Clinical research

Versatile solutions for human leukocyte antigen testing with peripheral blood

Introduction

The human leukocyte antigen (HLA) genes are located in the major histocompatibility complex and are considered the most polymorphic genetic system in humans, with more than 35 thousand alleles described and high levels of diversity inside and between human populations. They encode surface proteins responsible for recognizing self versus nonself and play an essential role in the immune response. Consequently, HLA typing is an important test for stem cell and solid organ transplantation, various disease associations, and pharmacogenetics to screen for drug hypersensitivity.

HLA mismatches between donors and recipients are the leading cause of graft rejection in tissue and organ transplantation. There is a correlation between the matching based on the precise identification of the HLA allele's sequences (high-resolution typing) and a decreased risk of rejection. However, the high diversity of the HLA system poses an increased challenge for the unambiguous typing of HLA alleles. Historically, sequence-specific oligonucleotide (SSO) and sequence-specific primer (SSP) assays have been widely used for HLA typing but fail to achieve high resolution. Alternatively, sequence-based typing (SBT) assays allow the generation of high-resolution results but provide limited throughput and require the utilization of numerous reflexive tests, increasing turnaround time and associated costs.

Next-generation sequencing (NGS) technologies triggered a revolution in the HLA typing field by providing the means to generate high-resolution data and resolve most of the allelic ambiguities without the use of multiple reflexive tests.

These new technologies allow the simultaneous sequencing of different genomic regions in multiple samples, helping to improve throughput and turnaround time. However, many HLA laboratories might find NGS challenging to adopt because of the increased complexity, dedicated labor requirements, and high costs. One Lambda™ AllType™ and AllType™ FASTplex™ NGS assays address all of these challenges by offering a robust and simple solution, allowing the parallel high-resolution typing of the *HLA-A*, *HLA-B*, *HLA-C*, *DRB1*, *DRB3*, *DRB4*, *DRB5*, *DQA1*, *DQB1*, *DPA1*, and *DPB1* loci, solving most of the ambiguities and generating data with more than 99% concordance [1]. The AllType FASTplex NGS kit is the only product on the market featuring a single multiplex PCR followed by a one-tube workflow. This feature decreases hands-on and total turnaround time while improving the assay's reliability by decreasing the number of pipetting steps and the chances of introducing human error. Table 1 summarizes the differences between the AllType and AllType FASTplex NGS kits.

Table 1. Specifications for AllType and AllType FASTplex NGS kits.

	AllType FASTplex NGS 11 Loci kit	AllType NGS 11 Loci kit
Amplification method	11 genes, long-range multiplex	11 genes, long-range multiplex
Turnaround time	7 hr (1.5 hr hands-on time)	8.5 hr (3.5 hr hands-on time)
Library prep workflow	Single tube	Traditional
Resolution	High	High
Instruments	Ion GeneStudio S5, Ion GeneStudio S5 Plus systems; Illumina MiSeq, MiniSeq, and iSeq 100 systems	Ion GeneStudio S5, Ion GeneStudio S5 Plus systems; Illumina MiSeq, MiniSeq, and iSeq 100 systems

High-performance NGS HLA assays demand high-quality genomic DNA extracted from biological samples such as peripheral blood. With the innovative magnetic beads and reagents included with the Applied Biosystems™ MagMAX™ DNA Multi-Sample Ultra 2.0 Kit, DNA of top quality and purity can be purified for downstream sequencing applications, including HLA typing across various NGS platforms. Combining the MagMAX kit with the Thermo Scientific™ KingFisher™ Apex Benchtop Sample Prep System, the workload is simple and easy while maintaining the flexibility of throughput from 6 to 96 samples. One extraction event can be performed in 45 minutes with only 5 minutes of hands-on time.

Here we evaluate the performance of 96 DNA samples extracted from whole blood using the MagMAX kit on the KingFisher Apex system, for high-resolution HLA typing with AllType and AllType FASTplex NGS kits.

Experimental procedures

Genomic DNA isolation

Whole blood was obtained from 96 unique donors in 10 mL acid citrate dextrose (ACD) collection tubes through Tennessee Blood Services. Genomic DNA was extracted from all 96 blood samples using the MagMAX DNA Multi-Sample Ultra 2.0 Kit on the KingFisher Apex system with the 400 µL whole blood workflow. Following extraction, the DNA's integrity and purity were assessed with a Thermo Scientific™ NanoDrop™ Eight Spectrophotometer using A_{260}/A_{280} and A_{260}/A_{230} ratios. The integrity of the genomic DNA was analyzed using the Genomic DNA ScreenTape™ Device on the Agilent™ 4200 TapeStation™ System. Concentrations of genomic DNA were obtained using the Invitrogen™ Qubit™ dsDNA BR Assay Kit on the Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader.

