MONOTYPE HLA™
HLA-DQ
24 OR 96 SAMPLES
USER MANUAL

FOR RESEARCH USE ONLY
WITH KITS M23, M24, M25, M26

Protocol Version 3.0.1

IFU v1
Omixon Biocomputing Limited
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Table of Contents

Document History

Protocol Version	IFU V	Date	Description of Changes	Author	Approval Name
1.0	-	June, 2015	Initial Version	Efi Melista	Peter Meintjes
1.1	-	November, 2016	Increase of library preparation reagent volumes	Efi Melista	Peter Meintjes
2.0	-	April 2017	"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.	Efi Melista	Peter Meintjes
3.0.1	V1	12th August 2019	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, Bead based size selection introduced in core manual, 1:4000 dilution introduced in qPCR-based library quantitation, library denaturation concentration changed to 4nM, , known limitations added, transfer of indexed adaptors from Adaptor Plate added to ligation step, Amplicon purification changed to bead-based method, modified Fragmentation Master Mix volumes, modified Fragmentation Incubation time, modified Library Pooling volumes, wording and formatting changes, Technical Support contact update, IFU versioning introduced	Tunde Vago	Efi Melista

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 HLA-DQA1 is amplified for its entire coding length, including elements of the 5' and 3' end untranslated regions and HLA-DQB1 is amplified from intron 1 to the 3' end 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 less than 48 hours 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.

Monotype HLA Packing List

Primer Component Box for 24 samples

Primer mix	Rxns	Vol/tube	# Tubes	Color code
HLA-DQA1	*24*	*60 µL*	*1*	*Brown*
HLA-DQB1	*24*	*60 µL*	*1*	*Black*
Enhancer 1	*96*	*1100 µL*	*1*	*Clear*

Primer Component Box for 96 samples

Primer mix	Rxns	Vol/tube	# Tubes	Color code
HLA-DQA1	*96*	*220 µL*	*1*	*Brown*
HLA-DQB1	*96*	*220 µL*	*1*	*Black*
Enhancer 1	*96*	*1100 µL*	*1*	*Clear*

Library Preparation Reagents Component Box for 24 samples

Reagent	Rxns	Vol/tube	# Tubes	Color code
Fragmentation Enzyme (A)	*24*	*85 µL*	*1*	*Yellow*
Fragmentation Buffer (B)	*24*	*85 µL*	*1*	*Red*
End Repair Enzyme (C)	*24*	*50 µL*	*1*	*Green*
End Repair Buffer (D)	*24*	*95 µL*	*1*	*Orange*
Ligation Enzyme (E)	*24*	*81 µL*	*1*	*Blue*
Ligation Buffer (F)	*24*	*900 µL*	*2*	*Black*

Library Preparation Reagents Component Box for 96 samples

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 24 or 96 individual sequencing libraries.

Two (2) Adaptor Plate configurations are available containing 24 individual indices. Adaptor plates, that contain 24 indices only are available in the following configurations: A1 (i1-i24), A2 (i25-i48), A3 (i49-i72) and A4 (i73-i96).
Three (3) 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) and C (i193-i288).

Excel Workbook

An Excel Workbook is provided to support the Monotype HLA protocol with volume calculations, plate layouts, reagent traceability, record keeping and MiSeq Sample Sheet generation of all of the supported adaptor plate configurations (A1, A2, A3, A4, A, B, C). The Workbook version compatible with this User Manual is v3.0.1. If you do not have a copy, please contact support@omixon.com.

Software – Omixon HLA Twin

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

Product Known Limitations

Please refer to the Product Known Limitations Guide document for known ambiguities and known software limitation of the Monotype HLA product. The Guide may be found on the Omixon website under MyHolotype/Support and Services/HLA Twin Instructions for Use, or it may be requested via a request to support@omixon.com.

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-DQA1 and HLA-DQB1 a total of 200-300 ng 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 (Optional)
- 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

- Promega GoTaq 2X Long PCR Master Mix (Cat#M402A)
  - Each sample requires 14 µL of Promega GoTaq Master Mix for HLA-DQA1 and DQB1 loci
- QuantiFluor dsDNA System from Promega (Cat# E2670)
- Agencourt AMPure XP beads from Beckman Coulter (Cat# A63880, A63881, or A63882)
  - Each Monotype HLA run requires a maximum of 2270 µL of AMpure XP beads
  - Cat# A63880 contains 5 mL of AMPure XP beads
  - Cat# A63881 contains 60 mL of AMPure XP beads
  - Cat# A63882 contains 450 mL of AMPure XP beads
- Qubit dsDNA BR Assay Kit (Cat# Q32850 or Q32853)
  - Cat# Q32850 contains 100 assays
  - Cat# Q32853 contains 500 assays
- 1 X Tris-EDTA (pH 8.0)
- Tween 20 Molecular grade ethanol (Anhydrous Alcohol)
- Molecular grade water (DNase and RNase free)
- 10N Sodium hydroxide
- MiSeq Reagent Kit from Illumina
- 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) - optional
- Library Quantification Kit – Illumina/Universal from KAPA Biosystems (Cat# KK4824) - optional

