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Validation of a membrane touch biosensor for the qualitative detection of IgG class antibodies to herpes simplex virus type 2

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A novel type of biosensor was assessed for application to the qualitative determination of circulating antibodies to herpes simplex virus type 2 (HSV-2). The device utilises a high activity HSV-2 type specific gG2 antigen for antibody capture and commercially available ELISA reagents. The study compares the diagnostic performance of a prototype HSV-2 biochip to well-established *in vitro* tests routinely applied in clinical procedures. A panel of human serum samples (n = 60) previously characterised for HSV-2 serological status using the DiaSorin LIAISON® HSV-2 chemiluminescent immunoassay were assayed on the HSV-2 biochip and the Focus Diagnostics HerpeSelect® 2 ELISA IgG kit to determine concordance with the predicate test method. Sensitivity and specificity of the HSV-2 biochip were found comparable to both the DiaSorin and Focus test methods. Sample index values calculated from the immunoassay response of the biochip's coulometric sensors indicated a high degree of linear correlation of the dataset with the corresponding index values from the DiaSorin LIAISON® test (r^2 0.8799) and Focus HerpeSelect® test (r^2 0.8794). The HSV-2 biochip demonstrated excellent diagnostic performance in qualitative and semi-quantitative measurements, matching closely the performance of two diagnostic industry standard predicate methods.

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Introduction

Herpes Simplex Virus type 2 (HSV-2) is a large double-stranded DNA virus of the Herpesviridae family. HSV-2 is ubiquitous, affecting both urban and remote populations and, along with HSV-1, is the major causative agent of genital ulcers worldwide.[1,2] It is estimated there are greater than 500 million persons worldwide living with HSV-2 infection and greater than 20 million new infections occur annually.[3] In the United States, the HSV-2 seroprevalence rate in the general population (ages 14–49 years old) is 16%,[4] whereas in some regions of sub-Saharan Africa it can be as high as 80%.[5] Seroprevalence in women is more common than in men[6,7] and vertical transmission from mother to infant during childbirth is a rare but very serious consequence of genital herpes infection. HSV-2 infection increases the risk of HIV-1 acquisition up to 3-fold.[8] Seropositivity for HSV-2 is associated with viral shedding in the genital tract in both symptomatic and asymptomatic subjects.[9] The majority of individuals that are seropositive for HSV-2 are asymptomatic and have no reported history of genital lesions.[10,11] Consequently, most HSV-2 transmission occurs from those that are not aware that they are infected.[1,12]

The classical gold-standard for genital HSV diagnosis in the laboratory has been viral culture followed by species typing to determine if the causative agent is HSV-1 or HSV-2.[13] In asymptomatic individuals, serological testing can be used to determine previous HSV infection. HSV-1 and HSV-2 share significant identity with up to 83% sequence homology observed in the protein-coding regions of their genomes.[14] Due to the significant similarities between HSV types, serological distinction between them is primarily reliant on the immune reaction to glycoprotein G.[15–17] Accurate serological tests are based on high purity glycoprotein G-1 (gG1) and glycoprotein G-2 (gG2) antigens for HSV-1 and HSV-2 respectively.[18] A number of such tests are commercially available. The Focus Diagnostics HerpeSelect® 2 ELISA IgG test utilises a recombinant gG2 antigen and is approved by the Food and Drug Administration (FDA) for use in sexually active adults and expectant mothers. This test has been widely used in various studies and patient populations.[19–23] A constraint of contemporary HSV-2 serological tests in routine clinical use is the requirement of specialised accessory equipment and indeed highly skilled technicians for their operation. It can often be difficult to access

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these tests in resource-limited settings for example in developing countries. Therefore a current clinical need exists for an easy-to-use and reliable, cost-effective serological test for HSV-2 diagnosis.

Today the majority of HSV-2 serological testing is performed using optical or chemiluminescent enzyme immunoassays in diagnostic laboratories due to the requirement for specialised equipment and trained assay technicians. It would be preferable to move towards point-of-care (PoC)-style HSV-2 serological tests, the primary benefit being the rapid sample-to-answer time of minutes as opposed to hours. This permits immediate preliminary diagnosis in a doctor's office or STD clinic during patient visits. Additionally HSV-2 PoC tests could be used in resource-limited settings where HSV-2 is prevalent and there is no access to centralised diagnostic laboratory services. There are PoC tests available for the qualitative determination of circulating antibodies against HSV-2, such as the Uni-Gold™ HSV-2 Rapid Test or the Sure-Vue® HSV-2 Rapid Test. These tests utilise colloidal gold conjugates to detect bound anti-HSV-2 IgG. The formation of a coloured line or spot at a test region containing gG2 antigen determines whether the sample is positive or negative. Assays such as these are potentially subject to operator variability since they rely on visual assessment of the test area: a particular problem in cases of samples that may be borderline or low positive. By contrast, a potentiostat-based electronic reader for the HSV-2 biochip test, measures, calculates and displays a sample index number for HSV-2 leaving little ambiguity on the HSV-2 serological status of a test sample.

Under consideration in this work is an intriguing type of microfluidic biosensor technology, *i.e.* membrane-touch biochip, which offers promise as a portable instrument for out-of-laboratory diagnostic tests. The device is engineered as a flow-through microfluidic system composed of a reaction chamber linked to series coulometric sensors. The reaction chamber supports immunochemical reagents, *e.g.* an antigen, and can be integrated with any exterior liquid-handling device for the delivery of sample and immunoassay reagents. Coulometric sensors on the chip are electronically activated at the end of an assay sequence by an on-chip micro-hydraulic mechanism actuated by a membrane-touch pump.[24] The sensors are responsive to redox molecules emanating from peroxidase activity of the immunoassay. The chip's detection operates by a millivolt-coulometry technique, whereby a small potential difference is applied across two indicator electrodes of a sensor and an electrochemical charge measured by a potentiostat instrument.

