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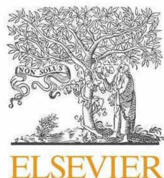
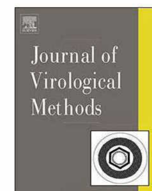
Journal of Virological Methods 252 (2018) 65–69



Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet



Expression and characterization of the soluble form of recombinant mature HSV-2 glycoprotein G for use in anti-HSV-2 IgG serodiagnostic immunoassay

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ARTICLE INFO

Keywords:
HSV-2
Glycoprotein G
gG-2
ELISA
Serodiagnosis

ABSTRACT

Herpes simplex virus type-2 (HSV-2) specific glycoprotein G (gG-2) is widely used as the antigen of choice for serodiagnosis of HSV-2. In order to develop an ELISA for serodetection of HSV-2 IgG in patient sera, the soluble form of the mature gG-2 antigen (mgG-2), gG283-649, was expressed using a baculovirus expression system. gG283-649 contains the complete extracellular domain of mgG-2 including the C-terminal region, which despite homology to gG-1, does not cross-react with HSV-1 antibodies present in HSV-1 positive patient sera. gG283-649 had increased performance compared to a previously described gG-2 fragment and showed high sensitivity and specificity in a method comparison with HerpeSelect 1 & 2 Immunoblot IgG, a commercially available FDA-cleared assay for serodetection of HSV-1 and 2 antibodies. A total of 234 clinical samples consisting of 134 high risk samples, including 45 samples from pregnant subjects, and a panel of 100 mixed diagnosis samples, spanning the measurable range were tested in the method comparison. Clinical sensitivity and specificity were determined to be 94.2% and 100%, respectively. We conclude that this soluble form of mgG-2 is a novel antigen of choice for developing an ELISA for type-specific serodiagnosis of HSV-2.

1. Introduction

Herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2) are double-stranded DNA viruses in the *Herpesviridae* family that cause orofacial and genital lesions, respectively. Genomic sequence homology between the two viruses is approximately 40% with up to 83% homology for protein coding regions (Whitley and Roizman, 2001). The similarities between the two viral genomes is cause for significant cross-reactivity when conducting serological diagnosis, and may result in improper treatments for patients.

HSV-1 is estimated to be found in the majority of the population of the United States while HSV-2 is found in a little more than 15% of the population. HSV-1 is frequently acquired in childhood while HSV-2 is transmitted sexually, although transmission through childbirth is a significant risk. The prevalence of the virus in the human population necessitates an accurate diagnosis for proper treatment and management (Bradley et al., 2014; Xu et al., 2006). Individuals can remain asymptomatic despite being infected with the virus, which poses a large risk for unintentional transmission and foregoing of treatment. This can prove problematic among high-risk patients (Corey et al., 1988; Mommeja-Marin et al., 2003; Whitley, 2006; Whitley et al., 1984), making it very important to accurately diagnose infection. Accurate serodiagnosis of the HSV infection can remain a challenge because of the homology of HSV-1 and HSV-2. Due to the high prevalence of HSV-1 in the general population, a cross-reactive immunoreagent for detecting HSV-2 would lead to unnecessary treatment for many individuals and thus necessitates a highly specific immunoassay.

HSV infection is confirmed by direct viral detection methods using cell culture-based, polymerase chain reaction-based methods, or by indirect serology-based detection methods (Ashley, 2001). Serological assays can detect latent as well as active infections and thus provide broader diagnostic usefulness. However, given the similarity between HSV-1 and HSV-2 genomes and their expressed proteins, serology-based detection can be difficult. For type-specific detection, envelope glycoprotein G (gG) used in serological assays distinguishes between HSV type 1 and 2 infections and is now the standard method for distinguishing serotypes (Ashley and Wald, 1999; Ho et al., 1992; Lee et al., 1985; Wald and Ashley-Morrow, 2002).

gG-2 is processed from an intact precursor into a soluble N-terminal fragment (sgG-2) and a membrane-bound mature antigen (mgG-2) derived from the C-terminal portion of the precursor protein (Su et al., 1993). While sgG-2 has been shown to have diagnostic potential

