Generate HLA PCR Amplicons

☐1 Add 5 µl of each HLA primer to the LRP plates.
☐2 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

☐3 Combine the following reagents in a 15 ml conical tube to create PCR master mix.

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

☐4 Add 40 µl PCR master mix. Pipette to mix.
☐5 Centrifuge at 280 × g for 2 minutes.
☐6 Place the LRP1 and LRP2 plates on a thermal cycler and run the PCR1 program.
☐7 Place the LRP3 plate on a thermal cycler and run the PCR2 program.
☐8 Centrifuge the plates at 280 × g for 2 minutes.
☐9 Label 2 new midi plates LRC1 and LRC2.
☐10 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

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

☐1 Add 45 µl SPB to the LRC1 and LRC2 plates.
☐2 Shake at 1800 rpm for 2 minutes.
☐3 Incubate at room temperature for 5 minutes.
☐4 Place on the magnetic stand and wait until the liquid is clear (~2 minutes).
☐5 Remove and discard all supernatant.
☐6 Wash 2 times with 200 µl 80% EtOH.
☐7 Use a 20 µl pipette to remove residual EtOH.
☐8 Air-dry on the magnetic stand for 5 minutes.
☐9 Add 30 µl RSB.
☐10 Shake at 1800 rpm for 2 minutes.
☐11 Incubate at room temperature for 2 minutes.
☐12 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
☐13 Transfer 20 µl supernatant to the LRB plates.

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

☐1 Add 8.8 ml LNA1 to a new 15 ml conical tube.
☐2 Pipette to further resuspend LNB1.
☐3 Transfer 1.6 ml LNB1 to the 15 ml conical tube that contains LNA1.
☐4 Invert the tube to mix.
☐5 Add 45 µl LNB1/LNA1 mixture to the LRB plates.
☐6 Shake at 1800 rpm for 30 minutes.
☐7 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
☐8 Remove and discard all supernatant.
☐9 Add 45 µl RSB.
☐10 Shake at 1800 rpm for 5 minutes.
☐11 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
☐12 Remove and discard all supernatant.
☐13 Add 40 µl HTB.
☐14 Shake at 1800 rpm for 5 minutes.

Tagment HLA PCR Amplicons

☐1 **[Option 1]** Using a thermal cycler:
- ☐a Transfer 40 µl to the TAG plates.
- ☐b Add 10 µl HTM to 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).

☐2 **[Option 2]** Using a TruTemp microheating system:
- ☐a Add 10 µl HTM to 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).

☐3 Transfer all supernatant the NTC plates.

Clean Up Tagmentation Reaction

☐1 Add 25 µl SPB to the NTC plates.
☐2 Shake at 1800 rpm for 2 minutes.
☐3 Incubate at room temperature for 5 minutes.
☐4 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
☐5 Remove and discard all supernatant.
☐6 Wash 2 times with 200 µl 80% EtOH.
☐7 Use a 20 µl pipette to remove residual EtOH.
☐8 Air-dry on the magnetic stand for 5 minutes.
☐9 Add 22.5 µl RSB.
☐10 Shake at 1800 rpm for 2 minutes.
☐11 Incubate at room temperature for 2 minutes.
☐12 Centrifuge at 280 × g for 2 minutes.
☐13 Place on a magnetic stand and wait for the liquid to clear (~2 minutes).
☐14 Transfer 20 µl supernatant to 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

☐1 Arrange the first Nextera XT Index Kit, as follows.
- Index 1 (i7) adapters in columns 1–12.
- Index 2 (i5) adapters in rows A–H.

☐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.
☐4 Using a multichannel pipette, add 5 µl of each Index 2 (i5) adapter to each column.
☐5 Add 20 µl NLM. 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

- ☐1 Transfer the PCR reactions to the NPC plates.
- ☐2 Add 25 µl SPB.
- ☐3 Shake at 1800 rpm for 2 minutes.
- ☐4 Incubate at room temperature 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 Wash 2 times with 200 µl 80% EtOH.
- ☐8 Use a 20 µl pipette to remove residual EtOH.
- ☐9 Air-dry on the magnetic stand for 5 minutes.
- ☐10 Add 32.5 µl RSB.
- ☐11 Shake at 1800 rpm for 2 minutes.
- ☐12 Incubate at room temperature for 2 minutes.
- ☐13 Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
- ☐14 Transfer 30 µl supernatant to 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

- ☐1 Transfer 5 µl from each well to the PHL tube.
- ☐2 Quantify the library .
- ☐3 Determine the library volume to denature:
  -0.67(X) + 11.9 = Y
  - X is the library concentration (ng/µl)
  - Y is the library volume (µl)
- ☐4 Transfer the volume determined by Y to the DHL tube.
- ☐5 Dilute with deionized water to a final volume of 10 µl.
- ☐6 Add 10 µl 0.1 N NaOH.
- ☐7 Vortex and then centrifuge briefly to mix.
- ☐8 Incubate at room temperature for 5 minutes.
- ☐9 Add 980 µl HT1, and then invert to mix.
- ☐10 Load 600 µl denatured library onto the thawed reagent cartridge.

Acronyms

Acronym	Definition
DHL	Diluted HLA Libraries
HLP	HLA Library Plate
HPM	HLA PCR Mix
HTB	HLA Tagmentation Buffer
HTM	HLA Tagmentation Mix
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