Development and Characterization of a Multiplex PCR Assay for Simultaneous Quantification of Herpes Simplex Virus 1 and 2 in Anogenital Swab Samples

Meghan Hart, Vinny Sanz, Vivian Morris, Melissa French, Lisa K. McNeil, Jessica Flechtner, Lizhi Yu

Genocea Biosciences, Cambridge, MA

ABSTRACT

Genital herpes is a common global disease caused by herpes simplex virus (HSV). Determination of viral shedding as a measure of viral reactivation has been used as a means of demonstrating the effectiveness of prevention or treatment of HSV, including prophylaxis and therapeutic vaccines. Although substantial morbidity of genital herpes is caused by HSV type 2 (HSV-2), an increasing number of genital herpes infections are caused by HSV-1. To determine the source of the disease in infected patients, we developed a multiplex qPCR assay to simultaneously detect and quantify HSV-1 and HSV-2 from human anogenital swab samples from patients enrolled in GEN-003 clinical trials. The impact of possible interference between HSV-1 and HSV-2 detection was carefully investigated by comparing the performance of a HSV-1 or HSV-2 singleplex assay to the HSV-1/2 duplex assay. Importantly, PCR linearity and efficiency values for each virus target were maintained in a duplex setting (HSV-1: 0.998 R^2 and 99.8% efficiency; HSV-2: 0.986 R^2 and 90.5% efficiency). Neither sensitivity nor linearity of HSV-1 was affected in the presence of a high amount of HSV-2 and vice versa, indicating the strong robustness of this duplex assay. Further testing of clinical samples containing either HSV-1 or HSV-2 showed that the detection of virus was 100% concordant with expected results. In the future, this duplex assay could be used as a comprehensive and cost-efficient method in clinical trials for investigating the viral shedding rate of patients infected by either type of virus after dosing with GEN-003, a therapeutic genital herpes vaccine. In addition, the use of this multiplex assay will enable dynamics of infection with HSV-1 and HSV-2 to be examined, especially in patients with recurrent lesions.

INTRODUCTION

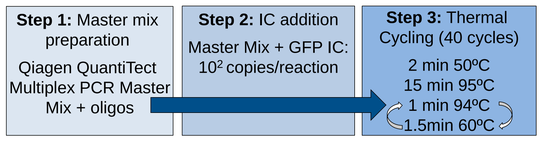
- Genital herpes, caused by herpes simplex virus, remains one of the most prevalent sexually transmitted infections.
- Substantial morbidity of genital herpes is caused by HSV-2, yet an increasing number of genital herpes infections are caused by HSV-1.[1]
- Determination of viral shedding has been used as a method of demonstrating the effectiveness of treatment of HSV.
- GEN-003, a therapeutic subunit vaccine, is in clinical development and has been shown to reduce viral shedding and lesion rates in HSV-2 subjects with genital herpes for up to 12 months with an acceptable safety profile.[2]
- A multiplex qPCR assay was developed with the following purposes:
  - Simultaneous detection/quantification of both HSV-1 and HSV-2 targets in clinical samples
  - Verification of the integrity of the entire extraction/PCR process of each sample by internal control (IC)
  - Investigation of the cause of genital herpes infection, clinical efficacy of GEN-003 on both HSV subtypes, dynamics of co-infection of genital herpes

Materials/Methods

A

DNA target	Oligo	Sequence
HSV-1 gG1	Forward 5'-3'	GCCTGGCTATCCGGAGAAAC
	Reverse 5'-3'	GCGCAGAGACATCGCGA
	Probe 5' Q670, 3' BHQ2	AGCACACGACTTGGCGTTCTGTGTG
HSV-2 gG2	Forward 5'-3' [3]	CGGAGACATTCGAGTACCAGATC
	Reverse 5'-3' [3]	GCCCACCTCTACCCACAACA
	Probe 5' FAM, 3' NFQ	CACGTGCAGCTCGCC
GFP (IC)	Forward 5'-3'	CCAAGGGTGACCTCCAGTTC
	Reverse 5'-3'	TGGAAGCCATACCCGATATGA
	Probe 5' VIC, 3' NFQ	CCCCTGGATTCTGG

B



C

Acceptance Criteria	
slope	-3.6≤x≤-3.1
R^2	≥0.98
% Efficiency	90-110

Figure 1: Multiplex assay design and criteria for acceptance. (A) Primer and probe sequences. (B) Assay workflow. (C) Assay performance was evaluated by previously set criteria.

