Challenge
High quality long-range PCR and library preparation for Human Leukocyte Antigen (HLA) typing using next-generation sequencing (NGS)

Solution
The Biometra TAdvanced thermal cycler ensures high temperature accuracy and uniformity, minimizing the risk of PCR bias and evaporation for optimal PCR specificity and product yield

Intended audience
Diagnostic laboratories performing HLA typing using next-generation sequencing

HLA Typing Using Long-Range PCR and Next-Generation Sequencing

Introduction

The major histocompatibility complex (MHC) or often referred to as Human Leukocyte Antigen (HLA) complex plays a crucial role in immunorecognition, organ and stem cell transplantation as well as disease association such as autoimmune diseases.[1] HLA genes encode for surface marker proteins of leukocyte cells. These genes are closely linked and highly polymorphic, meaning they exist in many different forms (alleles) in the population. Currently, more than 40,000 HLA alleles are described by the HLA nomenclature included in the IPD-IMGT/HLA database (access: 22.07.24). Each allele differs in the nucleotide sequence which can lead to changes in the amino acid sequence of the encoded protein and ultimately affects its function.[2]

HLA typing is crucial for eligible transplants between recipient and donor, where HLA settings have to match to minimize the risk of rejection from an immune response.

Several methods can be applied for HLA typing such as endpoint PCR, qPCR, Sanger sequencing, and next-generation sequencing (NGS). Throughout the accelerated capabilities of NGS, HLA typing did become easily accessible and offers high-resolution analysis of the target sequences.[3]

NGS library preparation for HLA typing can be conducted from long-range PCR amplicons of several kilo base pairs covering the whole gene sequence and enabling HLA typing at high-resolution level. However, long-range PCR entails an enlarged risk of PCR errors and unspecific products due to kilo bases in length. Longer PCR products have a higher probability for GC-rich sequences which impede the progress of the DNA polymerase during the PCR. In addition, the yield of long-range PCR products is usually lower compared to standard length PCR of several hundred base pairs. Since HLA genes are highly polymorphic, multiplex PCR is an essential procedure for typing different HLA loci

from one sequencing run and for covering all base pair differences as specific as possible. Along with long-range PCR for HLA genes, allele typing using NGS is an essential immunodiagnostic procedure.

Typical NGS sample preparation, including library preparation, requires PCR thermal cyclers. The precise temperature control, efficient heating and cooling, and excellent temperature uniformity across the entire block lead to a reproducible generation of amplicons and sequencing libraries without unspecific PCR products. The Biometra TAdvanced from Analytik Jena is a high-performance thermal cycler which optimally meets the requirements of PCR applications including long-range PCR and NGS library preparation.

This application note describes the HLA typing workflow using the GenDx NGSgo Library Full Kit and NGSgo-MX11-3 amplification kit. The initial long-range PCR and all library preparation steps were performed using the Biometra TAdvanced 96 SG. Figure 1 gives an overview of all steps for the NGS-based HLA typing workflow.