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http://dx.doi.org/10.1016/j.jviromet.2017.10.021
Received 17 July 2017; Received in revised form 23 October 2017; Accepted 25 October 2017
Available online 28 October 2017
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(Görander et al., 2003; Liljeqvist et al., 2002) the majority of epitopes recognized by HSV-2 specific antibodies found in patient sera are located in mgG-2 (Görander et al., 2006; Liljeqvist et al., 1998, 2000; Tunbäck et al., 2005). The identification of immunodominant regions have proven useful for diagnostic and therapeutic applications (Grabowska et al., 1999; Liljeqvist et al., 1998; Marsden et al., 1998; Tunback et al., 2000). Subsequently, several group have developed truncated antigens or peptides narrowing around the immunodominant region to address the limitations of serological assays based on the intact gG-2 antigen (Ikoma et al., 2002; Korshun et al., 2013; Levi et al., 1996; Liu et al., 2015). These studies have focused on only the immunodominant region of gG-2 or truncated the mgG-2 protein to exclude regions that are highly homologous to gG-1. However, a soluble form of the complete extracellular domain of mature gG-2 protein has not been characterized.

In this study we describe the expression and characterization of a soluble form of the mature gG-2 protein, $gG_{283\text{-}649}$, and its application in a bead-based indirect ELISA for detecting anti-HSV-2 IgG in patient sera. We compare the performance of the $gG_{283\text{-}649}$ to HerpeSelect 1 & 2 Immunoblot IgG. We have found that it performs with high specificity and sensitivity in a bead-based indirect ELISA for serodiagnosis of HSV-2 with no cross-reactivity to HSV-1 and thus may have the potential to be used as a highly specific and sensitive immunoassay reagent.

2. Materials & methods

2.1. HSV-2 gG fragment cloning

The HSV-2 gG-2 constructs, derived from the HG52 strain (P13290, UniProtKB), were codon optimized for *Spodoptera frugiperda*, commercially synthesized (DNA 2.0, Newark, CA), and cloned into pAcGP67a vector (BD Biosciences, San Jose, CA). An 8 x His tag was included on the C-terminal end of the construct for affinity purification. Sequences of the gG-2 constructs can be found in Supplemental Fig. 1. The sequence verified gG-2 gene in pAcGP67a vector was transformed into NEB Turbo chemically competent *E.coli* cells (New England Biolabs, Ipswich, MA) and maxiprep scale DNA was purified using HiPure Maxiprep purification kit (Life Technologies, Carlsbad, CA) and resuspended at a concentration between 1 and 2 μg/mL.

2.2. Protein expression & purification

Baculovirus was produced by transfecting constructs into SF9 cells (Life Technologies, Carlsbad, CA) with Sapphire Baculovirus DNA (Allele Biotech, San Diego, CA) and Cellfectin II (Life Technologies, Carlsbad, CA) transfection reagent. Baculovirus was amplified in two rounds in SF9 cells, multiplicity of infection (MOI) of 0.1 for the first round and 0.01 for the second round, and used to infect High Five cells (Life Technologies, Carlsbad, CA) at an MOI of 3 for protein production. All viral titers were determined using the BacPAK Baculovirus Rapid Titer kit (Takara Bio USA, Mountain View, CA). Seventy-two hours post-infection, cultures were clarified and proteins secreted into the supernatant were purified using immobilized metal affinity chromatography with Ni-NTA agarose (Qiagen, Hilden, Germany). Immobilized antigen was eluted using TBS pH 8.0 with 250 mM imidazole pH 8.0, dialyzed to removed imidazole, and concentrated using an Amicon centrifuge concentrator with 10 kDa MWCO (EMD Millipore, Hayward, CA). Purified antigen was analyzed by SDS-PAGE and Western Blot using primary antibodies anti-HSV2 gG antibody (clone: 3D4, Virusys, Taneytown, MD) and anti-His antibody (clone:HIS.H8, Thermo Fisher, Waltham, MA). The secondary antibody used was an anti-mouse antibody conjugated to peroxidase (Sigma-Aldrich, St. Louis, MO).

2.3. Microtiter-based direct ELISA

Direct ELISA was performed on purified gG-2 antigen by serially diluting in carbonate-bicarbonate buffer, pH 9.4 from 10 μg/mL down to 10 ng/mL and coating the dilutions onto a microtiter plate overnight at 4 °C. Coated plates were washed three times with TBST pH 8.0, blocked with 0.03% BSA in TBS pH 8.0, washed again, and incubated with 10 ng/mL solution of anti-gG2 (clone: 3D4, Virusys, Taneytown, MD) conjugated to alkaline phosphatase (AP) using an SH AP conjugation kit (Dojindo Molecular Technologies, Rockville, MD). ALP substrate was added to microtiter plate and luminescence was measured using a FLUOstar Omega microtiter plate reader (BMG Labtech, Ortenberg, Germany).

