Challenge
Need for automation due to increased sample numbers while maintaining consistency of next generation sequencing results

Solution
CyBio Felix automates size selection of 96 amplified NGS libraries simultaneously, increasing the efficiency of the library preparation and reproducibility of the size selection

Intended audience
Diagnostic labs, hospitals and transplant centers interested in HLA genotyping

Automated size selection of NGS libraries for HLA diagnostics

Introduction

HLA genotyping is an important procedure in the preparation for stem cell transplants. It is essential for determining the genetic compatibility of the human leukocyte antigens (HLA) between donor and recipient.[1] A high degree of compatibility significantly reduces the risk of rejection and enhances transplantation success. Next-generation sequencing (NGS) offers high-resolution, precise identification of HLA alleles, making it an invaluable method in stem cell transplantation.[2] Accurate HLA genotyping is paramount for successful outcomes in this procedure.

Automating the sample preparation for HLA genotyping provides numerous advantages, such as increased efficiency, reduced error rates, and minimized manual handling time. During the library preparation stage for NGS, the cleanup step is crucial. This step involves replacing the buffer containing DNA fragments and removing remaining enzymes and components from previous preparation stages, like primers and adapters. Various methods have been developed for DNA fragment cleanup, with the use of magnetic beads being the most straightforward.

Magnetic beads with their specific coating and optimized buffer conditions allow for the binding of DNA molecules. The size of the bead-bound DNA fragments largely depends on the concentration of the buffer components the beads are stored in. By adjusting the bead-to-sample ratios, DNA fragments within a user-defined size range can be selectively isolated. In this context, a double-sided size selection was performed using the liquid handling robot CyBio FeliX (Figure 1) to extract fragments between 500 bp and 800 bp for subsequent sequencing.

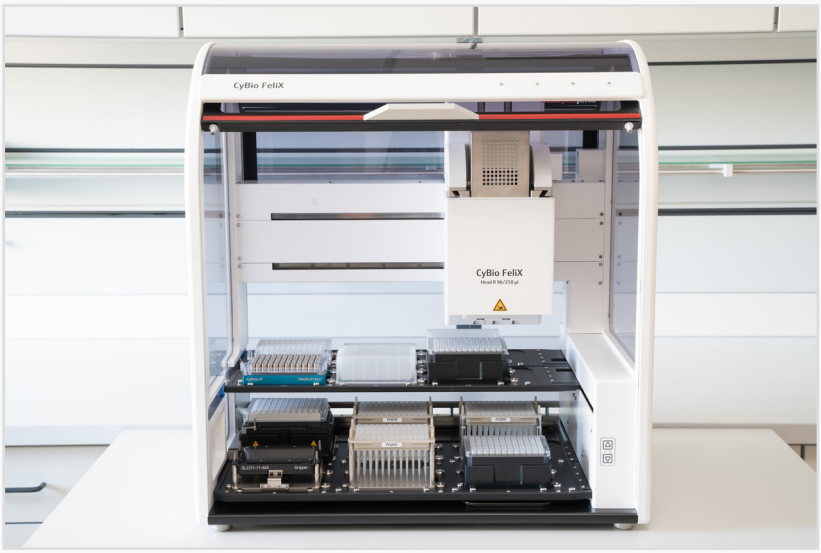

Figure 1: Analytik Jena CyBio FeliX hardware configuration. Decks equipped for double-sided size selection of NGS libraries

Materials and Methods

Reagents and consumables

- NGSgo® Library Full Kit (GenDx)
- NGSGo® Ampx v2 HLA-A, B, C, DRB1, DQB1, DPB1 (GenDx)
- AMPure XP Reagent (A63881, Beckman Coulter)
- Freshly prepared 80% Ethanol (9065.3, Carl Roth GmbH &CO.KG)
- Freshly prepared 0.1x Tris-EDTA buffer (93283-100ML, Sigma Aldrich)
- 96 deep well plate as waste reservoir, 12-column reservoir for beads storage, 2x One-well reservoir
- 96 well PCR plate containing DNA fragments
- 2x 96 well PCR plate
- Optional for non-skirted and half-skirted 96 well PCR plates: 2x Adapter 96, passive cooling function (844-00135-0, Analytik Jena)
- CyBio RoboTipTray 96/250 µL; DW PCR-certified, pre-sterilized, filter (OL3810-25-669, Analytik Jena)
- Optional: Protective Plate (OL3317-25-125, Analytik Jena)
- dsDNA BR Assay (Q32850, Thermo Fisher Scientific)
- QInstruments BioShake 3000-T elm (QINSTRUMENTS-2016-0517, Analytik Jena)
- QINSTRUMENTS Adapter for 96/200 µL Bio-Rad PCR plate (848-2016-1042, Analytik Jena)
- Mounting Kit; BioShake 3000 Series (OL3317-23-692, Analytik Jena)
- ALPAQUA® MAGNUM FLX™; Universal Magnet Adapter (OL3317-11-285, Analytik Jena)
- Gripper (OL3317-14-800, Analytik Jena)
- Support; 70 mm height (OL3317-11-110, Analytik Jena)
- Endpoint thermal cycler, e.g. Biometra T Advanced 96 S (846-x-070-251, x = 2 for 230 V, 4 for 115 V, 5 for 100 V, Analytik Jena)
- InvitrogenTM QubitTM 3 (Thermo Fisher Scientific)
- NovaSeq (Illumina®)

