Certificate of Analysis of the Holotype HLA 24/7 Configuration A4 & CE v2

Product name	Holotype HLA 24/7 Configuration A4 & CE v2
Reference number	H60
LOT number	0000459173 (N9/013- P5.1/014-E1/012-R2/018)
Kit Assembling Date	2019.07.15
Expiration data	2020.04.14

1 Quality control application overview

Reagents from Holotype Kits (HLA locus specific primers and PCR enhancers) are combined with Qiagen Long Range PCR Kit reagents for amplification of HLA-A, B, C, DRB1, DQB1, DQA1 and DPB1 genes for next-generation sequencing. Amplicons for all loci from each sample are combined in a roughly equimolar amount. Library preparation reagents (Fragmentation, End repair, and Ligation enzymes and buffers) are used to create libraries for sequencing from the pools of amplicons. Sample HLA typings are derived from the sequencing data. Indexed adaptor plates are tested for contamination and variability in a separate set of experiment.

2 Kit Components

2.1 Primer Component Box

The primer component provides specific ready to use primer solutions for targeted Long Range PCR amplification of HLA genes A, B, C, DPB1, DQA1 and DQB1, and DRB1. Additionally, it also contains two types of PCR additives (Enhancer 1 and Enhancer 2).

Primer mix	REF #	Rxns	Vol/tube	# Tubes	Color code
HLA-A	*P013*	*24*	*60 µL*	*1*	*Yellow*
HLA-B	*P023*	*24*	*60 µL*	*1*	*Red*
HLA-C	*P033*	*24*	*60 µL*	*1*	*Orange*
HLA-DRB1	*P043*	*24*	*60 µL*	*1*	*Green*
HLA-DQB1 Set 3	*P123*	*24*	*60 µL*	*1*	*Blue*

HLA-DQA1	*P083*	*24*	*60 µL*	*1*	*Brown*
HLA-DPB1	*P073*	*24*	*60 µL*	*1*	*Purple*
Enhancer 1	*E01*	*24*	*1100 µL*	*1*	*Clear*
Enhancer 2	*E02*	*24*	*300 µL*	*1*	*Clear*

2.2 Library Preparation Reagents Component Box

The library preparation component box provides reagents for library preparation (fragmentation, blunt-end and adenylate the ends of the amplicons and ligate indexed adaptor sequences to them) from HLA amplicons.

Reagent	REF #	Rxns	Vol/tube	# Tubes	Color code
Fragmentation Enzyme (A)	*R12*	*24*	*85 µL*	*1*	*Yellow*
Fragmentation Buffer (B)	*R22*	*24*	*85 µL*	*1*	*Red*
End Repair Enzyme (C)	*R32*	*24*	*50 µL*	*1*	*Green*
End Repair Buffer (D)	*R42*	*24*	*95 µL*	*1*	*Orange*
Ligation Enzyme (E)	*R52*	*24*	*81 µL*	*1*	*Blue*
Ligation Buffer (F)	*R62*	*24*	*900 µL*	*2*	*Black*

2.3 96-well Indexed Adaptor Plate

The 96 well indexed adaptor plate component contains ready to use indexed NGS adaptors (double stranded DNA oligonucleotides) in 5 µL solution for generating individual NGS libraries. The 96 well indexed adaptor plate contains sufficient kind of indexed adaptors for 24 individual NGS library generation and for downstream sample identification.

Product type	Associated Reagent	REF #	Rxns	Vol/well	# Plates
Holotype HLA 24/7 Configuration A4 & CE v2(REF:H60)	**Adaptor Plate A4 (i73-96)**	**N9**	**24**	**5 µL**	**1**

3 Summary of Quality Control testing

Evaluation/Assessment*	Pass/Fail
Physical inspection	Pass

Qualitative assessment of amplification by gel electrophoresis	Pass
Quantitative assessment of amplification by picogreen	Pass
Assessment of mappability of sequences	Pass
Assessment of amplification bias	Pass
Performance specifications: accuracy, precision, sensitivity, specificity	Pass
Assessment of fragmentation reagents	Pass
Assessment of end repair and ligation reagents	Pass
Assessment of adaptors for index sequence and location	Pass
Assessment of adaptors for cross-contamination	Pass
Assessment of adapter variability	Pass

* * See individual sections for details.

