analytical chemistry

Article

Fiber-optic immunosensor for detection of Crimean-Congo Hemorrhagic fever IgG antibodies in patients

Firoz Algaar, Evgeni Eltzov, Marina M. Vdovenko, Ivan Yurievich Sakharov, Luka Fajs, Manfred Weidmann, Ali Mirazimi, and Robert S. Marks

Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.5b01728 • Publication Date (Web): 07 Jul 2015

Fiber-optic immunosensor for detection of Crimean-Congo Hemorrhagic fever IgG antibodies in patients

Firoz Algaar. [a], Evgeni Eltzov [b,c], Marina M. Vdovenko[d], Ivan Yu. Sakharov[d], Luka Fajs[c], Manfred Weidmann [e], Ali Mirazimi [f,g,h,j], and Robert S. Marks[a,b,c,k,m *]

[a] Department of Environmental Engineering, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

[b] Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

[c] School of Material Science and Engineering, Nanyang Technology University, Nanyang Avenue, 639798, Singapore

[d] Department of Chemistry, Lomonosov Moscow State University, 119991 Moscow, Russia

[e] Institute of Aquaculture, University of Stirling, Stirling, United Kingdom

[f] Public Health Agency of Sweden, Stockholm, Sweden

[g] Karolinska Institute, Stockholm, Sweden

[h] Institute for Clinical and Experimental Medicine, Linköping University, Linköping, Sweden

[j] National Veterinary Institute, Uppsala, Sweden

[k] National Institute of Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva, Israel

[m] The Ilse Katz Center for Meso and Nanoscale Science and Technology, Ben-Gurion University of the Negev, Beer-Sheva, Israel

* Corresponding author

e-mail: rsmarks@bgu.ac.il

Tel: +972-8-6477182

Abstract

Crimean-Congo hemorrhagic fever (CCHF) is a severe viral disease with high fatality rate. CCHF virus is endemic in parts of Africa, Asia, Middle East and southeastern Europe. Rapid diagnostics of CCHF is vital for appropriate clinical management and prevention of secondary spread from human-to-human. Currently, diagnostics relies on Real-Time RT-PCR and antibody or antigen detection using ELISA. These methods require trained personnel and expensive equipment and are not appropriate for point-of-care (POC) diagnostics. Furthermore there are no POC assays available for CCHF.

We developed a fiber-optic biosensor for the detection of CCHF IgG antibodies. In order to improve sensitivity, we optimized both the bioreceptor immobilization protocol and the chemiluminescence substrate formulation. The resulting protocol showed a 100-fold greater sensitivity for detection of CCHF antibodies. Finally, we evaluated the fiber-optic biosensor with two CCHF patient sera. We showed that the fiber-optic biosensor is 10-times more sensitive that colorimetric ELISA and is able to detect both patients with high and low levels of IgG antibodies. We believe that the fiber-optic biosensor is a suitable alternative to ELISA as it is much more sensitive and makes it possible to detect small amount of antibodies at an early stage of infection, and can be integrated as a point-of-care diagnostic system of CCHF.

Introduction

Crimean-Congo hemorrhagic fever (CCHF) is a severe viral disease with an average case fatality rate of 5-30%. CCHF virus is the most widespread tick-borne virus in the world, with cases reported from Africa, Middle East, Asia, Russia and southeastern Europe. The virus is transmitted to humans mainly by infected ticks from the genus Hyalomma or by contact with blood or tissues from infected humans and animals. CCHF virus is classified as a BSL-4 agent due to its high pathogenicity, possibility of spread from human to human and because there is no effective treatment or vaccine available [1]. Rapid diagnostics are essential for effective infection control and prevention of spread of the disease from human to human. Namely, healthcare professionals are at high risk of

infection due to their close contact with patients especially during invasive and aerosol-forming interventions and many nosocomial transmissions have been reported [1].

