# Development and Qualification of a Real-Time PCR Assay for the Quantitative Detection of HSV-2 DNA in Anogenital Swabs

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## Introduction

Genital HSV shedding can be used to confirm viral infection, is a predictor of disease severity, and is associated with the risk of HSV transmission. Determination of the frequency of viral shedding, as a measure of viral reactivation, has been used as a means of demonstrating the effectiveness of antiviral therapy[1,2]. GEN-003 is an candidate therapeutic HSV-2 vaccine containing a fragment of ICP4 (ICP4.2) and a deletion mutant of gD2 (gD2ΔTMR), adjuvanted with Matrix-M2. To investigate the efficacy of the GEN-003 vaccine on genital HSV-2 shedding in clinical trial participants, we developed a sensitive and specific quantitative real-time PCR assay for the detection of HSV-2 DNA in anogenital swabs. Multiple assay performance parameters were evaluated including specificity, range, linearity, limit of quantitation (LOQ), assay precision and inter-laboratory reproducibility.

## HSV Shedding Assay

DNA was extracted from a 200µl aliquot of PCR buffer (100mM KCl, 10mM Tris-Cl pH 8, 25mM EDTA, 0.5% Igepal) either spiked with known quantities (PFU/mL) of HSV-2 strain 333 or containing human genital swab samples from HSV-2-infected subjects (collected in a total volume of 1.0 ml), using a QIAamp DNA Blood MDx 96 well kit and Qiagen Universal Robot. DNA was eluted in 100µl of water. HSV-2 specific gG primers and TaqMan probe (**Table 1**) were designed by Primer Express software (Applied Biosystems). The TaqMan probes were labeled at the 5' end with 6-carboxyfluorescein (FAM) as the reporter fluorophore, and at the 3' end with carboxytetramethylrhodamine (TAMRA) as the fluorescent quencher.

**Table 1. HSV-2 specific gG primers and probe for viral shedding assay.**

| HSV serotype | Probe | Forward primer | Reverse primer |
|---|---|---|---|
| HSV-2 | FAM-ACCCACGTGCAGCTCGCCG-TAMRA | CGGAGACATTCGAGTACCAGATC | GCCCACCTCTACCCACAACA |

PCR was carried out in a 20µl volume containing 5µl of DNA template, TaqMan Gene Expression Mastermix (Life Technologies), 45µM of each primer and 10µM of probe. Amplification reactions were run at 50°C for 2min and 95°C for 10min and then subjected to 45 cycles at 94°C for 15s, 60°C for 60s in a StepOne Plus real-time PCR unit (Life Technologies). The PCR threshold cycle (Ct) was determined automatically by the software, and defined as the cycle number at which the fluorescent signal of the reaction crosses the threshold, or background fluorescence measured in early cycles of the PCR reaction. As the template amount decreases, the Ct value for a reaction increases.

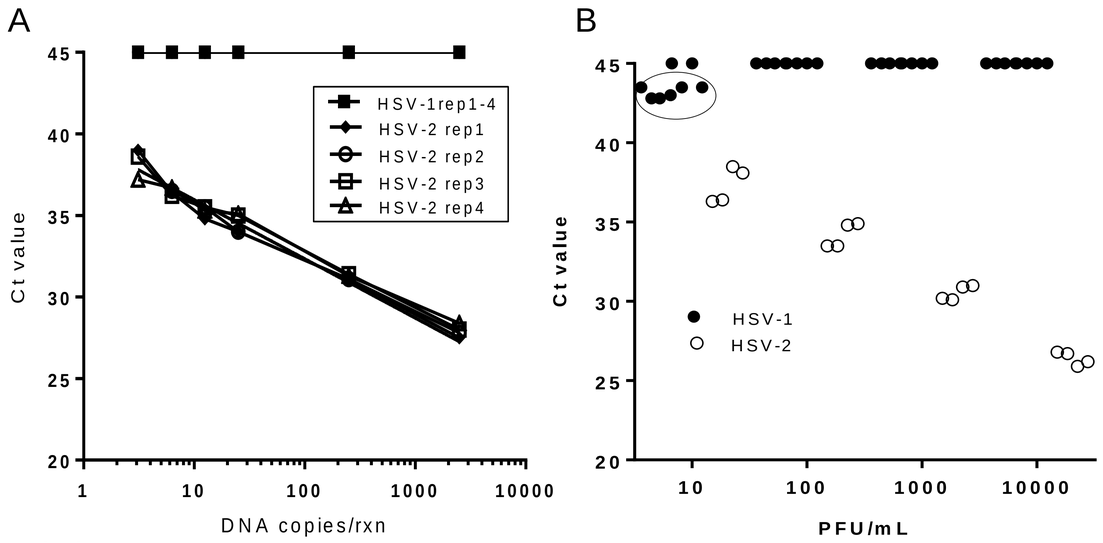
*[1]Diaz-Mitoma F, et al. 1998. JAMA 280:887*
*[2]Gupta R, et al. 2004. J Infect Dis 190:1374*

