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A Rapid Allele-Specific Assay for *HLA-A*32:01* to Identify Patients at Risk for Vancomycin-Induced Drug Reaction with Eosinophilia and Systemic Symptoms

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A Rapid Allele-Specific Assay for *HLA-A*32:01* to Identify Patients at Risk for Vancomycin-Induced Drug Reaction with Eosinophilia and Systemic Symptoms

A Rapid Allele-Specific PCR for *HLA-A*32:01* Typing

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ABSTRACT

Human leukocyte antigen (HLA) alleles have been implicated as risk factors for immune-mediated adverse drug reactions. We recently reported a strong association between *HLA-A*32:01* and vancomycin-induced drug reaction with eosinophilia and systemic symptoms (DRESS). Identification of individuals with the risk allele prior to or shortly after the initiation of vancomycin therapy is of great clinical importance to prevent morbidity and mortality, improve drug safety and antibiotic treatment options. A prerequisite to the success of a pharmacogenetic screening tests is the development of simple, robust, cost-effective single HLA allele test that can be implemented in routine diagnostic laboratories. In this study, we developed a simple, real-time allele-specific PCR for typing the *HLA-A*32:01* allele. Four-hundred and fifty-eight DNA samples including thirty *HLA-A*32:01*-positive samples were typed by allele-specific PCR. Compared to ASHI accredited sequence-based high-resolution, full allelic HLA typing, this assay demonstrates 100% accuracy, sensitivity of 100% (95% CI: 88.43% to 100%) and specificity of 100% (95% CI: 99.14% to 100%). The lowest limit of detection of this assay using the Power Up SYBR Green is 10 ng of template DNA. The assay demonstrates a sensitivity and specificity to differentiate *HLA-A*32:01* allele from closely related non-*HLA-A*32* alleles and may be used in clinical settings to identify individuals with the risk allele prior or during the course of vancomycin therapy.

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INTRODUCTION

Human leukocyte antigens (HLA) play a key role in the development of severe immune-mediated adverse drug reactions (IM-ADRs) [1]. Multiple phenotypically distinct IM-ADRs have been associated in particular with the carriage of specific HLA class I risk alleles [2]. HLA alleles are highly polymorphic genes and are mainly involved in antigen recognition as a trimolecular complex of MHC–Peptide–T-cell receptor. Thus, the presence of a specific HLA allele determines the repertoire of epitopes that can be presented, restricting the specificity of reacting T cells [3].

We recently reported a strong association between *HLA-A*32:01* and vancomycin-induced drug reaction with eosinophilia and systemic symptoms (DRESS) in patients of European ancestry and showed that approximately 20% of subjects exposed to vancomycin for more than 2 weeks can be expected to develop DRESS [4]. Vancomycin is a widely used antibiotic for the treatment of frequently encountered serious Gram-positive bacterial infections, including methicillin resistant *Staphylococcus aureus*. In reports of life-threatening T-cell–mediated reactions, such as DRESS, vancomycin features prominently in causality and recent studies suggest that vancomycin is the most common cause of antibiotic-related DRESS [5, 6]. DRESS is a rare but potentially life-threatening T-cell–mediated and multi-system disorder characterized by the delayed onset of fever, a widespread rash, white cell abnormalities, and the involvement of internal organs following administration of a drug [7, 8]. The mortality rate of DRESS is up to 10% and long- term sequalae have been described up to 4 years following acute disease [9]. Given that vancomycin is often initiated empirically as part of combination therapy in an emergent setting of severe infection and that DRESS typically takes two or more weeks of drug exposure to manifest clinically, it is proposed that the

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antibiotic should not be delayed but that *HLA-A*32:01* testing be ordered immediately and that consideration be given to substituting vancomycin for an alternative antibiotic if the test result returns positive. *HLA-A*32:01* testing may also have diagnostic utility and assist in drug causality assignment in subjects who have symptoms in keeping with DRESS improving the safety of future antibiotic therapy. A single allele assay for *HLA-A*32:01* with a turnaround time of less than two days would hence be of clinical utility and a potentially cost-effective option to improve the safety of this antibiotic that is in widespread global use.

