the **Journal of Molecular Diagnostics**

jmd.amjpathol.org

Check for updates

# 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

Francois X. Rwandamuriye,* Abha Chopra,*† Katherine C. Konvinse,‡ Linda Choo,* Jason A. Trubiano,‡§¶ Christian M. Shaffer,∥ Mark Watson,* Simon A. Mallal,*†‡ and Elizabeth J. Phillips*,**††

*From the Institute for Immunology and Infectious Diseases,\* Murdoch University, Murdoch, Western Australia, Australia; the Departments of Pathology, Microbiology and Immunology,‡ Medicine,\*\* and Pharmacology, †† and the Divisions of Infectious Diseases† and Clinical Pharmacology,∥ Vanderbilt University Medical Center, Nashville, Tennessee; the Department of Infectious Diseases and Centre for Antibiotic Allergy and Research,§ Austin Health, Heidelberg, Victoria, Australia; and the Department of Medicine,¶ University of Melbourne, Parkville, Victoria, Australia*

Human leukocyte antigen (HLA) alleles have been implicated as risk factors for immune-mediated adverse drug reactions. The authors recently reported a strong association between *HLA-A\*32:01* and vancomycin-induced drug reaction with eosinophilia and systemic symptoms. Identification of individuals with the risk allele before or shortly after the initiation of vancomycin therapy is of great clinical importance to prevent morbidity and mortality, and improve drug safety and antibiotic treatment options. A prerequisite to the success of 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, the authors developed a simple, real-time allele-specific PCR for typing the *HLA-A\*32:01* allele. Four-hundred and fifty-eight DNA samples including 30 *HLA-A\*32:01*—positive samples were typed by allele-specific PCR. Compared with American Society for Histocompatibility and Immunogenetics—accredited, sequence-based, high-resolution, full-allelic HLA typing, this assay demonstrates 100% accuracy, 100% sensitivity (95% CI, 88.43% to 100%), and 100% specificity (95% CI, 99.14% to 100%). The lowest limit of detection of this assay using PowerUp SYBR Green is 10 ng of template DNA. The assay demonstrates a sensitivity and specificity to differentiate the *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 before or during the course of vancomycin therapy. *(J Mol Diagn 2019, 21: 782—789; https://doi.org/10.1016/j.jmoldx.2019.04.006)*

Human leukocyte antigens (HLA) play a key role in the development of severe immune-mediated adverse drug reactions.[1] Multiple phenotypically distinct immune-mediated adverse drug reactions 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]

The authors 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 multisystem 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 sequelae 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 2 or more weeks of drug exposure to manifest clinically, it is proposed that the 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 <2 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 or sequence-based typing (SBT) techniques. Standard serological approaches lack specificity, because commercially available monoclonal antibodies cross-react with different HLA alleles.[10] HLA typing using sequence-specific oligonucleotide probes 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 analyze 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 and Methods

### DNA Samples

DNA samples were drawn from the Vanderbilt BioVU biorepository, which represents DNA linked to a deidentified electronic health record. Genomic DNA is extracted from discarded EDTA blood samples using the Qiagen automated DNA purification kit (Qiagen, Valencia, CA).

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 (F.X.R.) at the time of the validation of the assay. The DNA samples were of good quality with a mean DNA concentration of 50 ng/μL and a 260/280 ratio over 1.7. For this assay, the DNA concentration of samples were normalized to a concentration of 25 ng/μL with sterile deionized water (Cat# W3500; Sigma-Aldrich, Castle Hill, NSW, Australia). 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 the *HLA-A* locus. Exon 2 DNA sequences of *HLA-A\*32* group and other closest alleles (Table 1) from the IMGT/HLA database were aligned with *HLA-A\*32:01:01* using the

**Table 1**   HLA Genotypes of Samples Eliminated by the Present Assay (*HLA-A\*32:01* AS-PCR)

| HLA-A specificities | n |
| --- | --- |
| 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 |

HLA, human leucocyte antigen.

