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Multipurpose high-throughput filtering microarrays (HiFi) for DNA and protein assays

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ABSTRACT

We are reporting here a low cost colorimetric device for high-throughput multiplexed blood group genotyping and allergy diagnosis, displayed as an automated 96-well microtiter plate format. A porous polymeric membrane sealed at the bottom of each well accounts for the sensor support. For each sensing unit, a 6×6 matrix of specific probes is spotted on the external surface of the membrane resulting in 5 mm^2 microarrays. Thanks to the membrane porosity, reagents dispensed into the well can be eliminated through vacuum soaking. This unusual design drastically reduces the assay background signal.

The system was first validated on robust models composed of either two complementary oligonucleotide sequences or one allergen/specific rabbit IgG pair. The quality of both oligonucleotide and protein immobilisation on the membrane substrate was then demonstrated together with the capacity to use the arrayed biomolecules as probes for the quantitative detection of specific targets (respectively complementary oligonucleotide and specific antibody).

On the basis of these good results, two multiplex assays were developed for crude biological samples testing, focussing on two human in vitro diagnosis applications: a hybridisation assay for multiplex blood group genotyping and a multiparametric immunoassay for allergy diagnosis. In both cases, the transfer to crude biological samples testing was successful i.e. high signal to noise ratio of the stained membranes, reproducibility and good correlation with results obtained using routine testing procedures.

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1. Introduction

Expectations of biological analysis have reached a new level of complexity: the diagnostic tests are now required to be multiparametric in order to produce more information within a reduced time. Moreover, the number of samples to test keeps increasing, creating a real need for new high-throughput platforms for multiplex diagnosis. In addition to these technical considerations, the economical and social context reflects a rising demand from health authorities upon cost reduction and improved security of diagnosis/prognosis making tools.

Blood donation testing is an eloquent example of that complexity. The search for blood group antigens, infectious agents, microorganisms and other various analytes requires multiple testing procedures (involving immunoassays as well as nucleic acids assays) on million samples a year (Petrik, 2010). This panel of targets is constantly expanding with not less than 300 blood group

antigens identified as for now (Daniels, 2005), and more than 40 new infectious diseases described during the past generation according to World Health Organisation (WHO Report, 2007b).

With the persistent outburst of microarrays, ideal for multiparametric assays, many diagnostic biosensors have been developed based on that parallelised technology. However, even though many of those testing procedures are scientifically mature, this type of device is still not currently available for the diagnosis laboratories. Indeed, most of existing microarray based systems have been developed in a technological driven context (adapting end-user needs to the technology), creating a gap between end-user and technology.

We have been developing a multipurpose microarray based platform for multiparametric and high-throughput filtration assays (HiFi-assays), dedicated to the characterisation of biological samples at reduced cost and time. Combining the multiparametric performances of microarray technology with a 96-well format standard for robotics, our fully automated diagnostic tool permits very high-throughput processing of both nucleic acid assays and immunoassays, in clinical laboratories conditions.

This device is based on a classical 96-well microtiter filtration plate (e.g. Multiscreen HTS-HA, Millipore). The polymeric mem-

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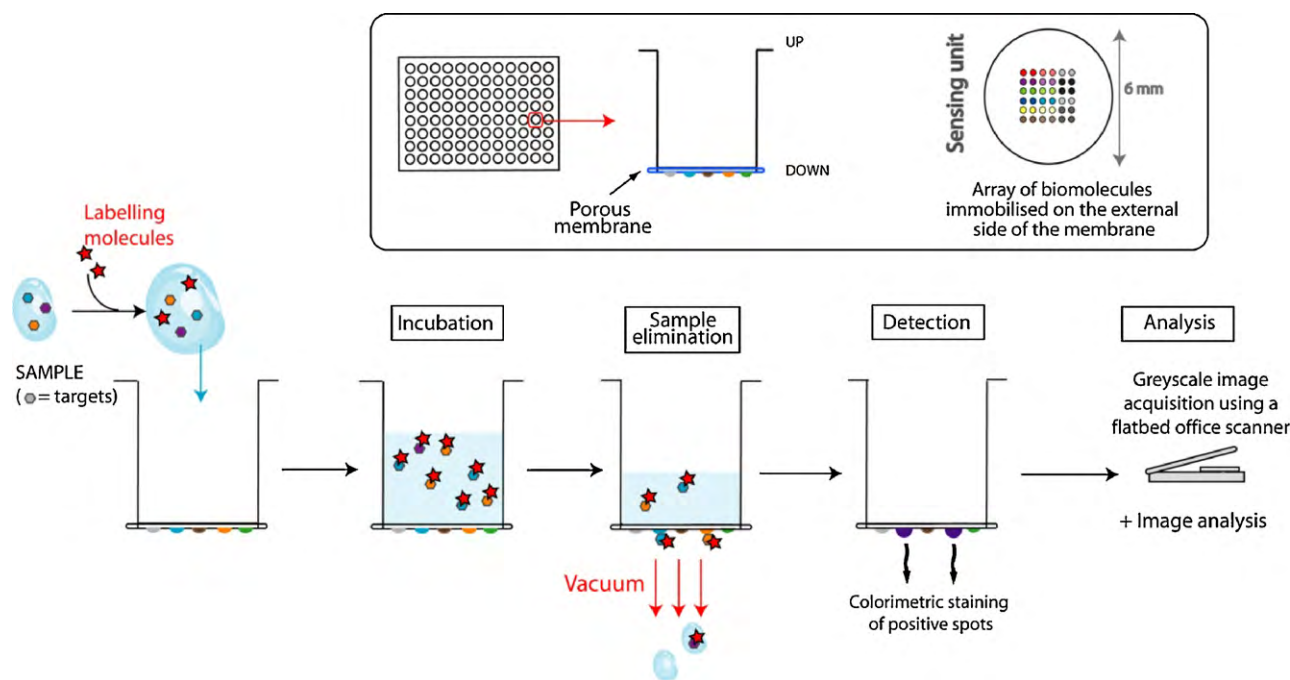


Fig. 1. HiFi-plate design and HiFi-assay filtering protocol. Probe molecules can be oligonucleotides as well as proteins or antibodies. HiFi-assays are based on a filtering protocol: thanks to vacuum soaking, reagents dispensed in wells are loaded through the membrane bottom. After staining of the positive spots, a greyscale image of the membrane bottom is acquired with a simple office scanner and analysed.

brane (mixed cellulose ester) sealed at the bottom of each well enables to eliminate reagents thanks to vacuum soaking. However, in HiFi-plates the membrane is not only used as a filter, but also as support for the immobilisation of an array of biomolecules, which constitutes the multiparametric sensing unit (Fig. 1). The distinctive feature of the plate is the immobilisation of probes on the backside of the membrane, and not inside the well as usually done (Perrin et al., 2003). To our knowledge, this non-conventional design was only mentioned once in the literature for chemiluminescent immunoassays (McBride et al., 2008).

It will be demonstrated how various biomolecules, such as oligonucleotides and proteins, can be immobilised on the substrate as medium density arrays, depending on the application and the target analyte. Our first interest was in the immobilisation of oligonucleotides on the membrane-bottomed plate in order to elaborate a multiplex blood group genotyping tool. In a second time, we successfully transferred the technology to protein immobilisation and developed a multiplex immunoassay for allergy diagnosis.

2. Materials and methods

2.1. Reagents

Synthetic oligonucleotides (Biotin–TTTTTTTTTTTTT–C₆–NH₂, NH₂–C₆–TTGAGTGCATGTTGTGCC, Biotin–GGCACAACATGCACCTCAA and all genotyping probes and primers) were supplied by Eurogentec (Belgium).