Conventional HLA typing is done by serological or sequence-specific typing methods such as PCR amplification with use of sequence-specific oligonucleotide probes (SSO) or sequence-based typing (SBT) techniques. Standard serological approaches lack specificity, as commercially available monoclonal antibodies cross-react with different HLA alleles [10]. HLA typing using SSO sometimes results in low resolution products that are unable to resolve some HLA alleles [11]. HLA typing by SBT is able to resolve HLA alleles with high resolution but is comparatively expensive, requires specialized DNA sequencing equipment and a skilled operator for analysis, and is time-consuming to prepare and analyse results. This study tested the potential of using a simple and fast PCR assay that utilizes allele-specific PCR (AS-PCR). This AS-PCR method should be less susceptible to laboratory or analysis errors, and be easier and less expensive to implement as a clinical test for the presence and absence of carriage of *HLA-A*32:01*.

MATERIALS & METHODS

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DNA Samples

DNA samples were drawn from the Vanderbilt BioVu which represents DNA linked to a de-identified electronic health record. Genomic DNA is extracted from discarded EDTA blood samples using the Qiagen automated DNA purification kit (Qiagen Inc., USA).

The study population was selected from the Vanderbilt Electronic Systems for Pharmacogenomic Assessment (VESPA) cohort [12]. For this study, 458 DNA samples from this databank were analyzed by AS-PCR/melting curve. These DNA samples from the VESPA cohort had previously undergone high resolution, full allelic HLA typing by next-generation sequencing and in depth genotyping with structured race assignment [13]. DNA sample identity was blinded to the operator at the time of the validation of the assay. The DNA samples were of good quality with a mean DNA concentration of 50 ng/uL and a 260/280 ratio over 1.7. For this assay, the DNA concentration of samples were normalized to a concentration of 25 ng/uL with sterile deionized water (Sigma, Cat# W3500). The study sample contained a good representation of the *HLA-A*32:01* allele (n=30) with a broad range of *HLA-A*32*-closely related alleles as listed in **Table 1**.

Primers

Primers (**Table 2**) were designed within the exon 2 of *HLA-A* locus. Exon 2 DNA sequences of *HLA-A*32* group and other closest alleles (**Table 1**) from IMGT/HLA database were aligned with *HLA-A*32:01:01* using the IMGT/ HLA alignment tool (IMGT/ HLA database: https://www.ebi.ac.uk/ipd/imgt/hla/align.html, last accessed on 8th March 2019). The forward primer is a locked nucleic acid primer (LNA) and contains the sequence unique to the allele groups *HLA-A*32* (except *A*32:04* and *A*32:01:18*), *A*29* and *A*74*. The reverse primer contains the sequence of the *HLA-A*32* group (except *A*32:03*) and of the *HLA-A*25* allele group. This primer set combination specifically amplifies all *HLA-A*32* alleles, except

*A*32:03, A*32:04* and *A*32:01:18,* yielding a 157-bp product. *HLA-A*29:13* allele is the only non-*HLA-A*32* group allele that can be amplified with this primer set combination.(Fig. 1A). Alignments of exon 2 of all *HLA-B* and *HLA-C* alleles (Fig.1B) show that no other class I alleles are targeted by this primer combination, except for *HLA-B*07:27* and *HLA-C*06:02:15* which could potentially be amplified. However, there are quite a number of mismatches in the reverse primer and the frequency of these alleles is very low in the global population . Internal control primers were designed to amplify the highly conserved housekeeping gene galactosylceramidase (*GALC;* HGNC:4115), yielding a 352-bp product.

Allele-Specific PCR for Detection of *HLA-A*32:01*

The real-time PCR reaction contained 2uL (50 ng) of total DNA, 1X Power Up SYBR Green Master Mix (Thermo Fisher Scientific, Australia), 250 nM of each *HLA-A*32* specific primer, and 50 nM of each *GALC* primer in a 10-µL final volume. The master mix was dispensed on a 96 or 384-well qPCR plate using the Mantis® Liquid Handler (Formulatrix®, MA) by using a high-volume chip. DNA samples were stamped on a 96- or 384-well qPCR plate straight from the DNA storage plates using the Biomek® FXP liquid handler (Beckman Coulter, Australia).

The real-time PCR was performed in 96-or 384 well optical plates on Bio-Rad CFX96/384 qPCR machine (Bio-Rad Laboratories, Australia) using the following cycling conditions: Initial denaturation at 96 °C for 6 min to allow polymerase activation, followed by 35 cycles at 96 °C for 30 seconds and 62 °C for 1 minute. This was followed by a melting curve cycle from 65 °C to 95 °C with 0.5 °C increment for 5 seconds. The conditions of the PCR, such as primer concentrations and cycling conditions, were optimized to enable a clear separation of both *HLA-A*32* specific Tm peak compared to the internal control Tm peak during melt curve analysis.