## Results

### Figure 1. HSV-2 gG Primers and Probe Set are Specific for the Target Sequence.

To verify the specificity of the gG2 primers and probe, experiments were performed with purchased genomic HSV-1 and HSV-2 DNA (Advanced Biotechnologies) and DNA extracted from plaque-titered viral stocks.

(A) A standard curve was prepared by making three 10-fold dilutions followed by three 2-fold dilutions of genomic HSV-2 DNA, ranging from 3.1 to 2,500 copies per reaction, and amplified in four wells (rep) using the gG2 primers and probe described in **Table 1**. Ct values are plotted on the y-axis and DNA copy number on the x-axis. Each line represents one of the four replicate curves. The $R^2$ value range from 0.968 to 0.996. **(B)** DNA was extracted from four 10-fold dilutions of HSV-1 and HSV-2 plaque titered viral stocks and used as template for the gG2 primers and probe set. Ct values are shown on the y-axis and are plotted against the stock HSV dilution on the x-axis. Each symbol represents eight HSV-1 and four HSV-2 extraction wells PCR amplified in duplicate.



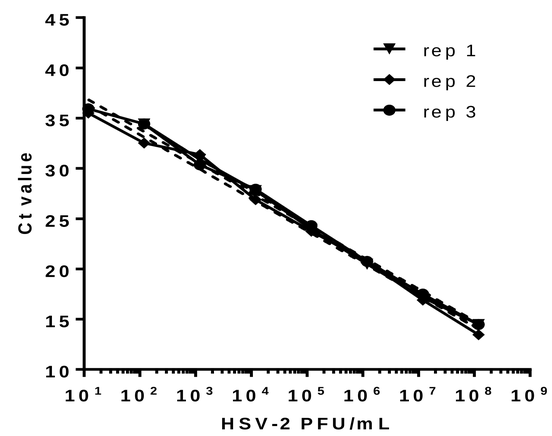

**Conclusions:**

1) Using a gG2 primer/probe set, PCR amplification of a standard curve, ranging from 3.1 to 2,500 copies and prepared from genomic HSV-2 DNA is linear, with a mean $R^2$ value of 0.986 ± 0.012.
2) The gG2 real-time PCR primers/probe set is specific for HSV-2 DNA and not DNA from HSV-1.
3) Some non-specific reactivity was observed for HSV-1 samples (panel B, circled, 6 of 8) with gG2 primers and probes but additional studies demonstrated that the positive PCR wells observed in this study were due to a robotic error during the DNA extraction step that cross-contaminated these samples with HSV-2.