The utility of the membrane-touch biochip as a reliable immunoassay device was demonstrated in HSV-2 antibody measurements using a high purity recombinant gG2 protein to functionalise the biochip reaction chamber. The analytical performance of this HSV-2 biochip was assessed in terms of assay sensitivity and specificity against FDA-cleared, market leading HSV-2 serological tests from DiaSorin (LIAISON® HSV-2 IgG) and Focus Diagnostics (HerpeSelect® 2 ELISA IgG). The study's findings and consequences for the biochip design as a practical immunoassay sensor are discussed.

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Baljit Singh

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Expertise: Advancement of chemical & bio-sensors technologies for human/animal diagnostics, dairy/food and water testing. Biomaterials fabrication and immunoassay techniques. Sensors for food allergens, contaminants/residues and rapid microbiology analysis. Carbon supported metallic nanocomposites, electroactive and nanoporous materials, plastic thin-film devices and printed electrode systems.

Experimental

Materials and methods

Components of the membrane-touch biochip were fabricated from plastic-film laminates based on proprietary polyester materials (Melinex, DuPont Teijin Films, USA). Coulometric sensors were patterned onto the chip by a screen-printing process (DEK248 semi-automatic screen printer, UK), utilising biosensor-grade carbon conducting ink (DuPont, USA). Microfluidic features were designed using CorelDraw drawing software and component parts machined by a CO_2 laser (Epilog Zing, USA). The base polymer chip was functionalised with a recombinant gG2 antigen (Aalto Bio Reagents, Ireland) diluted to 5 μg mL^{-1} in 50 mM carbonate buffer, pH 9.6 and 15 μL solution (containing 75 ng gG2 antigen) and injected into the reaction chamber. Chips were incubated at ambient temperature (20–25 °C) for 90 minutes to allow adsorption of the gG2 antigen to the reaction chamber surface, see Fig. 1 and 2. The gG2 solution was then aspirated and the biochip reaction chamber washed by injection of 50 μL phosphate buffered saline, pH 7.4 containing 0.05% (v/v) Tween 20 (PBST buffer). Wash buffer was completely aspirated from the reaction chamber and 15 μL of 1% (w/v) bovine serum albumin (BSA) in 50 mM carbonate buffer, pH 9.6 was injected to block potential non-specific binding sites on the reaction chamber surface. Following a 90 minutes incubation at ambient temperature (20–25 °C), the BSA solution was aspirated and the biochips were dried by incubation at 37 °C for 60 minutes. HSV-2 gG2 coated biochips were stored at 2–8 °C until required for immunoassay.

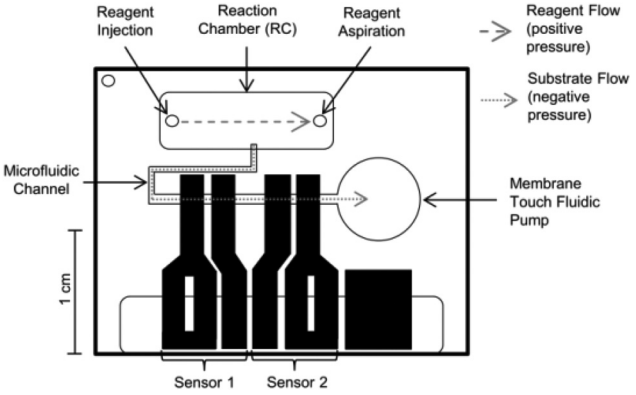

Fig. 1 A schematic representation of the biochip platform is shown. The reaction chamber capacity is 15 μL. Assay reagents are injected into the reaction chamber using a pipette, incubated for the required time to allow binding to occur, and aspirated before subsequent injection of the next reagent. The reaction chamber contents do not enter the microfluidic channel leading to the sensors during the immunoassay routine. Prior to injection of the substrate solution to indirectly detect the analyte of interest (*via* the captured enzyme conjugate), the membrane-touch device is engaged by application of a force. Upon incubation of the substrate solution in the reaction chamber, the force is removed from the membrane-touch pump; this creates negative pressure which draws the reaction chamber contents (*i.e.* the electrochemically active substrate solution) into the microfluidic channel and into contact with the sensors. The electrochemical charge is measured at the electrodes by millivolt coulometry.

HSV-2 immunoassay with biochip

The HSV-2 biochip assay was performed using commercially available ELISA reagents from the HerpeSelect® 2 IgG kit (Focus Diagnostics, CA, USA; product EL0920G). Control and serum samples were mixed thoroughly using a vortex mixer and diluted 51-fold in sample diluent. Diluted samples (15 μL) were injected into gG2 antigen-coated biochip reaction chambers and incubated at ambient temperature (20–25 °C) for 30 minutes to allow adsorption of specific anti-gG2 antibody in the sample to the functionalised reaction chamber surface. The sample was then aspirated and the reaction chamber washed twice with 50 μL PBST buffer per wash. Goat-anti-human IgG-peroxidase conjugate supplied with the ELISA kit was diluted 3-fold in 1% (w/v) BSA in PBST and 15 μL of diluted conjugate was injected into the washed biochip reaction chamber. The conjugate was incubated for 30 minutes at ambient temperature (20–25 °C) to allow binding to captured anti-gG2 antibody from the test sample. The conjugate was then aspirated and the reaction chamber washed twice with 50 μL PBST buffer per wash. Remaining wash buffer within the