3.1 Physical inspection

All contents of the kit are inspected for proper components, volumes and labeling. The condition of all reagents were inspected after packaging and shipping.

3.1.1 Results of physical inspection

Criteria for acceptability	Pass/Fail
Expected volumes in all tubes and wells	Pass
Proper labeling	Pass
Proper shipping condition (on dry ice)	Pass
Reagents clear and not discolored	Pass
Proper plate sealing	Pass

3.2 Amplification components quality control testing

3.2.1 Amplification

Amplification primers and enhancer reagents are tested on a selected panel of cell lines that have known typing information for the tested 7 HLA loci. Primers were used to amplify 12 samples in duplicate. All samples are assessed by gel electrophoresis for presence of amplicon at each loci and also through quantitative assessment of amplicon amount by picogreen.

3.2.1.1 Results of amplification

Criteria for acceptability	Pass/Fail
Quantitative assessment of amplification by picogreen : > 50 ng/µL for all amplicons (excluding FTA)	Pass
Qualitative assessment of amplification by gel electrophoresis	Pass

3.2.2 Assessment of amplification success and specificity

The primers used to amplify the HLA genes are intended to be specific to a given locus (or loci in the case of multiplexed primers). To assess the performance of the primers for specificity to the intended loci four measures are used:

- *Mapped read count per locus*: This measure estimates amplification success
- *Best quality mapped read count per locus*: This measure estimates amplification success
- Best quality mapped read count / Mapped read count per locus: This measure estimates amplification quality
- Reads mappable to the seven targeted loci / Processed read count for the sample: This measures estimates primer specificity

3.2.2.1 Results of amplification success and specificity

Criteria for acceptability	Pass/Fail
Amplification success: ***Pass*: At least 6000 mappable reads AND at least 5000 best mapping reads found for each loci in all samples.** ***Fail*: Less than 6000 reads OR less than 5000 best mapping reads found for at least one locus.**	Pass
Amplification quality: ***Pass:* At least 25% of mappable reads can be used for consensus generation.** ***Fail:* Less than 25% of mappable reads can be used for consensus generation.**	Pass
Primer specificity: ***Pass*: At least 60% of processed reads are mappable to the targeted loci.** ***Fail*: Less than 60% of processed reads are mappable to the targeted loci.**	Pass

3.2.3 Amplification balance assessment

The differences between the representations of each allele in the samples is evaluated for balance.

3.2.3.1 Results for amplification balance assessment

Criteria for acceptability	Pass/Fail
Amplification balance: ***Pass:* Minor allele should be no lower than 20% (i.e. the major allele shown in graph should be lower than 0.8)** ***Fail:* At least one minor allele goes lower than 20%.**	Pass

3.2.4 Genotyping performance assessment

Given a set of samples, it is expected that the Holotype kit that is being quality control tested produces the same genotyping results obtained using independent genotyping methods (e.g. SSO, SBT or previous NGS runs). Therefore, a set of performance measures was calculated for each locus independently to measure the performance of genotyping:

- Sensitivity
- Specificity
- Precision/Positive Predictive Value (PPV)
- Negative Predictive Value (NPV)
- Accuracy/Type Correctly Classified (TCC)
- Repeatability
- Reproducibility

Performance statistics were calculated on a three field level. Genotypes derived from both replicates in the pooled configuration were taken into account for the aforementioned calculations and were recorded separately.