MiSeq Reagent Kit capacity

Illumina MiSeq Reagent Kit	Time Hours	# of Samples
Std 300 Cycle (MS-102-2002)	~24	192
Micro 300 Cycle (MS-103-1002)	~19	192
Nano 300 Cycle (MS-103-1001)	~17	56
Std 500 Cycle (MS-102-2003)	~39	192
Nano 500 Cycle (MS-103-1003)	~28	80

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 or with the qPCR instrument
- Plate seals for general use
- 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 96-well plates
- 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 for 24 samples

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
- Purified Amplicon Pools – Plate containing all pooled amplicons after bead-based purification
- Standards Quantitation Plate – 96-well plate compatible with the plate fluorometer or qPCR machine where DNA standards are placed to allow for amplicon quantitation

Step 0 – Genomic DNA Preparation

Duration: ~up to an hour

Refer to Workbook: gDNA preparation tab

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/µl. 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-DQA1 and HLA-DQB1 a total of 200 - 300 ng of gDNA is required for each sample .

Step 1 – HLA Amplification Master Mix Preparation

Duration: ~15 minutes

Refer to Workbook: PCR preparation tab

The purpose of this step is to prepare locus-specific Master Mixes to amplify HLA-DQA1 and DQB1 for each sample.

Reagent list

Item	Storage	Supplied by
HLA-DQA1 Primer Mix	-20°C	Omixon
HLA-DQB1 Set 3 Primer Mix	-20°C	Omixon
Enhancer 1	-20°C	Omixon
Promega GoTaq 2X Long PCR Master Mix	-20°C	Promega
Molecular grade H_2O	Room temperature	User

Protocol

1.1 – Remove the HLA-DQA1 and HLA-DQB1 Primer Mixes, Enhancer 1 and the Promega GoTaq 2X Long PCR Master Mixes from storage and thaw at room temperature.

Note: The Taq polymerase in the Promega GoTaq 2X Long PCR Master Mix is a hot start enzyme, therefore it is not required to keep the Master Mix on ice.

1.2 – Vortex both primer mixes, Enhancer 1 and the Promega GoTaq 2X PCR Master Mixes for 5 seconds, then spin them down for 5 seconds (quick spin). Prepare a Master Mix for each Primer Mix according to the tables below (for other than 24/96 samples use the Excel Workbook to calculate the volumes):

Master Mix: HLA-DQA1

Reagent	Volume/sample/locus	Volume/24 samples/locus	Volume/96 samples/locus
Molecular grade H_2O	7.5 µL	195 µL	750 µL
Promega GoTaq 2X PCR Master Mix	7 µL	182 µL	700 µL
Primer Mix	1.5 µL	39 µL	150 µL
Total Volume	**16 µL**	**416 µL**	**1600 µL**

Master Mix: HLA-DQB1 set 3

Reagent	Volume/sample/locus	Volume/24 samples/locus	Volume/96 samples/locus
Molecular grade H_2O	5.5 µL	143 µL	550 µL
Promega GoTaq 2X PCR Master Mix	7 µL	182 µL	700 µL
Enhancer 1	2 µL	52 µL	200 µL
Primer Mix	1.5 µL	39 µL	150 µL
Total Volume	**16 µL**	**416 µL**	**1600 µL**

1.3 – Vortex each Master Mix for 5 seconds and spin them down for 5 seconds (quick spin).

1.4 – Dilute all gDNAs to a concentration of 20-30ng/µL (minimum volume is 12 µL per sample).

Note: Monotype HLA includes sufficient reagents for either 24 or 96 reactions (depending on the kit size) plus additional volume for pipetting loss and failed amplification.

Step 2 – HLA-DQA1 and HLA-DQB1 Amplification

Duration: ~6 hours 30 minutes

Refer to Workbook: Plate maps tab

The purpose of Step 2 is to amplify the HLA-DQA1 and HLA-DQB loci using optimized PCR conditions. Once the PCR reactions are completed, amplification is verified by agarose gel electrophoresis (optional).

Quick tip – The agarose gel electrophoresis, while recommended, is not required to successfully complete the Monotype 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 Monotype HLA protocol.

Reagent list

Item	Storage	Supplied by
HLA-DQA1 Master Mix	-20°C	Step 1
HLA-DQB1 Set 3 Master Mix	-20°C	Step 1
gDNA	4°C	User

Protocol

2.1 – Aliquot 16 µL of each Master Mix into separate wells of 96-well PCR plates.

2.2 – Add 4 µL of each diluted gDNA into the appropriate well of the plates prepared in the previous step. Mix by pipetting.

2.3 – Seal the plate with a thermal seal and visually inspect each well. Spin-down all Amplification Plates in a centrifuge for 5 seconds (quick spin).

