Journal of Immunological Methods 465 (2019) 72–76

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical Note

Simple, rapid and inexpensive typing of common HLA class I alleles for immunological studies

Soi Cheng Law[a,1], Oscar L. Haigh[b,2], Carina M. Walpole[b], Colm Keane[a], John J. Miles[c], Maher K. Gandhi[a,1], Kristen J. Radford[b], Raymond J. Steptoe[a,*]

[a] University of Queensland Diamantina Institute, The University of Queensland, Translational Research Institute, Woolloongabba, Queensland, Australia
[b] Mater Research UQ, Translational Research Institute, Woolloongabba, Queensland, Australia
[c] Australian Institute of Tropical Medicine & Health, James Cook University, Cairns, Queensland, Australia

ARTICLE INFO

Keywords:
HLA typing
PCR
Flow cytometry.

ABSTRACT

Current HLA-typing methods are typically designed to provide exquisitely-detailed identification of multiple HLA-alleles to satisfy the requirements for organ and bone marrow transplantation or genetic studies. Many human immunological studies, on the other hand, focus around only a small number of HLA alleles that are abundant or of relevance to specific diseases. Consequently, for such studies, many HLA typing approaches are not cost-effective and are potentially complicated, slow and not easily performed in-house. Work-flow would be streamlined by a simple, inexpensive and rapid typing method able to be performed in-house. We outline a straightforward approach that provides appropriate data for much immunological research. In a predominantly Caucasian population, flow cytometry using anti-HLA-A2, -B8 and -B7 antibodies consistently and accurately screened for samples carrying the highly-abundant HLA class I alleles HLA-A*02:01, -B*08:01 and -B*07:02 that form the focus of immunological studies. Next, we describe a straightforward and simple strategy for design and use of allele-specific PCR primers to identify, at high-resolution, alleles of interest. When combined with a simple gDNA extraction technique this provides reliable, simple and inexpensive in-house HLA typing demonstrated here for highly-abundant HLA class I alleles.

1. Introduction

Human leukocyte antigen (HLA) genes encode immunologically-important proteins that present antigens to T cells or play other important roles in immunity. For many immunological studies, including humanized mouse models, MHC multimer analysis of antigen-specific T cells and many *in vitro* assays, the samples used must express defined HLA alleles, therefore requiring some form of HLA typing to be performed. For HLA class I, the most frequently represented allele is HLA-A*02:01 (allele frequency: 45–60%) across many populations (Samandary et al., 2014), HLA-B*08:01 (14%–35%) and HLA-B*07:02 (9%–19%) are also highly represented in Caucasian, sub-Saharan and North American Indian populations (Samandary et al., 2014; Gonzalez-Galarza et al., 2015) and HLA-A*24:02 is common in Japanese populations (Eura et al., 1999). Immunological studies frequently focus around these most common alleles because sample collection is simplified and outcomes are applicable to a wide section of the relevant population. Additionally, reagents such as antigen-specific MHC tetramers and genetically-modified mice expressing human HLA are also typically focused on the more abundant alleles. Significant strides have been made in HLA typing that parallel and are a consequence of the advances in solid organ and bone marrow transplantation. In line with this most HLA typing methods are adapted to provide information for transplant as well as genetic studies where identification of the widest range of alleles possible is a priority.

DNA-based molecular methods currently predominate for HLA typing. Polymerase chain reaction (PCR) with sequence-specific primers (PCR-SSP) or sequence-specific oligonucleotide probes (PCR-SSO) have been widely-used. By multiplexing primers, PCR-SSP can be used to simultaneously screen for a large number of alleles (Bunce et al., 1995) either at high or low resolution (Bontadini, 2012). This method is most suited to rapid testing of small numbers of samples at low resolution, whereas PCR-SSO which uses oligonucleotide probes to detect polymorphisms within particular loci is more suited to high-throughput