Alkaline phosphatase-labelled streptavidin, alkaline phosphatase-labelled monoclonal anti-human IgE antibodies developed in mouse, *Arachis hypogaea* lectin (from peanut) and anti-*A. hypogaea* lectin antibodies developed in rabbit were purchased from Sigma–Aldrich (France). Alkaline phosphatase-labelled anti-rabbit IgG (H+L) antibodies developed in goat were supplied by Jackson Immuno-Research (USA).

Recombinant allergenic proteins (in PBS) from cherry (rPru a4: 0.42 mg mL^{−1}; rPru a1: 1.59 mg mL^{−1}; rPru a3: 1.86 mg mL^{−1}), apple (rMal d1: 0.11 mg mL^{−1}, rMal d3: 0.99 mg mL^{−1}, rMal d4:

1.15 mg mL^{−1}, hazelnut (rCor a1: 0.48 mg mL^{−1}) and birch tree pollen (rBet v1: 0.82 mg mL^{−1}) were kindly provided by Dr. Stephan Scheurer (Paul Ehrlich Institute, Germany) as well as apple (18 µg mL^{−1}) and cherry (419 µg mL^{−1}) extracts and reactive sera.

2.2. General protocol description

HiFi-plate preparation. Original membrane-bottomed 96-well plates (Multiscreen HTS-HA) were purchased from Millipore. When not mentioned, 2.4 nL drops of spotting solutions (pitch = 500 µm) were arrayed on the membrane backside using a BioChip Arrayer BCA1 (Perkin-Elmer).

Automated filtration assay and analyte detection. HiFi filtration assays were carried out on an EVO75 robot (TECAN, Switzerland) equipped with a plate filtration unit and a heater. The vacuum and the soaking duration were finely controlled in order to generate the most reproducible results. The filtering protocol involved the following steps. (i) The membranes were washed with PBS (100 mmol L^{−1}, pH 7.4) and saturated with PBSTA (PBS added with Tween 20 0.1% (v/v) and BSA 1% (w/v)). (ii) Meanwhile cocktail solutions of analyte and labelling molecules in PBSTA were pre-incubated on the heater unit. (iii) Samples were then transferred to HiFi-plate microwells and loaded on the membrane for interaction and labelling. (iv) The membranes were washed with veronal saline buffer (100 mmol L^{−1}, pH 8.5). (v) Finally, 200 µL of a BCIP/NBT ready-to-use solution (Sigma–Aldrich) were added per well for signal generation at room temperature (5 min to 1 h). The original colour of the BCIP/NBT solution was light yellow whereas the purple product of the enzymatic reaction precipitates on positive spots.

Image acquisition. The microtiter plate bottom was imaged using an ordinary flatbed scanner (HP Scanjet 3770, Hewlett-Packard) in greyscale (from 0 to 65,535 arbitrary units (a.u.)) with a 2400 dpi resolution.

Data analysis. Image analysis (signal quantification) was performed using GenePix Pro 6.0 software (Molecular Devices). The signal intensity per spot was calculated as the median intensity for all pixels included in a circular feature defining the spot and

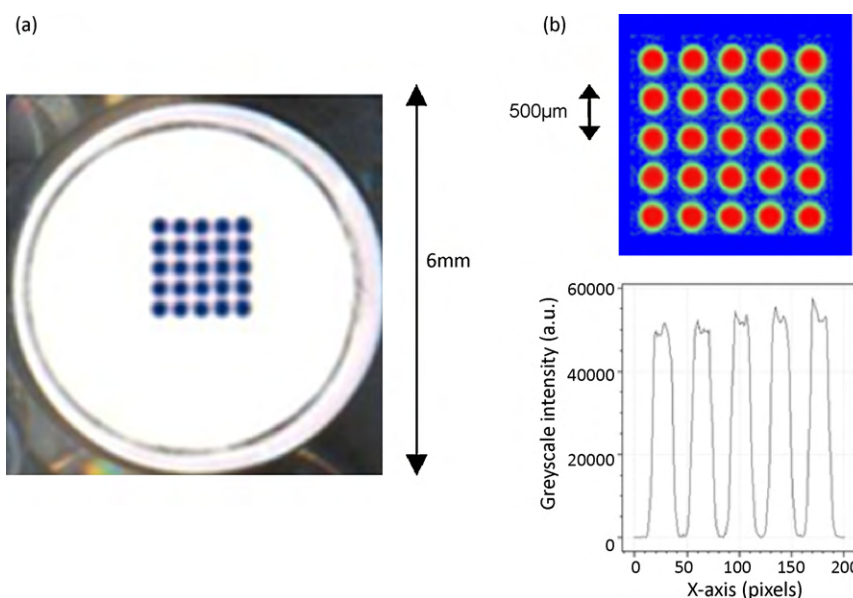


Fig. 2. Array characteristics. 5×5 microarray of replicate spots of a biotinylated 15^{mer} synthetic oligonucleotide (spotting solution: $10 \mu\text{mol L}^{-1}$, 2.5 nL drops, pitch = $500 \mu\text{m}$). (a) Real colour picture. The membrane bottom was imaged using an office scanner (2400 dpi) after staining of the positive spots. (b) Greyscale intensity image of the entire array (up) and the corresponding profile for one row of spots (down).

corrected using a local background evaluation. The resulting net specific intensity on a given probe was calculated as the mean intensity for all replicate spots.

2.3. Synthetic oligonucleotide immobilisation and detection

The synthetic oligonucleotides used as first and second model systems were Biotin–TTTTTTTTTTTTTT– $\text{C}_6\text{–NH}_2$ and $\text{NH}_2\text{–C}_6\text{–TTGAGGTGCATGTTGTGCC}$ /Biotin–GGCACAACATGCACCTCAA respectively.

The automated protocol took place as described above. Operating temperatures and durations were the following: (i) saturation: 10 min at room temperature; (ii) sample pre-incubation: 5 min at 50°C ; (iii) incubation on membrane: 10 min at 50°C . Alkaline phosphatase-labelled streptavidin was used at a concentration of $2 \mu\text{g mL}^{-1}$.

2.4. Multiplex blood group genotyping

Targeted SNPs selection. Six single nucleotide polymorphisms (SNPs) associated to 4 minor blood groups (Kell, Kidd, Duffy and MNS) were selected for their clinical relevancy. Those SNPs concern the following mutations and correspond to specific red blood cell surface antigens: Kell: T698C (KEL1/KEL2), T961C (KEL3/KEL4); Kidd: G838A (JK1/JK2); Duffy: G125A (FY1/FY2); MNS: C59T (MNS1/MNS2), T143C (MNS3/MNS4) (Fig. 3).

Whole blood collection. Whole blood samples (EDTA anticoagulant) were collected (with inform consent) from the Etablissement Français du Sang Rhone-Alpes. Samples were randomly picked among the blood bank and belonged in majority to the Caucasian ethnic group. Selected donors were phenotyped using standard serologic hemagglutination techniques at the Etablissement Français du Sang Rhone-Alpes.

Probe patterning. Fifteen probes were arrayed on membrane backside forming a 6×6 matrix composed of 12 synthetic oligonucleotide probes (from 17^{mer} to 23^{mer}) specific of the targeted alleles (2 replicates per probe) and 3 control probes (for spotting, hybridisation and negative control; 4 replicates per probe). The spotting procedure (sciFLEXARRAYER S3, SCIENION, Germany) was per-

formed using 2.5 nL drops of $50 \mu\text{mol L}^{-1}$ oligonucleotide solutions in PBS.

