HOLOTYPE HLA™
96/7
A & B
USER MANUAL

FOR RESEARCH USE ONLY

Protocol Version 2.1

IFU v2

Omixon Biocomputing Limited

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Document History

Protocol Version	IFU V	Date	Description of Changes	Author	Approval Name
1.5	-	January, 2015	Initial Version	Robert Pollok	Peter Meintjes
1.6	-	March, 2015	DQB Enhancer combination, general edits, Appendix 2: Sample Sheet	Robert Pollok	Peter Meintjes
1.7	-	June, 2015	ExoSAP-IT Step Change, DQB1 Set 1&2 pooling, DQB1 Set 1 optional, <16 samples/run, Pippin Prep 0.8	Robert Pollok	Peter Meintjes
1.7.1	-	June, 2015	DPB1 caps changed from Clear to Purple, DQA1 caps changed from Clear to Brown. MiSeq Reagent Nano Kit v2 requirements updated.	Robert Pollok	Peter Meintjes
1.7.2	-	July, 2015	MiSeq Reagent Kit v2 and MiSeq Reagent Nano Kit v2 requirements updated.	Robert Pollok	Peter Meintjes
1.7.3	-	November, 2015	Amplification verification and quantitation may be optional after sufficient and consistent experience.	Robert Pollok	Peter Meintjes
1.7.4	-	February, 2016	Increased volume of Enhancer 2, Enhancer 2 should now be saved after Combined DQB Enhancer is made, added new DRB4 Master Mix formulation, increased the volume used in Fragmentation and End Repair master mix for full plate, and rounded some values to the nearest tenth.	Robert Pollok	Efi Melista
1.7.5	-	April, 2016	Changed wording from “LR-PCR enzyme” to “Taq Polymerase” based on Qiagen’s documentation change. Removed X2 from Holotype HLA product configuration name. Approval name has been added on document history.	Robert Pollok	Efi Melista
1.7.6	-	April, 2016	DQB1 set 1 and set 2	Robert	Efi Melista

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			statement update	Pollok	
1.7.7	-	July, 2016	Updated library preparation reagent volumes, addition of adaptor plate A2 and B information.	Tunde Vago	Efi Melista
2.0	-	January, 2016	"DQB" removal from the DQB Enhancers, Gel verification after LR-PCR is optional, amplicon quantitation simplification, per-sample pooling volume change in 11-locus kits, ExoSAP-IT replacement by ExoSAP-IT Express, Qubit use for library quantitation.	Tunde Vago	Efi Melista
2.1	-	September, 2017	DQB1 set 1 and set 2 primer mix replaced by DQB1 set 3, library quantitation method wording update, end repair reaction time decrease, ligation reaction time decrease, Sample sheet removed from Appendices	Tunde Vago	Efi Melista
2.1	V2	9th August 2019	IFU versions introduced, general edits	Tunde Vago	Efi Melista

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The Principle of the Method: NGS-based HLA typing for the Illumina MiSeq

For many years the HLA community has been working toward a method that will accurately identify the extensive polymorphism of the HLA genes and of their gene products. The advent of PCR, combined with other technologies (Sanger sequencing, SSOP, SSP, Luminex), provided a formula for significantly improving the detection of HLA polymorphisms albeit with several limitations that continue to inhibit our ability to comprehensively characterize the HLA genes. Technologies developed over the last several years, cumulatively called Next Generation Sequencing (NGS), have provided new opportunities that allow the complete characterization of the HLA genes in haploid fashion. NGS has two distinct features, 1) clonal sequencing of DNA fragments, and 2) tremendously high throughput. NGS provides the capability to phase polymorphisms thereby eliminating all ambiguities and provides HLA typing at the three to four field level without reflexive testing, thereby introducing a potentially total solution to the HLA typing problem. The protocol described here takes advantage of this technology and combines long-range PCR amplification of HLA genes with sequencing on the Illumina MiSeq platform. More specifically the HLA genes A, B, C, DQA1 and DQB1 are amplified for their entire coding length, including elements of the 5' and 3' untranslated regions, while DRB1 is amplified from intron 1 to intron 4 and DPB1 is amplified from intron 1 to the 3' untranslated region. The amplicons are then processed through a series of steps that:

1. Fragment the amplicons to a size appropriate for sequencing on the Illumina platform,
2. Blunt-end and adenylate the ends of the fragmented amplicons and
3. Ligate adaptor sequences that are used throughout the process on the MiSeq to capture, amplify, and sequence the DNA. The adaptors also include an index which is a short sequence, unique to each adaptor, which identifies the origins of the library (sample/locus).

After pooling the indexed libraries, size selection and quantitation, the sample is loaded on the MiSeq for sequencing. The whole process takes 3-5 days depending on the selection of the flow cell on the Illumina platform. The generated data are analyzed using two different algorithms in HLA Twin™ (www.omixon.com). The use of two independent algorithms in HLA Twin provides the highest level of confidence so that the HLA genotyping results can be reported immediately without further attention. Samples with questionable or ambiguous genotypings are flagged by the software to be analyzed manually.

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Holotype HLA Packing List

Primer Component Box

Primer mix	Rxns	Vol/tube	# Tubes	Color code
HLA-A	*96*	*220 µL*	*1*	*Yellow*
HLA-B	*96*	*220 µL*	*1*	*Red*
HLA-C	*96*	*220 µL*	*1*	*Orange*
HLA-DRB1	*96*	*220 µL*	*1*	*Green*
HLA-DPB1	*96*	*220 µL*	*1*	*Purple*
HLA-DQA1	*96*	*220 µL*	*1*	*Brown*
HLA-DQB1 (Set 3)	*96*	*220 µL*	*1*	*Blue*
Enhancer 1	*96*	*1100 µL*	*1*	*Clear*
Enhancer 2	*96*	*300 µL*	*1*	*Clear*

Library Preparation Reagents Component Box

Reagent	Rxns	Vol/tube	# Tubes	Color code
Fragmentation Enzyme (A)	*96*	*278 µL*	*1*	*Yellow*
Fragmentation Buffer (B)	*96*	*278 µL*	*1*	*Red*
End Repair Enzyme (C)	*96*	*162 µL*	*1*	*Green*
End Repair Buffer (D)	*96*	*324 µL*	*1*	*Orange*
Ligation Enzyme (E)	*96*	*324 µL*	*1*	*Blue*
Ligation Buffer (F)	*96*	*1800 µL*	*2*	*Black*

96-well Adaptor Plate

Indexed adaptors in a 5 µL solution for generating 96 individual sequencing libraries.
Two (2) Adaptor Plate configurations are available containing 96 individual indices. Adaptor plates, that contain 96 indices only are available in the following configurations: A (i1-i96), B (i97-i192).

Excel Workbook

An Excel Workbook is provided to support the Holotype HLA protocol with volume calculations, plate layouts, reagent traceability, record keeping and MiSeq Sample Sheet generation of all of the supported adaptor plate configurations (A, B). If you do not have a copy, please contact support@omixon.com.

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Software – Omixon HLA Twin

Contact sales@omixon.com for Omixon HLA Twin license associated with your purchase of Holotype HLA.

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Recommendations

DNA Extraction Recommendations

- High quality genomic DNA (gDNA) extracted from whole blood, blood cells (B-cell lines, buffy coats, cord blood or any fraction of white blood cells), saliva and buccal swabs can be used. For the amplification of HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DPB1, HLA-DQA1 and HLA-DQB1 0.8 - 1.2 µg of gDNA is required.