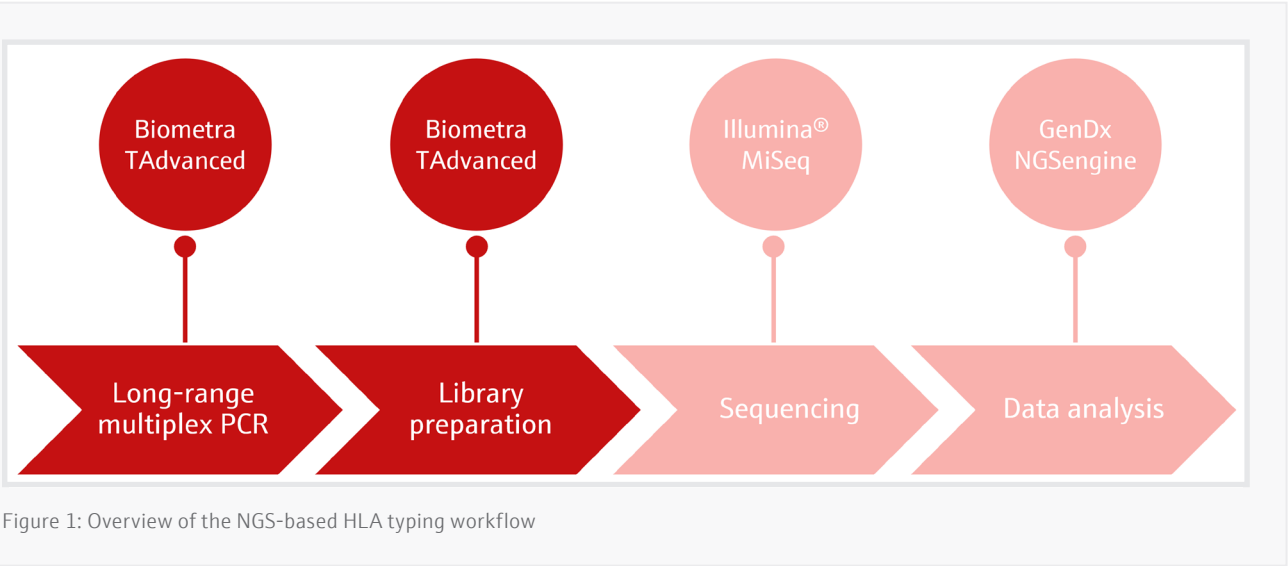

Figure 1: Overview of the NGS-based HLA typing workflow

Materials and Methods

Samples and reagents

- Purified DNA from blood samples or buccal swabs
- GenDx NGSgo-MX11-3, gene panel for multiplex long-range PCR
- GenDx NGSgo Library Full Kit, library preparation including random fragmentation, end-repair, adaptor binding compatible with Illumina® indexes

Instrumentation

- Biometra TAdvanced 96 SG thermal cycler, Analytik Jena
- Gel electrophoresis system, e.g. Biometra Compact Line, Analytik Jena
- Gel documentation, e.g. UVP GelStudio, Analytik Jena
- Illumina® MiSeq

Sample preparation

The concentration of purified DNA samples was in the range of 15 to 80 ng. According to the GenDx NGSgo-X11-3 manual, the PCR master mix and the amplification primers for each long-range PCR mix were combined. Each mix (A, B, and C) amplifies different HLA genes (Table 1) ranging from 2.5 to 6.7 kb in length.

Table 1: Amplicon length of PCR products from HLA gene loci from the GenDx NGSgo®-MX11-3 panel

Primer panel	Targets	Amplicon	Length
Mix A	HLA-A	whole gene	3.1 kb
	HLA-DRB1	whole gene*	2.5 + 5 kb
	HLA-DRB1	exon 1	5 kb
	HLA-DRB3	whole gene*	2.5 + 5 kb
	HLA-DQA1	whole gene	5.8 kb
Mix B	HLA-B	whole gene	3.4 kb
	HLA-DQB1	whole gene	6.7 kb
	HLA-DRB5	whole gene*	2.6 + 4.8 kb
Mix C	HLA-C	whole gene	3.4 kb
	HLA-DPB1	exon 2-5	5.7 kb
	HLA-DRB4	exon 2-5	4.3 kb
	HLA-DPA1	whole gene	5.5 kb

* except for part of intron 1

The HLA amplicons were generated following the PCR program for the long-range PCR as displayed in Table 2.

Table 2: Thermal cycler program for long-range PCR of HLA loci.

Step	Cycle	Profile	Temperature	Holding time	Ramp rates
1	1	Initial denaturation	95 °C	3 min	8 °C/sec
2	30	Denaturation	95 °C	15 sec	8 °C/sec
		Annealing	65 °C	30 sec	6 °C/sec
		Elongation	67 °C	6 min	8 °C/sec
3	1	Final elongation	67 °C	10 min	8 °C/sec
4	1	Cooling	15° C	∞	6 °C/sec

The PCR products from each mix of a sample were pooled for the library preparation with an input of 300 ng per sample. Library generation including fragmentation, end-repair, adaptor ligation, and index-PCR using the GenDx NGSgo Library Full Kit was performed following the manufacturer's guidelines. Libraries from all samples were then pooled and diluted to 4 nM concentration for loading on an Illumina® flow cell. The libraries were sequenced on an Illumina® MiSeq sequencing system using V3 chemistry.

