Published in final edited form as:

Methods Mol Biol. 2013 ; 1034: 197–219. doi:10.1007/978-1-62703-493-7_10.

Next-Generation HLA Sequencing Using the 454 GS FLX System

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Abstract

Next-generation sequencing (NGS) of HLA class I and II loci (HLA-A, HLA-B, HLA-C, DRB1, DRB3, DRB4, DRB5, DQA1, DQB1, DPB1) is described here in detail using the 454 Life Sciences GS FLX System and Titanium chemistry. An overview of the protocol with our experience on sequence performance efficiencies, read depth and ambiguity analyses using the GS FLX System are also presented. A total of 14 HLA primer pairs with multiplex identifiers (MIDs) are used in clonal, amplicon-based pyrosequencing of up to 44 samples per plate using the GS FLX. Genotype assignment and ambiguity reduction analysis is performed using Conexio Assign ATF 454 software. Clonal NGS gives a significant reduction in genotyping ambiguity during analysis of the highly complex HLA system.

Keywords

HLA class I and II sequencing; Clonal pyrosequencing; Next-generation sequencing (NGS); 454 Life Sciences GS FLX Titanium Chemistry; 454 Life Sciences GS FLX 454 System; HLA genotype ambiguity reduction

1 Introduction

High resolution HLA class I and II genotyping continues to be a challenge because of the extreme level of polymorphism in these loci. Allelic ambiguities, the biggest problem in high resolution HLA typing, stem from incomplete genomic coverage and inability to set phase, and are time consuming to resolve. Next-generation, clonal sequencing significantly resolves phase ambiguities and provides higher resolution HLA genotypes. This chapter describes next-generation HLA sequencing using the 454 Life Sciences GS FLX System (GS FLX) and Titanium chemistry, detailing test procedures, and listing manufacturer and laboratory provided materials and reagents needed for the testing. Further information for the protocols may be found by visiting the Web sites of the manufacturers of the instruments, reagents, and robotics.

1.1 Protocol Overview

In this protocol, an amplicon-based, clonal pyrosequencing strategy is used for HLA genotyping. 454 Life Sciences GS FLX [1] Titanium chemistry and primers are used to

sequence the HLA class I and II genes, HLA-A, HLA-B, HLA-C, DRB1/3/4/5, DQA1, DQB1, and DPB1 [2, 3]. To begin, genomic DNA samples are amplified with 14 HLA primer pairs to amplify the sequences, including exon and noncoding regions as illustrated in Fig. 1. Each primer includes a 5' 26 base pair (bp) adaptor (A or B), and 4 bp library key and a 10 bp multiplex identifier (MID), used to identify the sample, in addition to the HLA primer sequence. The adaptor sequence serves to bind the genomic-target portion of the amplicon to the complimentary A or B capture beads for recovery of forward and reverse sequence reads, respectively (Fig. 2). Following PCR amplification, nonspecific and primer-dimer artifact products are removed from the amplicons, which are quantified, diluted, and pooled for the secondary “clonal” amplification, or emulsion PCR (emPCR) step. The emPCR step includes sepharose beads that carry oligonucleotides complementary to either the A or B adaptor sequence. The primary amplicons are mixed with the beads in a ratio that will yield, on average, one DNA molecule bound per bead. These bead-bound sequences are emulsified with amplification reagents in an oil–water mixture. Each “microreactor” contains a single bead with a bound DNA fragment. After emPCR, each bead contains millions of identical DNA molecules. These beads, along with DNA polymerase and enzyme beads for the pyrosequencing reaction, are loaded onto a PicoTiterPlate™ (PTP). Each well contains a single bead with a clonally amplified sequence attached.

The Titanium PTP enables runs with an average of 600,000 high quality reads for amplicon sequencing. The MID tags in the GS GType HLA primer plates allow up to 44 samples to be sequenced at all 8 HLA loci (including DRB3/4/5) in one PTP. The GS FLX generates raw sequencing image files that are processed into sequence text files (.fna files in FASTA format). Next, sequence text files are transferred from the server to a PC for genotyping using Conexio Assign ATF 454 software (developed by Dr. Damian Goodridge, Conexio Genomics, Perth), which compares the sequence files to the most current, curated HLA sequence database (IMGT/HLA Database: <http://www.ebi.ac.uk/ipd/imgt/hla/>) and assigns HLA genotypes. In general, consensus sequences from 50 or more sequence reads per amplicon are considered “highconfidence” as are the genotype assignments based on these consensus sequences.

1.2 Experience with the System

In the Children’s Hospital & Research Center Oakland (CHRCO) laboratory, our hands on time to perform the Titanium chemistry is ~24 h for semi-automated versus ~35 h for manual (Table 1). Assay setup and run time for sequencing all HLA loci in 44 samples is 40 h (24 h hands-on) plus 1 h analysis time. Using robotics the assay and analysis time takes about 1/4 the time of Sanger sequencing. The average amplicon read depth of the GS FLX system is between ~250 and 650 reads in our hands. In an international alpha-trial testing of 20 blinded samples, the 454 Life Sciences GS FLX System sequencing method demonstrated 98 % concordance with known genotypes between eight participating labs [2]. In our other studies, using at least one control per PTP region per run, 100 % of control sample genotypes (previously characterized by Sanger Sequencing with ambiguity reduction) were concordant [4, 5]. The average rate of detection of novel alleles was ~0.04 % (8/19,069 loci) in Caucasoid study populations [4, 5].