2.4 – Place the Amplification Plates into thermal cyclers and run the program according to the tables below:

HLA-DQA1 and HLA-DQB1 Amplification

Number of Cycles	Temperature	Time
1	95°C	2 minutes
40	95°C	15 seconds
	65°C	30 seconds
	68°C	8 minutes
1	68°C	10 minutes
1	4°C	∞

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-DQA1	~5.5
HLA-DQB1 (Set 3)	~6.5

Safe stopping point. Amplicons can be stored at 4°C overnight or at -20°C for up to 3 months.

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

Duration: ~up to 45 minutes

Refer to Workbook: Amplicon quantitation tab

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 1 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 Monotype 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 Monotype HLA protocol.

Reagent list

Item	Storage	Supplied by
HLA-DQA1 and DQB1 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

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

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 (5 seconds) and spin down (for 5 seconds).

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 Amplicon Quantitation 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 in a range of 50-100 ng/µL.

- If DNA concentration is greater than 180 ng/µL: add 20 µL of H_2O
- If DNA concentration is 115-180 ng/µL: add 10 µL of H_2O
- If DNA concentration is less than 115 ng/µL: do not add any H_2O

Safe stopping point. Diluted amplicons can be stored at 4°C overnight or at -20°C for up to 3 months.

Step 4 – Amplicon Pooling and Purification

Duration: ~40 minutes

Refer to Workbook: Library preparation tab

In this step, the normalized amplicons are pooled for each sample. The amplicon pools are then purified using magnetic beads.

Reagent list

Item	Storage	Supplied by
Normalized HLA-DQA1 and DQB1 Amplification Plate(s)	4°C	Step 3
AMPure XP magnetic 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 – Remove the Agencourt AMPure XP Reagent from storage, and allow the beads to come to room temperature.

4.2 – Combine HLA-DQA1 and DQB1 loci for each sample into the wells of a fresh Pooled Amplicons Plate. Combine the volumes indicated for each locus according to the following table to obtain a final volume of 15 µL:

HLA locus	Pooled volume
DQA1	7.5 µL
DQB1 set 3	7.5 µL

4.3 – Cover the Pooled Amplicon Plate with a seal and centrifuge for 5 seconds (quick spin).

4.4 – Prepare fresh 80% Ethanol. Each sample requires 200µL. It's recommended to prepare enough for all the samples plus 10% extra. For volume calculation use the Excel Workbook.

4.5 – Vortex AMPure XP beads **thoroughly** to resuspend (for 30 seconds).

4.6 – Prepare a clean 8-tube strip or reservoir and aliquot the Agencourt AMPure XP beads. Each sample requires 9 µL. It's recommended to aliquot enough for all the samples plus 10% extra. For volume calculation use the Excel Workbook.

4.7 – Using a multichannel pipette, transfer 9 µL of AMPure XP beads into each well of the Pooled Amplicon Plate. Mix by pipetting until the beads are mixed homogenously.

4.8 – Incubate for 5 minutes at room temperature.

4.9 – Put the plate on the magnetic stand for 3 minutes.

4.10 – Set the multichannel pipette to 100 µL, then slowly remove and discard the supernatant. Be careful not to disturb the beads.

4.11 – Pour the freshly prepared 80% ethanol into a clean reservoir.

4.12 – Without removing the plate from the magnet, dispense 100 µL of the 80% ethanol into each well with a multichannel pipette. Be careful not to disturb the beads.

4.13 – Incubate on the magnet at room temperature for 30 seconds, then carefully remove and discard the supernatant. Be careful not to disturb the beads.

4.14 – Repeat steps 4.12 and 4.13 once.

4.15 – To remove residual ethanol, keep the plate on the magnet and carefully remove any remaining supernatant with a small multi-channel pipette without disturbing the beads.

4.16 – Leave the plate on the magnet for 5 minutes to air dry the beads.

4.17 – Prepare a clean 8-tube strip or reservoir, and aliquot molecular grade water sufficient for 17 µL per reaction. For volume calculation use the Excel Workbook.

4.18 – Keeping the plate on the magnet aliquot 17 µL water to each well.

4.19 – Remove plate from magnet and

a. Mix by pipetting 10 times (or until beads are fully resuspended), or
b. Cover the plate and vortex for 20 seconds (or until beads are fully resuspended).

4.20 – Cover the plate and centrifuge for 5 seconds (quick spin).

4.21 – Incubate at room temperature for 2 minutes.

4.22 – Place the plate back on the magnet and incubate for 2 minutes.

4.23 – Transfer all eluant (~17 µL) from the wells to the corresponding wells of a new 96-well plate. Be careful not to disturb the beads.

Safe stopping point. Purified Amplicon Pools can be stored at 4°C overnight or at -20°C for up to 3 months.

Step 5 – Library Preparation

Duration: ~1 hour 40 minutes

Refer to Workbook: Library preparation tab

During this step, the purified amplicon pools are prepared for sequencing on the Illumina MiSeq. The amplicons are enzymatically fragmented, the ends are repaired and adenylated, then indexed adaptors are ligated to the ends.