Multiplex PCR. Genomic DNA was extracted from the blood sample using a MagNa Pure LC pure robot and the MagNa Pure LC DNA Isolation Kit 1 (Roche Diagnostics). Twelve sequences hosting the 6 target polymorphisms were amplified and labelled using a single 96-well format multiplex PCR on a TProfessional thermal cycler (Biometra, Germany). Forward primers were labelled with biotin at their 5' end. PCR product quality was controlled using electrophoresis (2% agarose gel with 190 V for 20 min)

Filtration assay. The 96-well PCR plates were directly transferred to the EVO75 robot (without further purification) and samples were tested after a 1:5 dilution. Operating temperatures and durations were the following: (i) saturation: 10 min at room temperature; (ii) sample pre-incubation: 5 min at 50°C ; (iii) incubation on membrane: 10 min at 50°C . Alkaline phosphatase-labelled streptavidin was used at a concentration of $2 \mu\text{g mL}^{-1}$.

Genotype assignment. For each SNP associated to the two alleles A_1 and A_2 , the following discrimination parameter P_{score} was evaluated and compared to a set of cut-off values in order to assign genotype.

$$P_{\text{score}} = \frac{I(A_2) - I(A_1)}{I(A_2) + I(A_1)}$$

2.5. Peanut allergen/rabbit IgG monoparametric assays

The automated protocol took place as described above. Operating temperatures and durations were the following: (i) saturation: 10 min at room temperature; (ii) sample pre-incubation: 10 min at 37°C ; (iii) incubation on membrane: 60 min at 37°C . Alkaline phosphatase-labelled polyclonal anti-rabbit IgG antibodies were used at a concentration of $0.12 \mu\text{g mL}^{-1}$.

2.6. Allergy diagnosis multiplex immunoassays

Probe patterning. Twelve different probes were arrayed on the membrane backside resulting in a 37-spot matrix composed of 10 recombinant allergens and extracts (2 replicates per probe) and 2 control probes (WHO reference IgE and an alkaline phosphatase-labelled antibody; 3 replicates per probe).

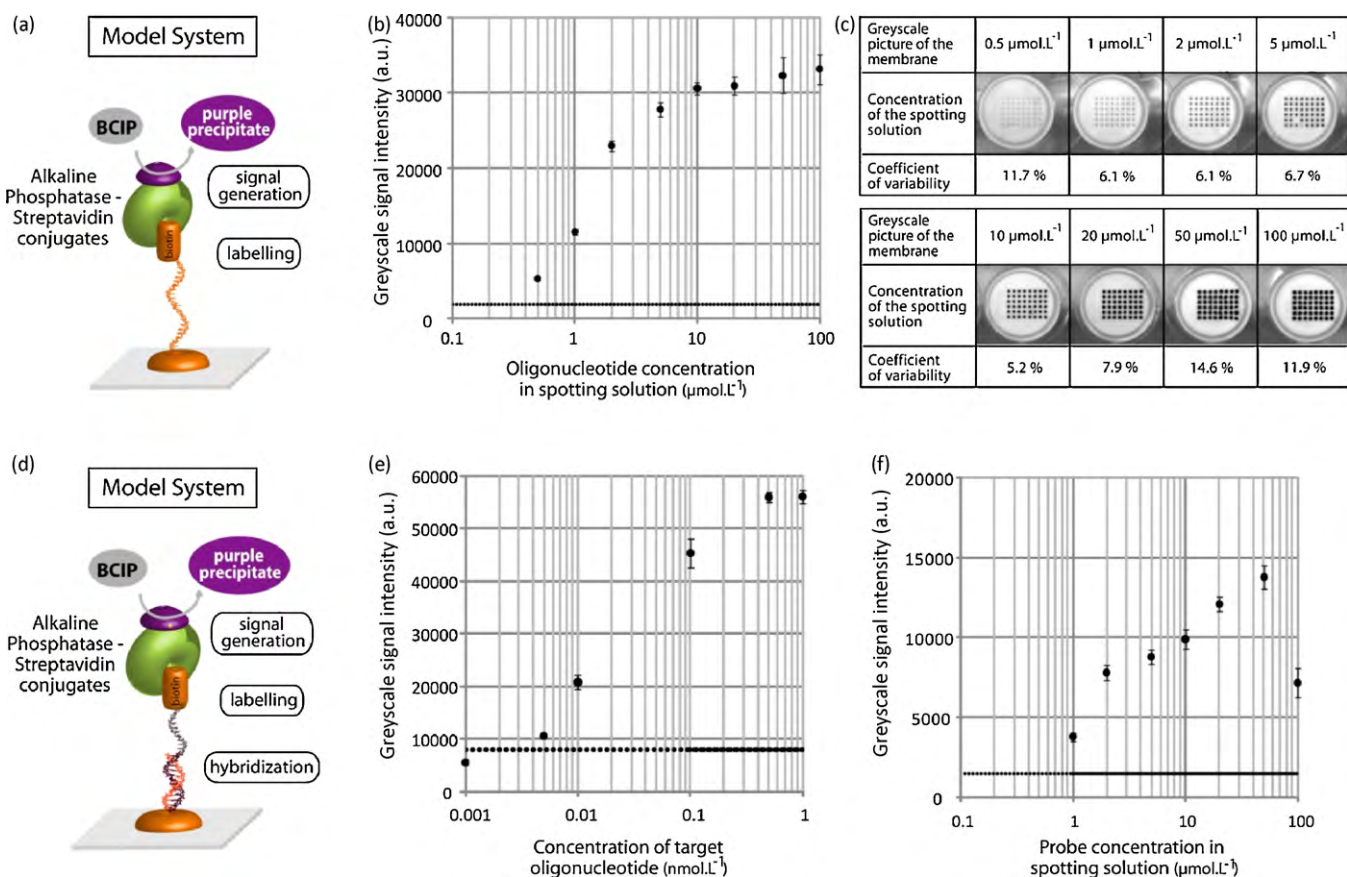


Fig. 3. Influence of probe concentration. For that whole set of experiments, alkaline phosphatase–streptavidin conjugates concentration was $2 \mu\text{g mL}^{-1}$ and the spotting pattern was an 8×6 monoparametric matrix (2.4 nL , pitch = $500 \mu\text{m}$). The dot lines indicate limits of detection. Upper part: effect of spotting solution concentration on oligonucleotide immobilisation. (a) The model system: Biotin–TTTTTTTTTTTT– $\text{C}_6\text{–NH}_2$. (b) Spot intensity as a function of the spotting solution concentration. (c) Images of the corresponding stained membranes (greyscale, 2400 dpi). Lower part: hybridisation assays using a couple of synthetic probes. (d) The model system: $\text{NH}_2\text{–C}_6\text{–TTGAGGTGCATGTTGTGCC/Biotin–GGCACAAACATGCACCTCAA}$. (e) Spot intensity as a function of the target concentration (probe concentration = $10 \mu\text{mol L}^{-1}$). (f) Spot intensity as a function of the probe concentration (target concentration = 2 nmol L^{-1}).

Filtration assay. The automated protocol took place as described above. Operating temperatures and durations were the following: (i) saturation: 10 min at room temperature; (ii) 1:10 dilution sample pre-incubation: 10 min at 37°C and (iii) incubation on membrane: 60 min at 37°C . Alkaline phosphatase-labelled monoclonal anti-human IgE antibodies were used at a concentration of $2 \mu\text{g mL}^{-1}$.

