads

TruSight HLA Sequencing Panel Library Preparation Kit Contents

The TruSight HLA Sequencing Panel Library Preparation is packaged and shipped on dry ice unless specified otherwise. Index kits are sold separately.

Each HLA locus requires a unique index combination or 8 unique index combinations per sample. Illumina offers 4 unique 96 index Nextera XT Index kits.

Illumina recommends running 24 samples of 8 loci per sample (192 libraries) using a standard MiSeq Reagent Kit v2 (flow cell and cartridge). Alternatively, you can run 6 samples (48 libraries) using a MiSeq Nano Reagent Kit v2 (flow cell and cartridge).

Consumable	Catalog #
TruSight HLA Sequencing Panel Library Preparation (24 Samples)	FC-142-1001
Nextera XT Index Kit v2 Set A (96 Indexes)	FC-131-2001
Nextera XT Index Kit v2 Set B (96 Indexes)	FC-131-2002
Nextera XT Index Kit v2 Set C (96 Indexes)	FC-131-2003
Nextera XT Index Kit v2 Set D (96 Indexes)	FC-131-2004

TruSeq Index Plate Fixture Kit

Illumina recommends using the index plate fixture to help with correctly arranging the index primers during the PCR Amplification steps.

Consumable	Catalog #
TruSeq Index Plate Fixture Kit	FC-130-1005

TruSight HLA Sequencing Panel Library Preparation Contents (FC-142-1001)

NOTE
Some components in the kit require a different storage temperature other than the temperature shipped. As soon as you receive your kit, store the kit components at the specified temperature.

- Box 1: Pre-PCR 2°C to 8°C

Quantity	Acronym	Reagent Name	Storage Temperature
2	SPB	Sample Purification Beads	2°C to 8°C

- Box 2: Pre-PCR -25°C to -15°C

Quantity	Acronym	Reagent Name	Storage Temperature
1	RSB	Resuspension Buffer	-25°C to -15°C
1	HLA-A	HLA-A Primers	-25°C to -15°C
1	HLA-B	HLA-B Primers	-25°C to -15°C
1	HLA-C	HLA-C Primers	-25°C to -15°C
1	DRB	HLA-DRB Primers	-25°C to -15°C
1	DQB1	HLA-DQB1 Primers	-25°C to -15°C
1	DQA1	HLA-DQA1 Primers	-25°C to -15°C
1	DPB1	HLA-DPB1 Primers	-25°C to -15°C

Quantity	Acronym	Reagent Name	Storage Temperature
1	DPA1	HLA-DPA1 Primers	-25°C to -15°C
5	MasterAmp	DNA Polymerase	-25°C to -15°C
1	HPM	HLA PCR Mix	-25°C to -15°C

▸ Box 3: Post-PCR 2°C to 8°C

Quantity	Acronym	Reagent Name	Storage Temperature
3	SPB	Sample Purification Beads	2°C to 8°C
3	LNB1	Library Normalization Beads	2°C to 8°C

▸ Box 4: Post-PCR -25°C to -15°C

Quantity	Acronym	Reagent Name	Storage Temperature
3	RSB	Resuspension Buffer	-25°C to -15°C
3	LNA1	Library Normalization Additives	-25°C to -15°C
2	HTM	HLA Tagmentation Mix	-25°C to -15°C
2	HTB	HLA Tagmentation Buffer	-25°C to -15°C
2	NLM	Nextera Library Amplification Mix	-25°C to -15°C

Consumables and Equipment

Make sure that you have the following user-supplied consumables and equipment before proceeding to library preparation. Consumables and equipment are recommended for the TruSight HLA Sequencing Panel Library Preparation protocols.

Table 1 User-Supplied Consumables

Consumable	Supplier
20 µl pipette filter tips	General lab supplier
20 µl multichannel pipettes	General lab supplier
20 µl single channel pipettes	General lab supplier
1000 µl pipette filter tips	General lab supplier
1000 µl multichannel pipettes	General lab supplier
1000 µl single channel pipettes	General lab supplier
200 µl pipette filter tips	General lab supplier
200 µl multichannel pipettes	General lab supplier
200 µl single channel pipettes	General lab supplier
96-well storage plates, round well, 0.8 ml (midi plate)	Fisher Scientific, part # AB-0859
Ethanol 200 proof (absolute) for molecular biology (500 ml)	Sigma-Aldrich, part # E7023
96-well PCR plates compatible with thermal cycler	General lab supplier
RNase/DNase-free multichannel reagent reservoirs, disposable	VWR, part # 89094-658
Tris-HCl 10 mM, pH 8.5	Prepared from Tris-HCl salt General lab supplier
Laboratory Grade water	General lab supplier
Foil seals	Beckman Coulter, part # 538619
Microseal 'B' adhesive seals	Bio-Rad, part # MSB-1001
MiSeq Reagent Kit v2 (500 cycles)	Illumina
1.5 ml microcentrifuge tubes	General lab supplier
2 N NaOH	Sigma Molecular Grade 10 N NaOH part # 72068 General lab supplier

Table 2 User-Supplied Equipment

Equipment	Supplier
96-well thermal cycler (with heated lid)	See table in *Thermal Cycler* section.
[Optional] Microheating System-SciGene TruTemp Heating System	Illumina, catalog # SC-60-503 (115 V) or catalog # SC-60-504 (220 V)
[Optional] midi plate insert for microheating system*	Illumina, catalog # BD-60-601
High-Speed Microplate Shaker (1800 rpm)	BioShake iQ, catalog # 1808-0505 or VWR, catalog # 14216-214 (230 V) or VWR, catalog # 13500-890 (110 V/120 V)
Magnetic stand-96	Ambion, part # AM10027
Microplate centrifuge	General lab supplier
Vortexer	General lab supplier

*For high throughput labs, use of the MIDI block instead of a thermal cycler. The MIDI block reduces pipetting steps and consumable use.

Thermal Cycler

The following table lists the recommended settings for selected thermal cycler models. Illumina recommends that you validate any thermal cyclers not listed. If your lab has not yet performed the TruSight HLA Sequencing Panel Library Preparation protocol.

Thermal Cycler	Lid Temp	Vessel Type
Bio-Rad C1000	Heated	Plate
Applied Biosystems GeneAmpPCR System 9700	Heated	Plate
ABI veriti	Heated	Plate
Bio-Rad DNA Engine Tetrad 2	Heated, Constant at 100°C	Polypropylene plates and tubes
Eppendorf Nexus	Heated	Plate

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 3 Illumina General Contact Information

Website	www.illumina.com
Email	techsupport@illumina.com

Table 4 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Australia	1.800.775.688	Netherlands	0800.0223859
Austria	0800.296575	New Zealand	0800.451.650
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

Safety Data Sheets

Safety data sheets (SDSs) are available on the Illumina website at support.illumina.com/sds.html.

Product Documentation

Product documentation in PDF is available for download from the Illumina website. Go to support.illumina.com, select a product, then select **Documentation & Literature**.

Illumina
5200 Illumina Way
San Diego, California 92122 U.S.A.
+1.800.809.ILMN (4566)
+1.858.202.4566 (outside North America)
techsupport@illumina.com
www.illumina.com