Note: Omixon recommends volumes greater than necessary for 24/96 samples because many of the enzymes and buffers are viscous, resulting in excess pipetting loss. It is also recommended to use a reagent plate for more than 8 samples. The reagent plate is a 96-well plate placed on a PCR cooler block and where each Master Mix is aliquoted into a clean column for dispensing with a multichannel pipette.

Reagent list

Item	Storage	Supplied by
Purified Amplicon Pools	4°C	Step 4
Molecular grade H_2O	Room temperature	User
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

Protocol

5.1 – Turn on the thermal cycler. Verify that the heated lid is warming up.

5.2 – Thaw the Fragmentation Buffer to room temperature and keep it on ice.

Note: Be sure to vortex the Fragmentation Enzyme **thoroughly** before use. Spin it quickly (5 seconds), then keep it on ice.

5.3 – Prepare Fragmentation Master Mix according to the table below (for other than 24/96 samples use the Excel Workbook to calculate the volumes):

Fragmentation Master Mix

Reagent	Volumes per sample (µL)	Recommended volumes for 24 samples (µL)	Recommended volumes for 96 libraries (µL)	Color code
Molecular grade H_2O	1.5 µL	42 µL	165 µL	
Fragmentation Buffer (B)	2 µL	56 µL	220 µL	Red
Fragmentation Enzyme (A)	1.5 µL	42 µL	165 µL	Yellow
Total Volume	**5 µL**	**140 µL**	**550 µL**	

5.4 – Vortex the Fragmentation Master Mix thoroughly (for 5 seconds) and spin down for 5 seconds (quick spin). Place the Fragmentation Master Mix on ice.

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 and cooler racks is recommended to minimize risk of excessive fragmentation.

5.5 – Centrifuge the Purified Amplicon Pools for 5 seconds (quick spin), and place it on ice or a cold block.

5.6 – Prepare a Reaction Plate: place a new 96-well PCR plate on a PCR cold block.

5.7 – Add 5 µL of Fragmentation Master Mix into the empty wells of the Reaction Plate, corresponding with samples in the Pooled Amplicons Plate. The use of a multi-channel pipette is recommended.

5.8 – Transfer 15 µL of each purified amplicon pool from the Pooled Amplicons Plate to the corresponding well of the Reaction Plate using a multi-channel pipette. Mix by pipetting. Keep the Reaction Plate on the cold block.

5.9 – Cover the Reaction Plate with a thermal seal and centrifuge for 5 seconds (quick spin).

5.10 – Incubate the Reaction Plate in a thermal cycler using the following program:

Fragmentation Program

Number of Cycles	Temperature	Time
1	37° C	8 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.

5.11 – Prepare the End Repair Master Mix according to the table below (for fewer than 24 samples use the Excel Workbook to calculate the volumes):

End Repair Master Mix

Reagent	Volumes per sample (µL)	Recommended volumes for 24 samples (µL)	Recommended volumes for 96 libraries (µL)	Color code
Molecular grade H_2O	1.25 µL	35 µL	150 µL	
End Repair Buffer (D)	2.5 µL	70 µL	300 µL	Orange
End Repair Enzyme (C)	1.25 µL	35 µL	150 µL	Green
Total Volume	**5 µL**	**140 µL**	**600 µL**	

5.12 – Vortex the End Repair Master Mix and spin down for 5 seconds (quick spin).

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

5.14 – Cover the Reaction Plate with a thermal seal and centrifuge for 5 seconds (quick spin).

5.15 – Incubate the Reaction Plate in a thermal cycler using 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.

5.16 – 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 1 minute at 3000 rpm.

5.17 – Carefully pull the seal off the Adaptor Plate. Do not shake the Adaptor Plate once the seal is removed to prevent index cross contamination.

5.18 – Transfer 5 µL from each well of the Adaptor Plate to the corresponding well of the Reaction Plate. Mix well by pipetting. Use the Reaction Plate for the remaining steps in the protocol.

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 5 µL from each well of the Adaptor Plate to the corresponding well of the Reaction plate, and mix well by pipetting.

b. Reseal the Adaptor Plate and return it to -20°C. Use the Reaction Plate for the remaining steps in the protocol.

5.19 – Prepare the Ligation Master Mix according to the table below (for fewer than 24 samples use the Excel Workbook to calculate the volumes):

Ligation Master Mix

Reagent	Volumes per sample (µL)	Recommended volumes for 24 samples (µL)	Recommended volumes for 96 libraries (µL)	Color code
Ligation Enzyme (E)	2.5 µL	70 µL	275 µL	Blue
Ligation Buffer (F)	30 µL	840 µL	3300 µL	Black
Total Volume	**32.5 µL**	**910 µL**	**3575 µL**	

5.20 – Vortex the Ligation Master Mix thoroughly (for 5 seconds) and spin down for 5 seconds (quick spin).

5.21 – Aliquot 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.

5.22 – Cover the Reaction Plate with a thermal seal and centrifuge for 5 seconds (quick spin).

