eppendorf

SHORT PROTOCOL No. 41

Automated Illumina® TruSight® HLA v2 Sequencing Panel Library Preparation with the ep*Motion*® 5075t

Introduction

This protocol describes the workstation configuration and pre-programmed sub-methods for the automated construction of 8 sequencing libraries with the TruSight HLA v2 Sequencing Panel. The TruSight HLA v2 Sequencing Panel provides a comprehensive human leukocyte antigen (HLA) typing solution for simple, rapid assessment of the 11 most commonly-typed HLA loci in a single assay.
The ep*Motion* method translates a pipetting-intensive protocol into a ready-to-run method requiring minimal interventions and setup time. The workflow allows maximum walk-away time and reduces human error. The overall hands-on time is less than 30 minutes and the total run time is about 4 hours. The workflow is divided into five sub-methods on the ep*Motion* 5075t (Figure 1). This protocol is optimized towards efficient processing of 8 samples with a multichannel tool. Therefore the number of samples, which can be processed in parallel, is fixed.

Materials and Methods

Methods

The Illumina TruSight HLA v2 Sequencing Panel targets 11 genetic loci for genotyping using 8 long-range PCR products. To increase the amplification efficiency and prevent oligo interference, each DNA sample is equally divided into 8 fractions that undergo separate, parallel preparations, which are pooled after tagmentation to simplify the workflow as well as conserving reagents. The protocol is split into 5 sub-methods (Figure 1), each stopping at a safe point according to the manufacturer's manual. In addition, to maximize the liquid handling speed, samples are laid out in a column instead of a row (instructed in Illumina's Reference Guide) to allow 8-channel pipetting. The following sections describe the setup for each of these sub-methods.

		Tip consumption	
		50 µL filter tips	300 µL filter tips
PCR Setup for HLA Amplicons	Sub-method I (20 min)	24	0
⬇			
Long-range PCR			
⬇			
Clean-up HLA PCR Amplicons	Sub-method II (55 min)	152	208
⬇			
Normalize HLA PCR Amplicons	Sub-method III (115 min)	256	96
⬇			
Tagment HLA PCR Amplicons			
⬇			
Amplicon Pooling and Clean-up			
⬇			
Amplify and Indexing PCR Setup	Sub-method IV (10 min)	17	0
⬇			
Indexing PCR			
⬇			
Clean-up	Sub-method V (30 min)	32	24
⬇			
Library Pooling & Loading			
Total	**Runtime: 230 min**	**481**	**328**

Figure 1: on the epMotion with each step in dark grey boxes.
These steps are grouped into ep*Motion* sub-methods shown in light blue boxes. Steps in green boxes are performed off deck.
Runtimes on the epMotion and tip consumption are also shown.

Sub-method I

Sub-method I features the setup of long-range PCR reactions to amplify HLA loci. Start with 8 DNA samples (>10 ng/µL) supplied in the "Sample Plate". To ensure the quality of libraries at least 45 µL of DNA with a minimal concentration of 10 ng/µL is required. DNA samples should be supplied in the first column (Wells A1-H1) of a skirted Eppendorf twin.tec® PCR Plate 96 LoBind. Locus-specific primer vials should be uncapped and placed in the Rack ILMN tubes as shown in figure 3. Freshly prepared master mix is recommended to be provided with a 10 % excess in an Eppendorf 5 mL tube placed in the Reservoir Rack as shown in figure 4. Please consult Illumina's Reference Guide for the composition of the master mix. The ep*Motion* will use the supplied reagents to set up a total of 64 PCR reactions in the "LRP1 plate" and "LRP2 plate". Following the setup on the system, these plates should be manually sealed and long-range PCR is performed on two thermal cyclers running "PCR1" and "PCR2" programs as described in the reference guide. This step may require over-night cycling as the amplification time is ~10 hours.

Worktable Layout

Position	Item
A2	50 µL Filtertips
A3	50 µL Filtertips
B3	Skirted Eppendorf twin.tec PCR Plate 96 LoBind with DNA template ("Sample Plate")
B4	Rack ILMN tubes with primers (see figure 3)
C2 (Temp2)	Reservoir Rack with 1x Reservoir Rack Module TC for Eppendorf Tubes® 5.0 mL (see figure 4)
C3	Thermoadapter PCR 96 + empty semi-skirted Eppendorf twin.tec PCR Plate 96 LoBind ("LRP1 plate")
C4	Thermoadapter PCR 96 + empty semi-skirted Eppendorf twin.tec PCR Plate 96 LoBind ("LRP2 plate")