Using this system, class I and II genotype calls are significantly reduced to a single allelic genotype call or to shorter allelic ambiguity strings than expected using traditional Sanger sequencing. Table 2 illustrates the fraction of unambiguous genotype calls and of genotype calls that consist of ambiguity strings of varying lengths observed using 454/Roche Applied Science primers for sequencing class I (exons 2–4) and class II DQB1 (exon 2 and 3) and DRB1, DQA1, DPB1 (exon 2) in a study of 1,000 samples [4, 5]. Note that non-Caucasoid populations would be expected to have fewer ambiguous genotypes, due to unique HLA sequences in these regions. Note also that when expression variants (e.g., null alleles) are taken into consideration (Table 2a versus b) there is little difference for most of the loci except for HLA-A and DQA1 (shaded); this illustrates the need to include additional exons, and in some cases noncoding sequence to further resolve the ambiguities. The inclusion of supplementary coding and noncoding regions in future versions of the typing system will significantly enhance the ability to resolve ambiguous sequences. In conclusion, HLA genotyping utilizing next-generation sequencing (NGS) methods provides a significant improvement in throughput with reduction of ambiguity afforded by clonal analysis of amplicons. The 454 GS FLX System, Titanium chemistry and HLA primers, and the Conexio Assign ATF 454 genotyping software are excellent for high resolution, high throughput analysis of samples for the HLA class I and II loci.

2 Materials

Prepare all solutions using analytical grade reagents and molecular biology grade water.

Follow all waste disposal regulations when disposing of waste materials. Prior to opening any tubes or microwell plates, solutions should be mixed by gentle vortexing and then spun down in a centrifuge to avoid loss of contents and cross-contamination. All disposable supplies including tubes, pipette tips (*see* Note 1) and microwell plates are DNase free and sterile. Facilities should allow for separation of prePCR functions from postPCR functions as well as pre-emulsion PCR (emPCR) functions from post-emulsion PCR functions. Throughout the entire protocol all TE (Tris–EDTA) buffer should be low EDTA as described under Genomic PCR Components. All quantities indicated are sufficient to genotype 40 samples and 4 negative controls (*see* Note 2) for 8 loci at high resolution. These samples are run on one GS FLX run using a four region PicoTiterPlate (PTP).

2.1 Sample Preparation Components

DNA may be purified from any of a variety of sources using any of the commercially available kits. At the end of the procedure the DNA sample should be in water or TE as described under Genomic PCR Components.

2.2 Genomic PCR Components

1. PCR Workstation (Dead air box): set up in a pre-PCR room (*see* Note 3).

¹To avoid contamination with small amounts of DNA that can result from handling, pipette tips should be purchased already in racks.

²For some applications, it may be sufficient to have one negative control per run on the GS FLX. In such cases, one negative control and 43 samples can be run. It is also advisable to have one or more samples be a positive control (for example a well characterized cell line with known genotypes) for each run.

2. Vortex: in PCR Workstation.
3. Minifuge: in PCR Workstation.
4. Pre-PCR multichannel pipettes (one of each): 8-channel multipipette, 20–200 μ l, 8-channel multipipette, 1–10 or 2–20 μ l, 12-channel multipipette, 2–20 μ l.
5. Pre-PCR single channel pipettes: various.
6. Pipetting reservoirs.
7. 96-well plate cooler (Eppendorf, Hamburg, Germany) or ice: if plate coolers are used, a quantity of at least four is advisable.
8. PCR primers dried on plates: to obtain high resolution typing, purchase both GS GType HLA MR and HR plates (1 box of each, Roche Applied Science, Indianapolis, IN, USA).
9. DNA sample in water or TE (*see* Note 4).
10. 10 $\times$ PCR Buffer II w/o $MgCl_2$ (Applied Biosystems), 6 ml.
11. 25 mM $MgCl_2$ (Applied Biosystems), 3 ml.
12. 80 % (w/v) Glycerol (*see* Note 5), autoclave, cool, and store at room temperature.
13. 100 mM dNTP blend, 25 mM each of dATP, dCTP, dGTP, dTTP (Applied Biosystems) GeneAmp dNTP mix in 350 μ l.
14. Plate thermoseals: BioRad microseal “B’ film” recommended.
15. Film sealing roller (or similar tool).
16. AmpliTaq Gold, 5 U/ μ l (Applied Biosystems), 1.3 ml.
17. Plate centrifuge in pre-PCR environment.
18. Thermal cyclers: the preferred model is the ABI GeneAmpTM PCR System 9700 with 96-well gold-plated silver block (Foster City, CA) (*see* Note 6). Four thermal cyclers run simultaneously provide adequate throughput, although eight provide maximum throughput. The thermal cycler should be in a post-PCR environment.

2.3 Amplicon Cleanup Components

1. Plate centrifuge in post-PCR environment.

³The work surface of all dead air boxes (both pre- and post-PCR and pre- and post-emPCR) and hoods should be wiped down with a mild detergent solution, followed by 10 % bleach, followed by 70 % ethanol and then 15' of UV light both before and after each use. This procedure should be followed for the vortex, minifuge, and pipettes that are used in each dead air box. Gloves and lab coats should be changed when moving between any of the following areas: pre-PCR, post-PCR, pre-emPCR, post-emPCR areas.

⁴We highly recommend that the TE solution used throughout the entire sequencing procedure have a low concentration of EDTA (10 mM Tris, 0.1 mM EDTA pH 8.0 $\pm$ 0.1). This assures the presence of adequate Mg^{2+} in PCR steps.

⁵The manufacturing source of glycerol is important. Aged or impure glycerol can cause general failure in PCR. Glycerol stock should be 100 %, Molecular Biology grade from Sigma Aldrich. To make 80 % (w/v) Glycerol: Place a 100 ml graduated cylinder with a stir bar in the bottom on a balance. Tare the balance. Slowly pour add 80 g of glycerol into the cylinder. Add water to 100 ml. Stir until homogeneous. Remove the stir bar. Add water to 100 ml. Cover with Parafilm[®] (SPI Supplies, West Chester, PA, USA) and mix. Transfer to a bottle with 150 ml volume. Only 6 ml is required but it is most convenient to make a larger volume for autoclaving.