Quick diagnostics provide the basis for the implementation of proper infection control measures and patient management [2–4]. Currently the diagnostics of acute CCHF relies mainly on the detection of viral RNA with Real-Time RT-PCR. This method enables quick, sensitive and specific detection, but is not suitable for point of care (POC) diagnostics, as it requires trained personnel and expensive equipment [5,6]. Besides, these criteria are mostly not met in rural areas where CCHF is endemic [7]. Diagnostics of acute CCHF is also performed with ELISA for detection of IgG and IgM antibodies as well as CCHF virus nucleoprotein (NP) and rarely by virus isolation. Current serological assays provide reliable results and offer adequate sensitivity in clinical settings, however they all require trained personnel, are time consuming and not appropriate for POC diagnostics [7]. An ideal POC test for detection of CCHF IgG would have to exhibit sensitivity and specificity similar to or greater than the current sensitivity and specificity of standard in-house and commercial ELISA tests that ranges between 66.9–90.2% and 95.8–100.0% respectively [5]. Besides it would ideally give quantitative results, be easy to use, robust and offer maximum operational safety to the user.

There is a need for point-of-care diagnostic assays allowing for quick and reliable, bedside diagnostics of CCHF. Fiber-optic biosensors present a suitable alternative to existing diagnostic options for CCHF due to their small size, diagnostic accuracy and low cost. Other POC technologies, such as lateral flow have similar advantages however they do not give quantitative results and have limited multiplexing capabilities. Optic fiber biosensors are quantitative and can easily be multiplexed, a feature that would enable the design of a multiple-target POC assay. The core of optic-fiber biosensor development is the immobilization of capture affine biomolecules (eg. antigen, antibodies, nucleic acids) at the endface and the tip of an optical fiber. Target molecules then bind to the capture biomolecules and these can also bind to complementary labeled detection biomolecules. After exposure to a substrate, the marker enzyme oxidizes it and a chemiluminescent glow is produced as a side reaction that is collected

by the optical fiber and transduced to the detector [8]. Previously we developed fiber-optic biosensors for sensitive detection of antibodies against Ebola virus, Rift Valley Fever virus, Dengue virus, West-Nile and Hepatitis C [9–13]. The aim of this study is to develop a fiber-optic biosensor for accurate detection of antibodies against CCHF virus NP and to optimize the fiber-optic immobilization procedures so as to enhance overall signal.

Materials and methods

Materials

Methyl alcohol (136805) was purchased by Bio-Lab (Israel), acetic acid (45731, 99.8% (v/v)) was purchased from Fluka, sulfuric acid (76649390, 95% (v/v)), hydrochloric acid (7647010, 37% (v/v)) and hydrogen peroxide solution (7722841, 35%(v/v)) were purchased from Acros Organic (USA). 3-(Glycidoxypropyl)trimethoxysilane (GPTMS)(440167, 98%(v/v)), sodium m-periodate (S1878), phosphate buffer saline (PBS)(P4417), sodium cyanoborohydride (71435), glycine (G7403), skim milk powder (SM)(70166), Luminol (A8511), 4-morpholinopyridine (MORP) (480657) and polyoxyethylene-sorbitan monolaurate (Tween® 20) (P7949) were purchased from Sigma Aldrich (Israel).

CCHFV-N protein expression and purification

For expression 1-50ng of plasmid CCHFNhispET19# (provided by Ali Mirazimi) containing the nucleocapsid gene of CCHFV strain Ibr10200 was transformed into BL21 and plated out for overnight culture on LB amp plates at 37°C. Clones were picked and subsequently cultured overnight in 5ml LB amp at 37°C, 50ml LB amp culture for 24h at 28°C after transfer of 1 ml, and 500ml LB amp culture after transfer of 10 ml at 28°C during the following day until an OD260nm between 0.4-0.6 was reached (NanoDrop). Expression was induced with 1 mM IPTG and the culture continued at 28°C overnight. A bacterial pellet was harvested by centrifugation at 4000g / 20min, washed with PBS,