3. Results and discussion

Each membrane-bottomed well of HiFi-plate represents a single multiparametric sensing unit, so that processing the whole plate enables to test 96 biological samples simultaneously for multiple parameters. The combination of HiFi-assay's automated filtering protocol and specific layout (backside spotting) is the key to the sensor's efficiency. First of all, the porous membrane (pore diameter = $0.45 \mu\text{m}$) filters dust and micrometer-sized contaminants, which get trapped on its upper surface. Since such particles tend to concentrate the coloured precipitate used for revelation, generating then strong interfering noise signals, this filtration lowers partially the background on the underside of the membrane. Moreover, the filtering protocol enables to avoid the diffusion constrain: during the loading of the sample through vacuum soaking, the analyte molecules are forced to pass through the membrane, which favours the interactions of targets with the immobilised probes and improves the sensor's sensitivity. Finally, the resulting background observed on the membrane underside is lower than the upperside

one and much more homogeneous. In the end, the overall signal appears much “cleaner” under the plate, with a strong specific signal and a homogenous background which facilitates a lot the signal analysis.

The level of interactions on each spot is quantitatively detected through a colorimetric signal generation using alkaline phosphatase labelling. The exposition of the membrane to a BCIP/NBT solution (enzyme substrate) generates a purple precipitate on positive spots (Fig. 2). After this staining step, the microtiter plate bottom can be easily imaged using low cost equipment such as a flatbed office scanner (greyscale imaging, 2400 dpi).

Before developing an assay for complex biological samples, such as blood or sera, preliminary studies were performed in order to optimise the biomolecules immobilisation on the membrane substrate and to investigate the possibility of a quantitative detection of the interaction taking place between target and probe molecules.

3.1. Oligonucleotide immobilisation and detection

In order to investigate the quality of the oligonucleotide immobilisation on the polymeric membrane substrate, synthetic biotinylated oligonucleotides (Biotin–TTTTTTTTTTTT– $\text{C}_6\text{–NH}_2$) were arrayed using a piezoelectric spotter and labelled with streptavidin–alkaline phosphatase conjugates (Fig. 3(a)). Immobilisation did require neither surface pre-treatment nor functionalisation of biomolecules, suggesting a non-covalent interaction with

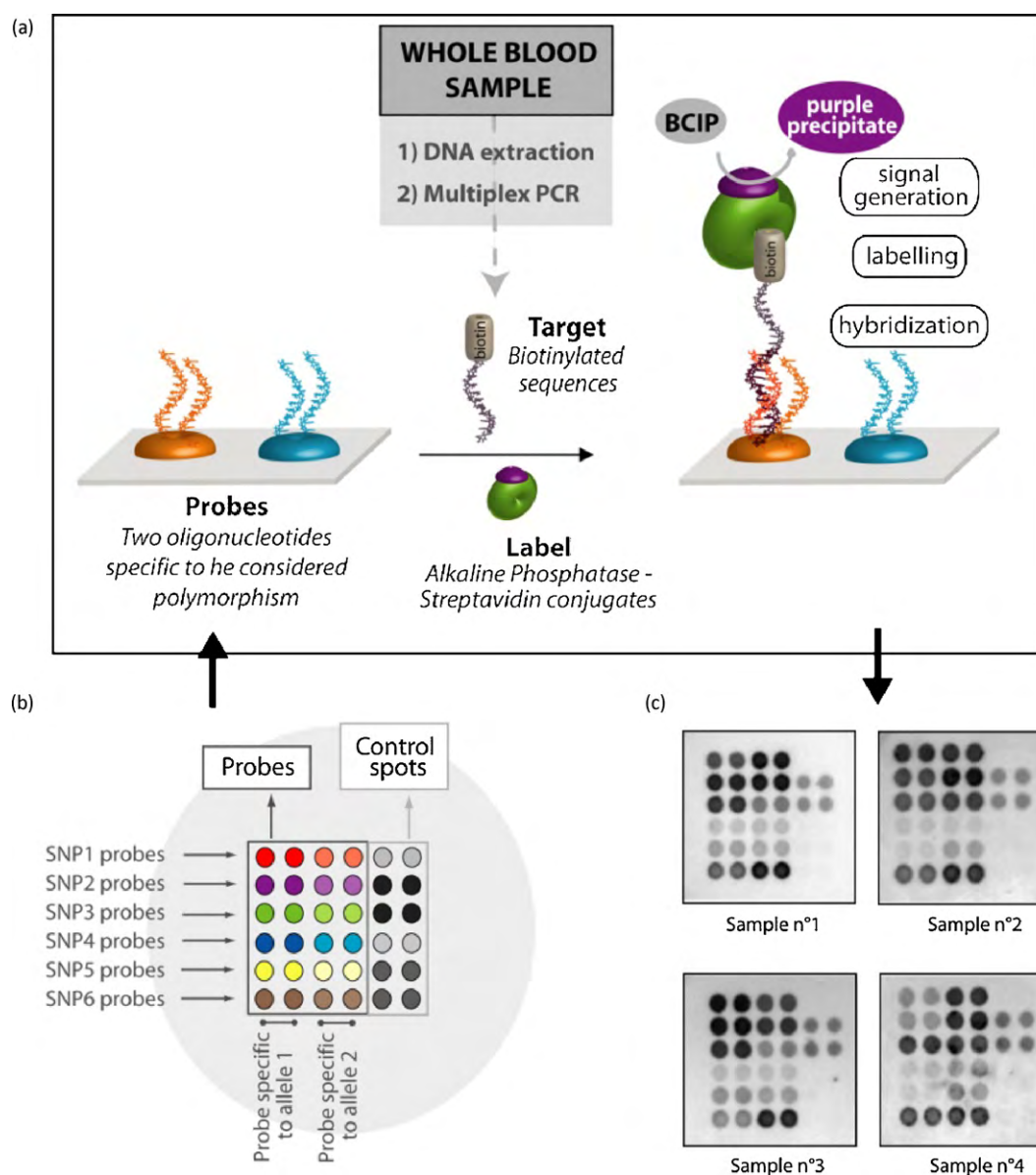


Fig. 4. HiFi-assay for multiplex blood group genotyping. (a) HiFi-assay strategy (simplified scheme: case of a single SNP). (b) Spotting pattern: 50 $\mu\text{mol L}^{-1}$ (in PBS) solutions of the 12 specific probes and 3 controls probes were spotted on the membrane (2.4 nL, pitch = 500 μm). (c) Greyscale images of 4 representative stained membranes resulting for 4 different samples testing (2400 dpi).

the substrate. Fig. 2(a) presents a real-colour picture of a stained array of 25 replicate spots.

Oligonucleotides were thus successfully immobilised as medium density microarrays (10–100 spots per well, up to 1000 spots cm^{-2}), with an average spot diameter of $300 \pm 50 \mu\text{m}$. Spots were homogeneous and highly reproducible (Fig. 2(b)) with coefficients of variability yielding between 5 and 10%. The membrane porosity enables a 3D immobilisation of the probes (Supplementary Fig. 1), which results in an increased amount of oligonucleotides per spot and consequently an enhanced sensor sensitivity.

