

Performance Characteristics of MultiCode®-RTx PCR Assay for the Quantitative Detection of Human Herpesvirus 6 DNA in Plasma Samples



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Abstract

Human Herpesvirus 6 (HHV-6) is a lymphotropic, beta-herpesvirus, known to frequently reactivate in immunosuppressed solid organ and hematopoietic transplant recipients. Although the causative role of HHV-6 in any specific disease manifestation in these patient populations is yet to be definitively established, HHV-6 reactivation is clearly associated with increased risk of adverse clinical events in transplant patients. Monitoring for the presence of circulating HHV-6 DNA by quantitative PCR may, therefore, be useful tool for detecting at risk patients and evaluating the success of therapeutic interventions. We report here on the performance characteristics of a quantitative PCR assay for HHV-6 (qHHV6-PCR) that utilizes MultiCode-RTx® technology (EraGen Biosciences, Madison, WI) for detecting PCR products in real-time. One of the advantages of MultiCode-RTx® is that, since tagged-primers rather than probes are used for amplicon detection, amplification efficiency is the sole variable influencing the kinetics of signal generation resulting in improved precision of target quantitation. The qHHV6-PCR assay described enables multiplexed detection of HHV6-DNA and an extractable internal control target. Quantitation is accomplished via interpolation of results into externally-generated, lot specific, calibration curves. We have established the analytical performance metrics of the qHHV6-PCR assay, including the lower limit of quantitation (LLOQ) and detection (LOD), and delineated the sample storage requirements, reproducibility, specificity and internal control performance of this novel assay. The results of these experiments indicate that this assay is capable of highly reproducible quantitation of HHV6-DNA in plasma samples over a range of at least 4 logs. Utilizing samples submitted for quantitative monitoring of CMV infection, we were also able to examine the prevalence and viral dynamics of HHV-6 infection in patients with or without concomitant CMV infection.

Methods

In brief, the qHHV6-PCR assay was performed as follows. Nucleic-acid was recovered from clinical samples (500 µL initial volume) using the QIAamp virus kit (QIAGEN). Co-extraction and recovery of the internal control target (ICT) was performed via addition of ICT to lysis buffer (designed to result in a final [ICT] in PCR of 250 cp/rxn). Amplification and detection was performed on an ABI 7900HT instrument with 2-channel detection (FAM for HHV-6 target and JOE for ICT). Positive reactions were considered to be those that yielded Ct values of ≤40 cycles with peak Tm values of 78.9°C – 80.9°C for HHV-6 target and 76.7°C – 78.7°C for ICT. Quantitation of target was accomplished via interpolation of Ct values into externally-generated standard curves constructed using quantitated DNA as the calibrating material.

LOD and LLOQ values for the qHHV6-PCR assay were determined by testing and analyzing multiple replicates (n=24) of a limiting dilution series of HHV-6 reference material (ZeptoMetrix Corp.) diluted in pooled negative plasma samples. Linearity of the assay was assessed by testing serially diluted HHV-6 reference material.

To establish qHHV6-PCR specific conditions for sample storage, samples (n=3) were spiked with HHV-6 at a final concentration approximately 1 log₁₀ above the LLOQ (1,000 cp/mL) and tested in triplicate after storage for 0, 1, 3, 5 and 7 days either refrigerated (2°C to 8°C) or frozen (-15°C to -30°C).

Exclusivity was established by testing high-titers of related human herpesviruses, as well as representative viruses and micro-organisms potentially present in samples analyzed in the qHHV6-PCR assay.