HSV-1/2 multiplex assay performed similarly to singleplex assays

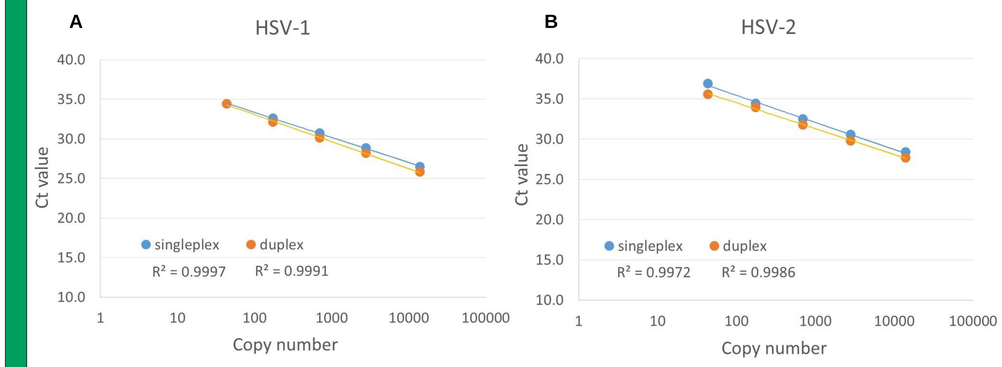


Figure 2: HSV-1 and HSV-2 standard curves made from commercially available gDNA (Vircell) were detected at similar levels in both singleplex and multiplex qPCR formats. PCR efficiency values for each are reported as follows: (A) HSV-1- singleplex: 107%, multiplex: 96%; (B) HSV-2- singleplex: 98%, multiplex: 104%

HSV-1/2 multiplex assay remained functional with addition of GFP internal control

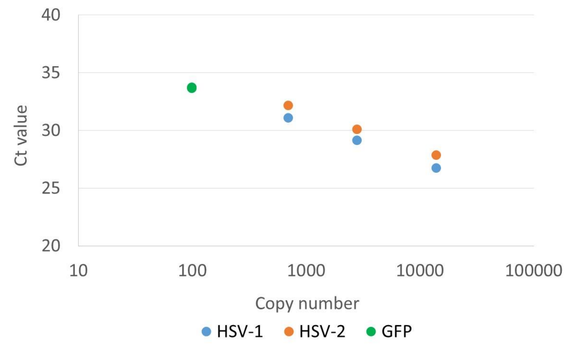


Figure 3: Feasibility of adding an internal control into the multiplex assay. HSV-1 and HSV-2 gDNA were titrated at three concentration points. Master mix containing primers and probes along with GFP internal control was used for simultaneous target detection without observation of cross-talk between dye channels. HSV-1 and HSV-2 data are shown as the mean of triplicate wells, GFP is plotted as a mean value for each concentration point (n=3).

Assay linearity was not affected by the presence of a highly concentrated second target

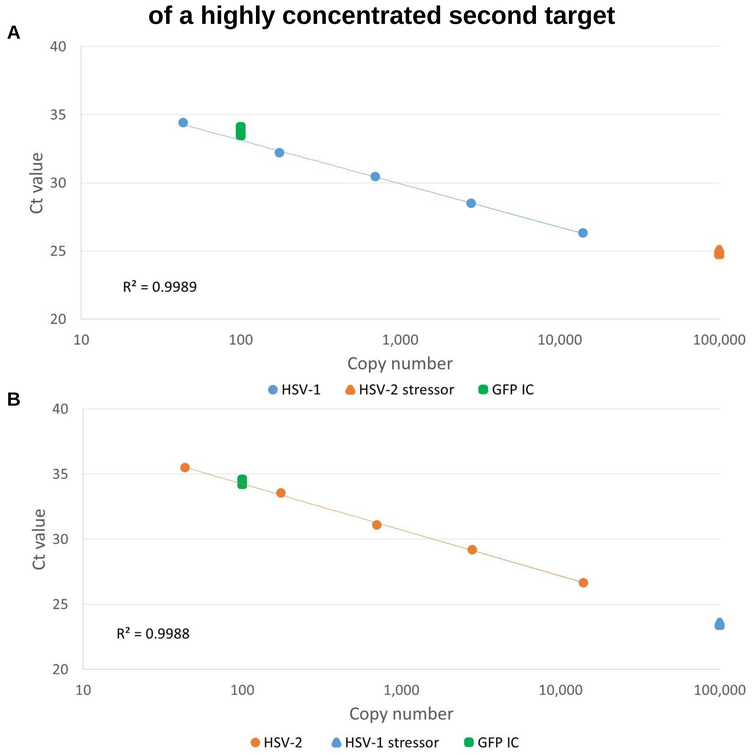


Figure 4: Standard curves tested in the presence of a highly concentrated second target and GFP IC. Standard curves for either HSV-1 or HSV-2 were prepared using gDNA. A highly concentrated stressor target (either HSV-1 or HSV-2) was added to each standard curve concentration point along with GFP IC at a set concentration. Standard curve data are shown as the mean of duplicate wells, IC and stressor data are plotted as a mean value for each concentration point (n=5). (A) HSV-1 standard curve: R^2 = 0.9989, GFP IC, and HSV-2 stressor = 100,000 copies/reaction. (B) HSV-2 standard curve: R^2 = 0.9988, GFP IC, HSV-1 stressor = 100,000 copies/reaction.