5.23 – Incubate the Reaction Plate in the thermal cycler using 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 up to 3 months.

Library Pooling

5.24 – Create the final library by combining an aliquot from each pooled amplicon, now a sample-specific library, into a 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 2.0 mL low bind tube that will contain the pooled **Library**.

For Library Size Selection using AMPure XP magnetic beads, please proceed to Step 5. For Library Size Selection using the Pippin Prep, please proceed to Appendix 2.

Step 6 – Bead-based Library Size Selection

Duration: ~1 hour 20 minutes

Refer to Workbook: Library preparation tab

Step 6 describes the protocol for size selection of a single pool of indexed sample libraries using AMPure XP magnetic beads. Agencourt AMPure XP beads use solid phase reversible immobilization chemistry to bind specific sizes of DNA to the beads based on a bead/sample (v/v) ratio. Lower ratios of beads (0.2X- 0.8X) bind larger fragments of DNA leaving smaller fragments in solution. Increasing the bead/sample ratio (1X – 2X) allows the beads to bind smaller fragments as well.

Reagent list

Item	Storage	Supplied by
Pooled Library	4°C	Step 5
AMPure XP beads	4°C	Beckman Coulter
1X Tris-EDTA	20°C to 25°C	User
Tween 20	20°C to 25°C	User
80% ethanol (freshly prepared)	20°C to 25°C	User
Molecular grade Water	20°C to 25°C	User

Note: The following protocol is written for libraries containing at least 12 samples where the library pool volume is 700 µL. If your Pooled Library is less than 700 µL, use the Excel Workbook to calculating the volumes or use the following ratios:

	Library : Beads ratio
First Selection	1 : 0.2
Second Selection	1 : 1
Third Selection	Always fixed

Protocol

Reagent Preparation:

6.1 – Prepare the Bead Wash Buffer according to the table below:

Reagent	Volume
1X Tris-EDTA	40 mL
Tween 20	20 µL
Total Volume	40.02 mL

Note: The Bead Wash Buffer can be stored at room temperature for up to 3 months.

6.2 – Freshly prepare 5 ml of 80% ethanol (4 ml EtOH + 1 ml H_2O).

6.3 – Vortex AMPure XP beads **thoroughly** to resuspend (for 30 seconds).

6.4 – Aliquot 840 µL of AMPure XP beads in a 2 ml low bind tube and **bring to room temperature**. Keep the stock AMpure beads at room temperature for use in Selection 3 step below.

6.5 – Add 840 µL of bead wash buffer to the aliquoted beads. Vortex thoroughly for 10 seconds. Spin for 5 seconds (quick spin).

6.6 – Incubate for 1 minute at room temperature.

6.7 – Put the tube on the magnetic stand for 2 minutes or until solution is clear (typically no more than 4 minutes).

6.8 – Carefully remove and discard the supernatant. Be careful not to disturb the beads.

6.9 – Add 840 µL of bead wash buffer to the beads. Vortex thoroughly for 10 seconds. Spin for 5 seconds (quick spin).

6.10 – Incubate for 1 minute at room temperature.

6.11 – Vortex the beads for 10 seconds to resuspend and aliquot 140 µL in a clean 2 ml low bind tube. *If using fewer than 12 samples, see Excel Workbook for the volume of beads to use.*

6.12 – **KEEP** the remaining 700 µL of beads in the bead wash buffer aside for the 2nd selection step.

1st Selection:

6.13 – Place the tube containing 140 µL of beads in bead wash buffer on a magnetic stand for 2 minutes. *For fewer than 24 samples see Excel Workbook for the volume of beads to use.*

6.14 – Carefully remove and discard supernatant. Remove the tube from the magnet.

6.15 – Add **700 µL** of the pooled library to the beads. Vortex beads for 5 seconds to resuspend and spin for 5 seconds (quick spin). *If using fewer than 24 samples see Excel Workbook for the volume of Pooled Library to use.*

6.16 – Incubate at room temperature for 5 minutes.

6.17 – Place the tube back on the magnetic stand for 4 minutes to pellet the beads.

6.18 – **COLLECT the eluate (library)** into a clean 1.5 ml low bind tube.

2nd Selection:

6.19 – Place the tube containing 700 µL of beads in bead wash buffer on a magnet for 2 minutes to pellet the beads. Carefully remove and discard the supernatant. *If using fewer than 24 samples see Excel Workbook for the volume of beads to use.*

6.20 – Remove the tube with the beads from the magnet.

6.21 – Add the library eluate collected in the 1st selection step 5.18 to the beads.

6.22 – Mix thoroughly by vortexing for 10 seconds to resuspend beads. Spin for 5 seconds (quick spin).

6.23 – Incubate at room temperature for 5 minutes.

6.24 – Place the tube back on the magnetic stand for 4 minutes to pellet the beads.

6.25 – Carefully remove and discard the supernatant.

6.26 – Add ~0.7-1 mL of 80% freshly prepared ethanol to the tube while still on the magnetic stand. Volume should be sufficient to cover the beads.

