
**Challenge**
Ensuring consistent quality of Next Generation Sequencing results and increasing efficiency with a growing number of samples in large genome projects

**Solution**
170-fold increase in efficiency and excellent quality through automated library preparation (purification, pooling and quantification) with the CyBio FeliX Liquid Handler

**Intended audience**
Biotech and pharma companies constructing Next Generation Sequencing libraries in high throughput

# Semi-Automated High-Throughput Workflow for the Preparation of NGS Libraries in Ancient Genomics

## Introduction

At the end of 2018, the new Lundbeck Foundation GeoGenetics Centre was established. One of its missions is to explore the evolutionary history of neurological and mental diseases/disorders over the past 10,000 years. To gain an understanding of the biological factors underlying these diseases/disorders a total of 5,000 genomes from ancient materials (bones and teeth) – equaling 20,000 ancient DNA (aDNA) Next Generation Sequencing (NGS) libraries – had to be generated. The challenge of this genomics project is not only the quantity, but also the quality of the samples, as aDNA can be highly fragmented.
To accomplish this ambitious task, a high-throughput semi-automated pipeline for producing NGS libraries has been established. In dedicated clean labs aDNA is extracted (see Figure 1, Step (2)) and the setups of qPCR and indexing PCR are performed (3a), each in a 96-well format on a pipetting robot. All downstream lab work is done in PCR labs. To keep up with the high sample volume and production speed, subsequent steps have been automated as well.
Thus, the liquid handling robot CyBio FeliX was integrated into the pipeline to perform three steps of the overall workflow (see Figure 1, marked in red). The flexibility of the CyBio FeliX allowed for configurations to be tailored to the specific needs of each step. In the bead purification step (4) high-throughput is of the utmost importance, which is accomplished by using a multichannel pipetting head to process up to 96 samples in parallel. On the other hand, the library pooling (6) and qPCR setup (7) steps require a high degree of flexibility with regard to the volume ranges to be transferred. Using the CHOICE Head with low as well as high volume liquid handling adapters ensures precise transfers of all required volumes in these steps of the workflow. Altogether, this serves to prepare samples as efficiently as possible to allow for fast and reliable sequencing data generation (8).

Due to its two-level moveable deck design, the CyBio FeliX offers enough deck positions to automate complex workflows on a very small footprint. All automated steps greatly benefit from the increased reproducibility and the reduced potential for human error. However, they prove most profitable in especially error-prone pipetting tasks – which are usually either the very complex or overly monotonous ones. In addition, relieving staff of highly repetitive or static manual activities, benefits their long-term health as well as freeing them up to focus on analytical work.

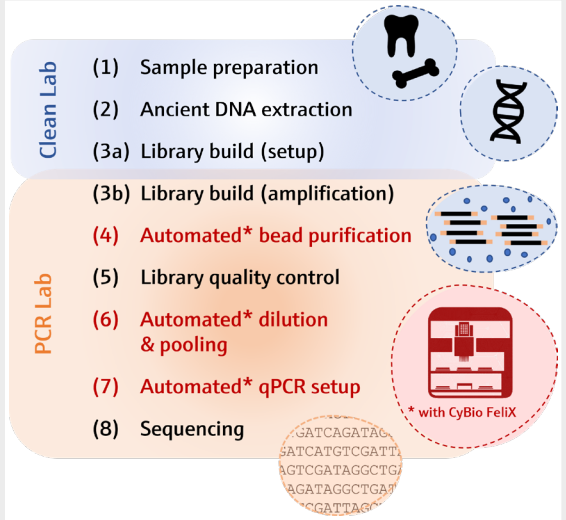


Figure 1: Schematic overview of the NGS library construction workflow.

