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Novel sensitive, specific and rapid pharmacogenomic test for the prediction of abacavir hypersensitivity reaction: *HLA-B*57:01* detection by real-time PCR

Aim: International HIV treatment guidelines recommend *HLA-B*57:01* typing before abacavir administration, in order to reduce the incidence of abacavir hypersensitivity reactions, the major cause of early therapy discontinuation. A fast, sensitive and specific test for *HLA-B*57:01* detection has been developed in the present study. **Materials & methods:** Two sets of sequence-specific primers were designed, and amplification rapidly detected by real-time PCR. **Results:** A total of 108 samples were analyzed in a single-blind fashion, and 41 samples were identified as positive. Complete agreement, with $\kappa = 1$ (standard error = 0.0962, $p < 0.0001$), was found, with a validated methodology used in the EPI109367 clinical trial funded by GlaxoSmithKline, and consisting of low-resolution sequence-specific oligonucleotide PCR, followed by high-resolution sequence-specific oligonucleotide PCR carried out on the *HLA-B*57*-positive samples. **Conclusion:** We provided a detailed characterization of a novel *HLA-B*57:01* screening test, which can be easily implemented by those laboratories already involved in the detection of viral load and virus genotyping.

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KEYWORDS: abacavir ■ HIV-1 ■ *HLA-B*57:01* ■ hypersensitivity reaction ■ pharmacogenomic test ■ real-time PCR

Abacavir (ABC) is a nucleoside reverse transcriptase inhibitor used as part of combination antiretroviral therapy in HIV-infected patients [1]. It has been extensively evaluated in clinical studies and is currently recommended as a preferred or an alternative nucleoside reverse transcriptase inhibitor in antiretroviral-naïve patients according to international HIV guidelines [10]. It is generally a very well-tolerated drug, even though an increased risk of myocardial infarction in patients exposed to ABC within the preceding 6 months has been recently reported [2], but not confirmed by other studies [3]. The major reason for early discontinuation of ABC remains the occurrence of an immunologically mediated hypersensitivity reaction (HSR) [4]. ABC-HSR has been observed in 5–8% of treated patients, appears within the first 6 weeks of therapy and requires an immediate and permanent discontinuation of the drug [5]. A strong association between the ABC-HSR and MHC class I allele *HLA-B*57:01* (locus position 6p21.3 and OMIM: 142830) has been observed in Caucasian HIV-infected patients [6,7]. Subsequently, skin patch testing demonstrated the presence of such an association across racial and geographic populations [8,9]. Finally, a powered, double-blind, prospective, randomized study (Prospective Randomized Evaluation of DNA Screening in

a Clinical Trial [PREDICT-1]) demonstrated the clinical utility of *HLA-B*57:01* screening in reducing ABC-HSR in HIV-infected patients. HSR was clinically diagnosed in 93 patients from a total of 1650 patients, with a significantly lower incidence (3.4%) in the prospective screening group than in the control group (7.8%; $p < 0.001$). Interestingly, *HLA-B*57:01* screening eliminated immunologically confirmed HSR, with a negative predictive value of 100% [9]. Therefore, international HIV treatment guidelines now recommend *HLA-B*57:01* typing before ABC administration, in order to reduce the incidence of ABC hypersensitivity [10,11,101]; while the screening is also part of the license of ABC-containing products.

Different technologies are available to detect individual HLA types that are routinely used for the characterization of tissue compatibility during organ transplantation, such as DNA sequence-based typing or the sequence-specific oligonucleotide probe method with additional DNA sequencing for patients screened as positive with the probes. However, these approaches are time-consuming, expensive and not readily available [12]. An alternative method has been developed by Mallal's group, based on the identification of the *HLA-B*57:01* allele by sequence-specific primer (SSP) by

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semiquantitative PCR [12]. This methodology has been validated, and it has been widely diffused for the rapid screening of *HLA-B*57:01*-positive patients [13]. The assay was recently improved by Giardina and collaborators, using the same primer sets in a PCR assay based on fluorescence detection of the amplified products through capillary electrophoresis [14]. However, considering that most of the standard semiquantitative PCR techniques have been replaced by real-time (Q)-PCR, in the present study we have developed and characterized an alternative screening test based on the SSP method but using Q-PCR to detect the amplification products. We report the detailed methodology of this technique and the results of its validation by comparison with a reference method.

Materials & methods

■ DNA samples

A first group of 28 blood samples was provided by the Institute of Clinical Infectious Diseases, Catholic University Medical School of Rome (Italy) and the Department of Infectious Diseases, University of Sassari (Italy). These patients were originally recruited and thus genotyped as described below in the EPI109367 clinical trial funded by GlaxoSmithKline (GSK; Brentford, UK) [15]. They were readmitted at the outpatient clinics (after institutional review board approval of an amended research protocol for *HLA-B* genotyping with a different method), whole blood samples were collected in EDTA-treated tubes and used in the present study. These samples were initially used to optimize the experimental conditions, as detailed below.