⁶Use of other thermal cyclers *may* be possible. However, it is well known that performance of different brands/models of thermal cyclers vary even if the thermal profile is programmed to be the same.

2. Agencourt SPRIPlate 96R magnet plate (Beckman Coulter, Indianapolis, IN).
3. Pipetting reservoirs.
4. Post-PCR multichannel pipettes (one of each): 8-channel multipipette, 20–200 μ l, 8-channel multipipette, 1–10 or 2–20 μ l, 12-channel multipipette, if preferred over 8-channel.
5. Agencourt AMPure XP Kit (Beckman Coulter), 60 ml.
6. 70 % Ethanol, 600 ml (*see* Note 7).
7. TE (Tris–EDTA buffer) (*see* Note 4).
8. Heat block.

2.4 Amplicon Quantification Components

1. Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, CA), 1 kit.
2. Pipetting reservoirs.
3. Post-PCR multichannel pipettes (one of each): 8-channel multipipette, 20–200 μ l, 8-channel multipipettor, 1–10 or 2–20 μ l, 12-channel multipipette, if preferred over 8-channel.
4. Post-PCR single channel pipettes: various.
5. 96-well Flat Bottom Black Polystyrene 96-well plates, 8 (*see* Note 8).
6. 50 ml BD Falcon tube.
7. Microplate fluorimeter, 96 wells.

2.5 Amplicon Quality Check Components

1. 96-well E-gels, or 2 % Agarose (Invitrogen).
2. Post-PCR 12-channel multipipette (non-electronic).
3. 10× Blue Juice Gel Loading Dye (Invitrogen), 150 μ l.
4. E-gel 96-well mother base + daughter base (Invitrogen).
5. Digital imager (e.g., AlphaImager HP, Protein Simple, Santa Clara, CA) or transilluminator and digital camera.

2.6 Amplicon Dilution and Pooling

1. Pipetting reservoirs.
2. 2 ml deep, square well plates.
3. TE (*see* Note 4).

⁷Dilute 420 ml of 100 % non-denatured alcohol to 600 ml with water.

⁸The specific type of plate required will depend on the fluorimeter used, see specific fluorimeter manual for details. For Molecular Devices Spectra MAX: Costar or VWR plates are suitable.

4. Post-PCR single channel pipette: various.
5. 1–20 μ l 8 or 12 channel multipipette. (Optional) Rainin Liquidator 96 or Liquid handler (e.g., BioMek).
6. 1.5 ml microfuge tubes.
7. 1.5 ml tubes with O-ring cap.

2.7 Emulsion PCR Setup and Thermal Cycling

1. PCR Workstations (dead air boxes) designated for emPCR set up, in a post-genomic PCR, pre-emPCR room/space: one for emulsion preparation, one for emulsion dispensing (*see* Note 9).
2. Vortex: 2, one in each PCR workstation.
3. Minifuge: 2, one in each PCR workstation.
4. TissueLyser or TissueLyser II (QIAGEN, Valencia, CA).
5. MV Tube rack adapters matched to TissueLyser or TissueLyser II (Roche Applied Science).
6. LabQuake Roller (Thermolyne).
7. Pre-emPCR multichannel pipettes (one of each): 8-channel multipipettor, 20–200 μ l, 8-channel multipipettor, 1–10 or 1–20 μ l, 12-channel multipipettor.
8. Pre-emPCR single channel pipettes: various.
9. Repeat pipette (Eppendorf): to accommodate 1 ml Combitips®.
10. Heat block, set at 65 °C (LabNet Accublock Digital DryBath).
11. emPCR Lib A MV Kit (Roche Applied Science).
12. 1.0 ml Combitips® plus tips (Eppendorf).
13. Thermal cyclers, 4 (*see* Note 10).

2.8 Emulsion Breaking

1. Vented hood or ductless fume hood: designated for emulsion breaking, in a post-PCR room (*see* Note 11).
2. Disposable lab coats (*see* Note 12).

⁹Since emulsion dispensing may produce aerosols, emulsions may be dispensed in a dead air box separate from the one in which the emulsions are made. These dead air boxes can be in the same post-genomic PCR room. This room is pre-emPCR. At CHRCO, one post-genomic PCR room dead air box is used for making the emulsions, and the emulsions are carefully dispensed on a bench.

¹⁰We have used both the Bio-Rad Tetrad DNA Engine with four Alpha unit reaction modules (i.e., one Tetrad has four blocks and is equivalent to four individual thermal cyclers) and the ABI GeneAmp™ PCR System 9700 with 96-well gold-plated silver block, set on the ABI 9600 simulation mode. Use of other thermal cyclers is not recommended as the performance of different brands/models of thermal cyclers does vary, even if the thermal profile is programmed to be the same.

¹¹If possible, emulsion breaking should take place in a post-PCR room separate from the post PCR room where emulsion preparation takes place.

¹²Since emulsion breaking can produce aerosols, disposable lab coats should be used.

3. GS FLX Titanium emPCR Breaking Kit, LV/MV, 12 pcs (Roche Applied Science).
4. Vacuum source with liquid trap.
5. Conical tubes with caps: 50 ml, quantity 4.
6. Post-PCR multichannel pipettes: 8-channel multipipette, 20–200 μ l (Rainin EDP3-Plus; Eppendorf “Repeater Plus” pipettes).
7. Post-PCR pipette: 100–1,000 μ l.
8. 100 % Isopropanol, 200 ml.
9. 100 % Ethanol, non-denatured, 100 ml.
10. 1.7 ml tubes, eight siliconized.
11. Centrifuge and rotor with two swinging buckets and 2–50 ml tube adaptors (*see* Note 13).
12. Minifuge.
13. Vortex.
14. Serological pipettes: 5, 10, 25 ml (Rainin Glassmaster and Greiner Bio-One Cellstar serological pipettes).