* Corresponding author at: UQ Diamantina Institute, Level 6, Translational Research Institute, 37 Kent Street, Woolloongabba, Queensland 4102, Australia.
E-mail address: r.steptoe@uq.edu.au (R.J. Steptoe).
[1] Present address: Mater Research UQ, Translational Research Institute, Woolloongabba, Queensland, Australia.
[2] Present address: Infectious Disease Models and Innovative Therapies (IDMIT), CEA, 18 rue Panorama - BP 6, 92,265, Fontenay-aux-Roses Cedex, France.

https://doi.org/10.1016/j.jim.2018.12.002
Received 14 August 2018; Received in revised form 3 December 2018; Accepted 5 December 2018
Available online 08 December 2018
0022-1759/

typing of large numbers of samples, but is more complex and slower (Bontadini, 2012). Sanger and next-generation sequencing (NGS) provide exquisite resolution of HLA alleles down to single nucleotide polymorphisms and are increasingly employed (Bontadini, 2012; Erlich, 2015). But, for some immunological studies, commercially-available kits screen for a vastly more diverse range of alleles than required or use specialized equipment not typically available in most laboratories whereas sequencing provides a level of detail and operates on a scale and at a cost typically beyond that suited to many immunological studies. For immunological studies, many researchers need only focus on a single allele or small number of alleles of interest. Under these circumstances widely-used, standard HLA typing procedures, often accessible only through service providers, are not cost-effective, potentially cumbersome and inefficient and can requiring 'batching' of samples with slow turn-around times for results. Together, this impairs ready access to typing or introduces unnecessary costs and complications. To expedite small-scale immunological studies, there is a need for a reliable, rapid, simple and cost-effective method that can be performed in-house. Additionally, many researchers new to human immunological research are often uncertain how to efficiently proceed through developing this aspect with minimal published literature to guide them. Here, focused around a Caucasian population relevant to many studies, but also relevant to other populations with some adaptation we collated simple procedures and describe an approach for typing common HLA class I alleles which is rapid and convenient and that is suitable for many immunological studies.