A standard AS-PCR was performed using the same reaction conditions as for the real-time PCR. The standard AS-PCR was performed in 96-well half skirt PCR plates (AXYGEN Scientific, Australia) on the Bio-Rad C1000 thermocycler (Bio-Rad Laboratories). PCR products were analyzed by electrophoresis on a 1% agarose gel containing 0.2 μg/mL ethidium bromide and run at 115V for 30 minutes at room temperature. The gel was visualized by a transilluminator (ChemiDoc XRS+, Bio-Rad Laboratories).

Data Analysis

Raw real-time PCR data were analyzed using CFX Manager Software 3.0 (Bio-Rad Laboratories). Statistical analyses for validation studies to calculate sensitivity, specificity, and confidence intervals were performed using GraphPad Prism 5.02 for Windows (GraphPad Software Inc., San Diego, CA).

RESULTS

To validate this *HLA-A*32:01* typing assay, 458 samples previously typed using American Society for Histocompatibility and Immunogenetics (ASHI) accredited sequence-based high-resolution, full allelic HLA typing were analyzed using the real-time PCR with Power Up SYBR Green master mix. All 30 samples out of the 458 samples were accurately identified as positive for *HLA-A*32:01* allele.

Samples were called positive or negative for *HLA-A*32:01* based on the presence or absence of *HLA-A*32:01* specific melt peaks. *HLA-A*32:01*-positive samples (n=30) showed two peaks at 88.5 ℃ ± 0.0 ℃ (mean ± standard error of the mean; range: 88.50 ℃ to 88.50 ℃) for the *HLA-A*32:01* allele and 76.05 ℃ ± 0.03 ℃ (mean ± standard error of the mean; range:

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76.00 ºC to 76.50 ºC) for *GALC*. The other 428 non-*HLA-A*32* samples showed a single peak at 76.07 ºC ± 0.01 ºC (mean ± standard error of the mean; range: 75.50 ºC to 76.50 ºC) for *GALC* (Fig. 2). No melting curves were detectable for the non-template negative controls (**Table 3**).

For the standard AS-PCR, samples were called positive or negative based on the presence or absence of the *HLA-A*32:01* specific PCR product after agarose gel electrophoresis. *HLA-A*32:01* positive samples showed two bands of 157-bp (*HLA-A*32:01*) and 352-bp (*GALC*). Non-*HLA-A*32* samples showed only one band of 352-bp (*GALC*) (Fig. 2). No product was detectable for the non-template negative controls. Thus, the sensitivity and specificity of this assay for *HLA-A*32:01* allele in these 458 DNA samples were 100% (95% CI: 88.43% to 100%) and 100% (95% CI: 99.14% to 100%), respectively (**Table 4**).

DISCUSSION

This study describes a simple, fast, and inexpensive PCR assay that utilizes AS-PCR with melt curve analysis for the detection of *HLA-A*32:01* allele. By using a combination of primers that included a LNA forward primer, the *HLA-A*32:01* allele was specifically amplified in a real-time PCR. The assay was both 100% sensitive and specific making it safe and appropriate for clinical use. The assay was able to exclude all non-*HLA-A*32:01* alleles included in the validation of this study. It is worth mentioning that the primer set combination could amplify the *HLA-A*29:13* allele. However the frequency of this allele is very low at around 0% in both Caucasian and African American population (http://www.allelefrequencies.net, last accessed on 8th March 2019).

In addition, alignments of exon 2 of all *HLA-B* and *HLA-C* alleles (Fig.1B) show that no other class I alleles are targeted by this primer combination, except for *HLA-B*07:02:27* and

*HLA-C*06:02:15* which may be amplified. However, there are quite a number of mismatches in the reverse primer which may prevent their amplification. Futhermore, the frequency of these alleles is very low within the global population (http://www.allelefrequencies.net, last accessed on 15 January 2019). Samples were not found in our biorepository for the validation of these two non- *HLA-A* alleles.