2.9 Bead Washing, Enrichment, Counting, and Sequencing Primer Annealing

1. Vortex (for preparation of enrichment beads).
2. Minifuge (for preparation of enrichment beads).
3. 10 N Sodium hydroxide.
4. Serological pipettes: 5, 10, 25 ml (Rainin Glassmaster and Greiner Bio-One Cellstar serological pipettes).
5. Post PCR pipettes: 1–20 μ l, 20–100 μ l, 100–100 μ l.
6. Magnetic Particle Concentrator (Invitrogen) or REMe unit (Roche Applied Science) and liquid handler (*see* Note 14).
7. LabQuake Roller (Thermolyne).
8. Heat block, set at 65 °C for manual; or the REMe unit (Roche Applied Science).
9. Ice if performing without REMe.
10. Bead Counter: CC Size Standard L10, 15 ml (Beckman Coulter, Carlsbad, CA).
11. Isoton II: 20 ml (Beckman Coulter).

¹³We use the Beckman Coulter X-12 or X-15, the Allegra X-15R centrifuge, or the Eppendorf Centrifuge 5430 for this step.

¹⁴Performing bead washing and enrichment manually, employing the Magnetic Particle Concentrator, is laborious. We recommend the use of the robotic enrichment module, i.e., REMe (Roche Applied Science), which requires a liquid handler for use. The REMe is compatible with numerous liquid handlers including, but not limited to, Hamilton Star or Starlet, Biomek Fx, Tecan Genesis, HP Multiprobe. Some liquid handlers may require modification of the deck to accommodate the REMe’s height while still allowing use of 1 ml tips to pipette the large volumes involved. Our first hand experiences have been with the HP Multiprobe, the Biomek Fx, and the Tecan Genesis robots.

12. Accuvettes: 4 (Beckman Coulter) (1 per sequencing pool per region).

2.10 Sequencing

1. 454 Life Sciences Genome Sequencer GS FLX Instrument (*see* Note 15).
2. GS FLX Titanium Sequencing Kit XLR70.
3. GS FLX Titanium PicoTiterPlate Kit 70 × 75, including the four region gasket.
4. Bead Deposition Device (70 × 75).
5. Bead Deposition Device Counterweight.
6. Microplate Centrifuge: Beckman Coulter Allegra X-15 and rotor, with microplate carriers and covers.
7. Microcentrifuge.
8. Lens paper.

2.11 HLA Genotyping

1. Conexio Genomics Assign ATF 454 software and PC (Windows XP or Windows 7).

3 Methods

3.1 Sample Preparation

Prepare high quality DNA (40–150 ng) in water or TE. We recommend 10 ng of DNA per genomic PCR with a minimum of 2 ng if DNA is limiting.

3.2 Genomic PCR

Note that high resolution typing is achieved only by typing a given sample using both the medium resolution (MR) and the high resolution (HR) GS GType HLA primer plates. One “set” of plates is defined as 1 MR plate plus 1 HR plate (*see* Table 3). Genomic target specific primers are arranged in rows, while Multiplex Identifiers (MIDs) that allow identification (or “tagging”) of samples are arranged in columns. Column 12 is intentionally left blank on each plate to make liquid handling easier when performing quantitation of amplicons. Rows G and H are blank on the HR plates.

1. Wipe the pre-PCR dead air box work surface, pipettes, minifuge and vortex with a laboratory wipe moistened with mild detergent solution, then 10 % bleach, then 10 % ethanol.
2. Turn on the UV light for 10' in the dead air box, with all equipment, including pipettes within. After this decontamination is complete, turn off the light.
3. Label one set of GS GType HLA primer plates as MR-1 and HR-1. Label a second set as MR-2 and HR-2, and two additional sets as MR-3 and HR-3, and MR-4 and

¹⁵The GS FLX Plus or GS Junior may also be used. In the latter case, the appropriate reagents and equipment (e.g., Bead Deposition Device) must be used and 5–6 samples may be genotyped per run.

HR-4. Arrange the plates so that MR-1 and HR-1 plates are adjacent to one another with columns aligned. Likewise, MR-2 and HR-2 should have their columns aligned. Remove the seals carefully (*see* Note 16).

4. Using a 12 channel micropipette and working by rows, add 10 μ l of water to well 1–11, rows A–H on the MR plates and rows A–F on the HR plates. Use different pipette tips each time so as not to cross-contaminate the wells. Let the plates sit at room temperature for 10' (*see* Note 17). Place the plates on ice.
5. While the plates in **step 4** are incubating at room temperature, prepare enough PCR master mix for eight plates: To a 15 ml tube on ice, add in order: 3,618 μ l of water, 2,250 μ l of 80 % glycerol, 1,800 μ l of 10 $\times$ PCR buffer without Mg²⁺, 108 μ l of 100 mM dNTP blend (25 mM each dA, dC, dG, dT), 288 μ l of AmpliTaq Gold DNA polymerase (5 U/ μ l), 1,296 μ l of 25 mM MgCl₂ (*see* Note 18).
6. To Column 11 of each plate add 2 μ l of TE for a negative control (*see* Notes 19 – 20).
7. To each well of columns 1–10 of plates MR-1 and HR-1 (rows A–F only for the latter), add 2 μ l of DNA sample numbers 1–10, respectively. DNA should be at a concentration of 5 ng/ μ l (*see* Note 21). Use a fresh tip for each sample to avoid cross-contamination of samples.
8. Repeat **step 7** three times. Pipetting 2 μ l of DNA samples numbers 11–20, 21–30, and 31–40 into MR-2 and HR-2, MR-3 and HR-3, and MR-4 and HR-4, respectively.
9. Using a 12-channel multipipette, pipet 23 μ l of PCR master mix into each well of the first 11 columns of the MR and HR plates (only rows A–F of the latter). It is important to use a fresh set of tips for each set of plates in order to avoid cross-contamination of primers and samples (*see* Note 22)

¹⁶This genomic PCR procedure is for processing eight plates; it is assumed there are eight thermal cycler blocks available. If there are fewer, adjust the procedure accordingly.