Subsequently, a panel of 80 additional DNA samples genotyped in the same clinical trial of the previous 28 were directly provided by GSK (Verona, Italy). These samples came from a multiethnic population and were genotyped by low-resolution sequence-specific oligonucleotide PCR, followed by high-resolution sequence-specific oligonucleotide PCR carried out on the *HLA-B*57* positive samples (Laboratory of Tissue Genotyping, Immunohematology and Transfusion Medicine Service, Fondazione IRCCS, Centro San Raffaele del Monte Tabor, Milan, Italy). This method was validated during the EPI109367 clinical trial, comparing results from 776 blood samples analyzed with this method with results obtained by LabCorp (NC, USA) on buccal swabs collected from the same subjects by sequence-specific oligonucleotide probe PCR [15]. The latter method was validated during the PREDICT-1 clinical trial on 1956

samples [9], by comparison with the gold standard technique based on DNA sequencing. This panel of samples was enriched in *HLA-B*57:01*-positive samples (46.3%); moreover, six alleles belonging to the *B*57* family were deliberately included. These alleles are discriminated on the basis of two mismatches in the exon-3-specific PCR primers, thus they were considered 'difficult' to detect in comparison to other alleles discriminated on the basis of >2 mismatches. The exact number of positive samples, the number of 'difficult' alleles, together with the correct genotype of the samples included in the panel analyzed by our laboratory, were disclosed at the end of our analysis. These data are summarized in TABLE 1.

■ Total DNA extraction.

Total DNA was prepared from whole blood samples, using the QIAamp® DNA Blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, whole blood was collected in EDTA-treated tubes and stored at -20°C until the day of the extraction. Aliquots of 200 µl for each whole blood sample were used, and total DNA was eluted in 200 µl elution buffer (10 mM Tris HCl; 0.5 mM EDTA; pH 9.0; provided by the kit). DNA concentration was measured using the Quant-iT™ PicoGreen® DNA Assay Kit (Invitrogen Corporation, Paisley, Scotland). A standard curve in the range of 0–10 ng was run in each assay using Lambda DNA standards, provided by the kit. Consistently, with data reported in the kit handbook, final DNA yield was approximately 5 µg per sample.

■ HLA-B-specific amplification by PCR

HLA-B-specific amplification between the first and the third intron was carried out by PCR, using the following validated primers: 5BIn1–57 forward primer (forward: 5'-GGG AGG AGC GAG GGG ACC G/CCA C-3'; intron 1: 36–57) and 3BIn3–37 reverse primer (reverse: 5'-GGA GGC CAT CCC CGG CGA CCT AT-3'; intron 3: 37–59), yielding a 922 bp product [16]. The PCR reaction contained 50 ng total DNA, 1X PCR buffer, 300 nM of each primer, 1.25 U AccuPrime™ Pfx DNA Polymerase (Invitrogen Corporation) in 50 µl final volume. After initial denaturation (10 min at 95°C), a total of 35 PCR cycles were conducted, using the following two-step PCR conditions: denaturation at 95°C for 20 s and annealing/extension at 68°C for 1 min, in a MasterCycler ep thermocycler® (Eppendorf,

Hamburg, Germany). The amplicons were separated by electrophoresis through 1.5% agarose gels containing 0.1 µg/ml ethidium bromide. This step allowed us to verify the efficiency of DNA amplification, in order to avoid the processing of incorrect preamplified samples, thus reducing the incidence of false-negative results.

■ *HLA-B*57:01* typing by SSPs Q-PCR

The Immunogenetics project (IMGT)/HLA database was used to align the *HLA-B*57:01* DNA sequence to other known *HLA-B* allele sequences [17]. The highest variability was found in the exon 2 and exon 3 sequences, which are regions that encode the peptide-binding domains of the MHC class I molecules. In this database, we initially found three different sequences relative to the *HLA-B*57:01* allele, named progressively as *57:01:01*, *01:02* and *01:03*, which differed for one mismatch on exon 3, as indicated in FIGURE 1 (red circles). These two SNPs were not included in the product of amplification when SSPs (black rectangles, FIGURE 1) were designed, so that all three *HLA-B*57:01* alleles could be detected. By 15 October 2010 (the last released update), seven additional sequences had been stored in the IMGT/HLA database [17]. The *HLA-B*57:01:04* allele, which differs by an A to G mutation at position 648 (AJ458991 sequence, NCBI GenBank database), is located within the PCR product amplified by exon-2-specific PCR (FIGURE 1A). This mutation does not interfere with the alignment of the primers, but may cause a small change in the melting temperature (T_m) of the amplification product. Nevertheless, the allele *HLA-B*57:01:04* will be detected by amplifications in the two Q-PCRs. The point mutations found in the exon 2 sequences of the newly identified *HLA-B*57:01:08* and *01:09* are not included in the amplified Q-PCR product, while the other newly identified sequences are identical in the exon 2 region (FIGURE 1A). The *HLA-B*57:01:05–09* point mutations identified on the exon 3 region (FIGURE 1B) are not included in the product amplified by the exon-3-specific Q-PCR. Finally, the point mutation found in the exon 3 sequence of the *HLA-B*57:01:10* allele is within the amplified Q-PCR product, and it is a C to G substitution, which does not change the T_m of the amplicon (FIGURE 1B). Taking all these observations together we can conclude that the additional *HLA-B*57:01* alleles will be detected by our assay.

In order to encompass the highest variable regions of the *HLA-B* DNA sequence, two sets of primers were designed. The first set consisted

Table 1. Allele distribution in the *HLA-B*57:01*-enriched panel of 80 single-blind analyzed samples.