3.2.4.1 Results for genotyping performance assessment

Criteria for acceptability	Pass/Fail
Sensitivity: ***Pass:*** **Sensitivity is 100% for all loci.** ***Fail:*** **Sensitivity is less than 100% for one or more loci.**	Pass
Specificity: ***Pass:*** **Specificity is 100% for all loci.** ***Fail:*** **Specificity is less than 100% for one or more loci.**	Pass
Precision/PPV: ***Pass:*** **Positive predictive value is 100% for all loci.** ***Fail:*** **Positive predictive value is less than 100% for one or more loci.**	Pass
NPV: ***Pass:*** **Negative predictive value is 100% for all loci.** ***Fail:*** **Negative predictive value is less than 100% for one or more loci.**	Pass
Accuracy/TTC: ***Pass:*** **Type correctly classified is 100% for all loci.** ***Fail:*** **Type correctly classified is less than 100% for one or more loci.**	Pass
Repeatability: ***Pass:*** **Results are identical between repeats within a QC run.** ***Fail:*** **There is at least one difference between repeats within a QC run.**	Pass
Reproducibility:	Pass

Pass: **Results of the current QC run and the QC run of the previous manufacturing lot are identical.**
Fail: **Results of the current QC run and the QC run of the previous manufacturing lot are not identical.**

3.3 Library Preparation reagent quality control testing

3.3.1 Assessment of fragmentation

The 12 sample pools are inspected to assess that the fragmentation reagents were functioning properly, breaking the DNA into a wide range of sizes. Amplicons were pooled together prior to fragmentation and a small amount of each pool was used to assess the proper fragmentation through gel electrophoresis.

3.3.1.1 Results of fragmentation

Criteria for acceptability	Pass/Fail
All samples should produce a wide range of DNA fragments	Pass
Similar size profile as the previous LOT	Pass

3.3.2 Assessment of End Repair and Ligation Reagents

To assess the end repair reagents and the ligation reagents during library preparation, qPCR is performed to ensure that there is library present. This is performed after size selection to ensure that adapter dimers are removed and the concentration of the library is based upon fragments with DNA from the amplicons generated.

3.3.2.1 Results of fragmentation

Criteria for acceptability	Pass/Fail
qPCR value should exceed 1 nM (1,000 pM)	Pass

3.4 Indexed Adapter Plate quality control testing

3.4.1 Assessment of adapters for index sequence and location

The test is performed on a specific sample panel developed for this purpose. 24 different libraries are prepared where no library possesses the same allele(s). Each sample has a unique HLA allele and the placement of the sample on the plate is known, the location of the indexed adapter can be confirmed with genotyping each of the samples.

Criteria for acceptability	Pass/Fail
Index sequence and location:	Pass

Pass: All allele calls match the expected reference genotyping.
Fail: At least one allele call does not match the expected genotyping.

3.4.2 Assessment of indexed adapters for cross-contamination

The test is performed on a specific sample panel developed for this purpose. 24 different libraries are prepared where no library possesses the same allele(s). Index cross- contamination is assessed by looking for unexpected allele calls. Cross contamination would be evident through the detection of unexpected alleles and the contaminating adaptors identified.

3.4.2.1 Results of indexed adapters cross-contamination

Criteria for acceptability	Pass/Fail
A sufficient amount of reads is available: ***Pass:*** **For every tested index, at least 4000 read pairs were mappable to the expected allele.** ***Fail:*** **For at least one tested index, less than 4000 read pairs were mappable to the expected allele.**	Pass
A sufficient amount of reads is only available for the tested indices: ***Pass:*** **More than 1000 read pairs is only available for the tested indices.** ***Fail:*** **More than 1000 read pairs is available for at least on not tested index.**	Pass
Contamination with a single index is not observed: ***Pass:*** **No index was identified with more than 1% contamination from another single index. The criterion must be true for all different contamination % estimates.** ***Fail:*** **At least one index was found with more than 1% contamination from another index.**	Pass

Authorization for release			
Name	**Zoltán Simon - Omixon**	**Function**	**COO**
Signature		**Sign date**	**2019.07.17**
Name	**Gabriella Adlovits - Omixon**	**Function**	**RAQS Manager**
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