¹⁷This step helps to solubilize the primers, which are dried down in the microwell plates. It helps to reduce the number of amplicon dropouts and the amount of primer dimer formed.

¹⁸The formulation nominally makes enough for 720 reactions. The difference from the GS GType HLA protocol is as follows: (1) less water is used in the master mix due to the presolubilization of the primers in the previous step, and (2) we use a 100 mM dNTP blend (each nucleotide at 25 mM) rather than a 40 mM dNTP blend (each nucleotide at 10 mM). If the 40 mM dNTP solution is substituted, be sure to make the appropriate adjustment.

¹⁹While it is desirable to include at least one negative sample per plate when beginning to perform runs, thereafter it is sufficient to include one negative sample per run. Column 11 can then, instead, be used for a sample; there is no qualitative or quantitative difference in the primers in column 11 that make them especially suitable for a negative control. In fact, it is preferable in successive runs, to rotate the position that will be occupied by the negative control. This will make it more likely that any contamination (while extremely rare in our hands) can be detected.

²⁰We recommend inclusion of at least one positive control (e.g., a cell line of known HLA genotype). This can be placed in any column of a plate set (MR + HR). We recommend rotating its position from run to run.

²¹If the amount of DNA available is limiting, much less can be used for each well. We have observed robust amplification using 2 ng. If necessary, you may increase the number of PCR cycles to 35. With less DNA, more primer dimer may initially be formed but can be removed by the PicoGreen procedure described in this protocol. Be sure the DNA is added to the master mix in a 2 μ l volume.

²²If an 8-channel micropipette is used to pipette by columns (instead of rows), it is imperative that tips be changed after each transfer to avoid cross-contamination of samples.

10. Cover each plate with a fresh seal and seal very tightly (*see* Note 23). Vortex gently.
11. Centrifuge the plates for 30 s at $900 \times g$.
12. Place each plate in a thermal cycler block and run the following PCR thermal cycler program: 95 °C—10'; 31 cycles of 95 °C—15", 60 °C—45", 72 °C—15"; 72 °C—5', 4 °C—hold (*see* Note 24).
13. If you are not proceeding with the next step immediately, store the plates on ice (for processing the same day) or at -20 °C.

3.3 Amplicon Purification by AMPure

Processing plates two at a time is recommended, multiples are possible if you have multiple Agencourt SPRIPlate 96R magnet plates. Be sure to change pipette tips after transfers (*see* Note 25).

1. Thaw the plate with the PCR products (if necessary), vortex gently and centrifuge for 30 s at $900 \times g$.
2. Using an 8-channel multipipette and working by row, transfer 20 μ l PCR product (*see* Note 26) from the PCR plate to each well of a new 96-well PCR plate keeping the layout the same for each of the eight plates. Change tips after each transfer.
3. Add 20 μ l of water and pipet up and down to mix. Change tips after each transfer/mix.
4. Mix the Agencourt AMPure XP beads thoroughly by shaking the bottle to resuspend.
5. Transfer 6.3 ml (enough for one plate) or 12.4 ml (enough for two plates) of the Agencourt AMPure XP beads to a reagent reservoir.
6. Using an 8-channel multipipette and working by row, add 64 μ l of SPRI beads to each well and mix thoroughly by pipetting up and down at least ten times until the SPRI bead/PCR mixture is homogeneous. Change tips after each transfer/mix.
7. Incubate the plate for 10 min at room temperature.
8. Place the plate on a 96-well ring magnet plate and incubate for 5 min at room temperature until supernatant is clear.

²³It is of utmost importance to seal plates tightly. If this is not achieved, evaporation will occur in the wells during thermal cycling and amplicons may drop out. Pay strict attention to the recommendations made regarding brand of seals to use.

²⁴Note that, compared to the thermal cycling protocol in the GS GType HLA primer manual we recommend use of a modified thermal profile. We have decreased the number of cycles to reduce the amount of DRB crossover products generated, and added a 72 °C extension step during cycling. The latter changes are useful for more robust generation of long amplicons. The DRB crossover products are a minor in vitro PCR artifact that is revealed by NGS; the ability to detect this readily identifiable population of sequences increases with clonal sequencing because we can detect very minor sequence populations [5].

²⁵It is of utmost importance, during handling amplicons, to avoid cross-contamination between plates (e.g., contamination of MR-1 with MR-2) because the same MIDs are used for sample identification on each MR plate and each HR plate. ALWAYS change tips between plates.

²⁶The GS GType protocol specifies use of 22.5 μ l. We recommend a slightly smaller volume since less AMPure XP reagent can be used, giving a cost savings.

9. With the plate still on the ring magnet plate, use an 8 or 12-channel micropipette to carefully remove and discard the supernatant without disturbing the beads.
10. Using an 8 or 12-channel multipipette, add 200 μ l of freshly prepared 70 % ethanol.
11. Incubate 30 s at room temperature while still on the magnetic ring plate.
12. While the plate is still on the magnetic ring plate, carefully remove and discard the clear supernatant without disturbing the beads.
13. Repeat **steps 7 – 9**. Remove as much of the supernatant as possible.
14. Place the 96-well plate and magnetic ring plate together on a heat block set at 40 °C until all pellets are completely dry (10–20 min). Do not leave the plate on the heat block longer than necessary.
15. Keep the plate on the magnetic ring stand while removing it from the heat block.
16. Using the 8 or 12 channel multipipette, add 25 μ l of TE to each well (*see* Note 27). Remove the 96-well plate from the magnetic plate and tap the 96-well plate containing the samples until all pellets are dispersed (about ten times).
17. Place the 96-well plate briefly on the magnetic ring plate, remove and tap again until all pellets are dispersed. This ensures efficient elution of PCR products from the beads.
18. Place the 96-well plate on the magnetic ring plate and incubate for 2 min.
19. Using the multichannel pipette, pipet 20 μ l of the supernatant from each well into a fresh 96-well plate (*see* Note 28).
20. Cover the plate with a plate seal and store at –20 °C until ready to proceed to E-gel quality check and quantification by Fluorometry using Quant-iTTMPicoGreen[®] dsDNA Assay Kit.