The density of probes arrayed on the membrane can be finely tuned through the control of the spotting parameters. On the one hand, an increase in drop volume results in a stronger signal and a slight enlargement of spot (Supplementary Fig. 2). On the other hand, as shown in Fig. 3(b) and (c), the concentration of spotting solutions can be directly correlated to the probe density in each spot. Indeed, a direct correlation between spotting concentrations and spot signal intensities was observed. The spot signal intensities

increased linearly with the spotting concentration before reaching a plateau for concentrations higher than $10 \mu\text{mol L}^{-1}$. Such a plateau can be explained either by a saturation of the immobilisation sites or by a labelling limitation through steric hindrance between neighbour streptavidins.

On the basis of these good spotting results, monoparametric hybridisation assays were performed in order to evaluate the capacity of quantitatively detect the level of interactions on each spot. In that case, two complementary synthetic oligonucleotide sequences were used as a robust model of probe/target couple. The target oligonucleotide was functionalised with a biotin group acting as an anchor for streptavidin–alkaline phosphatase conjugate (Fig. 3(d)). The apparition of a blue precipitate on a spot indicates that hybridisation took place.

Furthermore, in order to evaluate the effect of probe density on the spot intensities, a constant concentration of target was hybridised on arrays prepared using increasing probe concentrations (Fig. 3(f)). At very high probe concentration in the spotting solution, a very high probe density on the surface leads to a steric

Table 1
 Comparison between the HiFi-assay results and the phenotypes determined by classical serology on a set of 16 patients. For each sample, both phenotypes determined by serology (hemagglutination techniques) and deduced from HiFi-assay resulting genotype are compared. Each column corresponds to an existing phenotype. Discordances are indicated in bold.

		SNP1			SNP2			SNP3			SNP4			SNP5			SNP6		
		KEL:1,-2	KEL:1,2	KEL:-1,2	JK:1,-2	JK:1,2	JK:-1,2	FY:1,-2	FY:1,2	FY:-1,2	MNS:1,-2	MNS:1,2	MNS:-1,2	MNS:-3,-4	MNS:3,4	MNS:-3,4	KEL:3,-4	KEL:3,4	KEL:-3,4
Sample 1	Serology			X		X		X			X			X					
	HiFi-assay			X		X		X			X			X					
Sample 2	Serology		X				X			X		X				X			X
	HiFi-assay		X				X			X		X				X			X
Sample 3	Serology	X			X			X				X		X					X
	HiFi-assay	X			X			X				X		X					X
Sample 4	Serology			X			X		X				X			X		X	
	HiFi-assay			X			X		X				X			X		X	
Sample 5	Serology			X		X		X					X	X					X
	HiFi-assay			X		X		X					X	X					X
Sample 6	Serology			X		X			X		X			X					X
	HiFi-assay			X		X			X		X			X					X
Sample 7	Serology			X		X			X			X				X			X
	HiFi-assay			X		X			X			X				X			X
Sample 8	Serology	X			X					X		X			X				X
	HiFi-assay	X			X					X		X			X				X
Sample 9	Serology			X		X			X		X			X					X
	HiFi-assay			X		X			X		X			X					X
Sample 10	Serology		X				X		X		X				X		X		
	HiFi-assay		X				X		X		X				X		X		
Sample 11	Serology			X			X			X		X				X			X
	HiFi-assay			X			X			X			X			X			X
Sample 12	Serology		X			X			X			X			X				X
	HiFi-assay		X			X			X			X			X				X
Sample 13	Serology			X		X			X			X				X			X
	HiFi-assay			X		X			X			X				X			X
Sample 14	Serology			X		X			X				X			X			X
	HiFi-assay			X		X			X				X			X			X
Sample 15	Serology			X	X			X						X			X		X
	HiFi-assay			X	X			X						X			X		X
Sample 16	Serology			X	X				X			X				X			X
	HiFi-assay			X	X				X			X				X			X