HLA typing

Extracted genomic DNA from 96 unique blood samples was assayed on Ion Torrent™ and Illumina™ sequencing platforms, for both AllType and AllType FASTplex NGS kits (Table 2, Figure 1).

Table 2. Summary of NGS performed with genomic DNA extracted from 96 unique whole blood samples.

Sample source	Assay kit	Sequencing instrument
Whole blood	AllType NGS 11 Loci kit for Ion Torrent platforms	Ion GeneStudio S5 System
	AllType FASTplex NGS 11 Loci kit for Ion Torrent platforms	
	AllType NGS 11 Loci kit for Illumina platforms	MiSeq System
	AllType FASTplex NGS 11 Loci kit for Illumina platforms	

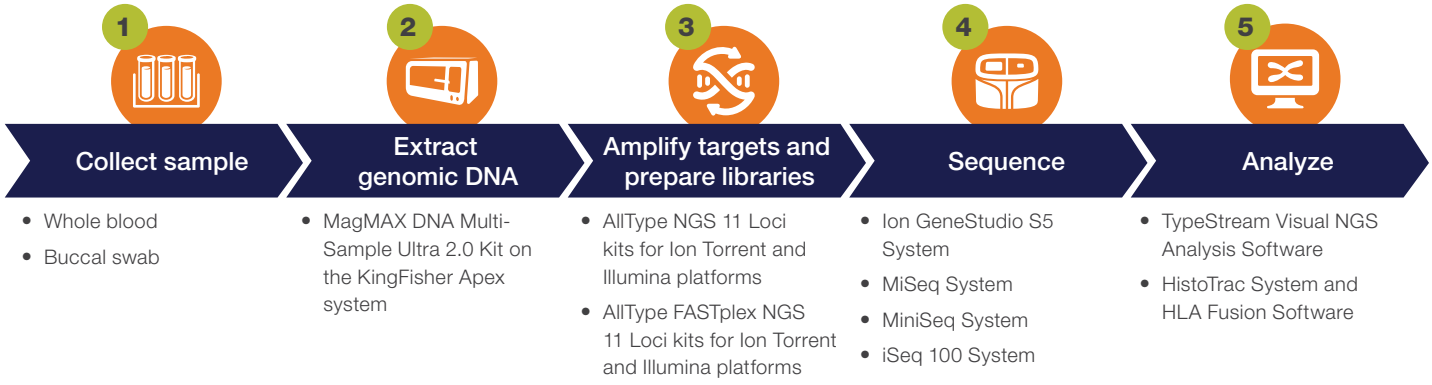


Figure 1. Overall workflow for genomic DNA extraction followed by NGS-based HLA typing.

Results

Genomic DNA isolation and quality

Nucleic acid extracted from 96 blood samples had concentrations ranging from 57 ng/µL to 365 ng/µL, which are suitable for many downstream sequencing applications (Figure 2). The average A_{260}/A_{280} ratio across all 96 unique samples was 1.89 (Figure 3), indicating highly pure DNA [2]. The integrity of the extracted nucleic acid was analyzed using the Genomic DNA ScreenTape Device on the Agilent 4200 TapeStation System. All 96 samples displayed a sharp single band above 51 kb in the genomic DNA region, as shown in the gel image from the Agilent 4200 TapeStation System (Figure 4). The DNA integrity number (DIN) was above 9 on average, indicating highly intact genomic DNA [3]. Table 3 shows the genomic DNA quality summary from the 96 unique isolates. Standard deviation (SD) and coefficient of variation (CV) are higher when reporting out concentration yield (ng/µL), because of natural donor variation across the 96 samples. Still, smaller SD and CV for DINs and constant A_{260}/A_{280} ratios indicated consistent quality across all 96 samples extracted with the MagMAX kit on the KingFisher Apex system.