## Materials and Methods

### Reagents and consumables

- HighPrep™ PCR Clean-up System MagBio Beads (AC-60500, MagBio Genomics)
- Qiagen Elution Buffer (19066, Qiagen)
- Molecular biology grade water (dilution of amplified libraries)
- Freshly prepared 80% ethanol
- KAPA Library Quantification Kit (7960298001, Roche)
- HS NGS Fragment Kit (1-6000bp), 500 (DNF-474-0500, Agilent)
- Tris-Tween Solution (Tween 20, P1379-1L, SigmaAldrich)
- Reservoir stand (372795, Ramcon) with modular reservoir quarter module (372790, Ramcon) and modular reservoir half reservoir (372786, Ramcon) (see „Bead Purification - Figure 3“)
- Eppendorf 96 PP U bottom plate (737-0167, VWR) Cleanup Plate (see „Bead Purification“)
- 96-well BC deep square plate (140504, Ramcon) Waste Plate (see „Bead Purification“)
- VWR non-skirted PCR plate on black 96-well PCR rack (211-0262, VWR) – Purification Input Plate (see „Bead Purification“) and Dilution Output Plate (see „Pooling“)
- Nunc™ 96-well Polypropylene Storage Microplates (267245, ThermoFisher) – Wash Plate (see „Bead Purification“)
- SX300 2D barcoded tubes in SBS96 format (2DSC-X03-BL-NS-SLC-S, LVL) – Final Elution Plate (see „Bead Purification“) and Pooling Input Plate (see „Pooling“)
- 1.5 mL tubes (Eppendorf) – Final pool tube (see „Pooling“)
- 0.2 mL PCR strips in plastic rack (miscellaneous vendors) (see „PCR“)
- LightCycler 480 Multiwell Plate 96, clear (05102413001, Roche) – for qPCR (see „PCR“)
- Abgene™ 96-well 1.2mL Polypropylene Deepwell Storage Plate (AB1127, ThermoFisher Scientific) – Dilution Plate (see „qPCR“ setup)
- Fisherbrand™ 96-well PCR rack (05-541-85, Fisher Scientific)
- CyBio TipRack 96/1000 µL tips (OL 3811-25-939-F, Analytik Jena)
- CyBio RoboTipTray 96/250 µL DW; PCR-certified, pre-sterilized, filter (OL3810-25-669, Analytik Jena)
- TipBox 96/50 µL; PCR-certified, pre-sterilized, filter (30-102-860, Analytik Jena)
- Protective Plate, 50 pieces (OL3317-25-125, Analytik Jena)
- Waste bag; Waste Box (10-406-342, Analytik Jena)

## Hardware

- CyBio FeliX Basic Unit with Enclosure (OL5015-24-100, Analytik Jena)
- CyBio FeliX Head R 96/1000 µL (OL3316-14-950, Analytik Jena)
- 8-Channel Adapter; Head R 96 (OL3317-11-330, Analytik Jena)
- CyBio FeliX CHOICE Head (OL3316-14-250, Analytik Jena)
- 8-Channel CHOICE Adapter; 1 µL – 50 µL (OL3316-14-332, Analytik Jena)
- 8-Channel CHOICE Adapter; 10 µL – 1000 µL (OL3316-14-330, Analytik Jena)
- Gripper (OL3317-14-800, Analytik Jena)
- Adapter 24 tubes, passive cooling function (844-00136-0, Analytik Jena)
- Adapter 96, passive cooling function (844-00135-0, Analytik Jena)
- 2x Height Adapter 40 mm (844-00445-0, Analytik Jena)
- 2x Support; 70 mm height (OL3317-11-110, Analytik Jena)
- Waste Box (844-00430-0, Analytik Jena)
- ALPAQUA® MAGNUM FLX™; Universal Magnet Adapter (OL3317-11-285, Analytik Jena)
- TipRack 96/1000 µL (OL3317-11-140, Analytik Jena)
- Biomek i5 (Beckman Coulter) for extraction and first steps of library generation (see Figure 1; (2), (3a))
- 5300 Fragment Analyzer System (Agilent) (see Figure 1; (5))
- Light Cycler 480 (Roche) (see Figure 1; (3b), after (7))
- NovaSeq 6000 (Illumina), chemistry version 1.5 (see Figure 1; (8))
- Applied Biosystems 2720 Thermal Cycler (ThermoFisher Scientific) (see Figure 1; (3b))
- Automatic 96-Channel Capper/Decapper for SAFE® screw caps with external thread (LVL technologies)

## Methods on CyBio FeliX

Within the overall workflow, the CyBio FeliX was used for the three automated steps that are performed in the PCR lab facilities (see Figure 1, steps (4), (6), (7)). To optimize each step, different CyBio FeliX configurations were used. The bead purification (4) was done with an R96/1000 µL Head for maximum through put due to 96-channel parallel pipetting. The pooling (6) as well as qPCR setup (7) steps are implemented using the CHOICE Head for maximum flexibility in terms of volume range.