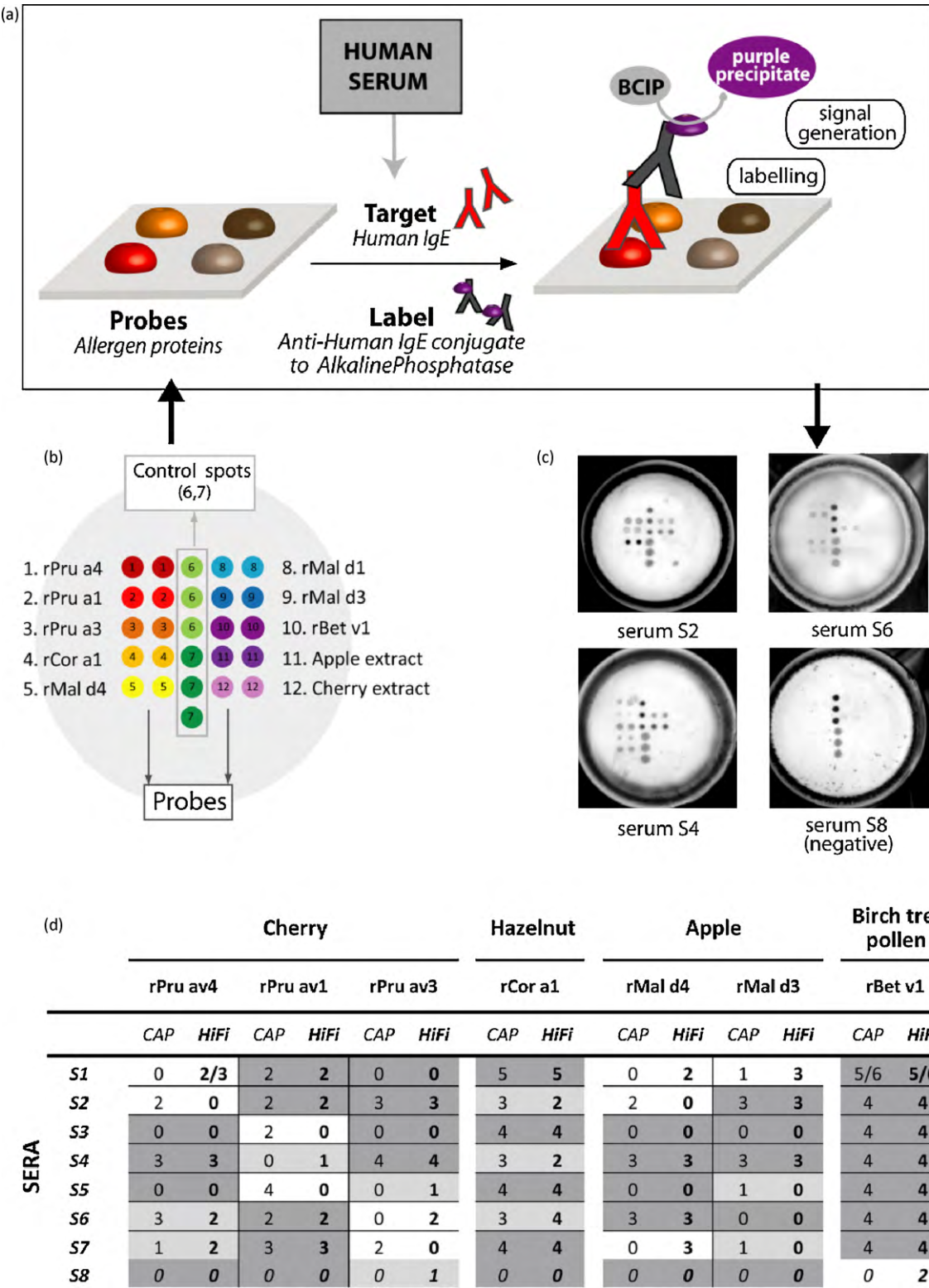


Fig. 5. HiFi-assay for multiplex allergy diagnosis. (a) HiFi-assay strategy for IgE detection in serum. (b) Spotting pattern: 10 specific probes (average concentration = 1 mg mL⁻¹ in PBS) and 2 controls probes were arrayed on the membrane (2.4 nL, pitch = 500 μm). (c) Greyscale pictures (2400 dpi) of 4 representative stained membranes corresponding to 3 allergen-reactive sera (S2, S4 and S6) and a negative serum (S8). (d) Comparison of HiFi-assay results with phenotypes determined by serology on a set of 16 patients. ImmunoCAP classification was used to compare the results of the 2 methods (corresponding reactivities: class 0 absent or undetectable, class 1 low, class 2 moderate, class 3 high, classes 4–6 very high). Specific IgE levels are calculated from CAPunit results as following (Fall et al., 2003): class 0 <0.35 kU L⁻¹; class 1 0.35–0.70 kU L⁻¹; class 2 0.70–3.5 kU L⁻¹; class 3 3.5–17.5 kU L⁻¹; class 4 17.5–50 kU L⁻¹; class 5 50–100 kU L⁻¹; class 6 > 100 kU L⁻¹.

hindrance between single strands resulting in a signal decrease (Ramsay, 1998).

This first raw of results validated both the oligonucleotide immobilisation protocol (with a probe density on the membrane easily tunable) and the quantitative detection of target oligonucleotides. The next step was to use the HiFi-nucleic acids assay for real sample characterisation in developing a reliable tool for multiplex blood group genotyping.

3.2. Application to multiplex blood group genotyping

Blood group typing of patient and donor is a critical step for the prevention of transfusion adverse reactions and blood compatibility can become very challenging in complex cases such as polytransfused patients. If the well-known ABO and Rhesus blood groups are currently typed in routine clinical laboratories, many other minor blood groups (29 blood groups identified) are also associated to clinically relevant red blood cell surface antigens (Quill, 2008). Extension of typing to other blood groups would increase transfusion safety by enabling a better characterisation of blood pouches and consequently a better matching between donor and patient. With average numbers of blood donations of 14 M a year in Europe and 20 M in the US (WHO Report, 2007a), blood qualification centres have to face a high-throughput demand. However, the serologic tests routinely performed in clinical laboratories rely on heavy monoparametric procedures, unsuitable for high-throughput and multiplexing formats (Petrik, 2010).

On the contrary, DNA microarray-based tests were identified as the alternative for bringing multiple blood group typing in blood donation qualification centres (Denomme and Flegel, 2008; Veldhuisen et al., 2009). Most of the already identified blood group antigens are associated to single nucleotide polymorphisms (SNPs) (Daniels, 2005), i.e. the variation of a unique nucleotide in the sequence of the coding gene. Those SNPs give the possibility to directly identify blood groups at the DNA level, through the determination of patient's genotype, i.e. the identification of the 2 copies of the considered gene displayed in patient's genome. Since each SNP is associated to 2 polymorphic alleles, the patient can exhibit 3 different genotypes: 2 homozygote configurations (2 copies of one allele) and one heterozygote configuration (one of each).

We have been developing a multiplex assay for the genotyping of 4 clinically relevant blood groups (Kell, Kidd, Duffy, MNS) (Poole and Daniels, 2007) associated to 6 single nucleotide polymorphisms. In order to target the sequences specific to the 12 considered alleles, 12 oligonucleotide probes were arrayed on membrane-bottomed wells (2 replicates per probe). For each array, 3 controls probes (4 replicates) completed the 6×6 sensor matrix (Fig. 4(b)). Since the starting material is a whole blood sample, it is necessary to extract genomic DNA and to amplify the target sequences before the hybridisation assay. The 12 target sequences were simultaneously amplified and labelled with a biotin group during a multiplex PCR step. These PCR products were directly dispensed in HiFi-plate wells without further purification. As described in the preliminary studies, streptavidin–alkaline phosphatase conjugate was used for a colorimetric signal generation on the hybridised spots (Fig. 4(a)). Using a 4-h-protocol duration, 96 whole blood samples can be fully processed on a single plate.

Fig. 4(c) presents 4 typical greyscale images of stained membranes, corresponding to 4 different blood samples. As can be seen, specific reactivity patterns were obtained for each sample. An image of the entirely stained plate is reported in Supplementary Fig. 3.

Ideally, a qualitative test might be sufficient to identify a patient's genotype, a positive coloration appearing only in the presence of the allele in the patient's genome. However, because those

blood group polymorphisms concern a single nucleotide (SNP), a non-negligible percentage of mismatch events occurs, resulting in “false positive” signals. The absence of fully negative result (white spot of probe) in Fig. 4(c) illustrates that fact. To optimise the genotyping efficiency, we investigated the effect of the hybridisation temperature on the match/mismatch signal ratio. The optimal operating temperature was 50 °C. Nevertheless, genotype assignment for each SNP still requires a semi-quantitative analysis performed through a quantitative comparison of the greyscale intensity levels of both alleles. Thus, a P_{score} parameter was calculated for each SNP of each sample (see Section 2) and compared to a set of cut-off values specific to each genotype (data not shown).

In order to evaluate the accuracy of HiFi genotyping assay, parallel serological tests were performed on every sample for phenotype determination. Table 1 compares results from HiFi-assay and serology technique for a set of 16 patients. A phenotype result was deducted from HiFi resulting genotype in order to enable a direct comparison of both techniques. Results were in excellent agreement (98% of concordance) with only 2 discordant results (for MNS blood group system) over 96 distinct parameters. It is likely that the signal weakness of MNS associated probes reduces sensitivity and implies a loss of accuracy.

Multiplex genotyping results were excellent with 100% matching with serological test results for 3 of the studied blood groups and an overall 93% matching for the 4 blood groups. Compared to other commercially available genotyping techniques, HiFi-assay is clearly fitting the blood bank requirements in terms of safety, automation and throughput. Indeed, most of the numerous microarray-based genotyping techniques developed over the past few years (Di Cristofaro et al., 2007; Hashmi et al., 2007; Hopp et al., 2010; Karpasitou et al., 2008) are based on technologies often expensive, seldom easy to handle and difficult to integrate within high-throughput industrial blood supply processes. On the contrary, HiFi-technology main added-value remains its transfer to a fully automated, well-proven and secure high-throughput protocol. This full automation (including genomic DNA extraction, multiplex PCR and filtering hybridisation assay) guarantees that HiFi-technology constitutes a secured and cost-efficient technique for blood qualification centre environment and requires a minimum technician intervention which lowers human error hazard. Besides, the convenient elimination of reagents through vacuum soaking during the filtration assays and the unidirectional fluids handling ensures reduced contamination. Last but not least, the long term stability of the colorimetric staining enables the end-user to store result plates in addition to in silico data storage, leading to better traceability.

3.3. Multiplex allergy diagnosis

Taking advantage of the successful development of the genotyping HiFi-assay, we transferred the technology to protein immobilisation, aiming to develop an immuno HiFi-assay for multiplex allergy diagnosis. Indeed, although classical monoparametric assays such as a classical ELISA and even more the high-capacity solid phase test ImmunoCAP (Phadia(ImmunoCAP(R) InVitroSight TM, 2007)) remain the gold standards for routine allergy diagnosis, recent literature indicates an increasing interest of the field for multiparametric assays based on microarray technologies (Cretich et al., 2009; Fall et al., 2003; Jahn-Schmid et al., 2003; Lebrun et al., 2005; Ott et al., 2008; Tai et al., 2009). Here, HiFi-assay can be a promising candidate thanks to its microarray-based multiparametric format, its high-throughput capacities and its significant background signal reduction obtained from using a filtering protocol. Undeniably, for immunoassays dedicated to the characterisation of crude serum, this last point can be really advantageous.