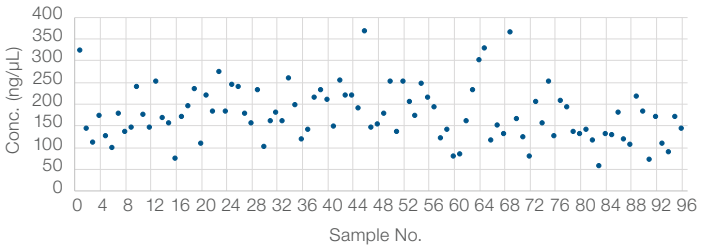


Figure 2. Concentrations of genomic DNA from 96 blood samples processed with the MagMAX kit on the KingFisher Apex system. DNA concentrations were obtained using the Qubit dsDNA BR Assay Kit on the Varioskan LUX Multimode Microplate Reader.

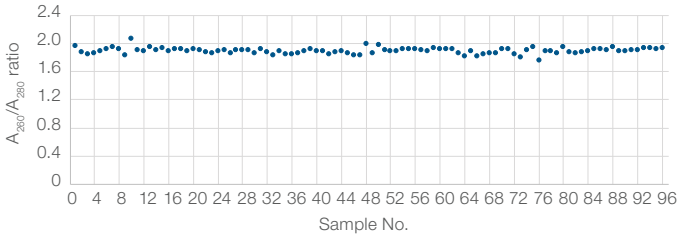


Figure 3. Purity of genomic DNA from 96 blood samples processed with the MagMAX kit on the KingFisher Apex system. DNA purity ratios were obtained using the NanoDrop spectrophotometer.

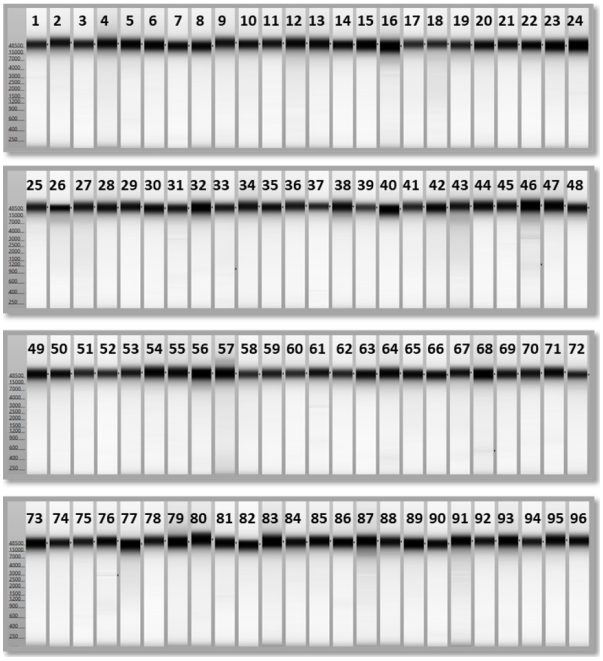


Figure 4. Genomic DNA bands from the 96 samples processed with the MagMAX kit on the KingFisher Apex system. DNA bands and profiles were obtained using the Genomic DNA ScreenTape Device on the Agilent 4200 TapeStation System.

Table 3. Nucleic acid quality summary for genomic DNA isolated from peripheral blood samples.

	gDNA concentration (ng/µL)*	Size (bp)**	DIN score**	A_{260}/A_{280} †	A_{260}/A_{230} †
Average	176.17	59,248.70	9.25	1.89	2.22
Standard deviation (SD)	62.70	1,983.32	0.43	0.04	0.25
Coefficient of variation (CV)	36%	3%	5%	2%	11%
Range	57–365				
Number of samples	96				

* Concentration obtained using the Qubit dsDNA BR Assay Kit on the Varioskan LUX Multimode Microplate Reader.
** Size and DIN score obtained using the Genomic DNA ScreenTape Device on the Agilent 4200 TapeStation Platform.
† Purity and quality absorbance ratios (A_{260}/A_{280} and A_{260}/A_{230}) were obtained using the NanoDrop spectrophotometer.

Quality and concentrations of amplicons generated with AllType NGS 11 Loci kits

The quality of the amplicons generated with the AllType NGS 11 Loci kits for Ion Torrent and Illumina sequencing platforms was evaluated using the Agilent 4200 TapeStation System. Expected ranges of approximately 5–6 kb were obtained from all 96 samples (Figure 5). Average concentrations of 55.6 ng/µL and 50.0 ng/µL were obtained for the Ion Torrent and Illumina platforms, respectively, satisfying the requirements for downstream post-amplification sequencing (Figure 6).