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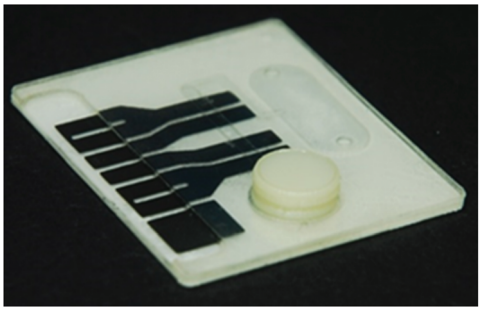

Fig. 2 Photograph of the generic membrane-touch biochip used for the HSV-2 ELISA. Chip dimensions: 25 mm × 30 mm. Reaction chamber 5 mm × 14 mm and membrane-touch pump 7 mm diameter. The indicator electrodes of sensors 1 and 2 have an area of 2 mm^2. The membrane-touch pump can be seen in the foreground of the image as a button elevated from the chip's base.

reaction chamber was aspirated and the membrane-touch device engaged by applying an external force. A volume of 15 μL of peroxidase substrate solution, 3′3′5′5′-tetramethylbenzidine (TMB), was then injected into the reaction chamber to detect the captured peroxidase conjugate. Following 5 minutes incubation, the applied force was removed from the membrane-touch device to create negative pressure. This action filled the microfluidic channel (shunt) leading to the sensors with reacted TMB substrate. Sensors were then activated by a 50 mV potential difference from a CH660 potentiostat (CH Instruments Inc., TX, USA). The electrochemical charge from the reacted substrate in contact with both sensors was measured by millivolt-coulometry. Coulometric data in immunoassay studies are tabulated in units of microcoulombs (μC): charge is sampled from charge-time measurements at 10 seconds (Q_{10}) for both sensors on the biochip.

Clinical sample analysis

A panel of 60 human serum samples previously screened using the LIAISON® HSV-2 Type Specific IgG chemiluminescent immunoassay test (DiaSorin, MN, USA) was purchased from a commercial vendor (Cerba Specimen Services, France) in order to validate the HSV-2 biochip assay. This sample panel comprised 30 positive samples (LIAISON® assay index >1.1), 29 negative samples (LIAISON® assay index <0.9) and one equivocal sample (LIAISON® assay index = 1.0). The samples were collected from a predominantly French population, with 49 (81.7%) collected from mainland France. Nine of the remaining 11 samples (15.0%) were collected in various French oversea territories (three samples from Mayotte, two samples each from Martinique and French Guiana, one sample each from French Polynesia and Reunion Island). The remaining two samples (3.3%) were from Madagascar and Morocco. The samples were from a predominantly female population (42 of 60 samples (70%)). The median age of the entire patient population was 40.5 years with patients ranging in age from 15–85 years. The sample index values on the DiaSorin LIAISON® HSV-2 Type Specific IgG test were supplied by Cerba Specimen Services. No other patient information was supplied. The majority of negative samples were supplied with reported index values of <0.5. Each sample was assayed in triplicate on the HSV-2 biochip assay as described above. Strongly reactive samples (LIAISON® index >4.0) were additionally retested on the HSV-2 biochip at increased sample dilution factors (102-fold or 204-fold). All serum samples were assayed using the Focus Diagnostics HerpeSelect® 2 IgG ELISA kit according to the manufacturer's instructions.

Data analysis

The average electrochemical charge value for test samples and the cut-off control were calculated from the available 6 replicate measurements (3 biochips per sample, 2 sensors per biochip). Sample index values were calculated by expressing the mean charge as a ratio of the average charge of the cut-off calibrator sample assayed during the same testing run. Samples with index values >1.1 were classified as positive for anti-HSV-2 antibody. Index values for high positive samples were corrected for increased sample dilution factor relative to the cut-off calibrator. Similarly, optical absorbance values for the Focus ELISA assay were converted to index values by expressing the average test sample OD as a ratio of the mean OD of the cut-off calibrator sample assayed during the same testing run.

The cut-off calibrator is a commercially available component of the HerpeSelect 2 IgG ELISA kit (Focus Diagnostics, cat. # EL0920G). This ELISA test is both CE *in vitro* diagnostic (IVD) and Food & Drug Administration approved as an *in vitro* diagnostic for the qualitative determination of human IgG class antibodies to HSV-2 in human sera and is one of the market leading tests for HSV-2 antibody qualitative determination. Therefore the qualitative index for HSV-2 antibody positivity or negativity is regarded as accurate and extensively applied in immunoanalysis. Moreover, the Focus Diagnostics cut-off calibrator is a human serum matrix formulated to contain a level of anti-HSV-2 antibodies to give the optimum differentiation between negative and positive sera. In designing the assay, Focus validated the cut-off calibrator against a reference lab-developed Western blot method using five serum panels (n = 383) tested on both ELISA and Western blot methods. In ELISAs, samples yielding an absorbance measurement ≥10% lower than the cut-off calibrator are defined as HSV-2 antibody negative. Samples yielding an absorbance measurement ≥10% higher than the cut-off calibrator are

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defined as HSV-2 antibody positive. Samples that are within the ±10% margin of the within-run cut-off calibrator are regarded as equivocal and are either re-tested or the patient is resampled after a period of weeks to re-test the immune response. The same classification for sample positivity or negativity for anti-HSV-2 IgG in the biochip test is utilised; that is if a sample is either <90% or >110% of the within-day HSV-2 biochip calibrator signal, the sample is classed as negative or positive respectively.

