Automation of library preparation for high resolution HLA typing by next generation sequencing

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Abstract

Implementation of NGS for routine HLA typing is challenging since library preparation for hundreds of samples is time-consuming and labor-intensive, requiring many error prone pipetting steps and repetitive tasks.
Here, we describe an automated NGS workflow utilizing Hamilton´s Microlab® STARlet liquid handling systems for library preparation for high-resolution HLA typing on the Illumina MiSeq Sequencing System. Samples are analyzed for exon 2 and exon 3 of HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 in one sequencing run with 2 x 300 bp paired-end reads. We integrated the complete sample processing from sample registration, liquid handling processing, sequencing and analysis into our LIMS. The liquid handling steps for PCR amplification, pooling and PCR purification are fully automated. Compared to classical Sanger sequencing methods, this automation solution provides a reliable, high throughput and cost-efficient genotyping approach.

- ▶ Speed up the NGS library construction process and reduce hands-on time
- ▶ Increase process stability and security by automating error-prone pipetting steps
- ▶ Flexibly process 8 – 48 samples or full plates with 96 samples

Introduction

For several decades, Sanger Sequencing has been the method of choice and gold standard for many HLA typing laboratories, replacing conventional methodologies such as sequence-specific primers (SSP), and sequence-specific oligonucleotide probes (SSOP).

With the arrival of massively parallel sequencing technologies about 10 years ago, the possibilities in basic and clinical research have dramatically changed.

Technological progress has enabled that the first commercially available NGS method – the Roche 454 technology – is now being more and more replaced in diagnostic laboratories by Illumina sequencing technology.

The Illumina sequencing-by-synthesis (SBS) technology offers simplified sample preparation processes, higher data output, and lower costs per base sequenced.

Due to the various benefits, HLA typing is now performed using the Illumina MiSeq platform.

The Hamilton Microlab® STARlet

HAMILTON

Workflow

HLA typing is performed for exon 2 and 3 on the 5 HLA loci HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1. The DNA amplicon library is sequenced with the Illumina MiSeq Sequencing System using MiSeq Reagent Kit v3 chemistry. PCR amplicons are generated with 12 target specific primer pairs. Index and adaptor sequences are introduced by a second outer primer pair. Two different setups were established:

▶ low throughput setup: flexible amplification of 8-48 donor registry samples in 12 reactions per sample, e.g. for urgent samples with short processing times, resulting in one or two 384-well plates

▶ high throughput setup: 96 donor registry samples in matrix plate format are amplified in 12 reactions per sample, resulting in three 384-well PCR plates

Amplicons of the same target region from up to 96 samples are then pooled and subsequently purified with SPRIselect® beads. Hereafter, amplicon pools are quantified by PicoGreen, diluted to 4nM, mixed together and denatured, resulting in a ssDNA library, ready for sequencing.

The liquid handling steps for reaction setup, pooling, and PCR purification are automated on Microlab® STARlet instruments. In the pre-PCR area, one workstation is used for PCR setup. In the post-PCR area, a second workstation is used for automated pooling and purification of PCR products.
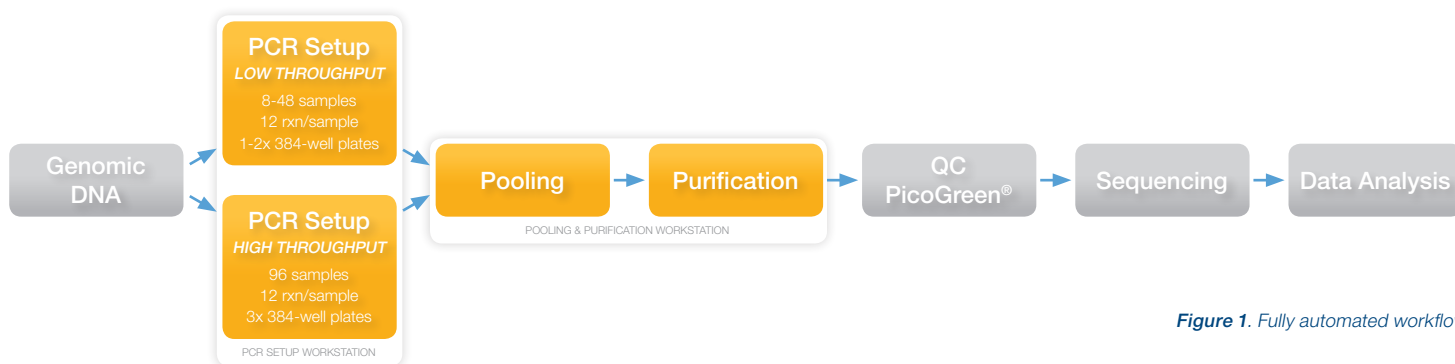
Figure 1. Fully automated workflow

