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Analytical Biochemistry 322 (2003) 148–155
ANALYTICAL BIOCHEMISTRY
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A combined oligonucleotide and protein microarray for the codetection of nucleic acids and antibodies associated with human immunodeficiency virus, hepatitis B virus, and hepatitis C virus infections

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Received 4 April 2003

Abstract

A multiplexed assay based on the codetection of nucleic acids and antibodies in human serum infected by human immunodeficiency virus (HIV), hepatitis B virus (HBV) or hepatitis C virus was proposed. The combined immuno- and oligosorbent array (CombOLISA) microarray is prepared in 96-well standard microplates by spotting (1) nucleic probes specific for a virus genome, (2) viral proteins for the capture of serum antibodies, and (3) nonspecific proteins for verifying specificity. Experimental assay conditions were optimized so that both DNA hybridization and immunological reactions can be achieved simultaneously in the same well and buffer and all at the same temperature. A generic detection system based on the precipitation of an insoluble colorimetric substrate in the presence of enzyme-labeled antibodies or streptavidin was proposed. The optical density of each spot was correlated to the corresponding analyte concentration. The influence of critical parameters on CombOLISA performance such as serum concentration was studied. Calibration curves and sensitivity thresholds were established for each parameter. Serial dilutions of serum were correlated to results obtained with validated immunoassay platforms such as a microplate enzyme-linked immunosorbent assay or the VIDAS automat. Also, several HIV- and HBV-infected serum samples were tested independently by CombOLISA and VIDAS. Coefficients of variation for genomic and proteomic parameters vs spot density were below 15%.

Keywords: Hybridization; DNA; Microtiterplate well; Densitometry; Enzyme substrate; Alkaline phosphatase; Immunoassays; ELISA; Complexity; Multidetection

Coinfections by hepatitis B (HBV)[1] and C (HCV) viruses are frequent in seropositive patients infected with human immunodeficiency virus (HIV) since the same routes of transmission are shared by these viruses (drug abusers, blood transfusion, etc.) [1]. Diagnosis and therapy follow-up of such associated diseases are possible by the combination of several individual assays for testing pertinent parameters.

The immune response to HIV type 1 (HIV-1) is oriented mainly against *gag* and *env* glycoproteins, but a period of about 3 weeks is observed between contamination and appearance of anti-HIV antibodies. During this period, p24 protein is present in the serum of most patients. The recent emergence of combined assays for the codetection of p24 antigenemia and anti-HIV antibody titer—e.g., HIV Duo assay (bioMérieux)—allows

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[1] *Abbreviations used:* HBV, hepatitis B virus, HCV, hepatitis C virus; HIV, human immunodeficiency virus; COMBOLISA, combined immuno- and oligosorbent array; ELISA, enzyme-linked immunosorbent assay; AP-GαH IgG, alkaline phosphatase-labeled goat anti-human IgG; AP-SA, alkaline phosphatase- labeled streptavidin; NSP, nonspecific proteins; PBS, phosphate-buffered saline; PBS-T, PBS-Tween 20; PEG, polyethylene glycol; HS, horse serum; SPR, solid-phase receptacles.

0003-2697/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2003.08.002

reducing the delay between contamination and diagnosis [2]. Quantification of HIV-1 genome is achieved by molecular techniques, which take on more importance since they are extremely sensitive [3], viral load RNA being predictive of CD4 decline, acquired immune deficiency syndrome progression, and patient survival [4].

In the case of HBV infection, the presence of plasma hepatitis B surface antigen (HBs-Ag) indicates an active HBV infection [5]. Furthermore, testing HBV DNA levels during therapy may allow early recognition of patients who do not respond to therapy [3], as both the DNA and the protein are often associated for HBV follow-up [6]. On the other hand, appearance of anti-HBs antibodies is an indicator of patient recovery.

Detection of HCV infection by α HCV positivity has been facilitated by the development of antibody assays [7]. However, these methods are of restricted use due to the period of several weeks between infection and seroconversion [8]. Alternatively, amplification of viral nucleic acid is an effective means for direct HCV quantification [9].

Many commercial tests currently available permit the detection of each of these parameters in separate assays. Emerging protein microarray technology enabling one to set up more complex systems such as antigen microarrays for serodiagnosis of several infectious diseases [10] has been proposed. Other generic array formats designed for the detection of a wider range of infectious or toxic substances have been proposed, notably by Lee et al. [11] or Yang et al. [12]. These chips could be used indiscriminately for either immunoassays or DNA hybridization. Multiplexed assays based on tagged microspheres are also well adapted for versatile applications targeting proteomics or genomics [13,14]. But to our knowledge, no description of a technique allowing the simultaneous, real-time codetection of immunological and DNA hybridization reactions has been made in the literature.