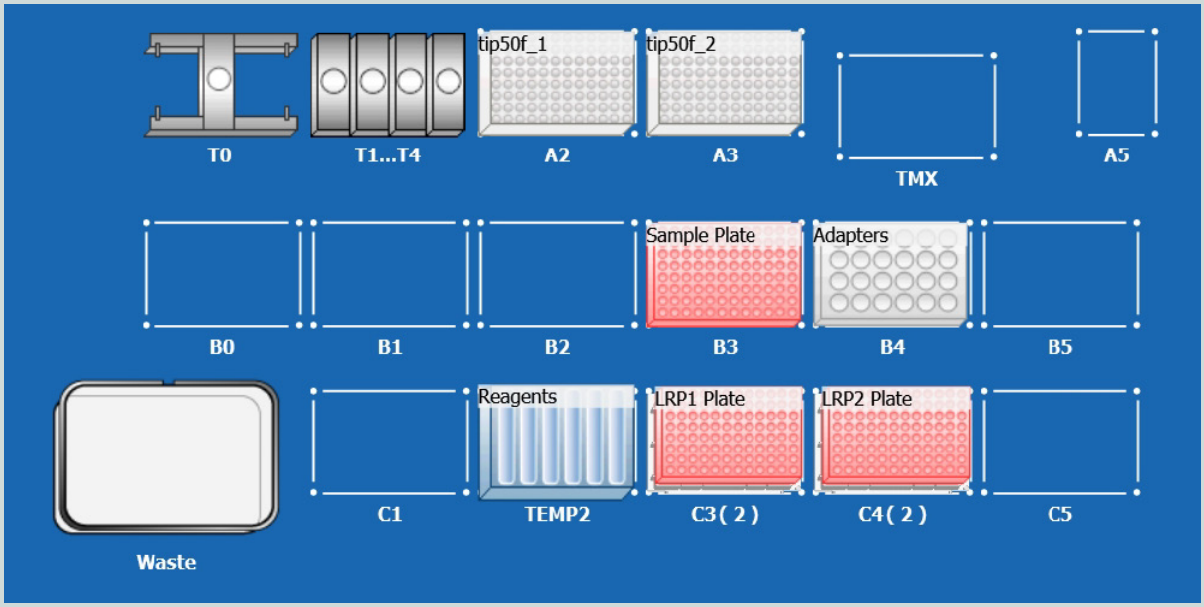

Figure 2: ep*Motion* worktable layout for sub-method I as described in the text.

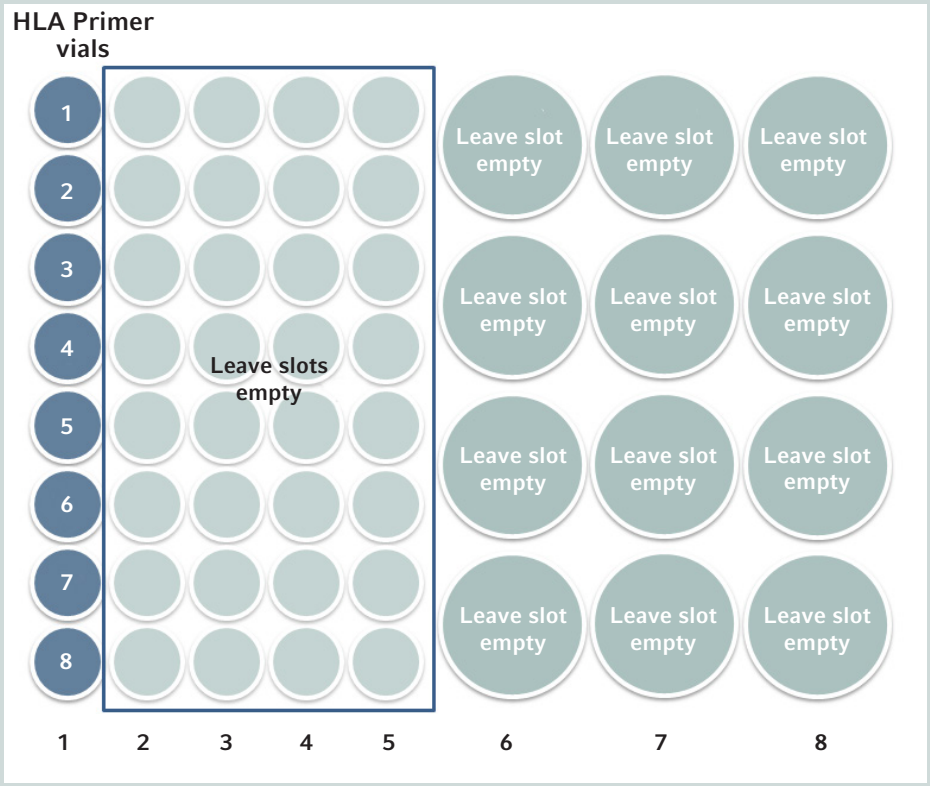

Figure 3: Rack ILMN tubes layout for sub-method I. Numbers in the first column correspond to the following primers from the kit:
1: Primers HLA-A
2: Primers HLA-B.2
3: Primers HLA-C
4: Primers DPA1
5: Primers DPB1.1
6: Primers DQA1;
7: Primers DRB.2
8: Primers DRB.2

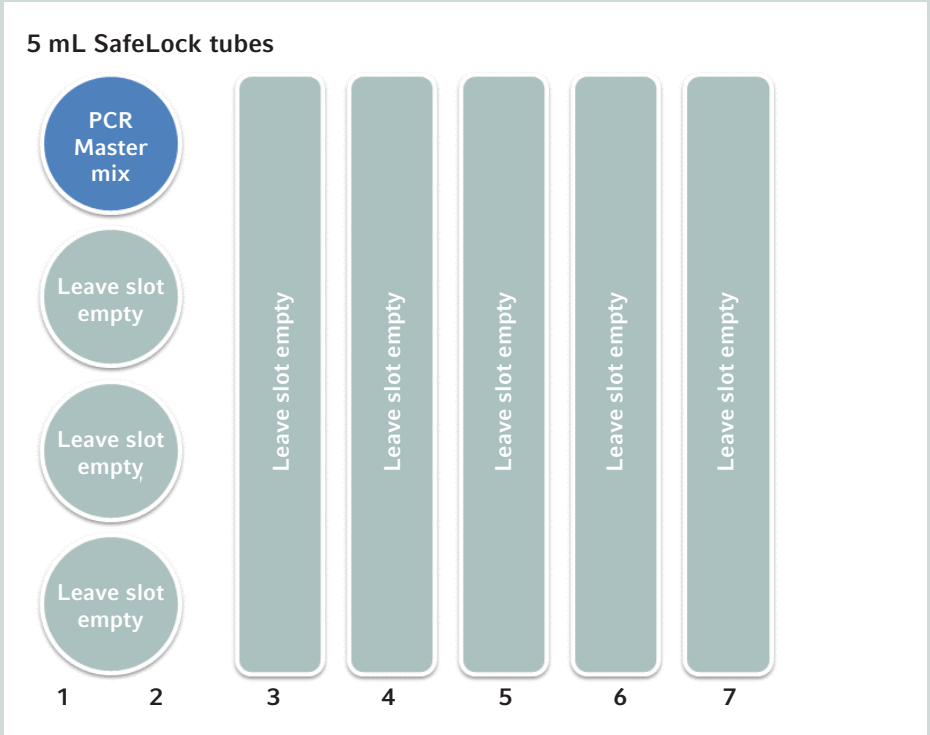

Figure 4: Reservoir Rack layout for sub-method I as described in the text.