2.4. Bead-based indirect ELISA

Bead-based indirect ELISA was performed using magnetic M-270 epoxy beads (Life Technologies, Carlsbad, CA) conjugated with purified antigen. Samples were diluted 10-fold in protein-free assay buffer (Surmodics, Eden Prairie, MN) supplemented with 400 μg/mL heterophilic blocking reagent (Scantibodies, El Cajon, CA) and incubated with antigen-coated magnetic beads. Beads were washed with TBST pH 8.0 three times before incubation with detection solution. The detection solution consisted of 100 ng/mL anti-human IgG (Novus Biological) conjugated to AP using an alkaline phosphatase conjugation kit (Dojindo Molecular Technologies, Rockville, MD) using SH conjugation. Beads were washed and incubated with ALP substrate and luminescence was measured using an Infinite M1000 PRO microplate reader (Tecan, San Jose, CA).

2.5. Cut-off index (COI)

The cut-off index (COI) was determined using assay calibrators derived by serially diluting a high HSV-2 IgG sample obtained from a HSV-1/2 mixed titer panel (SeraCare, Milford, MA) in HSV-2 negative serum. Calibrators were tested on LIAISON HSV-2 Type Specific IgG chemiluminescent immunoassay (DiaSorin, Saluggia, Italy). Antibody index of calibrators were assigned based on the results from Diasorin Liaison method. A cut-off sample, which was determined as antibody index of 1.0, a positive control sample and a negative control sample were run on each plate of bead-based ELISA. The antibody index of each sample for the bead-based ELISA was calculated by sample RLU divided by cut-off sample RLU. The non-diluted high HSV-2 IgG sample was used as a positive control and HSV-2 negative serum was used as a negative control. Antibody index greater than 1.1 was determined as positive and less than 0.9 was determined as negative. Samples with antibody index between 0.9 and 1.1 were reported as "equivocal" and were re-tested.

2.6. Method comparison

The HerpeSelect 1 and 2 Immunoblot IgG test (Focus Diagnostics, Cyprus, CA) was used as the reference method. The test was performed using the manufacturer's recommended protocol.

2.7. Patient sera

Patient sera were obtained from Discovery Life Sciences (Los Osos, CA), SeraCare (Milford, MA), and Cureline (South San Francisco, CA), a commercial biobank working under the Colleagues of American Pathologists (CAP) guidelines for human research tissue biobanks and using the ISBER Best Practices for biobanks. Sera were obtained from unique high risk adults, individuals who were suspected to have been exposed to HSV-1/2 and/or other sexually transmitted infections (STIs) and had been previously tested for HSV-1 or HSV-2. The subjects also included pregnant individuals who were tested for HSV-1 or HSV-2. Mixed diagnosis samples, consisting of samples obtained from individuals for various diagnostics tests that may or may not have included HSV-1/2, were obtained from the Center for Disease Control

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(CDC, Atlanta, GA). All samples were obtained from consenting patients under respective institutional review board (IRB) approved protocols. HSV-1/2 diagnosis was determined using the reference method.

Sample acquisition, processing, shipment, and storage conditions were designed to minimize pre-analytical variabilities and to preserve sample integrity. Obtained samples were aliquoted in order to minimize freeze-thaw cycles and stored long-term at −80 °C. Freezer temperature was monitored using a temperature probe and third-party facilities monitoring service, SensoScientific (Simi Valley, CA), to ensure storage temperature remained within range to maintain sample integrity.

2.8. Statistical analysis

Sensitivity and specificity for method comparison were calculated as positive percent agreement (PPA) and negative percent agreement (NPA), respectively. PPA was calculated using the following formula: $PPA = \frac{TP}{TP+FN}$. Where true positive (TP) was the number of samples called as positive by both the reference method and bead-based indirect ELISA. False negative (FN) was the number of samples called as positive by the reference method but called as negative or equivocal by the bead-based indirect ELISA. NPA was calculated using the following formula: $NPA = \frac{TN}{TN+FP}$. Where true negative (TN) was the number of samples called as negative by both the reference method and bead-based indirect ELISA. False positive (FP) was the number of samples called as negative by the reference method but called as positive or equivocal by the bead-based indirect ELISA. Samples that were called as equivocal by the reference method were excluded from the PPA and NPA calculations.