<i>HLA-B</i> status	Replicates (n)
*07	6
*08	5
*13	2
*14	5
*15	5
*18	10
*27	4
*35	15
*38	9
*39	3
*40	4
*44	10
*47	1
*48	1
*49	3
*51	11
*53	2
*56	2
*57:01	37
*57:02	1
*57:03	5
*58	9

HLA-B typing was carried out by low-resolution sequence-specific oligonucleotide PCR, followed by high-resolution sequence-specific oligonucleotide PCR carried out on the *HLA-B*57*-positive samples (Laboratory of Tissue Genotyping, Immunohematology and Transfusion Medicine Service, Fondazione IRCCS, Centro San Raffaele del Monte Tabor, Milan, Italy), a technique validated during the GlaxoSmithKline-funded EPI109367 clinical trial [15]. Nine alleles were not univocally genotyped and were thus not included in this table. Among these, one allele was reported as *HLA-B*57:01* positive, even though data were also compatible with the *HLA-B*57:14* genotype. Most patients were heterozygous, while one patient was homozygous for *HLA-B*57:01*, one for *HLA-B*18* and one for *HLA-B*38*.

of forward primer 345F (5'-GTC TCA CAT CAT CCA GGT GAT GT-3') and reverse primer 419R (5'-GAC TGG TCA TGC CCG CGG AG-3'), allowing for the amplification of a 75 bp amplicon, corresponding to nucleotides 1020–1094 on exon 3 of the AJ458991 sequence, NCBI GenBank database (FIGURE 2A). This product is similar but not identical to that previously described by Mallal and collaborators [12]. The second set of primers consists of forward primer 193F (5'-GCG AGT CCG AGG ATG GCG CC-3') and reverse primer 319R (5'-GGA GCG CGA TCC GCA GGT TCT-3'), which yield a 127 bp product, corresponding to nucleotides 622–748 on exon 2 of the AJ458991 sequence (NCBI GenBank database, FIGURE 2B). In FIGURE 2, the sequence of the *HLA-B*57:01:01* is aligned to the official reference sequence *HLA-B*07:02:01*,

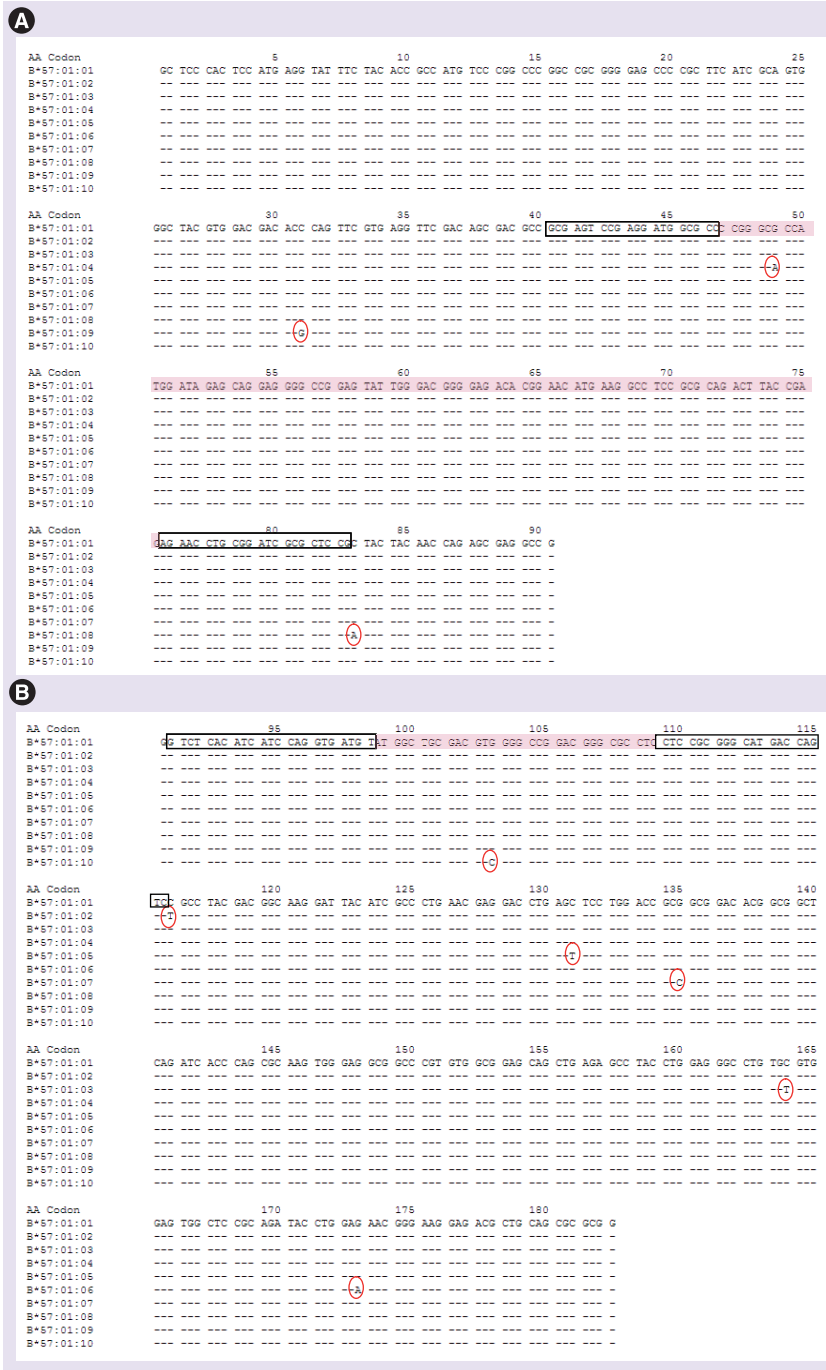


Figure 1. DNA sequences relative to HLA-B*57:01. DNA sequences relative to the (A) exon 2 and (B) exon 3 regions of HLA-B*57:01 are aligned. When the sequence-specific primer (black rectangles) were designed in October 2007, the first three DNA sequences were found in the Immunogenetics project (IMGT)/HLA database [17]. These sequences differ for one mismatch (red circles), that was not included in the DNA region amplified by sequence-specific primer sets. However, at present, seven additional sequences are stored in the database. Pink rectangles refer to the sequences amplified between each primer set.