## Summary

- The HSV-2 shedding assay is highly specific for its viral target: no cross-reactivity of HSV-1 with HSV-2 gG2 primers/probe was detected.
- The assay exhibited a log-linear response characteristics with HSV-2 input levels of up to $10^8$ PFU/ml.
- Limit of quantitation (LOQ) is 11 HSV-2 DNA copies/reaction, 50% of samples show a positive PCR signal at 2.7 copies/reaction.
- Intra-assay variability (%CV) for 7 operators was 14.3% ± 2.5, indicating a high level of assay precision. The overall inter-assay variability was 32.4% ± 11.8.
- Inter-laboratory reproducibility was highly correlated. Relative inter-lab sensitivity and specificity were 100% in both directions for negative samples and for mid or high positive samples

### Figure 2. PCR Amplification is Linear from 10 to $10^8$ PFU/mL of HSV-2.




Eight 10-fold dilutions of plaque-titered HSV-2, from $10^8$ to 10 PFU/mL, were prepared in PCR buffer and then DNA was extracted in triplicate (rep 1, 2, 3). PCR amplification was performed in duplicate for each extraction well using gG2 primer and probe set. Data are plotted as the mean Ct value of duplicate amplifications from each extraction. NTC samples showed no PCR amplification. **Conclusions:** $R^2$ values demonstrate excellent linearity for the HSV-2 shedding assay (**Table 2**).

**Table 2. Shedding assay linearity.**

| Sample | $R^2$ |
|---|---|
| rep 1 | 0.9986 |
| rep 2 | 0.9915 |
| rep 3 | 0.9954 |

### Table 3. Interoperator, Inter- and Intra-assay Results are Comparable

The intra-assay and inter-assay coefficient of variation (CV% = Mean/SD x 100) was calculated for the DNA copies/reaction from three consecutive assays run over three days and performed by seven operators (OP1-OP7). Each PCR was run with a seven point genomic DNA standard curve from 2.7 to 14,000 copies per reaction. Four 10-fold or seven 5-fold dilutions of HSV-2 spiked into PCR buffer were assayed as previously described. Intra-assay (>30 to 2.3 x $10^6$ DNA copies; 40 wells/day) and inter-assay (1 to 2.23 x $10^6$ copies; 3 days) are shown.

| Parameter | OP1 | OP2 | OP3 | OP4 | OP5 | OP6 | OP7 | Mean ± SD |
|---|---|---|---|---|---|---|---|---|
| Intra-assay | 9.7 | 14.3 | 18.0 | 14.7 | 13.0 | 15.6 | 14.8 | 14.3 ± 2.5 |
| Inter-assay | 50.2* | 22.5 | 40.0 | 26.9 | 27.8 | 18.1 | 42.8 | 32.4 ± 11.8 |

*inter-assay CV% of 50.2 for OP1 was due to a 3-fold difference in DNA copies on Day 2 for 2 of 4 dilutions of virus.

### Figure 3. The Viral Shedding Assay has Acceptable Precision, with an LOQ of 11 Copies Per Reaction.

**(A)** An example of intra-assay variability for OP5 on Day 1. Seven 5-fold dilutions of HSV-2 were analyzed in each of ten wells per assay plate. **(B)** An example of assay performance for clinical swab samples. Analysis of the same set of samples in duplicate was carried out by two operators (e.g. OP1 vs OP2, OP2 vs OP3, etc). These repeatability studies were performed by all seven operators as an assay training prerequisite. **Conclusion:** samples containing >50 DNA copies/rxn varied < 2-fold. Samples containing < 50 DNA copies/rxn varied on the average by <3-fold.