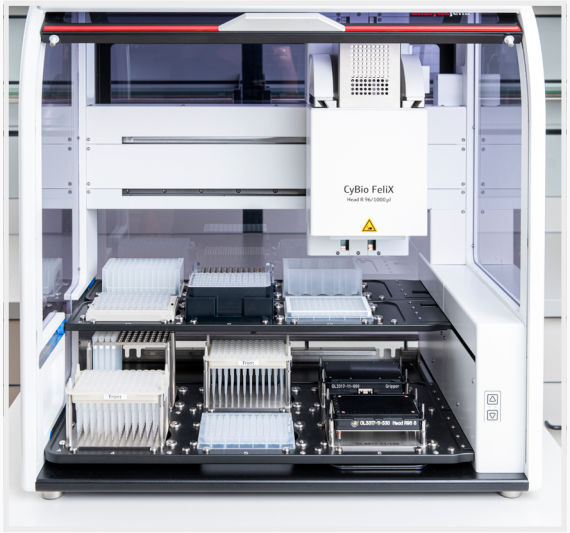


Figure 2: Analytik Jena CyBio FeliX hardware configuration. Decks equipped for bead-based purification of NGS libraries.

### Detailed Protocol – Bead Purification of NGS libraries

The protocol described here details the processing of 100 µL NGS libraries but can be scaled up or down: e.g., using half the amount of beads for 50 µL libraries. (see Figure 2)

**Step 1: Setting up cleanup.** From the quarter reservoir 160 µL of MagBio beads are distributed 8-channel wise into a 96-deepwell cleanup plate.

**Step 2: Sample transfer.** 100 µL of amplified NGS libraries are transferred from the input to the cleanup plate in one pipetting step. Beads and PCR product are then mixed 10 times by pipetting. Afterwards, the plate is incubated for 5 minutes at room temperature.

**Step 3: Washing beads.** The cleanup plate containing the DNA-bead mix is transferred to a magnet adapter using the Gripper. After a 3-minute incubation period, the supernatant is cleared of beads and can be removed in a 96-channel parallel pipetting step. 200 µL of freshly prepared 80% Ethanol is added to the cleanup plate using the 8-channel adapter. Once 80% EtOH is added to all columns it is removed after 30 s of incubation. Then, the residual EtOH is removed from all 96 wells at once. This washing step is repeated once more.

**Step 4: Drying beads.** The beads are left to dry for 2 minutes at room temperature.

**Step 5: Elution.** The cleanup plate is removed from the magnet using the Gripper. 35 µL of Qiagen Elution Buffer from the reservoir stand is added column-wise to the beads and mixed 10 times by pipetting. The mix is then incubated at room temperature for 2 minutes.

**Step 6: Final transfer.** The cleanup plate is moved back to the magnet for 2 minutes to collect the beads and clear the supernatant. The supernatant from all 96 wells is then transferred to 300 µL 2D barcoded LVL tubes in a single pipetting step.

**Step 7: Fragment analyzer.** In a parallel pipetting step, 2 µL of each purified NGS library are then transferred to a fresh 96-well plate containing Fragment Analyzer reagents. Sample quantification is carried out on the Fragment Analyzer 5200. According to the results, samples are pooled equimolarly (see „Pooling").