2. Results & discussion

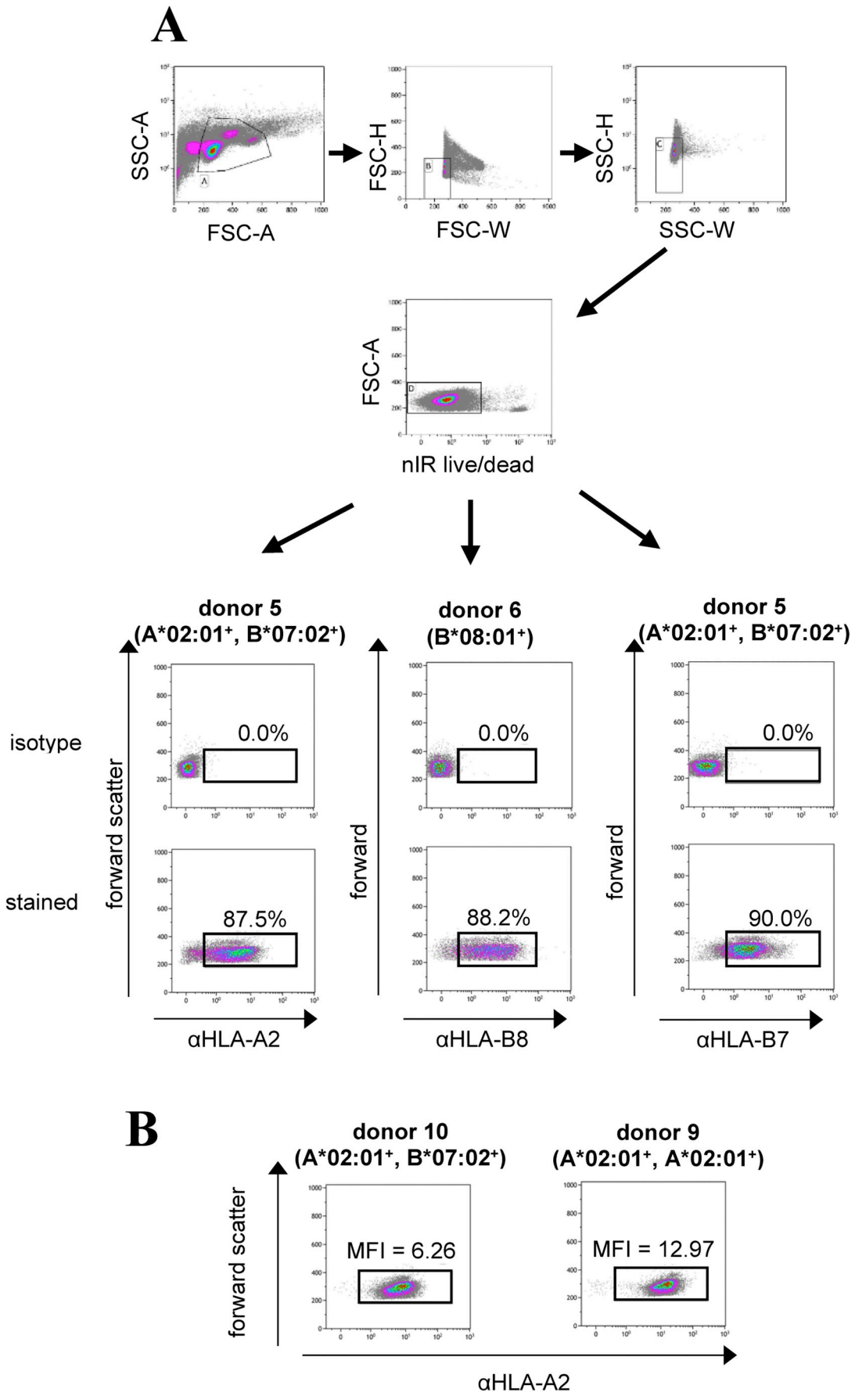
2.1. Flow cytometry-based typing

We first tested the effectiveness of typing using flow cytometry and anti-HLA antibodies. Peripheral blood mononuclear cells (PBMC) were prepared by Ficoll-Paque density separation from 19 buffy coats (Australian Red Cross Blood Bank), 29 lymphoma patients and 12 healthy donors. In some instances, frozen PBMC were thawed for analysis (Supplementary Methods). PBMC were stained with near-infrared live/dead stain (Life Technologies, 10 min on ice), washed with FACS wash (PBS/0.1% BSA/2 mM EDTA), stained (20–30 min on ice) with combinations of αHLA-A2-FITC (Biolegend, BB7.2, supplementary methods), αHLA-B8-APC (Miltenyi Biotec, REA145) and αHLA-B7-PE (Biolegend, BB7.1), relevant isotype control antibodies or left unstained as required, washed again (FACS wash) and fixed (4% paraformaldehyde). Cytometry data were acquired using a BD LSRFortessa X-20 (BD Bioscience) and analyzed (Supplementary Fig. 1) using Kaluza software (Beckman Coulter). Staining of HLA-A2, HLA-B7 and HLA-B8 was consistently distinguishable from background staining (Fig. 1A, Supplementary Fig. 1). To validate the flow-cytometry we analyzed 9 previously-typed samples (supplementary methods) and found complete concordance between the previously-defined HLA-class I typing for HLA-A2, HLA-B8 and HLA-B7 (4, 3, 2 samples respectively). Interestingly, for 3 HLA-A*02:01 homozygous individuals tested, A2 staining was more intense (Fig. 1B) than for HLA-A*02:01 hemizygous and presumptive hemizygous individuals analyzed in parallel (MFI: 13.1 ± 1.0, $n = 3$ *vs* 4.7 ± 1.2, $n = 4$ respectively). While not extensively tested here, this may aid in distinguishing HLA-A2 homozygous and hemizygous individuals. For samples we tested that were not previously typed, of 60 analyzed for HLA-A2 and HLA-B8, 36 (60.0%) were deemed HLA-A2^{+ve} and 13 (21.7%) HLA-B8^{+ve}. Of 31 samples tested for HLA-B7, 9 (29%) showed positive staining. Overall, the frequencies of each allele detected conformed to expectations for the population tested (Samandary et al., 2014; Gonzalez-Galarza et al., 2015). For some studies, or for screening a large number of samples, antibody-based approaches that provide two-digit HLA allele resolution may be sufficient to identify those samples appropriate for use or to be further processed for higher-resolution typing. An advantage of flow cytometry-based typing is its convenience for many immunological laboratories and, as results can be obtained under ideal circumstances within 1–2 h, its rapidity.

