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Research paper

Unambiguous high resolution genotyping of human leukocyte antigens

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ABSTRACT

We have developed a high resolution sequencing based typing method for genotyping Human Leukocyte Antigens (HLA) over a period of twenty years. The methods are based upon the separation of HLA alleles per locus at the initial amplification to simplify the analysis post-sequencing. The increasing discovery of polymorphism in HLA, manifested in new alleles, has necessitated the continuing development of this method. Here we present methods for the high resolution Sequence Based Typing of HLA-A, B, C (class I) and HLA-DQB1 and DRB1 (class II). The purpose of this article is to provide a valuable resource of methods and primers for other laboratories engaged in HLA typing.

1. Introduction

The "Classical" Human Leukocyte Antigen system comprises six loci divided into class I (HLA-A, -B, and -C) and class II (HLA-DP, -DQ, and -DR). The principal function of these proteins is to present peptides which are derived from viral and intracellular proteins (class I) or those derived from processing of pathogens and extracellular proteins (class II) (McCluskey and Peh, 1999). HLA are the most polymorphic protein and genetic systems found in humans and represent a significant challenge for successful allogeneic transplantation of an organ or stem cells (Tait, 2011; Tiercy, 2002) and in stem cell banking and future applications (Taylor et al., 2011).

The complexity and expanse of HLA polymorphism has attracted the adaption and development of new technologies in tissue typing laboratories (Dunn 2011, 2015). Various techniques have been developed and modified in the post-PCR era, including PCR sequence-specific primer (PCR-SSP) (Bunce et al., 1995), PCR-sequence-specific oligonucleotide probe (PCR-SSOP) (Cao et al., 1999), PCR-sequencing-based typing (PCR-SBT) (Dunn et al., 2003), oligonucleotide arrays (Guo et al., 1999), and, more recently, next generation sequencing (NGS) (Holcomb et al., 2011). The un-abating march of HLA polymorphism, mainly discovered through DNA sequencing, has revealed the inadequacies of many of these technologies (Dunn, 2011).

DNA sequencing, using the Sanger method of di-deoxy chain termination (Sanger et al., 1977), for HLA typing became the preferred method for allelic HLA typing through the discovery of locus- and antigen-specific polymorphisms in the noncoding introns flanking the polymorphic exons (Cereb et al., 1995; Cereb and Yang, 1997; Kotsch et al., 1997, Kotsch et al., 1999; Dunn et al., 2005). The methods described here have evolved since 2000 in response to the discovery of increasing levels of HLA polymorphism. The initial methodology used amplification of exon 2 in class II genes (HLA-DPB1, DQB1 and DRB1) and exons 2 and 3 in class I genes (HLA-A, B, C), amplifying and sequencing both alleles together. As new alleles were reported with polymorphisms outside of these amplified regions we included these exons. Full amplification of HLA class I genes was commenced in 2000 and this was used as the template for sequencing reactions. This has proved more difficult for class II genes because of their size but we have now included exons 2 and 3 for sequencing HLA-DQB1 and HLA-DRB1. As the numbers of HLA polymorphisms have increased so the number of ambiguities has increased. Allele ambiguities are due to polymorphisms outside of the region analysed and genotype ambiguities are combinations of alleles which have identical heterozygous sequences in the region analysed. Our strategy then changed to combine a group-specific primer with a locus-specific primer followed by cycle-sequencing the desired number of exons (on both strands). Ideally, each allele will be amplified and sequenced separately providing an unambiguous allelic HLA type.

The number of exons sequenced depends on the locus: e.g. HLA-A, B exons 1–4; HLA-C exons 1–7; HLA-DQB1, DRB1 exons 2 and 3. We have not extended this strategy to include other loci such as HLA-DPA1, DPB1, DQA1 or DRB3/4/5 as typing resolution using Luminex is usually sufficiently high enough.

Here we present a comprehensive HLA sequencing strategy for use in Sanger sequencing but could also be applied to current NGS technologies. The strategy includes all amplification and sequencing primers, in Supplementary Tables 1 and 2, to enable unambiguous high resolution typing of HLA-A, B, C, DRB1 and DQB1.

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2. Materials and methods

- Samples received were whole blood or DNA. DNA was extracted from whole blood by various conventional methods and used at 20–50 ng/μl. Absorbance at A260/A280 was in the range of 1.5–2.0. 1st-field ('low') resolution HLA types were obtained by Luminex-based LifeCodes PCR-SSO (Immucor).
- The class I PCR enzyme is a combination of proof reading and standard Taq DNA polymerases ('Expand', Roche Diagnostics) whilst class II uses AmpliTaq Gold DNA polymerase (Thermo Fisher). The PCR conditions and parameters are shown in Table 1. Many different thermal cyclers have been used for these protocols but must be optimised for each laboratory's use.
- Primers were designed against IMGT Alignment 3.23 and manufactured by Thermo Fisher custom DNA oligo service (Thermo Fisher Scientific Inc., Waltham, MA, USA). Amplification primers are recorded in Supplementary Table 1 with the list of allele groups predicted to be amplified by a given primer set according to sequence matches at the 3′ primer ends. Annealing locations are given according to the IMGT genomic DNA alignment for each gene. HLA class I sense-strand primers are group-specific and anneal within exon 1, intron 1 or the 5′UTR. The antisense primer is gene-specific (universal) annealing within the 3′UTR. HLA class II amplification primer pairs amplify exons 2 and 3 separately by using group-specific primer pairs in introns 1 and 2 (exon 2) and introns 2 and 3 (exon 3). The majority of HLA-DRB1 sense primers are mismatched to the target sequence at the 5th nucleotide from the 3′ end to increase stringency as according to Kotsch et al. (1999). Cycle sequencing primers are described in Supplementary Table 2. HLA class I primers are universal for all allele groups. Class II primers may differ depending on the allele group of the fragment to be sequenced.
- Excess primers and dNTPs are removed from amplicons with exoSAP-IT (Affymetrix) using the manufacturer's protocol with amplicons diluted 1:10 (class I) or 1:4 (class II). Cycle sequencing of treated amplicon (3 μl) with dye-labelled di-deoxy chain terminating inhibitors was carried out according to manufacturer's instructions (Thermo Fisher) with 1 μl 3.2 μM sequencing primer. The cycle sequencing protocol was the 'standard' one recommended by the manufacturer: 96 °C for 1 min followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Unincorporated dyes were removed using CleanSeq™ beads (Beckman Coulter) according to the manufacturer's protocol automated with a Biomek 3000 robot (Beckman Coulter). Sequenced fragments were separated using an ABI3130 Genetic Analyser.
- A number of different software tools have been used to compile and analyse HLA nucleotide sequences derived from the strategies described here including MatchTools (Applied Biosystems), Assign (Conexio) and SBTengine (GenDx). Validation of the most recent update to this method has been performed with SBTengine v3.11.