Results and discussion

Biochip operation

A schematic illustration and photographic image of the membrane-touch biochip are presented in Fig. 1 and 2. Biochip architecture includes a micro-volume flow-through reaction chamber with a t-connector fluidic link to two series coulometric sensors. A membrane-touch pump communicates liquid held in the reaction chamber to the sensors *via* a microfluidic shunt channel. The sensors themselves consist of an anode and cathode pair, which are operated by the application of a millivolt potential difference. In this study, the surface of the reaction chamber is functionalised with recombinant HSV-2 gG2 protein in order to selectively capture anti-HSV-2 antibodies contained within test samples. Control or serum samples are diluted in assay diluent and injected into the gG2 reaction chamber and incubated to allow binding of IgG in the test sample to the reaction chamber surface. Bound IgG is detected by subsequent injection and incubation of an anti-human IgG antibody-peroxidase conjugate. The horseradish peroxidase (HRP) label is measured through its oxidation of the substrate TMB in the presence of hydrogen peroxide. The rate of oxidation of TMB is proportional to the surface bound peroxidase and thus to the amount of anti-gG2 IgG captured from the original test sample. Peroxidase-mediated oxidation of TMB in the presence of hydrogen peroxide yields a blue coloured product which is generated by way of a two-electron reaction mechanism.[25] The electron release in the enzyme oxidation reaction can be measured by polarised electrodes and therefore by the printed electronic sensors on the biochip. It should be realise that the entire fluidic sequence of the ELISA takes place only within the reaction chamber of the biochip and not at the coulometric sensors.

The isolation of the sensors on biochip from the reaction chamber is a significant design improvement, having the effect of reducing background interferences common to printed immunoelectrodes. At the stage where the substrate is injected onto the biochip, the membrane-touch microfluidic is engaged by the application of an external force. After a sufficient substrate-reaction time, this force is removed from the membrane-touch device creating a negative pressure which diverts oxidised TMB substrate into a shunt channel making contact to the sensors.

The electrochemical charge is measured once a small potential difference is applied across an electrode pair – a technique referred to as potential-step coulometry. For analysis of HSV-2 biochip performance, electrochemical charge values were sampled at 10 seconds (Q_{10}) from charge-time plots.

One aspect of the sensor response which should be recognised is the parabolic dependence of Q_{10} on peroxidase concentration. This is shown in eqn (1). An equation adapted for coulometric peroxidase electrodes from the original work of Kies on a theoretical approximation for dead-stop end-point amperometry.[26]

$$Q = k \cdot \left[1 \pm \sqrt{1 - 4\left(\frac{\Delta - 1}{\Delta + 1}\right)^2 \times (1 - x)}\right] \quad (1)$$

$$k = \frac{1}{2} nFAC \cdot \sqrt{D \cdot t} \quad (2)$$

In this case, Q represents the electrochemical charge flowing across the indicator electrodes of a coulometric sensor and k is an electrochemical parameter related to redox molecule properties and the electrode reaction. Δ is a function of the potential difference applied to the sensor and t, the coulometric measurement time. Where eqn (1) is applied to peroxidase immunoassays measured *via* TMB redox processes, the x term is a function of peroxidase kinetics; *i.e.* enzyme concentration and reaction time. For the peroxidase reaction time leading to the condition, $C_{\mathrm{TMB_{red}}} = C_{\mathrm{TMB_{ox}}}$, ($x = 0.5$) the sensor response (sampled charge) takes the limiting form:

$$Q_{\max} = k \cdot \frac{\sqrt{\Delta} - 1}{\sqrt{\Delta} + 1} \quad (3)$$

Here, $Q_{\max}$ is the maximum electrochemical charge delivered by the sensor. The sensor response to blank samples is established by the surface binding characteristics of the peroxidase conjugate to the functionalised reaction chamber. High baseline readings are also observed with excessive peroxidase reaction times. Both factors strongly influence the coulometry leading to $Q \rightarrow Q_{\max}$ ($x < 0.5$). The effect of this is to "compress" the charge readings for any set of samples or standards. In an extreme case, where $x > 0.5$ (high conjugate adsorption or long peroxidase reaction time), the output of the sensor for a high analyte concentration can indeed fall below a sample of lower concentration, and could potentially be assigned as a low positive or even a negative.

HSV-2 biochip and control samples

The HerpeSelect® 2 IgG ELISA kit from Focus Diagnostics is approved by the Food & Drug Administration for clinical use and has been extensively studied and is widely available.[19–23] The ELISA kit contains a number of control samples; these were tested repeatedly on the HSV-2 biochip to determine the reproducibility of the coulometric enzyme immunoassay. Three controls (negative, low positive and high positive) and the cut-off calibrator were each tested across 10 separate runs, with 2 biochips per sample per run and 2 sensors per biochip, yielding 40 measurements per sample in total. Results are summarised in Table 1 and demonstrated the HSV-2 biochip

Table 1 HSV-2 biochip inter-assay reproducibility

Sample	Mean charge (μC)	SD	CV	Mean index value	*N*
HSV-2 cut-off calibrator	0.855	0.151	17.7%	1.00	40
HSV-2 negative control	0.301	0.133	44.2%	0.35	40
HSV-2 low positive control	1.204	0.197	16.4%	1.41	40
HSV-2 high positive control	2.055	0.284	13.8%	2.40	40

SD, standard deviation; CV, coefficient of variation.

assay was reproducible in terms of its qualitative performance. A representative coulometry trace for each HSV-2 control and calibrator is presented in Fig. 3.