Software

- CyBio Composer Software (Analytik Jena)
- NGSengine software (GenDx)

Methods on CyBio FeliX

The protocol described here shows the automated size selection of DNA libraries for NGS with magnetic beads using the CyBio FeliX liquid handling platform (Figure 1).
The automated steps were embedded in the library preparation process of the NGSgo® Library Full Kit (GenDx) for NGS HLA typing. Prior to library preparation, singleplex PCR were conducted for six different HLA loci using the NGSGo® Ampx v2 kit (GenDx).
After PCR amplification, the DNA concentrations were measured with a Qubit assay and all PCR products were pooled for each sample. The library preparation was continued according to the manufacturer's manual. The automated library cleanup was performed after the adapter ligation. The size selection was conducted after the indexing PCR using the deck layout displayed in Figure 2.
The complete workflow is illustrated in Figure 3, which shows each single step of the fragment size selection and the library cleanup. Steps 1-14 are only required in case of a double-sided size selection. For a cleanup of amplified libraries in which only small fragments are to be removed from the samples, steps 4-8 are omitted.

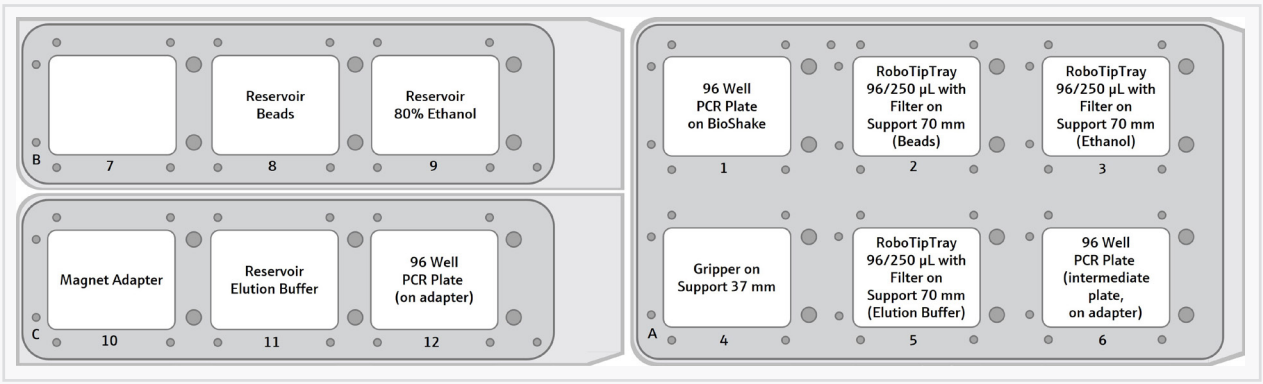

Figure 2: Graphical deck layout for Protocol – Double-sided Size Selection. The CyBio FeliX provides 12 deck positions on 3 movable decks. The upper decks B and C equipped with the required accessories are shown on the left, the lower deck A is shown on the right. The 12-column reservoir on position 8 is equipped with 2 mL AMPure XP beads per column.

The CyBio FeliX performed the size selection steps described in the instructions below:

Step 1: Setup of large fragment removal
From the beads reservoir 10.4 µL of AMPure XP beads are distributed directly into each well of the PCR plate located on the BioShake. This plate already contains 26 µL of DNA fragments per well which are mixed with the beads by pipetting up and down five times. Remaining beads in the tips are discarded into a 96 deep well waste plate.

Step 2: Incubation DNA Collection
Beads and DNA fragments are incubated for five minutes at room temperature while being mixed at 1600 rpm.
Note: After the incubation, the beads should be distributed evenly within the solution.

Step 3: Bead Separation
Using the gripper, the PCR plate containing DNA fragments and beads is placed on the magnet adapter. After being incubated for five minutes at room temperature, no beads should remain in suspension.
Note: Prolong incubation period if necessary.