6.27 – Incubate on the magnet at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

6.28 – Repeat steps 6.26 and 6.27 once.

6.29 – Spin the tube for 3 seconds in centrifuge (quick spin), quickly place back on the magnet, and remove residual ethanol with a pipette.

6.30 – Leave the tube on the magnet with the lid open for 5 minutes to air dry the bead pellet.

6.31 – Remove the tube from the magnet and elute the DNA by adding **200 µL** molecular grade water to the beads.

6.32 – Mix thoroughly by vortexing for 10 seconds to resuspend beads. Spin for 5 seconds (quick spin).

6.33 – Incubate at room temperature for 2 minutes.

6.34 – Place the tube on the magnetic stand for 2 minutes.

6.35 – **TRANSFER the 200 µL of eluate (library)** into a clean 2 ml low bind tube.

3rd Selection:

6.36 – Mix **thoroughly by vortexing** (for 30 seconds) the room temperature stock AMPure XP beads, then aliquot 160 µL to the 200 µL of library from step 5.35 of the 2nd selection.

6.37 – Mix thoroughly by vortexing for 5 seconds to resuspend beads in the library. Spin for 5 seconds (quick spin).

6.38 – Incubate at room temperature for 5 minutes.

6.39 – Place the tube back on the magnetic stand for 4 minutes to pellet the beads.

6.40 – Carefully remove and discard the supernatant.

6.41 – Add ~0.5-1 mL of 80% freshly prepared ethanol to the tube while still on the magnetic stand. Volume should be sufficient to cover the beads.

6.42 – Incubate on the magnet at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

6.43 – Repeat steps 6.41 and 6.42 once.

6.44 – Spin the tube for 5 seconds in centrifuge (quick spin), quickly place back on the magnet, and remove residual ethanol with a pipette.

6.45 – Leave the tube on the magnet with the lid open for 5 minutes to air dry beads.

6.46 – Remove the tube from the magnet and elute the library by adding **50 µL** molecular grade water to the beads.

6.47 – Mix thoroughly by vortexing for 10 seconds to resuspend beads. Spin for 5 seconds (quick spin).

6.48 – Incubate at room temperature for 2 minutes.

6.49 – Place the tube on the magnetic stand for 2 minutes.

6.50 – **TRANSFER the 50 µL of eluate (library)** into a clean 1.5 ml low bind tube.

Safe stopping point. Libraries can be stored at -20°C for up to 3 months.

Step 7 – Library Quantitation using an intercalating dsDNA fluorescent dye

Duration: ~15 minutes

Refer to Workbook: Library quantitation tab

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) and the Quantus reader by Promega (uses the Quantifluor dsDNA fluorescent dye). 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 method 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 6

Protocol

7.1 – 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 the standards and the library for 5 seconds and spin them for 5 seconds (quick spin).

7.2 – Add 995 µL Buffer and 5 µL dye to a 1.5 ml centrifuge tube. Vortex for 2 seconds and spin down for 5 seconds (quick spin).

7.3 – 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.

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

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

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

7.7 – Switch on Qubit machine and choose BR protocol.

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

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

7.10 – 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”.

7.11 – Using the results from the Qubit measurement, dilute 10 µL of the Size Selected Library to a concentration of 4 nM with molecular grade H_2O in a new 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 up to 3 months. In case of long-term storage, re-quantification of the library is highly recommended before running it on the MiSeq.

Step 8 – Sequencing on Illumina MiSeq

Duration: ~24 hours

Refer to Workbook: Sequencing tab and Sample Sheet tab

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 run. 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 4nM	4°C	Step 7
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	96
Micro 300 Cycle (MS-103-1002)	~19	24	24	48	32	24
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

8.1 – Prepare the MiSeq machine according to standard Illumina protocols.

8.2 – *Denature the 4nM Library*: Combine 5 µL of freshly prepared 0.2 N NaOH and 5 µL of the 4 nM Diluted Size Selected Library in a new 1.5 mL low bind microcentrifuge tube. Vortex for 5 seconds and spin down for 5 seconds (quick spin). Incubate this 2 nM Denatured Library at room temperature for 5 minutes.

8.3 – *Prepare a 20 pM Denatured Library*: Add 990 µL of chilled HT1 to the 10 µL of the 2 nM Denatured Library. Vortex for 5 seconds and spin down for 5 seconds (quick spin).

8.4 – *Prepare a 9 pM Denatured Library*: Add 550 µL of chilled HT1 and 450 µL of the 20 pM Denatured Library to a new 1.5 mL low bind microcentrifuge tube. Vortex for 5 seconds and spin down for 5 seconds (quick spin).

8.5 – 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 (A1, A2, A3 or A4) is selected on the Sequencing tab that contains the appropriate indexed adaptor sequences.

Step 9 – 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 user 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.