Q-PCR conditions were set using two positive controls (data not shown). Q-PCR reactions were carried out in a 20 µl reaction volume, using 163 nM of each primer and the Brilliant SYBR® Green Q-PCR Master Mix 2X (Stratagene, CA, USA) in a MX3000P Q-PCR machine (Stratagene). Reaction efficiency of approximately 98% was found in the dilution range of 1:100 and 1:5000 using HLA-B preamplified DNA, while a significantly lower efficiency was detected when crude DNA was directly used as template in the Q-PCRs (data not shown). Thus HLA-B preamplified DNA diluted 1:1000 was chosen for subsequent assessments.

The first group of 28 blood samples were analyzed in a single-blind fashion using the two Q-PCRs described above, with the following cycling conditions: 30 cycles of denaturation at 95°C for 20 s; annealing at 64°C for the exon-3-specific primers and 65°C for the exon-2-specific primers for 30 s; and extension at 72°C for 30 s. Data were analyzed using the amplification-based threshold and the adaptive baseline algorithms provided by the software analysis system of the MX3000P Q-PCR machine. The first algorithm automatically sets the threshold fluorescence for each dye, based on the amplification properties of each reaction; while the adaptive baseline algorithm calculates the best baseline for each amplification plot, thereby providing the most accurate fluorescence take-off point of reaction per sample, named threshold cycle (Ct). Using the automated algorithm to calculate the Ct values and detect amplifications by Q-PCRs, we identified four positive and 24 negative samples (TABLE 2), in complete concordance with results of the previous genotyping. Data obtained with this first set of samples were used to further standardize Q-PCR conditions as follows: 20 cycles for the exon-3-specific Q-PCR, taking Ct values <15 to determine positive amplifications; 15 cycles for the exon-2-specific Q-PCR, taking Ct values <14 as positive amplifications (as described in detail in the 'Results' section). Using these Q-PCR parameters, we carried out a single-blind analysis of a panel of 80 additional DNA samples, directly provided by GSK.

■ Data analysis

Results obtained with the analytical method for HLA-B*57:01 screening generated in our laboratory were compared with results obtained using a previously validated method. The κ-statistic measure of inter-rater agreement for two unique raters and two ratings was calculated, according to the method described by

corresponding to the AJ292075 sequence (NCBI GenBank database).

HLA-B preamplified DNA was used for HLA-B*57:01 allele detection by Q-PCR, performed with the two set of primers described above.

Cohen [18]. κ is a general measure of agreement for categorical data and it is computed using the following equation:

$$K = \frac{\text{Pr}(a) - \text{Pr}(e)}{1 - \text{Pr}(e)}$$

where $\text{Pr}(a)$ is the relative observed agreement among raters, and $\text{Pr}(e)$ is the hypothetical probability of chance agreement. κ can vary between -1 (complete disagreement) to +1 (complete agreement). Results were analyzed by Stata 10.1 (Stata Statistical Software 10.1, StataCorp LP, TX, USA).

Results

■ Analysis of the first 28 samples

DNA samples from 28 individuals were analyzed in a single-blind fashion using a HLA-B-specific amplification followed by two sequence-specific Q-PCRs. Using the automatic algorithms provided by the Q-PCR software (see 'Materials & methods' section), four positive samples were identified, that is amplification in both Q-PCRs (TABLE 2). However, the Q-PCR designed for the exon 2 allowed for the identification of five positive samples (average Ct = 11.7) (TABLE 2). This primer set does not differentiate some alleles of the *HLA-B*57* group, thus suggesting that the genotype of the additional positive sample belongs to the *B*57* group. At present the *HLA-B*57* group includes 37 different alleles, identified progressively with two numbers, from *B*57:01* to *B*57:37*. Moreover, with the exon-2-specific Q-PCR we identified five additional samples with an average Ct value of approximately 14.9, three of which had similar Tm to the ones reported in TABLE 2. However, these higher Ct values are not compatible with possible differences in the amount of initial DNA template owing to the homo- or hetero-zygous status, which can only account for no more than ± 1 cycle difference (one cycle = double amount of DNA), thus they were considered nonspecific amplifications. For the same reason, all the amplifications detected in both Q-PCRs with Ct values higher than 20 cycles were considered nonspecific.

■ Standardization of Q-PCR cycling conditions & data analysis

Results from the analysis of the first 28 samples were compared with the previous genotyping, and demonstrated a complete concordance; thus, they were used to standardize the Q-PCR profile setting and the analysis parameters in order to better compare data from different Q-PCR runs. Only the first 20 cycles were taken into account



Figure 2. HLA-B*57:01 alignment with the official reference sequence B*07:02:01. *HLA-B*57:01* is aligned with the official reference sequence. The location of the sequence-specific primers within (A) exon 2 and (B) exon 3 is indicated (rectangles), and was selected in order to include the variable regions of the *HLA-B* DNA sequence to discriminate the allele of interest (*B*57:01*) and exclude other B alleles.

for a second analysis, which was carried out using a higher fluorescence threshold, manually adjusted to a fixed value arbitrarily chosen in the exponential phase of the amplification plot, which was equal to 0.05 for the exon 3 Q-PCR. This value was equal to 0.15 for the exon 2 Q-PCR. Moreover, the background correction was carried out using fluorescence values from cycle 3 to 10. Data from 21 samples analyzed with these parameters are shown in FIGURE 3. Briefly, the Ct values

Table 2. Positive results from the first set of 28 patients who were analyzed in a single-blind fashion.