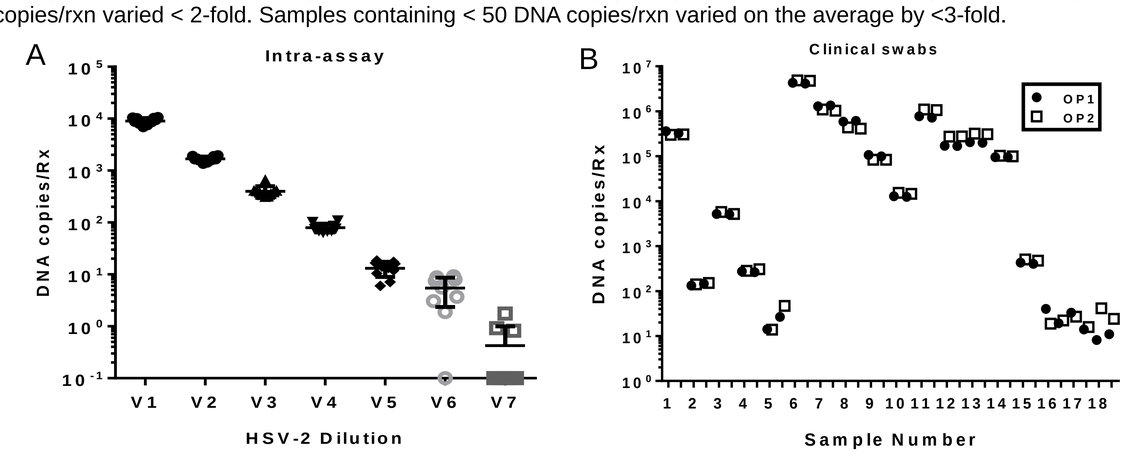



**Table 4** shows the percent of wells that gave a positive or negative PCR result calculated using 200 data points from duplicate wells for the two most dilute points on the HSV-2 genomic DNA standard curve (10.9 and 2.7 copies/reaction).

**Table 4. Limit of Quantitation (LOQ).**

| DNA copies | Rep. wells pos | One well pos | Rep. wells neg |
|---|---|---|---|
| 10.9 | 95% | 100% | 0 |
| 2.7 | 34% | 50% | 19% |

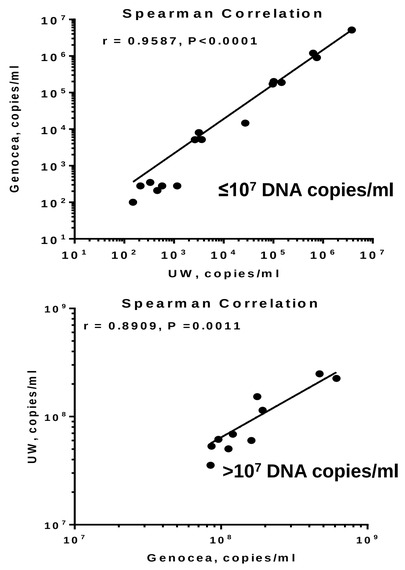
### Figure 4. Viral Shedding Assay Results Are Consistent Between Laboratories

To ensure reproducibility of results between independent laboratories, Genocea Biosciences and University of Washington exchanged three sets of samples: 50 negative, ten low positives* (<1000 copies/ml), and ten mid- or high-positives (>1,000 copies/ml). Each laboratory assayed the samples with their own viral shedding assay and results were compared by University of Washington. The results are shown in the tables below, with corresponding correlation coefficients for low (top) and mid or high (bottom) copy number shown in the graphs to the right.

| Genocea originally assigned category of results | UW subsequent evaluation | | | Total |
|---|---|---|---|---|
| | Negative | Below LOD | Positive | |
| Negative | 49 (96%) | 2 (4%) | 0 (0%) | 51 |
| Positive-low | 1 (11%) | 2(2%) | 6 (67%) | 9 |
| Positive-mid | 0 (0%) | 0 (0%) | 10 (100%) | 10 |
| Total | 50 | 4 | 16 | 70 |

| UW originally assigned category of results | Genocea subsequent evaluation | | | Total |
|---|---|---|---|---|
| | Negative | Below LOD | Positive | |
| Negative | 50 (100%) | 0 (0%) | 0 (0%) | 50 |
| Positive-low | - | - | - | No samples* |
| Positive-high | 0 (0%) | 0 (0%) | 10 (100%) | 10 |
| Total | 50 | 0 | 10 | 60 |

*Note: UW did not sent 10 low HSV-2 positive samples to Genocea for testing.




**Conclusion:** Inter-laboratory viral shedding assay results are highly reproducible.

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