3.4 E-Gel Check for Amplicon Generation and Removal of Primer Dimer

Dilution of loading dye

1. Place approximately 50 ml of TE in a 50 ml Falcon tube. Add 125 μ l of “10 $\times$ ” Blue Juice.

E-gel loading and running

1. Remove the 96-well E-gel from the pouch, and snap it into place on the E-gel mother base (*see* Note 29). If you have daughter bases or additional mother bases you may run four gels simultaneously.

²⁷We prefer elution with this larger volume, rather than the 10 μ l specified in the GS GType HLA primer protocol, since it assures that the pellets on the magnetic ring stand are fully covered and, thus, complete elution is favored. In addition, a greater volume of eluent makes handling in successive steps easier.

²⁸A small amount of transfer of pellet in some of the wells is difficult to avoid and is acceptable. In subsequent steps, including gels, quantification and dilution, take care to avoid, as best as possible, any beads that may have carried through.

²⁹Alternatively, you may perform gel electrophoresis of the amplicons on a standard agarose gel set-up. However, since E-gels are very thin we find them to be much more sensitive. Additionally, the buffer-less system is neat and convenient.

2. Using a non-electronic 12 channel multipipette, place 9 μ l of each AMPure purified sample into the corresponding well on the E-gel.
3. Add 11 μ l diluted loading dye to each well.
4. Press the start button and set time for 7–8 min.
5. Press the start button until the run indicator light turns green.

Digital imaging of the agarose gels

1. Image the E-gels with a digital imager or digital camera equipped with a transilluminator and a filter appropriate for ethidium bromide stained gels. Save the image for analysis (*see* Note 30).

3.5 Choosing the Amplicons for the Final Emulsion PCR Pool

For each sample a total of 14 amplicons are generated, each of which, ideally, should be mixed in equimolar amounts for the emPCR reaction. Not all amplicons are generated with equal efficiency and very occasionally there is very little amplicon made but a large amount of primer dimer. To achieve optimal sequencing results it is important to only use well-quantified and relatively pure amplicons for the final mix for each sample.

1. Determine which amplicons to use based on the following criteria:
 - a. If the amplicon detected on the E-gel is much smaller than expected, it is primer-dimer. DO NOT use it for the emPCR pool.
 - b. If the molar ratio of primer-dimer to amplicon is estimated to be 3:1 or more (bands of roughly equal intensity on an E-gel). DO NOT use it in the emPCR pool. Repeat the AMPure procedure on these samples as in Subheading 3.3, **steps 2 – 18** except: use 10 μ l of sample (already AMPure purified once) diluted with 10 μ l of water, with the addition of 32 μ l of SPRI beads. Reanalyze the product by E-gel as in Subheading 3.4 and determine if it should be included in the amplicon pool as described in Subheading 3.5.
 - c. If a full-length amplicon is completely undetectable on the E-gel and no product is detected by quantification with PicoGreen when performed as in Subheading 3.6, assign this amplicon a concentration value of 0.1 ng/ μ l and include this reaction in the amplicon pool (*see* Note 31). This is the treatment that should be applied to negative controls as long as primer dimer has been successfully removed.

3.6 Quantification by PicoGreen Fluorescence

Preparation of DNA serial dilution standard—This procedure makes enough standards for quantification of four plates of amplicons (*see* Note 32).

³⁰This purpose of these gels is only to determine if amplicon has been made and if it is free of primer dimer (not to estimate the size of amplicons). The amplicons run approximately to the top of the well in the adjacent lane. Primer-dimer runs substantially below this.

³²Although it is advisable to make batches of standards in these quantities, do not process more than two amplicon plates at a time because the PicoGreen reagent is light sensitive.

1. Thaw an aliquot of the DNA standard provided with the PicoGreen reagent (100 ng/μl)
2. Transfer TE to ten 1.5 ml microcentrifuge tubes as follows:
 Tube 1: 1,188 μL TE
 Tubes 2–10: 600 μL TE
3. Prepare a 1:100 dilution of the standard by transferring 12 μl of DNA standard to Tube 1 and vortexing for 10 s.
4. Transfer 600 μL from Tube 1 to Tube 2 and vortex for 10 s. Complete a dilution series by transferring 600 μL from tube 2 to tube 3, and vortexing for 10 s, then transferring 600 μl from tube 3 to tube 4 and vortexing and so on until Tube 9 has been vortexed. Tube 10 is the no DNA control (*see* Note 33).
5. For dilutions from tubes 3–10, transfer 100 μL to the wells of column 12 in a 96-well fluorimeter plate. Since samples are diluted 1:50 in the final assay plate, the values of the standards on the axis of the linear regression plot will take this calculation into account as shown in Table 4

Preparation of DNA samples—Process no more than two amplicon plates at a time.

1. Using a multichannel pipette, transfer 98 μL of TE to the appropriate columns and rows of the 96-well black fluorimeter plate already containing standards; one well for each DNA sample to be measured, i.e., for an MR plate this will be columns 1–11, all rows; for an HR plate this will be columns 1–11, rows A–F.
2. Add 2.0 μL of DNA sample to wells containing the TE and mix by pipetting up and down.

Preparation and addition of diluted PicoGreen reagent—Only make enough PicoGreen reagent to perform the assay on two plates at a time. Protect the second plate from light while reading the first plate.