Sample number	Threshold	Average Ct \pm SEM (n = 3)	Average Tm \pm SEM (n = 3)
Q-PCR exon 3			
1	0.024	13.4 \pm 0.1	85.45 \pm 0.023
2	0.024	13.5 \pm 0.3	85.51 \pm 0.020
3	0.020	10.6 \pm 0.3	85.97 \pm 0.001
4	0.020	10.9 \pm 0.1	85.90 \pm 0.183
Q-PCR exon 2			
1	0.053	11.9 \pm 0.1	85.90 \pm 0.202
2	0.053	12.4 \pm 0.1	86.30 \pm 0.001
3	0.136	11.5 \pm 0.04	86.47 \pm 0.001
4	0.136	11.6 \pm 0.1	86.08 \pm 0.001
5	0.136	10.9 \pm 0.1	85.95 \pm 0.001

Samples were analyzed in two different PCR runs carried out per set of primers, as shown by differences in the threshold value. The latter was set by automatic algorithms provided by the Q-PCR software analysis system. Data, average Ct (each sample measured in triplicate) and average Tm values, relative to positive amplifications are reported.
 Ct: Threshold cycle; Q-PCR: Real-time PCR; SEM: Standard error of the mean;
 Tm: Melting temperature.

for the positive samples were as expected; higher than those reported in TABLE 2. In fact, the average Ct value of the positive samples measured in the exon 3 Q-PCR was 13.4 ± 0.7 (mean \pm standard error of the mean [SEM], $n = 4$) with three cycle differences between the lower, and the highest Ct value; and a value of 12.8 ± 0.05 (mean \pm SEM, $n = 4$) with two cycle differences between the lowest and the highest sample detected in positive assays using the exon 2 Q-PCR.

Taking these data into account, subsequent Q-PCRs were run and analyzed as follows:

- Exon-3-specific Q-PCR: 20 cycles, cycle 3–10 fluorescence values for background correction, fluorescence threshold of 0.05, and cut-off Ct value <15 to determine positive amplifications. The cut-off value was calculated considering the average Ct calculated for the positive samples
- Exon-2-specific Q-PCR: 15 cycles, cycle 3–7 fluorescence values for background correction, fluorescence threshold of 0.15, and cut-off Ct value <14 to determine positive amplifications. The cut-off value was calculated considering the average Ct calculated for the positive samples

(13.4 ± 1.5 cycle, since three cycle differences were measured among the lowest and the highest positive sample.

However, this Q-PCR will be used to confirm positive results obtained with the first PCR, and to exclude the presence of the *55:14*, *57:16*, *57:34* and *58:14* alleles, which would be detected as positive with the exon-3-specific Q-PCR but negative with the exon-2-specific Q-PCR.