Our proposal in this work is a microarray based on a standard 96-well microplate format for which the potential as a protein microarray has already been demonstrated [15]. Each well is functionalized by 16 spots comprising nucleic acids and viral proteins, each of these probes allowing the detection of a parameter relevant for the diagnosis or follow-up of three frequently associated viral infections (HIV, HBV, HCV). Immunological models are chosen so that a systematic comparison is possible between CombOLISA and validated immunoassay platforms such as ELISA in microtiter plates or the VIDAS automat.

Materials and methods

Nucleic acid probe and DNA targets

HIV

HIV-1 RNA was bought from Ambion (Austin, TX, USA). Biotinylated primers for amplification (SK431: TGCTATGTCAGTTCCCCTTGGTTCTCT and SK462: AGTTGGAGGACATCAAGCAGCCA TGCAAAT) [15] and 5′-aminated probe for amplified products' capture (C_{HIV}: GAGACCATCAATGAGGA AGCTGCAGAATGGGAT) [16] were synthetized by Eurogentec (Seraing, Belgium) as were all other oligonucleotides.

HCV

RNA targets from HCV were extracted from serum of chronically infected patients using Nucleospin RNA Virus Kit (Macherey-Nagel, Hoerdt, France) and amplified by RT-PCR with 5′-biotinylated primers (RC21: CTCCCGGGGCACTCGCAAGC and RC1: GTGTA GCCATGGCGTTAGTA) [17]. The 5′-aminated probe C_{HCV} (CATAGTGGTCTGCGGAACCGGTGAGT) [18] was designed to capture biotinylated amplified products.

HIV and HCV targets were amplified by RT-PCR under the following conditions using an Access kit from Promega (Madison, WI, USA): 1× AMV/Tfl reaction buffer, 1.8 mM $MgSO_4$, 0.2 mM dNTP, 1 μM primers, 1 U of AMV reverse transcriptase, and 5 U of Tfl DNA polymerase; RT cycle 48 °C for 45 min; 35 PCR cycles (94 °C for 30 s 60 °C for 1 min, 68 °C for 2 min); final extension at 68 °C for 7 min. PCR templates were analyzed on agarose gels stained with ethidium bromide and revealed under UV illumination. Concentrations of amplified products were evaluated by comparison to band density of a mass ladder (Eurogentec). HIV and HCV amplicons were 46 and 23 nM, respectively.

HBV

A synthetic single-stranded nucleic target (74 bp) (CCCAGTAAAGTTCCCCACCTTATGAGTCCAAG GAATTACTAACATTGAGATTCCCGAGATTGAG ATCTTCTGCGA) from the HBV genome [19], a 5′-aminated capture probe for target hybridization (C_{HBV}: ATCTCGGGAATCTCAATGTTAG), and a 5′-biotinylated detection probe that also hybridizes to the synthetic target (D_{HBV}: TATTCCGACTCATAAGGTG) were synthetized.

Immunoassay

Recombinant HCV core protein, whose synthesis is described elsewhere [20], and HIV envelope glycoprotein GP160 were obtained from bioMerieux. HBs antigens were obtained from Hytest (Turku, Finland) for the Ay subtype and from Cliniqa (Fallbrook, CA, USA) for the Ad subtype. GP160 and HBs antigens were the same as those used for adsorption on receptacles of the VIDAS instrument in the HIV Duo kit and in the Anti-HBs Total kit, respectively. Two proteins (NSP_1, NSP_2) having no affinity in the present study were also spotted to verify immunological reaction specificity. Infected human sera were kindly provided by the Croix-Rousse

Hospital (Lyon, France). Alkaline phosphatase-labeled goat anti-human IgG (AP-GαH IgG) was from Jackson Immunoresearch (West Grove, PA, USA) and alkaline phosphatase-labeled streptavidin (AP-SA) was from Sigma (St. Quentin, France).