resuspended im 5ml buffer A2, disrupted at level 3.5 (Sonifier II W-250/W-450, Branson, USA) for 30 sec for 7 times on ice and finally centrifuged at 12.000g, 4°C for 25 min. The supernatant was transferred to equilibrated Ni-agarose (1.4ml Ni-agarose (Qiagen, Germany) washed twice with 10ml water and twice with buffer A1) and incubated for 2h at 4°C on a shaker. After one additional centrifugation step the agarose was resuspended in 1ml buffer A1 and transferred into an empty column (Biorad, Germany). The column was then successively washed with 20ml buffer B, 10ml buffer E1, and the protein was eluted with 8 fractions of 500µl buffer E2. (Buffer A (50 mM Tris (pH 8,0), 500 mM NaCl , 5 mM MgCl , 10 % Glycerin, 0,1 % NP-40, 2mM Imidazol), buffer A1 (bufferA + 140mM ß-mercaptoethanol), buffer A2 (BufferA + 0.1mM PMSF), buffer B (buffer A1 + 28mM Imidazol), elution buffer E (20 mM Tris (pH 8.0), 100 mM NaCl, 5 mM MgCl, 20 % Glycerin), buffer E1 (buffer E, 2mM Imidazol), buffer E2 (buffer E, 250mM Imidazol). The protein concentration was determined by the Bradford method (BCA-Assay, Pierce, UK) after confirming a pure band on an SDS page-gel.

CCHF sera and antibodies

Inactivated sera from two confirmed CCHF cases, CCHFV positive rabbit sera against N protein (Andersson et.al 2004 J. Med Virol), recombinant CCHFV N protein (Andersson et.al 2004 J. Med Virol), anti-CCHFV monoclonal antibody against N protein and anti-CCHF polyclonal rabbit antibodies were all received from the Public Health Agency of Sweden. Human IgG was detected with donkey HRP-coupled anti-human IgG antibodies. Rabbit antibodies were detected with HRP-coupled goat anti-rabbit IgG HRP (Jackson ImmunoResearch Laboratories, USA).

Optical fiber immobilization

We used SFS400/440B black Tefzel®Superguide G UV-Vis (Fiberguide Industries, Stirling, NJ, USA) optic fibers. The length of any single fiber used in the experiments was approximately 25 cm. Tefzel® jacket and silicon buffer were removed with a fiber

stripping tool (Micro-Strip®, from Micro-Electronics Inc., MA, USA), leaving a 0.5 cm nude optical fiber core tip.

Optic fibers were prepared according to the reference protocol described by Liebes et al. [14]. Briefly, the fibers were cleaned by sonication in 1:1 (v/v) methanol/ 97% HCl solution for 10 min at 90ºC and treated with piranha solution (7:3 [v/v] H_2S0_4/H_20_2) to produce surface hydroxyl groups, rinsed with nanopure water and dried with nitrogen gas. Fiber surfaces were then silanized with (3-glycidoxypropyl)trimethoxysilane for 60 min at 90ºC. Fibers were treated with 11.6 mM hydrochloric acid for 60 min (formation of vicinal diols) and 100 mM sodium m-periodate dissolved in 10% (v/v) acetic acid for 60 min at room temperature (oxidation to aldehyde). Fibers were then rinsed with deionized water and incubated with 1ml of 0.43µg/ml HRP (for assay optimization) or CCHFV NP (for assay validation) overnight at 4ºC. Un-reacted aldehyde groups were blocked using 1 M glycine for 30 min at RT and the unsaturated amines were stabilized by a Schiff base with 0.3 M sodium cyanoborohydride for 60 min at RT.

Fiber-optic chemiluminescent and Colorimetric immunoassay

Instrument set-up for chemiluminescence measurements was performed as previously described [22, 23]. A Hamamatsu HC135-01 Photo Multiplier Tube (PMT) Sensor Module was used for chemiluminescence measurements in a light-tight box. The far end of the fiber was held by a fiber holder (FPH-DJ, Newport) and placed into an adjustable single-fiber mount (77837, Oriel). Real-time data acquisition was performed using LabView (version 3.1, National Instruments Corporation). Chemiluminiscence detection was performed with the Immuno-star HRP chemiluminescent kit (170-5040, Bio-Rad Laboratories, France). Chemiluminescence readings were integrated for 1 s, measuring a mean value of photon counts during 15 s. Results are presented as the mean and standard deviation of relative light units (RLU). Colorimetric detection was performed using 100µl 3,3,5,5-tetramethylbenzidine (TMB) (T0440, Sigma Aldrich, Israel) and 50µl of 2N H_2SO_4 afterwards in each well. Optical density readings at 450 were determined with a Labsystems Multiscan RC ELISA reader.