Technical and Equipment Recommendations

- Thermal cycler with 96-well format
- Plate fluorometer (or any instrument capable of fluorescence detection in 96-well plate format, for use with the Promega QuantiFluor dsDNA System)
- Pippin Prep (Cat# PIP0001) or Blue Pippin (Cat# BLU0001) by SAGE Science
- Qubit fluorometer (Cat# Q33216, Thermo Fisher Scientific)
- qPCR instrument with 96 or 384-well plate format (optional)
- Illumina MiSeq (Cat# SY-410-1003)
- 64-bit computer with minimum 4 Cores and 16 GB of RAM
- Long-term data storage (approximately 2 TB of data per MiSeq per year)

Associated Reagent Recommendations

- LongRange PCR kits from Qiagen (Cat# 206401, 206402 or 206403)
  - Each sample requires 3.2 µL of Taq Polymerase
  - Cat# 206401 LongRange PCR kit (20) contains 8 µL of Taq Polymerase
  - Cat# 206402 LongRange PCR kit (100) contains 40 µL of Taq Polymerase
  - Cat# 206403 LongRange PCR kit (250) contains 100 µL of Taq Polymerase
- ExoSAP-IT Express from Affymetrix (Cat#75001-200, 75001-1ML, 75001-4X-1ML or 75001-10ML)
  - Each pooled sample requires 4 µL of ExoSAP-IT Express enzyme
  - Cat# 75001-200 contains 200 µL of ExoSAP-IT Express enzyme
  - Cat# 75001-1-ML contains 1 mL of ExoSAP-IT Express enzyme
  - Cat# 75001-4X-1ML contains 4 mL of ExoSAP-IT Express enzyme
  - Cat# 75001-10ML contains 10 mL of ExoSAP-IT Express enzyme
- Qubit dsDNA BR Assay Kit (Cat# Q32850 or Q32853)
  - Cat# Q32850 for 100 assays
  - Cat# Q32853 for 500 assays
- Library Quantification Kit – Illumina/Universal from KAPA Biosystems (Cat# KK4824) (optional if using a qPCR instrument)
- QuantiFluor dsDNA System from Promega (Cat# E2670)
- Agencourt AMPure XP beads from Beckman Coulter (Cat# A63880, A63881, or A63882)
  - Each Holotype HLA run requires a maximum of 900 µL of AMpure XP beads
  - Cat# A63880 contains 5 mL of AMPure XP beads

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- Cat# A63881 contains 60 mL of AMPure XP beads
- Cat# A63882 contains 450 mL of AMPure XP beads

- Gel cassette, 1.5% agarose, dye free with internal standard (Marker K/R2), for the Pippin Prep/Blue Pippin (Cat# CDF1510 for Pippin Prep and BDF1510 for Blue Pippin)
- Molecular grade ethanol (Anhydrous Alcohol)
- Molecular grade water (DNase and RNase free)
- Sodium hydroxide
- 1× TE buffer (pH 8.0)
- MiSeq Reagent Kit from Illumina

MiSeq Reagent Kit capacity

Illumina MiSeq Reagent Kit	Time Hours	24/7 Samples	24/11 Samples	96/5 Samples	96/7 Samples	96/11 Samples
Std 300 Cycle (MS-102-2002)	~24	24	24	96	96	72
Micro 300 Cycle (MS-103-1002)	~19	24	20	40	28	20
Nano 300 Cycle (MS-103-1001)	~17	6	4	8	6	4
Std 500 Cycle (MS-102-2003)	~39	24	24	96	96	96
Nano 500 Cycle (MS-103-1003)	~28	12	8	16	12	8

Recommended Supplies

- 1.5 mL microcentrifuge tubes
- 1.5 mL low-bind microcentrifuge tubes
- 2.0 mL low-bind microcentrifuge tubes (Eppendorf DNA LoBind Cat# 022431048 recommended)
- 0.5 ml thin wall tubes for Qubit instrument (Qubit Assay tubes Cat# Q32856 recommended)
- Adjustable volume pipettes (1.0 – 1000 µL capacity)
- 8-channel adjustable volume pipettes (1.0 - 100 µL capacity)
- 96-well plates compatible with the thermal cycler
- 96-well optical plates compatible with the plate fluorometer
- 96-well plates compatible with the qPCR instrument (optional)
- Plate seals compatible with the thermal cyclers (tested for long range PCR)
- Optical plate seals compatible with the qPCR instrument (optional)
- Magnetic stand compatible with 2 mL microcentrifuge tubes
- 96-well cooler racks (2 pieces)
- 50 mL conical tubes
- 50 mL reservoirs

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Summary of Steps

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Glossary/Definitions

- Amplicon Plate – Alternative name for an Amplification Plate
- Amplicon Quantitation Plate – 96-well plate compatible with the plate fluorometer or qPCR machine where the amplicons are quantitated.
- Amplification Plate – 96-well PCR plate used to amplify the HLA loci.
- Final Library – Library that includes all Sample Libraries ready to be sequenced in a single MiSeq run.
- Reaction Plate – Plate where the sequential reactions that fragment, end repair, and ligate the indexed adaptors to the Sample Libraries are performed.
- Reagent Plate – Plate used to aliquot the various reagents used to prepare the Libraries
- Sample Library – A library prepared by combining (pooling) all HLA loci for a given sample.
- Pooled Amplicons Plate: Plate containing a sample library (all loci combined) per well.
- Standards Quantitation Plate – 96-well plate compatible with the plate fluorometer or qPCR machine where DNA standards are placed to allow for amplicon quantitation.

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Step 0 – Genomic DNA Preparation

Duration: ~1 hour 45 minutes

Isolate gDNA from whole blood, blood cells (B-cell lines, buffy coats, cord blood or any fraction of white blood cells), saliva and buccal swabs. gDNA should be dissolved in water as the EDTA in TE buffer can inhibit long-range PCR reactions and its recommended concentration is 20-30ng/ul. We highly recommend to use a fluorescence-based quantitation method to determine the gDNA concentration.
Its quality, as evaluated by spectrophotometry, should be:

1. A 260nm/280nm absorbance ratio between 1.7 and 1.9.
2. A 260nm/230nm absorbance ratio of 1.7 or greater.
3. Minimal degradation. DNA that is old or has gone through repeated freeze/thaws will suffer from more degradation.

For the amplification of HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DPB1, HLA-DQA1 and HLA-DQB1, 0.8 - 1.2 µg of gDNA is required for each sample.

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Step 1 – HLA Amplification Master Mix Preparation

Duration: ~1 hour 50 minutes

The purpose of this step is to prepare locus-specific Master Mixes to amplify each targeted HLA locus individually. The loci amplified by this protocol are HLA-A, HLA-B, HLA-C, HLA-DRB1, HLA-DPB1, HLA-DQA1 and HLA-DQB1.

Note: The Master Mixes prepared in this step include all reagents needed for amplification except the Taq Polymerase.

Reagent list

Item	Storage	Supplied by
HLA-A Primer Mix	-20°C	Omixon
HLA-B Primer Mix	-20°C	Omixon
HLA-C Primer Mix	-20°C	Omixon
HLA-DRB1 Primer Mix	-20°C	Omixon
HLA-DPB1 Primer Mix	-20°C	Omixon
HLA-DQA1 Primer Mix	-20°C	Omixon
HLA-DQB1 (Set 3) Primer Mix	-20°C	Omixon
Enhancer 1	-20°C	Omixon
Enhancer 2	-20°C	Omixon
LongRange PCR Buffer (10×)	-20°C	Qiagen
dNTPs (10 mM each)	-20°C	Qiagen
Molecular grade H_2O	-20°C	Qiagen

Protocol

1.1 - Remove all primer mixes, Enhancer 1 and 2, the dNTPs and the Long-Range PCR Buffer (10×), from storage and thaw at room temperature.