Amplification of low-level nonspecific products in real-time PCR has been previously reported. The formation of these nonspecific products can be influenced by annealing temperature, primer concentration, magnesium concentration, and DNA inputs [14]. In this optimized assay, non-specific amplification was not detected. Also, a LNA primer that specifically target *HLA-A*32* alleles was used. The incorporation of a LNA primer into oligonucleotide primers provides an increase of specific binding strength for target DNA amplification [15]. In addition, a commercial optimized real-time PCR master mix (Power Up SYBR Green master mix), which is reported to be formulated for maximum specificity and reproducibility, was used. The detection range for template DNA in this assay was between 10 ng to 100 ng. However, the assay optimal DNA concentration was between 25 ng and 50 ng.

The specificity of the assay was assessed for only the *HLA-A*32:01* allele. The primer set combination was designed to specifically amplify all HLA-A*32 alleles, except *A*32:03, A*32:04* and *A*32:01:18.* Given the low frequency of other *HLA-A*32* alleles (eg, *A*32:02*, *A*32:03, A*32:04,* and *A*32:01:18*), especially in those of European ancestry[16] (http://www.allelefrequencies.net, last accessed on 15 January 2019), other non-*HLA-A*32:01* alleles were not found in our biorepository of samples primarily from those of European ancestry. Notably, across the entire Vanderbilt BioVU cohort with imputed HLA typing (N=65,638), the only *HLA-A*32* allele imputed was *HLA-A*32:01*. European ancestry

represented 85% of this cohort. Furthermore, it is currently unknown whether *HLA-A*32* alleles other than *HLA-A*32:01* are associated with vancomycin DRESS or whether there are associations between other HLA alleles and vancomycin DRESS in those of non-European ancestry. It is known for instance that the carriage rate of *HLA-A*32:01* is approximately 2% to 3% in African Americans which is half that carried in European Americans. This would mean that if the positive predictive value is equivalent to the almost 20% in European Americans, that approximately twice as many African Americans would need to be tested to prevent or pre-empt one case of vancomycin DRESS which is still a cost-effective ratio. It will be important to determine if alleles other than HLA-A*32:01 are associated with vancomycin DRESS in non-European race.

As for previous associations between drug hypersensitivity and HLA alleles such as abacavir and *HLA-B*57:01*, an allele also primarily represented in those of European ancestry, the association was specific for *HLA-B*57:01* and to-date *HLA-B*57:01* screening has a 100% negative predictive value for abacavir hypersensitivity. It has been shown that HLA-B*58:01, HLA-B*57:03, and HLA-B*57:02 which differ by as few as 2 amino acids in the antigen binding cleft are not associated with abacavir hypersensitivity [17]. HLA-A*32:01 has a leucine at position 156 which is a key peptide binding residue which differs from the glutamine at 156 for HLA-A*32:02 and HLA-A*32:03 (IPD-IMGT/HLA database; https://www.ebi.ac.uk/cgi-bin/ipd/imgt/hla/align.cgi; last accecced on 4 January 2019). On the other hand, it has been demonstrated that in some drug hypersensitivity phenotypes different alleles of the same family confer susceptibility to adverse drug reaction. This is the case of carbamazepine SJS/TEN in which in addition of the *HLA-B*15:02* risk allele, members of the same HLA-B*75 serotype could also present CBZ to activate $CD8^+$ T cells [18]. Wei et al found that three amino acid residues (Asn63, Ile95, and Leu156) in the peptide-

binding groove of *HLA-B*15:02* were involved in the presentation of CBZ to $CD8^+$ T cells. Asn63 which is shared by members of the *B*75* family was the key residue to confer this specificity [18].

High resolution HLA typing is performed by direct sequencing of HLA class I and class II sequences. Although sequence-based typing (SBT) remains the gold standard for HLA typing, it is significantly more expensive, requires significant expertise and labor, and has a longer turnaround time compared to this assay in terms of time, cost, and labor and remains the domain of specialty immunogenetics and transplant laboratories. SBT also requires sophisticated equipment, highly-trained staff, and a robust informatics and quality assurance infrastructure which might not be available in most of the clinical settings. HLA typing by hybridization of PCR amplicon with sequence-specific oligonucleotide probes (SSO) is an alternative method, but this method shows low resolution typing [19].