Microarray setup

Capture probes C_{HIV}, C_{HBV}, and C_{HCV} were diluted at 10 μM in a coating buffer (150 mM Na_2HPO_4/NaH_2PO_4, 450 mM NaCl, 1 mM EDTA, pH 7.4). Nonspecific proteins (NSP_1, bovine serum albumin; NSP_2, human chorionic gonadotropin) were diluted at 50 μg/ml in 50 mM carbonate buffer, pH 9.3. GP160, HBs antigens, and HCV core proteins were diluted at 10 μg/ml in phosphate-buffered saline (PBS; 50 mM Na_2HPO_4/NaH_2PO_4, 150 mM NaCl, pH 7.4). Spotting was carried out with the Biochip Arrayer (Perkin–Elmer, Boston, MA, USA), which is based on a submicroliter noncontact, drop-on-demand piezoelectric dispensing technology providing a typical spot diameter of 250 μm. Each probe was deposited in duplicate spots in a circular format in white, high binding 8-well modules or in full 96-well microplates (Fig. 1A). After probe spotting and drying under controlled pressure and temperature conditions, plates were rinsed with PBS–Tween 20, 0.05% (PBS-T).Then 30 μl of a preservative solution containing 1% bovine serum albumin and 5% saccharose was incubated for 1 h in each well. Liquid was discarded and microarrays were dried under vacuum and stored.

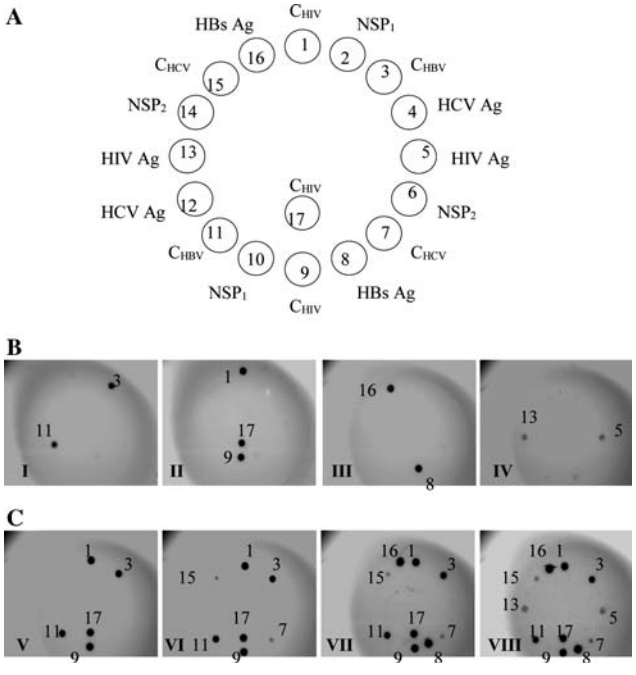


Fig. 1. (A) Map of the CombOLISA microarray (duplicated spots). C_{HIV} [1,9,17], C_{HBV} [3,11], and C_{HCV} [7,15]: nucleic acids for viral DNA hybridization. HIV Ag [5,13], HBs Ag [8,16], and HCV Ag [4,12]: viral protein for serum assay. NSP_1 [2,10] and NSP_2 [6,14]: nonrelevant proteins for array specificity evaluation. (B) CombOLISA array at the issue of various assays, analytes being added independently. I: HBV DNA. II: HIV DNA. III: HBV serum. IV: HIV serum. (C) Analytes added consecutively in the same well. V: HBV and HIV DNAs. VI: HBV, HIV, and HCV DNAs. VII: HBV, HIV,and HCV DNAs and HBV serum. VIII: HBV, HIV, and HCV DNAs and HBVand HIV sera.

DNA and serum assay on CombOLISA

The three types of assays carried out on CombOLISA array are summarized in Fig. 2.

Capture

Each analyte, either separately or combined in pairs or in a polyanalyte grouping, was diluted in 30 μl of reaction buffer (0.1 M Na_2HPO_4/NaH_2PO_4, (1) 0.5 M NaCl, 0.6% Tween 20, 2% PEG 4000, pH 7): HIV or HCV biotinylated amplified products (5% v/v) were previously denatured with 0.2 N NaOH for 5 min and/or (2) 1 nM synthetic HBV DNA (3) and/or HIV-, HBV-, or HCV-infected human sera (0.3% v/v).

The mixture was incubated in the well spotted with the CombOLISA array for 1 h at 37 °C. The well was rinsed twice with PBS-T.