**Table 2** Primer Sequences for HLA Typing of *HLA-A*32:01* Allele

| Primer | Description | Sequence | 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* |

Plus sign marks the location of a locked nucleic acid in the primer sequence.
HLA, human leucocyte antigen.

IMGT/HLA alignment tool (IMGT/HLA database, https://www.ebi.ac.uk/ipd/imgt/hla/align.html, last accessed March 8, 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 (Figure 1A). Alignments of exon 2 of all *HLA-B* and *HLA-C* alleles (Figure 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*, that 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.
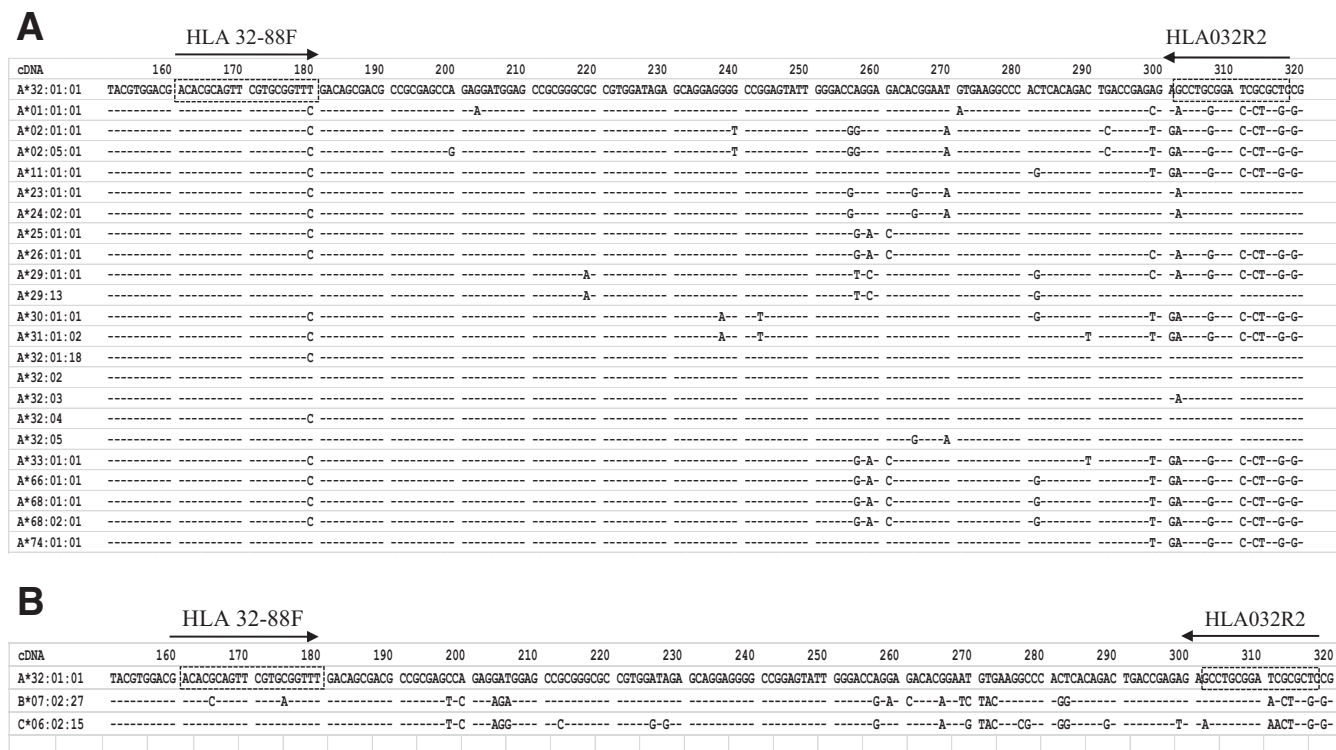


**Figure 1** Binding sites of the set of primers and comparison of the different allele sequences of the target region. Exon 2 DNA sequences of *HLA-A*32* group and other closest alleles from IMGT/HLA database were aligned with *HLA-A*32:01:01* using the IMGT/HLA alignment tool (IMGT/HLA database, https://www.ebi.ac.uk/ipd/imgt/hla/align.html, last accessed March 8, 2019). Primer positions are marked by **dashed boxes**. The **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* (plus sign marks the location of a locked nucleic acid in the primer sequence). 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 that may prevent the amplification.