PCR setup Workstation

Microlab® STARlet with CO-RE 96 Probe Head, dedicated to rapid library construction (Figure 2). DNA samples, target specific primers, Index primers and PCR master mix are loaded in 96 well plates on the deck. Depending on the setup, one, two or three 384-well PCR plates per run are prepared with 10µl total PCR volume per reaction in about 40 - 70 minutes.
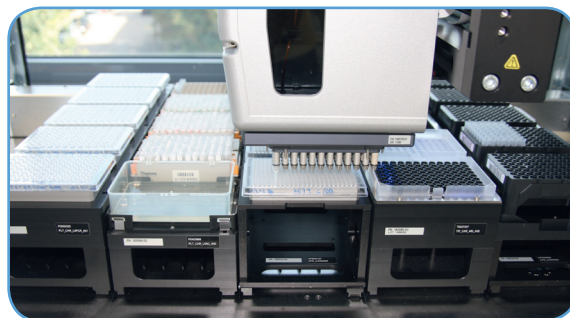
Purification and Pooling Workstation

Microlab® STARlet with 8x1000µl pipetting channels, dedicated to amplicon pooling and purification.

Application Software

Methods are programmed using the Hamilton VENUS Two software. It reads work lists, reads and tracks barcodes from samples, primers, reagents and consumables. The software provides full flexibility for adaptations of the processes with regard to changes in workflow or use of different kits. Workstations for PCR setup/library preparation and PCR purification can be used for other non-NGS methods as well simply by creating different methods in the software.

Figure 2. The Microlab® STARlet PCR Setup Workstation

Figure 3. The Microlab® STARlet Purification & Pooling Workstation

Results

The workflow is optimized for 1 - 2 sequencing runs per week. Accordingly, 380 - 760 samples can be typed for HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 in this time-frame. Due to the clonal amplification, high resolution typing results can be achieved; most of the results can be reported with suffix "G".

Quality of fragment libraries

The library consists of PCR products in the size of about 520 to 690 bp, depending on the target region.

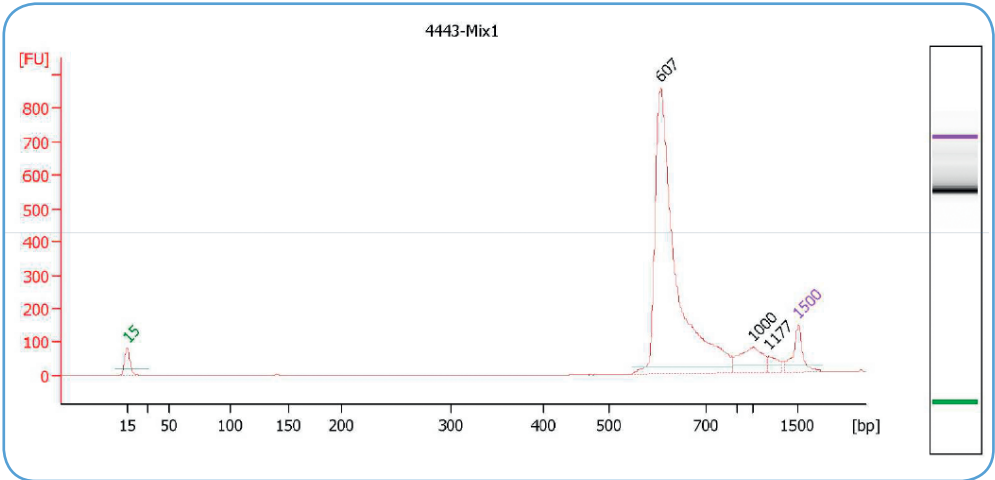Short undesirable fragments were excluded effectively with SPRIselect® beads (Figure 4).

Figure 4. Representative result for automated PCR of fragment libraries; Bioanalyzer 2100 analysis after purification

Sequence metrics

Read length is sufficient to cover complete exons, up to 276 bp, of all loci.

A minimum sequencing depth of 50 was aimed to achieve for distinctive allele assignment and could be reached for >98% of samples (Table 1).