After sequencing, the data analysis was performed using the GenDx NGSengine software to determine the allele types for all analyzed HLA genes.

Results and Discussion

Long-range PCR poses a critical step of sample preparation for sequencing workflows as the risk for unspecific amplification is increased due to longer PCR products. Using the Biometra TAdvanced thermal cycler, consistent and reproducible PCR products were obtained for all HLA genes across 24 samples from two separate preparation runs (Figure 2 (a) and (b)) without any unspecific PCR products.

(a) (b)

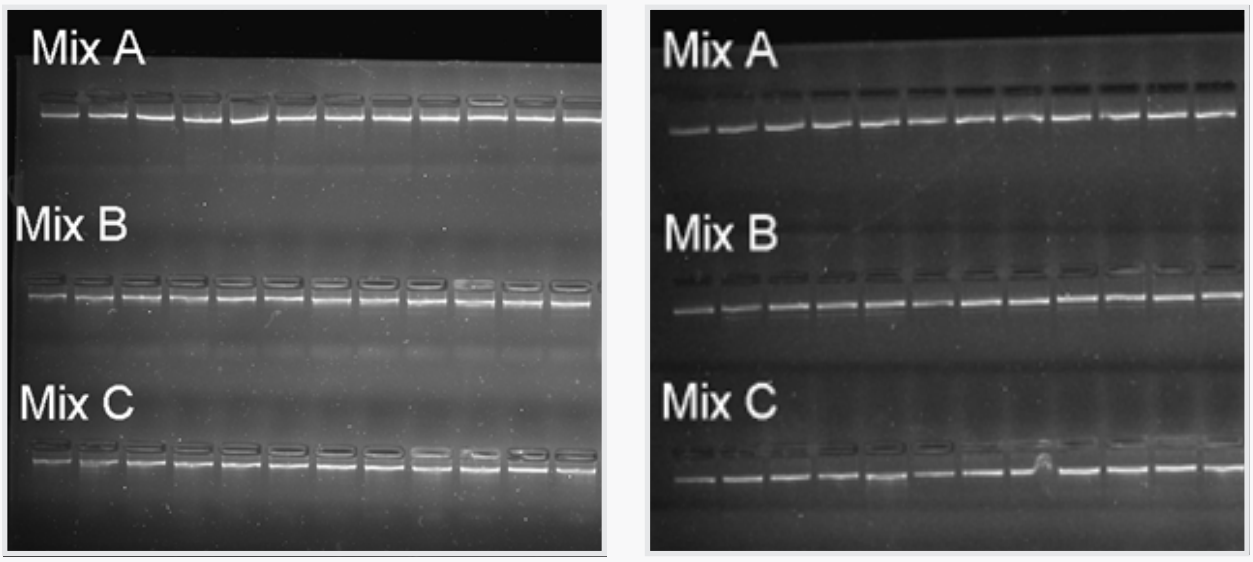

Figure 2: Gel images showing constant fragment size and stability for the long-range PCR products from all three multiplex primer combinations of two PCR runs ((a) and (b)) for following NGS library preparation and HLA gene sequencing.

NGS-based HLA allele determination was conducted using the GenDx NGSengine software. Figure 3 shows an exemplary data overview from the two sample preparations ((a) and (b)). The sequencing reads were aligned to the reference reads. The mappability (top panel), meaning, how many sequencing reads were assigned to the reference sequences, was at high level with over 92% across all genes. Then, the heterozygous positions were phased to determine the HLA type. Phasing describes the process of determining which specific alleles are paired together on the same chromosome.

Figure 3 (middle panel) shows the depth of coverage (number of times a nucleotide was sequenced) for the HLA-A gene from the two samples. The average read coverage across the analyzed sequence was 2098 for preparation (a) and 2006 for preparation (b), which represents a high-resolution level suitable for HLA typing. Base variation (bottom panel) shows the level of variance across all base positions to classify homogeneous and heterogenous HLA alleles. For both HLA-A genes allele determination was optimally depicted by a high signal-to-noise ratio of below 5% base variation across all nucleotide positions.
Based on these results, the HLA patterns from recipients and donors can be determined to achieve an optimal match for transplantation with minimal risk of transplant rejection.

