Development and Optimization of a Real-Time PCR Assay for Detection of Herpes Simplex and Varicella-Zoster Viruses in Skin and Mucosal Lesions by Use of the BD Max Open System

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We transitioned laboratory-developed PCR assays for herpes simplex virus 1 (HSV-1), HSV-2, and varicella-zoster virus (VZV) to the BD Max system by using BD Max open system reagents. After optimization, the agreement with the reference PCR assay was 100% (123/123) for HSV-1, 96.7% (119/123) for HSV-2, and 100% (60/60) for VZV using retrospective clinical samples.

Molecular technologies have largely replaced culture in the virology laboratory. While many commercial platforms exist for the detection of respiratory viruses, hepatitis viruses, human immunodeficiency virus (HIV), and cytomegalovirus (CMV) (1–4), there are few FDA-approved tests for the detection of herpes simplex virus (HSV) or varicella-zoster virus (VZV), especially from skin and mucosal lesions. Therefore, the laboratory either relies on less sensitive methods, such as culture and direct fluorescent-antibody assay (DFA) testing, or develops its own PCR assay. The BD Max (BDM) system (BD Diagnostics, Sparks, MD) is an automated molecular platform that combines specimen extraction and real-time PCR with a number of FDA-cleared assays. In addition, their generic extraction and PCR reagents for the “open platform” allow users to design their own assays using their own primers and probes (5, 6). We transitioned our existing laboratory-developed assays (LDAs) for HSV and VZV from a QIAcube extractor (Qiagen, Valencia, CA) and ABI 7500 real-time PCR instrument (Applied Biosystems, Carlsbad, CA) to the BDM. The current work describes our optimization steps for transitioning the LDA to the BDM and details the performance of the new assay.

Archived frozen (−70°C) patient specimens from skin and mucosal lesions in viral transport medium (VTM) and with known LDA results were used to determine the performance of the optimized BDM assays. Previously, LDA-positive specimens, representing a wide range of threshold cycle ($C_T$) values (14 to 35), and a random selection of previously LDA-negative specimens were tested. The limit of detection (LOD), expressed in log copies/ml, was determined by diluting purified and quantified DNA in a negative patient sample in VTM and analyzing via the probe method (7).

The LDA uses DNA extracted from clinical specimens in viral transport medium with the QIAcube extractor (custom protocol ID2822) and the QIAamp minikit using the blood and body fluid protocol (Qiagen). The purified DNA was eluted in 200 μl of buffer AE, with 5 μl of template added to the PCR. The PCR used TaqMan universal PCR master mix (Applied Biosystems).

The VZV LDA used a 0.9 μM final concentration of each primer (VZV forward [5′-TCT TGT TCC ACC GGA GGC TTC TG-3′] and VZV reverse [5′-TGG GTG TCC ACC GGA TGA T-3′]) and 0.2 μM probe (6-FAM-TCT CGA CTG GGT GGG ACT TGC G-TAMRA [6-FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine]) (8). The PCR was performed using an ABI 7500 instrument as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. No cross-reactivity was observed in HSV-1- or HSV-2-positive samples, and in silico analysis demonstrated no cross-reactivity with other herpesviruses or lesion-causing bacteria. The limit of detection for the LDA was 2.90 log copies/ml.

The VZV assay on the BD Max system used the same primer and probe concentrations that were used in the LDA, the BDM ExK DNA-3 extraction kit, and BDM DNA MMK (specimen-processing control [SPC]) master mix. Use of the BDM DNA MMK master mix with or without the SPC yielded equivalent results (data not shown). Cycling parameters on the BDM were 96°C for 10 min followed by 40 cycles of 96°C for 17.7 s and 60°C for 1 min, with the fractional seconds added to allow for adequate time for reading the reactions, as required by the BD Max assay software. Because the maximum signal output of many of our known positive specimens saturated the FAM channel detector, we investigated the effect of changing the detector gain from its default setting of 60 to as low as 20. While maximum curve amplitudes decreased with the lower gain settings, no positive specimens became negative at the lowest gain setting. We therefore chose a setting of 20 to minimize the number of positive specimens that generated a saturated signal. We kept the default setting of 60 for the SPC channel. These parameters proved to be appropriate for the transfer of the VZV LDA to the BDM, as there was 100% agreement with 22 positive and 48 negative specimens (Table 1). The LOD was determined to be 3.05 log copies/ml.