Step 4: Supernatant Transfer
The supernatant containing unbound DNA fragments is transferred in a single pipetting step into a fresh 96 well PCR plate using a RoboTipTray 96/250 µL. This plate is then placed on the BioShake with the gripper.

Step 5: Tip Exchange
Tips used to distribute the beads and transfer the supernatant are replaced with fresh tips manually.

Step 6: Setup of Bead Cleanup
From the beads reservoir, 3.9 µL are transferred into each well of the 96 well PCR plate containing the supernatant. The beads are mixed with the DNA containing solution by pipetting five times.

Step 7: Second Incubation DNA Collection
Beads and DNA fragments are incubated and mixed for five minutes at room temperature and 1600 rpm.
Note: After the incubation, the beads should be distributed evenly.

Step 8: Second Bead Separation
PCR plate containing DNA fragments bound to the beads is placed on the magnet adapter with the gripper and incubated for five minutes at room temperature.
Note: No beads should remain in the solution after incubation.

Step 9: Discard Supernatant
In a single pipetting step, the supernatant is discarded into the waste plate using a RoboTipTray 96/250 µL.

Step 10: Ethanol Wash
100 µL 80% ethanol is added to the beads using a fresh RoboTipTray 96/250 µL. The beads are incubated with ethanol for 30 seconds while they remain collected at the wall of the well. After incubation, the supernatant is removed using the tips previously used for bead transfer. The supernatant is then discarded in the waste plate. This ethanol wash is repeated another two times.

Step 11: Bead Drying
Beads are incubated for three minutes at room temperature to allow remaining ethanol to evaporate.

Step 12: DNA Fragment Elution
PCR plate containing dried beads is placed on the BioShake with the gripper. From the elution buffer (0.1x Tris-EDTA) reservoir 25 µL of elution buffer is added to the dried beads using fresh tips. To ensure efficient resuspension, DNA fragments are eluted from the beads by pipetting five times and subsequent shaking for five minutes at 1600 rpm.

Step 13: Final Bead Separation
PCR plate containing resolved beads is placed on the magnet adapter with the gripper and incubated for five minutes at room temperature. *Note:* No beads should remain in the solution after incubation.

Step 14: Elution Buffer transfer
20 µL of the supernatant containing the target size DNA fragments is transferred into a fresh PCR plate.