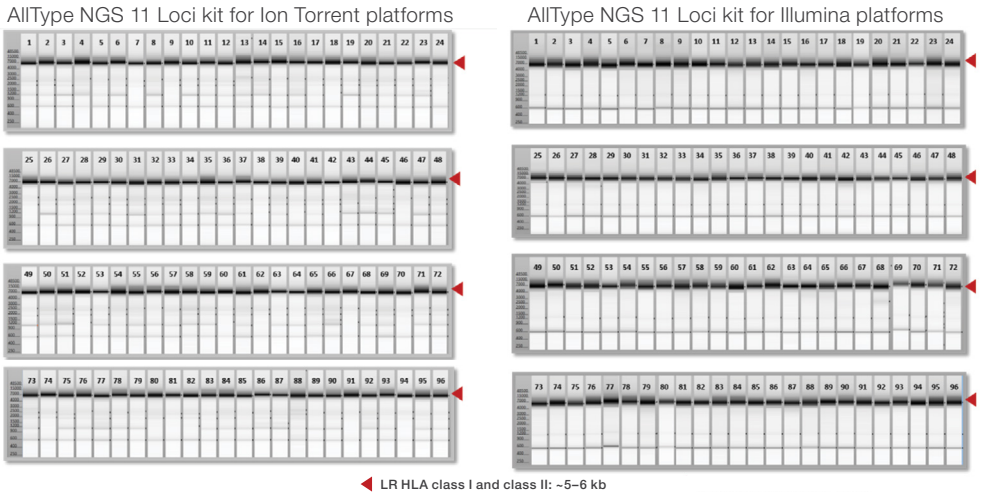
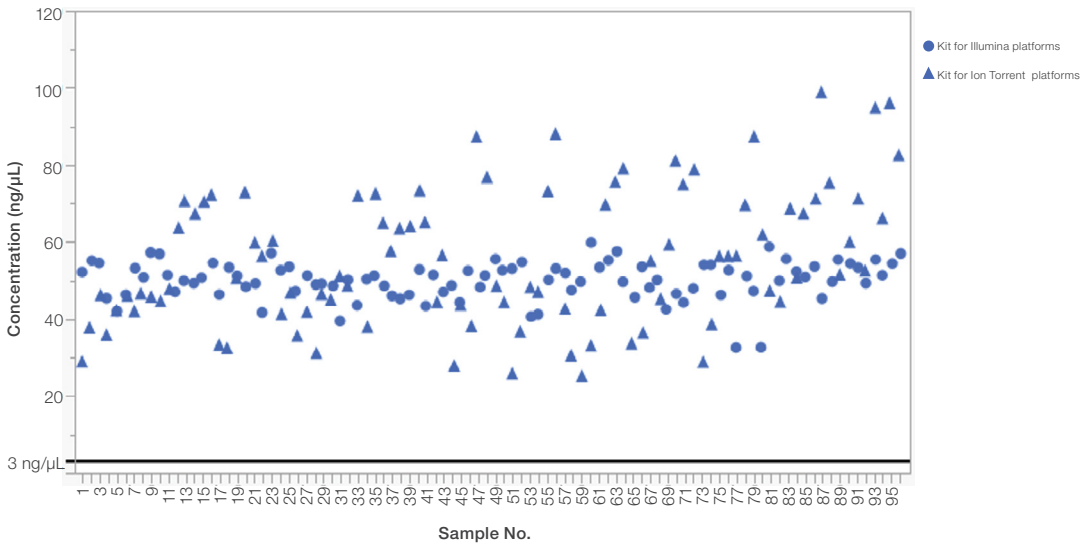


Figure 5. Profile of amplicons generated with AllType NGS 11 Loci kits for Ion Torrent and Illumina platforms and evaluated on the Agilent 4200 TapeStation System. Amplicons were generated from genomic DNA extracted from peripheral blood samples with the MagMAX kit on the KingFisher Apex system. The red triangles indicate approximately 5–6 kb sizes for HLA class I and class II amplicons across all 96 samples.



	Amplicons generated with AllType NGS 11 Loci kit for Ion Torrent platforms	Amplicons generated with AllType NGS 11 Loci kit for Illumina platforms
Average concentration (ng/µL)	55.6	50.0
Standard deviation (ng/µL)	17.5	5.0
Coefficient of variation	32%	10%

Figure 6. Concentrations of amplicons generated with AllType NGS 11 Loci kits for Ion Torrent and Illumina platforms. Amplicons were generated from genomic DNA extracted from blood with the MagMAX kit on the KingFisher Apex system. Concentrations were measured using the Qubit dsDNA BR Assay Kit. The minimum concentration required for this panel workflow is 3 ng/µL, indicated on the graph by the threshold line; all amplicons exceeded the concentration requirement.