Detection

The array was incubated for 30 min at 37 °C with 30 μl of 0.2 μM of D_{HBV} diluted in the reaction buffer, rinsed, and then exposed for 30 min at 37 °C to a solution comprising a mixture of AP-SA at 0.5 μg/ml (nucleic acids detection) and AP-GαH IgG at 1 μg/ml (human antibodies detection) diluted in the reaction buffer. After extensive well rinsing with PBS-T, 30 μl of BM-purple-AP substrate (Roche, Basel, Switzerland) was added to each well, catalyzing the precipitation of an insoluble purple product in the presence of AP. The array was imaged with the automated Apimager system (Apibio, Grenoble, France). Spot density was analyzed by calculation of the mean gray level of each spot minus that of the crown surrounding the spot and correlated to nucleic acid concentration and/or serum dilution. Pixel density is scaled from 0 (white, no signal) to 65, 536 (black, saturating signal) and expressed in arbitrary units (a.u.).

Reference immunoassays

ELISA in microtiter plate

The assay of anti-HCV antibodies was carried out in a transparent 96-well microtiterplate following a standard sandwich ELISA procedure described elsewhere [21]. Briefly, 100 μl of recombinant HCV core proteins diluted at 10 μg/ml in 0.1 M carbonate buffer, pH 8.3, was coated per well during 2 h at 37 °C. Plates were rinsed with PBS-T and exposed for 1 h at 37 °C to HCV

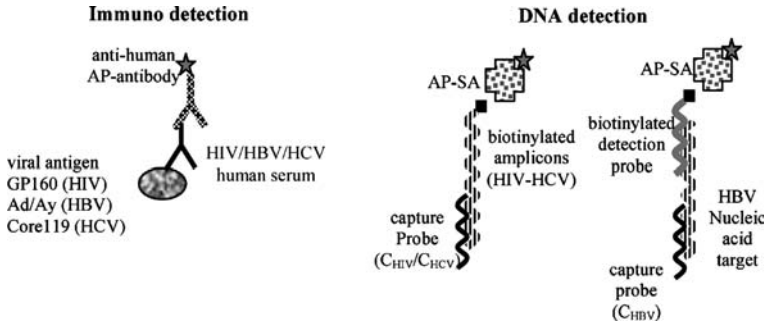


Fig. 2. Illustration of sandwich assays set up on CombOLISA for the detection of anti-viral antibodies (anti-HIV, anti-HBs, and anti-HCV) and viral nucleic acid targets (HIV, HBV, and HCV). HIV and HCV amplicons were biotinylated during amplification. HBV target was hybridized to a biotinylated detection probe. Alkaline phosphatase enzyme common to both assay formats allowed the simultaneous detection of nucleic acids and proteins.

sera diluted in PBS-T + 10% horse serum (PBS-T-HS). Finally, 100 µl of AP-GαH IgG diluted at 0.5 µg/ml in the PBS-T-HS was incubated per well for 30 min at 37 °C. After extensive washes with PBS-T, 100 µl of a 2 mg/ml solution of *p*-nitrophenyl phosphate was added to each well. After 30 min, enzymatic reaction was stopped with 1 N NaOH and optical density was read at 405 nm on a microplate spectrophotometer (µQuant, Biotek, Winooski, USA).

VIDAS

Assays were carried out on the commercial kits HIV Duo and Anti-HBs Total under conditions recommended by the manufacturer. Two hundred µl of serum pure or diluted in the appropriate buffer provided with the kit was introduced in the sample well, to be incubated on antigen- or antibody-coated solid-phase receptacles (SPR).

HIV Duo

The test combines two enzyme immunoassays (anti-HIV-1/anti-HIV-2 IgG and p24 antigens) with a final fluorescent revelation. AP-GαH IgG and a combination of biotinylated mouse anti-p24 antibodies and AP-SA are added for the detection. The introduction of the fluorescent AP substrate methyl-umbelliferyl phosphate into the SPR reveals the presence of complexes.

Anti-HBs Total

Serum antibodies bind with the SPR-coated antigens and are revealed by a mixture of biotinylated Ad and Ay antigens mixture and by SA-AP. Fluorescent detection occurs as above.