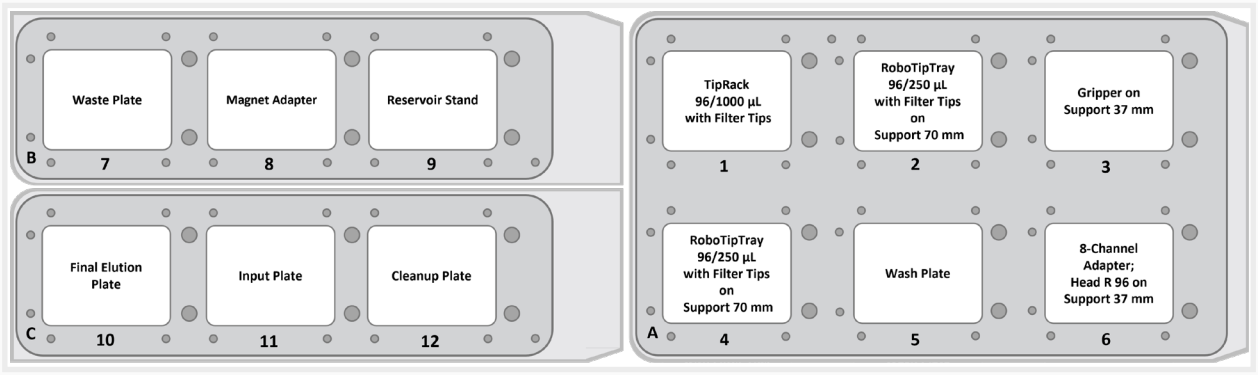


Figure 3: Deck layout for Protocol – Bead Purification. The CyBio FeliX provides 12 deck positions on 3 movable decks. The upper decks B and C equipped with the required accessories are shown on the left, the lower deck A is shown on the right. The reservoir stand (position 9) is equipped with MagBio beads (quarter reservoir), Elution Buffer (quarter reservoir), Ethanol (half reservoir).

## Detailed Protocol – Pooling of NGS libraries

**Step 1: Import CSV file.** A semicolon separated CSV file is read into the protocol. The file contains information pertaining to step 3 below: the sample name, pipetting volume, and information if diluted or undiluted sample needs to be transferred (well info).

**Step 2: Preparation of Dilutions.** Molecular grade water is distributed to a non-skirted 96-well plate on a cooling block (see Figure 4, Dilution Output Plate). Subsequently, amplified NGS libraries from the Input Plate are added to the water and mixed 10 times. The volumes of water and libraries can be set at the beginning of the run.

**Step 3: Pooling of samples.** According to volumes stated in the CSV file each sample is added individually to a 1.5 mL low bind Eppendorf tube, to achieve normalization of samples. The volumes to be transferred determine the order in which samples are added to the tube – samples with the highest transfer volumes are added first.

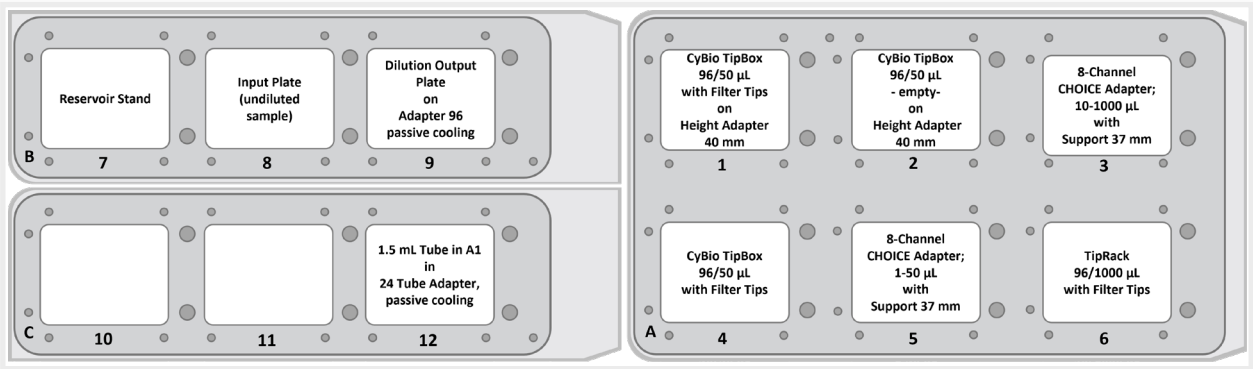


Figure 4: Deck layout for Protocol – Dilution & Pooling. The CyBio FeliX provides 12 deck positions on 3 moveable decks. The upper decks B and C equipped with the required accessories are shown on the left, the lower deck A is shown on the right. The deck layout shown here comprises all accessories needed for the dilution as well as the pooling of samples. For higher throughput, two Input Plates may be processed in parallel. However, this requires two separate runs with separate deck layouts – one for the dilution step, one for the pooling step.

## Detailed Protocol – Setup of quantitative real-time PCR

**Step 1: Transfer Tris-Tween** to Dilution Plate. 597 µL of Tris-Tween are transferred to each well of the columns for the first dilution (columns 1-5) and 594 µL are transferred to each well of the columns for the second dilution (columns 7-11) on the dilution plate.