Table 1
PCR conditions and parameters for HLA class I and class II amplifications.

HLA-A, B, C		HLA-DRB1, DQB1	
Reagent	Volume (μl)	Reagent	Volume (μl)
Expand 10× buffer	2.5	AmpliTaq 10× buffer	2.5
dNTP 10 mM each	1.0	dNTP 10 mM each	1.0
25 mM $MgCl_2$	1.8	25 mM MgCl2	1.8
DMSO	2.5	Betaine 5 M	2.5
Expand Polymerase	0.4	AmpliTaq Gold Polymerase	0.2
DNA 20 ng/μl	6.0	DNA 20 ng/μl	6.0
Primers 5 μM each	1.0	Primers 5 μM each	1.0
H_2O	9.8	H_2O	10.0
TOTAL	25	TOTAL	25

Class I PCR program	Class II PCR program
Initial denaturation at 94 °C, 2 min	Initial hot start 96 °C, 12 min
5 cycles of	10 cycles of
94 °C, 20 s	96 °C 30 s
70 °C, 15 s	65 °C 30 s
72 °C, 3 m, 30s	72 °C 1 min
5 cycles of	15 cycles of
94 °C, 20 s	96 °C 30 s
68 °C, 15 s	61 °C 30 s
72 °C, 3 m, 30s	72 °C 1 min
25 cycles of	15 cycles of
94 °C, 20 s	96 °C 30 s
66 °C, 15 s	57 °C 30 s
72 °C, 3 m, 30s	72 °C 1 min
Final extension 72 °C, 10 min	Final extension 72 °C, 5 min

3. Results

In the early incarnations of this sequencing strategy, both HLA alleles were amplified together in a gene-specific manner, exons 2 and 3 for class I and exon 2 for class II. In this updated strategy, heterozygous alleles are amplified in isolation using group-specific primers. Exons 2–4 of HLA-A and B, exons 2–6 of HLA-C and exons 2 + 3 of HLA-DRB1 and DQB1 are sequenced in both directions. HLA-C exon 7 is sequenced twice on the sense strand, and HLA-A exon 4 is partially sequenced on the antisense strand as the antisense primer anneals within the 3′ end of the exon.

The methods and strategies described here have been evaluated using Quality Assurance samples and the methods finally presented here have been evaluated using DNA provided by UK NEQAS for H&I Educational typing scheme (https://neqas.welsh-blood.org.uk), templates that had previously been HLA typed to at least 2nd field and assessed by NEQAS according to the consensus of all participating laboratories. This method was also validated against 10 samples of unknown HLA type provided by the UCLA International DNA Exchange Scheme (http://pathology.ucla.edu/) August 2016 and November 2016 send-outs. These validations showed 100% concordance between our SBT results and the consensus results from the Quality Assurance schemes.

Despite best efforts, ambiguous allele combinations may still be found which require additional primer sets to resolve. Allele ambiguities arise when one or more alleles differ outside of the region sequenced while genotype ambiguities are heterozygous combinations of alleles which have the same nucleotide sequence in the region sequenced. Fig. 1 shows an example of genotype ambiguities and how to resolve these for a sample which is HLA-A*02, 24. This sample was sequenced, exons 1–4, in 2011 with both alleles present (Fig. 1A) or the A*02 allele was amplified and then sequenced in isolation (Fig. 1B). Sequencing both alleles gives 11 possible heterozygous combinations which is equivalent to a low resolution result (Fig. 1A). Sequencing the amplified A*02 allele in isolation shows that A*02:01:01 is present and therefore the full result for this sample is A*02:01:01/02 L, 24:02:01:02L. Admittedly, with this sequencing strategy we cannot exclude A*02:01:01:02L but this is a very rare allele.

4. Discussion

Over a period of almost 20 years variations of the SBT strategy described here have been used to unambiguously type HLA alleles and characterize new alleles. Incarnations of the methodology have been fully validated in laboratories in UK and New Zealand and passed EFI and ASHI Accreditations there, respectively.

In the initial development of this sequencing based typing strategy between 1998 and 2003 many samples were received from UK HLA laboratories which gave equivocal PCR-SSP types. In this period such laboratories were experiencing DNA typing for the first time, many following the "Phototyping" SSP protocol developed in Oxford (Bunce et al., 1995) and comparing their DNA results with their established serological techniques. Such a 'Reference Service' helped these laboratories identify and characterize many new alleles which are listed in