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A vertical flow paper-microarray assay with isothermal DNA amplification for detection of *Neisseria meningitidis*

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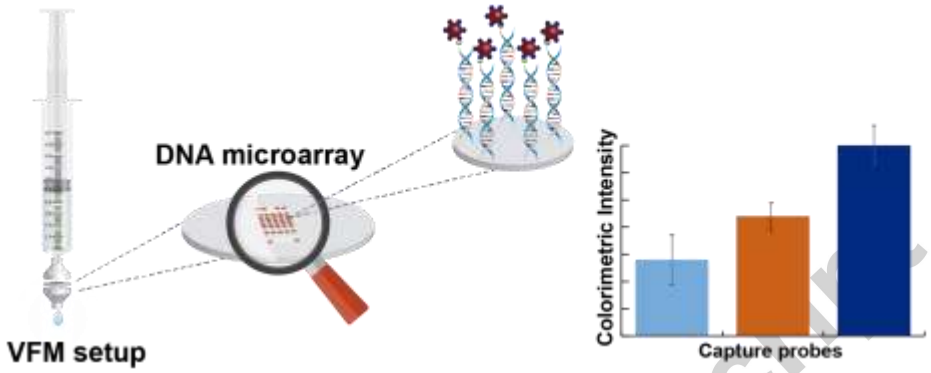
Abstract

Paper-based biosensors offer a promising technology to be used at the point of care, enabled by good performance, convenience and low-cost. In this article, we describe a colorimetric vertical-flow DNA microarray (DNA-VFM) that takes advantage of the screening capability of DNA microarrays in a paper format together with isothermal amplification by means of Recombinase Polymerase Amplification (RPA). Different assay parameters such as hybridization buffer, flow rate, printing buffer and capture probe concentration were optimized. A limit of detection (LOD) of 4.4 nM was achieved as determined by tabletop scanning. The DNA-VFM was applied as a proof of concept for detection of *Neisseria meningitidis,* a primary cause of bacterial meningitis. The LOD was determined to be between 38 and 2.1×10^6 copies/VFM_{assay}, depending on the choice of DNA capture probes. The presented approach provides multiplex capabilities of DNA microarrays in a paper-based format for future point-of-care applications.

Graphical Abstract



Keywords: microarray, paper-based biosensor, point-of-care, gold nanoparticles, *Neisseria meningitidis*.

1. Introduction

Nucleic acid testing (NAT) comprises different technologies for the detection and characterization of DNA and RNA. The main applications have been oriented towards the fields of biotechnology and medicine; from gene expression profiling to clinical diagnostics.[1,2] For instance, DNA microarray technology has been established as a multiplexed and high throughput tool for screening large-scale genomic data sets in the same experiment (*e.g.* gene expression analyses and single nucleotide polymorphisms detection).[2,3] NAT also plays an important role in clinical applications where such techniques allow rapid, specific and sensitive detection of pathogens.[4,5] Despite the wide implementation of these technologies, they are typically performed in centralized laboratories due to the need of sophisticated detection equipment and skilled personnel. Such requirements limit their application in near-patient and low-resource settings. Nevertheless, in recent years, many efforts have been devoted to redeveloping NAT technologies towards rapid and field-friendly point-of-care (POC) devices, with high usability to clinicians and other end-users in decentralized clinical settings.[6,7]

In this context, paper as substrate has gained popularity due to its low-cost, ubiquitous availability, biodegradability, biocompatibility, and ease to which chemical reagents may be deposited to create microfluidic channels.[8,9] Lateral flow assays (LFA) are the prime example of paper-based POC devices that have been used for decades in multiple fields, such as: human and veterinary health care, food safety, environmental control, among others. LFAs have numerous advantages as POC tests, even though their sensitivity and multiplexing capability are still limiting factors.[10,11] In efforts to couple the advantages of these devices with multiplex detection, several studies have reported lateral flow microarray assays for protein[12,13] and DNA detection.[14] Other examples of POC such as: flow-through or vertical flow assays have also been described in the literature. In these devices, analytes pass vertically through a (bio)functionalized porous membrane into an absorbent paper or are pulled

through by external forces (*e.g.* vacuum, manual pressure). High-throughput flow-through multiplexed assays have been reported as an alternative for ELISA tests.[15–17] In addition, tridimensional paper microarrays created by means of wax-printed patterns on stackable membranes have been described. [18,19] Further, alternative vertical-flow microarrays have recently been investigated in our group to avoid downstream effects, control the reagent flow speed and enable more rapid detection as compared to diffusion-based techniques.[20,21]

