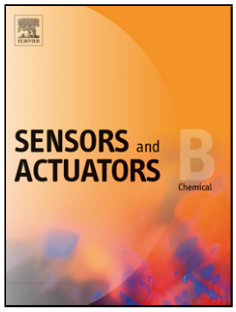

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Laser-induced fluorescence integrated in a microfluidic immunosensor for quantification of human serum IgG antibodies to *Helicobacter pylori*

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Abstract

A portable immunosensor coupled to laser-induced fluorescence (LIF) detection system has been successfully developed. It was applied to the quantitative determination of specific IgG antibodies against *H. pylori* present in human serum samples. This system is a relevant alternative tool for the diagnostic of peptic ulcer and gastritis diseases produced by *H. pylori*, which are recognized risk factors for the development of gastric mucosa associated lymphoid tissue lymphoma and gastric adenocarcinoma. More than 50% of the population has been affected by *H. pylori*, one of the most widespread infections around the world.

Our proposed system combines the LIF detection, which offers great sensibility with the specificity of the immunological reactions and the microfluidic technology. The device has a central channel (CC) with packed *H. pylori* antigen immobilized on 3-aminopropyl-modified controlled pore glass (AP-CPG). Antibodies in serum samples reacted immunologically with the immobilized antigen and then, they were determined using alkaline phosphatase (AP) enzyme-labeled second antibodies specific to human IgG. The 4-methylumbelliferyl phosphate (4-MUP), employed as enzymatic substrate, was converted to soluble fluorescent methylumbelliferone by AP, and this fluorescent product was finally quantified by LIF detection system. The calculated detection limits for LIF detection and the ELISA procedure were 0.17 and 2.1 UmL^{-1}, respectively, and the within- and between-assay coefficients of variation were below 5.1%.

Keywords: Microfluidic system; enzyme immunoassays; LIF detection; *Helicobacter pylori*; immunosensor; gastric diseases.

1. Introduction

Helicobacter pylori is a Gram-negative spiral-shaped bacterium which was first described by Warren and Marshall in 1983 [1, 2]. This microorganism, classified as a class I carcinogen by the World Health Organization [3], plays a causative role in the development of gastritis (80–85%), peptic ulcer (10–15%), gastric adenocarcinoma (1–2%) and mucosa associated lymphoid tissue (MALT) lymphoma (< 0.01%) [4-7]. More than 50% of the world's population have *H. pylori* in their upper gastrointestinal tract, making it the most widespread infection in the world [8].

According to published articles, the prevalence of this relevant infection has been associated with poor social and economic development [9, 10]; low education level; poor hygiene practices during childhood; absence of sanitary drinking water; absence of a sewage disposal facility during childhood and [11] inadequate food handling [12]. However, is important to take in account that the severity of the gastric damage and the clinical outcome of *H. pylori* infection is a consequence of a combination of several factors, including host genetic, bacterial virulence, environmental influence and lifestyle.

The transmission of *H. pylori* infection is still poorly understood, but faecal–oral, oral–oral and gastro–oral are the most consensual routes of transmission [13, 14]. *H. pylori* infection is usually acquired in childhood and it can persist for the lifetime of the host if it is not treated [15].

The standard recommended treatment for the eradication of this microorganism consist in the combination of a proton pump inhibitor and two antibiotics, clarithromycin with either amoxicillin or metronidazole [16]. For previously described therapy an eradication rate of >90% has been reported [17]. However, the widespread use of antibiotics has generated a relatively high failure rate of about 20-40% of the

patients [18, 13] mainly due to antibiotic resistance [19], but also because the bacteria may be present in a protective environment like the stomach mucosal [20].

Regarding to the diagnosis of *H. pylori* infection, invasive methods like endoscopy with biopsies for histology, culture, rapid urease test and the noninvasive tests including serological tests and the urea breath test have been reported [21, 22]. Serological tests are based on the detection of IgG antibodies against *H. pylori* present inhuman serum samples. This determination proved to be of considerable value in the diagnosis of active *H. pylori* infection due to the reliable correlation between the presence of this antibodies and gastric mucosal colonization [23, 24]. As mentioned above, one tool for serologic diagnostic of *H. pylori* infection is the determination of anti-*H. pylori* IgG antibodies, which is performed using enzyme immunoassay (EIA) in human serum samples to provide an early diagnosis [25].

Due to the prevalence of the *H. pylori* infection, the development of a simple, rapid, accurate, and cost-effective diagnostic methods to achieve: ulcer healing, prevention of peptic ulcer recurrence and the reduction of the prevalence of gastric cancer in high-risk populations, becomes important [26].

The miniaturization of heterogeneous enzyme immunoassays using microfluidic technology [27] as platform represents an interesting alternative tool, due to its advantages, such as high degree of integration, remarkable sensitivity and low regent consumption between them [28].

With the aim to recognize the antibody/antigen binding event into microfluidic systems, different transduction pathway scan be used, anyway, laser-induced fluorescence (LIF) offers good sensitivity with low detection limits and provides the possibility of make detections in very small sample volumes. In addition, the miniaturization of the excitation source and the detection device has permitted that the

whole device can be made easily portable [29]. For these features, LIF detection has become a particularly suitable tool for the detection of several analytes on microfluidic devices in length scales of micrometers or smaller.

In the presented work, we established a LIF immunosensor incorporated into microfluidic analytical system for a rapid and sensitive quantification of human serum IgG antibodies to *H. pylori*, based on the use of purified *H. pylori* antigen that was immobilized on 3-aminopropyl-modified controlled pore glass (AP-CPG). Antibodies in the serum samples were allowed to react immunologically with the antigen, and the bound antibodies were quantified by alkaline phosphatase (AP) enzyme-labeled second antibodies specific to human IgG. 4-methylumbelliferyl phosphate (4-MUP) was converted to soluble fluorescent methylumbelliferone by AP [30, 31] and it was quantified using LIF detection system. The response obtained from the product of enzymatic reaction was proportional to the activity of the enzyme and consequently, to the amount of IgG antibodies to *H. pylori* antigen in serum.