Protocol optimization

The initial immobilization protocol (Protocol 1) [14] was optimized in order to achieve maximum output chemiluminescence signal levels. For protocol optimization we immobilized 200 U/ml HRP on the fiber optic. Optimization steps are shown in Table 1. Steps with the highest chemiluminescence output comprise the optimized protocol (Protocol 2), which we used for the optic-fibre immobilization with CCHF virus NP.

We also evaluated the effect of two different HRP substrate compositions. We compared the output levels of the Immuno-star-HRPTM substrate (Bio-Rad), that includes 1:1 luminol/enhancer and stable peroxide buffer, and a substrate described by Vdovdenko et al. [15] that is based on the addition of two enhancers: SPTZ (sodium salt of 3-(10'-phenothiazinyl)propane-1-sulfonate) and MORP (4-morpholinopyridine). A total of 240µl substrate was added to each well.

Fiber-optic immunoassay for detection of CCHF antibodies

Assay's sensitivity was evaluated with a 10^2 to 10^6 dilution range of Polyclonal Rabbit anti-CCHF virus antibodies. Briefly, 200 µl diluted antibodies were added per well (five sets of triplicates) and incubated for 60 min.

Sensitivity of the assay was further evaluated with two confirmed samples from two CCHF patients from Turkey. The samples were collected for routine diagnostic purposes in Turkey and development of diagnostic tools and their collection was approved by the governing ethical committee (Turkish Public Health Institution). Serum samples were chosen based on their initial serology results: high-level IgG for patient 1 (CDC ELISA, OD: 2) and low-level IgG for patient 2 (OD: 0,4).

Optic fibers were incubated with 200µl diluted serum (dilutions from 10^3 to 10^6) for 60 min (three sets of triplicates). Unmodified fibers were used to check the level of background signal. Optic fiber tips were washed four times with PBST–BSA for a one-time 10 min, followed by a three time 3 min protocol. Fibers were then dipped into

200µl donkey anti-human IgG peroxidase-labeled antibodies (1/500) for 60 mi and washed with PBS-Tween before chemiluminiscence detection as described above.

CCHF IgG ELISA

Fiber-optic immunoassay for detection of CCHF virus antibodies performance was compared to the performance of CCHF IgG ELISA. MaxiSorp 96-well microtiter plates (Nunc) were coated with 100µl/well of CCHF virus NP. Plates were placed overnight at 4ºC. Plates were then washed three times with 100µl of PBS each. Each well was blocked by 150µl/well of blocking solution (1M glycine (w/v) in PBS buffer, pH 7.2) for 1 h at 37ºC. Plates were washed three times with 100µl 0.1%PBST containing 5% (w/v) skimmed milk (washing step). Serial dilutions (10^{-2}-10^{-6}) of anti-CCHF antibodies (100µL/well) were added in triplicates and incubated for 1 h at 37ºC followed by a washing step. Goat anti-mouse IgG peroxidase-labeled antibodies (100 µl/well) (10^{3}) were added and incubated for 1 h at 37ºC, followed by a washing step. Two different substrates were used. For colorimetric ELISA, we added 3,3',5,5' tetramethylbenzidine (TMB) (100 µl/well) and incubated at room temperature for 30 min in the dark. The reaction was stopped with 2N H_2SO_4 (50 µl/well). Optical density reads at 450 nm (OD_{450}) were determined with the Labsystems Multiscan RC ELISA reader. Results are presented as the mean and standard deviation of OD_{450}.

