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High-resolution, high-throughput HLA genotyping by next-generation sequencing

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Abstract

The human leukocyte antigen (HLA) class I and class II loci are the most polymorphic genes in the human genome. Hematopoietic stem cell transplantation requires allele-level HLA typing at multiple loci to select the best matched unrelated donors for recipient patients. In current methods for HLA typing, both alleles of a heterozygote are amplified and typed or sequenced simultaneously, often making it difficult to unambiguously determine the sequence of the two alleles. Next-generation sequencing methods clonally propagate in parallel millions of single DNA molecules, which are then also sequenced in parallel. Recently, the read lengths obtainable by one such next-generation sequencing method (454 Life Sciences, Inc.) have increased to >250 nucleotides. These clonal read lengths make possible setting the phase of the linked polymorphisms within an exon and thus the unambiguous determination of the sequence of each HLA allele. Here we demonstrate this capacity as well as show that the throughput of the system is sufficiently high to enable a complete, 7-locus HLA class I and II typing for 24 or 48 individual DNAs in a single GS FLX sequencing run. Highly multiplexed amplicon sequencing is facilitated by the use of sample-specific internal sequence tags (multiplex identification tags or MIDs) in the primers that allow pooling of samples yet maintain the ability to assign sequences to specific individuals. We have incorporated an HLA typing software application developed by Conexio Genomics (Freemantle, Australia) that assigns HLA genotypes for these 7 loci (HLA-A, -B, -C, DRB1, DQA1, DQB1, DPB1), as well as for DRB3, DRB4, and DRB5 from 454 sequence data. The potential of this HLA sequencing system to analyze chimeric mixtures is demonstrated here by the detection of a rare HLA-B allele in a mixture of two homozygous cell lines (1/100), as well as by the detection of the rare nontransmitted maternal allele present in the blood of a severe combined immunodeficiency disease syndrome (SCIDS) patient.

Keywords

454; human leukocyte antigen; sequencing

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Introduction

The human leukocyte antigen (HLA) class I and class II loci are the most polymorphic genes in the human genome, with a complex pattern of patchwork polymorphism localized primarily to exon 2 for the class II genes and exons 2 and 3 for the class I genes. For current HLA typing methods, allele-level resolution of HLA alleles, which is clinically important for hematopoetic stem cell (HSC) transplantation in the unrelated donor setting, is technically challenging (see below). Several large-scale studies have demonstrated that precise, allele-level HLA matching between donor and patient significantly improves overall transplant survival by reducing the incidence and severity of both acute and chronic GVHD (graft versus host disease) and improving the rates of successful engraftment (1–15).

Currently, bone marrow donor registries contain data on millions of potential donors who have been analyzed, for the most part, at an intermediate level of resolution for HLA- A, -B, and DRB1 loci. Multiple potentially matched unrelated donors are selected, based on this initial typing, and then these donor samples are reanalyzed at allele-level resolution at these and additional HLA loci to identify the donor best matched to the recipient.

Currently, the highest resolution HLA typing is obtained with fluorescent, Sanger-based DNA sequencing using capillary electrophoresis. Ambiguities in the HLA typing data may still persist due to multiple polymorphisms shared between alleles and the resultant phase ambiguities when both alleles are amplified and sequenced together. Resolving these ambiguities requires time-consuming approaches such as amplifying and then analyzing the two alleles separately.

An alternative approach to the phase ambiguity problem is clonal sequencing. Next-generation DNA sequencing (16) provides orders of magnitude increases in the number of reads of contiguous sequence obtainable in a short time. Sequencing hundreds of millions of bases from amplified single DNA molecules is possible within a few days. To date, however, read lengths that would allow the resolution of phase ambiguities in HLA alleles have been achieved only with the clonal pyrosequencing-based method developed by 454 Life Sciences, Inc (17). The Roche GS FLX genome sequencer generates sequence read lengths greater than 250 nucleotides. The average HLA exon encoding the peptide binding groove is approximately 270 base pairs; the range is 239–242bp for DQA1 exon 2 to 276bp for exon 3 of class I genes. As our amplicons are only slightly longer than the exons, each exon can be sequenced completely by sequencing both strands with sufficient overlap between the reads that specific HLA alleles can be unambiguously assigned. We have chosen to analyze HLA polymorphism by isolating the relevant exons through specific polymerase chain reaction (PCR) amplification, prior to emulsion PCR and pyrosequencing rather than capturing by hybridization and then sequencing the relevant genomic DNA.

Here, we demonstrate that this approach allows the rapid, accurate determination of HLA type at allelic resolution for many individuals at multiple HLA loci simultaneously. This approach features novel HLA genotyping software developed by Conexio Genomics, Inc. (Freemantle, Australia) for analyzing sequence read data from the Roche GS FLX instrument (specifically, the fna files). This software compares the sequence reads to the

database of known HLA allele sequences and assigns a genotype for each locus for each individual.

The very large number of sequence reads (n = 300 – 400K) generated in a single run makes possible the detection of rare sequence variants present in individual samples. For example, maternal cells can be found in low frequencies in the blood of some severe combined immunodeficiency disease syndrome (SCIDS) patients; these chimeric mixtures, consequently, contain rare nontransmitted maternal alleles. Here, we demonstrate this capability of 454 sequencing through the analysis of DNA mixtures from two homozygous cell lines, as well as through the analysis of DNA from an SCIDS patient. In this case, rare copies of the maternal nontransmitted allele could be detected, in addition to the inherited paternal and maternal alleles at the HLA-B and HLA-C loci.

Materials and methods

Primer design and PCR conditions

The 454 HLA fusion primers consist of four main parts (Figure 1). Starting from the 5’ end, the primer contains a 19-base adapter sequence, which is responsible for capture of PCR amplicons by DNA capture beads. Adapter sequences end with a 4-base library key tag (TCAG), which allows the 454-genome sequencer software to differentiate HLA amplicon derived sequences from internal control sequences. We added 4-base multiplex identifier (MID) sequences (18) immediately following the library key tag to allow for multiplexed sequencing of HLA amplicons. The locus-specific sequence for amplification of the target genomic region follows the MID sequence (see Table S1, Supporting Information) for the HLA locus-specific primer sequences). Fusion primers were designed in sets of 12, with each primer having a unique MID sequence. The design of these primers involves the usual ‘trade-offs’ for HLA amplification; the primers should be specific to the locus, to the extent possible, and also be capable of amplifying all alleles at that locus with comparable efficiency. If the 454 HLA fusion primers are not completely specific (for example, an HLA-A exon 4 primer pair could also amplify HLA-E, -F or -G), then, unlike the case with Sanger sequencing or SSOP typing methods where sequences of related genes adds ‘noise’ to the typing system, these sequence reads can be filtered out such that the genotype assignment is unaffected. In some cases, however, as in the coamplification of DRB3, DRB4, and DRB5 together with the DRB1 locus using generic DRB primers, these additional sequence reads can serve as potentially important genetic markers and provide additional valuable genotypes.