Technical Assistance

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

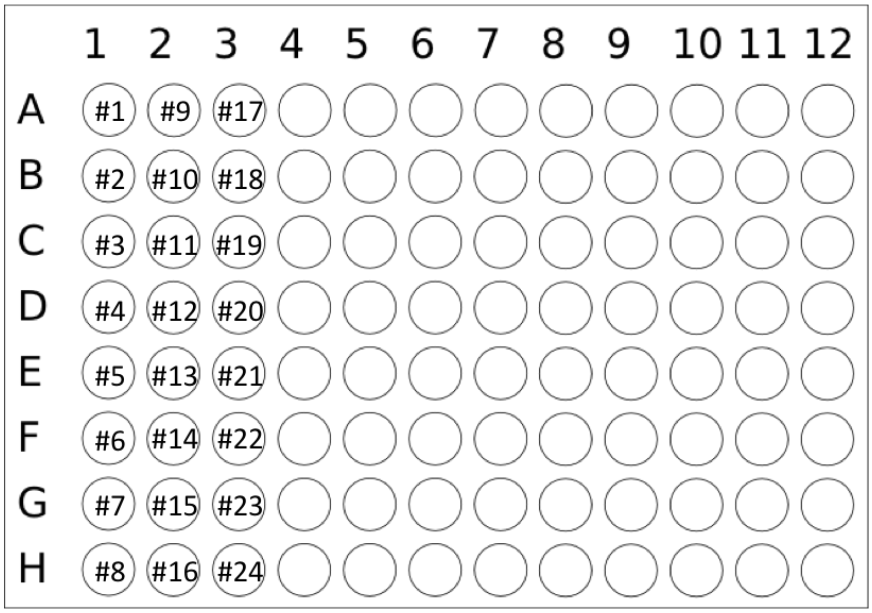
Material Safety Data Sheets are available on MyHolotype upon registration.
https://www.omixon.com/my-holotype/login/

Phone Support

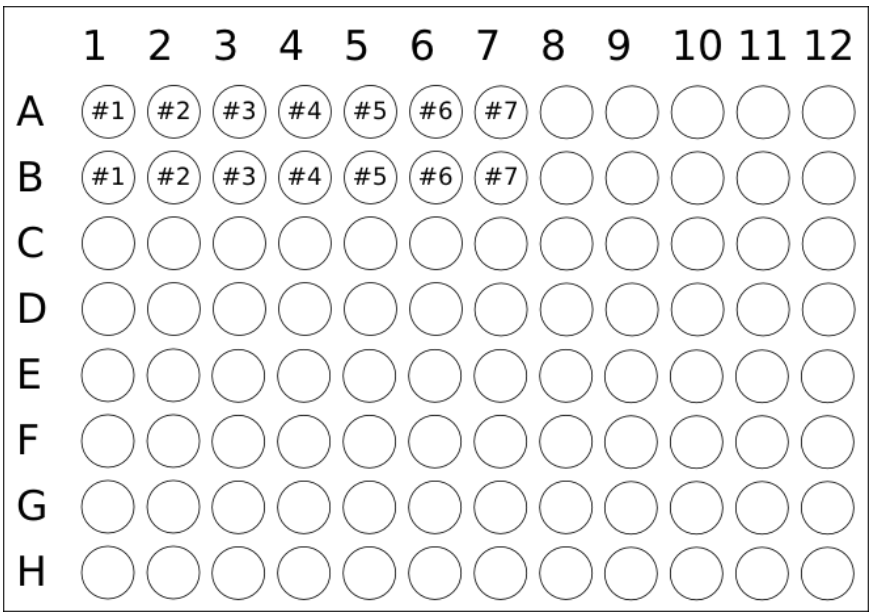
+1 (617) 500-0790

Supplemental Figures

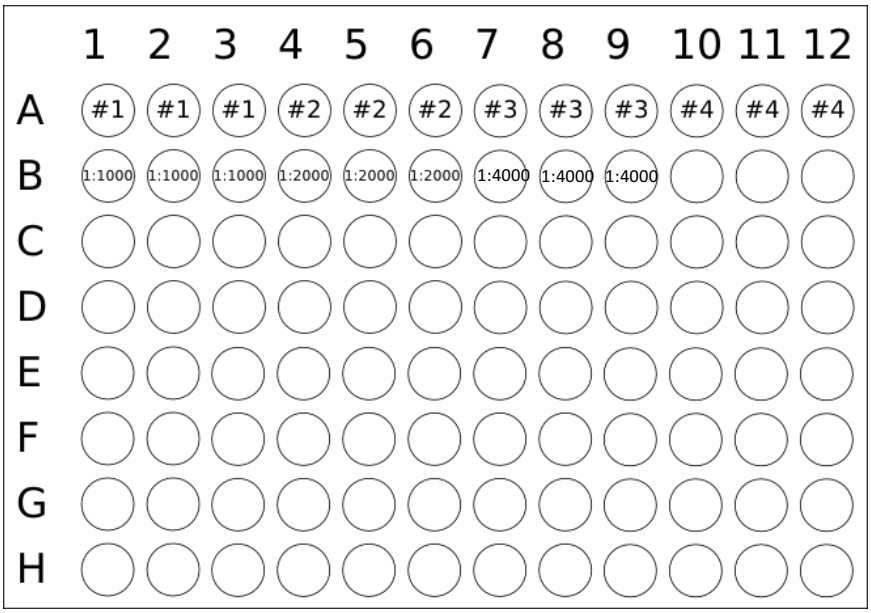
Plate example for Amplicon Plate, Amplification Plate, Dilution Plate, Amplicon Quantitation Plate (for an individual locus) & Reaction Plate