2.2. PCR-based typing

Although, HLA-A*02:01 is highly represented in most populations (Samandary et al., 2014; Gonzalez-Galarza et al., 2015), HLA-A2^{+ve} samples identified by flow cytometry might contain HLA-A2 alleles other than HLA-A*02:01 at a low frequency. This also extends to lower-frequency alleles for HLA-B7 and HLA-B8. Therefore, to provide higher resolution and validate flow-based identification of HLA-A2^{+ve} HLA-B7^{+ve} and HLA-B8^{+ve} samples, we set out to define a simple PCR-based approach for this purpose. As individual allele-specific PCRs would be suitable for most users and simplifies primer design, PCR primers (Table 1) were designed to amplify products from HLA-B*08:01 and HLA-B*07:02. Nucleotide sequences for the target HLA class I alleles were obtained from IPD-IMGT/HLA database and an NCBI BLAST search was used to define HLA alleles with closely related sequences. Genomic DNA sequences for the target alleles were then compared to those of the closely-related HLA-alleles defined by the BLAST search and primers designed for highly polymorphic regions such that primers unique to the target alleles were generated. Primer sequences were designed either manually or using Primer-BLAST (full details in Supplementary Methods). Primers for HLA-A*02:01 were as reported previously (Najima et al., 2016) but subject to the testing we describe for the primers designed here. Primers were tested and selected as described in Supplementary Methods and Supplementary Fig. 2. Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as an internal control. PCRs used individual 25 µl reactions (2.5 µl 10× Coral Load PCR buffer [Qiagen], 0.5 µl 10 mM dNTPs [Fisher Biotec], 0.5 U Taq polymerase [Qiagen], 0.5 µl each of 10 pmol/µl forward and reverse primers [IDT], 2 µl (20 ng) gDNA template and 18.9 µl ultrapure water [Invitrogen]). Reactions were denatured (95 °C, 5 min), amplified (30 cycles x [denaturation: 30s @ 95 °C; annealing: 30s as per Table 1; extension: 60s @ 72 °C]) and held at 4 °C (Eppendorf Pro S). Products were separated on a 2% agarose gel (90 V, 35 min) and visualized with GelRed dye (Fisher Biotec). The HLA class I primer sets selected (Table 1) from several designed each showed specificity for the targeted HLA class I allele and amplified a product of the expected size across 7 samples previously typed by Sanger sequencing without evidence of cross-reactivity (*e.g.* Supplementary Fig. 2) and with strong product amplification at optimized annealing temperatures (Fig. 2A, Supplementary Fig. 2).