The PCR amplifications of 14 exons from the 24 cell-line DNAs were all carried out individually. The thermal cycling conditions are as follows: 95°−10’, 95°−15”, 60°−45”, 72°−15”; 35 cycles, 72°−5’. We note that our HLA-C-specific exon 3 primers used in this

Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Sequence of HLA-specific 454 fusion primers (target PCR segment only)

Figure S1 Screenshot of Conexio genotyping software for HLA-A of the cell-line DBUG

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experiment generate a 653-bp amplicon. This amplicon is too long to allow complete sequencing of exon 3 by the GS FLX (average read length is 250 bases). Using this amplicon as the template for nested PCR with primers FDB1180 and RHLACE3 (Table S1, Supporting Information) generates a 381-bp amplicon from which full coverage sequencing can be achieved. Currently, we use only the second 'internal' primer pair yielding a 381-bp amplicon directly from genomic DNA, so that a nested PCR is not necessary.

Each of the 336 PCR reactions (25 ul) was prepared using a standard master mix that consisted of 10 mM Tris-HCl buffer, pH 8.3, 50 mM KCl, 1.5 mM MgCl, 150 uM dNTPs, Glycerol 10% v/v, AmpliTaq Gold (2 units). This mix was then added to each well of a microtiter plate containing 10 ng of cell-line genomic DNA, and forward/reverse fusion primers (10 pmoles each). Following PCR amplification, nonspecific and primer–dimer artifact products were removed from the amplicons using the Agencourt AMPure system (Agencourt Bioscience Corp., Beverly, MA). Aliquots from purified amplicons are further evaluated by electrophoresis on eGel® s (Invitrogen Corporation, Carlsbad, CA). The amplicons are then quantified by Quant-iT™PicoGreen® assay (Invitrogen Corporation) on a Molecular Devices Gemini XS microplate fluorimeter (Molecular Devices, Sunnyvale, CA) and subsequently diluted to 200 000 molecules/μl. With 12 MIDs available per amplicon, the maximum number of samples that can be sequenced in a picotitre plate region is 12. Thus, in our 24-sample runs, we created two pools of amplicons for all loci, one pool for samples 1–12 and another for samples 13–24. Emulsion PCR, bead recovery, and pyrosequencing were carried out as described (Roche Applied Science GS FLX emPCR Method Manual USM-00033.A-December 2007, Roche Applied Science GS FLX Sequencing Method Manual USM-00035.A-December 2007).

Results

Multiplex pyrosequencing

The analysis of multiple HLA loci for multiple samples in a single 454 run is facilitated by the incorporation of MID tags into the PCR fusion primers (18). Figure 1 illustrates the 454 fusion primer structure, and Table S1 (Supporting Information) presents the sequences of the HLA-specific primers (without MID or adapter sequences) that were used to generate the data reported here. Fourteen primer pairs were designed for exons 2, 3, and 4 of HLA-A, B, and C, exon 2 of DRB1, DPB1, DQA1, and exons 2 and 3 of DQB1. Primers with 12 different MID tags for each target sequence were designed for a total of 168 (14 × 12) primer pairs. The primers for exon 2 of DRB1 also amplify the DRB3, DRB4, and DRB5 loci, genes that are present on specific DRB1 haplotypes (http://www.ebi.ac.uk/imgt/hla/).

Following amplification of the various samples, the PCR products were quantified by PicoGreen fluorescence, diluted to the appropriate concentration, and pooled for emulsion PCR. Pyrosequencing runs of 24 and 48 individuals were achieved using 2 or 4 picotiter plate regions, respectively. Average read depths for each exon (per individual sample) are shown in Table 1. Overall average read depths per amplicon range from 500 to 700 reads (forward sequences + reverse sequences). Typical HLA amplicon passed filter sequence read yields and read length distributions for amplicons in a 24-sample run (336 amplicons) are shown in Figure 2(A) and (B) for a 48-sample run (672 amplicons). Read length

distributions are centered around the 250 bases. This length is sufficient for forward and reverse sequence reads to overlap, allowing unambiguous assignment of sequences to each exon and, ultimately, to each allele. The alignment of HLA sequences to the database of known HLA alleles and assignment of HLA genotypes is accomplished with the Conexio Genomics HLA genotyping software. In the sequence read length distribution from the 48-sample run (Figure 2B), a proportion of reads are short, ranging in size from 50 to 180 bases. The most numerous of these are in the 60–80 base range. These short sequences are the consequence of primer–dimer artifact from the initial PCR reactions that was carried into emulsion PCR with the diluted amplicon pools. The Agencourt AMPure system (Agencourt Bioscience Corp., Beverly, MA) was not used to purify the primer–dimer artifact from the amplicons used in this particular experiment, while it was used to purify the amplicons sequenced in the 24-sample run. A comparison of the read distributions between these two runs reveals the efficiency of the Agencourt AMPure system to remove primer–dimer artifact and any other shorter nonspecific PCR products, from amplicons being prepared for 454 sequencing.

Genotyping software

The GS FLX data processing software filters the hundreds of thousands of individual sequence reads generated in each sequencing run based on sequence quality length minimums resulting in sets of sequence reads that constitute the ‘passed filter’ reads. To facilitate HLA genotype assignment from 454 sequence data files, Conexio Genomics’ HLA genotyping software application compares the passed filter forward and reverse 454 sequence reads derived from each exon to the current IMGT-HLA sequence database (EMBL-European Bioinformatics Institute, Cambridge, UK). The database also contains the sequence of HLA pseudogenes and related genes, allowing the filtering out of sequences generated from pseudogenes or from nonclassical HLA class I genes (e.g. HLA-,E,F,G, and H). Screenshots of the software displaying the analysis of sequence reads for exon 2 of the DRB1 gene, and exons 2, 3, and 4 of the HLA-B genes for the cell-line DBUG are shown in Figures 3(A) and (B). The number of different forward and reverse sequence reads for each exon is shown in the upper panel. For the DRB1 exon 2, there were 82 forward reads of one allele (designated as sequence 1.1) and 75 forward reads of the other allele (designated as sequence 1.4) and 81 reverse reads of one allele (designated as 1.2) and 69 reads of the other allele (designated as 1.3). The genotype assignment is shown to the right, along with the number of mismatches of the sequence file to the HLA alleles in the database. In some cases, a unique genotype (top line in the right panel; DRB1*070101/1105) is assigned with 0 mismatches, as in Figure 3(A) for DRB1; other closely related potential genotype assignments having one or more mismatches are shown just below the 0 mismatch genotype assignment. In other cases, more than one possible genotype is consistent with the sequence data. In the sequence reads for DBUG for HLA-B exons 2, 3 and 4, two genotypes are assigned with 0 mismatches (Figure 3B). In this case, the polymorphism that distinguishes the two genotype assignments (B*070501 and B*070601) is located in exon 5 (not sequenced in this panel).