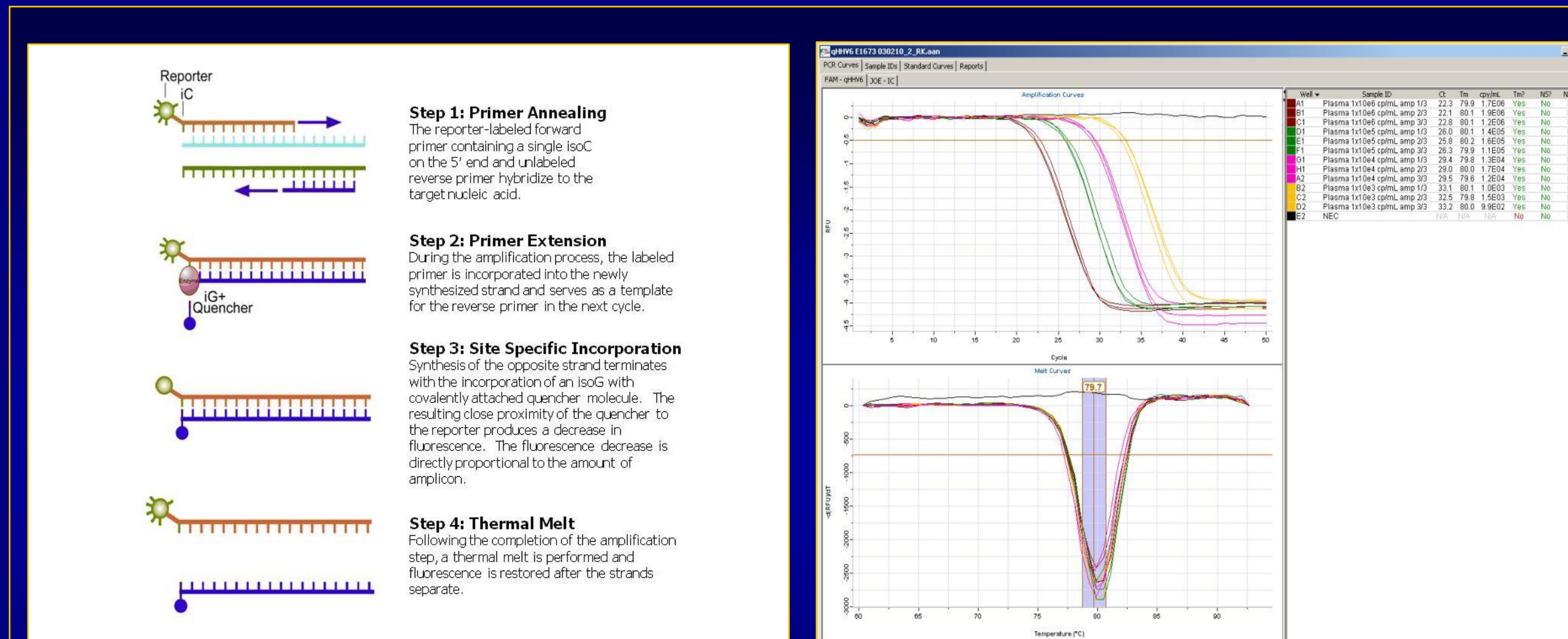


Figure 1. Schematic illustration of the MultiCode®-RTx system and instrument readout from qHHV6-PCR target dilution series.

Results

Nominal conc. (cp/mL)	No. tested	No. positive	% positive
500	24	24	100
200	24	24	100
100	24	24	100
50	24	23	60
10	24	12	46

Table 1. Determination of LOD for qHHV6-PCR assay on plasma samples. Probit (XLSTAT) analysis on data shown indicated an LOD value for plasma of **42 cp/mL**, based on a 95% probability of a positive result.

Nominal conc. (Log ₁₀ cp/mL)	Mean conc. (Log ₁₀ cp/mL)	SD ¹	% CV ²
500 (2.7)	578 (2.8)	0.08	19.3
200 (2.3)	225 (2.4)	0.10	21.6
100 (2.0)	122 (2.1)	0.19	38.6

Table 2. Establishment of LLOQ for the qHHV6-PCR assay. Analysis of quantitative values obtained for a subset of samples in Table 1 indicated reproducible results could be obtained for plasma samples containing a minimum of 100 cp HHV-6 DNA/mL.