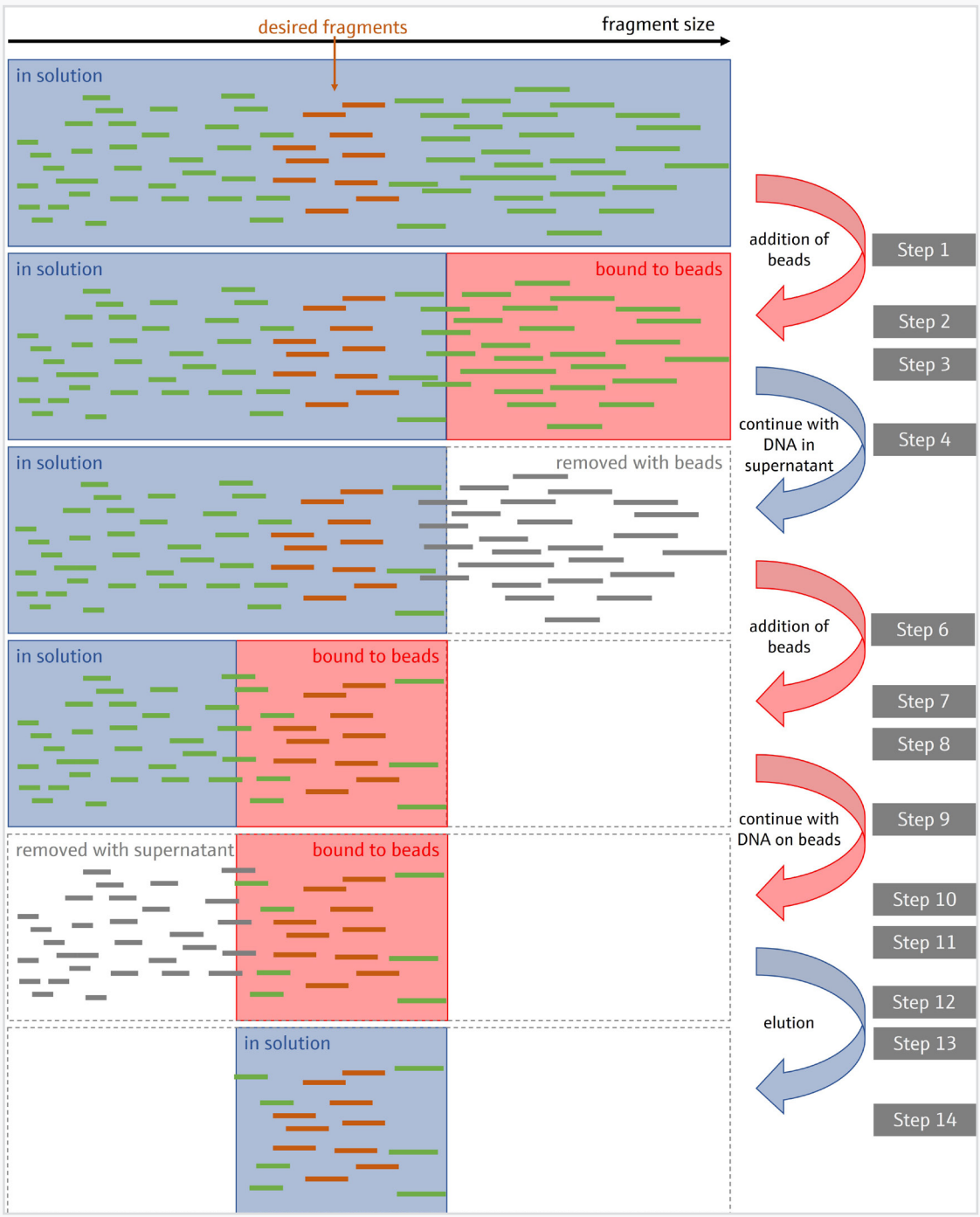

Figure 3: Illustration of the size selection process
Double-sided size selection in NGS library preparation using magnetic beads to selectively bind and isolate DNA fragments within a desired size range. By performing two rounds of bead cleanup with different bead-to-sample ratios, fragments outside the target size range are effectively removed, ensuring a library of appropriately sized DNA fragments for sequencing.

Results and Discussion

During library preparation, DNA concentration measurements were performed before and after size selection as well as fragment size assessments after sequencing, using the NGSengine software (GenDx) to evaluate the efficacy of the size selection steps. The reduction in DNA concentration following the 0.7x bead clean up and size selection procedures indicates successful removal of unwanted DNA fragments and contaminants. For the 0.7x bead cleanup, the average DNA concentration decreased significantly (t-test, $p > 0.05$), with differences ranging from -4.4 to -34.6 ng/µL across all

samples, demonstrating effective purification via the automated workflow (Figure 4). Similarly, size selection also resulted in a consistent reduction in DNA concentration, ranging from -2.4 to -15.4 ng/µL, targeting the isolation of DNA fragments within the desired size range (600-800 bp). These results confirm that the cleanup and size selection processes efficiently enrich the samples for high-quality and appropriately sized DNA fragments, essential for optimal sequencing performance.

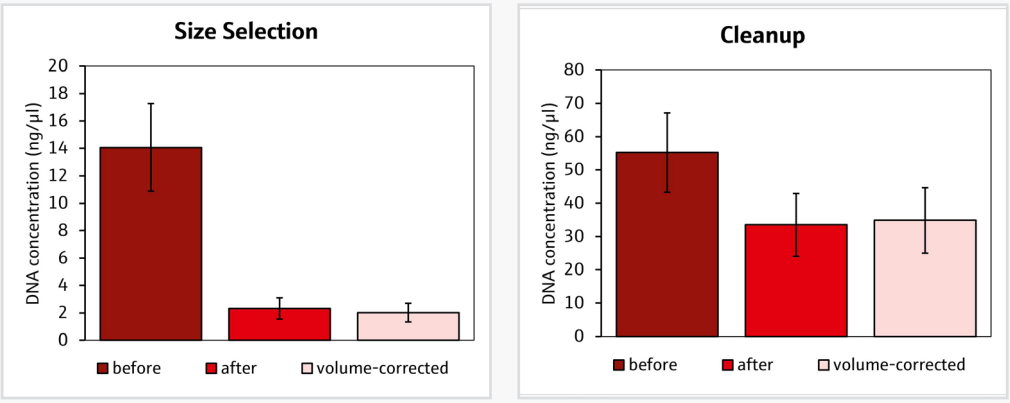

Figure 4: DNA concentrations of sequencing libraries before and after the A) size selection and B) clean up. Volume corrected concentrations were based on different elution volumes after the size selection and clean up.