Rare sequence reads, such as those derived from pseudogenes, related HLA genes, or from PCR amplification or pyrosequencing artifacts, that differ from consensus allele sequences,

are filtered into a ‘secondary alignment’. Analysis of these low-frequency sequences in the secondary alignment can prove instructive with regard to PCR primer specificity and systematic pyrosequencing errors. The most common, albeit still very rare, sequencing artifact we observed was variation in homopolymeric runs of G. For example, in the analysis of DNA from the AMALA cell line, we observed 283 reverse exon 2 HLA-A sequence reads for the consensus sequence of 4 Gs, while in the secondary alignment, we observed 9 reverse exon 2 reads for 3 Gs. Since the Conexio genotyping software filters these sequence reads into the secondary alignment, these rare sequence artifacts do not affect the accuracy and reliability of the HLA genotype assignments.

High-throughput HLA sequencing

A total of 24 cell-line derived DNA samples of known HLA type, based on previous analyses of probe hybridization HLA typing and Sanger sequencing results, were sequenced at all 7 loci (HLA-A, -B, -C, -DRB1, -DQA1, -DQB1, DPB1). Exon 2 sequences of DRB3, DRB4, and DRB5 were also identified in the amplicons generated by the generic DRB primer pair. Subsequently, a run of 48 samples (24 cell-line DNAs and 24 DNAs extracted from blood samples) were sequenced at the same loci and genotype assignments were generated from the sequence data by the Conexio Genomics HLA genotyping software. In general, consensus sequences derived from 50 or more sequence reads are considered ‘high confidence’ as are the genotype assignments based on these consensus sequences. In the 24-sample run, the concordance of software genotype calls and previously determined HLA types for all 7 loci was 99.4% (Table 2). The allele assignments for DRB3, DRB4, and DRB5, are not included in this Table. If more than one genotype was assigned (ambiguity string) for a given locus, only the top genotype in the list (see Figure 4) is shown in the Table. Of the 168 allele calls (7 loci×24 samples), one was incorrect. The HLA-A*2601 allele of cellline DBUG (A*1101,*2601) was not called in this experiment by the software. DNAs from 24 cell lines were amplified by 454 fusion primers containing MID tags and analyzed in two regions as described in the Materials and Methods. The genotype assignments for these cell lines, based on SSOP or Sanger SBT (sequence-based typing) are shown in the two left panels. The genotype assignments determined by 454 sequencing and Conexio software are shown on the right. If the software indicated more than one genotype was consistent with the sequence data (ambiguity) and the correct reference genotype was included, we called this typing ‘concordant’. Concordance was 99.4% for the 168 allele calls (7 loci × 24 samples). For the cell-line DBUG (A*1101,*2601), the software correctly called the A*1101 allele but not the A*2601 allele in this run. Of the 167 correct allele calls, 133 were called correctly by the software with no manual editing, 26 were called correctly after the manual editing, and 9 were called correctly following nontarget gene (pseudogene, related HLA gene) removal.

The failure to detect A*2601 in this particular 24-sample run was due to a problem with the initial version of the genotyping software in which exon 2 sequence reads that should have been sorted into the primary alignment appeared in the secondary alignment. This problem has been corrected in subsequent versions of the ATF software and we have been able to detect both alleles of this cell line in subsequent runs. Figure S1 (Supporting Information) shows a screen shot of the Conexio genotyping software in which both DBUG HLA-A

alleles were correctly assigned. PCR conditions that minimize differential amplification of alleles from genomic DNA should be used, as in all PCR-based typing methods. Also, it is advisable to aim for relatively high numbers of sequence reads per amplicon so that, even if preferential amplification occurs, a sufficient number of sequence reads are recovered to allow assignment of both alleles of a heterozygote by the genotyping software.

A genotype report listing the possible allele assignments (0 mismatches with the database) for all loci tested for each individual sample is generated by the Conexio software. An illustrative example of the genotype assignments for the cell line, RAJI, is shown in Figure 4. Genotype assignments with 0 mismatches of the consensus sequences to the database are shown beneath the locus designation. For those loci with multiple genotype assignments (ambiguity), the polymorphisms that distinguish the primary assignment (first genotype in list) from the other genotypes in the list lie outside of the regions sequenced in this study. The DRB3 locus is on the DRB1*0301 haplotype; the DRB1*1001 haplotype does not carry an additional DRB locus. For DRB1, DQB1, DPB1, and HLA-B, a unique set of two alleles is assigned. For the DRB3 locus, present on the DRB1*03 haplotype, two possible alleles are listed. At the HLA-A locus, the assignment includes several synonymous variants of the A*0301 allele (the first four digits refer to nonsynonymous variants) as well as a very rare ‘null’ variant, A*03010102N, which differs from A*0301 outside the genomic regions sequenced here. Similarly, the three rare alleles listed under HLA-C also differ from the primary assignment, Cw*0401, outside the sequenced region. The long list of possible DQA1 genotypes also reflects differences from the primary assignment, DQA1*0101/DQA1*0501 at polymorphisms that reside outside exon 2. These residual ambiguities can be resolved by additional analyses (i.e. SSP), if desired, or by incorporating additional genomic regions (exons and/or introns) in the 454 sequencing run.

As noted in the Table 2, most genotype assignments were performed by the software without any need for any manual editing, while a subset of samples required some additional intervention (see below). In these samples, the software provides no initial genotype assignment with 0 mismatches. Following manual ‘inactivation’ of rare sequences reads that had not been automatically filtered out, the software provided a genotype assignment with 0 mismatches. In some cases, the manual editing involved ‘trimming back’ bases from the ends of sequence reads that contained mismatches with the consensus sequence. These mismatches reflect pyrosequencing errors that can occur at the ends of reads due to the increased frequency of incomplete extension and carry-forward error that is associated with the incomplete removal of ATP from previous nucleotide incorporation cycles.