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

The real-time PCR reaction contained 2 µL (50 ng) of total DNA, 1× PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Scoresby, VIC, Australia), 250 nmol/L of each *HLA-A\*32*—specific primer, and 50 nmol/L of each *GALC* primer in a 10-µL final volume. The master mix was dispensed on a 96- or 384-well real-time quantitative PCR (qPCR) plate using the Mantis Liquid Handler (Formulatrix, Bedford, 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 FX$^P$ liquid handler (Beckman Coulter, Lane Cove West, NSW, Australia).

The real-time PCR was performed in 96- or 384-well optical plates on a Bio-Rad CFX96/384 qPCR machine (Bio-Rad Laboratories, Gladesville, NSW, Australia) using the following cycling conditions: initial denaturation at 96°C for 6 minutes to allow polymerase activation, followed by 35 cycles at 96°C for 30 seconds and at 62°C for 1 minute. This was followed by a melting curve cycle from 65°C to 95°C with 0.5°C increments for 5 seconds. The conditions of the PCR, such as primer concentrations and cycling conditions, were optimized to enable a clear separation of both the *HLA-A\*32*—specific melting point (Tm) peak compared with 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, Corning, NY) 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 115 V 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 version 3.0 (Bio-Rad Laboratories). Statistical analyses for validation studies to calculate sensitivity, specificity, and confidence intervals were performed using GraphPad Prism software version 5.02 for Windows (GraphPad Software, San Diego, CA).

## Results

To validate this *HLA-A\*32:01* typing assay, 458 samples previously typed using American Society for Histocompatibility and Immunogenetics—accredited, sequence-based, high-resolution, full-allelic HLA typing were analyzed using real-time PCR with PowerUp SYBR Green Master Mix.[13] All 30 positive samples of the 458 samples were accurately identified as positive for the *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°C ± 0.0°C (means ± SEM; range: 88.50°C to 88.50°C) for the *HLA-A\*32:01* allele and 76.05°C ± 0.03°C (means ± SEM; range: 76.00°C to 76.50°C) for *GALC*. The other 428 non—*HLA-A\*32* samples showed a single peak at 76.07°C ± 0.01°C (means ± SEM; range: 75.50°C to 76.50°C) for *GALC* (Figure 2). No melting curves were detectable for the nontemplate 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*) (Figure 2). No product was detectable for the nontemplate negative controls. Thus, the sensitivity and specificity of this assay for the *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 the *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 populations (Allele Frequency Net Database, *http://www.allelefrequencies.net*, last accessed March 8, 2019).