1.2 - Prepare the Combined Enhancer: add 132 µL of Enhancer 2 into the Enhancer 1 tube. Relabel Enhancer 1 tube as Combined Enhancer.

1.3 - Prepare a Master Mix for each Primer Mix according to the tables below:

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Master Mix: HLA-A, B, C, DRB1, DPB1, and DQA1

Reagent	Volume/sample/locus	Volume/96 samples/locus
Primer Mix	2 µL	204 µL
LongRange PCR Buffer (10×)	2.5 µL	255 µL
dNTP Mix (10 mM each)	1.25 µL	127.5 µL
Molecular grade H_2O	13.85 µL	1412.7 µL
Total Volume	19.6 µL	1999.2 µL

Master Mix: HLA-DQB1 Set 3

Reagent	Volume/sample/locus	Volume/96 samples/locus
Primer Mix	2 µL	204 µL
LongRange PCR Buffer (10×)	2.5 µL	255 µL
dNTP Mix (10 mM each)	1.25 µL	127.5 µL
Combined Enhancer	5.6 µL	571.2 µL
Molecular grade H_2O	7.85 µL	800.7 µL
Total Volume	19.2 µL	1958.4 µL

1.4 – Vortex each Master Mix and spin it down for 1 second. Place Master Mixes on ice.

1.5 - Dilute all gDNAs to a concentration of 30ng/µL (minimum volume is 45 µL).

Note: Holotype HLA includes sufficient reagents for 96 reactions plus additional volume for pipetting loss and failed amplification.

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Step 2 – HLA Class I and II Amplification

Duration: ~7 hours 50 minutes

The purpose of Step 2 is to amplify the HLA loci. HLA Class I and Class II amplifications have been optimized using two separate sets of PCR conditions. Once the PCR reactions are completed, amplification is verified by agarose gel electrophoresis (optional).

Quick tip – The agarose gel electrophoresis (step 2.5), while recommended, is not required to successfully complete the Holotype HLA protocol. When amplicons are quantitated (Step 3), any concentration above 50 ng/µL is considered a successful amplification. Agarose gel electrophoresis is an important quality control step and should not be skipped without sufficient experience with the complete Holotype HLA protocol.

Reagent list

Item	Storage	Supplied by
HLA-A Master Mix	-20°C	Step 1
HLA-B Master Mix	-20°C	Step 1
HLA-C Master Mix	-20°C	Step 1
HLA-DRB1 Master Mix	-20°C	Step 1
HLA-DPB1 Master Mix	-20°C	Step 1
HLA-DQA1 Master Mix	-20°C	Step 1
HLA-DQB1 (Set 3) Master Mix	-20°C	Step 1
Taq Polymerase	-20°C	Qiagen
gDNA	4°C	User
Molecular grade H_2O	20°C	User

Protocol

2.1 – Remove the Taq Polymerase from storage, spin it down, and add it to each Master Mix according to the tables below, rinsing the pipette tips thoroughly by pipetting:

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Taq Polymerase-Loaded Master Mix: HLA-A, B, C, DRB1, DPB1, and DQA1

Reagent	Volume/sample/locus	Volume/96 samples/locus
Master Mix from Step 1	19.6 µL	1999.2 µL
Taq Polymerase	0.4 µL	40.8 µL
Total	20 µL	2040 µL

Taq Polymerase-Loaded Master Mix: HLA-DQB1 Set 3

Reagent	Volume/sample/locus	Volume/96 samples/locus
Master Mix from Step 1	19.2 µL	1958.4 µL
Taq Polymerase	0.8 µL	81.6 µL
Total	20 µL	2040 µL

2.2 - Briefly vortex and spin down all Taq Polymerase-loaded Master Mixes. Aliquot 20 µL of each Taq Polymerase-loaded Master Mix into separate wells of 96-well PCR plates.

Note: Class I and Class II amplification have been optimized using two different PCR conditions, so Class I and Class II Master Mixes should not be in the same plate.

2.3 – Add 5 µL of each diluted gDNA into the appropriate well of the plates prepared in the previous step. Mix by pipetting. Seal them with a thermal seal and visually inspect each well. Spin-down all Amplification Plates in a centrifuge.

2.4 - Place the Amplification Plates into thermal cyclers and run the programs for Class I and Class II amplification according to the tables below:

Class I Amplification (HLA-A, B and C)

Number of Cycles		Temperature	Time
1		95°C	3 minutes
35		95°C	15 seconds
		65°C	30 seconds
		68°C	5 minutes
1		68°C	10 minutes
1		4°C	∞

Class II Amplification (HLA-DRB1, DPB1, DQA1 and DQB1)

Number of Cycles	Temperature	Time
1	95°C	3 minutes
35	93°C	15 seconds
	60°C	30 seconds
	68°C	9 minutes
1	68°C	10 minutes
1	4°C	∞

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Note: Amplification success can be verified by running 2 µL from each amplicon in a standard 2% agarose gel at 250 V for 30 minutes. (Optional)

Expected Amplicon Sizes

HLA locus	Expected amplicon size (kb)
HLA-A, B and C	~3
HLA-DRB1	~4.3
HLA-DPB1	~6.7
HLA-DQA1	~6
HLA-DQB (Set 3)	~6.6

Safe stopping point. Amplicons can be stored at 4°C overnight or at -20°C for longer.

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Step 3 – Amplicon Quantitation and Normalization (using a Plate Fluorometer)

Duration: ~1 hour 50 minutes

Amplicon Quantitation and Normalization is recommended to ensure precise input into the library preparation step (Optional). Amplicon concentration is measured using the QuantiFluor dsDNA System that contains a fluorescent DNA-binding dye and DNA standard for sensitive quantitation of small amounts of double-stranded DNA (dsDNA). Refer to Appendix 2 for Instructions on how to do the Amplicon Quantitation using a qPCR machine.

Quick tip – The amplicon quantitation, while recommended, is not required to successfully complete the Holotype HLA protocol. Amplicon normalization does not require precise measurement of amplicon concentration. An estimate of amplicon concentrations based on experience or agarose gel electrophoresis can be used instead. Amplicon quantitation should not be skipped without consistent experience with the complete Holotype HLA protocol.

Reagent list

Item	Storage	Supplied by
Class I Amplification Plate(s)	4°C	Step 2
Class II Amplification Plate(s)	4°C	Step 2
Lambda DNA Standard (100 ng/µL)	4°C	Promega
QuantiFluor dsDNA Dye (200×)	4°C	Promega
20× TE Buffer (pH 7.5)	4°C	Promega
Molecular grade H_2O	20°C to 25°C	User
ExoSAP-iT Express	-20°C	Affymetrix

Protocol

3.1 – Prepare DNA standards by serial dilution of the Lambda DNA standard (100 ng/µL) provided in the QuantiFluor kit according to the dilution table below:

Label on tube	Input DNA	Volume DNA (µL)	Volume 1x TE (µL)	Final Conc. (ng/µL)
Standard 1	Lambda DNA	7.5 µL	492.5 µL	1.5 ng/µL
Standard 2	Standard 1	250 µL	250 µL	0.75 ng/µL
Standard 3	Standard 2	250 µL	250 µL	0.38 ng/µL
Standard 4	Standard 3	250 µL	250 µL	0.19 ng/µL
Standard 5	Standard 4	250 µL	250 µL	0.09 ng/µL
Standard 6	Standard 5	250 µL	250 µL	0.05 ng/µL
Blank	Blank	0 µL	250 µL	0 ng/µL

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3.2 - Prepare the Amplicon Quantitation plates (see supplemental figures). Aliquot 99 µL 1x TE buffer to the wells of a 96-well optical plate for the total number of amplicons to be quantitated.