Another situation in which manual editing can be required is the genotype assignment of homozygous samples with low numbers of sequence reads; in these cases, the initial version of the software may take relatively rare sequences from the secondary alignment and assign two alleles to this homozygous sample. In all cases, the inspection of the genotyping software output and the manual editing described above, which takes 1–2 min per genotype, yielded the correct genotype assignments. Subsequent versions of the software have addressed these issues and significantly reduce the need for manual editing.

Analysis of mixtures (rare variant detection)

The very high number of sequence reads generated in a typical GS FLX run (300–400K), make possible the detection of rare variant sequences present in the sample. To estimate the sensitivity to detect such sequences, we prepared mixtures of PCR products for exons 2 and 3 of HLA-A and HLA-B from two HLA homozygous cell lines (AMAI, A*680201, B*530101 and SAVC, A*030101, B*70201) in various proportions (1/1, 1/10, 1/100). As can be seen in Table 3, the number of sequence reads for the two alleles of exon 3 of HLA-B were similar in the 1:1 mixture (forward: 1802 *vs* 1803 and reverse: 1248 *vs* 1133) as well as the HLA-A exon 2 sequence reads (forward: 1208 *vs* 1397 and reverse: 816 *vs* 1051). Since the efficiency of recovering forward and reverse allelic sequence reads for HLA-B exon 3 and HLA-A exon 2 was comparable, these exons were chosen to analyze the 1/10 and 1/100 mixtures. For the HLA-A 1/10 mixture, the ‘rare’ sequence reads (the A*30101 allele) represented 18% of the total forward strand reads and 15% of the reverse strand reads. In the HLA-B 1/10 mixture, the ‘rare’ sequence reads (the B*70201 allele) represented 11% of the total forward strand reads and 10% on the reverse strand reads. The rare variant in 1/100 mixtures could also be readily detected. It was present at a frequency of 0.9% and 1.6% in HLA-A forward and reverse strand reads, respectively, and 2.9% and 2.5% of HLA-B forward and reverse strands, respectively.

The blood of certain individuals is chimeric, with residual maternal cells present at very low levels in the child’s circulation or rare fetal cells maintained in the mother’s circulation (19). SCIDS patients often retain circulating maternal cells at very low levels and early detection of maternally derived immunologic cells is important after diagnosis for proper management of the patient. When such patients are recipients of hemapoetic stem cell transplants, characterizing the level of maternal microchimerism is clinically important; exposure to maternal antigens increases the possibility of severe GVHD using unmodified, HLA mismatched related, and unrelated donors in transplantation (20, 21).

Here, we describe the HLA profile of an SCIDS patient, F4R, who was the recipient of an hemapoetic stem cell transplant. The HLA-B and HLA-C types of this patient and his parents, determined by 454 sequencing and the Conexio HLA typing software, based on exon 2 and exon 3 sequence reads, are shown in Table 4. The presence of a ‘third’ HLA-B allele, the nontransmitted maternal allele (B*3512), could be identified in the ‘secondary alignment’ of exon 2 sequence reads. Along with other sequences that represented artifactual variants of the two inherited alleles, there were 11 reverse sequence reads for exon 2 for the B*3512 allele in this secondary alignment, compared with 290 reverse sequence reads for B*3905 and 280 reads for B* 390202 for exon 2 in the primary alignment. A nontransmitted maternal HLA-C* 0401 allele could also be detected in this sample. The HLA-C type of F4R is *0702 homozygous. For exon 3, 1153 copies of a forward sequence read corresponding to the two copies of HLA-C*0702-as well as 10 copies of a forward exon 3 sequence read corresponding to HLA-C*0401 were detected in the primary alignment. In this case, the rare nontransmitted maternal allele is found in the primary rather than the secondary alignment, as in the HLA-B example, because F4R is homozygous at HLA-C so this ‘additional’ allele is the *second* rather than the *third* allele. The analysis of the HLA-B and -C sequence reads suggests that the maternal cells in this SCIDS patient’s blood are on

the order of 1–2%. A more detailed and systematic analysis by 454 HLA sequencing of microchimerism, examining additional exons and SCIDS patients will be the subject of a subsequent manuscript.

Discussion

Allele-level matching for many HLA loci (A, B, C, and DRB1) of donors and recipients is clinically critical for successful HSC transplantation (1–12). In some studies, matching for HLA-DQB1 and DPB1 also has a significant effect on transplant outcome (13–15). Currently, the highest resolution HLA typing is obtained with fluorescent, Sanger-based DNA sequencing using capillary electrophoresis. Even at this level of sequence resolution, ambiguities in the HLA typing data can persist due to multiple polymorphisms shared between alleles and the resultant phase ambiguities when both alleles are amplified and sequenced together. Resolving these ambiguities requires time-consuming approaches such as amplifying and then analyzing the two alleles separately. Clonal sequencing, the analysis of amplicons generated from individual DNA molecules amplified from HLA exons allows the unambiguous sequence determination of the exons and, by comparing these sequence files to an HLA sequence database, the unambiguous determination, in most cases, of the two HLA alleles.

The read lengths achieved by the GS FLX system (avg = 250 bp) allow sufficient overlap for this sequence determination for each exon. The assignment of genotypes at each locus based on the exon sequence data files is performed by a software application developed by Conexio Genomics (Freemantle, AU). A critical aspect of the software is the ability to filter out related sequence reads (pseudogenes and other unwanted HLA genes) that were coamplified along with the target sequence. In most HLA typing methods, such as Sanger sequencing or SSOP typing, these coamplified sequences would generate ‘noise’ and minimize ‘signal’. The software also filters out very rare sequence reads that may have been generated by an error in the initial PCR amplification of the target sequence from genomic DNA, errors in the emulsion PCR, or pyrosequencing errors, as discussed earlier. On the basis of a recent report (22), the pyrosequencing error rate was estimated, in an ultradeep sequencing study of HIV amplicons to be 0.01 in homopolymeric regions (3–5 nucleotides) and 0.002 in nonhomopolymeric regions. The overall error rate was 0.004.

The clonal sequencing property of the 454 GS FLX reveals PCR primer specificity by identifying the sequences of all coamplified genomic regions, in addition to the intended target region. This property is useful in optimizing primer specificity, and allows for the use of generic primers, such as our DRB primers, which amplify DRB3, DRB4, and DRB5, in addition to DRB1, to generate valuable sequence information at multiple loci.