HSV-2 biochip and Focus HSV-2 ELISA on patient samples

A total of 60 patient serum samples were each assayed on 3 separate HSV-2 biochips yielding 6 electrochemical charge measurement values per test sample. For each biochip assay run the cut-off calibrator was also tested in triplicate. Due to limitations in the manual processing of biochips, including provision of washing steps and maintaining consistent reagent incubation times across all devices, it was only possible to assay 5 samples during a given biochip assay run (*i.e.* 15 biochips tested in parallel). The index values for serum samples in the HSV-2 biochip dataset were each calculated from the mean electrochemical charge obtained for the cut-off calibrator sample during the same assay run. Where samples were tested at a non-standard dilution (102× or 204× serum dilution), the sample index was multiplied by the fold-difference between test sample dilution and calibrator dilution (calibrator was consistently assayed at 51× dilution). The data obtained for the serum analysis is summarised in Table 2.

All serum samples were additionally assayed by the Focus Diagnostics HerpeSelect® 2 IgG ELISA kit. These assays were run in accordance with the manufacturer's instructions. Each sample was run in duplicate and the entire sample panel was performed using two gG2-antigen coated microwell plates. ELISA HSV-2 index values for test serum samples were calculated relative to the cut-off calibrator sample from the same microwell plate. There was an excellent qualitative agreement between the results generated on the HSV-2 biochip assay and the supplied LIAISON® sample index values from Cerba Specimen Services. For the purposes of sensitivity and specificity determination, equivocal samples (index of ≥0.9 to <1.1) were considered as negative for anti-HSV-2 antibodies. The sensitivity and specificity of the HSV-2 biochip test were both 100% using the LIAISON® assay as the reference method. Using the ELISA assay as the reference method, the sensitivity and specificity of the HSV-2 biochip assay was 96.8% and 100% respectively. These data demonstrate that the HSV-2 biochip assay performed to the same diagnostic standard as the FDA-approved assays, albeit on a small sample panel.

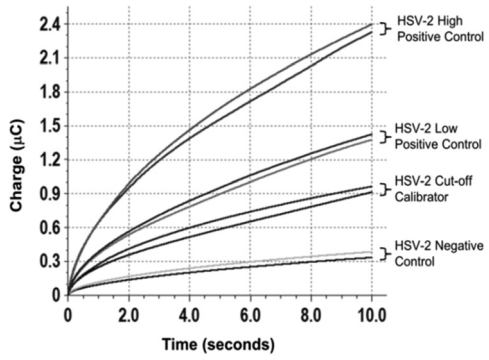

Fig. 3 HSV-2 control samples were tested as outlined in the materials and methods section. The charge-time plot above represents the sensor response from one HSV-2 biochip per sample. The two traces per sample type represent the electrochemical charge measurements from each sensor in a single biochip. The charge values used to calculate index values for patient samples in this study were all taken at the end-point of the coulometric measurement (*i.e.* after 10 seconds).

The sensitivity & specificity of the biochip relative to the ELISA assay was independent of the sample dilution factor applied for the high index positive samples; these represent a subset of 8 samples of the 60 sera panel assessed (13.3% of samples). The reported data for sensitivity and specificity includes all samples. Further, sensitivity and specificity relate to the correct classification of samples as being positive or negative for HSV-2 antibodies relative to a gold-standard method (in this study either the Focus or DiaSorin HSV-2 assays). The qualitative classification of a sample is determined by their response in the measuring system (biochip, ELISA or LIAISON) relative to the cut-off control assayed on the same plate (ELISA) or day (Biochip). Parallel assessment of the cut-off calibrator is required to minimise the impact of assay variances (*e.g.* sample incubation times, variations in dilutions, *etc.*). Whilst the HSV-2 assays, both biochip and ELISA, produce a continuous index score, they are ultimately qualitative in classifying samples as positive or negative, the magnitude of the index has no diagnostic significance.

The 8 'high index' sera identified by both Focus and DiaSorin test methods (index >4) yielded HSV-2 antibody positive index values when assayed at standard dilution factor of 51-fold. These index values ranged from 1.8 to 3.6 and are thus clearly positive for HSV-2. Therefore biochip sensitivity and specificity relative to the gold standard test methods are not impacted by the subsequent re-testing of these samples at increased sample dilution; this re-testing was performed solely to determine if the correlation between biochip and ELISA sample indices improved by increasing the measuring range of the biochip system. The linear correlation for non-corrected