2. Materials and methods

2.1. Reagents and solutions

All reagents used were of analytical reagent grade 3-aminopropyl-modified controlled pore glass (AP-CPG; 1,354 Å mean pore diameter, 19.7 m^2g^{-1} surface area) was purchased from Pure Biotech LLC, Glutaraldehyde (25% aqueous solution) was purchased from Merck,Darmstadt,4-methylumbelliferyl phosphate (4-MUP) and AP enzyme labeled second antibodies specific tohuman-chainwere purchased from Sigma Chemical (St. Louis, MO, USA). The Enzyme Immunoassay for the Quantitative Determination of IgG Class Antibodies to *Helicobacter pylori* was purchased from

EQUIPAR, Diagnostic, (Rome, Italy) and was used in accordance with the manufacturer's instructions [32]. All buffer solutions were prepared with Milli-Q water.

2.2. Instrumentation

The syringe pumps system (Baby Bee Syringe Pump, Bioanalytical Systems) was used for pumping, sample introduction, and stopping flow.

All solutions and reagent temperatures were conditioned before the experiment using a Vicking Masson II laboratory water bath (Vicking SRL, Buenos Aires, Argentina).

Absorbance was detected by Bio-Rad Benchmark microplate reader (Japan) and Beckman DU 520 general UV/VIS spectro photometer.

The optical system was constructed using the procedure of reference [33] with the following modifications. A 355 nm single-frequency DPSS laser (Cobolt Zouk™, USA) operated at 10 mW served as the fluorescence excitation source. It was focused onto the detection channel at 45° to the surface using a lens with a focal distance of 30 cm, as shown in Figure 1a. The relative fluorescence signal of 4-MUP was measured using excitation at 355 nm and emission at 440 nm.

The paths of the reflected beams were arranged, so that they did not strike the capillary channels elsewhere, in order to avoid photobleaching. The fluorescent radiation was detected with the optical axis of the assembly shown in Figure 1a, perpendicular to the plane of the device. Light was collected with a microscope objective (10: 1, NA 0.30, working distance 6 mm, PZO, Poland) mounted on a microscope body (BIOLAR L, PZO, Poland). A fiber optic collection bundle was mounted in a sealed housing at the exit of the microscope which was connected to

148 QE65000-FL Scientific-grade Spectrometer (Ocean Optics, Inc. USA). The entire
149 assembly was covered with a large box to eliminate the ambient light.
150 All pH measurements were made with an Orion expandable ion analyzer (Orion
151 Research Inc., Cambridge, MA, USA) Model EA 940 equipped with a glass
152 combination electrode (Orion Research Inc.).
153
154 *2.3. Preparation of the H. pylori antigens*
155 The antigens were prepared from a sonicated *H. pylori* culture strain. The *H.*
156 *pylori* were grown on blood agar plates at 37°C for3 days and then harvested, washed,
157 and resuspended in 0.01M phosphate-buffered saline (PBS, pH 7.2). This preparation
158 was subjected to sonication. The sonic amplitude level was set at 20%, and the machine
159 was operated using four cycles of 60 s regulated alternatively. The sonicated preparation
160 was centrifuged at 1000×*g* for 10 min, and the supernatant was stored in the 0.01M PBS
161 (pH 7.2), at −20 ◦C between uses.
162
163 *2.4. Immobilization of purified antigen of H. pylori on AP-CPG*
164 To carry out the process of modification, 1 mg of AP-CPG was allowed to react
165 with 1 ml of an aqueous solution of 5% w/w glutaraldehyde in 0.20 M CO_3^{2-}/ HCO_3^-
166 buffer at pH 10.00 (for 2 h at room temperature. After three washes with 0.10 M
167 phosphate buffer of pH 7.00, 100 μg mL^{-1} of *H. pylori* antigen solution was coupled to
168 the residual aldehyde groups overnight at 5°C. The immobilized antigens preparation
169 was finally washed three times with phosphate buffer (pH 7.00) and stored in the same
170 buffer at 5°C. The immobilized *H. pylori* preparation was perfectly stable for at least 1
171 month.
172

2.5. Design of microfluidic immunosensor system

Figure 1b shows a schematic representation of the design of our microfluidic immunosensor which was fabricated using commercially available 3.5 mm thick Poly methyl-methacrylate (PMMA) sheets. PMMA sheets were cut into plates measuring 20.0 mm × 50.0 mm (width × length) to form microchip substrates. The microfluidic pattern was designed and then it was sent to the laser scriber for direct machining on PMMA substrate. Once the microfluidic channels were formed, the device was thermally sealing by action of the temperature. The PMMA channel plate and the PMMA cover sheets were sandwiched between two glass plates (of the same dimensions), clamped together using laboratory clamps, and placed in a convection oven for 10 min at 110 °C. The bonded chip was then allowed to cool slowly and the clamps were removed.

The final device has a CC and accessory channels with diameters of 100 μm. In the CC of the device, *H. pylori* antigen-AP-CPG was packed and then kept in a fixed previous position of the detection zone because of the CC diameter's reduction of 100 μm to 30 μm, which avoids the loss of this modified support.