We next performed a specificity check for the primers selected after preliminary testing. Using Primer-BLAST (Supplementary Methods). This revealed a series of mostly rare or lowly-abundant alleles from which the primers would also amplify a product (Supplementary Fig. 3). To understand how this might influence the confidence of our typing outcomes we checked the relative abundance of the alleles for which there was 'potential cross-reactivity'. The closely-related alleles where 'cross-reactivity' was possible were almost all absent or present only at extremely low frequencies within the Caucasian or related populations (Supplementary Fig. 3). In particular this was noted for the HLA-A*02, HLA-B*07 and HLA-B*08 supertypes. Conveniently, use of the flow cytometric analyses described above would distinguish 'cross-reactivities' within other supertypes (e.g HLA*A01:01 and HLA A*26:03 for HLA A*02:01, Supplementary Fig. 3) and the combination of flow cytometry and simple PCR s facilitates a high degree of confidence that the intended HLA class I alleles have been identified. For the initial primer design steps we used the nucleotide (protein-coding) sequences from the IPD-IMGT/HLA database, but use of genomic sequences for this might facilitate ready primer design. Additionally, primer design might also be facilitated by seeking primer sites within the highly polymorphic α1 and α2 domains (Parham et al., 1988).



(caption on next page)

Fig. 1. Flow cytometry for low-resolution HLA typing. PBMC samples were stained and analyzed by flow cytometry as described. Shown are representative flow cytometry plots for staining obtained with (A) αHLA-A2 FITC, αHLA-B8 APC and αHLA-B7 PE and isotype control antibodies or (B) or HLA-A2 staining for HLA-A2 hemizygous (left) and homozygous (right) individuals.

2.3. Simple gDNA preparation

Several procedures can be used to isolate gDNA for analysis. Commercially-produced kits give gDNA of high quality, but the approach can be complicated, with an enzymatic digestion step that contributes additional complexity and cost. An alternative is the simple and fast HotSHOT method (Truett et al., 2000). This employs a 3-step process with inexpensive alkaline lysis and neutralising reagents and a short, 30 min incubation. To compare HotSHOT with a widely-used commercially-available kit, gDNA was extracted from duplicate PBMC samples using the DNeasy Blood and Tissue Kit (Qiagen) or the HotSHOT method. gDNA template from the HotSHOT or DNeasy Blood and Tissue Kit isolations gave comparable results across different primer sets and with perhaps marginally better PCR products from HotSHOT (Supplementary Fig. 4). We find that for HotSHOT, if gDNA archiving is required samples should be stored at -30 °C. Thus, gDNA isolation can be simplified with significantly (> 100-fold) reduced cost.

2.4. Conclusions

Across 60 PCRs performed using template from DNeasy (19 samples) or HotShot (41 samples) methods, we found 100% concordance between flow cytometry for HLA-A2 and PCR for HLA-A*02:01, HLA-B8 and HLA-B*08:01 and HLA-B7 and HLA-B7*07:02 (Fig. 2). The high concordance for HLA-A2/HLA-A*02:01, HLA-B8/HLA-B*08:01 and HLA-B7/HLA-B*07:02 likely reflects the preponderance of A*02:01, B*08:01 B*07:02 over other HLA-A2, HLA-B8 and HLA-B7 alleles within the predominantly Caucasian population tested. For example, in North American Caucasian populations, HLA-A*02:01 represents > 85–95% of HLA-A2 alleles, but is less in other populations (Player et al., 1996; Ellis et al., 2000; Cao et al., 2001), HLA-B*08:01 was the only HLA-B8 allele detected in one study (Cao et al., 2001) and HLA-B*07:02 may comprise ~90% of HLA-B7 alleles (Mack et al., 2009). We found that in the population tested, flow-cytometry based screening was effective at identifying at two-digit resolution HLA class I alleles that were common in the test population.