Sub-method II

Sub-method II consolidates and purifies the long-range PCR products after thermal cycling from "LRP1 Plate" and "LRP2 Plate". Sample Purification Beads (SPB), 80 % Ethanol and Resuspension Buffer (RSB) in the Reservoir Rack (Figure 6) should be freshly supplied in 30 mL reservoirs with at least 10 % excess. Make sure SPB are fully re-suspended before transfer to the reservoir. The sub-method ends with purified products in the "LRB" plate, which is used in sub-method III.

Worktable Layout

Position	Item
A2	50 µL Filtertips
A3	50 µL Filtertips
A4 (TMX)	Thermoblock PCR 96 OC
B0	Eppendorf 400 mL Reservoir for liquid waste
B2	300 µL Filtertips
B3	300 µL Filtertips
B4	300 µL FiltertipsC1 Alpaqua® MAGNUM FLX® Enhanced Universal Magnet Plate
C2 (Temp)	Reservoir Rack (see figure 6)
C3	Thermoadapter PCR 96 + semi-skirted Eppendorf twin.tec PCR plate ("LRP1 plate") from sub-method I
C4	Thermoadapter PCR 96 + semi-skirted Eppendorf twin.tec PCR plate ("LRP2 plate") from sub-method I
C5	Thermoadapter PCR 96 + semi-skirted Eppendorf twin.tec PCR plate ("LRB")

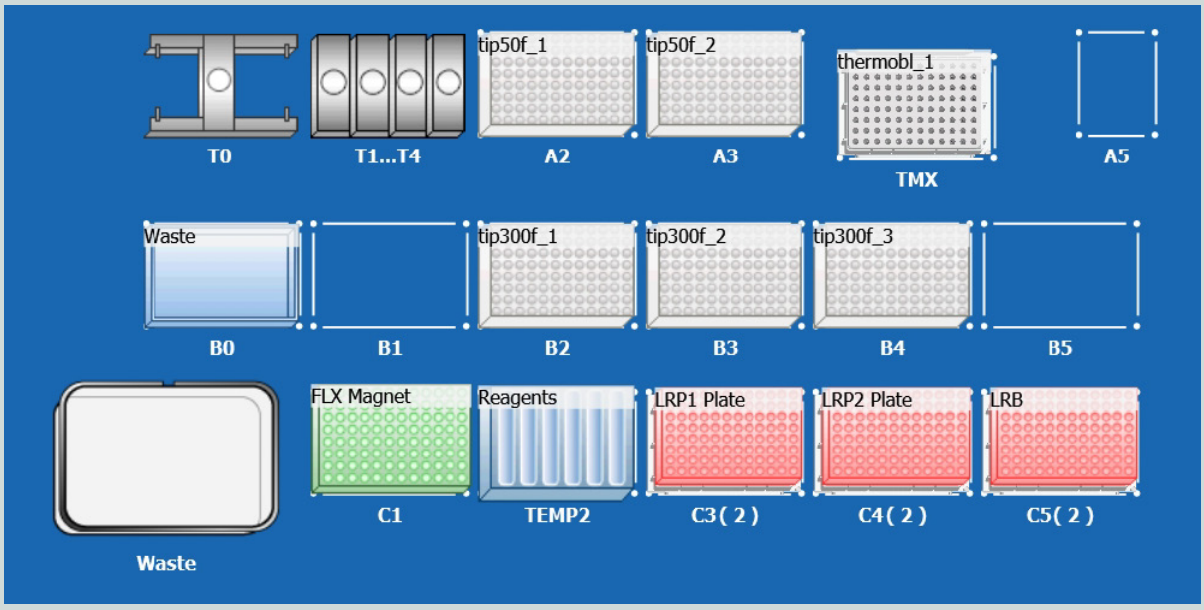

Figure 5: ep*Motion* worktable layout for sub-method II as described in the text.