In addition, alignments of exon 2 of all *HLA-B* and *HLA-C* alleles (Figure 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*, that may be amplified. However, there are quite a number of mismatches in the reverse primer that may prevent their amplification. Furthermore, the frequency of these alleles is very low within the global population (Allele Frequency Net Database, *http://www.allelefrequencies.net*, last accessed January 15, 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.[15] The formation of these nonspecific products can be influenced by
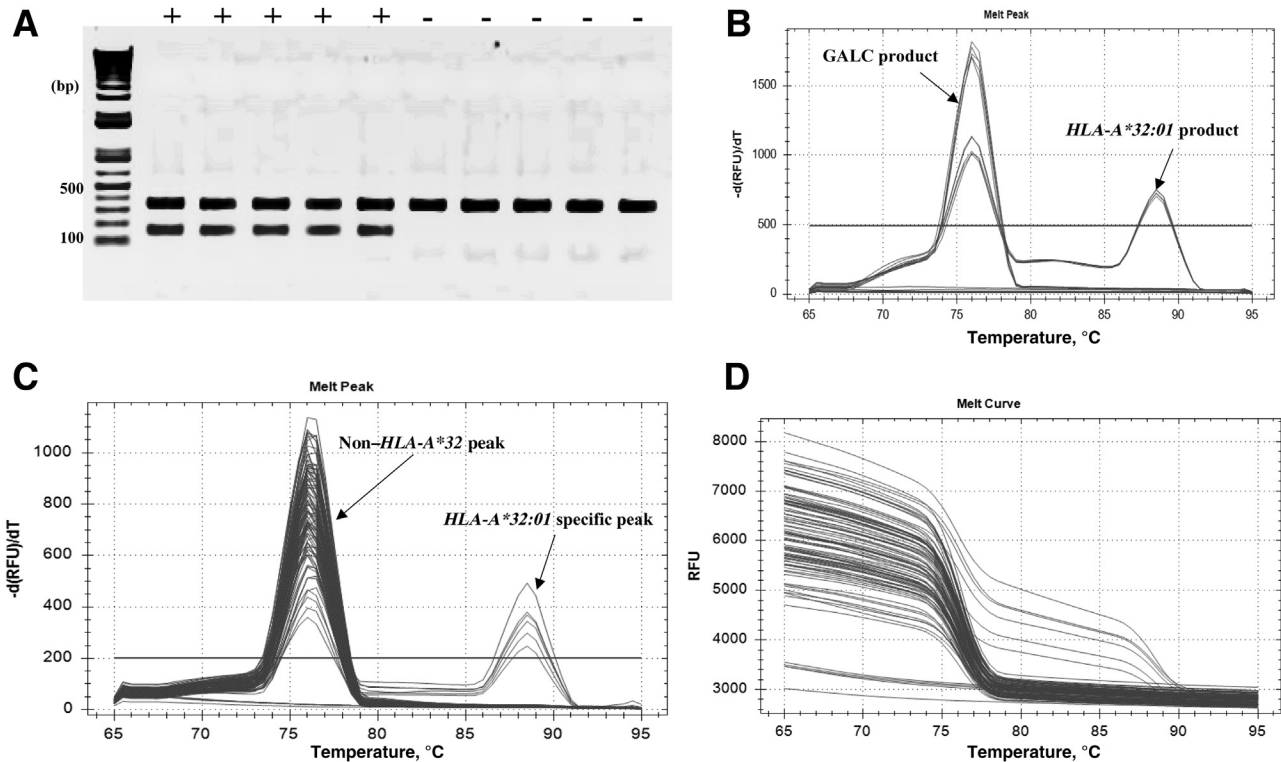
**Figure 2** AS-PCR results. **A:** *HLA-A*32:01*—positive (+) and non—*HLA-A*32* (−) samples were amplified by PCR using the following validated primers: HLA 32-88F forward primer and HLA032R2 reverse primer to amplify the *HLA-A*32* allele. GALC-F and GALC-R primers were used to amplify the internal control housekeeping gene in a multiplexed reaction. PCR products were run on a 1% agarose gel containing 0.2 μg/mL ethidium bromide at 115 V for 30 minutes. The gel was visualized by a transilluminator (ChemiDoc XRS+; Bio-Rad Laboratories). *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* and *GALC* housekeeping gene are shown by an **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 PowerUp SYBR Green are shown. Tm peaks of *HLA-A*32:01* and non—*HLA-A*32:01* alleles are shown by **arrows**. *HLA-A*32:01*—positive samples show double Tm peaks at 88.5°C ± 0.0°C (range: 88.50°C to 88.50°C) for the *HLA-A*32:01* allele and 76.05°C ± 0.03°C (range: 76.00°C to 76.50°C) for *GALC*. Non—*HLA-A*32* allele samples show a single Tm peak at 76.07°C ± 0.01°C (range 75.50°C to 76.50°C) for *GALC*. **D:** Melting curves for a subset of the 458 samples tested in real-time PCR. Data are expressed as means ± SEM. $n = 30$ (**C**, *HLA-A*32:01*—positive samples). RFU, relative fluorescence units; Tm, melting point.