The fragment sizes in the automated workflow were on average significantly larger (t-test, $p < 0.05$) ranging between 250 bp to over 350 bp compared to the manual process ranging between 210 bp to 320 bp, aligning more closely with the goal of size selection (Figure 5). Automating processes therefore ensure reliable and efficient selection of correct size fragments optimal for sequencing procedure downstream. The advantage of the automated process over the manual process on CyBio FeliX is that all samples can be processed simultaneously without any time delay. This prevents the excessive binding of smaller fragments to the beads. Accurate adherence to incubation times ensures correct size selection of the fragments.

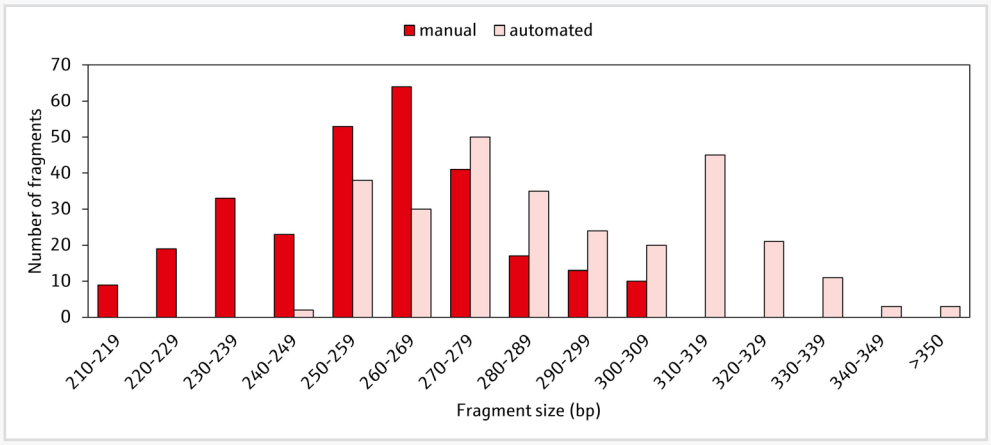

Figure 5: Comparison of the average sequence fragment sizes after manual and automatic size selection with CyBio FeliX. The average fragment sizes of six HLA loci from 48 samples are displayed after trimming of sequencing adapters and barcodes using the NGSengine software (GenDx). Fragment sizes in the automated workflow (light red) were generally larger compared to the manual process (dark red), which grants higher coverage to reference sequences and higher resolution for HLA typing.

Optimal conditions for sequencing are ensured by correct size selection, providing ideal sequencing conditions. The efficiency of the automated system is beneficial, as it can perform multiple cleanups sequentially, speeding up the process compared to manual methods. Improved accuracy is another advantage, as manual methods are prone to errors, whereas automation enhances reproducibility. Lastly, the ergonomics of automation reduce the physical strain associated with manual handling, making the process more user-friendly.

The automated workflow, with its ability to consistently produce larger DNA fragments and handle multiple samples efficiently, not only aligns with sequencing requirements but also offers significant benefits in terms of throughput, reproducibility, and error reduction. This ensures optimal conditions for sequencing and enhances overall process efficiency.

Summary

The use of the CyBio FeliX allows 96 samples to be processed simultaneously, in approximately 40 minutes, with the flexibility to process multiple runs during the day. The compact device reduces the bench space needed for an automated library preparation process for optimal sequencing on an Illumina® NovaSeq. Using the CyBio FeliX for these procedures makes them 4x faster compared to the manual workflow. In addition, automation with CyBio FeliX increases walk-away time significantly.

Figure 6: CyBio FeliX

Recommended device configuration

Table 1: Overview of devices, accessories, and consumables

CyBio FeliX Accessoires	Order number
CyBio FeliX Basic Unit with Enclosure	OL5015-24-100
CyBio FeliX Head R 96/250 µL	OL3316-14-850
QInstruments BioShake 3000-T elm	QINSTRUMENTS-2016-0517
QINSTRUMENTS Adapter for 96/200 µL Bio-Rad PCR plate	848-2016-1042
Mounting Kit; BioShake 3000 Series	OL3317-23-692
ALPAQUA® MAGNUM FLX™, Universal Magnet Adapter	OL3317-11-285
Gripper	OL3317-14-800
Support, 70 mm, 3x	OL3317-11-110
CyBio RoboTipTray 96/250 µL DW; PCR-certified, pre-sterilized, filter	OL3810-25-669
Optional:	
Adapter 96, passive cooling function, 2x	844-00135-0
Protective Plates	OL3317-25-125

Acknowledgements

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References

[1] Mayor, N. P. et al. HLA Typing for the Next Generation. PLOS ONE 10, e0127153 (2015)

[2] 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)

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