Validation of Real-Time PCR Assay for HLA-B*5701 Detection: A Novel and Rapid Pharmacogenomic Test Predicting Abacavir Hypersensitivity Reaction

CVS 2014
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Introduction

Abacavir, an effective drug used in the management of HIV-infected patients, has the potential to cause a drug hypersensitivity reaction in ~5% of patients who carry the HLA-B*5701 allele. To decrease the risk of a hypersensitivity reaction, screening for the HLA-B*5701 allele is now recommended for all patients prior to starting or restarting an abacavir-containing therapy.[1,2,3] Current technologies routinely used to detect the individual HLA types utilize DNA sequence-based typing or a sequence-specific oligonucleotide probe method with subsequent DNA sequencing for patients screened as positive. These technologies are laborious, time-consuming, expensive and not readily available. Here-in we describe the analytical performance of a real-time PCR assay for the detection of the HLA-B*5701 allele.

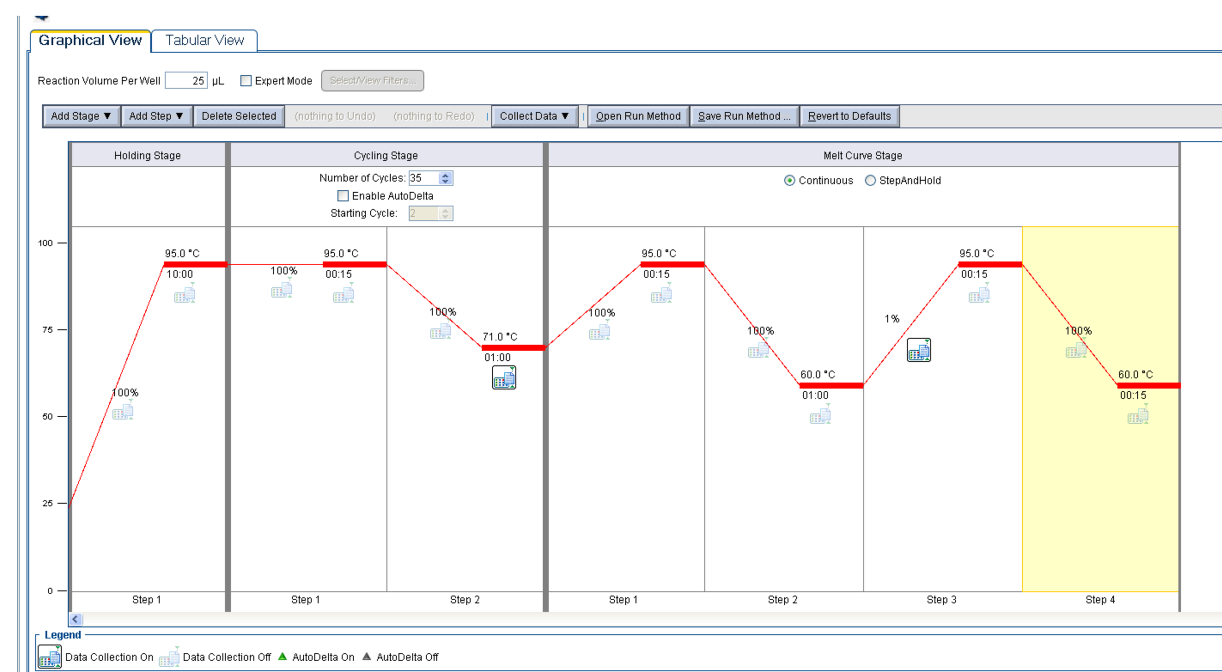
Method

Using the easyMAG® Specific B protocol (bioMérieux, Durham NC), DNA is extracted from 200 µL of whole blood. The assay utilizes DNA confirmed spectrophotometrically to have an OD 260/280 range of 1.7 to 2.0 and a concentration of ~12.5 ng/µl to ~50 ng/µl. Prior to rtPCR, samples >50ng/µl can be diluted with molecular grade water to obtain a concentration of ~40 ng/µl (alternate concentrations utilized for validation as described).