3.3 - Add 1 µL of amplicons from corresponding wells in the Amplicon Plates to individual wells in the Amplicon Quantitation Plates. Mix by pipetting

3.4 - Prepare 1× QuantiFluor Dye working solution using the following formula: 0.5 µL QuantiFluor Dye (200X) + 99.5 µL 1× TE buffer. Prepare sufficient 1× QuantiFluor Dye working solution so that each sample (total samples in Amplicon Plates) and standard (14 total) will receive a 100 µL aliquot.

3.5 - Prepare a Standards Quantitation Plate and Amplicon Quantitation plates. Aliquot 100 µL of 1× QuantiFluor Dye working solution to wells of the 96-well optical plate that will be the Standards Quantitation Plate and to the Amplicon Quantitation Plates from Step 3.2.

3.6 - Using the standards prepared above, add 100 µL of each standard, in duplicate, to individual wells in the Standards Quantitation Plate (14 wells total). Mix by pipetting.

3.7 - Vortex well to mix and spin down.

3.8 - Run the Standards Quantitation Plate on the plate fluorometer followed by the Amplicon Quantitation Plates.

3.9 - Calculate the concentration of DNA in the Amplicon Quantitation Plates using RFU data generated by the plate fluorometer. Refer to the Dilution Tab in the provided workbook for assistance with calculations.

3.10 - Dilute DNA in the Amplicon Plates with molecular grade H_2O so that the final concentration of DNA is approximately 67 ng/µL.

- If DNA concentration is 150 ng/µL or greater: add 25 µL of H_2O
- If DNA concentration is 100-150 ng/µL: add 10 µL of H_2O
- If DNA concentration is less than 100 ng/µL: do not add any H_2O

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Amplicon pooling

3.11 - Pool all loci for each sample into a single Pooled Amplicons Plate. Combine the volumes indicated for each locus as in the following table to obtain a final volume of 35 µL.

HLA locus	Pooled volume
A	5 µL
B	5 µL
C	5 µL
DRB1	5 µL
DPB1	5 µL
DQA1	5 µL
DQB1 set 3	5 µL

3.12 – Add 4 µL of ExoSAP-iT Express into each pooled amplicon. Rinse the pipette tips by pipetting. Seal the plate with a thermal seal and spin down.

3.13 - Place the Pooled Amplicons Plate into a thermal cycler and run the following program:

ExoSAP-IT Express PCR Purification

Number of Cycles	Temperature	Time
1	37°C	4 minutes
1	80°C	1 minutes
1	4°C	∞

Safe stopping point. Amplicons can be stored at 4°C overnight or at -20°C for longer.

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Step 4 – Library Preparation

Duration: ~3 hours

During this step, the pooled amplicons are prepared for sequencing on the Illumina MiSeq. The amplicons are enzymatically fragmented, the ends are repaired and adenylated, and indexed adaptors are ligated to the ends. The libraries are then pooled followed by a single cleanup and concentration step performed using AMPure XP beads.

Note: Omixon recommends volumes greater than is necessary for 96 samples because many of the enzymes and buffers are viscous, resulting in excess pipetting loss.

Reagent list

Item	Storage	Supplied by
Pooled Amplicons Plate	4°C	Step 3
Fragmentation Enzyme (A)	-20°C	Omixon
Fragmentation Buffer (B)	-20°C	Omixon
End Repair Enzyme (C)	-20°C	Omixon
End Repair Buffer (D)	-20°C	Omixon
Ligation Enzyme (E)	-20°C	Omixon
Ligation Buffer (F)	-20°C	Omixon
Adaptor Plate	-20°C	Omixon
AMPure XP beads	4°C	Beckman Coulter
80% Ethanol (freshly prepared)	20°C to 25°C	User
Molecular grade H_2O	20°C to 25°C	User

Protocol

4.1 - Turn on the thermal cycler. Verify that the heated lid is warming up.

Note: Be sure to vortex the Fragmentation Enzyme (A) thoroughly before use.

4.2 - Prepare Fragmentation Master Mix according to the table below:

Fragmentation Master Mix

Reagent	Volume per library (µL)	Recommended volumes for 96 libraries (µL)	Color code
Fragmentation Enzyme (A)	2 µL	220.8 µL	Yellow
Fragmentation Buffer (B)	2 µL	220.8 µL	Red
Total Volume	4 µL	441.6 µL	

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4.3 - Prepare a Reagent Plate: place a new 96-well PCR plate on a PCR cooler rack and aliquot and equal amount of the Fragmentation Master Mix into each well of a single column.

Note: The fragmentation reaction has been designed to provide ideally sized DNA for sequencing on the Illumina MiSeq. It is important to keep the reagents cold until the reaction is started in the thermal cycler to prevent excessive fragmentation. Use of multi-channel pipettes is recommended to minimize opportunities for excessive fragmentation.

4.4 - Centrifuge the Pooled Amplicons Plate for 10 seconds, and place it on ice or a cold block.

4.5 - Prepare a Reaction Plate: place a fresh 96-well PCR plate on a PCR cold block.

4.6 - Add 4 µL of Fragmentation Master Mix from the Reagent Plate into wells of the Reaction Plate, corresponding with samples in the Pooled Amplicons Plate. The use of a multi-channel pipette is recommended.

4.7 - Transfer 16 µL of each amplicon from the Pooled Amplicons Plate to the corresponding well on the Reaction Plate using a multi-channel pipette. Mix by pipetting.

4.8 - Cover the Reaction Plate with a thermal seal and centrifuge for 10 seconds.

4.9 - Incubate the Reaction Plate in a thermal cycler with the following program:

Fragmentation Program

Number of Cycles	Temperature	Time
1	37° C	10 minutes
1	70° C	15 minutes
1	4° C	∞

Safe stopping point. Libraries can be stored at 4°C overnight or at -20°C for longer.

4.10 - Prepare the End Repair Master Mix according to the table below:

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End Repair Master Mix

Reagent	Volume per library (µL)	Recommended volumes for 96 libraries (µL)	Color code
Molecular grade H_2O	1.25 µL	139.2 µL	
End Repair Enzyme (C)	1.25 µL	139.2 µL	Green
End Repair Buffer (D)	2.5 µL	278.4 µL	Orange
Total Volume	5 µL	556.8 µL	

4.11 - Aliquot an equal amount of End Repair Master Mix into a single unused column of the Reagent Plate.

4.12 - Centrifuge the Reaction Plate (containing the fragmented Samples) for 10 seconds. Add 5 µL of End Repair Master Mix from the Reagent Plate into each well of the Reaction Plate. The use of a multi-channel pipette is recommended. Mix by pipetting.

4.13 - Cover the Reaction Plate with a thermal seal and centrifuge for 10 seconds.

4.14 - Incubate the Reaction Plate in a thermal cycler with the following program:

End Repair Program

Number of Cycles	Temperature	Time
1	20°C	30 minutes
1	70°C	5 minutes
1	4°C	∞

Safe stopping point. Libraries can be stored at 4°C overnight or at -20°C for longer.

4.15 - Remove the Indexed Adaptors Plate from storage and thaw at room temperature after the End Repair Program starts in the thermal cycler. When the Adaptor Plate is at room temperature, centrifuge it for 3 minutes at 3000 rpm.

4.16 - Carefully pull the seal off of the Adaptor Plate. Do not shake the Adaptor Plate once the seal is removed to prevent cross contamination.

4.17 – Transfer the entire volume of each well from the Reaction Plate (25 µL) to the corresponding well in the Adaptor Plate.