1. Remove PicoGreen reagent from 4 °C storage and let thaw at room temperature protected from light (*see* Note 34).
2. Transfer 24.875 ml TE to a 50 ml Falcon tube.
3. Add 125 μL PicoGreen stock solution to the TE and mix by vortexing. This produces a 1:200 dilution of the original PicoGreen stock. Keep solution protected from light until ready to use (*see* Note 35).
4. Darken the room and, using a multichannel pipette, transfer 100 μL of diluted PicoGreen solution to each well containing reference standard or sample dsDNA.

³³Compared to the GS GType HLA protocol, we recommend making two lower concentration standards and, since the concentration of amplicons is generally well below 12 ng/μl, tubes 1 and 2 are not used. You may save the remainder in them to make a smaller amount of the more dilute standards in case a repeat assay of some samples is necessary.

³⁴Thawing may take greater than 30 min if the vial is full.

³⁵In order to minimize deterioration of the light sensitive PicoGreen dye, be sure the fluorimeter has been warmed up prior to dilution of the PicoGreen stock.

Fluorimetry

1. Turn on the fluorimeter to warm up for 10 min and load the appropriate software application (*see* Note 35).
2. Place the 96-well plate on the instrument and start the protocol.
3. Read the second plate immediately after the first.
4. Construct two standard curves to cover the entire range of concentrations. The first curve should include concentrations from 0 to 0.78125 ng/μl. The second curve should include 1.5625–12.5 ng/μl and the blank.
5. Ensure that the standard curve has an R^2 value of each curve is 0.98 or greater and that each point for which there is a visible band on the E-gel gives a positive value. If not, redo dilution of standards and samples and repeat fluorimetry.
6. Ensure that sample readings fall within the range of the standard curve. If any sample readings exceed the highest standard curve value, dilute those samples, re-assay them and take the dilution factor into account when calculating the final concentration.
7. Use the appropriate standard curve (as determined by the Relative Fluorescence reading) to calculate the concentration of each amplicon.

3.7 Amplicon Dilutions and Pooling for emPCR

Initial dilution

1. Use the GS GType HLA Assay Amplicon Dilution Calculator available at <http://www.454.com/my454> to calculate “custom” dilutions of amplicons. This tool allows the input of PicoGreen data and will output the amount of TE and amplicon necessary for the normalization of each sample, i.e., the dilution of each sample to 1×10^9 molecules/μl (*see* Note 36).
2. To a 2 ml deep well plate, add the specified amount of TE to each well, maintaining the MR and HR plate layout.
3. Add the specified amount of amplicon to each well, being sure to use a clean tip for each amplicon.
4. Perform dilutions for all plates.

Pooling

1. In a 1.5 ml microfuge tube, with an O-ring cap, make emPCR Pool 1 (initial dilution) by mixing 5 μl of each diluted amplicon from plate MR-1 plus HR-1. Vortex well.

³⁶Careful normalizations assure more closely balanced read depths during sequencing. Care should be taken in pipetting. This step can be performed by a liquid handler with a spaneight head that allows for “cherry picking.” If such an instrument is available, a file that enumerates the quantities of TE and each amplicon necessary for appropriate dilution can be generated using Microsoft Excel. This file, in turn, can be read by numerous brands of liquid handlers.

2. As in **step 1** above, make emPCR Pool 2 (initial dilution) by mixing 5 μ l of each diluted amplicon from plate MR-2 plus HR-2. Likewise, construct emPCR Pools 3 and 4 (initial dilution) using MR-3 plus HR-3 and MR-4 plus HR-4, respectively

Final dilutions

1. Pipet 495 μ l of TE into a new 1.5 ml microfuge tube with an O-ring cap. Add 5 μ l of the emPCR Pool 1 (initial dilution) made in **step 1**. Dilute emPCR Pools 2, 3 and 4 (initial dilutions) in a like manner to make these intermediate dilutions (1×10^7 molecules/ μ l).
2. Dilute the intermediate dilution pools one final time by pipetting 495 μ l of TE into a new 1.5 ml microfuge tube with an O-ring cap. Add 5 μ l of the emPCR Pool 1 (intermediate dilution) to make the final dilution. Dilute emPCR intermediate Pools 2, 3, and 4 in a like manner to make the final dilutions. Store these final dilutions (1×10^6 molecules/ μ l) at -20°C in a 1.5 ml tube with an O-ring cap (*see* Note 37).

3.8 Emulsion PCR, Emulsion Breaking, Sequencing and Genotyping Guidelines

1. For *each* of the four final pools, make 8 A and 8 B emulsions, each with 0.4 cpb (copies per bead) i.e., 3 μ l of the above dilution per emulsion (6.8×10^6 beads) following the directions in the GS FLX emPCR Method Manual—LibA MV from 454 (*see* Note 38).
2. After enrichment and processing for sequencing, following the GS FLX Sequencing Method Manual, load 590,000 beads per region (*see* Note 39) on a 70×75 PTP fitted with a four-region gasket. Load Pool 1 in region 1, Pool 2 in region 2, and similarly for Pools 3 and 4.
3. Place the PTP in the GS-FLX instrument and start the amplicon sequencing process.
4. Following sequencing genotyping can be performed by use of various software. We particularly recommend use of Conexio Genomics Assign ATF 454 which is available directly from Conexio Genomics (Perth Australia). 5. Information critical to high quality genotyping is found in the FAQ document that accompanies the GS GType HLA primer sets and is available at <http://www.454.com/my454>.

Acknowledgments

The authors would like to thank Henry Erlich, Martha Ladner, David Noonan, and Melinda Rastrou for their thoughtful reading of the manuscript. We would also like to acknowledge Damian Goodridge for the development of the genotyping software, and Kazu Osoegawa, Martha Ladner, Franziska Cohen, Sherry Hawbecker, David Noonan for setting this system up and sequencing all the samples in the CHORI lab. We would also like to

³⁷It is good to store initial, intermediate, and final dilutions for future use as these pools are of sufficient quantity for many emulsions.