Results and Discussion

The aim of this study was to develop and optimize a fiber optic biosensor for detection of CCHF virus antibodies. As a starting point we used a previously described immobilization protocol (Protocol 1) [14] and optimized each step in order to maximize the chemiluminescence output. In the cleaning step we tested the effects of sonication and duration of methanol washing. We showed that sonication significantly increased the biosensor end point signal output (RLU 6857 vs. 1114 without sonication) and that methanol washing for 20 min was the most effective. Next we wanted to assess the efficacy of optical fiber activation (production of surface hydroxyl groups) using different activating solutions. Namely, we wanted to replace the piranha solution with a safer alternative as it is highly toxic and dangerous, requires special handling and disposal measures. However, our results show that the piranha solution was by far the most effective activator, with the biosensor output of 16669 RLU. Neither HCL, H_2SO_4, CH_3COOH or H_3PO_4 had comparable results (RLU: 3598, 9625, 1991, 5681, respectively). Piranha solution was therefore used in the optimized protocol (Protocol 2). Next step was the optimization of the silanization process. We tested 3 different temperatures (30°C, 60°C and 90°C) and 3 different durations (30 min, 60 min, 90 min) of silanization. We obtained the highest biosensor output at 60°C for 60 min (8660 RLU). In the last step we optimized the temperature and duration of treatment with sodium m-peroxidate. The highest biosensor output was achieved at 25°C for 90 min (20594 RLU). The results of the protocol optimization are shown in Table 1.

Next, we compared protocols 1 and 2 for their overall performance in the immunoassay, and as seen in Figure 1, the sensitivity of protocol 2 is 100-fold higher (limit of detection for protocol 1 is antibody dilution 10^{-4} and for protocol 2 is 10^{-6}). The results show that optimization steps had great influence on the overall efficacy of the immunoassay. Protocol 2 was therefore used for validation of the fiber-optic biosensor using patient sera.

We also compared two different substrate formulations with respect to the overall biosensor output. We wanted to assess if enhancers (SPTZ or MORP) added to the luminol/peroxide substrate, would enhance the biosensor's output, and indeed, as seen in figure 2, the addition of the two enhancers did increase the biosensor's output by roughly 10-fold. An additional advantage in using SPTZ/MORP enhancers is that they provide a more durable light signal that increases the biosensor's sensitivity[16].

In the last step we evaluated the sensitivity of the developed fiber-optic biosensor for the detection of CCHF IgG antibodies in human sera. We tested the assay with both patients' sera. Fiber-optic biosensor detection was more sensitive than ELISAs. The lowest limit of detection of Patient 1 serum was 10-fold greater compared to ELISA, down to titer 1:1,000,000. The assay was also able to detect the low positive serum sample of Patient 2 down to titer 1:5,000 (Table 2).

Our results show that our fiber-optic biosensor is a suitable alternative for ELISA, for the detection of CCHF virus IgG antibodies. The assay enables rapid and sensitive detection of CCHF antibodies and can be manufactured as a semi-disposable, point-of-care device. The technology also enables us to modify the device to enable detection of other biomolecules (e.g. CCHF RNA). Another advantage of the proposed biosensor is that it does not require trained personnel or expensive equipment for its operation. Today's diagnostic assays lack these features and are therefore available only at select diagnostic centers. However, the endemic areas of CCHF are mainly rural areas with limited access to these high-end diagnostic services. High pathogenicity of CCHF and the risk of human-to-human transmissions, especially in hospital settings, warrant the development of point-of-care devices that would enable quick, safe and reliable diagnostics of CCHF. The main advantage of the fiber optic biosensor is that it provides sensitive results and can be designed as a point-of-care device at a cost comparable to current standard ELISA assays. The assay could serve as a rapid, primary diagnostic tool for bedside CCHF diagnostics in endemic areas where extensive diagnostic equipment and trained personnel are not available. The fiber-optic biosensor could provide the

necessary solution, however further studies are needed to determine the actual clinical sensitivity and specificity of the assay on a larger scale.