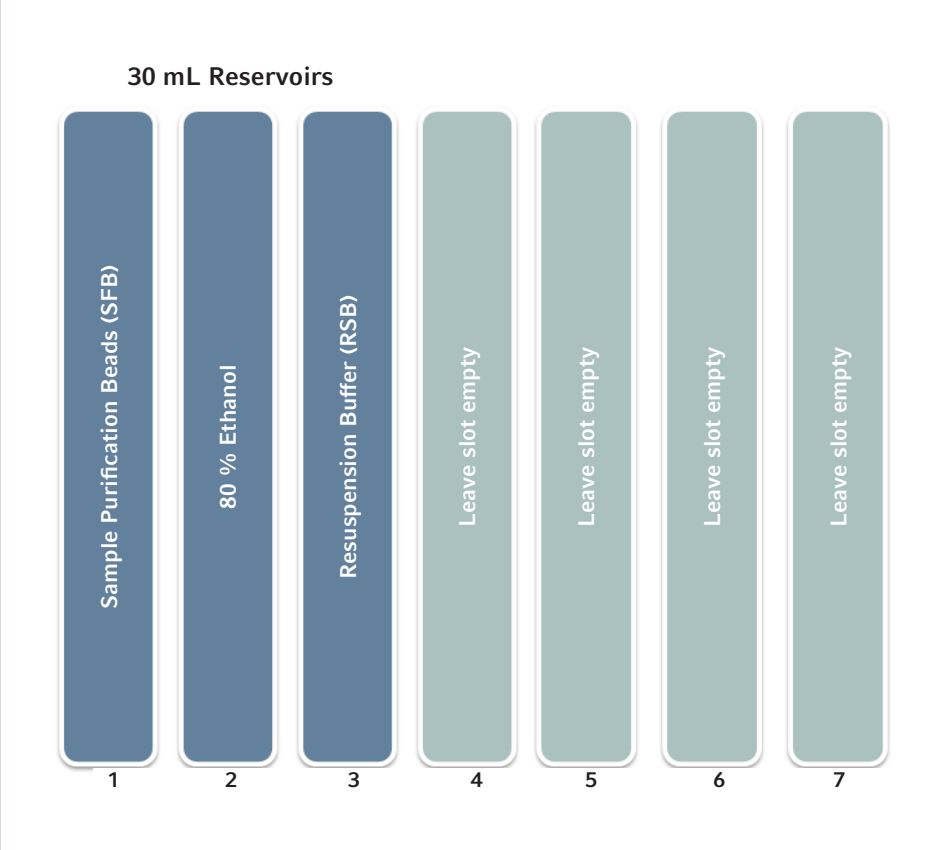

Figure 6: Reservoir Rack layout for sub-method II as described in the text.

Sub-method III

Sub-method III features on-deck normalization and Nextera® tagmentation, which fragments the DNA and adds PCR tags on both ends of the fragments. The sub-method begins with the "LRB" plate on the TMX position from sub-method II. SPB, 80 % Ethanol, RSB, LNA1 (Library Normalization Additives 1) and LNB1 (Library Normalization Beads 1), HLA Tagmentation Buffer (HTB) in the Reservoir Rack should be freshly supplied in 30 mL reservoirs (Figure 8) with at least 10 % excess. In addition, HLA tagmentation mix should be provided in Eppendorf 0.2 mL PCR tubes with at least 10 % excess in the reservoir rack (Figure 8). LNA1 and LNB1 should be mixed freshly in accordance to the Reference Guide. Make sure that SPB and normalization beads are fully re-suspended before transferring them to the reservoir. At the end of the sub-method, all DNA samples are pooled, purified and stored in the "TPP" plate according to their original sample identity.

Worktable layout

Position	Item
A2	50 µL Filtertips
A3	50 µL Filtertips
A4 (TMX)	Thermoblock PCR 96 OC + semi-skirted Eppendorf twin.tec® PCR Plate 96 LoBind with samples ("LRB") from sub-method II
B0	Eppendorf 400 mL Reservoir for liquid waste
B1	50 µL Filtertips
B2	300 µL Filtertips
B3	300 µL Filtertips
C1	Alpaqua MAGNUM FLX Enhanced Universal Magnet Plate
C2 (Temp)	Thermoadapter PCR 96
C3	Reservoir Rack (see figure 8)
C5	Thermoadapter PCR 96 + empty semi-skirted Eppendorf twin.tec PCR Plate 96 LoBind (TPP)

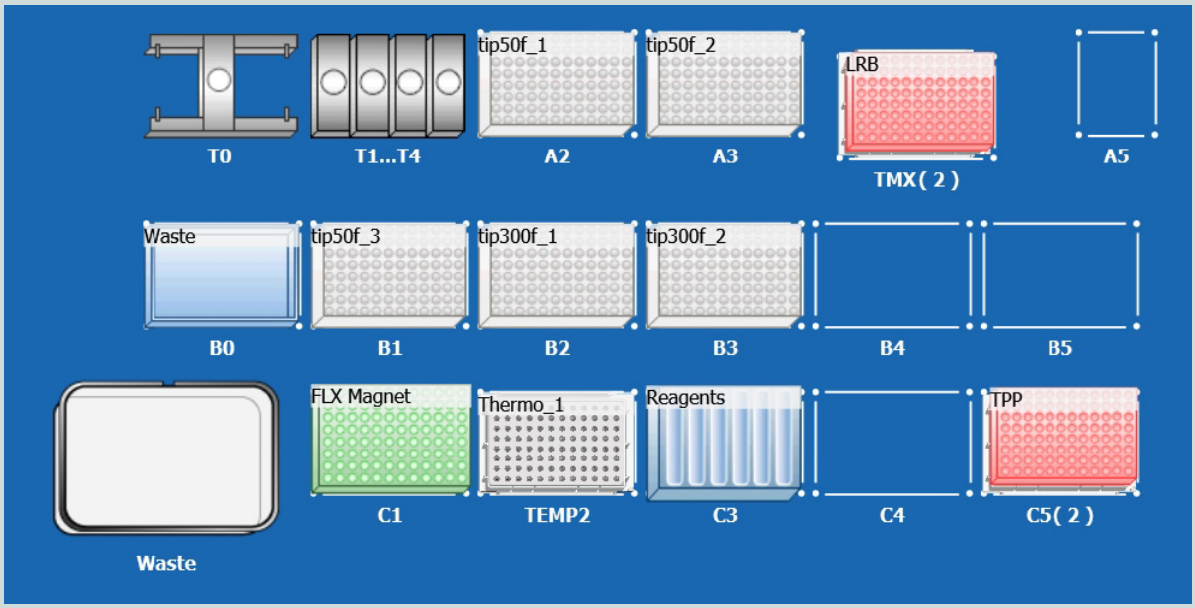

Figure 7: ep*Motion* worktable layout for sub-method III as described in the text.