Overall, we conclude flow cytometry serves as an excellent screening tool to define samples that might be progressed to expensive NGS typing and, as desired, to quickly allocate samples for experimental use when rapid typing outcomes are required. Under some circumstances, such as for HLA-A2, HLA-B8 and HLA-B7 in Caucasian populations, flow cytometry may provide sufficient confidence without molecular-level confirmation for small-scale immunology laboratory research studies not requiring variant subtyping. Further DNA-based techniques can be used for higher resolution allocation of alleles if required. We described primers for HLA-A*02:01, HLA-B*08:01 and HLA-B*07:02 and an approach to primer design that enables simple routine PCR screening. Because of the single-allele primer set approach, primers amplifying DNA sequences for each HLA allele of interest are readily identified and the likelihood of cross-reactivity reduced. Careful selection of primer sets, so that any cross-reactivities are limited to alleles that are either absent or very rare within the target sample population can significantly improve the degree of confidence for correct HLA allele identification. This worked very effectively for the predominantly Caucasian population studied here. Alternatively, if distinction is required between specific HLA class I alleles, additional PCRs using primers designed around polymorphisms that enable these distinctions to be made could be performed to enable this. It might be possible to further develop the approach outlined here for multiplexing if a small number of specific alleles were of interest, thereby generating an assay more akin to commercially-available multiplexed PCR-SSP approaches,

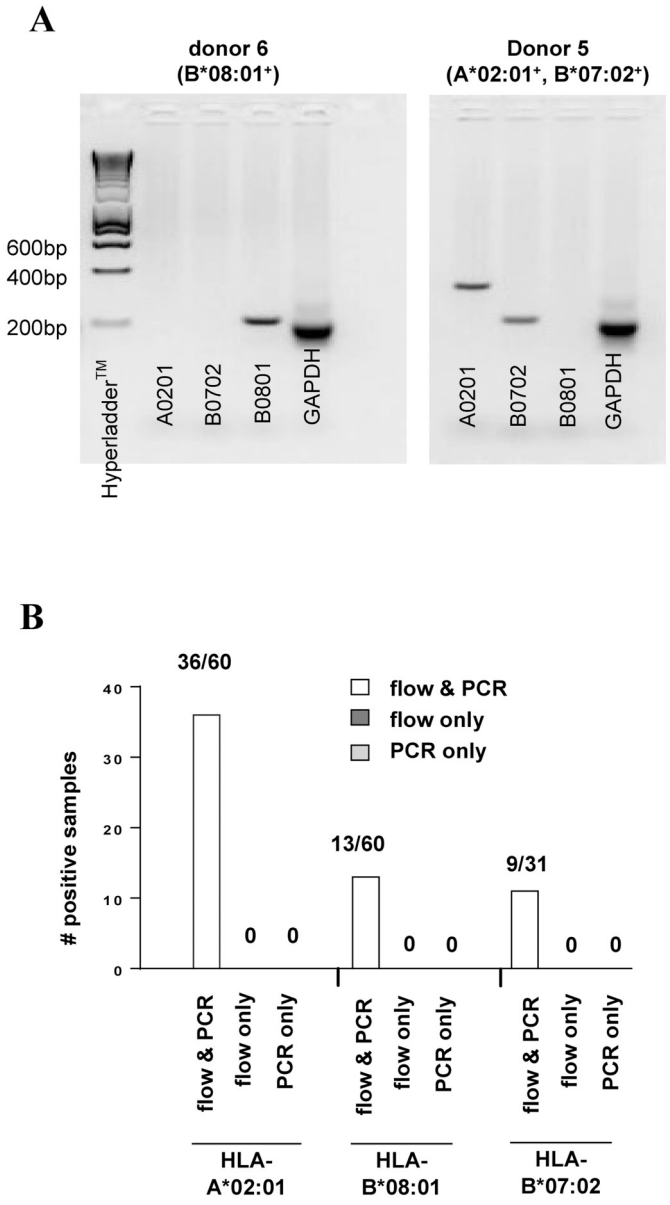


Fig. 2. Concordance of PCR typing for HLA-A*02:01, HLA-B*08:01 and HLA-B*07:02 with flow cytometry. A) gDNA was isolated and PCR performed and visualized as described. Shown are representative PCR gel images for samples extracted using the HotSHOT method. B) Extent of concordance between flow cytometry and PCR detection of common MHC class I alleles. Incorporated are data for PCR with gDNA isolated using DNeasy Blood and Tissue Kit (19 samples) or HotSHOT (41 samples) methods.

but this would make primer design significantly more complex, reducing the overall simplicity. The primer design procedure outlined here could be extended to allow rapid screening for more diverse HLA types that are potentially of interest to others, for example HLA-A*24:02 (Supplementary Fig. 5). Additionally, it is conceivable that by careful design (Dunkley, 2012), it might be possible to extend primer selection for six digit typing where single nucleotide polymorphisms define individual alleles, but this would require further development and validation.