Acknowledgements

This project was funded by the CCH Fever Network (Collaborative Project) supported by the European Commission under the Health Cooperation Work Program of the 7th Framework Program (Grant agreement no. 260427). We thank Doris Heidenreich for technical assistance and Gulay Korukluoğlu for the human serum samples.

References

(1) Tarantola, A.; Ergonul, O.; Tattevin, P.; Ergonul, O.; Whitehouse, C. A. Springer: Netherlands, 2006; pp. 281–294.
(2) Pshenichnaya, N. Y.; Nenadskaya, S. A. *Int. J. Infect. Dis.* **2015**, *33C*, 120–122.
(3) Papa, A.; Mirazimi, A.; Köksal, I.; Estrada-Pena, A.; Feldmann, H. *J. Clin. Virol.* **2014**.
(4) Conger, N. G.; Paolino, K. M.; Osborn, E. C.; Rusnak, J. M.; Günther, S.; Pool, J.; Rollin, P. E.; Allan, P. F.; Schmidt-Chanasit, J.; Rieger, T.; Kortepeter, M. G. *Emerging Infect. Dis.* **2015**, *21*, 23–31.
(5) Vanhomwegen, J.; Alves, M. J.; Zupanc, T. A.; Bino, S.; Chinikar, S.; Karlberg, H.; Korukluoğlu, G.; Korva, M.; Mardani, M.; Mirazimi, A.; Mousavi, M.; Papa, A.; Saksida, A.; Sharifi-Mood, B.; Sidira, P.; Tsergouli, K.; Wölfel, R.; Zeller, H.; Dubois, P. *Emerging Infect. Dis.* **2012**, *18*, 1958–65.
(6) Escadafal, C.; Olschlager, S.; Avsic-Zupanc, T.; Papa, A.; Vanhomwegen, J.; Wolfel, R.; Mirazimi, A.; Teichmann, A.; Donoso-Mantke, O.; Niedrig, M. *PLoS neglected tropical diseases 6*, e1706.
(7) Bente, D. A.; Forrester, N. L.; Watts, D. M.; McAuley, A. J.; Whitehouse, C. A.; Bray, M. *Antiviral Res.* **2013**, *100*, 159–89.
(8) Collings, A.; Caruso, F. *Reports on Progress in Physics 60*, 1397–1445.
(9) Sobarzo, A.; Paweska, J.; Herrmann, S.; Amir, T.; Marks, R.; Lobel, L. *Journal of Virological Methods 146*, 327–334.
(10) Konry, T.; Novoa, A.; Shemer-Avni, Y.; Hanuka, N.; Cosnier, S.; Lepellec, A.; Marks, R. S. *Anal. Chem.* **2005**, *77*, 1771–9.
(11) Atias, D.; Liebes, Y.; Chalifa-Caspi, V.; Bremand, L.; Lobel, L.; Marks, R.; Dussart, P. *Sensors and Actuators, B: Chemical* **1899**, *140*, 206–215.

(12) Herrmann, S.; Leshem, B.; Landes, S.; Rager-Zisman, B.; Marks, R. S. *Talanta* **2005**, *66*, 6–14.
(13) Petrosova, A.; Konry, T.; Cosnier, S.; Trakht, I.; Lutwama, J.; Rwaguma, E.; Chepurnov, A.; Mühlberger, E.; Lobel, L.; Marks, R. *Sensors and Actuators B: Chemical* **2007**, *122*, 578586.
(14) Liebes, Y.; Amir, L.; Marks, R.; Banai, M. *Talanta* **1901**, *80*, 338–345.
(15) Vdovenko, M.; Ciana, L.; Sakharov, I. *Analytical Biochemistry 392*, 54–58.
(16) Vdovenko, M.; Ciana, L.; Sakharov, I. *Biotechnology Journal 5*, 886–890.