Results and discussion

Reaction buffer optimization

Several buffers, all suitable for hybridization, of various ionic strengths (0.4–1 M), salt nature, and pH [7,8] were tested, and density obtained in each case on antigen spots was estimated (Table 1). Buffers A (Tris–HCl/NaCl), B (Tris–HCl/EDTA/NaCl), and C (NaH_2PO_4, EDTA, NaCl) give very weak signals. Buffer D (Hepes/LiCl) is slightly better performing. The buffer that gives the best results is the selected reaction buffer (Na_2HPO_4/NaH_2PO_4/NaCl). The ionic strength is an important parameter. It must be high enough, above 0.5 M, for double-stranded DNA stabilization [23]. On the other hand, salt molarity should be less than 0.6 M to allow the formation of immune complexes [24]. Thus, signals with buffers B and C are affected by the excessive salt content. Furthermore, salt nature influences immune reaction yields. For example, buffers A and D having almost the same pH (≈7) and ionic strength (≈0.6 M) as the reaction buffer, show significantly lower signals, especially for protein spot density (−30 to −40%), but also at a lower extent for nucleic probes spots (−14%). Finally, the reaction temperature (37 °C) is chosen so that proteins are protected from heat denaturation and

Table 1
Salt composition, ionic strength, and pH of buffers tested on CombOLISA format and comparison of intensities obtained on protein spots

Buffer	Composition	pH	Protein spot density (% of reaction buffer signal)
Buffer A	Tris–HCl 10 mM, NaCl 0.5 M, Triton X-100 0.05%	7.4	<10%
Buffer B	Tris–HCl 10 mM, EDTA 1 mM, NaCl 1 M, Triton X-100 0.05%	8.0	<10%
Buffer C	NaH_2PO_4 60 mM, EDTA 6 mM, NaCl 0.9 M, Triton X-100 0.05%	7.4	<10%
Buffer D	Hepes 160 mM, LiCl 0.5 M, Tween 20 0.05%	7.5	60–70%
Reaction buffer	Na_2HPO_4/NaH_2PO_4 0.1 M, NaCl 0.5 M, Tween 20 0.65%, PEG 4000 2%	7.0	100%

the specificity of double-stranded DNA is ensured. Importantly, incubation temperatures are well below melting temperatures of nucleic capture probes, 59.5 °C for C_{HIV}, 49.3 °C for C_{HBV}, and 58.1 °C for C_{HCV}.

Specificity

Using CombOLISA arrays (Fig. 1A) a selection of analytes was assayed, separately or simultaneously: HIV, HBV, and HCV nucleic targets and HIV- and HBV-infected human sera. Arrays obtained are reported in Fig. 1B (individual assays) and Fig. 1C (combined assays). All spots of the arrays, visible or not after the enzyme reaction, are quantified with regard to relative pixel density (Table 2). Density below 1200 a.u., which corresponds to the minimal significant density, is three times the standard deviation on 10 background spots. Detected spots are relevant to the nature of incubated analytes. One single type of probe is detected in Fig. 1B when a single analyte was assayed per array: C_{HBV} (spots 3, 11) in image I (array incubated with 1 nM HBV DNA), C_{HIV} (spots 1, 9, 17) in image II (5% HIV RT-PCR product), HBs Ag (spots 8, 16) in image III (0.3% HBV serum) and HIV Ag (spots 5, 13) in image IV (0.3% HIV serum). Array complexity increases in Fig. 1C when analytes are assayed simultaneously. *Two components* , C_{HIV} and C_{HBV} in image V (array incubated with HIV and HBV DNAs); *three components* , C_{HIV}, C_{HBV}, and C_{HCV} in image VI (HIV, HBV, and HCV DNAs); *four components*, C_{HIV}, C_{HBV}, C_{HCV}, and HBs Ag in image VII (HIV, HBV, and HCV DNAs and HBV serum). *five components*, C_{HIV}, C_{HBV}, C_{HCV}, HBs, and HIV Ag in image VIII (HIV, HBV, and HCV DNAs and HBV and HIV sera). Thus, both DNA hybridization and immunological reactions are achievable under identical conditions, notably in the same buffer and at the same temperature. Nonspecific protein spots are not detected, demonstrating reaction specificity. Spots are homogeneous and no ring effect appears, contrary to that sometimes observed on antigen microarrays [10,21,22].

Hybridization reactions are slightly affected by the presence of human serum due to high DNA concentration. Signals obtained on C_{HBV} for a target concentration of 1 nM are reported as a function of noninfectious human serum concentration (Fig. 3). A pixel density decrease of less than 7% is observed for serum concentration variations from 0.003 to 50%. The presence of 0.6% detergent in the reaction buffer avoids many nonspecific interactions between immobilized oligonucleotides and serum proteins. On the other hand, high human serum concentration significantly influences the relative pixel density of protein spots. The relative signal of HBs antigen spots decreases for serum concentration above 1% because of nonspecific adsorption of human protein onto recombinant protein spots. Thus, experiments were generally carried out with less than 1% serum to avoid such undesired interactions.