Paper-based POC tests could be useful in early diagnostics of acute infectious diseases in the central nervous system. One example is meningitis, a severe inflammatory process affecting the membranes (meninges) that cover the brain and spinal cord, caused by a disseminated infection by virus (*e.g.* Herpes Simplex Virus), parasite (*e.g.* Plasmodium falciparum), or bacteria (*e.g. Neisseria meningitidis -N. meningitidis*). In the case of acute bacterial meningitis, clinical symptoms of such an infection can be discrete and non-specific, and if left untreated, can result in death or disability.[22]

According to the World Health Organization, during the last twenty years, 900.000 suspected cases of bacterial meningitis were reported in Sub-Saharan Africa, with a mortality rate of up to 10%.[23] It is critical to distinguish between viral and bacterial causes in order to determine appropriate treatment plan and care level. Nucleic acid amplification methods (*e.g.* thermal cycling-PCR- and isothermal reactions) have been used as alternative to culture-based methods for bacterial meningitis detection.[24–26] Recombinase polymerase amplification (RPA) is emerging as an attractive isothermal method for use in the field, because of its minimal instrumentation, single operating temperature (*e.g.* body heat), and shorter amplification times (*i.e.* 15-40 min) resulting in comparable performances with PCR.[27–29]

Here, we present a colorimetric vertical-flow DNA microarray (DNA-VFM) for detection of *N. meningitidis* as a proof of concept. Our approach relies on the isothermal amplification of a nucleic acid combined with colorimetric microarray detection by using capture probes complementary to different segments of the amplified target gene.

2. Materials and methods

2.1. Reagents and instrumentation

Amersham Protran 0.1 µm nitrocellulose membrane and all the reagents for preparing the buffers: Phosphate-buffered saline (PBS, pH 7.4), Saline Sodium Citrate (SSC, pH 7.0), Tris Buffer Saline (TBS, pH 7.6), betaine and glycerol as additives for printing buffers, Bovine Serum Albumin (BSA), and Sodium dodecyl phosphate (SDS) were purchased from Sigma Aldrich (Germany). All solutions were prepared with mQ water produced by MilliQ-system (>18.2 MΩ/cm) purchased from Millipore (Sweden). 40 nm monoclonal anti-biotin gold nanoparticles (AuNPs) were purchased from BBI Solutions (UK). Lambda Exonuclease, storage buffer for DNA, Tris-EDTA buffer (TE, pH 8.0), and UltraPure™ Agarose-1000 were provided by Thermo Fischer (Sweden). Primers and hybridization probes were purchased from Biomers (Germany). Recombinase polymerase amplification (RPA) was used as isothermal DNA amplification method and a TwistAmp® Basic kit was purchased from Twistdx (UK). DNA purification kit, MinElute Reaction Cleanup Kit, was provided by Qiagen (Sweden). Nano-Plotter GeSim NP 2.1 (Germany), a robotic printer, was used for printing the microarray on the nitrocellulose membrane. A PHD ultra syringe pump, was provided by Harvard Apparatus (Sweden) for controlling the flow-rate on the vertical-flow assay. Ultra-violet (UV) crosslinker Hoefer UVC500 (USA) was used to attach DNA onto nitrocellulose membranes. A flatbed scanner, CanonScan 9000F Mark II (Canon), was used for scanning the microarrays.

2.2. DNA sequences: Primers and capture probes design

We used an *in silico* approach to design RPA primers and capture probes specific for the *ctrA* gene,[25] a gene that is specific for *N. meningitidis*.

Sequences of different *N. meningitidis* strains were screened with Blast[30] and then aligned using Jalview, a bioinformatic tool.[31] Based on the multi-aligned sequence (*i.e.* most conserved region), primers were designed using PrimerBLAST,[32] following the recommendations provided by the manufacturer of the RPA amplification kit. In addition, the most conserved region worked also for designing the capture probes by means of the probe design free software Picky.[33] Secondary structures of capture probes were calculated by Mfold, a web-based software simulating experimental conditions (25°C; 0.150 M [Na^+]). For the development and optimization of the DNA-VFM, we used synthetic sequences (target and hybridization probe) already available in our lab (see **Table 1**).

Specificity of probes and primers towards *N. meningitidis* was assessed with *Haemophilus influenzae (H. influenzae),* another causative pathogen of meningitis.

2.3. Microarray printing

Capture probes were printed on the nitrocellulose membrane using the Nanoplotter where 4 nL (10 droplets/spot) of oligonucleotide solutions were spotted at several concentrations. After printing, membranes were dried at room temperature, and exposed to 254 nm UV light with a total energy imparted of 125 mJ/cm^2. To minimize non-specific bindings, membranes were blocked by immersion in a BSA 3% aqueous solution for 15 min, followed by two washing steps with PB 5 mM, 0.05 % Tween-20 for other 15 min each, and finally membranes were used or stored at room temperature until use. In addition, printing buffer was supplemented with additives to improve the printing conditions, as well as the signal-to-background values. A non-related biotinylated sequence was used as positive control. The same sequence with an amine group instead of biotin was used as negative control.