Two simultaneous PCR reactions are conducted on each DNA sample. One reaction amplifies the HLA-B*5701 allele and the other amplifies an internal control gene (ASR from Pharmigene, Inc., Taiwan[4] and iTaq™ Universal SYBR® Green Supermix, Bio-Rad[5]). Samples are analyzed using the ABI 7500 FAST Real Time PCR instrument programmed with the PCR amplification profile followed by a dissociation stage (See Figure 1).

Two Ct values are obtained for each sample, one from the HLA-B*5701 detection mix (Ct_{geno}) and the other from the Internal Control detection mix (Ct_{IC}). The difference ($\Delta Ct = Ct_{geno} - Ct_{IC}$) for each sample is determined. Result interpretation is described in Table 1.

Figure 1. Amplification Parameters for the ABI 7500 FAST RealTime PCR System



Results

Table 1. Results Interpretation

<table>
<tr><td rowspan="3">$Ct_{IC} \le 27$</td><td rowspan="2">$Ct_{geno} \le 35$</td><td>$\Delta Ct \le 7$</td><td>HLA-B*5701 positive</td></tr>
<tr><td>$\Delta Ct > 7$</td><td>HLA-B*5701 negative</td></tr>
<tr><td colspan="2">$Ct_{geno} > 35$ (undetermined)</td><td>HLA-B*5701 negative</td></tr>
<tr><td rowspan="2">$Ct_{IC} > 27$</td><td colspan="2">PCR inhibitors may be present</td><td rowspan="2">Retest</td></tr>
<tr><td colspan="2">Insufficient template quantity</td></tr>
</table>

To demonstrate analytical performance, eighty-one samples including 64 HLA-B*5701 positive samples and 17 HLA-B*5701 negative samples were analyzed.

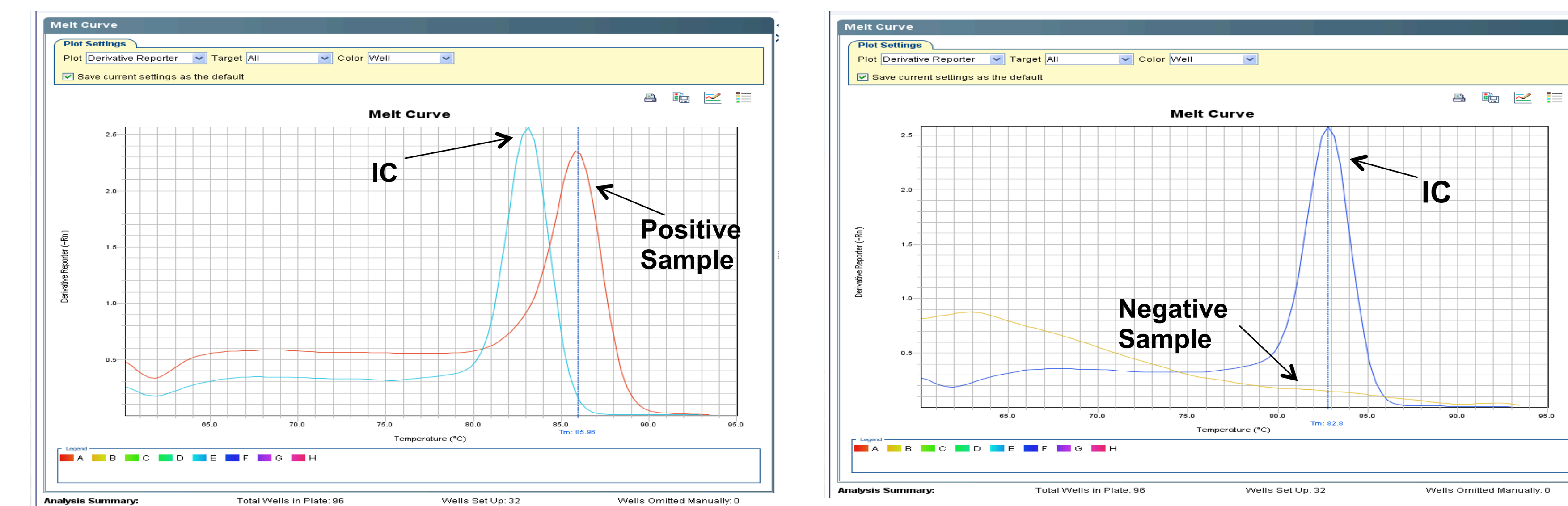
- For analytical sensitivity, extracted DNA from 5 unique samples diluted to ~12.5 ng/µl (Actual range 8.5 ng/µl to 15.3 ng/µl) was tested in duplicate on 2 separate runs (n = 20 data points). All samples yielded positive results.
- For precision, 3 samples of varying concentration (~15 ng/µl, ~25 ng/µl and ~50 ng/µl) were analyzed in triplicate over 3 runs by 2 different operators (n = 27 data points). All samples yielded positive results. Analysis of Ct values is shown in Table 2 and a representative melt curve is shown in Figure 2.
- To demonstrate accuracy, parallel testing of 20 unique samples tested by an independent laboratory using an alternate method, yielded the same interpretative results when analyzed with the bioMONTR assay (Data not shown).
- For analytical specificity, 5 unique negative samples were analyzed in duplicate (n = 10 data points). All samples yielded negative results. A representative melt curve is shown in Figure 2.
- A four member proficiency panel was analyzed and results returned to Pharmigene for evaluation. All samples yielded the correct interpretative results (Data not shown).