2.6. Detection procedure

The developed method was based on the principle of a heterogeneous non-competitive fluorescence immunoassay (Fig. 2). The carrier buffer was 0.01M PBS, pH 7.2.The immunological procedure was as follows: unspecific binding was blocked by a 5-min treatment at room temperature with 3% skim milk in a 0.01 M phosphate-buffer saline (PBS) pH 7.2 at flow rate of 2.0 μL min^{-1} and then it was rinsed with 0.01M PBS (pH 7.2) for 5 min at a flow rate of 2.0 μL min^{-1}. After that, serum sample, firstly diluted 100 fold with 0.01 M PBS (pH 7.2), was injected into the PBS carrier stream at

198 flow rate of 2.0 μL min^{-1} for 5 min. Consequently IgG anti-*H. pylori* antibodies present
199 in it reacted with *H. pylori* antigen on the surface of the support (*H. pylori antigen*-AP-
200 CPG). Then the device was washed with 0.01M PBS (pH 7.2) at a flow rate of 2.0 μL
201 $min^{-1}$for 5 min to remove the exes of sample. Bound antibodies were quantified using
202 alkaline phosphatase enzyme-labeled second antibodies specific to human IgG (dilution
203 of 1/1.000 in 0.01 M PBS, pH 7.2) injected at flow rate of 2.0 μL min^{-1} for 5 min. Then,
204 the microfluidic system was washed with 0.01 M PBS (pH 7.2) for 4 min.
205 The relative fluorescence signal, which corresponded to the IgG specific *H.*
206 *pylori* antibodies-alkaline phosphatase enzyme-labeled second antibodies complex was
207 measured 'in-situ' in the immune-microfluidic device. 4-MUP fluorescence was
208 measured using excitation at 355 nm and emission at 440 nm. DEA buffer (100 mM
209 diethanolamine, 50 mM KCl, 1 mM $MgCl_2$, pH 9.6) was used to prepare the 4-MUP
210 solution. Finally, 5 μL of substrate solution (2.5mM 4-MUP in DEA buffer, pH 9.6)
211 were injected into the carrier stream at a flow rate of 2 μL min^{-1}, and the enzymatic
212 product was measured by LIF.
213
214 **3. Results and discussion**
215 *3.1. Immunosensor optimization*
216 Systematic studies of relevant parameters that affect the performance of
217 immunological reactions were performed.
218
219 *3.2. Concentration of immobilized H. Pylori antigen*
220 One of the most important parameter was the antigen concentration employed to
221 the immobilization procedure. The amount of antigen is an important factor, due to it
222 affects the sensitivity of the immunoassay. For this study, the Horseradish peroxidase

(HRP) saturation method was used. The optimum value of *H. pylori* antigen was 100 μg mL^{-1}.

3.3. Flow rate and sample volume

Other factor studied was the flow rate, which affects the dispersion of the analytes and the yield of the immunological reaction [34]. Pressure-driven flow is one the most widely used mode of fluid delivery for microfluidic immunosensor. In our case the immunoreactants were injected into the microfluidic sensor with a syringe pump system. This parameter was optimized by studying the relative fluorescence obtained for a standard of 100 UR mL^{-1} at different flow rates between 1-15 μL min^{-1} (Fig. 3). Flow rates from 1 to 3 μL min^{-1} had a little effect over signals obtained, when the flow rate exceeded 4 μL min^{-1}, the relative fluorescence was reduced. Then, the flow rate used was 2 μL min^{-1}.

Another optimized parameter was the sample size, which was analyzed using a standard of 100 UR mL^{-1}, in a range of 1–20 μL. The relative fluorescence increased linearly when the sample size rises from 1 to 10 μL. Insignificant differences were observed when sample size was greater than 10 μL (Fig. 4). Then, a sample size of 10 μL was used.

3.4. Optimum pH values

The influence of pH on the antigen–antibody binding, the immunosensor regeneration and the enzymatic response were carried out varying the pH value of: carrier solution, desorption solution, and substrate solution within a range of 6.5–7.5, 2.0–3.0 and 8–10 respectively. The optimum pH range of antigen–antibody binding was

247 7.0–7.4 in PBS solution, the optimum pH value for desorption solution was 2.0 and the
248 rate of enzymatic response showed a maximum value of activity at pH 9.6.
249
250 *3.5. Substrate concentration*
251 An evaluation of the effect of 4-MUP concentration over the enzymatic response
252 was carried out in a range of 0.1–5.0 mM. The optimal 4-MUP concentration found was
253 2.5mM. This concentration was then used.
254
255 *3.6 Analytical performance*
256 The proposed method was applied in the quantification of anti-*H. pylori* IgG
257 antibodies concentration in 22 serum human samples under the conditions described
258 above, and the relative fluorescence response of the enzymatic product is proportional
259 to the concentration of anti-*H. pylori* specific IgG antibodies in serum samples of
260 infected patients.
261 A linear calibration curve to predict the concentration of *H. pylori*-specific IgG
262 antibodies in serum was produced within the range of 0–100 UmL^{-1}. The linear
263 regression equation was $RFU=2.834+5.263*C_{H.\ pylori}$, with the linear regression
264 coefficient r = 0.998. The coefficient of variation (CV) for the determination of 20
265 UmL^{-1}*H.pylori*-specific antibody was below 3.2% (six replicates).
266 The ELISA procedure was also carried out following the manufacturer's
267 protocol [32], absorbance changes were plotted against the corresponding *H. pylori*-
268 specific IgG antibody concentration and a calibration curve was constructed. The linear
269 regression equation was $A = 0.137 + 0.029*C$ *H. pylori*, with the linear relation
270 coefficient r= 0.995, the CV for the determination of 20 UmL^{-1}*H. pylori* specific
271 antibodies was 4.7% (six replicates).