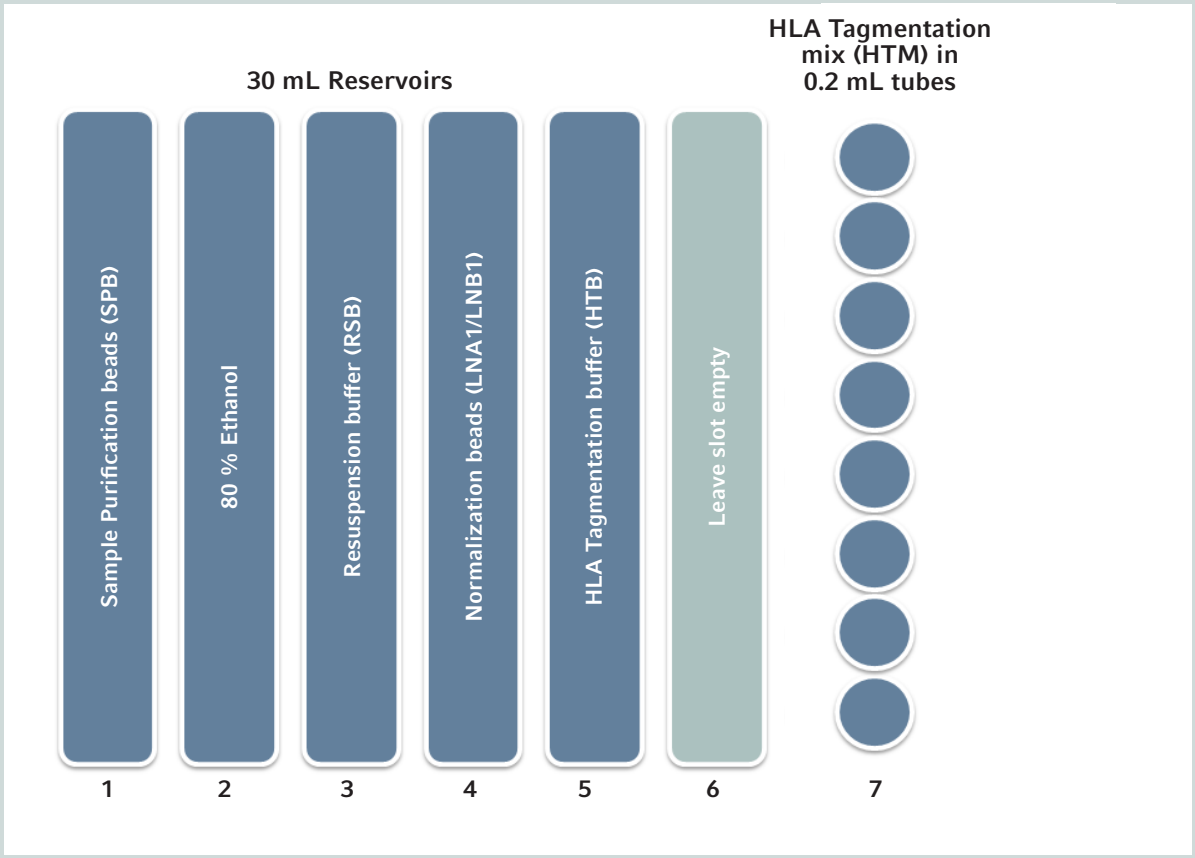

Figure 8: Reservoir Rack layout for sub-method III as described in the text.

Sub-method IV

Sub-method IV prepares the library amplification PCR, which uniquely adds indexes to samples and generates sequencing-ready DNA libraries. The method begins with the "TPP" plate from method III. Remove the caps from the kit-supplied i7- and i5-index tubes and place them according to figure 10. 4 i5-index tubes and 2 i7-index tubes should be provided as shown in figure 10. Additionally, Nextera Library Amplification (NLM) mix should be supplied in an Eppendorf 1.5 mL Safe-Lock tube with at least 10 % extra (Figure 10). The samples will be indexed as shown in Table 1. At the end of the sub-method, the "TPP" plate should be placed in a thermal cycler to run the "IndexAmp" program as described in the reference guide.

Worktable layout

Position	Item
A2	50 µL Filtertips
A3	50 µL Filtertips
A4 (TMX)	Thermoblock PCR 96 OC
B3	Rack ILMN tubes with index primers (see figure 10)
C1	Alpaqua MAGNUM FLX Enhanced Universal Magnet Plate
C2 (Temp)	Thermoadapter PCR 96 + semi-skirted Eppendorf twin.tec PCR Plate 96 LoBind with samples (TPP) from sub-method III