To make the GS FLX system cost-effective for highresolution clinical HLA typing, multiple samples must be analyzed at multiple loci in a single run. The use of MID tags, and multiple picotiter plate regions, makes running 24 or 48 samples analyzed at 7 loci possible and practical (see Table 1). Clearly, larger numbers of samples could be analyzed in a single run using additional MID tags and regions, provided that fewer amplicons per individual were sequenced.

It is the very large number of sequence reads generated in parallel that allows this multiplex analysis of multiple individuals at multiple loci, which also creates the opportunity to detect rare variant sequences. In mixtures of PCR products from two different genomic DNA samples, we were able to reliably detect HLA exon sequences present at a 1/100 dilution (Table 3). The challenge in this application is to filter out related but unwanted sequences, as well as rare sequences containing errors, yet retain and identify the rare allelic variant sequences. HLA sequences are well suited to this kind of analysis because most HLA alleles differ from one another by multiple polymorphisms while the sequences containing errors typically differ from the correct sequence by only one nucleotide.

In addition to the analysis of these cell-line DNA mixtures, the ability to detect rare HLA sequences present in mixtures was demonstrated in the analysis of blood from a SCIDS patient (Table 4). In this patient, the rare nontransmitted maternal allele could be detected along with the inherited maternal and paternal alleles. The potential to analyze chimeric mixtures may have important applications in clinical research (19).

In conclusion, we believe that this high throughput clonal sequencing system can provide cost-effective, reliable, highresolution HLA typing for clinical transplantation, as well as for research studies.

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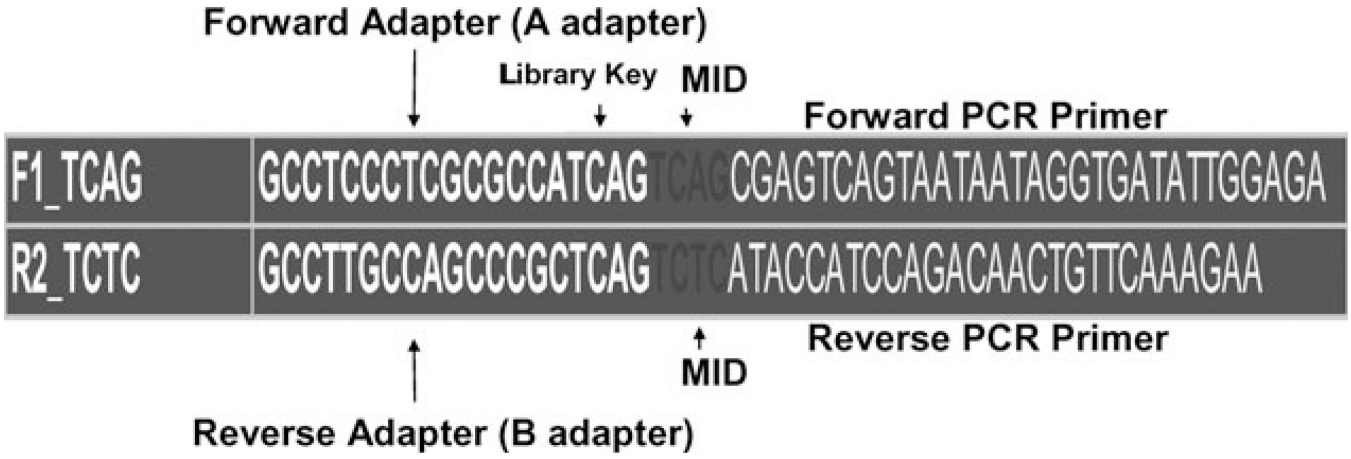
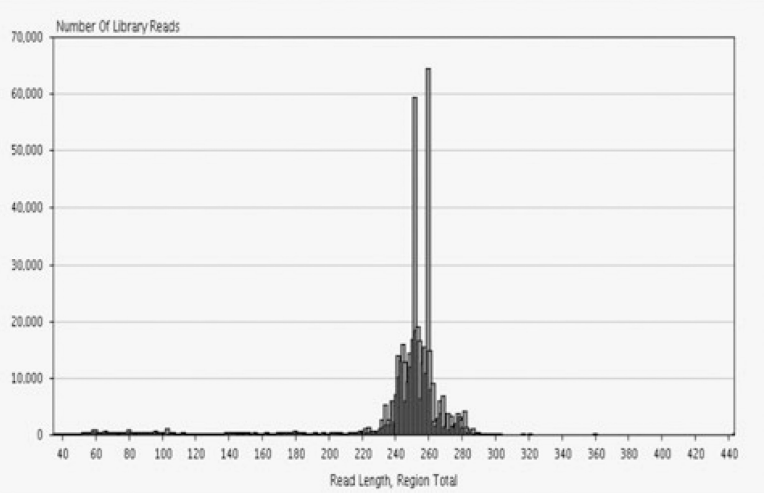


Figure 1.
Schematic of 454 sequencing fusion primer pair with 4-base multiplex identifier (MIDs).

A

	Region 1	2	Total
Passed Filter Wells	272.394	277.515	549.909



B

	Region				
	1	2	3	4	Total
Passed Filter Wells	81.395	57.752	83.652	55.056	277.855

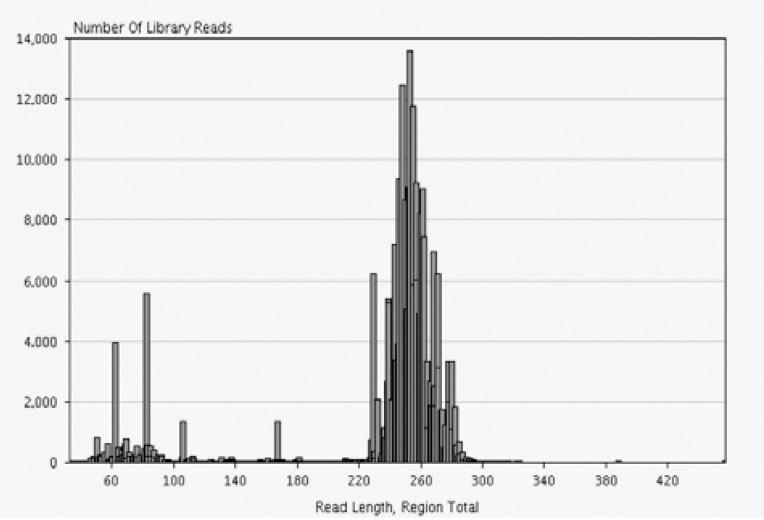


Figure 2.

