High Resolution HLA Typing of Blood Stem Cell Donors by Next Generation Sequencing

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Introduction

Recent advances in Next-Generation Sequencing (NGS) technologies have enabled NGS as a proven alternative for classical Sanger sequencing for the characterization of the human leukocyte antigens (HLA). However, HLA typing is still a time-consuming, labor-intensive and expensive process with limitations to discriminate alleles at high-resolution level. Here, we describe an automated high-throughput workflow using the Hamilton Microlab STAR line of liquid handling systems and the Roche 454 GS FLX system for initial typing of blood stem cell donors. High quality DNA is extracted on a chemagic STAR liquid handling system using chemagen's nucleic acid purification system. On the second workstation, PCRs are automatically prepared in 384-well plates, and eight PCRs per sample are used to cover exon 2 and 3 of HLA-A and -B genes and exon 2 of HLA-DRB1 gene. PCR products are pooled and cleaned up with AMPure beads applying an automated protocol. After emulsion PCR, enriched beads are collected with the Roche 454 REM e system, integrated on the third workstation. In total, 380 samples are sequenced in one run on GS FLX system by multiplexing 96 samples and using 4 separated plate regions. Sequences are assigned with SeqHLA 454 software (JSI medical systems). In a cohort of 475 donors, clinically relevant ambiguities were observed only in HLA-B locus (in 1.3% of donors). In our experience, NGS allows a high resolution typing of HLA alleles in combination with high throughput of samples.

Method description

HLA typing is performed for exon 2 and 3 of HLA-A and HLA-B and for exon 2 of HLA-DRB1 locus. DNA library construction and sequencing with the 454 GS FLX Sequencing System using Titanium chemistry is performed as described by Bentley et al. (Bentley G, et al. Tissue Antigens;2009;74(5):393–403). Briefly, a dsDNA amplicon library is prepared by PCR with 8 target specific primer pairs containing unique multiplex identification tags (MID). The adaptor sequence for bead capture is included in each primer. In every PCR run, 5 HLA exons of 95 donor samples and 1 negative control are amplified in 8 PCRs per donor sample, resulting in generation of two 384-well PCR plates.

Amplicons are then pooled together, resulting in 8 x 1.5 ml tubes, each containing the same target region from 95 donor samples and one negative control. Agencourt® AMPure® XP beads are used to purify the amplicons. Hereafter, amplicon pools are quantified by PicoGreen® and diluted to the appropriate concentration of 1 Mio molecules/µl for the following clonal amplification in emulsion on capture beads.

Eight pools are then mixed together and prepared for emulsion PCR (emPCR). The emPCR is performed in a 96-well PCR plate, resulting in clonal amplification to produce one fragment - one bead - one read. After emulsion breaking and recovery of the beads, DNA-carrying beads are enriched using the Roche 454 REM e System to yield between 5-20% enrichment of the beads. Finally, sequencing primers are annealed to the fragments on the beads to prepare sequencing-ready samples.

Using 96 MIDs, up to 96 samples can be sequenced per PicoTiterPlate™ region. Beads are loaded on a PicoTiterPlate™ using a 4-region gasket followed by sequencing according to the vendor's protocol. Thus, 380 donor samples plus 4 negative controls can be analyzed in one sequencing run.

System description

chemagic STAR DNA extraction workstation

Microlab STAR combined with chemagen nucleic acid purification instrument in a single housing. 96 rod head, 4 x 1ml + 4 x 5ml channels, barcode reader, and LIMS conncetion. DNA isolation is performed typically from 200µl or 400µl blood using chemagic DNA blood kits. The system is adapted to process variable sample numbers in different vial sizes up to 96 samples in total (→ WS1).