The detection limit (DL) was considered to be the concentration that gives a signal three times the standard deviation (SD) of the blank. For LIF detection and EIA procedure the DLs were 0.17 and 2.1 UmL^{-1}, respectively. This result shows that LIF detection was more sensitive than spectrophotometric method.

The precision of the proposed method was carried out with control serum at 20, 50 and 100UmL^{-1}*H. pylori* specific antibody concentrations. The within-assay precision was tested with five measurements in the same run for each serum. These series of analyses were repeated for three consecutive days in order to estimate the between-assay precision. The obtained results are shown in Table 2. The *H. pylori* assay showed good precision; the CV within-assay values were below 3.2% and the between-assay values were below 5%. The accuracy was tested with a dilution test. It was performed with 100 UmL^{-1}*H. pylori* specific antibodies control sera (Fig. 5). The life-time of the immunosensor was about 30 continuous working days, and it allowed a sample speed about 2 samples h^{-1}.

The microfluidic system with LIF detection was compared with a commercial spectrophotometric system for the quantification of *H. pylori* specific IgG antibody in serum samples. The slopes obtained were reasonably close to 1, indicating high correspondence between the two methods (Fig. 6).

Our group had previously developed bioanalytical sensors for the electrochemical determination of H. pylori specific IgG antibodies in human serum samples [35-38], in comparison, the new microfluidic device was based on LIF detection system with incorporated optics fibers. Moreover, the achieved DL and the employed sample volume (10 μL) were lower than that obtained by the sensors previously reported [35-38]. In addition, no clean-up of the detection system was needed between analyses in comparison to electrochemical detectors, which make an

297 improvement in time consuming analysis, reproducibility and lifetime of the
298 immunosensor. The advantages above mentioned for our system, combined with its
299 portability and adaptability make our system an attractive and adequate tool to
300 accomplish automated analysis in health references centers.
301
302 **4. Conclusions**
303 This article describes the design, development, and application of a microfluidic
304 immunosensor coupled to LIF detection for the quantitative determination of specific
305 IgG antibodies present in serum samples of patients with *H. pylori* infection.
306 Our developed system combines: the high sensitivity of LIF detection and the
307 inherent properties of optical fibers, such as the chemical inertness of the surface, high
308 transmission, flexibility and low cost, with microfluidic technology features as
309 reduction in liquid volumes required for sampling, fluid transfer, and washing of sensor
310 surfaces, which translates into a more rapid manipulations, lower power requirements,
311 and increased portability of the device.
312 This biosensor shows major advantages over previously developed bioanalytical
313 sensors for the determination of *H. pylori* specific IgG antibodies in serum samples,
314 namely better sensitivity provided reaction-detection integration, which is faster,
315 required minimal sample handling and no clean-up of the detection system was required
316 or needed.
317 The obtained results employing our system showed that it has significant
318 potential for performing bioanalytical analysis aimed at the determination of low
319 concentrations of several analytes in relevant fields.
320
321

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Figure captions

Figure 1. a) Schematic representation of the optical detection system. DPSS laser light was focused with a lens (1) onto the detection channel (2), which was held in place with a Plexiglas holder (3). Fluorescence emission (4) was collected with a microscope objective (5), focused onto fiber optic collection bundle (6) and then detected with a QE65000-FL Scientific-grade Spectrometer. ***b) Schematic representation of the microfluidic immunosensor***. Central channel. (CC), controlled pore glass (CPG), Detection area (DA). All measurements are given in millimeters.

Figure 2. Schematic representation of the immunological reaction

Figure 3. Effect of flow rate analyzing a 100 U mL^{-1} *H. pylori*-specific antibodies standard at different flow rates. 2.5 mM of 4-MUP in DEA buffer pH 9.6 were injected into the carrier stream at different flow rates, and the enzymatic product was measured by LIF using excitation wavelength 355 nm and emission wavelength 440 nm

Figure 4. Effect of sample size for a standard of 100 U mL^{-1} *H.pylori*-specific antibodies. 2.5mM of 4-MUP in DEA buffer, pH 9.6 were injected into the carrier stream at a flow rate of 2 μL min^{-1}, and the enzymatic product was measured by LIF using excitation wavelength 355 nm and emission wavelength 440 nm. Each value of current is based on five determinations.

Figure 5. Dilution test results for 100 U mL^{-1} *H. pylori*-specific antibodies. 2.5mM of 4-MUP in DEA buffer, pH 9.6 were injected into the carrier stream at a flow rate of 2

494 μL min^{-1}, and the enzymatic product was measured by LIF using excitation wavelength
495 355 nm and emission wavelength 440 nm. Each value of current is based on five
496 determinations. Each value of RFU is based on five determinations.
497
498 ***Figure 6.*** Correlation between proposed method and commercial photometric assays.
499
500