**Step 2: Transfer samples** to dilution plate. 3 µL of sample are transferred in duplicate (see Figure 5, sample X is transferred as X1, X2) to the prefilled columns for the first dilution (columns 1-5). Up to 20 samples can be processed per run.

**Step 3: Transfer MasterMix** to qPCR plate. 16 µL of MasterMix are transferred to the qPCR plate.

**Step 4: Finalize sample dilution.** First, on the dilution plate 6 µL pre-diluted samples from step 2 are transferred from first dilution wells (columns 1-5) to the second dilution wells (columns 7-11). After thorough mixing, 4 µL of each sample of the second dilution are transferred to the qPCR plate in duplicates (see Figure 5, X1 is diluted once and then transferred to PCR plate twice, the same is done for X2).

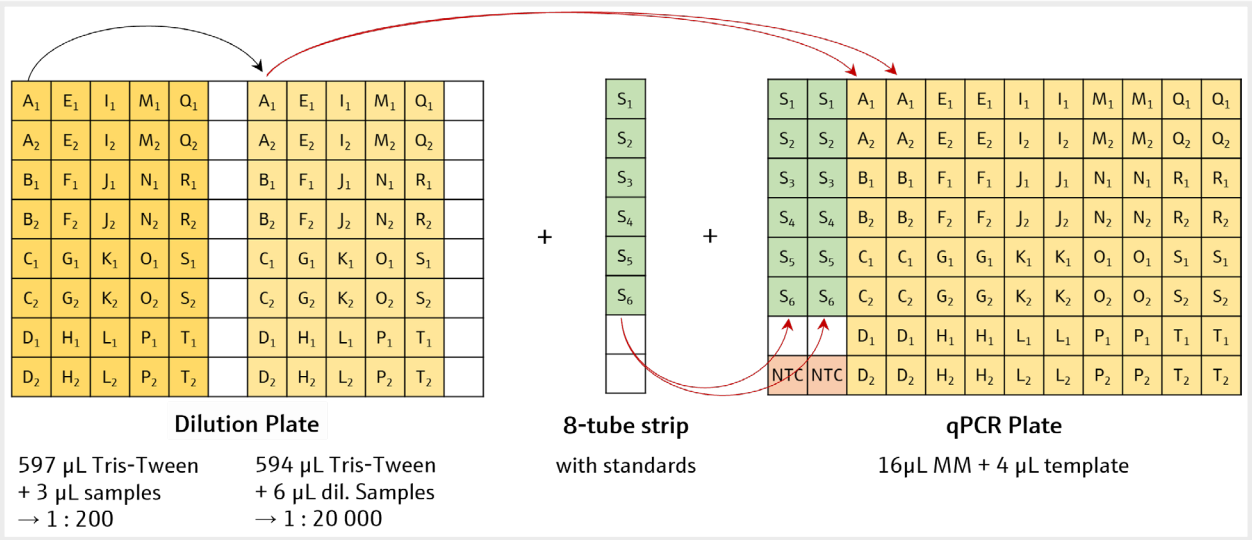


Figure 5: Setup of qPCR plate. Samples are transferred to the dilution plate in duplicate (X1, X2). After a two-step dilution (1:200 in columns 1-5; 1:20 000 in columns 7-11) diluted samples are transferred to the qPCR plate in quadruplicates (X1, X1, X2, X2). Standards and controls are transferred to the qPCR plate as well. A maximum of 20 samples can thus be processed in parallel.

**Step 5: Transfer standards** and negative controls. 4 µL of standards and negative control are transferred to the qPCR plate in duplicate.

**Step 6: Start qPCR run:** Upon sealing, the qPCR plate is transferred to the real-time PCR cycler. The temperature-time protocol is programmed according to the instructions of the kit (KAPA Library Quantification Kit, Roche).