Pooling and REM e workstation

Microlab STARlet with 8 x 1ml pipetting channels and integrated Roche 454 REM e system (Figure 2). Amplicon pools are created here in 1.5ml tubes. Fully automated enrichment and sequence primer annealing steps are performed on the REM e module (→ WS3).
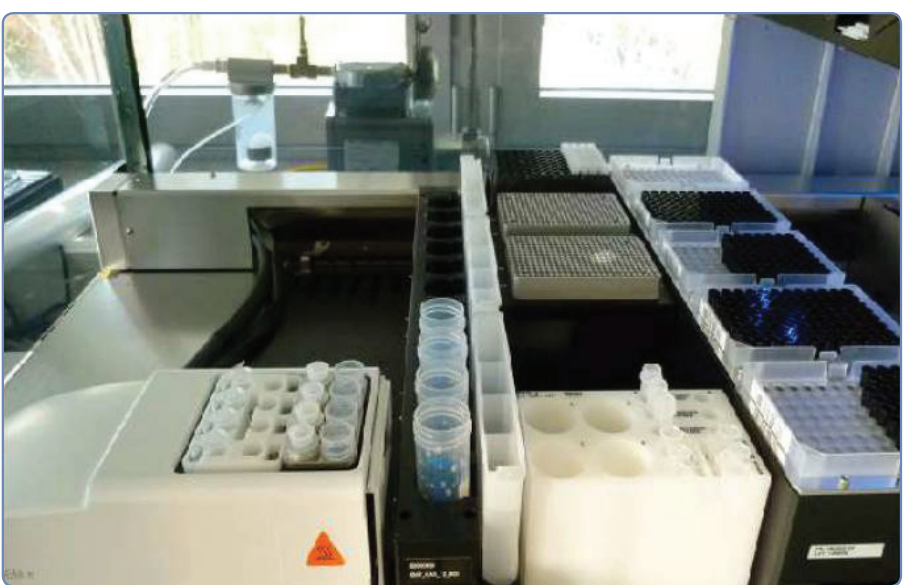

Figure 2: Deck of the Microlab STARlet pooling and REM e workstation.

PCR set-up workstation

Microlab STARlet with CO-RE 96 Probe Head, dedicated to rapid library construction (Figure 1). DNA samples, primers, MIDs and PCR master mix are loaded onto the deck in 96-well plates. Two 384 well PCR plates per run are prepared with 10µl total PCR volume per reaction in about 40 minutes (→ WS2).
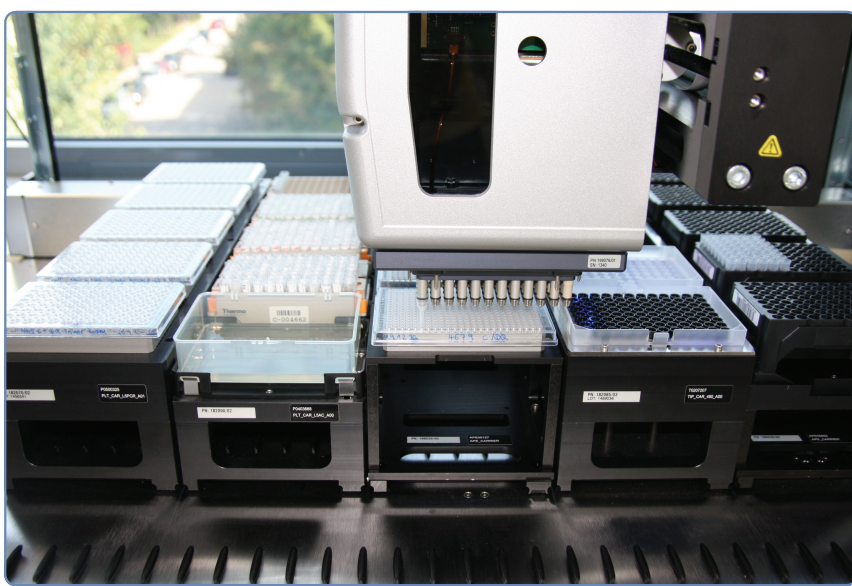

Figure 1: Microlab STARlet PCR set-up workstation.

Purification workstation

Microlab STARlet with 8 x 1ml pipetting channels, shaker and magnets for processing of Agencourt® AMPure® XP magnetic beads (Figure 3). Up to 32 pooled samples are introduced and processed in 96-well plates. Plate transfers between shaker and magnets are done with the CO-RE gripper (→ WS4).

Figure 3: Microlab STARlet purification workstation.