¹Using lognormal distribution
²Using normal distribution

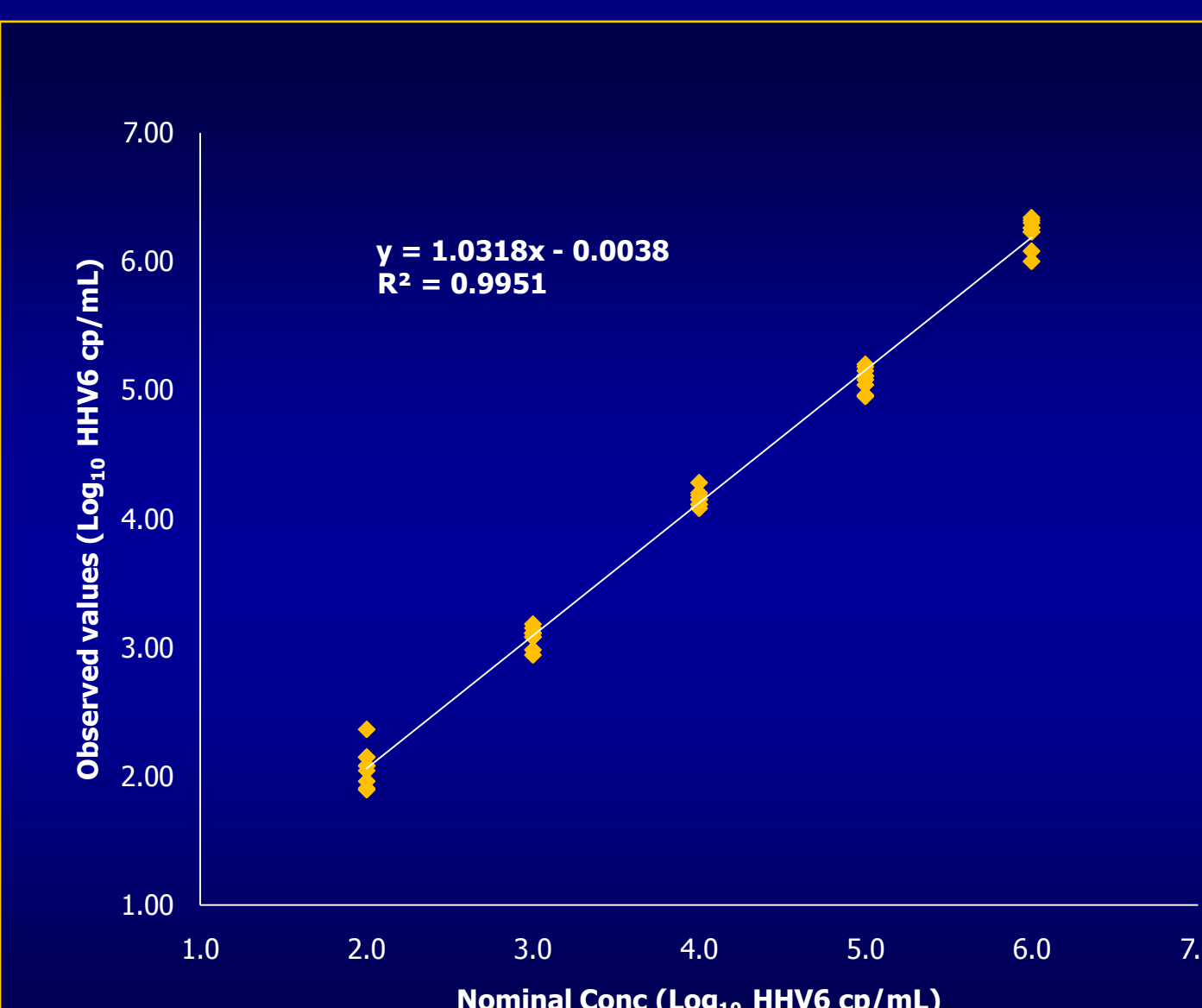


Figure 2. Demonstration of linearity and repeatability of the qHHV6-PCR assay for plasma samples. The panel consisted of a dilution series of high concentration HHV-6 Reference material (ZeptoMetrix Corp.) in sample matrix.

	HHV6 + (n=8)	HHV6 - (n=93)
CMV + (n=46)	4 (8.7%)	42 (91.3%)
CMV - (n=55)	4 (7.3%)	51 (92.7%)

Table 3. Frequency of HHV-6 PCR positive results in plasma samples from patients with and without CMV viremia (as determined by a positive qCMV-PCR result).