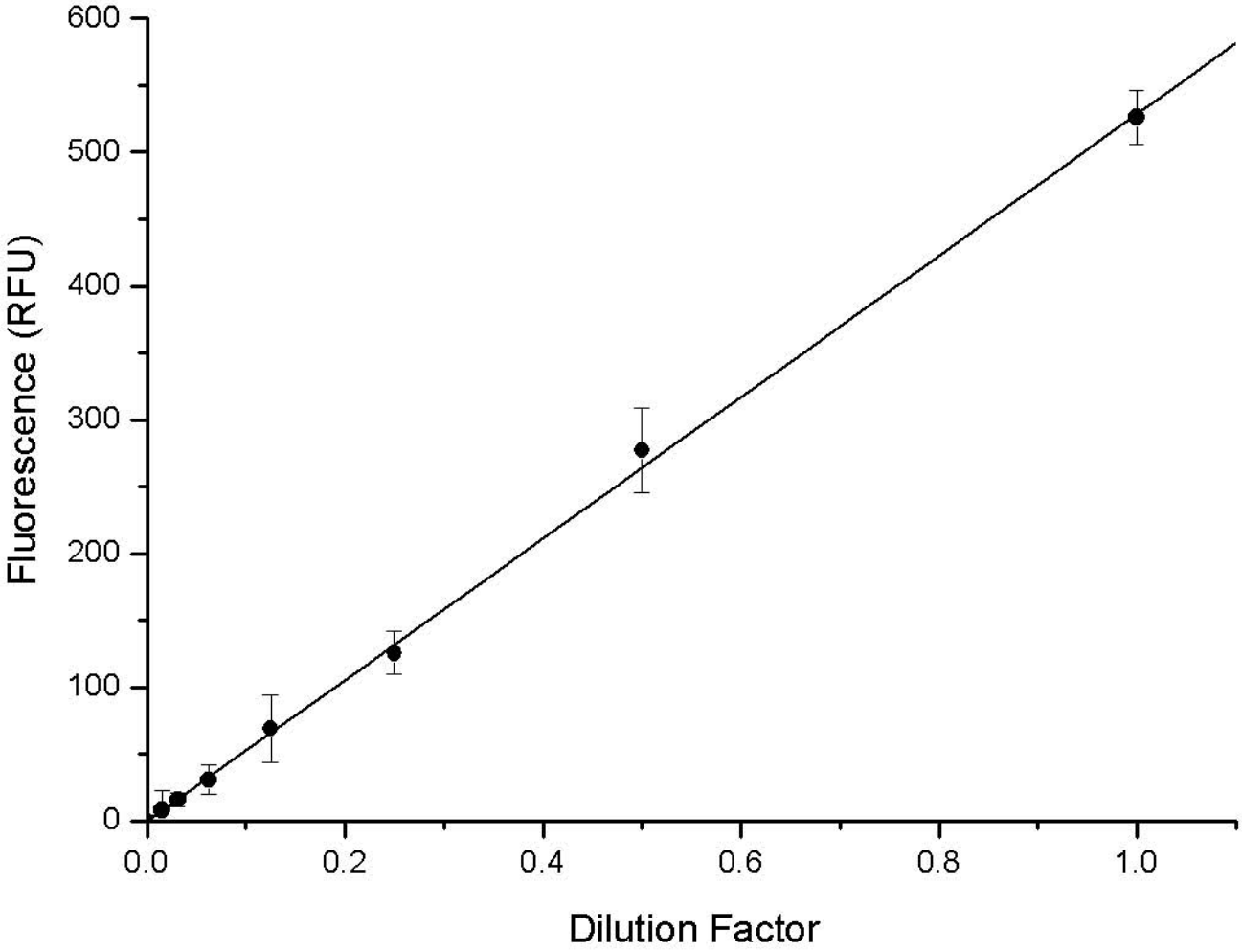
Table 1. Summary of optimum conditions for IgG anti-*H. pylori* antibodies fluoroimmunosensor.

Sequence	**Condition**	**Time**
Blocking solution	3% skim milk (PBS, pH 7.2) 2.0 μL min^{-1}	5 min
Washing buffer	Flow rate: 2.0 μL min^{-1} (PBS, pH 7.2)	2 min
Serum samples	diluted 100-fold 2.0 μL min^{-1}	5 min
Washing buffer	Flow rate: 2.0 μL min^{-1} (PBS, pH 7.2)	4 min
Enzyme conjugated	AP-conjugated (dilution of 1/2000) 2.0 μL min^{-1}	5 min
Washing buffer	Flow rate: 2.0 μL min^{-1} (PBS, pH 7.2)	4 min
Sustrate	2.5mM 4-MUP in a DEA buffer, pH 9.6	1 min
LIF detection	Excitation wavelength: 355 nm Emission wavelength: 440 nm.	2 min

Table 2. Within-assay precision (five measurements in the same run for each control serum) and between-assay precision (five measurements for each control serum, repeated for three consecutive days).

[a] Control sera	Within-assay		Between-assay	
	Mean	**CV %**	**Mean**	**CV %**
20 U mL^{-1}	20.7	3.7	21.0	3.2
50 U mL^{-1}	49.7	1.5	49.5	4.3
100 U mL^{-1}	101.3	2.9	98.8	3.7