Table 2. Results of HLA B*5701 Precision Testing_Analysis of C_T Values

HLA B*5701 RealTime PCR_Intra-Assay and Inter-Assay Precision (HLA B*5701 Ct)												
	Intra-Assay Precision									Inter-Assay Precision		
DNA Concentration	Low (~15 ng/µl)			Mid (~25 ng/µl)			High (~50 ng/µl)			Low (~15 ng/µl)	Mid (~25 ng/µl)	High (~50 ng/µl)
Run #	1	2	3	1	2	3	1	2	3			
Replicates (n)	3	3	3	3	3	3	3	3	3	9	9	9
Mean (Ct)	23.908	23.302	23.950	23.633	23.466	23.592	21.896	21.507	22.045	23.720	23.564	21.816
Std Dev	0.051	0.703	0.139	0.057	0.087	0.106	0.031	0.370	0.068	0.477	0.106	0.306

Results (cont'd)

Figure 2. Melt Curves: Positive and Negative Samples with Associated Internal Controls



Conclusions

- bioMONTR Lab's new Research Use Only Real-Time PCR assay for detection of HLA-B*5701 demonstrates acceptable analytical performance,
- Exhibits 100% sensitivity and specificity for the HLA B*5701 allele when compared to alternate technologies,
- Performs across a dynamic range of ~12.5 to 50 ng/µl of DNA extracted from whole blood,
- A pharmacogenomic test such as bioMONTR Lab's HLA-B*5701 assay will be a valuable tool in HIV clinical trial programs and for screening HIV positive patients prior to initiation of abacavir containing regimens.

References

1. Mallal, S. et al. (2008) HLA-B*5701 Screening for Hypersensitivity to Abacavir, N Engl J Med. 358(6):568-579.
2. Saag M., et al. (2008) High Sensitivity of Human Leukocyte Antigen-B*5701 as a Marker for Immunologically Confirmed Abacavir Hypersensitivity in White and Black patients. Clinical Infectious Diseases (CID), 46:1111-1118.
3. Ziagen® prescribing information. Research Triangle Park, NC GlaxoSmithKline, 2008 and FDA (July 2008) Important safety-related label update for Ziagen (http://www.fda.gov/forconsumers/byaudience/forpatientadvocates/hivandaidsactivities/ucm121646.htm).
4. PG5701 Detection Kit (Part Number PG-5701C-024), Pharmigene, Inc. Taiwan.
5. iTaq™ Universal SYBR® Green Supermix (Catalog # 172-5120), Bio-Rad Laboratories, Hercules, CA.

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