Representative sequence read alignments are shown in Figure 5.

Yield total (GB)	10,3
≥ Q30 (%)	75,2
Reads (M)	17,9
Read passed filter (M)	16,7
Reads identified (%)	80,5

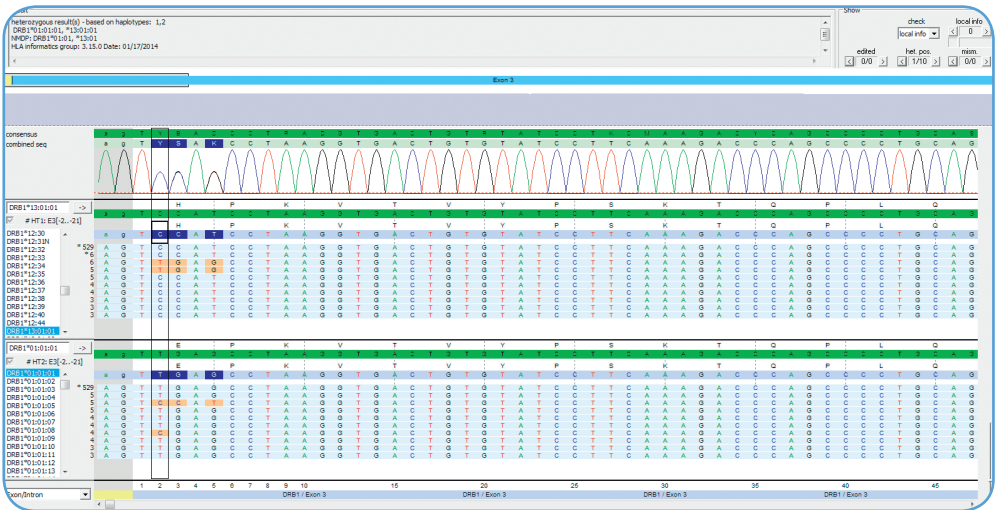*Table 1. Average run characteristics for 380 donor samples in one run*

Figure 5. Representative sequence read alignments

Genotype assignment

As a result of clonal amplification of the target region, an unambiguous genotype assignment is possible in most of cases by using a suffix "G" for alleles identical over clinically relevant exons 2 and 3 (HLA-A, -B and -C) or exon 2 (HLA-DRB1 and -DQB1). In very few cases ambiguities were observed due to the recombination events between exon 2 and 3 (Table 2).

	Unambiguous/G Groups		Ambiguous	
HLA-A	474	99.8%	1	0.2%
HLA-B	465	97.9%	10	2.1%
HLA-C	459	96.6%	16	3.4%

Table 2. *Genotype assignment for a cohort of 475 donors*

Discussion

High resolution typing for HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 is established in our laboratory with full automation of a significant part of the workflow. In our experience, 380 samples can be analyzed in one sequencing run. We successfully integrated the complete sample processing from sample registration, liquid handling processing, sequencing and analysis into our LIMS. The workflow is optimized for sequencing on the Illumina MiSeq System. Compared to classical Sanger sequencing methods, this automation solution provides a reliable genotyping method and enables to increase throughput and to reduce costs at the same time.

Pre and post PCR steps are separated for enhanced safety and prevention of cross-contamination. Individual process steps are designed as complete walk-away solutions enabling significant reduction of manual intervention.
The high flexibility of both liquid handling workstations allows automation for other applications as well.

System Configuration

PCR Set-up Workstation	Microlab STARlet, manual load
	CO-RE 96 Probe Head
	carriers for tips, reagents, 96well/384well plates, and 800µl Matrix tubes
Purification and Pooling Workstation	Microlab STARlet, manual load
	8 x 1000µl channels
	carriers for tips, reagents, 96well plates, and magnet
Sample Preparation	Custom-made target specific primers and index primers (Metabion)
Reagents and Accessories	Agencourt® SPRIPlate® 96R-Ring Super Magnetic Plate
	(A32782 / Beckman Coulter)
	SPRIselect® beads (Beckman Coulter)
Sequencing	Illumina MiSeq Sequencing System
	Illumina MiSeq Reagent Kit v3
Data Analysis	SeqNext-HLA (JSI medical systems)

References

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[3] Erlich RL, et al. Next-generation sequencing for HLA typing of class I loci. BMC Genomics. 2011 Jan; 18(12):42-55.