Table 2 HSV-2 assay index values

Sample ID	Gender	Age (years)	Origin	LIAISON HSV-2 IgG assay index	HSV-2 biochip assay sample dilution	Biochip mean charge (μC)	Biochip SD	Biochip CV	Biochip cut-off mean charge (μC)	Biochip sample index[a]	ELISA mean optical density$_{450/630\ nm}$	ELISA SD	ELISA CV	ELISA cut-of f mean optical density$_{450/630\ nm}$	ELISA sample index
13 T0445114	Male	35	Morocco	<0.5	51	0.288	0.097	33.7%	0.790	0.4	0.103	0.004	3.4%	0.304	0.3
13 T0464169	Female	19	France	<0.5	51	0.342	0.048	14.2%	0.648	0.5	0.030	0.001	4.7%	0.304	0.1
13 T0466262	Female	38	Reunion Island	<0.5	51	0.283	0.122	43.0%	0.648	0.4	0.021	0.001	3.4%	0.304	0.1
13 T0470653	Male	39	France	<0.5	51	0.244	0.083	34.0%	0.766	0.3	0.031	0.001	2.3%	0.304	0.1
13 T0470927	Female	48	France	<0.5	51	0.728	0.087	11.9%	0.766	1.0	0.197	0.004	1.8%	0.304	0.6
13 T0475236	Female	42	France	<0.5	51	0.252	0.099	39.5%	0.891	0.3	0.025	0.000	0.0%	0.304	0.1
13 T0475390	Female	30	France	<0.5	51	0.542	0.161	29.7%	0.891	0.6	0.046	0.001	3.1%	0.304	0.2
13 T0480781	Female	44	French Polynesia	<0.5	51	0.501	0.103	20.6%	0.891	0.6	0.091	0.004	3.9%	0.304	0.3
13 T0482332	Female	31	France	<0.5	51	0.068	0.032	47.6%	0.376	0.2	0.061	0.008	12.9%	0.304	0.2
13 T0482367	Female	30	Martinique	<0.5	51	0.032	0.023	72.6%	0.376	0.1	0.051	0.009	18.2%	0.304	0.2
13 T0487266	Male	25	France	<0.5	51	0.035	0.017	48.5%	0.376	0.1	0.148	0.006	4.3%	0.304	0.5
13 T0490897	Male	51	France	<0.5	51	0.388	0.105	26.9%	0.648	0.6	0.020	0.001	7.1%	0.298	0.1
13 T0491281	Female	16	France	<0.5	51	0.190	0.077	40.6%	0.790	0.2	0.056	0.001	1.3%	0.298	0.2
13 T0496713	Female	44	France	<0.5	51	0.217	0.087	40.3%	0.626	0.3	0.034	0.005	14.8%	0.298	0.1
13 T0498896	Female	57	France	<0.5	51	0.417	0.126	30.3%	0.648	0.6	0.197	0.018	9.3%	0.298	0.7
13 T0500895	Male	32	France	<0.5	51	0.504	0.051	10.2%	0.626	0.8	0.117	0.001	1.2%	0.298	0.4
13 T0506737	Male	83	France	<0.5	51	0.253	0.072	28.5%	0.626	0.4	0.081	0.000	0.0%	0.298	0.3
13 T0507029	Male	85	France	<0.5	51	0.276	0.130	47.0%	0.710	0.4	0.031	0.001	2.3%	0.298	0.1
13 T0509304	Female	21	France	<0.5	51	0.547	0.116	21.2%	0.710	0.8	0.086	0.002	2.5%	0.298	0.3
13 T0511323	Male	35	France	<0.5	51	0.330	0.091	27.6%	0.710	0.5	0.050	0.001	2.8%	0.298	0.2
13 T0511392	Male	35	France	<0.5	51	0.481	0.206	42.8%	1.091	0.4	0.044	0.005	11.4%	0.298	0.1
13 T0513104	Male	23	France	<0.5	51	0.310	0.047	15.0%	0.790	0.4	0.060	0.004	7.1%	0.298	0.2
13 T0522750	Female	23	France	<0.5	51	0.905	0.080	8.8%	1.091	0.8	0.109	0.005	4.6%	0.298	0.4
13 T0527146	Female	26	France	<0.5	51	0.227	0.046	20.4%	0.554	0.4	0.038	0.001	1.9%	0.298	0.1
13 T0527211	Male	33	France	<0.5	51	0.214	0.122	57.0%	0.554	0.4	0.075	0.000	0.0%	0.298	0.3
13 T0529135	Female	24	France	<0.5	51	0.277	0.092	33.2%	0.554	0.5	0.023	0.001	3.1%	0.298	0.1
13 T0537549	Male	15	France	<0.5	51	0.706	0.152	21.6%	1.040	0.7	0.197	0.007	3.6%	0.298	0.7
13 T0549351	Female	40	France	<0.5	51	0.508	0.117	23.0%	0.946	0.5	0.056	0.004	7.6%	0.298	0.2
14 D0391962	Male	64	France	0.7	51	0.492	0.125	25.4%	0.604	0.8	0.475	0.011	2.2%	0.298	1.6
13 T0530393	Male	78	France	1.0	51	0.783	0.243	31.1%	0.790	1.0	0.041	0.001	3.4%	0.298	0.1
13 T0470933	Male	38	France	1.2	51	1.228	0.198	16.1%	0.766	1.6	0.975	0.017	1.7%	0.304	3.2
13 T0434783	Female	24	Mayotte	1.4	51	1.471	0.112	7.6%	0.946	1.6	1.031	0.012	1.2%	0.304	3.4
13 T0517588	Female	37	France	1.5	51	1.395	0.254	18.2%	1.040	1.3	0.957	0.023	2.4%	0.298	3.2
13 T0528664	Female	23	France	1.5	51	2.140	0.307	14.3%	0.946	2.3	1.601	0.025	1.6%	0.298	5.4
13 T0565931	Male	62	France	1.6	51	0.883	0.055	6.2%	0.604	1.5	1.183	0.018	1.6%	0.298	4.0
13 T0395081	Female	64	France	1.7	51	1.985	0.146	7.4%	1.040	1.9	1.091	0.013	1.2%	0.304	3.6
13 T0065482	Female	43	France	1.8	51	1.343	0.096	7.1%	0.891	1.5	0.990	0.016	1.6%	0.304	3.3
13 T0086841	Male	55	Mayotte	1.8	51	0.845	0.134	15.8%	0.376	2.2	1.174	0.004	0.3%	0.304	3.9
13 T0087037	Male	55	Mayotte	1.8	51	2.187	0.297	13.6%	0.648	3.4	1.248	0.037	3.0%	0.304	4.1
13 T0103221	Female	48	France	1.8	51	1.638	0.235	14.4%	0.626	2.6	1.688	0.042	2.5%	0.304	5.6
13 T0123866	Female	30	France	1.8	51	0.985	0.050	5.1%	0.710	1.4	1.377	0.050	3.6%	0.304	4.5
13 T0162515	Male	70	Martinique	1.8	51	2.031	0.310	15.3%	1.091	1.9	1.261	0.024	1.9%	0.304	4.1
13 T0167364	Female	43	French Guiana	1.8	51	1.148	0.130	11.3%	0.554	2.1	1.021	0.006	0.6%	0.304	3.4
13 T0022372	Female	52	France	1.9	51	1.877	0.147	7.8%	0.766	2.4	1.286	0.011	0.8%	0.304	4.2
13 T0026255	Female	41	Madagascar	1.9	51	1.133	0.156	13.8%	0.648	1.7	0.881	0.023	2.7%	0.304	2.9
13 T0033959	Female	32	France	2.5	51	1.882	0.295	15.7%	0.766	2.5	1.300	0.062	4.8%	0.304	4.3
13 T0140910	Female	60	France	2.7	51	1.926	0.212	11.0%	0.554	3.5	1.977	0.077	3.9%	0.304	6.5