For many studies, depending on the desired goals, rapid sample

Table 1
Primer sequences for HLA-A*02:01, HLA-B*08:01, HLA-B*07:02 and GAPDH. Primers were designed, checked and optimized as detailed in Supplementary Methods.

Primer[a]	Sequence (5′-3′)	Anneal	Expected product
A0201_717F	AGACTCACCGAGTGGACCTG	67.5 °C	333 bp
A0201_1049R	GTCGCAGCCATACATCCTCT		
B0801_1009F	CACCCTCCAGAGCATGTACG	67.5 °C	209 bp
B0801_1217R	TGCCCTCCAGGTAGGCTCTGTC		
B0702_492F	ACTCCATGAGGTATTTCTACACCT	63.2 °C	210 bp
B0702_701R	TCTGTGCCTGGGCCTTGT		
GAPDH_F	GGTATCGTGGAAGGACTCATGAC	61 °C	380 bp
GAPDH_R	ATGCCAGTGAGCTTCCCGTTCAGC		

[a] Primer positional annotations are denoted for genomic sequence.

screening with flow cytometry, followed by confirmatory PCR where desired, would rapidly, cost effectively and with high-specificity define the common HLA class I alleles we tested here. Importantly, these alleles are the focus of many *ex-vivo* studies. The value of the typing approach we describe here is, that by combining a series of relatively simple procedures and focusing on common HLA class I alleles used for many immunological studies, we estimate the cost per sample can be reduced up to one twentieth compared to commercial outsourcing of typing or one-percent of the cost for NGS-based typing (that also requires bioinformatics support), whilst maintaining sensitivity and specificity. Advantageously, no special equipment not readily accessible to most immunology-focused laboratories is required and the procedures are readily performed in-house maximizing flexibility. Overall, based on our studies we provide details of primer sets we believe can be used effectively to provide rapid and simple identification of HLA-A*02:01, HLA-B*08:01 and HLA-B*07:02 depending on the end-user's requirements. Our findings simplify, expedite and reduce cost for typing of common HLA class I alleles for immunological studies and will be suitable for many studies.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2018.12.002.

Author contributions

SCL, OLH and CMW designed and performed experiments. CK, JJM, and MKG provided samples. SCL, KJR, RJS wrote the manuscript.

Funding

This work was supported by the Cancer Council Queensland (1098690). RJS was supported by a University of Queensland Vice Chancellor's Senior Research Fellowship. KJR was supported by a Mater Foundation Senior Research Fellowship. OLH was supported by US Department of Defense grant W81XWH-15-0451. CK and MKG were supported by the Leukemia Foundation.

Competing interests statement

The authors declare no competing interests.

Acknowledgements

We thank Professor Scott Burrows and Dr. Rebekah Brennan (QIMRB) for providing gDNA or samples from HLA-typed individuals. The authors thank the TRI Flow Cytometry Core Facility and Dr. David Sester for excellent flow cytometry support.