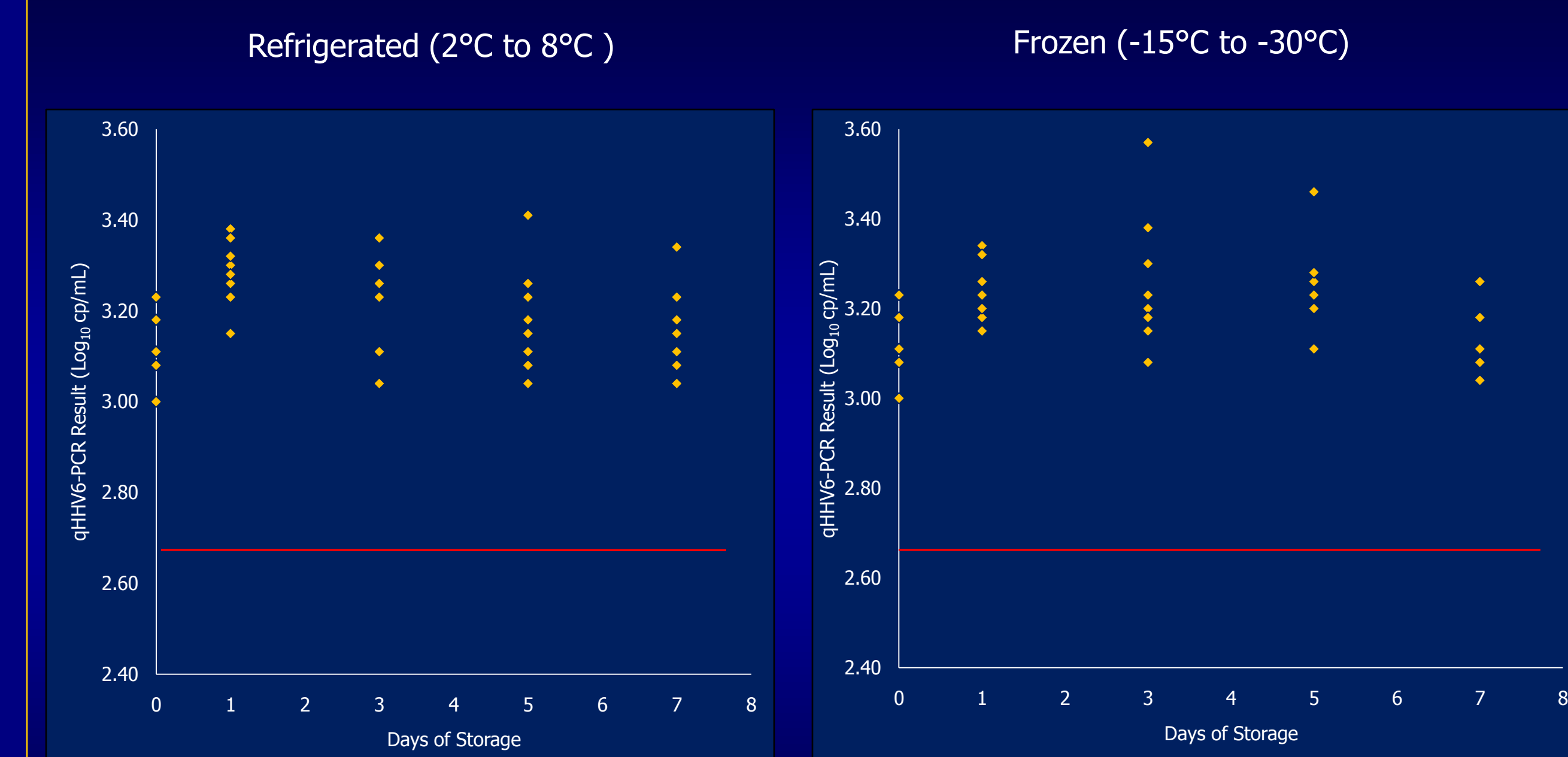


Figure 4. Stability of HHV-6 DNA in plasma samples at refrigerated and frozen temperatures. Samples (n=3) were analyzed in triplicate at each time point. Red bar indicates stability guard band (-0.5 Log₁₀ cp/mL below mean value at time zero).

Table 4. Exclusivity panel. Testing was performed either on high-titer cultured organisms* or on viral nucleic-acid. All the analytes tested generated negative results.

CMV	EBV	VZV	HSV 1
HSV2	BK Virus	HBV	HCV
HIV-1	<i>K. pneumoniae</i> *	<i>S. aureus</i> *	<i>C. albicans</i> *

Conclusions

- ❖ The results contained here demonstrate the performance characteristics of a qHHV6-PCR assay using MultiCode®-RTx chemistry (EraGen Biosciences). The assay, as configured at ViroMed Labs., has an LOD of approximately 42 cp/mL for plasma, with an LLOQ of approximately 100 cp/mL based on an acceptable total precision of SD ≤0.25 log₁₀ HHV6-DNA cp/mL.
- ❖ The assay was demonstrably linear over at least 4 logs of target input DNA conc. resulting in an upper limit of quantitation of at least 1 million cp/mL.
- ❖ Values obtained in the qHHV6-PCR assay were not adversely affected by storage of samples for up to 7 days under either refrigerated or frozen conditions.
- ❖ Analysis of unselected plasma samples submitted to ViroMed Laboratories for quantitative CMV PCR revealed an overall incidence of concomitant HHV-6 infection of 7.9% (8/101) with no difference in incidence in samples determined to be positive or negative for CMV DNA.