(A) Typical HLA amplicon passed filter wells (sequence reads) by picotiter plate region and by read length (336 amplicons). (B) Typical HLA amplicon passed filterwells (sequence reads) by picotiter plate region and by read length (672 amplicons) for 48-sample run.

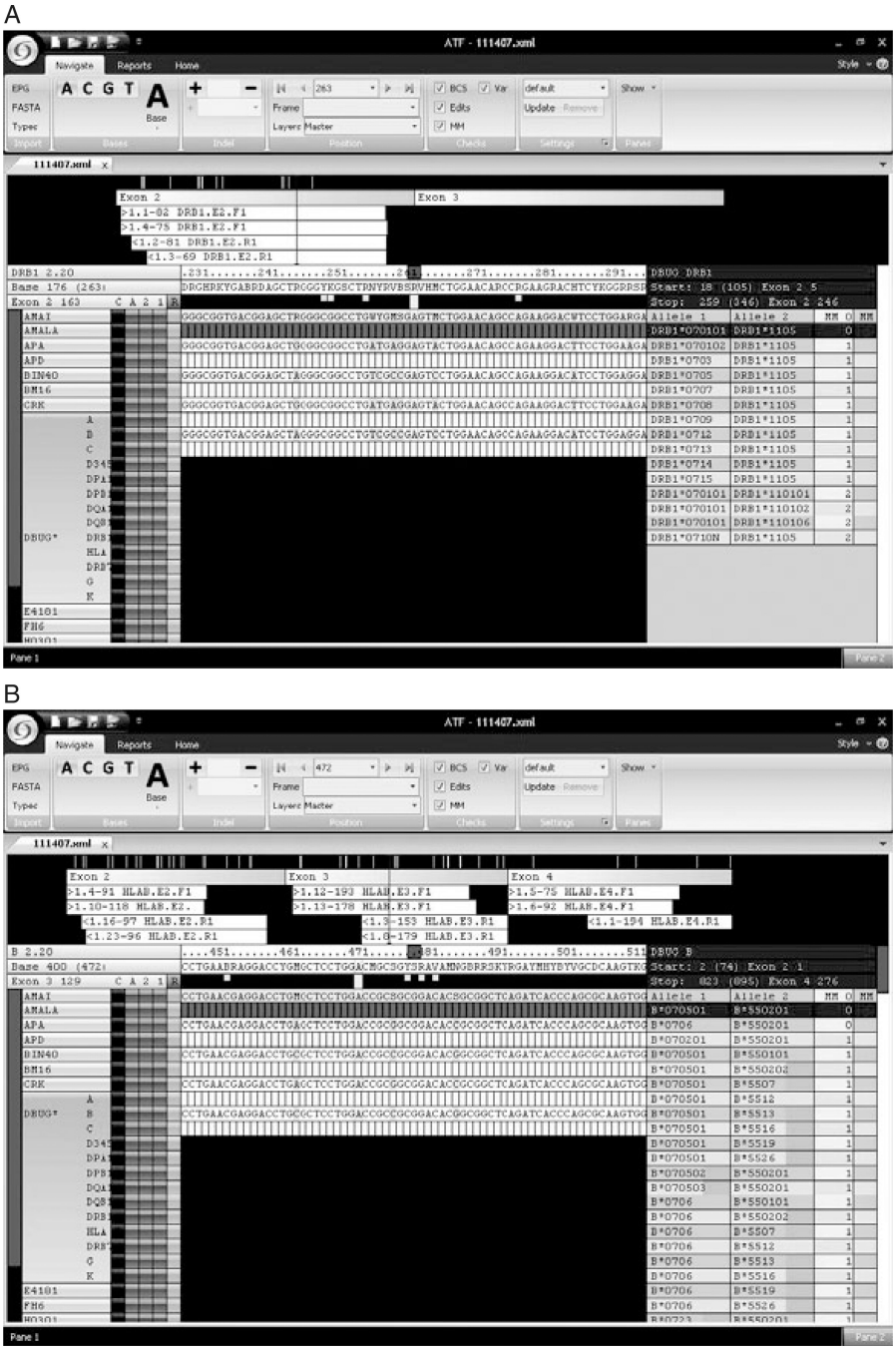


Figure 3.

(A) Screenshot of Conexio genotype assignment: DRB1 for DBUG. (B) Screenshot of Conexio genotype assignment: HLA-B for DBUG.

Sample: 14-RAJI

HLA-A		HLA-B		HLA-C	
A*03010101	A*03010101	B*1510	B*1510	Cw*030402	Cw*04010101
A*03010101	A*03010102N			Cw*030402	Cw*04010102
A*03010101	A*03010103			Cw*030402	Cw*0409N
A*03010102N	A*03010102N			Cw*030402	Cw*0420
A*03010102N	A*03010103			Cw*030402	Cw*0430
A*03010103	A*03010103				

DR345	DPB1		DQA1		DQB1		DRB1	
DRB3*020201	DPB1*010101	DPB1*010101	DQA1*010101	DQA1*050101	DQB1*020101	DQB1*050101	DRB1*030101	DRB1*100101
DRB3*0212			DQA1*010101	DQA1*0503				
			DQA1*010101	DQA1*0505				
			DQA1*010101	DQA1*0506				
			DQA1*010101	DQA1*0507				
			DQA1*010101	DQA1*0508				
			DQA1*010101	DQA1*0509				
			DQA1*010102	DQA1*050101				
			DQA1*010102	DQA1*0503				
			DQA1*010102	DQA1*0505				
			DQA1*010102	DQA1*0506				
			DQA1*010102	DQA1*0507				
			DQA1*010102	DQA1*0508				
			DQA1*010102	DQA1*0509				
			DQA1*010401	DQA1*050101				
			DQA1*010401	DQA1*0503				
			DQA1*010401	DQA1*0505				
			DQA1*010401	DQA1*0506				
			DQA1*010401	DQA1*0507				
			DQA1*010401	DQA1*0508				
			DQA1*010401	DQA1*0509				
			DQA1*010402	DQA1*050101				
			DQA1*010402	DQA1*0503				
			DQA1*010402	DQA1*0505				
			DQA1*010402	DQA1*0506				
			DQA1*010402	DQA1*0507				
			DQA1*010402	DQA1*0508				
			DQA1*010402	DQA1*0509				
			DQA1*0105	DQA1*050101				
			DQA1*0105	DQA1*0503				
			DQA1*0105	DQA1*0505				
			DQA1*0105	DQA1*0506				
			DQA1*0105	DQA1*0507				
			DQA1*0105	DQA1*0508				
			DQA1*0105	DQA1*0509				
			DQA1*0107	DQA1*050101				
			DQA1*0107	DQA1*0503				
			DQA1*0107	DQA1*0505				
			DQA1*0107	DQA1*0506				
			DQA1*0107	DQA1*0507				
			DQA1*0107	DQA1*0508				
			DQA1*0107	DQA1*0509				

Figure 4.
Conexio genotype assignments for the cell line RAJI report.