Multiplex assay can detect HSV-1 or HSV-2 from clinical samples

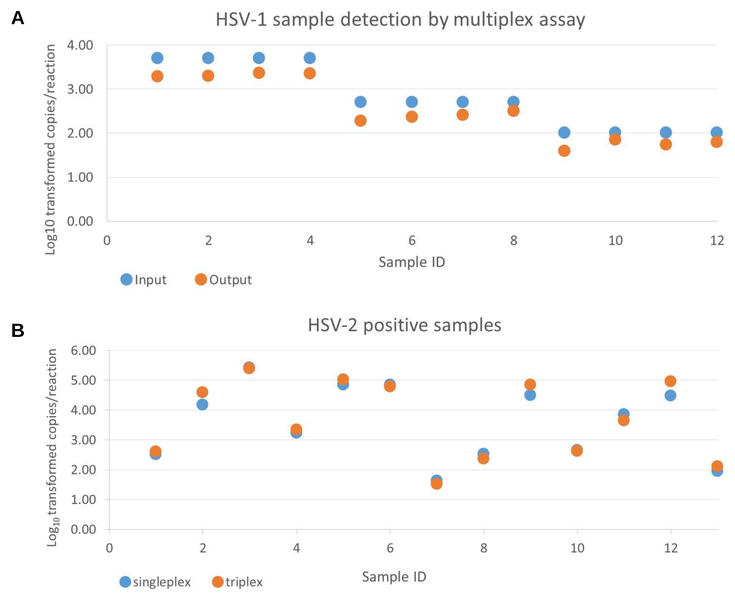


Figure 5: HSV-1 and HSV-2 detection of clinical samples using the multiplex assay. (A) HSV-1 samples were contrived by spiking a known amount of HSV-1 gDNA into sample transport medium. gDNA was extracted, quantified, and output values were compared to input values. (B) HSV-2 positive genital swabs collected from human subjects were extracted and quantified by singleplex qPCR. Samples were then tested by multiplex PCR and values were compared.

Co-infection of HSV-1 and HSV-2 in clinical anogenital samples

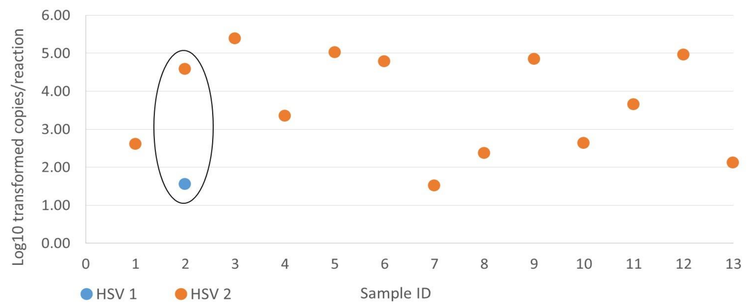


Figure 6: Multi-target detection of HSV-1 and HSV-2 in GEN-003-002 clinical anogenital swab samples. Genital lesions from HSV-2 infected subjects were swabbed during the time of an outbreak. DNA was extracted and HSV-1 and HSV-2 gDNA was quantified using the multiplex qPCR assay. One case of co-infection was identified by the multiplex assay and was further confirmed by HSV-1 and 2 singleplex assays (data not shown).

SUMMARY

- The multiplex PCR assay is robust, linear, and accurately quantitates samples from subjects infected with HSV-1 or HSV-2.
- This assay performs similarly to both HSV-1 and HSV-2 singleplex assays.
- Assay performance was not lost upon addition of an internal control.
- The presence of a highly concentrated second target does not affect assay efficiency or linearity.
- A complete characterization of the HSV-1/2 multiplex assay including limit of detection, lower limit of quantification, reproducibility and precision is currently underway.
- The multiplex assay will help assess dynamics of infection and co-infection with HSV and will be a valuable tool in assessing infection and vaccine efficacy in future GEN-003 clinical trials.

References

[1] Roberts et al, "Increasing Proportion of Herpes Simplex Virus Type 1 as a Cause of Genital Herpes Infection in College Students." Sexually Transmitted Diseases, 2003

[2] Fife et al, GEN-003, a Therapeutic Vaccine for Genital Herpes, Significantly Reduces Viral Shedding and Lesions for at Least 6 months. Presented at: ASM Microbe, 2016

[3] Long et al, Development and Qualification of a Real-Time PCR Assay for the Quantitative Detection of HSV-2 DNA in Anogenital Swabs. Presented at: International Herpes Workshop, 2015