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Note: If the entire Adaptor Plate is NOT going to be used, it is possible to use only the necessary number of adaptors. Cut the plate seal between the wells to be used and the wells to be kept. Carefully pull the seal off the Adaptor Plate, leaving the seal in place over the wells to be kept.

a. Transfer 25 µL from each end-repaired sample of the Reaction Plate to a well in the Adaptor Plate, mixing well with a pipette.

b. Transfer the entirety of each sample from the Adaptor Plate into the original well of the Reaction Plate.

c. Reseal the Adaptor Plate and return it to -20°C. Use the Reaction Plate instead of the Adaptor Plate for the remaining steps in the manual.

4.18 - Prepare the Ligation Master Mix. Prepare enough Ligation Master Mix for each sample.

Ligation Master Mix

Reagent	Volume (µL)	Recommended volumes for 96 libraries (µL)	Color code
Ligation Enzyme (E)	2.5 µL	252.5 µL	Blue
Ligation Buffer (F)	30 µL	3030 µL	Black
Total Volume	32.5	3282.5 µL	

4.19 - Aliquot the Ligation Master Mix into 3 unused columns of the Reagent Plate. The use of a multi-channel pipette is recommended.

4.20 - Add 32.5 µL of Ligation Master Mix into each well of the Reaction Plate. The use of a multi-channel pipette is recommended. Mix by pipetting.

4.21 - Cover the Reaction Plate with a thermal seal and centrifuge for 10 seconds.

4.22 - Incubate the Reaction Plate in the thermal cycler with the following program:

Ligation Program

Number of Cycles	Temperature	Time
1	25°C	10 minutes
1	70°C	10 minutes
1	4°C	∞

Safe stopping point. Libraries can be stored at 4°C overnight or at -20°C for longer.

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Pooling and library purification

4.23- Allow AMPure XP beads to come to room temperature. Ensure they are homogeneous (no clumps or pellets). Prepare freshly made 5 mL of 80% ethanol (4 mL EtOH + 1 mL H_2O).

4.24 - Create the Library by combining an aliquot from each pooled amplicon, now a sample-specific library, into a single 2.0 mL low bind microcentrifuge tube.

I. **For 16 or more samples** - Calculate the amount of each sample library to pool together as a single **Library** of 900 µL total volume. Divide 900 µL by the number of sample libraries. This is the volume of aliquot to be taken from each sample library and pipetted into the Library.
II. **For fewer than 16 samples** – Transfer 60 µL of each sample library into a **Library**.

4.25 - Add 900 µL of AMPure XP beads to the Library tube. Mix thoroughly by vortexing and centrifuge briefly. Do not allow the beads to separate. Incubate the Library for 10 minutes at room temperature.

Note: If there is less than 900 µL of library in the Final Pool, add an equivalent amount of AMPure XP beads. There should be a 1:1 ratio of Final Pool and AMPure XP beads.

4.26 - Place the Library tube onto a magnetic stand and incubate for 10 minutes.

4.27 – Keeping the tube on the magnetic stand, carefully remove and discard the supernatant from the Library tube, without touching the beads.

4.28 - Keeping the tube on the magnetic stand, add ~1.5–2 mL of freshly prepared 80% ethanol to the Library tube. The volume of ethanol added should be sufficient to cover the beads.

Note: Apply the ethanol to the side of the tube without beads.

4.29 - Incubate the Library tube at room temperature for 30 seconds; afterwards, carefully remove and discard the supernatant.

4.30 - Repeat steps 4.28 and 4.29.

4.31 - Quickly spin down the Library tube and place it back on the magnetic stand with the lid open. Remove residual ethanol with a pipette. Do not touch the beads.

Note: Ensure the bead pellet does not contain residual ethanol. This may require rotating the tube on the magnetic stand to remove ethanol without disturbing the bead pellet.

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4.32 - Allow the beads to air dry for 5-8 minutes on the magnetic stand until the bead pellet is dry.

4.33 - Remove the Library tube from the magnetic stand and elute the Library with 31 µL molecular grade water. Do not let the pipette tip touch the beads, as they will stick to it.
4.34 - Vortex the Library to fully resuspend the beads. Centrifuge briefly if some droplets remain on the side walls. Ensure the beads remain in suspension.

4.35 - Incubate the Library at room temperature for 2 minutes.

4.36 - Place the Library tube on the magnetic stand for 2 minutes.

4.37 - Collect the Library: keeping the Final Library tube in the magnetic stand, collect 31 µL of the supernatant into a new 1.5ml low bind microcentrifuge tube.

Safe stopping point. Libraries can be stored at -20°C for extended periods of time.

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Step 5 – Library Size Selection

Duration: ~1 hour

Step 5 takes the Library from Step 4 and performs size selection using the Pippin Prep. The Pippin Prep can automatically select a range of DNA fragment sizes and elute them into a collection chamber. Note: Blue Pippin may be used instead of the Pippin Prep. Refer to Appendix 1 for Pippin Prep Instructions for Use.

Reagent list

Item	Storage	Supplied by
1.5% Agarose Gel Cassette, Dye Free	20°C to 25°C	Sage Science
Pippin loading solution/marker mix (labeled K)	4°C	Sage Science
Pooled Library	4°C	Step 4

Note: Marker K is used with the Pippin Prep. The Blue Pippin uses Marker R2.

Protocol

5.1 - Bring the Marker K loading solution to room temperature.

5.2 - Combine 31 µL of the Pool with 10 µL of Marker K loading solution.

5.3 - Mix by vortexing and spin down.

5.4 - Configure the Pippin Prep to collect DNA fragments between 650 and 1300 bps. Load the 40 µL sample into the sample port and run. Run time is 45-50 minutes.

5.5 - Collect the whole content (approximately 40 µL) from the elution port of the Pippin Prep and transfer it to a new 1.5ml low bind microcentrifuge tube. This is the size-selected library.

Safe stopping point. Libraries can be stored at -20°C for extended periods of time.

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Step 6 – Library Quantification using an intercalating dsDNA fluorescent dye

Duration: ~15 minutes

It is necessary to quantify the size-selected library in order to optimally use the output of the Illumina MiSeq sequencer. The concentration of the size-selected library can be accurately measured by an intercalating dsDNA fluorescent dye, such as SYBR green or equivalent. Commercially available kits and instruments for this purpose include, but are not limited to, the Qubit reader by Thermo Fisher (uses the Qubit Broad-Range dsDNA assay kit), the Quantus reader by Promega (uses the Quantifluor dsDNA fluorescent dye) and others. Here, the Qubit method is described as it is the most commonly used instrument. In case another instrument and kit is used, follow the manufacturer's standard instructions.

Note: This dsDNA fluorometric method is a quick but accurate enough way to determine the concentration of the final size-selected library. It measures all of the dsDNA that is present in the library. Optionally you may use the KAPA Biosystems Library Quantitation kit and qPCR machine for a more specific measurement of the library concentration. For this protocol See Appendix 3.

Reagent list

Item	Storage	Supplied by
Qubit dsDNA BR Assay Kit	room temperature	Thermo Fisher
Qubit dsDNA BR Standards	4°C	Thermo Fisher
Size Selected Library	4°C	Step 5

Protocol

6.1 – Bring the Qubit Standards to room temperature. Prepare Qubit assay tubes (500 µL, thin-walled) for your library in duplicate and the two standards. Vortex and centrifuge the standards and the library.

6.2 – Add 995µL from Buffer and 5µL from dye to a 1.5 ml centrifuge tube. Vortex and spin down.

6.3 – Transfer 190 µL from the reagent mix to the Qubit tubes for the two standards. Transfer 198 µL from the reagent mix to the two Qubit tubes for the duplicates of the library.