Workflow diagram

EDTA Blood
- WS 1 — DNA extraction
- WS 2 — PCR (3 HLA loci, primer and adapter)
- WS 3 — Pooling
- WS 4 — Purification
- QC: Bioanalyzer, PicoGreen®
- emPCR - Amplification
- emPCR - Breaking
- WS 3 — emPCR - Enrichment
- QC: Bead count
- Sequencing
- Data processing, filtering
- Data analysis
- Data export, reporting

Results

The workflow is optimized for one sequencing run per week. Accordingly, 380 samples can be typed for HLA-A, HLA-B and HLA-DRB1 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". By introducing automated steps into the workflow, the sample throughput was multiplied by 2 to 3 in comparison to Sanger sequencing.

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 Agencourt® AMPure® XP beads (Figure 4).
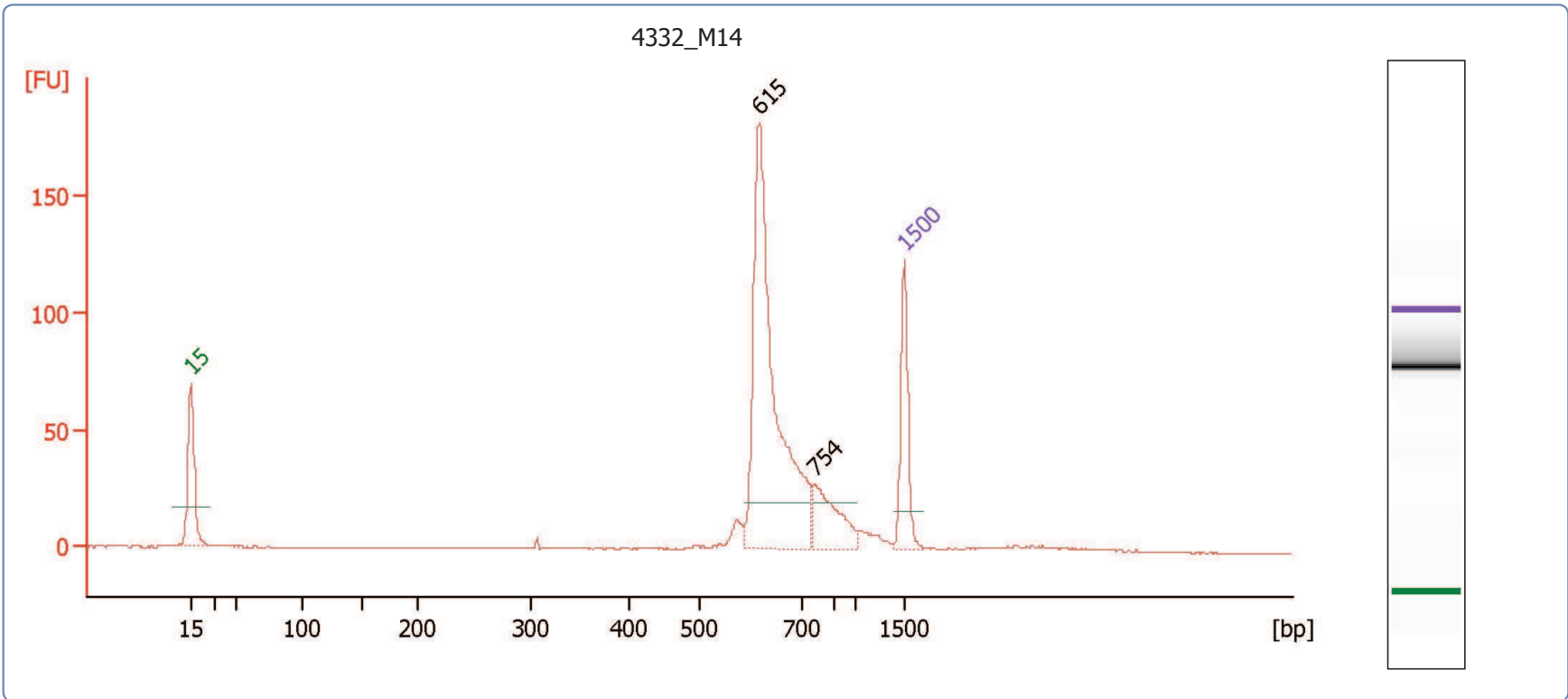

Figure 4: Representative result for automated PCR of fragment libraries; Bioanalyzer 2100 analysis after bead enrichment and purification.