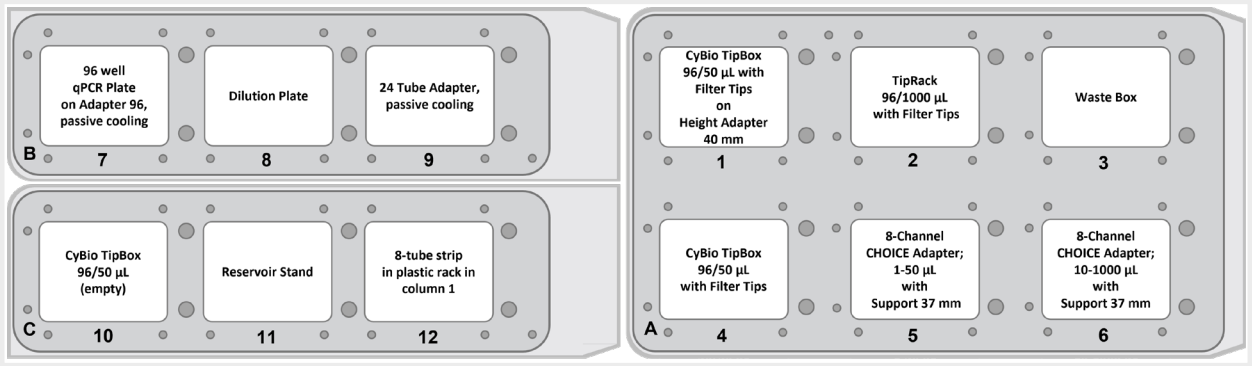


Figure 6: Deck layout for Protocol – Setup quantitative real-time PCR. The CyBio FeliX provides 12 deck positions on 3 moveable decks. The upper decks, B and C, equipped with the required accessories are shown on the left, the lower deck A is shown on the right. The Tube Adapter (position 9) holds the MasterMix and PCR grade water. Tris-Tween for the dilution is provided in the reservoir on position 11. The 8-tube strip (position 12) contains the standards.

# Results and Discussion

Out of the 20, 000 libraries prepared and sequenced during the project two-thirds have been generated using the automated workflow. Due to the nature of the workflow and fragility of the sample material, automation does not cut down on the overall time-to-result – it takes about 3 weeks from extraction to sequencing. However, efficiency has been greatly improved by automating certain parts of the workflow. When processing samples predominantly manually, 10 people were involved in the laboratory work, generating about 24-48 libraries each. After the automation of key sub-steps on the CyBio FeliX, only one person was needed for the laboratory work, who created 400 libraries in the same period.

**Bead Purification:**
As depicted in Figure 7, automated and manual bead purification are of the same quality and result in identical fragment lengths and the same concentrations of purified libraries. The bead purification on the CyBio FeliX equipped with CHOICE Head is not faster than the manual process but can be repeatedly done without a decline in performance quality. However, it was found that upgrading to a multi-channel head (CyBio Head R96/1000 µL) cut down the processing time to 50%, making it the perfect choice for higher throughput.

**Pooling:**
The automation of the dilution and pooling of libraries improved error rates as well as cutting down the processing time. As libraries must be individually diluted (if necessary) and transferred, this step was especially error prone. Automating this step and using a CSV file with data generated based on the library quality control, benefited not only the data quality but staff health as well, as it eliminated this tedious and unergonomic pipetting task from their workload. Not only personnel, but also material costs were reduced using automation. As up to 192 libraries can be pooled for sequencing, reliability and robustness of the devices are paramount. Upon sequencing it was found that equimolarity of pools was more consistently achieved with the automated setup than with the manual one (data not shown). The reduction in error rates and thus fewer re-runs of samples cut down spendings on flow cells significantly.