References

Bontadini, A., 2012. HLA techniques: typing and antibody detection in the laboratory of immunogenetics. Methods 56, 471–476.
Bunce, M., O'Neill, C.M., Barnardo, M.C., Krausa, P., Browning, M.J., Morris, P.J., Welsh, K.I., 1995. Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). Tissue Antigens 46, 355–367.
Cao, K., Hollenbach, J., Shi, X., Shi, W., Chopek, M., Fernandez-Vina, M.A., 2001. Analysis of the frequencies of HLA-A, B, and C alleles and haplotypes in the five major ethnic groups of the United States reveals high levels of diversity in these loci and contrasting distribution patterns in these populations. Hum. Immunol. 62, 1009–1030.
Dunkley, H., 2012. HLA Typing by SSO and SSP Methods. In: Christiansen, F.T., Tait, B.D. (Eds.), Immunogenetics: Methods and Applications in Clinical Practice, Methods in Molecular Biology. Vol. 882. Springer Science+Business Media, New York, pp. 9–25.
Ellis, J.M., Henson, V., Slack, R., Ng, J., Hartzman, R.J., Katovich Hurley, C., 2000. Frequencies of HLA-A2 alleles in five U.S. population groups. Predominance Of A*02011 and identification of HLA-A*0231. Hum. Immunol. 61, 334–340.
Erlich, H.A., 2015. HLA typing using next generation sequencing: an overview. Hum. Immunol. 76, 887–890.
Eura, M., Katsura, F., Oiso, M., Obata, A., Nakano, K., Masuyama, K., Ishikawa, T., 1999. Frequency of HLA-A alleles in Japanese patients with head and neck cancer. Jpn. J. Clin. Oncol. 29, 535–540.
Gonzalez-Galarza, F.F., Takeshita, L.Y., Santos, E.J., Kempson, F., Maia, M.H., Silva, A.L., Silva, A.L., Ghattaoraya, G.S., Alfirevic, A., Jones, A.R., Middleton, D., 2015. Allele frequency net 2015 update: new features for HLA epitopes, KIR and disease and HLA adverse drug reaction associations. Nucleic Acids Res. 28, D784–D788.
Mack, S.J., Tu, B., Lazaro, A., Yang, R., Lancaster, A.K., Cao, K., Ng, J., Hurley, C.K., 2009. HLA-A, -B, -C, and -DRB1 allele and haplotype frequencies distinguish eastern European Americans from the general European American population. Tissue Antigens 73, 17–32.
Najima, Y., Tomizawa-Murasawa, M., Saito, Y., Watanabe, T., Ono, R., Ochi, T., Suzuki, N., Fujiwara, H., Ohara, O., Shultz, L.D., Yasukawa, M., Ishikawa, F., 2016. Induction of WT1-specific human CD8+ T cells from human HSCs in HLA class I Tg NOD/SCID/IL2rgKO mice. Blood 127, 722–734.
Parham, P., Lomen, C.E., Lawlor, D.A., Ways, J.P., Holmes, N., Coppin, H.L., Salter, R.D., Wan, A.M., Ennis, P.D., 1988. Nature of polymorphism in HLA-A, -B, and -C molecules. Proc. Natl. Acad. Sci. U. S. A. 85, 4005–4009.
Player, M.A., Barracchini, K.C., Simonis, T.B., Rivoltini, L., Arienti, F., Castelli, C., Mazzocchi, A., Belli, F., Parmiani, G., Marincola, F.M., 1996. Differences in frequency distribution of HLA-A2 subtypes between north American and Italian white melanoma patients: relevance for epitope specific vaccination protocols. J. Immunother. Emphasis Tumor Immunol. 19, 357–363.
Samandary, S., Kridane-Miledi, H., Sandoval, J.S., Choudhury, Z., Langa-Vives, F., Spencer, D., Chentoufi, A.A., Lemonnier, F.A., Benmohamed, L., 2014. Associations of HLA-A, HLA-B and HLA-C alleles frequency with prevalence of herpes simplex virus infections and diseases across global populations: implication for the development of an universal CD8+ T-cell epitope-based vaccine. Hum. Immunol. 75, 715–729.
Truett, G.E., Heeger, P., Mynatt, R.L., Truett, A.A., Walker, J.A., Warman, M.L., 2000. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). BioTechniques 29, 52, 54.