[a] U mL^{-1} *H. pylori* specific antibodies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500
400
300
200
100
0
Fluorescence (RFU)
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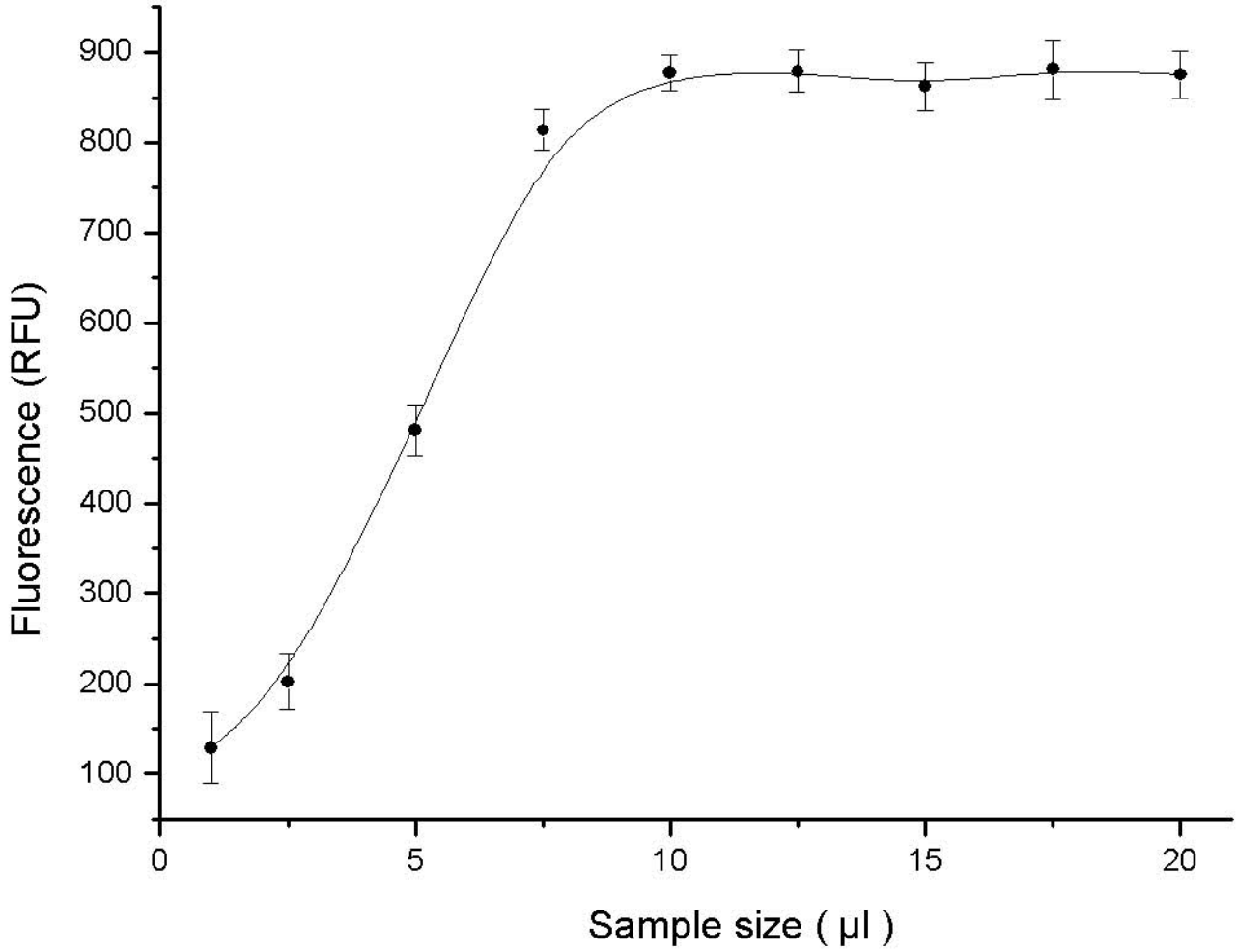
0.2
0.4
0.6
0.8
1.0
Dilution Factor