annealing temperature, primer concentration, magnesium concentration, and DNA inputs.[14] In this optimized assay, nonspecific 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 (PowerUp 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 and 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] (Allele Frequency Net Database, *http://www.allelefrequencies.net*, last accessed January 15, 2019),

**Table 3** Melting Curve Analysis of *HLA-A*32:01* and *GALC* Amplicons

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

*GALC*, galactosylceramidase; Tm, melting point.

**Table 4** Comparison of the Present Assay with SBT

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

qPCR, real-time quantitative PCR; SBT, sequence-based typing.

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, 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 whether alleles other than HLA-A*32:01 are associated with vancomycin DRESS in non-European races.

As for previous associations between drug hypersensitivity and HLA alleles such as between 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.[17] 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.[18] *HLA-A*32:01* has a leucine at position 156 that is a key peptide binding residue that 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 accessed January 4, 2019). On the other hand, it has been demonstrated that in some drug hypersensitivity phenotypes, different alleles of the same family confer susceptibility to an adverse drug reaction. This is the case of carbamazepine-induced Stevens-Johnson syndrome/toxic epidermal necrolysis in which in addition to the *HLA-B*15:02* risk allele, members of the same HLA-B*75 serotype could also present carbamazepine to activate CD8^{+} T cells.[19] Wei et al[19] 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 carbamazepine to CD8^{+} T cells. Asn63, which is shared by members of the *B*75* family, was the key residue to confer this specificity.

High-resolution HLA typing is performed by direct sequencing of HLA class I and class II sequences. Although 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 with 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 that might not be available in most of the clinical settings. HLA typing by hybridization of the PCR amplicon with sequence-specific oligonucleotide probes is an alternative method, but this method shows low-resolution typing.[20]

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, calculations show that it costs around 20-fold less using this real-time PCR assay in comparison to HLA typing by SBT. Time for operator setup and analysis also favors this rapid real-time assay due to the higher number of steps required for SBT. A single sample can be typed for *HLA-A*32:01* with a turnaround time of <3 hours, with <1 hour of operator hands-on time with real-time PCR, whereas SBT such as Sanger sequencing has a turnaround of at least 8 to 10 hours with 3 hours of hands-on time. Ideal batch scale for SBT 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 next-generation sequencing might not be available in most of the clinical settings.

The assay described here appears to give both a specificity and sensitivity of 100%, with the advantage of being very fast and cheaper compared with 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 a qPCR platform can use the standard AS-PCR followed by agarose gel electrophoresis. Although limited testing was conducted on a standard PCR machine with the PowerUp SYBR Green Master Mix followed by agarose gel electrophoresis analysis and gel visualization, this assay was found to successfully adapt in settings that do not have a qPCR platform.

The considerable reduction of operator manual handling of post-PCR amplicon and manipulation of results also reduces the potential risk of sample mix-up as well as contamination. Where testing a large number of samples is needed, the use of a 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 this study's 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*.[21–26] Most of these single-allele assays have been advocated as a pre-prescription screening strategy with specific drugs and specific populations.[27,28] The association of *HLA-A\*32:01* and vancomycin DRESS has only been recently reported. Currently, the authors 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 a population of predominant European ancestry of 6.8% and estimates from that, that approximately 20% of those carrying *HLA-A\*32:01* and exposed to vancomycin for at least 2 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.