In comparison with current HLA typing methods in terms of time and cost, this *HLA-A*32:01* screening assay seems to be appropriate for use in clinical settings. On the cost of reagents alone, our cost calculation shows that it costs around 20 times less using this real-time PCR assay in comparison to HLA typing by sequence based typing. Time for operator setup and analysis also favors this rapid real-time assay due to the higher number of steps required for sequence based typing. A single sample can be typed for *HLA-A*32:01* with a turnaround time of less than three hours, with less than one hour of operator hands-on time with real-time PCR, whereas sequence based typing such as Sanger sequencing has a turnaround of at least eight to 10 hours with three hours of hands-on time. Ideal batch scale for sequence based typing is approximately 90 samples and adds 10 hours of data analysis whereas for real-time PCR, the reaction can scale to approximately 384 sample batches with

minimal addition of operator time when robotic setup is available. In addition, the level of operator expertise and sophisticated sequencing equipment needed for NGS might not be available in most of the clinical settings.

The assay described here appears to give both specificity and sensitivity of 100%, with the advantage of being very fast and cheaper compared to other HLA typing methods. The flexible methodology means that this could be set-up in a variety of specialty immunogenetics or non-specialty laboratory settings that have access to a PCR platform that can perform melting curve analysis. Those settings without qPCR platform can use the standard AS-PCR followed by agarose gel electrophoresis. Although limited testing was conducted on standard PCR machine with the Power Up SYBR green master mix followed by agarose gel electrophoresis analysis and gel visualization, this assay was found to successfully adapt in settings which do not have qPCR platform.

The considerable reduction of operator manual handling of post PCR amplicon and manipulation of results also reduce the potential risk of sample mix-up as well as contamination. Where testing a large number of samples is needed, the use of robotic liquid handling system for transferring master mixes and DNA samples could provide some benefits in reducing turnaround time and human errors. In cases where unusual melt curves are found due to poor purity of DNA or operator technical error, samples should be further evaluated by using conventional PCR genotyping or SBT or by obtaining another sample. However, such cases were not encountered during our validation experiments.

Currently single allele assays exist or have been published for *HLA-B*57:01, HLA-B*15:02, HLA-B*58:01, HLA-B*13:01,* and *HLA-A*31:01* [20-25]. Most of these single allele assays have

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been advocated as a pre-prescription screening strategy with specific drugs and specific populations [26, 27]. The association of *HLA-A*32:01* and vancomycin DRESS has only been recently reported. Currently, we are unaware of the existence of other available PCR-based methods specifically for the detection of *HLA-A*32:01*.

This study is therefore novel in both reporting a new single-allele test specific for *HLA-A*32:01* and also in proposing a new use approach to pharmacogenetic testing that is practical and convenient for both the clinician and the laboratory. Based on a prevalence of *HLA-A*32:01* in population of predominant European ancestry of 6.8% and estimates from that approximately 20% of those carrying *HLA-A*32:01* exposed to vancomycin for at least two weeks will develop vancomycin DRESS, it can be estimated that 75 patients would need to be tested for *HLA-A*32:01* to prevent one case of DRESS. Given the low cost and relative convenience of testing in this clinical context and the severe implications of DRESS, this number needed to test should be sufficient to justify testing from a cost-effectiveness standpoint in most populations with carriage frequencies of *HLA-A*32:01* similar to the population tested in this study. Furthermore, it would also be feasible to use this rapid-turnaround single-allele assay as an ancillary diagnostic test in patients who have developed DRESS on multiple antibiotics including vancomycin.

In conclusion, this AS-PCR is a fast and reliable method for typing the *HLA-A*32:01* allele. This assay demonstrates the sensitivity and specificity needed for the assignment of the *HLA-A*32:01* allele but caution that these assay characteristics may not be maintained with any modification to the method. In addition as per our experience with HLA-B*57:01, the development of an allele specific international quality assurance program will help minimize false positive and negative errors that may significantly impact patient safety [28].