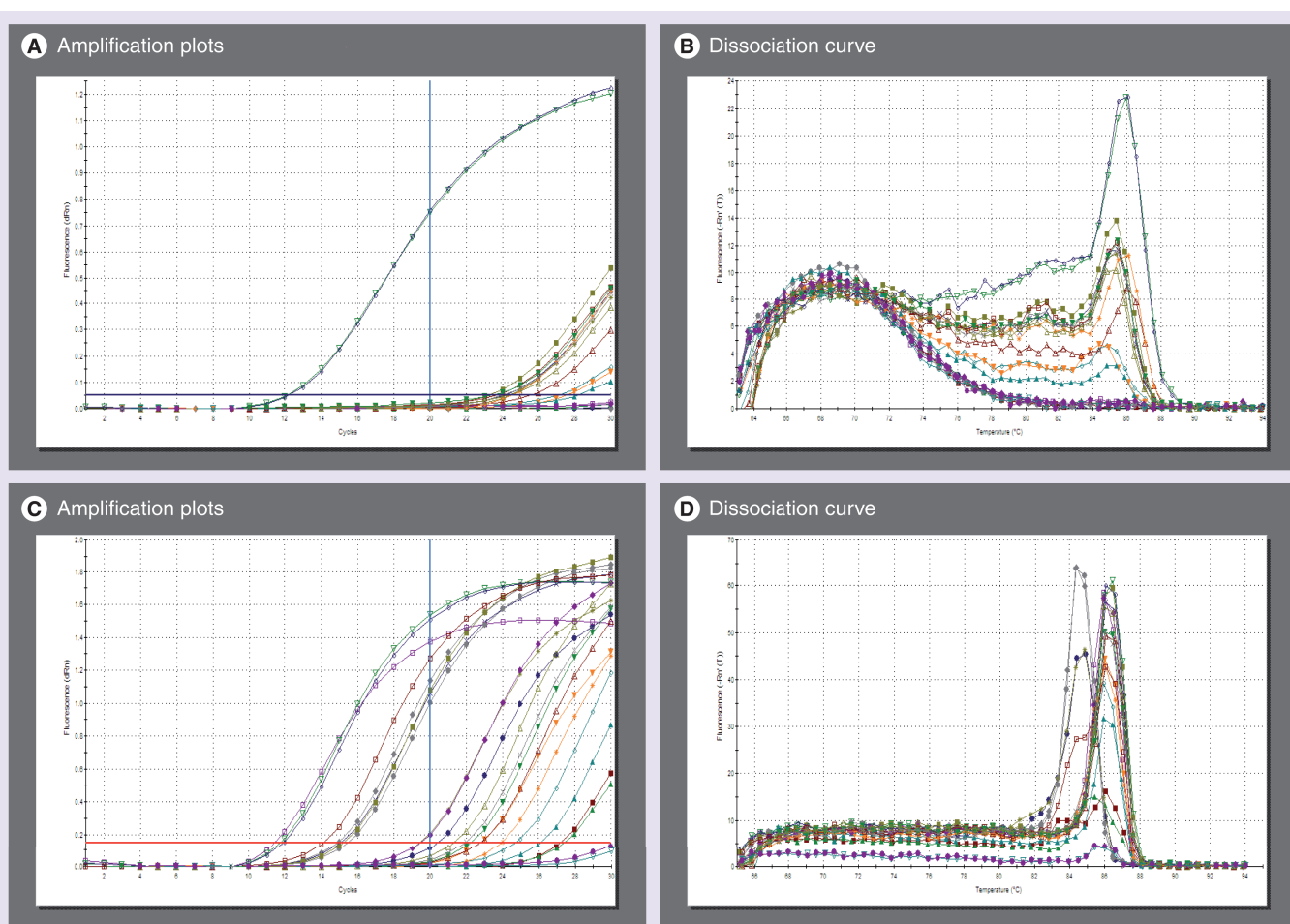


Figure 3. Identification of *HLA-B*57:01* by real-time PCR. (A & B) Exon-3- and (C & D) exon-2-specific real-time (Q)-PCR were run for 30 cycles on 21 samples; data were analyzed using fluorescence values detected within cycles 3–10 as background and a fixed threshold level. The first 20 cycles were considered for data analysis. In the exon-3-specific Q-PCR, two positive samples were detected with an average threshold cycle (Ct) of 12.2 (A) and a melting temperature (Tm) of 85.90 (B). In the exon-2-specific Q-PCR, three positive samples were detected with an average threshold cycle (Ct) of 11.8 (C) and a Tm of 86.17 (D). Five other samples had an average Ct value of 15, which was considered not compatible with possible variation in the initial quantity of DNA template owing to the homo- or hetero-zygous status. The latter would explain a difference of one cycle in the Ct value (one cycle = double DNA quantity).

In order to assess the error rate owing to possible changes in the PCR efficiency, one positive sample was measured in triplicate in eight different PCR runs. The average Ct value was 12.7 ± 0.3 (mean \pm SEM, $n = 8$) for the exon-3-specific PCR, and 11.9 ± 0.4 (mean \pm SEM, $n = 8$) for the exon 2 PCR. Ct values measured in each PCR run were found consistently below the cut-off Ct value, indicating the high reproducibility of the results in different PCR runs.

With these settings, we have analyzed in a single-blind fashion 80 DNA samples provided directly by GSK and previously genotyped in the EPI109367 trial. We identified a total of 37 *HLA-B*57:01*-positive samples by detecting positive amplifications in both Q-PCRs. In particular, in the exon-3-specific Q-PCR, we identified 37 positive samples, with an average Ct value of 12.9 ± 0.1 (SEM), and an average Tm of the amplification product equal to 85.48 ± 0.03 (SEM). By contrast, in the exon 2 Q-PCR, 43 positive samples were identified, with an average Ct value of 12.8 ± 0.1 (SEM), and an average Tm equal to 86.3 ± 0.03 (SEM). In FIGURE 4, Q-PCR fluorescence plots and Tm for the first ten samples analyzed with the parameters described above are shown as an example of a typical Q-PCR assay. All data were compared with previous genotyping data and a 100% overlap was found.

In conclusion, we analyzed, with a Q-PCR screening test, a total of 108 samples, and identified 41 samples as positive and considered 67 as negative. Complete agreement was found with a validated methodology, with κ for concordance = 1 (standard error = 0.0962; $p < 0.0001$).

Discussion

In the present paper we have validated a rapid pharmacogenomic test for the screening of the *HLA-B*57:01* allele using SSPs and Q-PCR. Since the *HLA-B*57:01* sequence differs in 59 point mutations from the official HLA-B reference sequence, namely *HLA-B*07:02:01* (FIGURE 2), multiple sets of primers needed to be used in order to detect the allele of interest. We designed two sets of SSPs (approximately 20 bp oligonucleotides) that aligned the *HLA-B*57:01* sequence in two different regions; one in exon 3 and another in exon 2. In the presence of the *HLA-B*57:01* allele, primers bind DNA and a specific product can be amplified by Q-PCR and detected by SYBR Green fluorescence. In 2005, the group of Mallal described an allele-specific PCR method for the detection of *HLA-B*57:01*, based on the use of a panel of four SSPs; one forward and three reverse primers [12].