The immuno HiFi-assay building blocks for the development of an allergy diagnosis tool were here (i) the use of recombi-

nant proteins and crude allergen extracts as arrayed probes for the detection of (ii) allergen-specific immunoglobulins E (IgE) in crude human sera and (iii) a detection of the interaction using a secondary anti-human IgE antibody labelled with alkaline phosphatase (Fig. 5(a)).

In a preliminary study, monoparametric assays were performed on a fully-characterised peanut allergen/rabbit IgG system in order to validate the protein immobilisation protocol and to investigate the possibility of a quantitative immunoassay on the HiFi system. For that purpose, membrane bottomed wells were arrayed with *Arachis hypogaea* lectin (specific peanut allergen) spots and loaded with cocktail solutions composed of anti-*Arachis hypogaea* lectin rabbit IgG at different concentrations and alkaline phosphatase-labelled monoclonal anti-rabbit antibodies. The obtained calibration curve is presented in Supplementary Fig. 4. As observed for oligonucleotide-based assays, coefficients of variability yielded between 5 and 10% and a quantitative correlation between the resulting greyscale signal and the concentration of target molecules was demonstrated. A limit of detection of $0.2 \mu\text{g mL}^{-1}$ of specific antibodies was found with a linear detection ranging over two decades at least.

Given the successful results obtained using the peanut allergen/rabbit IgG system, we proceed with the development of a multiplex array for allergy testing on crude serum samples. A set of recombinant antigens associated to different allergens (birch tree pollen, hazelnut, cherry, apple) and crude allergen extracts (apple, cherry) were arrayed on the membrane (2 replicates per probe, average antigen concentration = 1 mg mL^{-1} in PBS). The spotted matrix was completed by 2 control probes (3 replicates each): a WHO reference IgE and an alkaline phosphatase-labelled antibody as positive control (Fig. 5(b)). Seven patient sera with a positive allergic reaction history and one patient serum without any known sensitivity were loaded on the membrane and incubated at 37°C for 1 hour. The cocktail solution was here composed of 1:10 dilution of patient serum together with anti-human IgE antibodies conjugated to alkaline phosphatase ($2 \mu\text{g mL}^{-1}$). Images of 4 representative resulting stained membranes are reported in Fig. 5(c).

As can be seen, specific reactivity patterns were obtained for each sample whereas the control spot signals remained stable. Moreover the image obtained from the control well, loaded with an allergen non-sensitive patient serum, confirms the high specificity of the system. Indeed, for this sample, only the control spots were positively stained. It is also worth noting that as expected, the filtering protocol enables a drastic reduction of the background on the external side on the membrane (see images of membrane bottom and top in Supplementary Fig. 5).

In order to validate the allergy diagnosis potentiality of the immuno HiFi-assay, the greyscale signal of every spot was quantified for each patient. The normalised signals were then converted into CAP unit and compared to the reactivity previously determined using the ImmunoCAP system. Fig. 5(d) presents a comparison of the two methods for a set of 7 allergen-sensitive sera (S1–S7) and one negative serum (S8). Because of the very low signals obtained on apple extract, cherry extract and rMal d1 protein spots, no reliable analysis was performed for those probes. For the full set of parameters, only 63% of the results were matching data from ImmunoCAP testing. However, as a matter of fact, both result sets were in very good agreement (92%) for sera characterised as highly reactive by ImmunoCAP system (classes 4–6). Indeed, within these 3 classes, 12 results over 13 were concordant. The weak correlation observed for low reactivity samples can be explained by the fact that class determination is based on an exponentially increasing CAPUnit scale. Actually, values obtained for class 1, 2 or 3-samples are extremely close in comparison with class 4, 5 and 6 data sets. The lower sensitivity of HiFi-test makes thus difficult the differentiation of class 1, 2 or 3-samples.

Nevertheless, it should be noted that only one parameter was totally misdiagnosed (very different reactivity levels): a negative response was obtained for serum S5 against rPru av 1 allergen, whereas ImmunoCAP characterised that sample as highly reactive.

For rBet v1 (birch tree pollen) and rCor a1 (hazelnut), the two allergens which yielded the highest HiFi signals, a quantitative comparison of both techniques was performed to validate the correlation between the normalised greyscale signal intensity of the spots and the ImmunoCAP system result. Supplementary Fig. 6 presents the correlation plots together with their linear regression. The correlation coefficients of the linear regressions were good, especially for rCor a1 (0.93), even if as explained above, the lower sensitivity of HiFi-assay increases the dispersion of values for weakly reactive samples.

Allergy diagnosis results were very encouraging with 63% matching the gold standard ImmunoCAP assays. Furthermore high correlation (92%) was obtained for highly reactive sera (Classes 4–6) and a quantitative correlation with ImmunoCAP results was demonstrated for two allergens. In addition to the many advantages described above for multiplex blood group genotyping, immuno HiFi-assay main added-value is the drastic reduction of background on the external side of the membrane thanks to the filtering protocol, which is an undeniable advantage for immunoassays dedicated to the characterisation of crude serum.

4. Conclusions

HiFi-technology provides a multipurpose platform for multiparametric high-throughput testing suitable for nucleic acids-based assays as well as immunoassays. Indeed, preliminary studies performed on robust model systems demonstrated (i) the quality of both oligonucleotide and protein immobilisation on the membrane substrate and (ii) the capacity to use the arrayed biomolecules as probes for the quantitative detection of specific targets (respectively complementary oligonucleotide and specific antibody).

Given the successful results obtained during these monoparametric assays, we proceeded with the development of multiplex assays for crude biological samples testing, focussing on two applications for human in vitro diagnosis: a hybridisation assay for multiplex blood group genotyping and a multiparametric immunoassay for allergy diagnosis. In both cases, the transfer to crude biological samples testing was successful i.e. high signal to noise ratio of the stained membranes, reproducibility and good agreement with results obtained using routine testing procedures.

The present report demonstrates proof of protocol for both blood group genotyping and allergy diagnosis assays. Next step will be further validation of assays reliability on a cohort of random patients. The resulting collected information will also be used as a feedback for optimisation of protocols.

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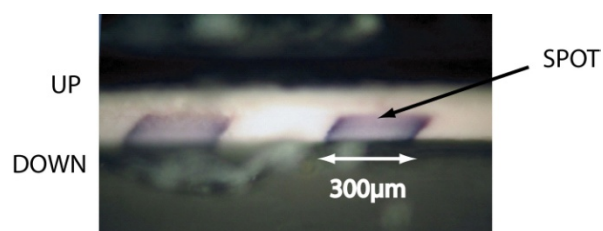
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.06.057.