Quality and concentrations of amplicons generated with AllType FASTplex NGS 11 Loci kits

High-quality amplicons were also obtained with the AllType FASTplex NGS 11 Loci kits for Ion Torrent and Illumina sequencing platforms. Average length ranges of 5–6 kb were obtained from all samples (Figure 7). Average concentrations of 67.4 ng/µL and 34.2 ng/µL were obtained for the Ion Torrent and Illumina platforms, respectively, satisfying the requirements for downstream post-amplification sequencing (Figure 8).

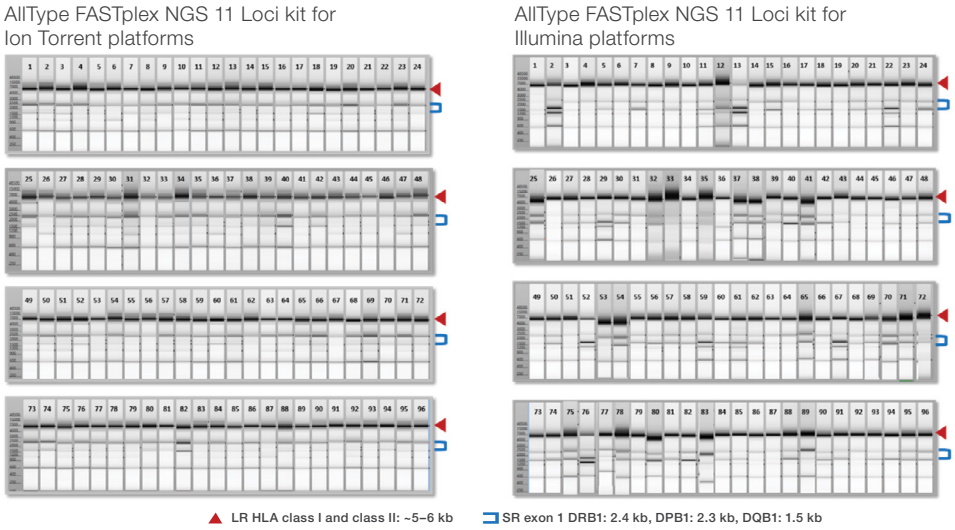
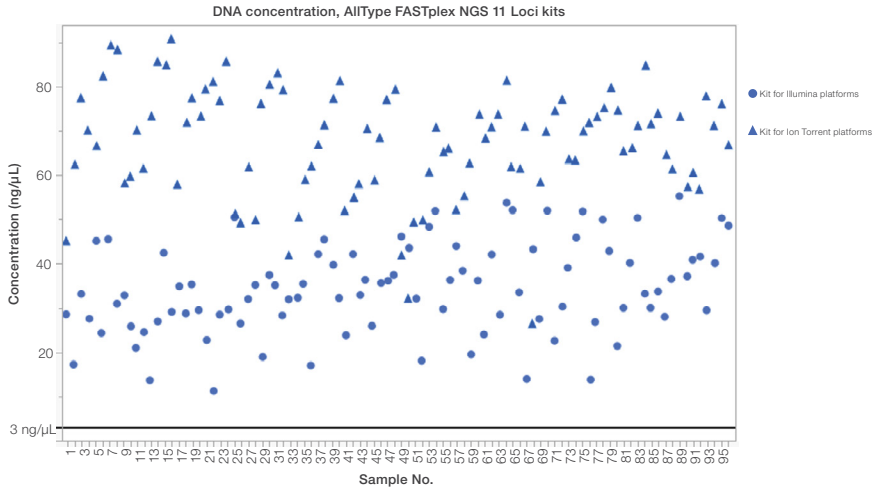


Figure 7. Profile of amplicons generated with AllType FASTplex NGS 11 Loci kits for Ion Torrent and Illumina platforms and evaluated on the Agilent 4200 TapeStation System. Amplicons were generated from genomic DNA extracted from peripheral blood with the MagMAX kit on the KingFisher Apex system. The red triangles indicate approximately 5–6 kb sizes for HLA class I and class II amplicons across all 96 samples. The blue brackets indicate approximately 1.5–2.4 kb sizes for the *DRB1*, *DPB1*, and *DQB1* amplicons.