Quantitative aspects and sensitivity

Sensitivity of DNA assay

Serial dilutions of HIV, HBV, and HCV nucleic acid targets (from 0.01 to 5 nM) were prepared and assayed simultaneously on the microarray for the evaluation of CombOLISA as a quantitative tool (Fig. 4). For all analytes, pixel density increases linearly in a semi-log scale as a function of DNA concentration, showing that enzyme-related detection with colorimetric-insoluble substrate is relevant for reaction follow-up and quantification.

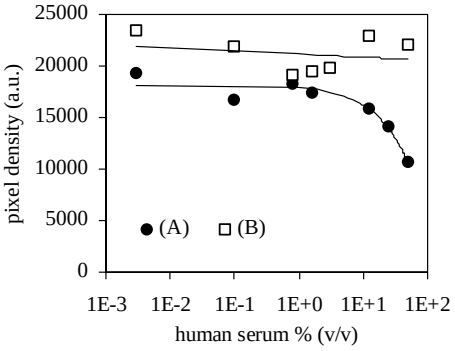


Fig. 3. Influence of human serum concentration on spot density of HBs antigen (A) and C_{HBV} (B).

Table 2
Pixel density measured on arrays in Fig. 1

Image spots	I	II	III	IV	V	VI	VII	VIII
1-9-17 C_{HIV}	<1200	**38144**	<1200	<1200	**49470**	**48832**	**42712**	**40290**
2-10 NSP_1	<1200	<1200	<1200	<1200	<1200	<1200	<1200	<1200
3-11 C_{HBV}	**34935**	2167	<1200	<1200	**43605**	**43350**	**36465**	**34807**
4-12 HCV core	<1200	<1200	<1200	<1200	<1200	<1200	<1200	<1200
5-13 HIV Ag	<1200	<1200	<1200	**12480**	<1200	<1200	<1200	**12240**
6-14 NSP_2	<1200	<1200	<1200	<1200	<1200	<1200	<1200	<1200
7-15 C_{HCV}	1530	<1200	<1200	<1200	<1200	**14917**	**11857**	**11730**
8-16 HBs Ag	<1200	<1200	**33664**	<1200	<1200	<1200	**45772**	**42840**

Density below 1200 is significant. Boldfaced numbers refer to specific spot density.

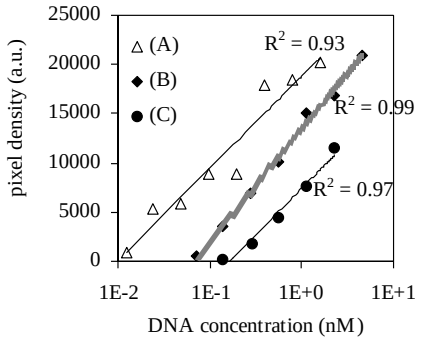


Fig. 4. Calibration curves for HBV (A), HIV (B), and HCV (C) nucleic acid targets assays carried out on CombOLISA. Pixel density measured on concerned spots is reported as a function of DNA concentration.

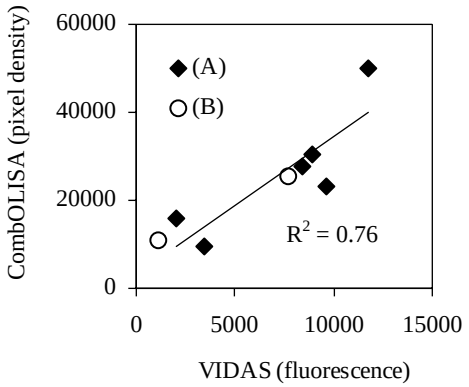


Fig. 5. Correlation graph of anti-HBV (A) and anti-HIV (B) antibody titer in eight human sera as determined by commercialized VIDAS, Anti-HBs Total, and HIV Duo tests (x axis) and independently by CombOLISA (y axis).