## Conclusions

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 the authors caution that these assay characteristics may not be maintained with any modification to the method. In addition as per the authors' 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.[29]

## Acknowledgments

## References

1. Redwood AJ, Pavlos RK, White KD, Phillips EJ: HLAs: key regulators of T-cell-mediated drug hypersensitivity. HLA 2018, 91:3−16
2. White KD, Chung WH, Hung SI, Mallal S, Phillips EJ: Evolving models of the immunopathogenesis of T cell-mediated drug allergy: the role of host, pathogens, and drug response. J Allergy Clin Immunol 2015, 136:219−234. quiz 235
3. Paul S, Weiskopf D, Angelo MA, Sidney J, Peters B, Sette A: HLA class I alleles are associated with peptide-binding repertoires of different size, affinity, and immunogenicity. J Immunol 2013, 191: 5831−5839
4. Konvinse KC, Trubiano JA, Pavlos R, James I, Shaffer CM, Bejan CA, Pilkinton MA, Rosenbach M, Zwerner JP, Williams KB, Jack Bourke J, Martinez P, Rwandamuriye F, Chopra A, Watson M, Mallal SA, Redwood A, White KD, Phillips EJ: HLA-A\*32:01 is strongly associated with vancomycin-induced drug reaction with eosinophilia and systemic symptoms. J Allergy Clin Immunol 2018, 144:183−192
5. Lin YF, Yang CH, Sindy H, Lin JY, Rosaline Hui CY, Tsai YC, Wu TS, Huang CT, Kao KC, Hu HC, Chiu CH, Hung SI, Chung WH: Severe cutaneous adverse reactions related to systemic antibiotics. Clin Infect Dis 2014, 58:1377−1385
6. Blumenthal KG, Peter JG, Trubiano JA, Phillips EJ: Antibiotic allergy. Lancet 2019, 393:183−198
7. Husain Z, Reddy BY, Schwartz RA: DRESS syndrome: part I. clinical perspectives. J Am Acad Dermatol 2013, 68:693.e1−693.e14. quiz 706-708
8. Pavlos R, Mallal S, Ostrov D, Buus S, Metushi I, Peters B, Phillips E: T cell-mediated hypersensitivity reactions to drugs. Annu Rev Med 2015, 66:439−454
9. Cacoub P, Musette P, Descamps V, Meyer O, Speirs C, Finzi L, Roujeau JC: The DRESS syndrome: a literature review. Am J Med 2011, 124:588−597
10. Carapito R, Radosavljevic M, Bahram S: Next-generation sequencing of the HLA locus: methods and impacts on HLA typing, population genetics and disease association studies. Hum Immunol 2016, 77: 1016−1023
11. Sayer D, Whidborne R, Brestovac B, Trimboli F, Witt C, Christiansen F: HLA-DRB1 DNA sequencing based typing: an approach suitable for high throughput typing including unrelated bone marrow registry donors. Tissue Antigens 2001, 57:46−54
12. Roden DM, Pulley JM, Basford MA, Bernard GR, Clayton EW, Balser JR, Masys DR: Development of a large-scale de-identified DNA biobank to enable personalized medicine. Clin Pharmacol Ther 2008, 84:362−369
13. Karnes JH, Shaffer CM, Bastarache L, Gaudieri S, Glazer AM, Steiner HE, Mosley JD, Mallal S, Denny JC, Phillips EJ, Roden DM: Comparison of HLA allelic imputation programs. PLoS One 2017, 12:e0172444
14. Ruiz-Villalba A, van Pelt-Verkuil E, Gunst QD, Ruijter JM, van den Hoff MJ: Amplification of nonspecific products in quantitative polymerase chain reactions (qPCR). Biomol Detect Quantif 2017, 14:7−18
15. Ballantyne KN, van Oorschot RA, Mitchell RJ: Locked nucleic acids in PCR primers increase sensitivity and performance. Genomics 2008, 91:301−305
16. Cao K, Hollenbach J, Shi X, Shi W, Chopek M, Fernández-Viña MA: 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 2001, 62:1009−1030
17. Mallal S, Nolan D, Witt C, MAsel G, MArtin A, Moore C, Sayer D, Castley A, Mamotte C, Maxwell D, James I, Christiansen F: Association between HLA-B\*57:01, HLA-DR7 and HLA-DQ3 and hypersensitivity to HIV-1 reverse transcriptase inhibitor abacavir. Lancet 2002, 359:727−732
18. Mallal S, Phillips E, Carosi G, Molina JM, Workman C, Tomazic J, Jagel-Guedes E, Rugina S, Kozyrev O, Cid JF, Hay P, Nolan D, Hughes S, Hughes A, Ryan S, Fitch N, Thorborn D, Benbow A; PREDICT-1 Study Team: HLA-B\*5701 screening for hypersensitivity to abacavir. N Engl J Med 2008, 358:568−579
19. Wei CY, Chung WH, Huang HW, Chen YT, Hung SI: Direct interaction between HLA-B and carbamazepine activates T cells in patients with Stevens-Johnson syndrome. J Allergy Clin Immunol 2012, 129: 1562−1569.e5