The LDAs used for the detection of HSV-1 and HSV-2 were singleplex assays with final primer and probe concentrations of 0.6 μM and 0.2 μM, respectively. The primers and probes used were HSV-1 forward (5′-GGC CTG GCT ATC CGG AGA-3′), HSV-1 reverse (5′ GCC GAG AGA CAT CGG GA-3′), HSV-1 probe (6-FAM-CAG CAC ACG ACT TGG CGT TCT GTG T-TAMRA),
TABLE 1 Performance of the BD Max system

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Agreement (% [no. of results/total no. of tests performed])</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>VZV</td>
<td>100 (22/22)</td>
</tr>
<tr>
<td>HSV-1</td>
<td>100 (23/23)</td>
</tr>
<tr>
<td>HSV-2</td>
<td>100 (28/28)</td>
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*Includes 5 samples that were classified as indeterminate (C<sub>F</sub> > 35) on the LDA.

HSV-2 forward (5′-AGA TAT CCT TTT TAT CAT CAG CAC CA-3′), HSV-2 reverse (5′-TTG TGC CAA GGC GA-3′), and HSV-2 probe (FAM-CAG ACA AAC GAA CGC CGC CGC CATMRA) (9). The PCR parameters used on the ABI 7500 for the two HSV assays were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 57°C for 1 min. No cross-reactivity was observed in the opposite HSV-type or VZV-positive samples, and in silico analysis demonstrated no cross-reactivity with other herpesviruses or lesion-causing bacteria. The LDA limits of detection were 2.54 log copies/ml for HSV-1 and 4.20 log copies/ml for HSV-2. When transferring the HSV LDA to the BDM, we used a multiplex assay that changed the HSV-2 probe to a VIC fluorophore. This resulted in signal bleed into the HSV-1 channel if the HSV-2 signal was strongly positive. To avoid this problem, we made a new HSV-2 probe using a ROX fluorophore and Black Hole Quencher-2 (Biosearch Technologies, Petaluma, CA). Similar to our approach with the VZV BDM assay, we started by changing the denaturation temperature from 95°C to 96°C. This worked well for the HSV-2 reaction, as 10 out of 10 positive samples were concordant. The HSV-1 assay, however, performed poorly, with only 7 out of 10 positive samples being concordant. We further adjusted the denaturation temperature to 99°C and tested different annealing temperatures ranging from 60°C to 64°C. Ultimately, an annealing temperature of 62°C worked best, detecting all positives with the best efficiency (lowest C<sub>F</sub> value). Our final cycling parameters were 99°C for 10 min followed by 40 cycles of 99°C for 15 s and 62°C for 53.8 s. For the HSV assay, no significant differences in specimen positivity rates or detector saturation were observed between gains of 40 to 80. The HSV and SPC targets were set to a gain of 60. The LOD were 2.6 and 3.84 log copies/ml for HSV-1 and HSV-2, respectively.

Using these parameters, the BDM HSV-1 reactions were 100% concordant with the LDA for 23 positive and 100 negative specimens (Table 1). The BDM HSV-2 reactions were 100% concordant with the LDA for 23 positive specimens. In addition, five specimens that were previously deemed indeterminate by the LDA because of high (≥35) C<sub>F</sub> values were positive in the BDM assay (Table 1); repeat testing of these five specimens with the LDA gave the same indeterminate results. Interestingly, 4 of the 95 specimens deemed negative by the HSV-2 LDA were positive for HSV-2 by the BDM assay. Repeat testing of these four specimens using the LDA gave negative results. Two of the four specimens were sent for testing at an outside laboratory that used a PCR assay different from either our LDA or the BDM assay. One specimen was reported positive and one as negative for HSV-2; the specimen reported as negative had undergone an additional freeze-thaw cycle before testing. The two other discrepant result specimens were not thought to require reference laboratory adjudication because they were collected from patients clinically diagnosed with HSV due to recurrent lesions; one of these specimens gave a visible amplification curve on the LDA that failed to cross the positive test threshold. We hypothesize that part of the reason for the increased HSV-2 detection in the BDM assay may be the 4-fold increase in the concentration of template added to the BDM reaction compared to that in the LDA.

In summary, we demonstrate that real-time PCR LDA for HSV and VZV can be transitioned to the BDM, given adequate assay and instrument optimization. Additionally, the change in concentration of template added to the BDM reaction may influence the results of any comparative study. Moving PCR assays to the BDM provides the advantage of one-step processing on a single instrument in a single location. Not only does this eliminate the need for separate processing and amplification areas and equipment, but it also allows for less technologist hands-on time and the ability to set up more tests in 1 day, ultimately improving efficiency in the laboratory.

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REFERENCES