	Amplicons generated with AllType FASTplex NGS 11 Loci kit for Ion Torrent platforms	Amplicons generated with AllType FASTplex NGS 11 Loci kit for Illumina platforms
Average concentration (ng/µL)	67.4	34.2
Standard deviation (ng/µL)	12.3	10.2
Coefficient of variation	18%	30%

Figure 8. Concentrations of amplicons generated with AllType FASTplex NGS 11 Loci kits for Ion Torrent and Illumina platforms. Amplicons were generated from genomic DNA extracted from peripheral blood with the MagMAX kit on the KingFisher Apex system. Concentrations were measured using the Qubit dsDNA BR Assay Kit. The minimum concentration required for this panel workflow is 3 ng/µL, indicated on the graph by the threshold line; all amplicons exceeded the concentration requirement.

Sequencing performance of amplicons generated with AllType and AllType FASTplex NGS kits

Post-amplification sequencing was performed on both Ion Torrent and Illumina platforms. The average read depth (Table 4) was estimated using One Lambda™ TypeStream™ Visual NGS Analysis Software 3.0 and used as quality metrics across all regions of the 11 HLA loci. The minimum recommended read depth for both AllType and AllType FASTplex NGS kits is 100. This requirement was surpassed with both kits and sequencing platforms (Table 4).

HLA genotypes were automatically estimated by TypeStream Visual NGS Analysis Software, and the accuracy of the test was determined by comparing results obtained with both AllType and AllType FASTplex NGS kits on the Ion Torrent and Illumina platforms. No discrepancies (i.e., concordance of 100%) were observed across both kits and both sequencing platforms, as expected when high-quality genomic DNA is used as initial input for both the AllType and AllType FASTplex NGS kits.

Conclusion

Automated DNA extraction using the MagMAX DNA Multi-Sample Ultra 2.0 Kit on the KingFisher Apex system consistently generated high-quality genomic DNA, suitable for downstream HLA typing with AllType and AllType FASTplex NGS kits on both Ion Torrent and Illumina NGS platforms. Sequencing analysis with individual allele assignments can be automatically generated by TypeStream Visual NGS Analysis Software and can provide results in less than 3 minutes.

Table 4. Average read depth for all regions across 11 HLA loci after sequencing performed with AllType and AllType FASTplex NGS kits on Ion Torrent and Illumina platforms.

HLA locus	Coverage in all regions			
	AllType NGS 11 Loci kit		AllType FASTplex NGS 11 Loci kit	
	Ion Torrent platform	Illumina platform	Ion Torrent platform	Illumina platform
HLA-A	730	591	553	403
HLA-B	884	942	558	547
HLA-C	835	834	543	479
DPA1	1,114	655	446	512
DPB1	1,367	1,404	541	558
DQA1	1,094	652	494	495
DQB1	1,573	1,133	533	569
DRB1	1,772	1,430	582	504
DRB345	1,435	1,291	562	532

Ordering information

Product	Cat. No.
Sample isolation reagents and equipment	
MagMAX DNA Multi-Sample Ultra 2.0 Kit	A36570
KingFisher Apex Benchtop Sample Prep System with 96 Deep-Well Head	5400930
HLA typing reagents	
AllType NGS 11 Loci Sample Prep Flex Kit for Ion Torrent platforms	ALL-PREP11LX
AllType NGS 11 Loci Sample Prep Flex Kit for Illumina platforms	ALL-PREP11LFX
AllType FASTplex NGS 11 Loci Kit for Ion Torrent platforms	ALL-FAST11LX
AllType FASTplex NGS 11 Loci Flex Kit for Illumina platforms	ALL-FAST11LFX
Quality control reagents and equipment	
NanoDrop Eight Spectrophotometer	840-343700
Varioskan LUX Multimode Microplate Reader	VL0000D0
Qubit dsDNA BR Assay Kit	Q32850

References

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2. Thermo Fisher Scientific. Interpretation of nucleic acid 260/280 ratios. Technical bulletin. **https://tools.thermofisher.com/content/sfs/brochures/T123-NanoDrop-Lite-Interpretation-of-Nucleic-Acid-260-280-Ratios.pdf**
3. Agilent Technologies. DNA integrity number (DIN) for the assessment of genomic DNA samples in real-time quantitative PCR (qPCR) experiments. Application note. **https://www.agilent.com/cs/library/applications/5991-6368EN.pdf**

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