3. Results

3.1. HSV-2 $gG_{283\text{-}649}$ expression

The soluble form of the mature gG-2 antigen, $gG_{283\text{-}649}$, was developed to include the entire extracellular portion of the mature membrane-bound gG-2 protein. Recombinant $gG_{283\text{-}649}$ was produced using the baculovirus expression system and purified at > 95% purity (Fig. 1a). Western blot using an anti-His antibody (Fig. 1b) and anti-HSV2 glycoprotein G antibody (Fig. 1c) confirmed that the purified antigen was the desired gG-2 antigen. The molecular weight of the expressed fragment is approximately 75 kDa based on SDS-PAGE and Western Blot analysis (Fig. 1a–c). A direct ELISA performed on the recombinant antigen using a gG-2 specific antibody also showed a dose response signal with increasing concentration of coated antigen (Fig. 1d).

3.2. Comparison of fragments in patient sera

The performance of $gG_{283\text{-}649}$ was compared to other baculovirus-expressed gG-2 antigens using a bead-based indirect ELISA on positive and negative control calibrators. Including among tested gG-2 antigens was $gG_{343\text{-}649}$, the soluble form of mgG-2 containing the sequence between the putative cleavage site and the transmembrane region (Liljeqvist et al., 2002) and a previously described gG-2 fragment—$gG_{681\text{-}594}$ (Ikoma et al., 2002). $gG_{283\text{-}649}$ and $gG_{343\text{-}649}$ had signal-to-background ratios of 37.7 and 36.5, respectively, which were higher than $gG_{281\text{-}594}$ (Fig. 2b), which had a ratio of only 1.9.

The $gG_{343\text{-}649}$ and $gG_{283\text{-}649}$ antigens were used in a bead-based indirect ELISA to test patient sera and compared to HerpeSelect 1 and 2 Immunoblot IgG (Focus Diagnostics), a gG-based qualitative diagnostic assay. The performance of $gG_{343\text{-}649}$ and $gG_{283\text{-}649}$ was tested in 112 patient sera samples and compared to the reference method and showed 88.9% and 98.6% specificity, respectively (Table 1). Both antigens had 100% sensitivity.

3.3. $gG_{283\text{-}649}$ performance in large cohort of high risk and mixed diagnosis patient sera

We selected to move forward with testing $gG_{283\text{-}649}$. The bead-based indirect ELISA was further optimized with the selected gG-2 antigen and re-tested with a larger cohort of 234 samples, including the original 112 samples. Of the cohort, 134 were high risk samples and 100 were mixed diagnosis samples (Supplemental Table 1). The high risk samples had 91.9% sensitivity and 100% specificity. The mixed diagnosis samples had 100% sensitivity and 100% specificity. Taken together, the $gG_{283\text{-}649}$ antigen had a sensitivity of 94.2% and specificity of 100% (Table 2).

The extended C-terminal portion covers a region of the antigen before the transmembrane region (Tunback et al., 2000) which has approximately 63% homology to gG-1 (Supplemental Fig. 2). Inclusion of this region has no negative impact on the cross-reactivity of $gG_{283\text{-}649}$ with HSV1-positive samples (Table 3). Among the high risk and mixed diagnoses samples, 74 were HSV1-positive/HSV2-negative as determined by the reference method. Among the HSV1-positive/HSV2-negative samples, $gG_{283\text{-}649}$ called none of the samples as false positives, suggesting no cross-reactivity with HSV-1 despite high homology between the $gG\text{-}1_{137\text{-}188}$ and $gG\text{-}2_{595\text{-}649}$ regions.