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FIGURE LEGENDS

Figure 1: Binding sites of the set of primers and comparison of the different allele sequences of the target region. Exon 2 DNA sequences of *HLA-A*32* group and other closest alleles from IMGT/HLA database were aligned with *HLA-A*32:01:01* using the IMGT/ HLA alignment tool (IMGT/ HLA database: https://www.ebi.ac.uk/ipd/imgt/hla/align.html, last accessed on 8th March 2019). **A-B**: Primer positions are marked by **dashed boxes.** The **solid arrows** show the direction of the forward primer: HLA 32-88F and reverse primer: HLA032R2. **A:** The forward primer: HLA 32-88F: 5'-ACACGCAGTTCGTGCGGTT+T-3' is a locked nucleic acid primer (locked at position 180) and contains the sequence unique to the allele groups *HLA-A*32* (except *A*32:04* and *A*32:01:18*), *A*29,* and *A*74*. The reverse primer: HLA032R2: 5'-GAGCGCGATCCGCAGGC-3' contains the sequence of the *HLA-A*32* group (except *A*32:03*) and of the *HLA-A*25* allele group. The combination of forward and reverse primers is specific for and amplify all HLA-A*32 alleles, except *A*32:03, A*32:04,* and *A*32:01:18*. It is worth noting that *HLA-A*29:13* allele is the only non-*HLA-A*32* group allele that can be amplified with this primer combination. **B:** Alignment of exon 2 of *HLA-B* and *HLA-C* alleles (IMGT/ HLA database) shows that only HLA-*B*07:02:27* and *HLA-C*06:15* may be amplified by this primer set combination; however, there are quite a number of mismatches within the reverse primer which may prevent the amplification.

Figure 2: AS-PCR results. A: *HLA-A*32:01*-positive (+) and non-*HLA-A*32* (-) samples were amplified by PCR using the following validated primers: HLA 32-88F forward primer and HLA032R2 reverse primer to amplify the *HLA-A*32* allele. GALC-F and GALC-R primers were used to amplify the internal control housekeeping gene in a multiplexed reaction. PCR products were run on a 1% agarose gel containing 0.2 μg/mL ethidium bromide at 115V for 30 minutes. The gel was visualized by a transilluminator (ChemiDoc XRS+, Bio-Rad, Australia). *HLA-A*32:01* positive samples show two bands of 157-bp (*HLA-A*32:01* product) and 352-bp (*GALC* product). Non-*HLA-A*32* samples show only one band of 352-bp (*GALC* product). **B**: Tm peaks of both *HLA-A*32:01*

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and *GALC* housekeeping gene are shown by **a solid line arrow**. Tm peaks for the *HLA-A*32:01* allele were clearly separate from the *GALC* Tm peak following melt curve analysis. **C:** Melting peaks for a subset of the 458 samples tested in a real-time PCR with Power Up SYBR Green are shown. Tm peaks of *HLA-A*32:01* and non- *HLA-A*32:01* alleles are shown by **a solid line arrow.** *HLA-A*32:01*-positive samples (n=30) show double Tm peaks at 88.5 ºC ± 0.0 ºC (mean ± standard error of the mean; range: 88.50 ºC to 88.50 ºC) for the *HLA-A*32:01* allele and 76.05 ºC ± 0.03 ºC (mean ± standard error of the mean; range: 76.00 ºC to 76.50 ºC) for *GALC*. Non-*HLA-A*32* allele samples show a single Tm peak at 76.07 ºC ± 0.01 ºC (mean ± standard error of the mean; range 75.50 ºC to 76.50 ºC) for *GALC*. **D:** Melting curves for a subset of the 458 samples tested in real-time PCR.

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Table 1. HLA genotypes of samples eliminated by the present assay (*HLA-A*32:01* AS-PCR).

HLA-A specificities	Number
*A*01:01*	123
*A*02:01, A*02:05*	202
*A*11:01*	45
*A*23:01*	9
*A*24:02*	41
*A*25:01*	12
*A*26:01*	18
*A*29:02*	28
*A*30:01*	21
*A*31:01*	31
*A*33:01*	7
*A*66:01*	2
*A*68:01, A*68:02*	49
*A*74:01:01*	3
*A*74:11*	1

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Table 2. Primer sequences for HLA typing of *HLA-A*32:01* allele.

Primer name	**Description**	**Sequence (5´ to 3´)**	**Target**
HLA 32-88F	*HLA-A*32* forward primer	**5´**-GACGACACGCAGTTCGTGCGGTT+T-**3´**	*HLA-A*32*
HLA 032R2	*HLA-A*32* reverse primer	**5´**-GAGCGCGATCCGCAGGC-**3´**	*HLA-A*32*
GALC-F:	*GALC* forward primer	**5´**-TTACCCAGAGCCCTATCGTTCT-**3´**	*GALC*
GALC-R:	*GALC* reverse primer	**5´**-GTCTGCCCATCACCACCTATT-**3´**	*GALC*

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Table 3. Melting curve analysis of *HLA-A*32:01* and *GALC* amplicons.