Table 2 *(Contd.)*

Sample ID	Gender	Age (years)	Origin	LIAISON HSV-2 IgG assay index	HSV-2 biochip assay sample dilution	Biochip mean charge (μC)	Biochip SD	Biochip CV	Biochip cut-off mean charge (μC)	Biochip sample index[a]	ELISA mean optical density$_{450/630\ nm}$	ELISA SD	ELISA CV	ELISA cut-of f mean optical density$_{450/630\ nm}$	ELISA sample index
13 T0058054	Female	45	France	2.8	51	1.343	0.235	17.5%	0.710	1.9	1.377	0.013	0.9%	0.304	4.5
13 T0027480	Female	31	France	3.2	51	1.377	0.111	8.1%	0.648	2.1	1.539	0.022	1.4%	0.304	5.1
13 T0056931	Female	60	France	3.9	51	1.532	0.129	8.4%	0.626	2.4	2.176	0.002	0.1%	0.304	7.2
13 T0048674	Female	35	France	4.0	51	2.281	0.219	9.6%	0.648	3.5	2.408	0.006	0.3%	0.304	7.9
13 T0158098	Female	52	France	4.2	102	1.238	0.106	8.5%	0.803	3.1	1.773	0.024	1.4%	0.304	5.8
14 P0498605	Female	55	France	4.3	102	2.274	0.340	14.9%	0.790	5.8	3.493	0.016	0.4%	0.298	11.7
13 T0034508	Female	27	France	4.8	102	2.070	0.271	13.1%	0.803	5.2	3.026	0.008	0.3%	0.304	10.0
13 T0035379	Female	63	France	6.8	102	2.271	0.179	7.9%	0.803	5.7	1.937	0.035	1.8%	0.304	6.4
13 T0155361	Female	50	France	7.6	102	2.467	0.203	8.2%	0.803	6.1	3.092	0.133	4.3%	0.304	10.2
13 T0167320	Female	27	France	8.1	204	0.753	0.070	9.3%	0.604	5.0	3.735	0.002	0.1%	0.304	12.3
13 T0111301	Female	58	French Guiana	8.6	102	2.389	0.137	5.7%	0.803	5.9	3.354	0.017	0.5%	0.304	11.0
13 T0155361	Female	50	France	12.8	204	1.837	0.177	9.6%	0.946	7.8	3.806	0.027	0.7%	0.304	12.5
13 T0147395	Female	68	France	14.9	204	2.182	0.278	12.7%	1.040	8.4	3.066	0.047	1.5%	0.304	10.1

[a] HSV-2 biochip index values for strongly reactive samples (LIAISON® index >4.0) were calculated by multiplying the determined index value based on the charge measurement by the fold-difference between the test sample dilution and cut-off calibrator sample dilution.

biochip index values is lower (r^2 0.36 or 0.71) *versus* correcting index values for sample dilution. This reflected the upper limit of charge detection in the HSV-2 biochip system.

Owing to the biochip geometry, the maximum electrochemical charge observed in the HSV-2 pilot trials to optimise the assay design was <3.0 μC. Using simple linear regression analysis, the coefficient of determination of the HSV-2 biochip sample index values with sample index values from the two reference methods was poor (r^2 0.3005 or 0.6237). Nevertheless, the HSV-2 biochip sensitivity and specificity in relation to both reference methods remained unaffected. The reason for the poor linear relationship between datasets was that the charge measurements for high positive samples did not truly reflect the intense peroxidase activity resulting from these samples. In order to account for the limitation in electrochemical charge generation on the HSV-2 biochip, samples that were strongly reactive on the LIAISON® system (index values >4.0) were re-assayed on the HSV-2 biochip at increased sample dilution and adjusting the sample index values accordingly. Scatter plots of HSV-2 biochip index values plotted against the LIAISON® HSV-2 index values (Fig. 4) and Focus HerpeSelect® 2 IgG index values (Fig. 5) are shown. Adjusting

Fig. 4 Sample index values on the DiaSorin HSV-2 LIAISON® assay are plotted (*x*-axis) against the index values on the HSV-2 biochip assay (*y*-axis). The linear-fit trendlines and coefficients of determination are shown.