6.4 – Add 10 µL from standard 1 to the corresponding Qubit tube and vortex it for 2 seconds. Repeat with standard 2.

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6.5 –Add 2 µL from the library to the corresponding Qubit tubes and vortex for 2 seconds.

6.6 – Incubate the Qubit tubes at room temperature for 2 minutes.

6.7 – Switch on Qubit machine and choose BR protocol.

6.8 – Put standard 1 Qubit tube in and push GO. Repeat with standard 2.

6.9 – Put the library tube in Qubit and push GO. Repeat for the replicate.

6.10 – To convert the Qubit result from ng/µL to nM concentration, enter the mean concentration of the two library replicates in the Omixon Workbook tab called “Library Quantitation”.

6.11 – Using the results from the Qubit measurement, dilute 10 µL of the Size Selected Library to a concentration of 2 nM with sterile H_2O in a fresh 1.5-mL low bind microcentrifuge tube. Store the remaining Size Selected Library at -20° C.

Safe stopping point. Libraries can be stored at -20°C for extended periods of time. In case of long-term storage, re-quantification of the library is highly recommended before running it on the MiSeq.

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Step 7 – Sequencing on Illumina MiSeq

Duration: ~24 - 40 hours

The Illumina MiSeq is an automated NGS instrument that can sequence the Size-selected Library prepared in the previous steps. De-multiplexing of the indexed samples is done automatically following completion of the sequencing run.

Quick tip – You can use a 1% PhiX spike-in as an additional control to monitor the sequencing reaction. Refer to Illumina documentation on the PhiX control for additional information.

Reagent list

Item	Storage	Supplied by
Reagent Cartridge	-20°C	Illumina
HT1	-20°C	Illumina
PR2	4°C	Illumina
MiSeq Flow Cell	4°C	Illumina
Library at 2nM	4°C	Step 6
NaOH 1 N or 2 N	20°C to 25°C	User
Molecular grade H_2O	20°C to 25°C	User

MiSeq Reagent Kit capacity

Illumina MiSeq Reagent Kit	Time Hours	24/7 Samples	24/11 Samples	96/5 Samples	96/7 Samples	96/11 Samples
Std 300 Cycle (MS-102-2002)	~24	24	24	96	96	72
Micro 300 Cycle (MS-103-1002)	~19	24	20	40	28	20
Nano 300 Cycle (MS-103-1001)	~17	6	4	8	6	4
Std 500 Cycle (MS-102-2003)	~39	24	24	96	96	96
Nano 500 Cycle (MS-103-1003)	~28	12	8	16	12	8

Protocol

7.1 - Prepare the MiSeq according to standard Illumina protocols.

7.2 - Prepare a 1 nM Denatured Library: Combine 10 µL of freshly prepared 0.2 N NaOH and 10 µL of the 2 nM Diluted Size Selected Library in a new 1.5 mL low bind microcentrifuge tube.

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Vortex and spin down. Incubate this 1 nM Denatured Library at room temperature for 5 minutes.

7.3 - Prepare a 20 pM Denatured Library: Add 980 µL of chilled HT1 to the 20 µL of the 1 nM Denatured Library. Vortex and spin down.

7.4 - Prepare a 9 pM Denatured Library: Add 550 µL of chilled HT1 and 450 µL of the 20 pM Denatured Library to a fresh 1.5 mL low bind microcentrifuge tube. Vortex and spin down.

7.5 – Transfer 600 µL of the 9 pM Denatured Library into the Load Samples reservoir of the MiSeq reagent cartridge.

Quick tip – It is advisable to use the provided workbook to create the Sample Sheet that is required by the Miseq. Ensure that the correct Adaptor Plate configuration (A or B) is selected on the Omixon Workbook Sequencing tab that contains the appropriate indexed adaptor sequences.

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Step 8 – Analysis of HLA Sequencing Data

The Illumina MiSeq will process the 9 pM Pooled Library and generate sequencing data as fastq files. Please refer to the HLA Twin manual for assistance with the correct installation of HLA Twin and for information on interpreting the genotyping analysis of your sequencing data. For implementing the Automated Protocol and issues relating to installation or analyzing data, contact support@omixon.com.

Automated Protocol

IT Setup and Configuration

1. Install HLA Twin Server on the Server
2. Install HLA Twin Client on a client computer – multiple HLA Twin Clients may connect to the server
3. Contact Omixon Support (support@omixon.com) for custom installation instructions for Automation

Protocol per Analysis

1. Launch HLA Twin Client and login
2. Data is already processed or is being processed. Review the results using the Traffic Light System in HLA Twin
3. Export the genotyping results and/or consensus sequences as required

Manual Server Protocol

IT Setup and Configuration

1. Install HLA Twin Server on the Server
2. Install HLA Twin Client on a client computer

Protocol per Analysis

1. Launch HLA Twin Client and login
2. Select the MiSeq data in fastq or fastq.gz format and start the Holotype HLA typing run
3. After the Holotype HLA typing has finished, review the results using the Traffic Light System in HLA Twin
4. Export the genotyping results and/or consensus sequences as required

Manual Desktop Protocol

IT Setup and Configuration

1. Install HLA Twin Desktop.

Protocol per Analysis

1. Launch HLA Twin and login
2. Select the MiSeq data in fastq or fastq.gz format and start the Holotype HLA typing run
3. After the Holotype HLA typing has finished, review the results using the Traffic Light System in HLA Twin
4. Export the genotyping results and/or consensus sequences as required

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Technical Assistance

For general assistance with this protocol contact support@omixon.com

Safety Data Sheets are available at www.omixon.com/holotype-hla-documentation/msds

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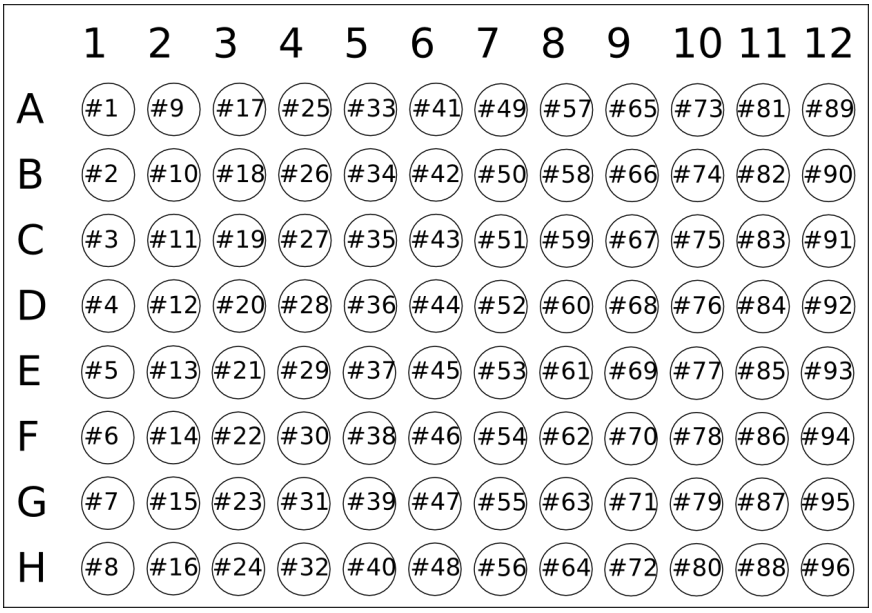
Supplemental Figures

Plate example for Amplicon Plate, Amplification Plate, Dilution Plate, Amplicon Quantitation Plate (for an individual locus) & Reaction Plate