4. Discussion

In this study, we characterize the soluble form of the mature gG-2 antigen, $gG_{283\text{-}649}$, with high signal-to-background ratio, high sensitivity and specificity, and low cross-reactivity to HSV-1. $gG_{283\text{-}649}$ covers the complete extracellular portion of the mature gG-2 antigen up

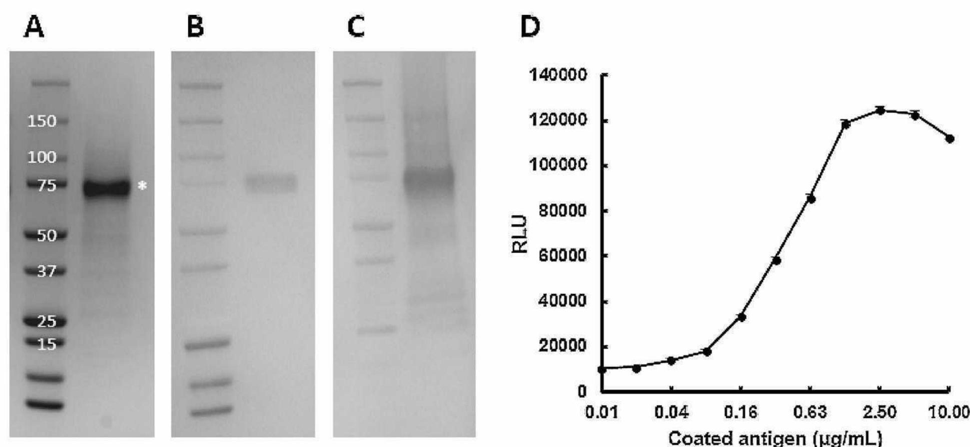

Fig. 1. HSV-2 $gG_{283\text{-}649}$ recombinant antigen. A.) Coomassie stained SDS-PAGE gel of purified antigen (white *) B.) Anti-His western blot (Clone: HIS.H8, Thermo Fisher Scientific) C.) Anti-HSV2 gG (Clone: 3D4; ViruSys): D.) direct ELISA with anti-HSV2 gG antibody conjugated to AP (10 ng/mL); error bars (SEM).

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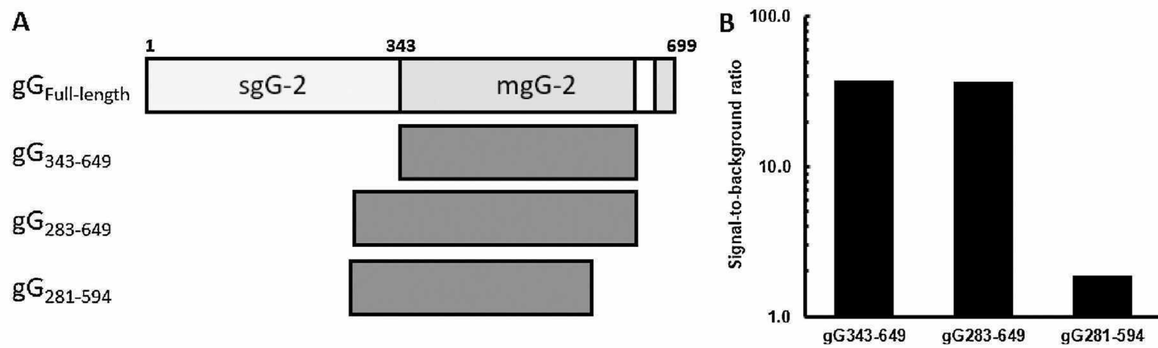

Fig. 2. HSV-2 gG fragment performance: A.) Various constructs (dark gray) derived from full length gG-2; sgG-2: soluble gG-2, mgG-2: mature gG-2, transmembrane domain: white B.) Signal-to-background ratio of recombinant gG-2 fragments using bead-based direct ELISA on positive and negative control calibrators.

Table 1
gG-2 fragment performance in IgG assay comparison. Truth table comparison of $gG_{343-649}$ and $gG_{283-649}$ to HerpeSelect 2 Immunoblot IgG as well as sensitivity and specificity.

		HSV-2 (HerpeSelect Immunoblot)				
		Positive	Equivocal	Negative	Sensitivity	Specificity
$gG_{343-649}$	Positive	40	0	8		
	Equivocal	0	0	0	100%	88.9%
	Negative	0	0	64		
$gG_{283-649}$	Positive	40	0	1		
	Equivocal	0	0	0	100%	98.6%
	Negative	0	0	71		

to the transmembrane region and includes two cleavage sites, R321/A322 and R342/L343, required for processing from the full-length precursor protein to the mature membrane bound antigen (Liljeqvist et al., 2002).