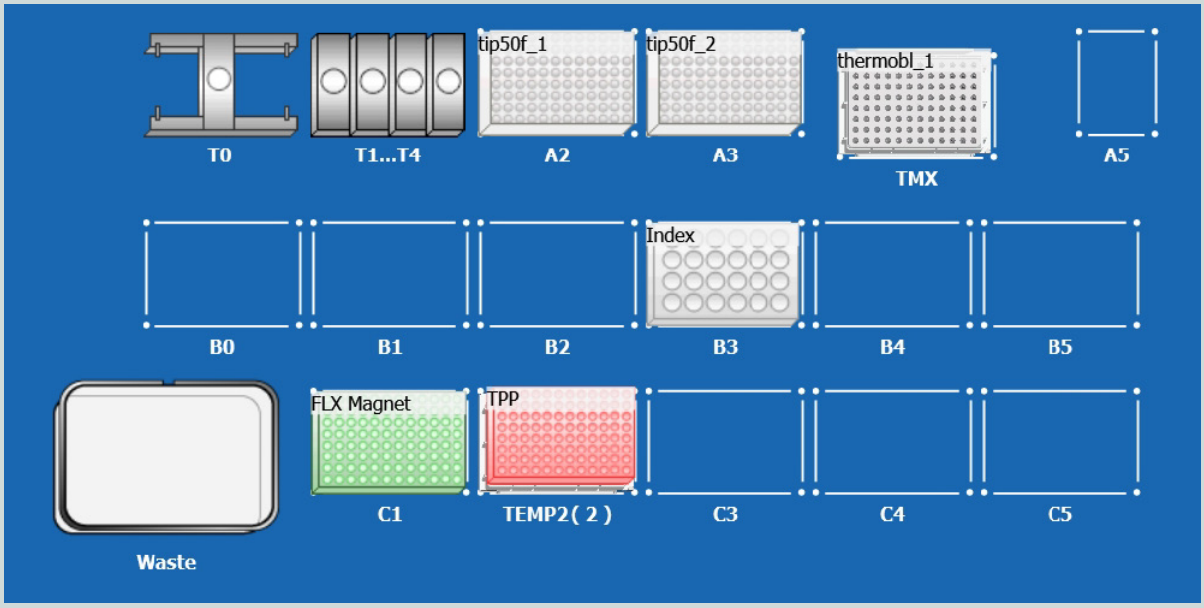

Figure 9: ep*Motion* worktable layout for sub-method IV as described in the text.

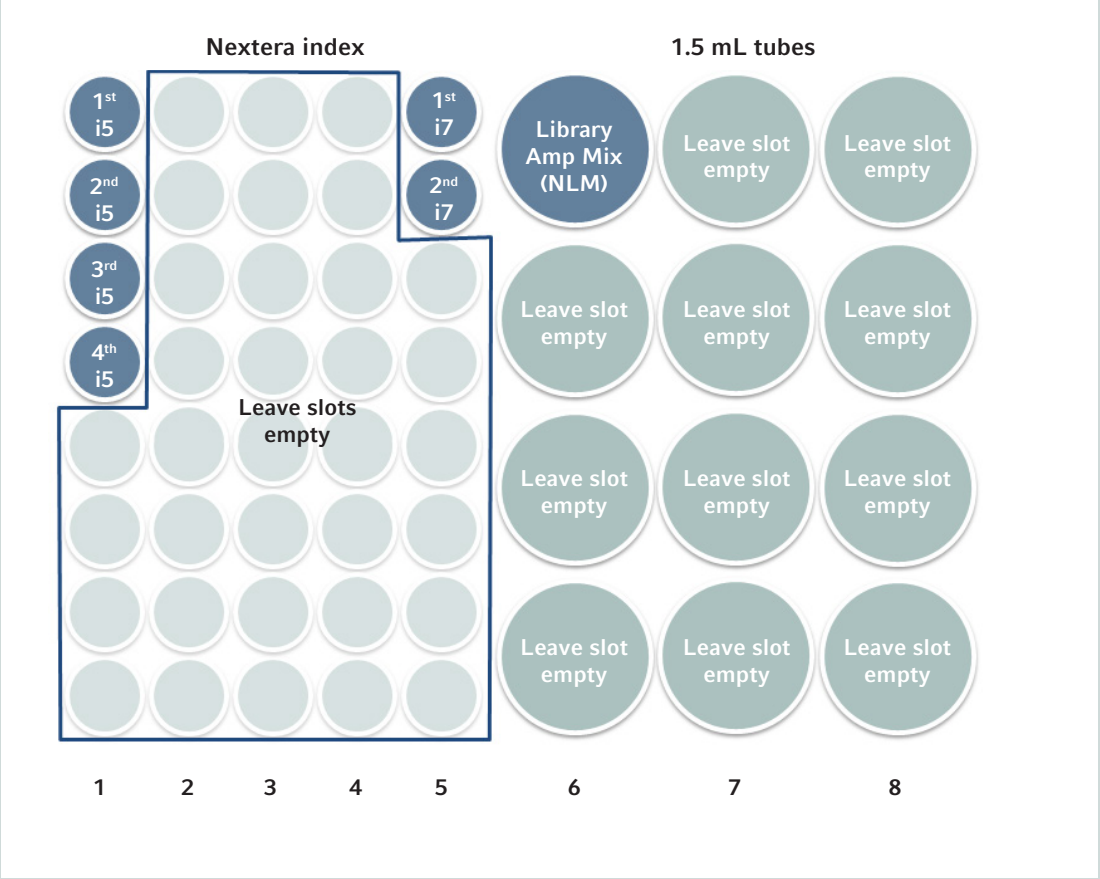

Figure 10: Rack ILMN tubes layout for sub-method IV as described in the text.

Table 1: Indexing scheme for the samples in the TPP plate.

Sample well in the "Sample plate"	i5 Index	i7 Index
A1	1st i5 Index	1st i7 Index
B1	2nd i5 Index	1st i7 Index
C1	3rd i5 Index	1st i7 Index
D1	4th i5 Index	1st i7 Index
E1	1st i5 Index	2nd i7 Index
F1	2nd i5 Index	2nd i7 Index
G1	3rd i5 Index	2nd i7 Index
H1	4th i5 Index	2nd i7 Index

Sub-method V

Sub-method V purifies the enriched, indexed libraries in the TPP plate. Sample Purification Beads (SPB), 80 % Ethanol and Resuspension Buffer (RSB) in the Reservoir Rack (Figure 12) should be freshly supplied in 30 mL reservoirs with at least 10 % excess. Make sure SPB are fully re-suspended before transfer to the reservoir. The sub-method ends with purified libraries in Column 1 (A1-H1) of the HLP plate, which can be further pooled for sequencing.