Amplification of the *HLA-B*57:01*-specific sequence was detected by standard semiquantitative PCR, a method presently used by several laboratories [13] which has been recently improved [14]. Fluorescence-based detection by capillary electrophoresis increases the sensitivity of Mallal's method, thus allowing the use of as low as 1 ng of DNA per sample. This allows the employment of a noninfective source of DNA, such as saliva and buccal swabs, the main advantage of this method [14]. The semiquantitative PCR technique has been progressively replaced by Q-PCR, a more rapid and sensitive method for the direct monitoring of DNA amplification during PCR cycling. In a subsequent study, the same research group described a faster test carried out in Q-PCR [19]. For this approach, they used only three of the four *HLA-B*57:01* SSPs previously characterized [12], thus missing the opportunity to discriminate the *HLA-B*57:06* allele [19]. Briefly, the authors used together with the *HLA-B*57:01*-specific primers, a set of primers specific for the housekeeping gene, the *HGH* gene, that yielded a 439-bp product. The authors set up a multiplex Q-PCR to favor amplification of the *HLA-B*57:01*-specific product when the allele is present instead of the amplification of the HGH-specific amplicon. The assessment of the *HLA-B*57:01* status was carried out by analyzing differences in the melting curves of the two different amplified products [19]. However, our preliminary data demonstrated a significantly lower Q-PCR efficiency and sensitivity when both primer sets were used directly on crude DNA preparation (data not shown); therefore, HLA-B-specific amplification using already validated primers was introduced in our strategy [16]. To prevent the introduction of nonspecific mutations during this amplification step, we used a high fidelity DNA polymerase as described in detail in the 'Materials & methods' section.

The allele-specific PCR method described by Mallal is based on the recognition of a high variable DNA sequence located on exon 3; this region has also been included in our strategy. The forward primer used in this Q-PCR is similar to the one described by Mallal *et al.*, however the reverse primers are located upstream, thus leading to the amplification of a shorter product of 75 bp [12]. This shift allowed us to discriminate the following alleles: *57:02*, *57:03*, *57:04*, *57:05*, *57:07*, *57:09*, *57:12* and the newly identified *57:17* allele, via one additional mismatch in comparison to the reverse primer used by Mallal's group [12]. In fact, we