Detection thresholds are estimated from initial slopes of calibration curves and their intersections with the minimal significant density (1200 a.u.). Levels of detectable DNA are directly related to molecule length. The shorter the amplicons or synthetic DNA, the lower the detection threshold. A 72-bp HBV target is detected above 18 pM while the threshold for HCV amplicons (220 bp) is more than 30 times higher (570 pM), the limit for HIV amplicons (142 bp) being intermediate (100 pM). Such results, in accordance with the literature [25], are explained by amplified hindrance generated at the liquid–surface interface by long-chain molecules. Indeed, steric factors are known to influence nucleic acid hybridization to oligonucleotide arrays [26]. CombOLISA sensitivity for DNA assays is also slightly limited by the presence of human serum protein, whose influence on hybridization reactions is more clearly evidenced at very low DNA concentrations than at a saturating concentration such as that in Fig. 3 (1 nM of HBV target). For example, the detection threshold for HIV cDNA shifts from 35 pM without human serum to 100 pM with 0.2% human serum.

Correlation between CombOLISA and validated immunoassays

Several HIV and HBV human sera were tested consecutively at the same dilution (0.3% v/v) by VIDAS and CombOLISA to evaluate the correlation of the two methods (Fig. 5). One serum was analyzed per microarray and per VIDAS test. For each serum the fluorescence signal obtained on VIDAS with HIV Duo or AntiHBs Total was plotted vs pixel density measured on the array for HIV or HBs antigens spots, respectively. Despite the limited number of available sera, a correct linear correlation is observed (regression coefficient: 0.76). Correlation of the two methods degrades for highly concentrated sera since the densitometry dynamic range of the microarray is below that of fluorescence [27]. Thus, signal saturation is reached for lower serum concentrations with CombOLISA.

The dynamic range of the assay can be significantly extended by exploiting enzyme kinetics, that is to say (1) reading the array response to a concentrated sample after a few minutes and (2) allowing spot color development progress up to several tens minutes for a very diluted serum. Also, it is clear from Figs. 1B and C that spot area is correlated to pixel density. The darker is the spot, the larger is its diameter. Plotting the product spot area × pixel density vs analyte concentration instead of pixel density vs analyte concentration induces one log enlargement of assay linearity (Fig. 6).

Sensitivity of serum titer assay

The sensitivity threshold of anti-viral antibodies assay was investigated with calibration curves derived from serial dilutions of three human sera either HIV, HBV, or HCV infected. Serum dilutions varied from 0.005 to 0.3% (v/v). All samples were assayed, consecutively, both on CombOLISA and on conventional immunoassay platform: HIV Duo for HIV sera, Anti-HBs Total for HBV sera, or in a microplate ELISA for HCV sera. Data are reported in Fig. 7. For each dilution of each serum, the pixel density measured on the

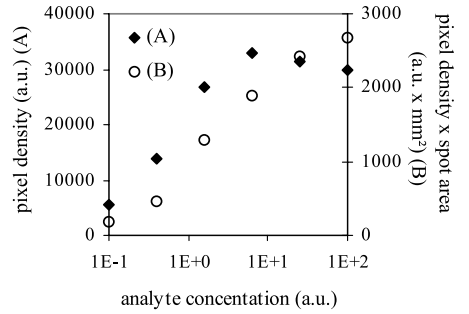
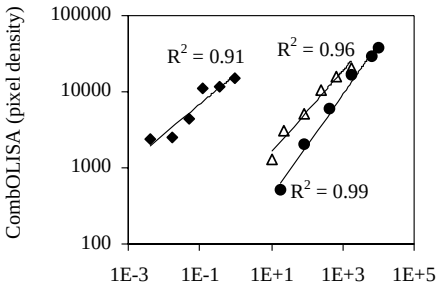


Fig. 6. Comparison of the general profile of calibration curves (pixel density vs analyte concentration) (A) and (pixel density × spot area vs analyte concentration) (B) obtained for all kind of analytes, showing the extension of the dynamic range in (B).



Detection limit (serum dilution)	◆ (A)	△ (B)	● (C)
CombOLISA	1/17000	1/3500	1/2100
reference assay	1/12600	1/7000	1/12000

Fig. 7. Calibration curves demonstrate the detection of αHCV (A), αHBs (B), and αHIV (C) antibodies in serial dilutions of infected human sera. CombOLISA assays (*y* axis) are reported as a function of reference immunoassays, microtiter plate ELISA for HCV (optical density) and VIDAS for HIV and HBV (fluorescence). Sensitivity threshold with regard to extreme sample dilution are compared for CombOLISA and for reference immunoassays.