Worktable layout

Position	Item
A2	50 µL Filtertips
A4 (TMX)	Thermoblock PCR 96 OC
B0	Eppendorf 400 mL Reservoir for liquid waste
B2	300 µL Filtertips
B3	300 µL Filtertips
C1	Alpaqua MAGNUM FLX Enhanced Universal Magnet Plate
C2 (Temp)	Thermoadapter PCR 96 + semi-skirted Eppendorf twin.tec PCR Plate 96 LoBind (“TPP”) from sub-method IV
C3	Reservoir Rack (see figure 12)
C5	Thermoadapter PCR 96 + semi-skirted Eppendorf twin.tec PCR Plate 96 LoBind (HLP)

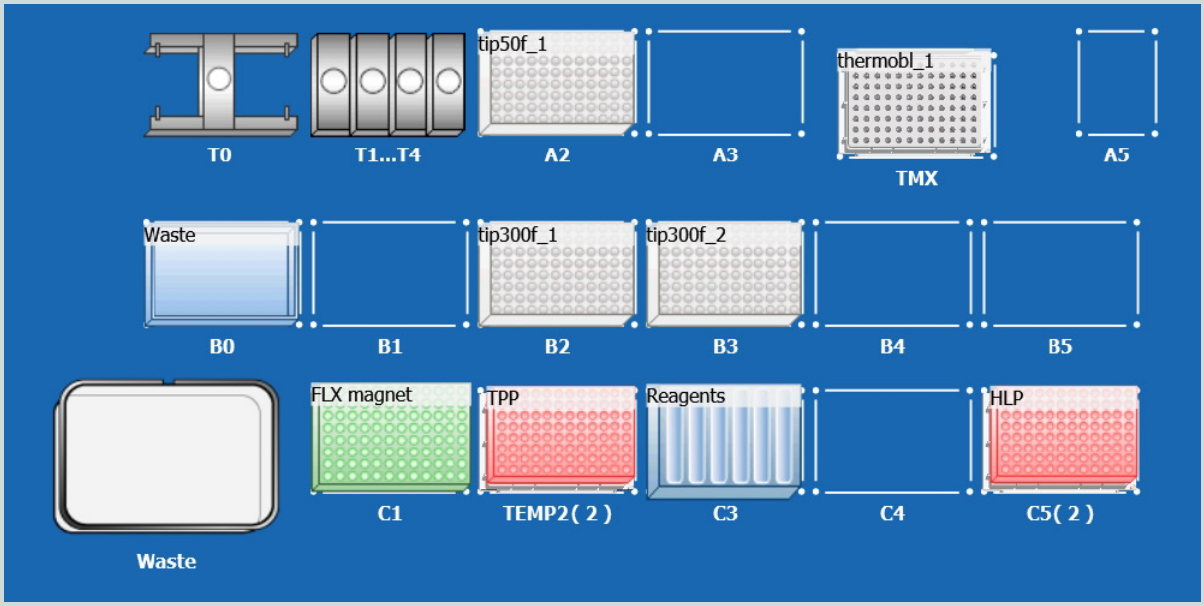

Figure 11: ep*Motion* worktable layout for sub-method V as described in the text.

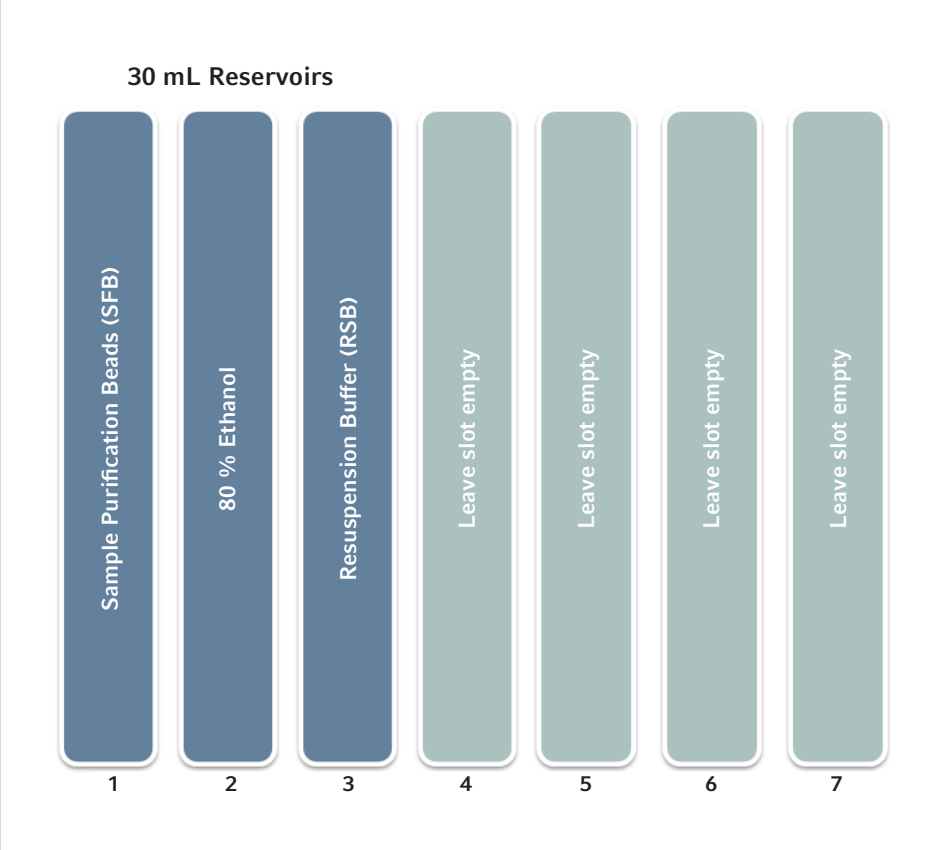

Figure 12: Reservoir Rack layout for sub-method V as described in the text.