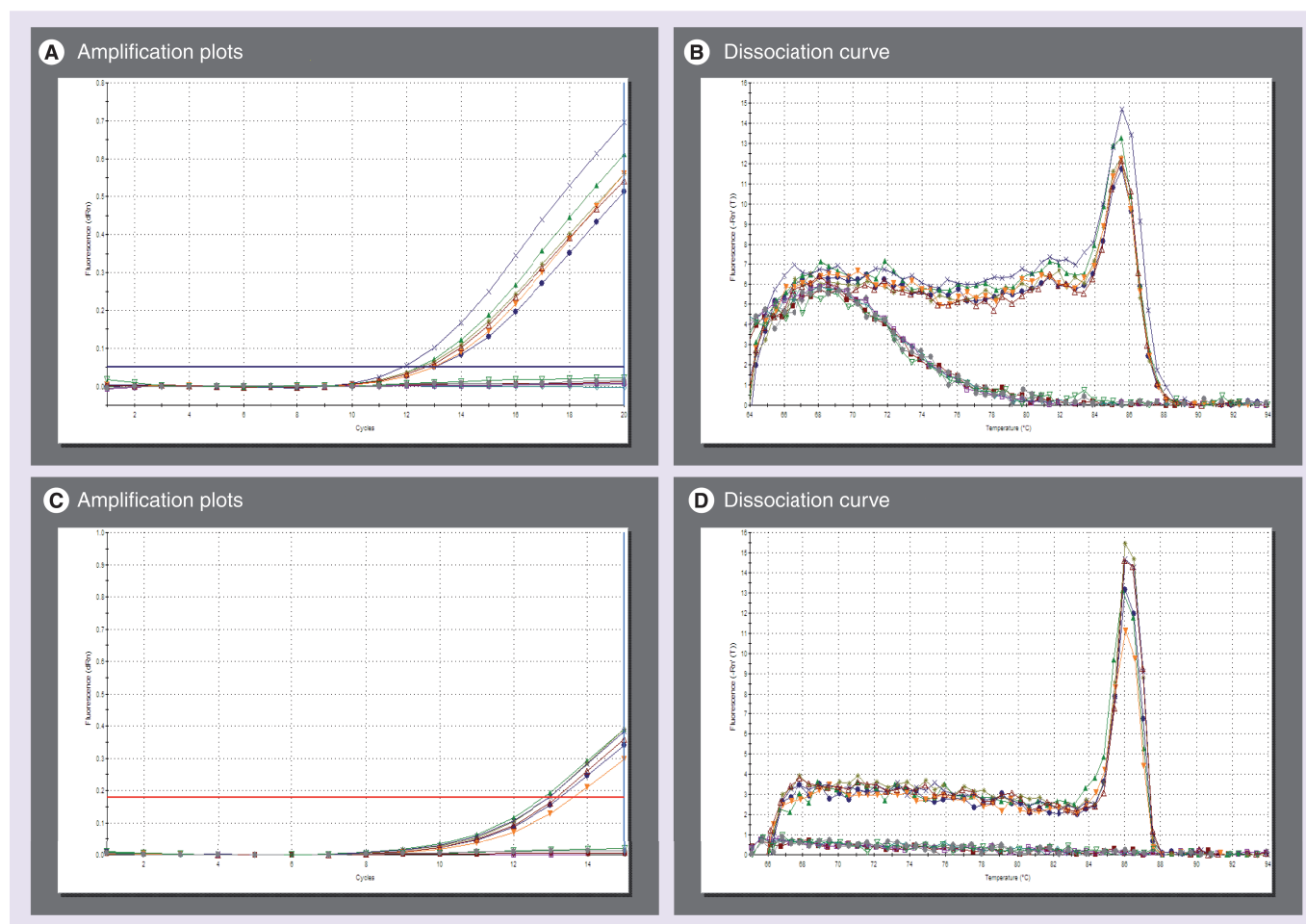


Figure 4. Use of standardized real-time PCR conditions for detection of *HLA-B*57:01*. (A & B) Exon-3- and (C & D) exon-2-specific real-time (Q)-PCR were run for 20 and 15 cycles, respectively, on the first ten GlaxoSmithKline samples (FIGURE 1); data were analyzed using fixed parameters for background correction and fluorescence threshold in order to compare different runs. In the exon-3-specific Q-PCR, six positive samples were detected with an average threshold cycle (Ct) of 12.6 (A) and a Tm of 85.47 (B). Similarly, in the exon-2-specific Q-PCR six positive samples were detected with an average Ct of 13.2 (C) and a Tm of 86.01 (D).

identified six samples as negative in the exon 3 Q-PCR and positive in the exon 2 Q-PCR, and hypothesized that they were alleles of the *B*57* group. These samples corresponded to one *HLA-B*57:02* allele and five *HLA-B*57:03* alleles included in the panel of known GSK samples. Based on these experimental data and the sequence analysis, we also expected to detect other alleles of the *B*57* family indicated above as negative on exon-3-specific Q-PCR and positive in the exon-2-specific Q-PCR. The latter reaction allows for the amplification of a second variable region within the exon 2 sequence, using a SSP set.

Finally, the amplification of a product within the two Q-PCRs allows for the identification of *HLA-B*57:01*, including all of the ten sequences deposited for this allele, along with *HLA-B*57:06*, *-57:08*, *-57:10* and *-57:13* alleles and the newly identified *HLA-B*57:14*, *-57:15*, *-57:18–20*, *-57:22–27*, *-57:29–33*

and *-57:35–37* which are absolutely identical in the regions examined. However, *B*57:06* and *B*57:08* alleles have a 0% frequency in the Caucasian population [20], and thus do not represent a significant confounding factor. There is no information available regarding the frequency of other alleles in different populations, thus suggesting that they may be very rare. Similar to our technique, the standard SSP-PCR method [12], widely used in other laboratories as a fast screening test as well as the newly described fluorescence method [14], does not discriminate these newly identified alleles, as well as the *B*57:16* and *B*57:34* alleles that are detected instead by our strategy using a mismatch in the forward primer of the exon-2-specific Q-PCR. However, the need for a continuous update of the primer sets based on newly identified DNA polymorphisms represents a limit of SSP-based techniques; thus, this work is an important update on the sequence studies

reported in the abovementioned papers [12,14]. Another advantage of our technique is the possibility to discriminate additional alleles, such as *HLA-B*55:14* and *-58:14*.

In this regard, we should also report that the validated methodology used to compare our results provided, with respect to the few alleles analyzed, results compatible with different genotyping studies (TABLE 1). Among these, the genotype of one *HLA-B*57:01*-positive sample was also compatible with the *HLA-B*57:14*-specific sequence. These data suggest that with respect to some *B*57* alleles, the only technique able to detect differences is the DNA sequence-based typing, which still remains the gold standard technique. However, since the frequency of such alleles seems to be extremely low, they should not represent a critical confounding factor.

Conclusion

In the present paper, we provided evidence to suggest the adoption of the test herein characterized into clinical practice as a fast method to screen for *HLA-B*57:01* status, particularly in those laboratories already involved in detection of viral load and virus genotyping. The test described may be considered as an alternative to Mallal's test for those laboratories that can have access to the Q-PCR technique. In particular, with respect to the validated methodology used to compare our method, the assay presented in this paper can allow the processing of 30 samples versus 10 samples in one single assay. The genotype results can be provided in 24 h (30 patients) versus 48 h of the reference

method (ten patients). The entire assay is run in approximately 6–8 h.

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Financial & competing interests disclosure

Dr Antonella Pirazzoli was employed by GlaxoSmithKline at the time of validation. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

- International HIV treatment guidelines recommend *HLA-B*57:01* typing before abacavir administration, in order to reduce the incidence of abacavir hypersensitivity reaction.
- Technologies available to detect individual HLA types, such as DNA sequence-based typing or the sequence-specific oligonucleotide probe method with additional DNA sequencing for patients screened as positive with the probes, are time-consuming, expensive and not readily available.
- In 2005, Mallal's group developed a quick method for *HLA-B*57:01* screening, based on the use of a panel of sequence-specific primers and standard PCR technique. This assay has been recently improved by fluorescence-based detection via capillary electrophoresis.

Results

- Two sets of sequence-specific primers have been designed and a novel test, based on real-time PCR technique, has been developed and validated in the present study.

Conclusion

- This test is rapid, allowing for the processing of 30 samples in 24 h, is sensitive and specific, inexpensive in comparison to already available alternatives, and can be easily implemented into routine clinical practice.
- The main limitation of this approach, and in general with sequence-specific primer techniques, is represented by the continuous need for sequence updating owing to the highly polymorphic nature of the *HLA-B* gene.
- Since the seminal work of Mallal's group, approximately 1000 new sequences have been identified for the *HLA-B* gene; thus, a useful update of sequence studies has been provided in the present paper.

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■ Website

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