Fig. 5 Sample index values on the HerpeSelect® 2 IgG assay are plotted (*x*-axis) against the index values on the HSV-2 biochip assay (*y*-axis). The linear-fit trendlines and coefficients of determination are shown.

the high positive sample index values by increasing sample dilution greatly increased the coefficient of determination between the HSV-2 biochip results and the two reference assay methods (r^2 0.8799 or 0.8794). These data demonstrate that the HSV-2 biochip assay performance closely mirrors the chosen *in vitro* diagnostic reference tests.

The poor correlation observed with respect to the full dataset, specifically the sensor response to very high positives for the 51-fold dilution data, is to a very great degree a direct consequence of the non-linear charge-concentration characteristic of the coulometric sensors. This means in practice it is possible in cases of extremely high positives to obtain an output from the sensors lower than less concentrated (HSV-2 antibody) samples. Rescaling the sensor for these high samples by dilution is therefore effectively decreasing the peroxidase activity and altering the concentrations of TMB_{ox} and TMB_{red}, upon which the electrodes of each sensor respond.

The generic membrane-touch biochip featuring an immunochemical reaction chamber, a hydraulic microfluidics pump and coulometric sensors was assessed for the qualitative determination of antibodies against HSV-2 in human serum. The assay comprises standard immunoassay reagents with a high activity recombinant gG2 antigen and electrochemical charge measurement. In terms of diagnostic performance, the HSV-2 biochip closely matched two FDA cleared HSV-2 serological diagnostic assays. The manufacturers of the chosen reference assay methods, namely DiaSorin and Focus Diagnostics, have both performed extensive assay validation as part of the IVD approval process. Both tests were validated against a gold standard HSV-2 Western blot method and in various populations have high sensitivities (94.8%–100%) and specificities (96.1%–100%) detailed in the assay product inserts. In fact, a comparative study between the Focus and DiaSorin HSV-2 tests revealed a 100% agreement in a panel of 247 samples from STD clinic patients and expectant mothers.[27] The HSV-2 biochip assay has high sensitivity (96.8%–100%) and specificity (100%) compared to these two reference assay methods. Therefore, by extension, the HSV-2 biochip assay is very likely to perform to a comparable standard against the HSV-2 serological gold standard Western blot method. The excellent correlation of the HSV-2 biochip is likely due to the use of a high activity recombinant HSV-2 gG2 antigen.

Further to the qualitative correlation of the HSV-2 biochip with the two reference assays, there was also a good semi-quantitative relationship. This is not unexpected since the biochip's coulometric sensors are quantitative for peroxidase activity. Using index values as a comparator, the HSV-2 biochip yielded a high linear correlation with the LIAISON® test (r^2 0.8799) and with the ELISA test (r^2 0.8794). In fact the correlation of the HSV-2 biochip dataset with the two reference assays was better than the linear correlation of the reference assays with one another (r^2 0.732). This indicates biochip could be adapted to perform quantitative assays as well as have broader utility for other diagnostic targets.

The HSV-2 biochip study has several limitations, which should be viewed in the context of an early phase technical development to bring a disruptive point-of-care technology to market. Firstly, the human panel used was small (60 sera) and collected from a narrow geographic range, originating predominantly in France. Secondly, only limited demographic data was available for the selected samples, namely gender and age of patients. Certain factors that are known to impact the accuracy of serological HSV-2 tests, such as decreased specificity in populations from sub-Saharan Africa or in HIV-positive patients may or may not have played a role in this study.[23] However, as such factors are known to impact on the analytical performance of other more established HSV-2 serological diagnostic tests, this bias would not be specific to the HSV-2 biochip test.

The rapid HSV-2 tests referenced in this work have typical assay times of between 5 and 15 minutes. By contrast the HSV-2 biochip, was optimised using well-characterised commercial immuno-reagents for a 65 minutes assay. This compares favourably to the Focus Diagnostics HerpeSelect® 2 ELISA IgG assay of 100 minutes. There is ample scope to reduce the assay time of the HSV-2 biochip by reconfiguring the chip's microfluidics and coulometric sensor architecture as well as other assay protocol optimisations.

Conclusion

A generation of physical chemists and biosensor developers have immobilised antibodies directly onto electrodes with varying degrees of success; often demonstrating poor analytical performance. The use of a shunt-fluidic mechanism actuated by a simple hydraulic pump (membrane touch) allows immunoassay reactions to proceed in isolation from the indicator electrodes of a coulometric sensor. This reduces significantly non-specific binding and background signals. In our laboratory no matter what blocking reagent strategy was attempted to printed carbon surfaces, background readings associated with non-specific adsorptions were consistently more than five-fold larger than any protein-blocked polymer surface.

The membrane-touch biochip is in its early development and the authors emphasise the many challenging technical issues to address before a HSV-2 biochip can become a meaningful out-of-laboratory ELISA technology. The device presented relies on manual sample delivery and reagent injection and so the chip would require an external fluidic device to complete these functions. The on-chip pump was controlled manually and would require an external electromechanical device to actuate its operation. The sensors of the biochip offer a quantitative measure over that of assigning only positive highs and lows or negatives to blood samples (qualitative), as shown in this work. In order for HSV-2 biochip to fulfil its promise the device would require extensive calibration for qualitative and quantitative applications against approved laboratory methods.

The application of a membrane-touch biochip to the qualitative HSV-2 immunoassay for human samples was demon-

strated in this work. Assay performance of a novel microfluidic coulometric sensor responsive to HSV-2 antibodies was validated against diagnostics industry standard methods. The results from a human panel confirm the potential of the biochip to fulfil an unmet need for rapid, accurate and cost effective clinical diagnosis and management of HSV-2 infection. Adaptation of this coulometric technology to diagnostic assays for emerging infectious diseases is now under consideration by the project team.

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