(b)

Figure 3: Two exemplary results (from preparations (a) and (b)) of HLA gene sequencing analysis using the GenDx NGSengine software. The top of both panels summarizes the analysis metrices for all sequenced genes from the two samples. The mappability for each gene to the reference sequences is displayed in the second table column.
The middle graph 'Depth of coverage' illustrates the number of times that a specific base was read during the sequencing process across all nucleotide positions.
The bottom graph 'Base variation' shows the frequency of variation in the bases at each position in the HLA gene and the determination between homozygous (dots) and heterozygous (cross) alleles. The horizontal line depicts 50 % base variation. The shaded yellow areas highlight the exon regions across the whole gene sequence. In this example, base variation below 10 % can be classified as signal noise (red dots), while higher base variation represents heterozygous bases (red and blue crosses) or homozygous positions on the top (blue dots).

Summary

HLA typing is an essential diagnostic tool to determine the specific inventory of human cell surface antigens. In this application note, an HLA typing workflow for next-generation sequencing (NGS) is described using the highly accurate and robust Biometra TAdvanced 96 SG thermal cycler from Analytik Jena and an HLA qualitative in-vitro diagnostic kit from GenDx. Sample preparation before library generation was based on multiplex long-range PCR of several kilo base pairs in length for high-resolution HLA typing. Reliable and reproducible PCR products were amplified without amplification bias and the generated libraries were suitable for Illumina® sequencing. The sequencing results showed very high mappability to reference sequences and sequence coverage to determine base variation for HLA typing which is crucial for successful organ and stem cell transplant or immunotherapies.

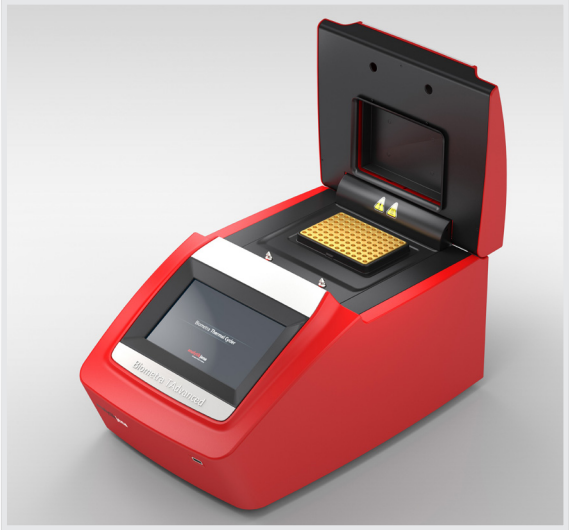

Figure 4: Biometra TAdvanced 96 SG

Recommended device configuration

Table 3: Thermal cycler for amplification and library preparation and electrophoresis instruments for fragment size validation.

Article	Article number	Description
Biometra TAdvanced 96 SG	846-x-070-241	End-point PCR thermal cycler
Biometra Compact Series	846-025-zzz	Horizontal gel electrophoresis family for gel sizes from mini to maxi gels and from low to high sample throughput

x = 2 for 230 V, x = 4 for 115 V, x = 5 for 100 V
zzz = different sizes

Acknowledgements

We acknowledge the provided data and contribution of the Laboratory Transplant Immunology/Molecular Genetics from the university hospital in Jena.

References

[1] Mayor, N. P. *et al.* HLA Typing for the Next Generation. *PLOS ONE* **10**, e0127153 (2015).
[2] Buhler, S. & Sanchez-Mazas, A. HLA DNA Sequence Variation among Human Populations: Molecular Signatures of Demographic and Selective Events. *PLOS ONE* **6**, e14643 (2011).
[3] Bravo-Egana, V., Sanders, H. & Chitnis, N. New challenges, new opportunities: Next generation sequencing and its place in the advancement of HLA typing. *Human Immunology* **82**, 478–487 (2021).

Headquarters
Analytik Jena GmbH+Co. KG
Konrad-Zuse-Strasse 1
07745 Jena · Germany

Phone +49 3641 77 70
Fax +49 3641 77 9279

info@analytik-jena.com
www.analytik-jena.com

Version 1.0 · Author: MaKr
en · 08/2024