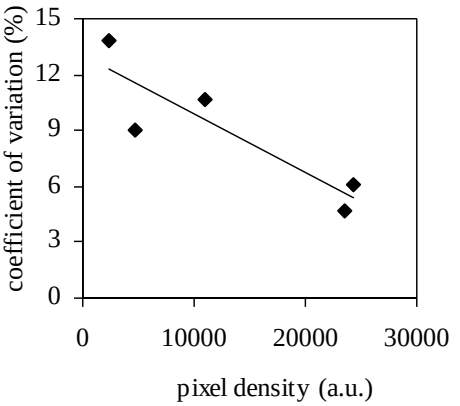


Fig. 8. Interwell coefficients of variation measured on a series of 22 spots related to three nucleic acid capture probes and two antigen spots at various density levels.

CombOLISA array (*y* axis) is plotted vs fluorescence for the VIDAS assay or optical density for the microplate ELISA (*x* axis) using a log–log scale. The three independent reference immunoassays are highly correlated with CombOLISA ($R^2 \geqslant 0.93$).

Sensitivity limits were estimated in each case. CombOLISA is half as sensitive as VIDAS Anti-HBs Total and one-fifth as sensitive as VIDAS HIV Duo. Indeed, the VIDAS detection protocol is based on a fluorescent substrate which is more sensitive than a colorimetric assay. Furthermore, HIV assay on VIDAS detects both p24 and anti-HIV antibodies and sums up signals, while only the latter analysis is performed with the CombOLISA. To improve protein microarray sensitivity, the use of antigens coupled to linear polymers which enhance protein stability and/or density has already demonstrated their efficiency [15]. On the other hand, CombOLISA on HCV serum is more sensitive that a standard microplate colorimetric ELISA, demonstrating that CombOLISA multiplexity is not detrimental to sensitivity.

Reproducibility

Interwell reproducibility was first investigated on dozens of nucleic acid and protein spots having the same average pixel density, close to saturation ($38{,}100 \pm 1400$). Coefficients of variation are below 7% and do not depend on the nature of the analyte (6.5% for C_{HBV} spots, 5.3% for C_{HIV} spots, 4.7% for HBs antigen spots). On the other hand, data scattering was measured on a series comprising 22 plots, consisting of five different types of spots (three nucleic acid probe spots and two antigen spots) of various density levels. In this case, an inverse correlation is found between signal intensity and reproducibility (Fig. 8). Coefficients of variation remain below 15% even in the case of low-density spots. Results are in accordance with the study of Tam et al. [28]. who obtained interwell variations ranging from 5 to 18% on a protein microarray. Spot-to-spot dispersion is linked to spot heterogeneity (shape, size, drop volume), biological reaction reproducibility, and precipitated product uniformity. Background variations measured on 80 spots extracted from 20 different arrays ranged between 3.3% inside the same well and 4.4% from well to well. These values have no influence on the overall reproducibility since the local background surrounding each spot is considered for relative density calculation.

Conclusion

An innovative microarray for multiplexed and simultaneous detection of proteins and nucleic acids has been evaluated by monitoring three human viral infections (HIV, HBV, and HCV) and several associated parameters for diagnosis and therapeutic follow-up. To detect viral antibodies and labeled cDNA in infected sera, purified protein antigens and nucleic acid probes were spotted in 96-well microplates. A reaction buffer was optimized so that both nucleic acid hybridization and immunological coupling were possible under the same experimental conditions, following the same protocol. Specificity was demonstrated by the consecutive addition of each analyte in the same well inducing the coloration of expected spots. Spot density was quantified by software analysis and was related to analyte concentration, demonstrating that CombOLISA was suitable for quantitative assays. CombOLISA results correlated well with values determined using standard commercial immunoassays and are comparable in sensitivity to a standard ELISA.

CombOLISA is a convenient and open microarray technology whose main advantage is to simplify and

considerably shorten multi-parametric analysis applicable to many fields including infectious disease diagnosis. A wide range of proteins and nucleic acids can be readily assayed, simultaneously, without significant loss of sensitivity. As for most microarrays, reagent volume—and, thereby, costs—are considerably reduced compared to those necessary for conventional monoparametric assays. The required serum volume is 7 times less than that of VIDAS for a single parameter, 14 times less for two parameters, and so on. The CombOLISA 96-well microplate format makes its use suitable for most laboratories despite the level of throughput or automation in place and has the capacity to interrogate 96×17 information points per plate.

Acknowledgments

We thank M. Jolivet, M. Quibriac, N. Piga, F. Merlier, G. Paranhos-Baccala, and V. Cheynet (bioMérieux) for the provision of viral proteins and infected human sera. We are very grateful to C. Roitsch for a critical reading of the manuscript.

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