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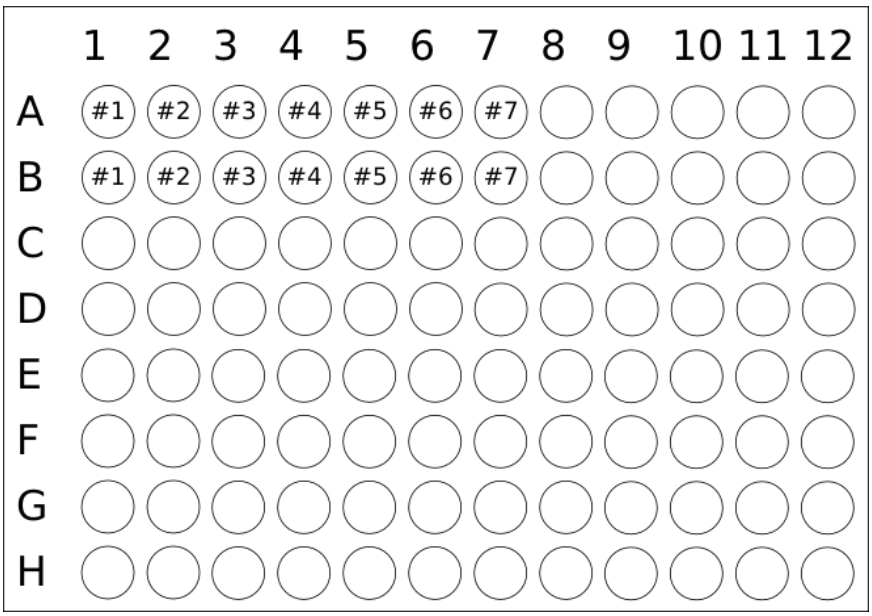
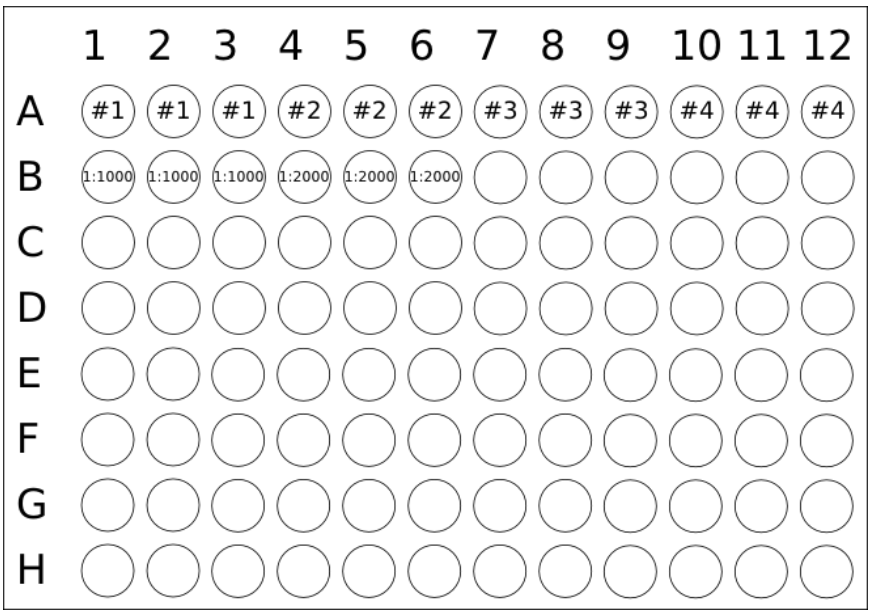
Standards Quantitation Plate Example



KAPA Library Quantitation qPCR Plate Example



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Appendix 1: Pippin Prep

Programming the Pippin Prep

1. Click the Protocol Editor Tab and click the “New” button.
2. Click the folder icon next to the Cassette field and select “1.5%DF Marker K” for the Pippin Prep or “1.5%DF Marker R2” for the Blue Pippin.
3. In the lane that you are programming:
    a. Highlight the “Range” field.
    b. Set the “Ref Lane” to match the lane number you are working in.
    c. Set the “Start*” field to 650.
    d. Set the “End*” field to 1300.
4. In the Reference Lane field, select the lane that you are working in.
5. Click the “Save As” button and name your program.

Running the Pippin Prep

1. Turn on the Pippin Prep by pushing the power button in the back of the device.
2. Visually inspect the Pippin Prep. Make sure the 5 LEDs are on and that the inside of the device is clean and dry.
3. Click on the Sage Science logo on the bottom right of the screen. This will allow you to enter a password. The factory default password is “pips”.
4. Click the Factory Setup Tab and make sure the Base-to-Threshold value is set to 0.02.
5. Place the calibration fixture inside the Pippin Prep, making sure the dark strip is face down and over the LED lights.
6. In the Main tab, click the “Calibration” button.
7. In the Calibration window, make sure the “Target I pH mA” field is set to 0.80 (0.60 for Blue Pippin) and then hit the “Calibrate” button.
8. Go to the Protocols Tab. Click the “Load” button and select the program for Holotype HLA and the specific lane you will be using. Make sure that:
    a. The correct lane is turned on
    b. Broad spectrum selection indicator is on
    c. The reference lane is the same lane that will be running.
9. Go to the Main Tab. Make sure that:
    a. The program you loaded is the one that is selected.

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b. The appropriate reference lane is selected and that it is also the lane the sample will be run in.

10. Inspect the cassette. Before taking off the tape sealing the wells, look to see if there are any bubbles behind the elution port. If there are any bubbles behind the elution port, gently tap and roll the cassette in your hand to work the bubbles out.
11. Place the cassette, with the tape still over the wells, inside of the Pippin Prep.
12. Carefully peel off the tape, making sure to remove the tape from the clean side of the cassette (lane 5) to the used side of the cassette (lane 1). Take care not to splash liquid when the tape is removed to prevent contamination.
13. Remove the entire volume of buffer from the elution port of the lane you will be using and add 40 µL of fresh electrophoresis buffer in that elution port.
14. Add a thin strip of tape over the elution ports.
15. Any reservoirs that are less than 3/4ths full should be topped off with electrophoresis buffer. Do not overfill the wells! The edge of the buffer should 'just' reach the plastic not higher to prevent dragging when the lid slides. Apply buffer from the clean wells (Lane 5) to the used wells (Lane 1).
16. Make sure each of the loading wells (wells with agarose) are filled with electrophoresis buffer. The buffer should be 'just' over the agarose, appearing completely flat.
17. Close the Pippin Prep slowly, watching to make sure no buffer is touching the lid as you are closing the device.
18. Perform the continuity test. When the sensors dry out slightly, it is common for the continuity test to fail once. If the continuity test fails, run the test one more time. Once the continuity test completes, open the Pippin slowly. Make sure no fluid is getting pulled across the cassette by the lid of the Pippin Prep.
19. Briefly vortex the Marker K loading solution and spin it down. Add 10 µL of Marker K loading solution to your ~30 µL of library.
20. Briefly vortex your library and spin it down.
21. Remove 40 µL of buffer from the sample well that you will be using.
22. Add ~40 µL of your library loaded with Marker K to the sample well that you will be using.
23. Mark the lane that you are using with the technician's initials and the date.
24. Close the Pippin Prep and click the "Start" button. Make sure the appropriate lane has been turned on. The sample should run for about 45 minutes.
25. After the run has completed, carefully open the Pippin Prep. Watch to see if the lid drags any liquid across the cassette.
26. Remove the tape over the elution ports, being careful not to flick any liquid.
27. Transfer all the volume from the elution port into a new 1.5 ml low bind tube.