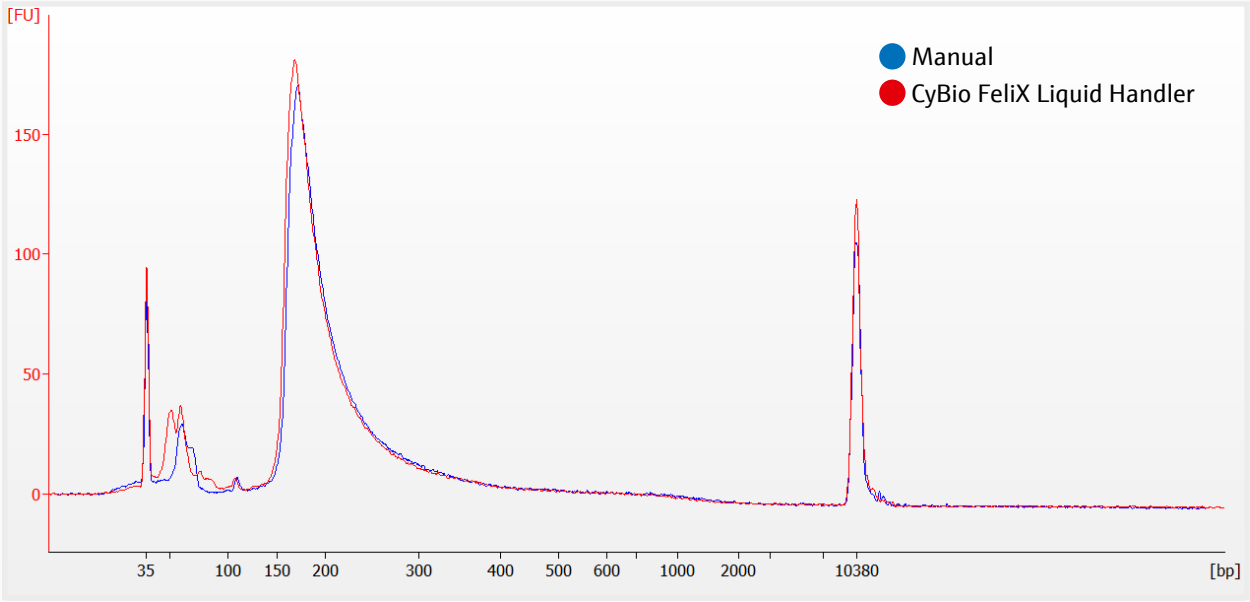


Figure 7: Library Quality Control. Exemplary data of a side-by-side comparison of automated (CyBio FeliX, red line) and manual (blue line) bead purification of the same library. Library concentration and fragment length distribution was determined using the 5300 Fragment Analyzer System (Agilent) with the HS NGS Fragment Kit (1-6000 bp).

**qPCR setup:**

As for all other automated steps, the greatest benefit for the qPCR setup is the reliability of the robotic setup. Table 1 shows the robustness of the qPCR setup of the CyBio FeliX. Deviations in crossing point (PCR cycles needed for the fluorescence signal to rise above the threshold, Cp) values were smaller than in the manual setup. It should be noted, that in most cases the manual setup results in data reproducibility like the one shown for CyBio FeliX. However, when conducting the side-by-side comparison, the day-to-day variability in human pipetting led to slightly worse results on this particular day. This shows that not only intra but also inter run reproducibility can be improved with an automated setup. Variations from the internal control as shown for the manual setup in Table 1, make a re-run of the qPCR necessary. Thus, again, automation can save time and money due to its reliability. In addition, it frees up time for laboratory staff to perform other tasks while the CyBio FeliX handles the preparation of the qPCR plate.

Table 1: qPCR setup reproducibility. qPCR setup (see Figure 5) of the same samples (A-G) was done manually as well as automated with CyBio FeliX. Sample C was the calibration control. Cp = crossing point, PCR cycles needed for the fluorescence signal to rise above the threshold; X = agreement within duplicates (X1/(X1+X2); perfect agreement = 50.0% per duplicate); Y = agreement between duplicates (mean of X1/mean of X2); perfect agreement = 100.0%)

| Sample | Manual | | | | CyBio FeliX | | | |
|---|---|---|---|---|---|---|---|---|
| | Cp | | X | Y | Cp | | X | Y |
| A | 13.67 | 13.70 | 49.9% | 105.7% | 14.00 | 14.01 | 50.0% | 100.4% |
| | 12.97 | 12.93 | 49.9% | | 13.94 | 13.95 | 50.0% | |
| B | 14.49 | 14.50 | 50.0% | 104.4% | 14.67 | 14.65 | 50.0% | 100.0% |
| | 13.87 | 13.89 | 50.0% | | 14.67 | 14.64 | 50.1% | |
| C | 13.92 | 14.04 | 49.8% | 101.6% | 14.23 | 14.19 | 50.1% | 101.0% |
| | 13.76 | 13.76 | 50.0% | | 14.09 | 14.06 | 50.1% | |
| D | 13.18 | 13.01 | 50.3% | 104.3% | 13.47 | 13.43 | 50.1% | 100.6% |
| | 12.56 | 12.54 | 50.0% | | 13.42 | 13.33 | 50.2% | |
| E | 11.87 | 11.86 | 50.0% | 102.6% | 12.54 | 12.46 | 50.2% | 100.0% |
| | 11.55 | 11.57 | 50.0% | | 12.50 | 12.51 | 50.0% | |
| F | 11.80 | 11.89 | 49.8% | 102.1% | 12.55 | 12.53 | 50.0% | 100.5% |
| | 11.62 | 11.59 | 50.1% | | 12.52 | 12.44 | 50.2% | |
| G | 13.87 | 13.88 | 50.0% | 103.5% | 14.45 | 14.44 | 50.0% | 99.3% |
| | 13.40 | 13.42 | 50.0% | | 14.51 | 14.58 | 49.9% | |