Table 1: Optimization steps for optical fiber immobilization (in bold are the parameters that define the optimized protocol 2)

Step	Optimization		Biosensor output [RLU]
Cleaning	Sonication	**With**	**6857 (39.72)**
		Without	1114 (15.92)
	Methanol	10 min	4710.17 (24.78)
		20 min	**13402.88 (16.86)**
		30 min	13141.38 (18.03)
Activation		HCL	3598 (7.6)
		Piranha	**16669 (31.8)**
		H_2SO_4	9625 (26.2)
		CH_3COOH	1991 (26.4)
		H_3PO_4	5681 (16.34)
Silanization	30 min	30ºC	1428 (32)
		60ºC	2760 (50)
		90ºC	1644 (33)
	60 min	30ºC	1864 (37)
		60ºC	**8660 (17)**
		90ºC	2941 (46)
	90 min	30ºC	3797 (28)
		60ºC	4198 (40)
		90ºC	1204 (3)
Sodium m-periodate	30 min	25ºC	1576 (31.5)
		30ºC	1870 (35.9)
		60ºC	1415 (16.4)
	60 min	25ºC	9086 (31.5)
		30ºC	3357 (15.4)
		60ºC	3465 (36.4)
	90 min	**25ºC**	**20594 (18.9)**
		30ºC	7884 (33.4)
		60ºC	3626 (26.1)

Table 2: Immunoassay results

Sample	Dilution	Fiber optic biosensor	ELISA
Patient 1	1:5000	+	+
	1:10000	+	+
	1:20000	+	+
	1:50000	+	+
	1:100000	+	+
	1:250000	+	-
	1:500000	+	-
	1:750000	+	-
	1:1000000	+	-
Patient 2	1:1000	+	ND
	1:5000	+	ND
	1:10000	-	ND
No serum control		-	-
Human serum control		-	-
Rabbit serum control		-	-
Positive control		+	+
No primary antibody		-	-
No secondary antibody		-	-

ND not determined

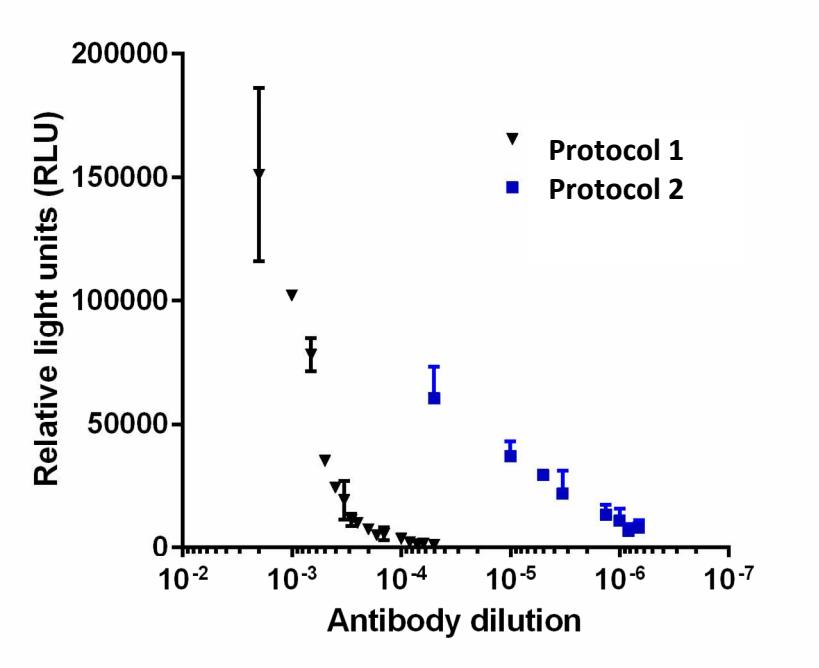


Figure 1: Immunoassay results for the comparison of immobilization protocols 1 and 2.