Results

Methods

The quality of enrichment steps and the final libraries can be demonstrated at different steps of the process on an Agilent® Technologies 2100 BioAnalyzer® or similar. An example of these QC steps is shown on the left hand-side in figure 13 starting from the 8 long-range PCR products for 1 sample to the final libraries from a typical 8-sample run on the right-hand side. In this example, 1 µL of the final libraries run on a BioAnalyzer using a High Sensitivity DNA chip. A distribution of DNA fragments with a size range up to ~2 kbp is expected.

The obtained results are highly concordant with manual library preparation. To demonstrate this, 24 samples were prepared using the method described here in 3 sets of 8 samples. These libraries were sequenced on a MiSeq® and genotyped using Illumina's Assign 2.0 TruSight HLA Analysis Software. Table 2 shows the concordance between manual and automated library preparation. 100 % concordance was obtained among 380 alleles analyzed.

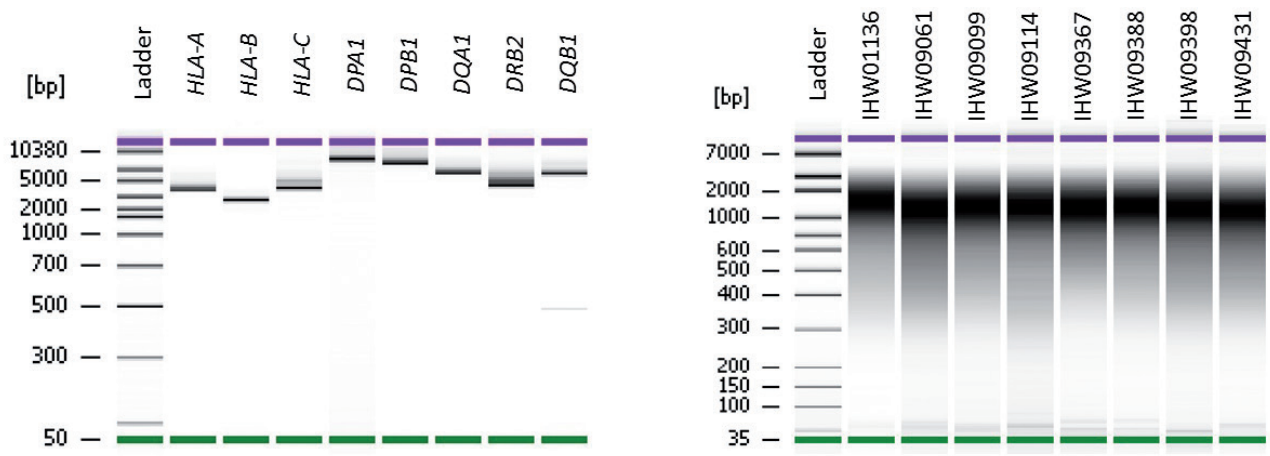

Figure 13: Example of a 2100 BioAnalyzer electropherogram of initial amplicons of one sample (left) and the post-enriched frament distribution obtained with automated library preparation of 8 samples on the ep*Motion* (right).

Table 2: Results comparison of manual and automated library prep.

	Total	*HLA-A*	*HLA-B*	*HLA-DPA1*	*HLA-DPB1*	*HLA-DQA1*	*HLA-DQB1*	*HLA-DRB1*	*HLA-DRB3*	*HLA-DRB4*	*HLA-DRB5*
Percentage concordant calls	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
Alleles analyzed	380	48	46	46	48	48	48	48	22	18	6

Ordering information

Ordering information

Description	Order no. international	Order no. North America
1x ep*Motion*® 5075t with Multicon	5075 006.022	5075006022
1x Thermal module on position C2	5075 757.001	960002181
1x TS 50 Dispensing Tool	5280 000.010	960001010
1x TS 300 Dispensing Tool	5280 000.037	960001028
1x TM50-8 Dispensing Tool	5280 000.215	960001044
1x TM300-8 Dispensing Tool	5280 000.231	960001052
1x Gripper	5282 000.018	960002270
3x Thermoadapter PCR 96	5075 787.008	960002199
1x Thermoblock PCR 96 OC	5075 751.666	5075751666
1x Reservoir Rack	5075 754.002	960002148
1x Reservoir Rack Module TC for PCR 0.2 mL tubes	5075 799.049	960002601
1x Reservoir Rack Module TC for Eppendorf Tubes® 5.0 mL	5075 799.340	5075799340
1x Rack ILMN Tubes	5075 751.747	5075751747
epT.I.P.S.® Motion, 50 µL, filtered	0030 014.413	0030014413
epT.I.P.S.® Motion, 300 µL, filtered	0030 014.456	0030014456
Reservoir 30 mL	0030 126.505	960051009
Eppendorf Reservoir 400 mL	5075 751.364	960002229
Eppendorf twin.tec® PCR Plate 96 LoBind, semi-skirted	0030 129.504	0030129504
Eppendorf twin.tec® PCR Plate 96 LoBind, skirted	0030 129.512	0030129512
Eppendorf Protein LoBind Tubes, 1.5 mL	0030 108.116	022431081
Eppendorf Protein LoBind Tubes, 5.0 mL	0030 108.302	0030108302
Eppendorf 0.2 mL PCR Tubes, thin-walled with hinged lid	0030 124.332	0030124707
Eppendorf PCR Foil, self-adhesive	0030 127.790	0030127790

Description	Ordering information
1x Alpaqua® MAGNUM FLX® Enhanced Universal Magnet Plate	Alpaqua order no. A000400
80% Ethanol	Any molecular biology grade
Illumina® TruSight® HLA v2 Sequencing Panel (24 samples)	Illumina order no. 20005170
Illumina® Nextera® XT Indexing kit	Illumina order no. FC-131-1001

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