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28. Cover all of the open wells with two pieces of plate sealing tape. Remember to leave a tab on the clean side. This will make it simple to remove the tape from clean to used.
29. Place the sealed cassette into its bag and set it aside.
30. Take the wash cassette and fill it with MiliQ water. Gently close the lid of the Pippin Prep, watching to see if you pull any liquid across the wash cassette.
32. Leave the Pippin Prep closed for several seconds.
33. Open the Pippin Prep, watching to see if you pull any liquid across the wash cassette.
34. Remove the wash cassette, empty it of water, and let it dry.
35. Clean any water off of the Pippin Prep and close it gently.
36. Select the “Shut Down” button in the Pippin Prep menu.

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Appendix 2: Amplicon Quantitation using a qPCR instrument

Duration: ~1 hours

Reagent list

Item	Storage	Supplied by
20× TE Buffer (pH 7.5)	4°C	Promega
Lambda DNA Standard (100 ng/µL)	4°C	Promega
200× QuantiFluor dsDNA Dye	4°C	Promega
Sterile H_2O	20°C to 25°C	User
Class I Amplification Plate(s)	4°C	Step 2
Class II Amplification Plate(s)	4°C	Step 2

Protocol

1. Create a serial dilution using 1.5 mL microcentrifuge tubes and the QuantiFluor Lambda DNA standard (100 ng/µL). Follow the dilution table below:

Label on tube	Input DNA	Volume DNA (µL)	Volume 1x TE (µL)	Final Conc. (ng/µL)
Standard 1	Lambda DNA	7.5 µL	492.5 µL	1.5 ng/µL
Standard 2	Standard 1	250 µL	250 µL	0.75 ng/µL
Standard 3	Standard 2	250 µL	250 µL	0.38 ng/µL
Standard 4	Standard 3	250 µL	250 µL	0.19 ng/µL
Standard 5	Standard 4	250 µL	250 µL	0.09 ng/µL
Standard 6	Standard 5	250 µL	250 µL	0.05 ng/µL
Standard 7, Blank	Blank	0 µL	250 µL	0 ng/µL

2. Prepare the Amplicon Quantitation plates (see supplemental figures). Aliquot 49.5 µL 1x TE buffer to the wells of a clean 96-well plate for the total number of amplicons to be quantitated.
3. Add 0.5 µL of amplicons from corresponding wells in the Amplicon Plates to individual wells in the Amplicon Quantitation Plates. Mix by pipetting.
4. Prepare 1× QuantiFluor Dye working solution using the following formula: 0.25 µL QuantiFluor Dye (200X) + 49.75 µL 1× TE buffer. Prepare sufficient 1× QuantiFluor Dye working solution so that each sample (total samples in Amplicon Plates) and standard (14 total) will receive a 50 µL aliquot.

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5. Prepare a Standards Quantitation Plate and Amplicon Quantitation plates. Aliquot 50 µL of 1× QuantiFluor Dye working solution to wells of the 96-well optical plates using the format of the Standards Quantitation Plate and the Amplicon Quantitation Plates (see supplemental figures).

6. Using the standards prepared above, add 50 µL of each standard, in duplicate, to individual wells in the Standards Quantitation Plate (14 wells total).

7. Vortex to mix thoroughly and spin down.

8. Put each Quantitation Plate in the qPCR machine one at a time and run the following program:

Number of Cycles	Temperature	Time
1	25°C	10 seconds
2	25°C	15 seconds
	25°C	30 seconds (data acquisition)

9. Calculate the concentration of DNA in the Amplicon Quantitation Plates using the raw RFU data generated by the qPCR instrument.

10. Dilute DNA in the Amplicon Plates with sterile H_2O so that the final concentration of DNA is approximately 67 ng/µL.

    - If DNA concentration is 150 ng/µL or greater: add 25 µL of H_2O
    - If DNA concentration is 100-150 ng/µL: add 10 µL of H_2O
    - If DNA concentration is less than 100 ng/µL: add 0 µL of H_2O

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Appendix 3: Library Quantitation using a qPCR instrument

Duration: ~1 hour

It is necessary to quantify the Size-selected Library in order to optimally use the output of the Illumina MiSeq sequencer. The concentration of the Size-selected Library can be accurately measured by qPCR.

Reagent list

Item	Storage	Supplied by
10× Illumina Primer Premix	-20°C	KAPA Biosystems
2× KAPA SYBR FAST qPCR Master Mix	-20°C	KAPA Biosystems
Std 1 (20.00 pM)	-20°C	KAPA Biosystems
Std 2 (2.00 pM)	-20°C	KAPA Biosystems
Std 3 (0.20 pM)	-20°C	KAPA Biosystems
Std 4 (0.02 pM)	-20°C	KAPA Biosystems
Illumina DNA Standards	-20°C	KAPA Biosystems
Molecular grade H_2O		User
1× TE Buffer (pH 8.0)		User
Size Selected Library	4°C	Step 5

Protocol

1 - Prepare the qPCR Primer Mix using the 10× Illumina Primer Premix and the 2× KAPA SYBR FAST qPCR Master Mix:

Note: The KAPA SYBR FAST qPCR kit reagents (qPCR Master Mix, Primer Premix and ROX solutions) are combined during the first use of the kit. This combined solution is stable for at least 30 freeze/thaw cycles. Follow KAPA documentation to determine if ROX is recommended for your qPCR instrument.

qPCR Primer Mix

Reagent	Volume (mL)
10× Illumina Primer Premix	1 mL
2× KAPA SYBR FAST qPCR Master Mix	5 mL
Total Volume	6 mL

2 – Prepare the qPCR Master Mix.

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qPCR Master Mix

Reagent	Volume (µL)
qPCR Primer Mix	228 µL
Molecular grade H_2O	76 µL
Total Volume	304 µL

3 - Prepare a serial dilution of the Size Selected Library.

a. Prepare a 1:1000 dilution by adding 1 µL of Size Selected Library to 999 µL of 1× TE buffer (pH 8.0), thoroughly rinsing the pipette tip. Vortex and spin down.

b. Prepare a 1:2000 dilution by adding 100 µL of the 1:1000 dilution to 100 µL 1× TE buffer (pH 8.0). Vortex and spin down.

4 - Prepare a qPCR Quantitation Plate in a fresh PCR plate compatible with your qPCR system.

5 - Aliquot 16 µL of the qPCR Master Mix in triplicate for standards 1-4, the 1:1000 dilution and the 1:2000 dilution (see supplemental figures).

6 - Aliquot 4 µL of standards 1-4, the 1:1000 dilution and the 1:2000 dilution into the corresponding wells.

7 - Seal the qPCR Quantitation Plate and centrifuge it for 10 seconds.

Note: Avoid creating bubbles in the qPCR Quantitation Plate wells. Centrifuge as needed to eliminate bubbles.

8 - Set the 1:1000 and 1:2000 triplicates as targeted samples, and define the standards (points: 4, starting concentration: 20pM, dilution: 1:10). Run the following program on the qPCR machine in order to determine the DNA concentration of the Size Selected Library:

Number of Cycles	Temperature	Time
1	95°C	5 minutes
25 (no melt curve)	95°C	30 seconds
	60°C	90 seconds (data acquisition)

9 – To convert the qPCR result from pM to nM concentration, enter the “Quantity mean” results of the two library replicates in the Omixon Workbook tab called “Library Quantitation”.

10 - Using the volumes from the workbook, dilute 10 µL of the Size Selected Library to a concentration of 2 nM with sterile H_2O in a fresh 1.5-mL low bind microcentrifuge tube. Store the remaining Size Selected Library at -20° C.

Safe stopping point. Libraries can be stored at -20°C for extended periods of time. In case of long-term storage, re-quantification of the library is highly recommended before running it on the MiSeq.

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