20. Dunn PP: Human leucocyte antigen typing: techniques and technology, a critical appraisal. Int J Immunogenet 2011, 38:463−473

21. Nguyen DV, Vida C, Chu HC, Fulton R, Li J, Fernando SL: Validation of a rapid, robust, inexpensive screening method for detecting the HLA-B*58:01 allele in the prevention of allopurinol-induced severe cutaneous adverse reactions. Allergy Asthma Immunol Res 2017, 9:79−84

22. Nguyen DV, Vidal C, Chu HC, Do NT, Tran TT, Le HT, Fulton RB, Li J, Fernando SL: Validation of a novel real-time PCR assay for detection of HLA-B*15:02 allele for prevention of carbamazepine-induced Stevens-Johnson syndrome/toxic epidermal necrolysis in individuals of Asian ancestry. Hum Immunol 2016, 77:1140−1146

23. Uchiyama K, Kubota F, Ariyoshi N, Matsumoto J, Ishii I, Kitada M: Development of a simple method for detection of the HLA-A*31:01 allele. Drug Metab Pharmacokinet 2013, 28:435−438

24. Chen P, Lin JJ, Lu CS, Ong CT, Hsieh PF, Yang CC, Tai CT, Wu SL, Lu CH, Hsu YC, Yu HY, Ro LS, Lu CT, Chu CC, Tsai JJ, Su YH, Lan SH, Sung SF, Lin SY, Chuang HP, Huang LC, Chen YJ, Tsai PJ, Liao HT, Lin YH, Chen CH, Chung WH, Hung SI, Wu JY, Chang CF, Chen L, Chen YT, Shen CY; Taiwan SJS Consortium: Carbamazepine-induced toxic effects and HLA-B*1502 screening in Taiwan. N Engl J Med 2011, 364:1126−1133

25. Hammond E, Mamotte C, Nolan D, Mallal S: HLA-B*5701 typing: evaluation of an allele-specific polymerase chain reaction melting assay. Tissue Antigens 2007, 70:58−61

26. Liu Z, Chen G, Kang X, Han M, Chen R, Chen C, Wang H: A multiplex allele-specific real-time polymerase chain reaction assay for HLA-B*13:01 genotyping in four Chinese populations. HLA 2016, 88:164−171

27. Phillips EJ, Mallal SA: Pharmacogenetics of drug hypersensitivity. Pharmacogenomics 2010, 11:973−987

28. Ferrell PB Jr, McLeod HL: Carbamazepine, HLA-B*1502 and risk of Stevens-Johnson syndrome and toxic epidermal necrolysis: US FDA recommendations. Pharmacogenomics 2008, 9:1543−1546

29. Hammond E, Almeida CA, Mamotte C, Nolan D, Phillips E, Schollaardt TA, Gill MJ, Angel JB, Neurath D, Li J, Giulivi T, McIntyre C, Koultchitski G, Wong B, Reis M, Rachlis A, Cole DE, Chew CB, Neifer S, Lalonde R, Roger M, Jeanneau A, Mallal S: External quality assessment of HLA-B*5701 reporting: an international multicentre survey. Antivir Ther 2007, 12:1027−1032