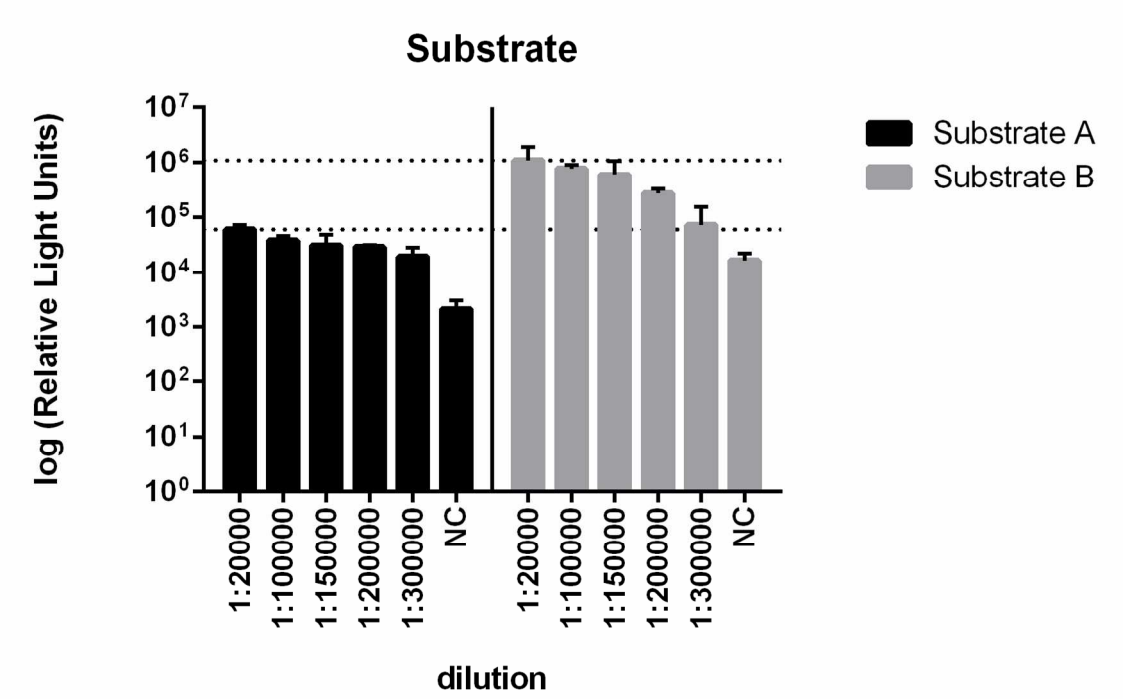
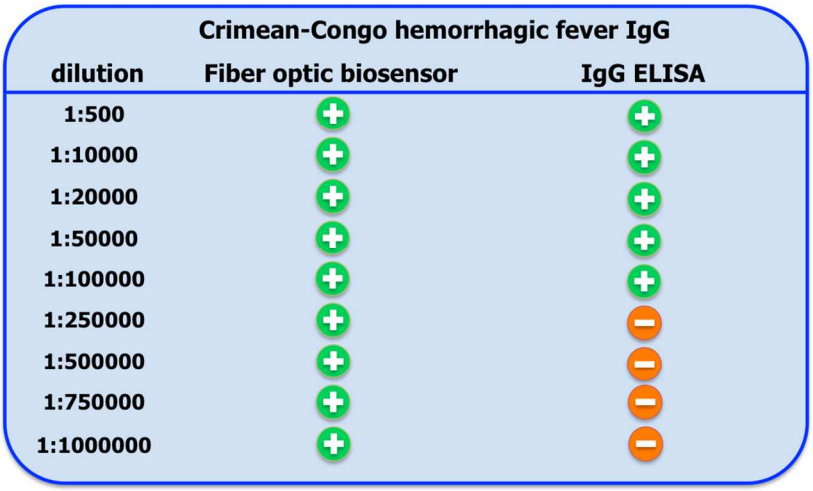


Figure 2: Immunoassay results for the comparison of two substrate formulations. Substrate A is a commercial luminol/peroxide formulation, substrate B includes two additional enhancers, SPTZ and MORP. NC= negative control.

For TOC only


Crimean-Congo hemorrhagic fever IgG
dilution
Fiber optic biosensor
IgG ELISA
1:500
1:10000
1:20000
1:50000
1:100000
1:250000
1:500000
1:750000
1:1000000