# Summary

Automating parts of any workflow holds a lot of potential for saving time and money, freeing up staff for analytical work while preserving their physical well-being, as well as increasing throughput and data reliability.
Within the presented NGS library preparation workflow, the use of the CyBio FeliX allows up to 96 samples to be processed simultaneously, while offering the flexibility to process multiple runs a day. The compact device reduces the bench space needed for an automated library preparation process. It can take on diverse tasks within the workflow – including library purification, dilution and pooling of libraries optimal for loading an Illumina® NovaSeq flowcell, as well as the setup of amplification and quantification reactions.
In summary, using the automated workflow, one staff member can now generate almost twice (1,7x) as much data as 10 staff members did before. When tackling projects that require large amounts of data, automation goes a long way towards realizing them.

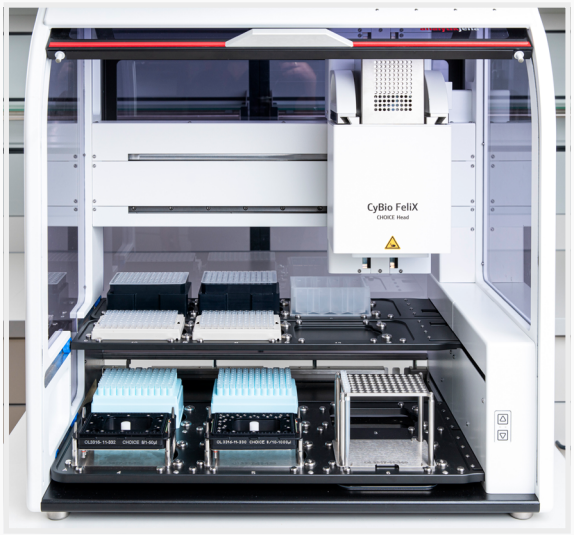


Figure 8: CyBio FeliX. Decks equipped for pooling of NGS libraries.

### Recommended device configuration

Table 2: Device and accessories used for bead purification.

| Article | Article number |
|---|---|
| CyBio FeliX Basic Unit with Enclosure | OL5015-24-100 |
| CyBio FeliX Head R 96/1000 µL | OL3316-14-950 |
| 8-Channel Adapter; Head R 96 | OL3317-11-330 |
| Gripper | OL3317-14-800 |
| ALPAQUA® MAGNUM FLX™; Universal Magnet Adapter | OL3317-11-285 |
| TipRack 96/1000 µL | OL3317-11-140 |
| 2x Support; 70 mm height | OL3317-11-110 |

Table 3: Device and accessories used for pooling and qPCR setup.

| Article | Article number |
|---|---|
| CyBio FeliX Basic Unit with Enclosure | OL5015-24-100 |
| CyBio FeliX CHOICE Head | OL3316-14-250 |
| 8-Channel CHOICE Adapter; 1 µL – 50 µL | OL3316-14-332 |
| 8-Channel CHOICE Adapter; 10 µL -1000 µL | OL3316-14-330 |
| Adapter 24 tubes, passive cooling function | 844-00136-0 |
| Adapter 96, passive cooling function | 844-00135-0 |
| 2x Height Adapter 40 mm | 844-00445-0 |
| TipRack 96/1000 µL | OL3317-11-140 |
| Waste Box* | 844-00430-0 |

*Waste Box only used for qPCR setup



**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 · Authors: AnKo, KaHa, FrHaPu
en · 01/2024