Sequence metrics

Read length is sufficient to cover complete exons, up to 276 bp, of all loci. It was aimed at achieving a minimum sequencing depth of 50 for distinctive allele assignment. This could be reached for >95% of samples (Table 1).

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Donor samples	380
Raw Wells	1.113.120
Key Pass Wells	1.035.262
Passed Filter Wells	448.350
Total Bases	208.395.659
Length Average	465

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 and -B) or exon 2 (HLA-DRB1). In very few cases ambiguities were observed due to the recombination events between exon 2 and 3 (Table 2).

	Unambiguous		Results with "G"		Ambiguous	
HLA-A	0	0.0%	475	100%	0	0%
HLA-B	2	0.4%	467	98.3%	6	1.3%
HLA-DRB1	77	16.2%	398	83.8%	0	0%

Time savings

In a typical run with 380 samples, significant time savings of the overall process as well as reduced hands-on time is observed (Table 3).

	Automated process / Hands-on time	Manual process
PCR setup	4 h / 25 min	14 h
Pooling	2 h / 5 min	8 h
Bead enrichment	3 h / 30 min	5 h
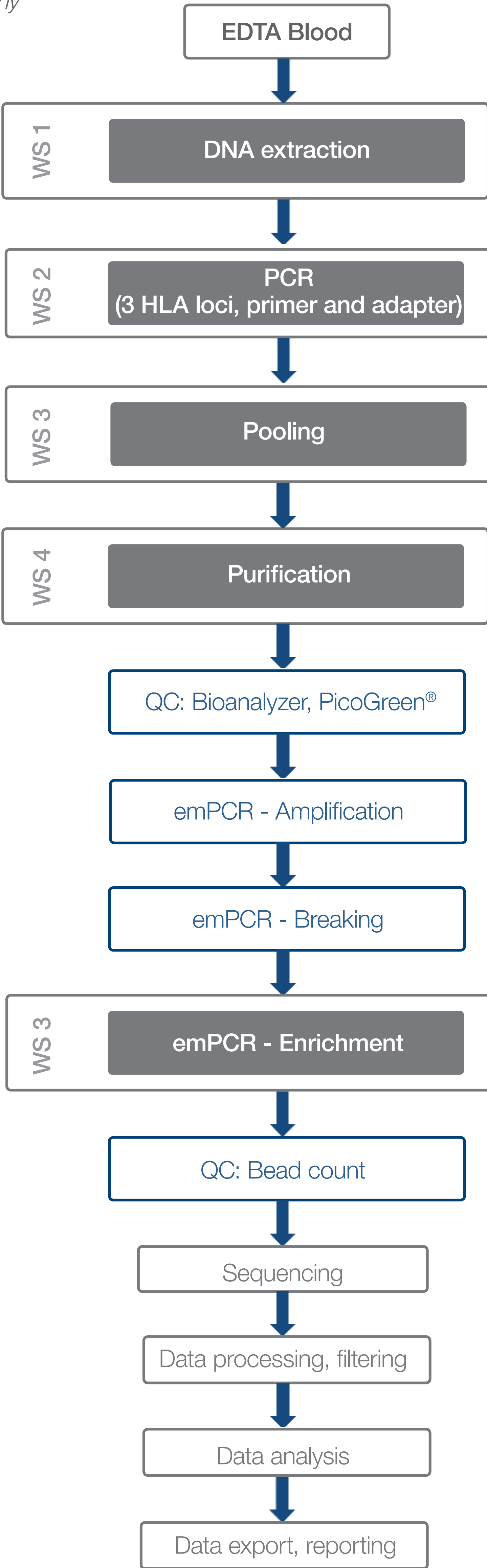
Summary

High resolution typing for HLA-A, HLA-B and HLA-DRB1 is established in our laboratory with full automation of 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 Roche 454 Titanium chemistry, adaptable for analysis on the Roche 454 GS FLX system or GS Junior system. Compared with classical Sanger sequencing methods, this automation solution provides a reliable genotyping method and enables increasing throughput and reducing 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 all three liquid handling workstations allows automation for other applications as well.

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