Table 1

Average number of forward (F) and reverse (R) sequence reads per amplicon per individual sample (24-sample run)

	HLA-A E2	HLA-A E3	HLA-A E4
F	277	205	114
R	256	182	108
	HLA-B E2	HLA-B E3	HLA-B E4
F	447	705	767
R	444	647	674
	HLA-C E2	HLA-C E3	HLA-C E4
F	234	161	345
R	226	146	231
	DQA1	DQB1 E2	DQB1 E3
F	634	595	576
R	575	506	576
	DPB1	DRB1	
F	290	206	
R	276	201	

Average read depths per amplicon (24-sample run).

Table 2

Concordance of 454 GS FLX determined human leukocyte antigen (HLA) genotypes with reference genotypes

Cell line	Locus	Allele1	Allele2	Allele1 454	Allele2 454	Cell line	Locus	Allele1	Allele2	Allele1 454	Allele2 454
JW5	DRB1	0103	03	0103	030101	E4181324	DRB1	150201	150201	150201	150201
JW5	DQA1	0101	0501	010101	050101	E4181324	DQA1	0103	0103	0103	0103
JW5	DQB1	0201/2	0501	020101	050101	E4181324	DQB1	060101	060101	060101	060101
JW5	DPB1	010101	020102	010101	020102	E4181324	DPB1	020102	0401	020102	040101
JW5	HLA-A	0101	2301	01010101	2301	E4181324	HLA-A	0101	0101	01010101	01010101
JW5	HLA-B	0801/4	18	080101	180101	E4181324	HLA-B	520101	520101	520101	520101
JW5	HLA-C	05	07	050101	070101	E4181324	HLA-C	1202	1202	120201	120202
RAJI	DRB1	0301	100101	030101	100101	SAVC	DRB1	0401	0401	040101	040101
RAJI	DQA1	0101	0501	010101	0501	SAVC	DQA1	0301	0301	030101	030101
RAJI	DQB1	0201/2	0501	020101	050101	SAVC	DQB1	0302	0302	030201	030201
RAJI	DPB1	010101	010101	010101	010101	SAVC	DPB1	1001	1001	1001	1001
RAJI	HLA-A	03	03	03010101	03010101	SAVC	HLA-A	0301	0301	03010101	03010101
RAJI	HLA-B	1510	1510	1510	1510	SAVC	HLA-B	0702	0702	070201	070201
RAJI	HLA-C	030402	04	030402	04010101	SAVC	HLA-C	0702	0702	07020101	07020101
NAMALWA	DRB1	0405	1503	040501	1503	LADA	DRB1	090102	1201/6	090102	120101
NAMALWA	DQA1	0102	0301	010201	030101	LADA	DQA1	0101	0301	010101	030101
NAMALWA	DQB1	0302	0602	030201	0602	LADA	DQB1	0201/2	0501	0202	050101
NAMALWA	DPB1	0101	0201	010101	020102	LADA	DPB1	0301	1701	030101	1701
NAMALWA	HLA-A	03	6802	03010101	68020101	LADA	HLA-A	0201	8001	02010101	8001
NAMALWA	HLA-B	0702	4901	070201	4901	LADA	HLA-B	0702	5703	070201	570301
NAMALWA	HLA-C	0701/6	0702/3	070101	07020101	LADA	HLA-C	0702/3	0802	07020101	0802
APA	DRB1	1405	150101/102	140501	150101	DBUG	DRB1	0701	1105	070101	1105
APA	DQA1	0101	0102	010101	010201	DBUG	DQA1	0101	0201	010101	0201
APA	DQB1	050301	0601	050301	060101	DBUG	DQB1	030302	0602	030302	0602
APA	DPB1	0501	0501	0501	0501	DBUG	DPB1	040101	0501	040101	0501
APA	HLA-A	2403	1101	110101	240301	DBUG	HLA-A	1101	2601	1101	
APA	HLA-B	1502	5502	1502	550201	DBUG	HLA-B	0705/6	55	070501	550201
APA	HLA-C	08	1203/6	080101	12030101	DBUG	HLA-C	010202	07020102	010201	07020101

Cell line	Locus	Allele1	Allele2	Allele1 454	Allele2 454	Cell line	Locus	Allele1	Allele2	Allele1 454	Allele2 454
MG	DRB1	0401/16	1001	040101	100101	AMAI	DRB1	1503	1503	1503	1503
MG	DQA1	0101	0301	010201	030101	AMAI	DQA1	0102	0102	010201	010201
MG	DQB1	0302/7	0501	030201	050101	AMAI	DQB1	0602	0602	0602	0602
MG	DPB1	0401	0601	040101	0601	AMAI	DPB1	0402	0402	0402	0402
MG	HLA-A	0101	0201	01010101	02010101	AMAI	HLA-A	6802	6802	68020101	68020101
MG	HLA-B	15	3701	15010101	370101	AMAI	HLA-B	5301	5301	530101	530101
MG	HLA-C	03	0602	030401	06020101	AMAI	HLA-C	0401	0401	040101	040101
TTL	DRB1	1301	1501	130101	150101	CRK	DRB1	0701	0701	070101	070101
TTL	DQA1	0102	0103	010201	0103	CRK	DQA1	0201	0201	0201	0201
TTL	DQB1	0502	0603	050201	060301	CRK	DQB1	0201/0202	0201/0202	0202	0202
TTL	DPB1	0201	1301	020102	1301	CRK	DPB1	010101	110101	010101	110101
TTL	HLA-A	1102	3303	110201	330301	CRK	HLA-A	2902/4	2902/4	290201	290201
TTL	HLA-B	51	5401	510101	5401	CRK	HLA-B	4403	4403	440301	440301
TTL	HLA-C	0102	0302	010201	030201	CRK	HLA-C	1601	1601	160101	160101
FH6	DRB1	160101	1001	160101	100101	H0301	DRB1	1302	1302	130201	130201
FH6	DQA1	0101	0102	010101	010201	H0301	DQA1	0102	0102	010201	010201
FH6	DQB1	0501	0502	050101	050201	H0301	DQB1	0609	0609	0609	0609
FH6	DPB1	020102	0401	020102	040101	H0301	DPB1	0501	0501	0501	0501
FH6	HLA-A	24	2901	24020101	29010101	H0301	HLA-A	0301	0301	03010101	03010101
FH6	HLA-B	0705/6	2702	070501	2702	H0301	HLA-B	1402	1402	1402	1402
FH6	HLA-C	0202	1505	020202	150501	H0301	HLA-C	0802	0802	080201	080201
JY	DRB1	0404	1301	0404	130101	OOS	DRB1	0101	0101	010101	010101
JY	DQA1	0103	0301	010301	030101	OOS	DQA1	0101	0101	010101	010101
JY	DQB1	0302	0603	030201	060301	OOS	DQB1	0501	0501	050101	050101
JY	DPB1	020102	0401	020102	040101	OOS	DPB1	020102	020102	020102	020102
JY	HLA-A	020101	020101	02010101	02010101	OOS	HLA-A	2601	2601/11N	260101	260101
JY	HLA-B	070201	070201	070201	070201	OOS	HLA-B	5601	5601	5601	5601
JY	HLA-C	0702	0702	07020101	007020101	OOS	HLA-C	0102	0102	010201	010201
BM16	DRB1	1201	1201	120101	120101	SSTO	DRB1	0403	0403	040301	040301
BM16	DQA1	0501	0501	050101	050101	SSTO	DQA1	0301	0301	030101	030101
BM16	DQB1	0301	0301	030101	030101	SSTO	DQB1	0305	0305	030501	030501