	Tm peaks			
HLA alleles	HLA-A*32:01	GALC	Amplicon length (bp)	Validation
*HLA-A*32:01*	88.5	76	157	Positive
Non-*HLA-A*32*	None	76	352	Negative

Tm: melting point; *GALC*: galactosylceramidase; bp: base pairs.

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Table 4. Comparison of the present assay with SBT.

	HLA SBT		
***HLA-A*32:01* qPCR**	*HLA-A*32*	Non-*HLA-A*32*	Total
Positive	30	0	30
Negative	0	428	428
Total	30	428	458

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Figure 1

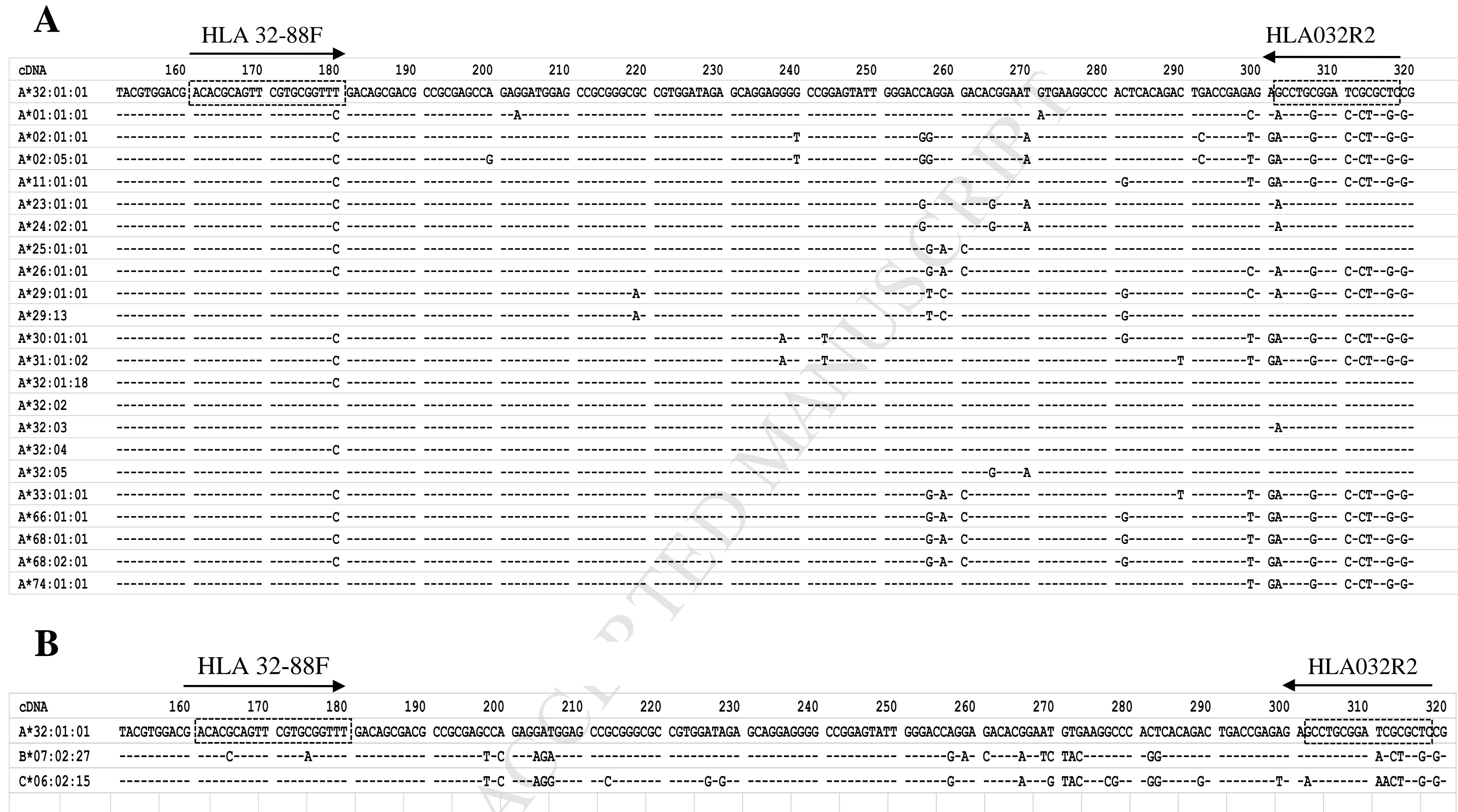

Figure 2