Both $gG_{343-649}$ and $gG_{283-649}$, which both contain the full extracellular domain of the mature gG-2 antigen, had higher signal-to-background ratios compared to the previously published gG-2 fragment by a factor of nearly 20-fold. Previous reports have shown that a high percentage of patient sera contain antibodies that were specific to the C-terminal end of the extracellular domain of the mature gG-2 antigen (Levi et al., 1996; Liljeqvist et al., 1998; Tunback et al., 2000). This increase in signal is likely due to including portions of the antigen that are recognized by antibodies found in patient sera. Interestingly, the post-cleavage version of the soluble mature antigen, $gG_{343-649}$, had a decrease in specificity compared to a version of the antigen, $gG_{283-649}$, that included the two putative cleavage sites required for processing into the mature gG-2 antigen.

While the C-terminal end of the antigen has high homology to the gG-1 antigen at 63%, the specificity for HSV-2 is retained with no cross-reactivity with HSV1-positive/HSV2-negative samples. Among the few false-negatives that were found in HSV2-positive samples, the majority of the false negative results occurred with HSV1-positive/HSV2-positive samples. Despite this occurrence, the sensitivity of the $gG_{283-649}$ antigen remained greater than 90% in HSV1-positive/HSV2-positive samples. Overall, the $gG_{283-649}$ antigen maintained high performance in patient sera from high risk individuals and mixed diagnosis samples with an overall sensitivity of 94.2% and specificity of 100%.

We have described the development of the soluble form of the mature gG-2 antigen—$gG_{283-649}$. Previous approaches to developing a gG-2 fragment for use as a diagnostic reagent have focused primarily around the immunodominant region or excluding regions with high homology to gG-1. Given that there is overlap between gG-1 homologous regions and epitopes recognized by antibodies in patient sera there is a risk of compromising sensitivity of the assay reagent. This could pose a potential risk of false negative results in HSV-2 patients resulting in unintended transmission of the virus and foregoing of necessary treatment, which is particularly dangerous in high-risk individuals such as pregnant women, neonates, and HIV-positive patients. As we have described, the inclusion of the complete extracellular domain of the mature gG-2 antigen does not lead to cross-reactivity in

Table 2
HSV-2 $gG_{283-649}$ performance in IgG assay comparison in high risk and mixed diagnosis patient sera. Truth table comparison of $gG_{283-649}$ to HerpeSelect 2 Immunoblot IgG as well as sensitivity and specificity.

		HSV-2 HerpeSelect Immunoblot IgG				
		Positive	Equivocal	Negative	Sensitivity	Specificity
High Risk	Positive	68	0	0		
	Equivocal	1	0	0	91.9%	100%
	Negative	5	0	60		
Mixed Diagnosis	Positive	30	0	0		
	Equivocal	0	0	0	100%	100%
	Negative	0	2	68		
Total	Positive	98	0	0		
	Equivocal	1	0	0	94.2%	100%
	Negative	5	2	128		

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Table 3
HSV-1 cross reactivity in patient sera using $gG_{283-649}$ in a bead-based indirect ELISA compared to the HerpeSelect 1 & 2 Immunoblot IgG.

		HerpeSelect 1 & 2 Immunoblot IgG								
		HSV-1 Positive			HSV-1 Equivocal			HSV-1 Negative		
		HSV-2								
		Positive	Equivocal	Negative	Positive	Equivocal	Negative	Positive	Equivocal	Negative
$gG_{283-649}$	Positive	48	0	0	0	0	0	50	0	0
	Equivocal	1	0	0	0	0	0	0	0	0
	Negative	4	0	74	0	2	0	1	0	54
	Sensitivity		90.6%						98.0%	
	Specificity		100%						100%	

HSV-1 patients in an indirect ELISA based serological assay. The antigen retains its specificity and also produces a more robust signal compared to a gG-2 fragment that excludes the HSV-1 homologous region. As a result, the $gG_{283-649}$ antigen can be used as a diagnostic reagent to develop a robust serodiagnostic ELISA without the risk of cross-reactivity with HSV-1 specific IgGs.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements

Disclosure Statement

All of the authors of this paper, as either current or former Theranos employees, either have received or are likely to receive in the future equity compensation from the company. As a result, all authors have an actual or potential financial interest in Theranos' success. WJ and PR are inventors on patents or patent applications related to the material presented here.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2017.10.021.

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HOLMES002152