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Supplementary information



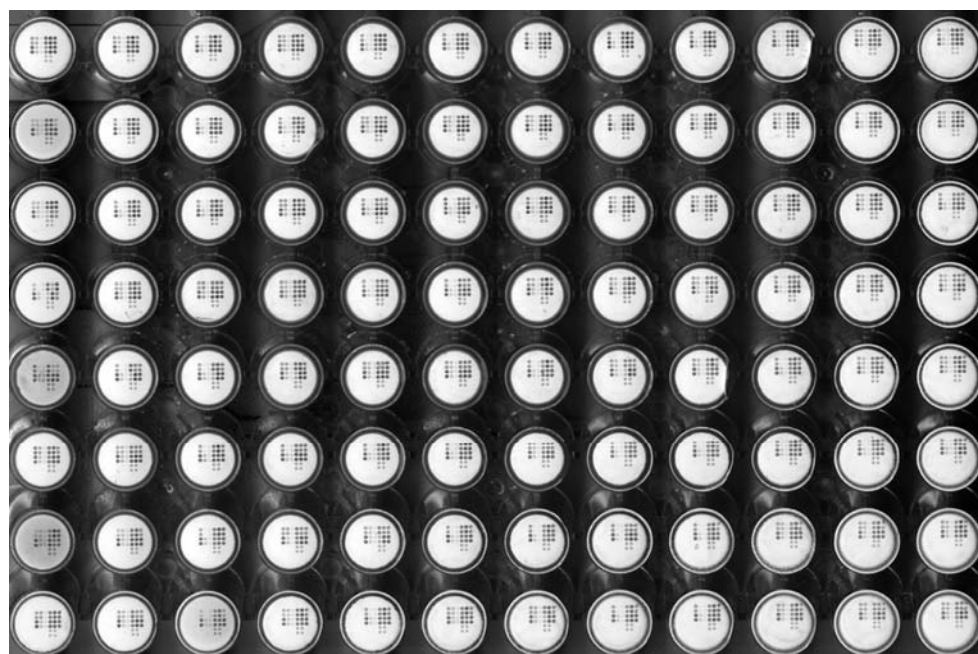
Supplementary Figure 1 | Traversal view of a stained membrane

(real colors optical microscopy picture)

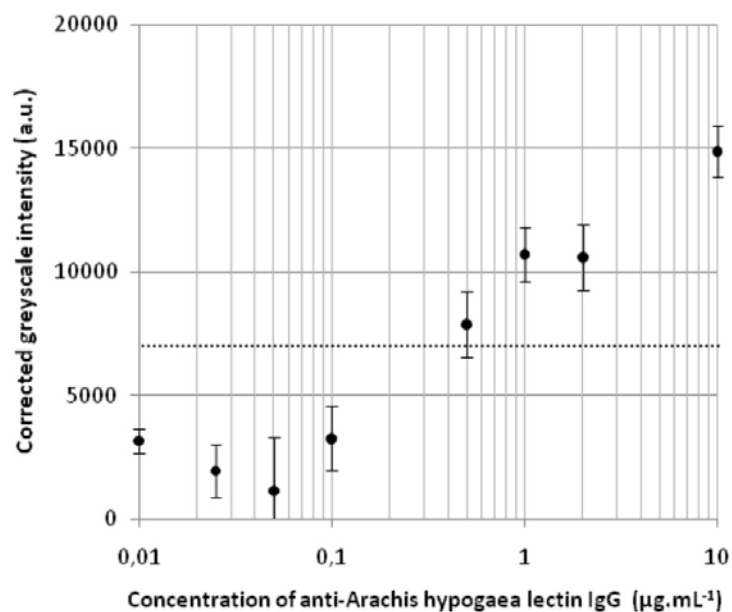


Supplementary Figure 2 | Effect of drop volume on probe density

5*5 microarrays of replicate spots of a biotinylated 15^{mer} synthetic oligonucleotide solution (10µmol.L⁻¹) were spotted with increasing drop volumes (0.7, 1.4 and 2.8nL). The membrane bottom was imaged with an office scanner (2400dpi) after staining of the positive spots (real colours).

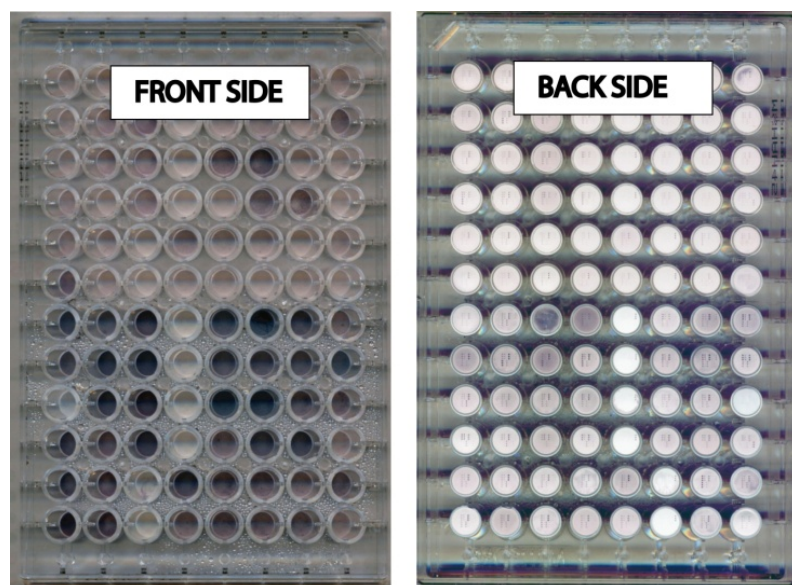


Supplementary Figure 3 | Greyscale picture of a stained HiFi-plate used for blood group genotyping of 96 different patients (2400 dpi)



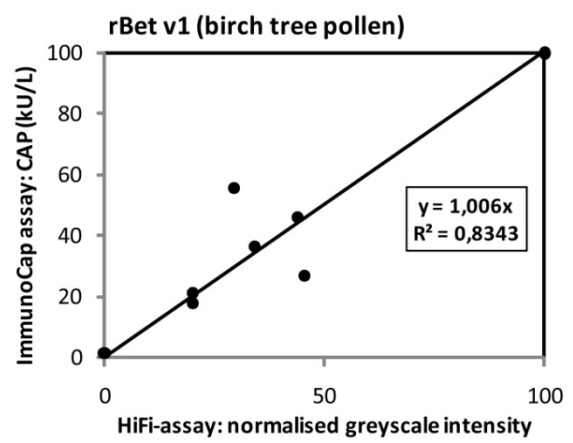
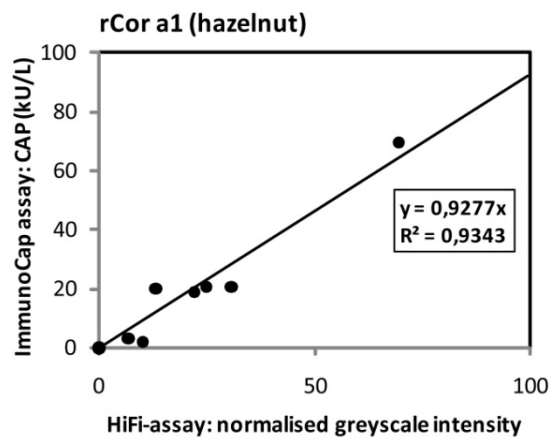
Supplementary Figure 4 | Quantitative detection of peanut allergen/rabbit IgG interaction

The immunoassay was performed using 8*6 arrays of replicates spots of 2.5 nL drops of a 1 mg.mL⁻¹ solution of peanut allergen. Alkaline phosphatase-labelled polyclonal anti-rabbit IgG antibodies were used at a concentration of 0.12 µg.mL⁻¹. The dot line indicates limit of detection.



Supplementary Figure 5 | Comparison of background levels on the inside and outside of wells (HiFi-allergy diagnosis assay)

HiFi filtering protocol enables a drastic reduction of background on the external side of the membrane, leading to highly improved signal to noise ratio of the stained membranes.



Supplementary Figure 6 | Correlation between immuno HiFi-assay and ImmunoCAP results for 2 allergens