³⁸The GS FLX emPCR Method Manual—LibA MV from 454 recommends the general use a much higher number of copies per bead [4–6]. In our experience, use of such high cpb with this HLA system gives an undesirably high percentage of dot and mixed reads during sequencing.

³⁹We load 75 % of the number of beads recommended in the GS FLX Sequencing Method Manual (v. Nov 2010). This lower bead density yields favorable numbers of sequence reads which is reflected in the percent passed filter. Our Percent Passed Filter ranges from approximately 28–55 %. Within this range (or higher), acceptable genotyping is obtainable using Conexio Assign ATF 454 v34 software.

acknowledge Priscilla Moonsamy and Bryan Hoglund for their contributions to initial development of primers and protocols. E. Trachtenberg is supported in part by NIH grants 2U01AI067068 and 2P01CA111412.

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Fig. 1.

HLA primer positions. Primer positions capturing coding and noncoding regions for genotyping HLA class I and II loci using the 454 Life Sciences GS GType HLA MR and HR primer sets are illustrated. *Notes* : Primers for DRB1 Exon 2 are generic and also amplify DRB3, DRB4, and DRB5 loci, and other related pseudogenes. *Boxes* in schematic indicate exons; *lines* indicate introns; *arrows* indicate primer positions. Not drawn to scale

Fig. 2.

The 454 Life Sciences GS FLX System Sequencing Procedure. Initial amplifications of HLA loci use primers with 454 Life Sciences Titanium 5' A or B adapters to allow for the recovery of forward and reverse sequence reads, and a multiplex identifier (MID) sample tags to allow for up to 44 samples to be sequenced per run. Following the primary PCR amplification, the amplicons are purified, quantified, diluted, and pooled (yielding the "amplicon library") for the emulsion PCR (emPCR) step. In this step, the amplicon library is mixed with the appropriate A or B complementary beads in a ratio designed to generate beads which, on average, have a single DNA fragment bound. These bead-bound sequences are emulsified with amplification reagents in an oil–water mixture to yield aqueous "microreactors," within which, each DNA is amplified to give millions of identical (i.e., "clonal") DNA molecules per bead. Beads that lack amplified DNA are washed away. The DNA containing beads, DNA polymerase and pyrosequencing enzymes are then loaded onto the PicoTiterPlate (PTP) for sequencing on the GS FLX, which generates runs with, on

average, 600,000 high-quality reads (reproduced with permission from 454 Life Sciences, a Roche Company: <http://www.454.com>)

Table 1

HLA sequencing hands on time using titanium chemistry: Manual versus semi-automated systems at two different sites

Process	Manual	CHRCO: Liquid handlers and REMe (semi-automated)	RMS: Liquid handlers and REMe (semi-automated)
PCR setup	2	2	0.5
AMPure Amplicon cleanup	4	3 (Qiagen Liquidator)	1
DNA quantitation	3–4	3 (Qiagen Liquidator)	1.5
Quality check	1	1	1
Dilution and pooling	8	4.5	3
emPCR setup	2.5–3	2.5	2.5
Emulsion break	3	3	2
Bead enrichment	8	0.25	0.25
Pyrosequencing setup	3–3.5	3	3
Total	34.5–36.5 h	22.25–24 h	14.75 h

Note: Comparison of hands-on time using manual versus semi-automated methods reveals an approximately 10 h difference per sequencing run with each increase in robotic sophistication in our laboratories. *CHRCO* Children's Hospital & Research Center Oakland, *RMS* Roche Molecular Systems

Table 2
Percent of two field (four digit) allelic HLA calls and percent of calls with >1 potential genotype

Genotype	% Class I			% Class II				
Calls	HLA-A	HLA-B	HLA-C	DRB1	DQB1	DQA1	DPB1	
(a)								
1	12	9	12	50	51	19	10	
2	44	3	26	39	24	0	36	
3	4	19	6	2	20	27	29	
4	40	5	24	8	5	0	11	
5+	0	64	32	1	0	54	14	
(b)								
1	93	8	12	50	51	19	10	
2	7	8	27	39	24	37	36	
3	0	17	5	5	20	22	29	
4	0	3	25	5	5	8	11	
5+	0	64	31	1	0	14	14	

For a given locus, the percentage of genotype calls that are unambiguous at the two field level (previously known as “four digit level”) (labeled 1) versus those that are composed of an ambiguity string of two through five or more genotype calls (labeled 2 through 5+) using the 454/Roche Applied Science HLA primers and GS FLX sequencing system in a study on 1,000 Caucasians [4, 5]. (a) Call percentages when expression variants are considered (as would be required for hematopoietic stem cell transplantation). (b) Call percentages when expression variants are not considered (as is often the case in a research study). The inclusion of supplementary coding and noncoding regions in future versions of the typing method would significantly enhance the ability to resolve ambiguous sequences

Table 3

GS GType HLA primer plates

MR	HR
HLA-A Exon 2	HLA-A Exon 4
HLA-A Exon 3	HLA-B Exon 4
HLA-B Exon 2	HLA-C Exon 4
HLA-B Exon 3	DPB1 Exon 2
HLA-C Exon 2	DQA1 Exon 2
HLA-C Exon 3	DQB1 Exon 3
DQB1 Exon 2	
DRB Exon 2	

Table 4

DNA quantification standards

Tube #	Well #	Concentration
1	N/A	50
2	N/A	25
3	A12	12.5
4	B12	6.25
5	C12	3.125
6	D12	1.5625
7	E12	0.78125
8	F12	0.396
9	G12	0.1953
10	H13	0