Cell line	Locus	Allele1	Allele2	Allele1 454	Allele2 454	Cell line	Locus	Allele1	Allele2	Allele1 454	Allele2 454
BM16	DPB1	020102	020102	020102	020102	SSTO	DPB1	0401	0401	040101	040101
BM16	HLA-A	0201	0201	02010101	02010101	SSTO	HLA-A	3201	3201	320101	320101
BM16	HLA-B	1801	1801	180101	180101	SSTO	HLA-B	4402	4402	440201	440201
BM16	HLA-C	0701	0701	070101	070101	SSTO	HLA-C	0501	0501	050101	050101
LH	DRB1	0301	0404	030101	0404	BIN40	DRB1	0404	0404	0404	0404
LH	DQA1	0301	0501	030101	050101	BIN40	DQA1	0301	0301	030101	030101
LH	DQB1	0201	0402	020101	0402	BIN40	DQB1	0302	0302	030201	030201
LH	DPB1	010101	0501	010101	0501	BIN40	DPB1	0301	0601	030101	0601
LH	HLA-A	2402	2402	24020101	24020101	BIN40	HLA-A	02	310102	02010101	310102
LH	HLA-B	0802	2708	080101	2701	BIN40	HLA-B	1401	4001	1401	400101
LH	HLA-C	0102	0701/6	010201	070101	BIN40	HLA-C	03	0802	030401/0301	0802
VOO	DRB1	0101	030101	010101	030101	APD	DRB1	1301	1301	130101	130101
VOO	DQA1	0101	0501	010101	050101	APD	DQA1	0103	0103	0103	0103
VOO	DQB1	0201/2	0501	020101	050101	APD	DQB1	0603	0603	060301	060301
VOO	DPB1	020102	0401	020102	040101	APD	DPB1	0402	0402	0402	0402
VOO	HLA-A	0101	0301	01010101	03010101	APD	HLA-A	0101	0101	01010101	01010101
VOO	HLA-B	0801	5601	080103	5601	APD	HLA-B	4001	4001	400101	400101
VOO	HLA-C	0102	0701/06/16	010201	070101	APD	HLA-C	0602	0602	06020101	06020101
AMALA	DRB1	1402	1402	1402	1402	HAR	DRB1	0301	0301	030101	030101
AMALA	DQA1	0501	0501	050101	050101	HAR	DQA1	0501	0501	050101	050101
AMALA	DQB1	0301	0301	030101	030101	HAR	DQB1	0201	0201	020101	020101
AMALA	DPB1	0402	0402	0402	0402	HAR	DPB1	040101	0401	040101	040101
AMALA	HLA-A	021701	021701	021701	21701	HAR	HLA-A	0101	0101	01010101	01010101
AMALA	HLA-B	1501	1501	15010101	15010101	HAR	HLA-B	0801	0801/5	080101	080101
AMALA	HLA-C	0303	0303	030301	030301	HAR	HLA-C	0701/6	0701/5	070101	070101

Correct calls by Conexio software without editing; Correct calls after minimal manual editing (highlighted); Correct calls after removal of nonspecific sequences (dark highlighted).

Table 3

Mixtures of homozygous cell-line DNAs (number of sequence reads)

Dilution ratio 1/1			Dilution ratio 1/10			Dilution ratio 1/100		
Type	**HLA-A**	**Reads**	**HLA-A**	**Reads**	**Frequency (%)**	**HLA-A**	**Reads**	**Frequency (%)**
A*680201	Exon 2 F	1208	Exon 2 F	2201		Exon 2 F	1348	
A*30101	Exon 2 F	1397	Exon 2 F	471	18%	Exon 2 F	12	0.9%
A*680201	Exon 2 R	816	Exon 2 R	1685		Exon 2 R	927	
A*30101	Exon 2 R	1051	Exon 2 R	304	15%	Exon 2 R	15	1.6%
Type	HLA-B		HLA-B			HLA-B		
B*530101	Exon 3 F	1802	Exon 3 F	4711		Exon 3 F	3794	
B*70201	Exon 3 F	1803	Exon 3 F	608	11%	Exon 3 F	112	2.9%
B*530101	Exon 3 R	1248	Exon 3 R	3295		Exon 3 R	2644	
B*70201	Exon 3 R	1133	Exon 3 R	363	10%	Exon 3 R	68	2.5%

HLA, human leukocyte antigen.

Tissue Antigens. Author manuscript; available in PMC 2014 October 21.

Table 4

SCIDs patient/recipient and mother and father : HLA-B and HLA-C genotypes

	HLA-B		HLA-C	
F4R	3905	390202	07020101	07020101
F4M	3905	3512	04010101	07020101
F4D	3503	390202	04010101	07020101

Alleles B*3512 and C*0401 are the nontransmitted maternal alleles.

HLA, human leukocyte antigen.