Fluorescence (RFU)
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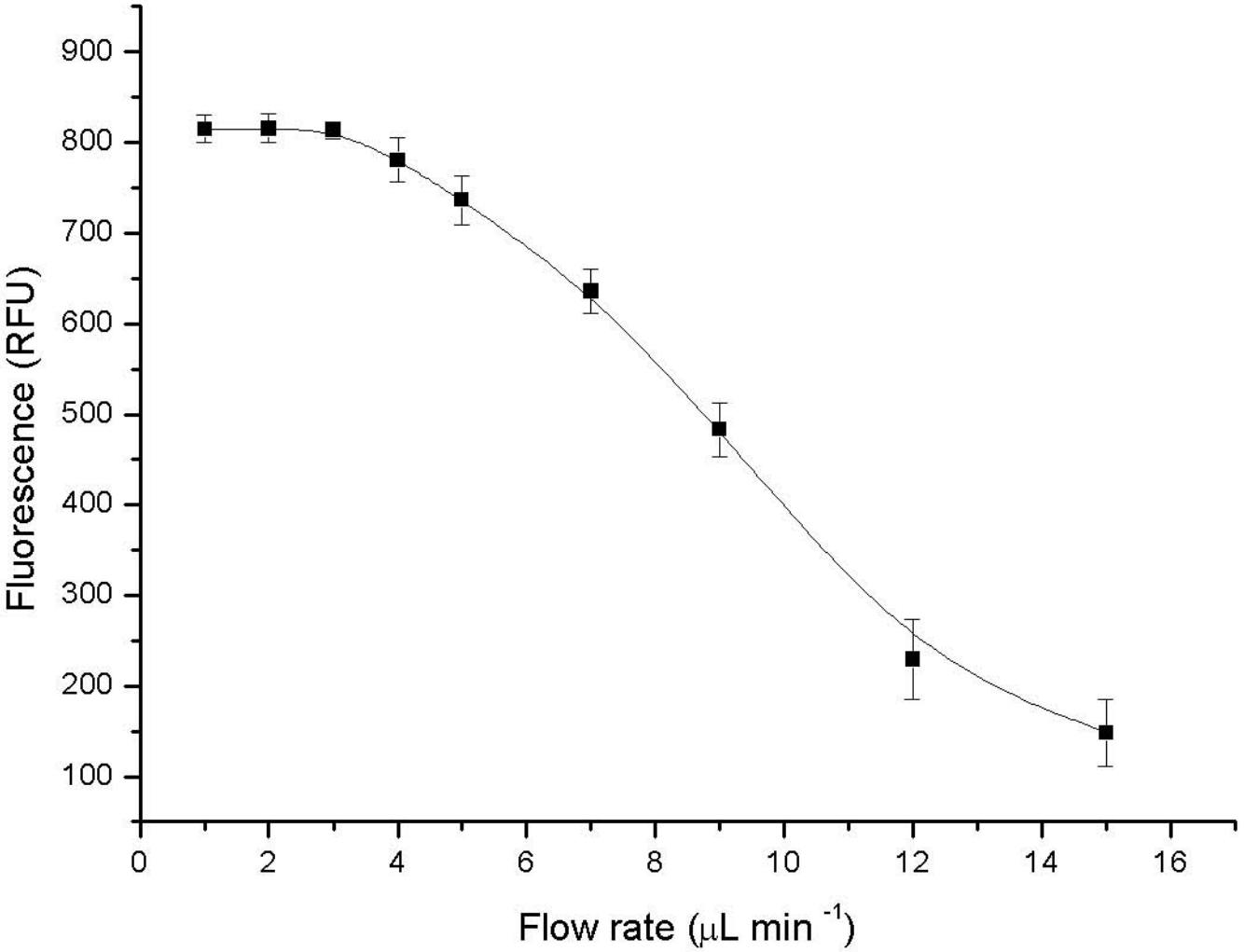
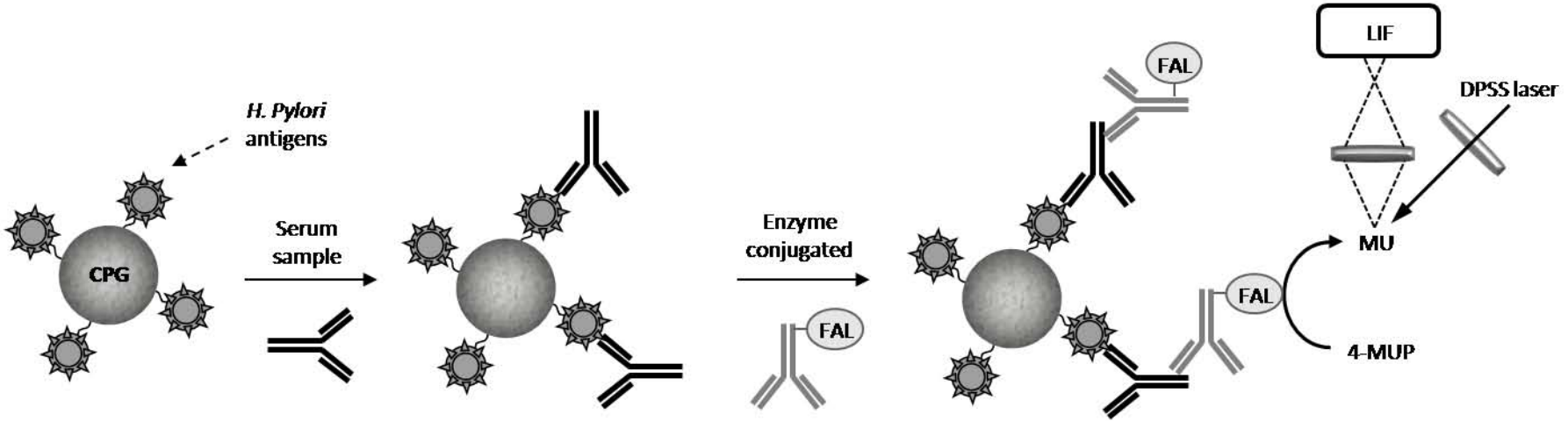
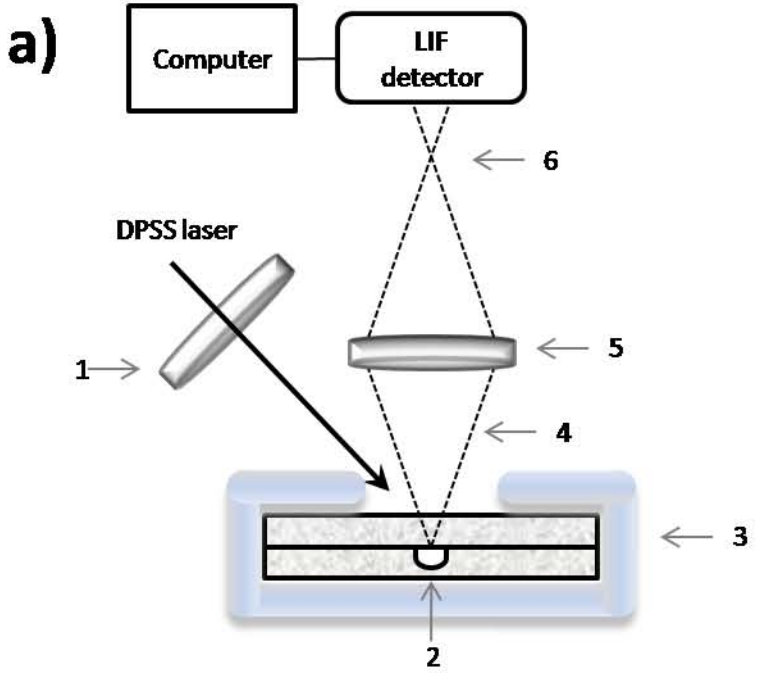
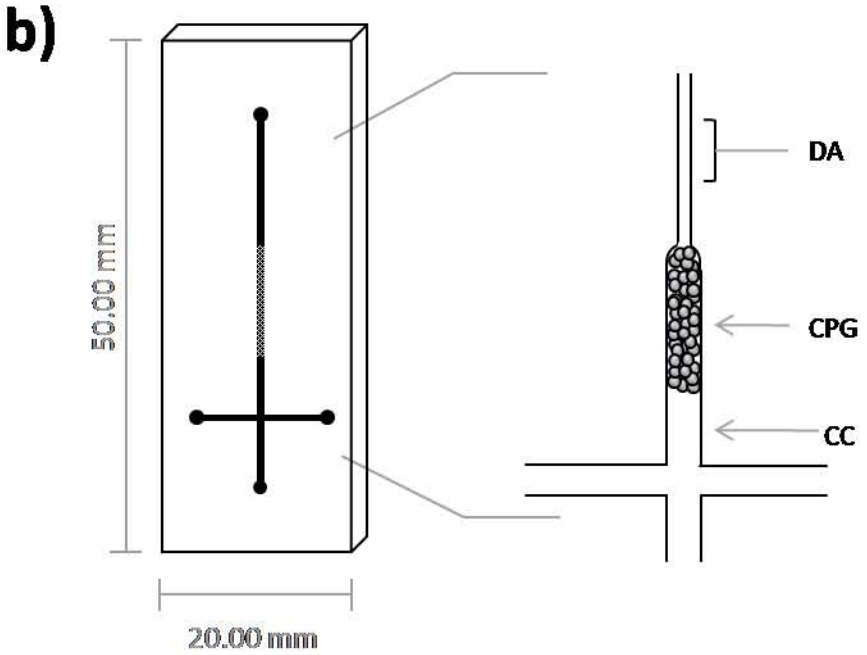
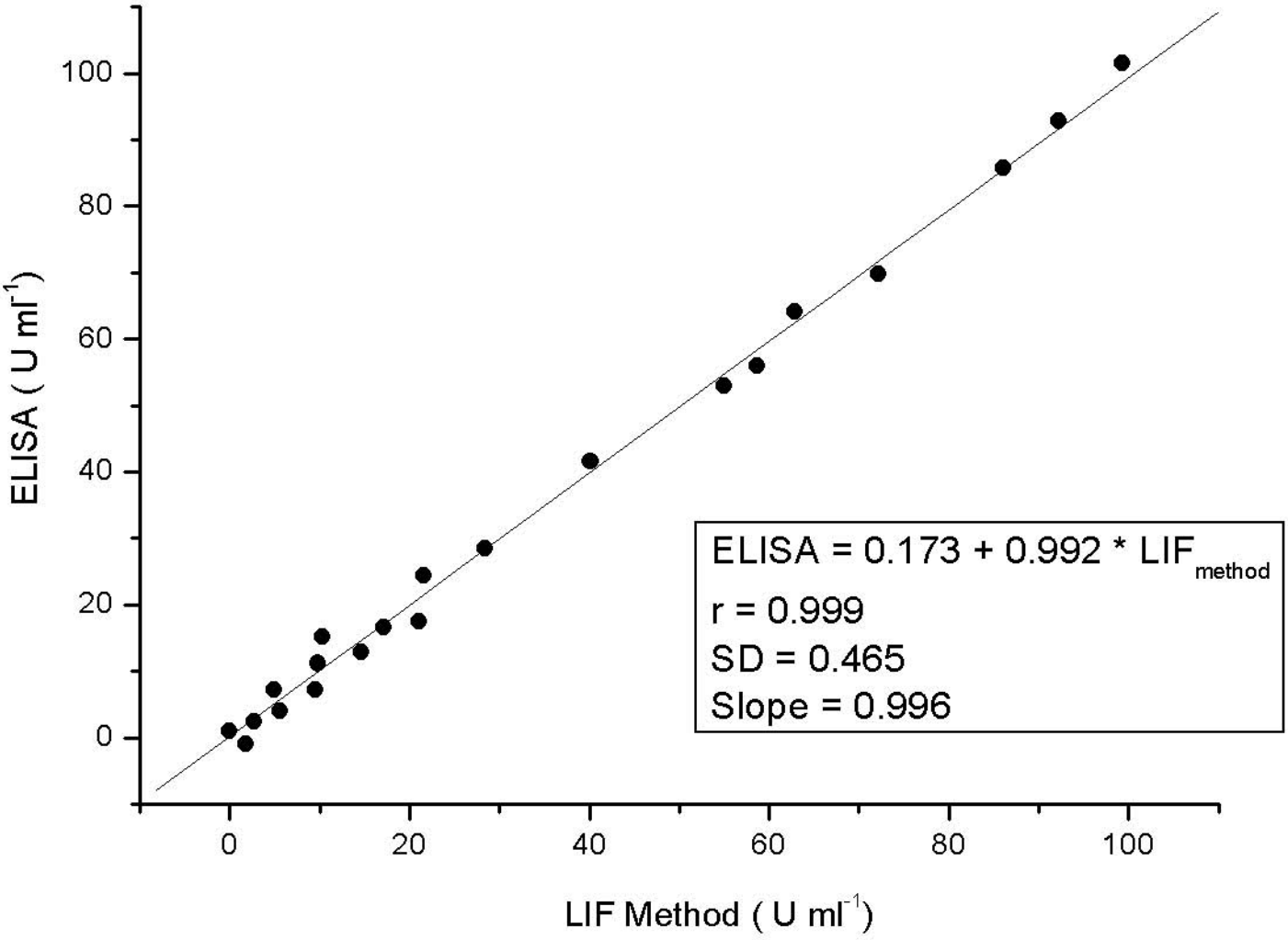
800
700
600
500
400
300
200
100
0
5
10
15
20
Sample size (µl)


Fluorescence (RFU)
900
800
700
600
500
400
300
200
100
0
2
4
6
8
10
12
14
16
Flow rate (μL min -1)


H. Pylori
antigens
CPG
Serum
sample
Enzyme
conjugated
FAL
FAL
FAL
LIF
DPSS laser
MU
4-MUP


a)
Computer
LIF detector
6
DPSS laser
1
5
4
3
2


b)
50.00 mm
20.00 mm
DA
CPG
CC


ELISA (U ml-1)
LIF Method (U ml-1)
0
20
40
60
80
100
ELISA = 0.173 + 0.992 * LIF method
r = 0.999
SD = 0.465
Slope = 0.996