Standards Quantitation Plate Example



KAPA Library Quantitation qPCR Plate Example



Appendix 1: Amplicon Quantitation using a qPCR instrument

Duration: ~40 minutes

Refer to Workbook: Amplicon quantitation tab

This procedure is a modified amplicon quantitation setup for qPCR machines with 100 µL capacity. For 200 µL volumes, see Excel Workbook.

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
Molecular grade H_2O	20°C to 25°C	User
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.

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 for 10 seconds to mix thoroughly and spin down for 5 seconds (quick spin).

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
	25°C	15 seconds
2	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 molecular grade H_2O so that the final concentration of DNA is in a range of 50-100 ng/µL.

    - If DNA concentration is greater than 180 ng/µL: add 20 µL of H_2O
    - If DNA concentration is 115-180 ng/µL: add 10 µL of H_2O
    - If DNA concentration is less than 115 ng/µL: do not add any H_2O

Appendix 2: Library Size Selection Using Pippin Prep

Duration: ~1 hour

Refer to Workbook: Library preparation tab

This step takes the Library from Step 4, performs purification with AMPure XP beads and 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.

Quick tip – For instructions on how to setup the Pippin Prep, refer to page 48.

Reagent list

Item	Storage	Supplied by
Pooled Library	4°C	Step 5
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
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

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

Protocol

Library Purification

1. – Allow AMPure XP beads to come to room temperature. Ensure they are homogeneous (no clumps or pellets) by vortexing for 30 seconds.

2. – Prepare freshly made 5 mL of 80% ethanol (4 mL EtOH + 1 mL H2O).

3. – Add 900 µL of AMPure XP beads to the tube containing 900 µL of Library. Mix thoroughly by vortexing for 10 seconds and centrifuge for 5 seconds (quick spin). 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 to the library. There should be a 1:1 ratio of Final Pool and AMPure XP beads.

4. – Place the Library tube on a magnetic stand and incubate for 10 minutes.

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

6. – 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.

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

8. – Repeat steps 6 and 7 above.

9. – Quickly spin down (for 3 seconds) the Library tube and place it back on the magnetic stand with the lid open. Remove any residual ethanol with a pipette. Do not disturb the beads.

Note: Ensure that 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.

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

11. – 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.

12. – Vortex the Library for 20 seconds to fully resuspend the beads. Centrifuge for 5 seconds (quick spin) if some droplets remain on the side walls. Ensure the beads remain in suspension.

13. – Incubate the Library at room temperature for 2 minutes.

14. – Place the Library tube on the magnetic stand for 2 minutes.

15. – 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 up to 3 months.

Library Size Selection

16. – Bring the Marker K loading solution to room temperature.

17. – Combine 31 µL of the Pool with 10 µL of Marker K loading solution.

18. – Mix by vortexing for 5 seconds and spin down for 5 seconds (quick spin).

19. – Configure the Pippin Prep to collect DNA fragments between 650 and 1300 bps. Set the reference lane to the one you are using.

20. – Load the 40 µL sample into the sample port and run. Run time is 45-50 minutes.

21. – 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 up to 3 months.

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.
    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.
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.

Appendix 3: Library Quantitation using a qPCR instrument

Duration: ~1 hour

Refer to Workbook: Library quantitation tab

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	20°C to 25°C	User
1× TE Buffer (pH 8.0)	20°C to 25°C	User
Size Selected Library	4°C	Step 6

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.

qPCR Master Mix

Reagent	Volume (µL)
qPCR Primer Mix	264 µL
Molecular grade H_2O	88 µL
Total Volume	352 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 for 5 seconds and spin down for 5 seconds (quick spin).
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 for 5 seconds and spin down for 5 seconds (quick spin).
c. Prepare a 1:4000 dilution by adding 20 µL of the 1:2000 dilution to 20 µL 1× TE buffer (pH 8.0). Vortex for 5 seconds and spin down for 5 seconds (quick spin).

4 – Prepare a qPCR Quantitation Plate in a new 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, the 1:2000 and the 1:4000 dilution (see supplemental figures).

6 – Aliquot 4 µL of standards 1-4, the 1:1000 dilution, the 1:2000 dilution and the 1:4000 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, the 1:2000 and 1:4000 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 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 4 nM with molecular grade H_2O in a clean 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 up to 3 months. In case of long-term storage, re-quantification of the library is highly recommended before running it on the MiSeq.