2.4. DNA extraction

N. meningitidis (CCUG 3269) and *H. influenzae* (CCUG 23946) were inoculated on GC-agar and was cultured for 2 days according to standard procedures at Dept. Clinical Microbiology, Akademiska Sjukhuset, Uppsala (Sweden). Bacteria were suspended in physiological NaCl to a density between 51-107 MacFarland determined in dilutions by DensiCHEK™ Plus. Bacterial suspensions were frozen 1:1 in freezing buffer at -70 °C. DNA was extracted from 3 x 200 µL of undiluted bacteria suspension by Qiagen's MagAttract DNA Mini M48 Kit. The concentration of pooled DNA was determined by NanoDrop® ND-1000 spectrophotometer. The measured concentration was used to calculate the copy number by using a web-based DNA calculator.[34] Extracted DNA was diluted and analyzed as template in real time-PCR.[35] The genome copy number was correlated to crossing points in PCR to enable a quantitative determination.

2.5. Isothermal amplification: Recombinase Polymerase Amplification (RPA)

RPA reactions were performed according to the protocol suggested by the manufacturer. A RPA reaction mixture contained: 2.4 µL 5'-biotin-labeled forward primer (10 µM), 2.4 µL 5'-phosphate-labeled reverse primer (10 µM), 29.5 µL supplied rehydration buffer, and 8.2 µL nuclease-free water. Dried pellets were rehydrated with 42.5 µL of reaction mixture and 5 µL of template. The resulting mixture was mixed and centrifuged and 2.5 µL of magnesium acetate (280 mM) were added to the lids of the tubes. They were closed and centrifuged to spin the magnesium into the reaction mixture and initiate the amplification. Then, the tubes were vigorously inverted 10 times to mix, and were centrifuged again. The reactions were incubated at 37°C for 20 min, without further agitation. Once the reaction was finished, RPA products were purified and verified via gel electrophoresis, using 2% UltraPure Agarose-1000 in TAE 1X buffer at 150 V for 60 min.

2.6. Single-stranded DNA (ssDNA) generation

To generate ssDNA, purified RPA product (40 µL) was digested in a final volume of 50 µL with 1U Lambda exo-nuclease at 37°C for 30 min, followed by heat deactivation at 80°C for 10 min. The reaction mixture was then diluted up to 500 µL with buffer for further detection in the microarray.

2.7. DNA vertical-flow microarray (DNA-VFM):

A 1.3 cm-diameter nitrocellulose disk containing the printed microarray was placed into a polypropylene filter holder (Sterlitech, USA), coupled with a 1 mL syringe, and a syringe pump was used to control the flow-rate of the different assay solutions. In brief, two main steps occur during the DNA-VFM assay: hybridization and colorimetric labeling, both performed at room temperature (approx. 23-25°). First, 500 µL of the selected buffer at 1 mL/min was added to wet the membrane. Then, hybridization was performed using 450 µL biotinylated synthetic DNA solution at several concentrations (in the case of amplified product, 50 µL digested dsDNA in 500 µL running buffer) at 150 µL/min, followed by a washing step with 1 mL of the selected buffer at 1 mL/min. After washing, a solution of monoclonal anti-biotin coated AuNPs 1:4 (optical density $\approx$ 2.5) was used as colorimetric label (450 µL, 1 mL/min). Finally, a last washing step to remove the unbound nanoparticles was performed. Once the DNA-VFM assay was done, the membrane was dried for 10 min at room temperature, and then scanned in a flatbed scanner in 16 bits grayscale.

2.8. Data analysis

Analyses of scanned images were performed with GenePix Pro 5.1 software (Axon Instruments). The mean spot intensity ($n \geq 3$) subtracted by the local background was used for data. When was needed, signal-to-background ratios (SBR) were calculated by dividing the corrected intensity of each spot with the mean of background spot intensities of the array. Image J was used in order to

generate 3D surface plots. GraphPad Prism software was used for fitting non-linear curves.

3. Results and Discussion

3.1. Principle of the assay

Our approach consists of a DNA-VFM that bridges the high-throughput screening of oligonucleotide arrays with isothermal DNA amplification and colorimetric read-out. Briefly, the principle of the assay is based on convection-controlled hybridization between two nucleic acids strands on a paper microarray within a filtration holder in a cross-flow manner. Within this holder, the amplified target (in solution) binds the capture probes immobilized on a piece of unbacked nitrocellulose, with subsequent colorimetric labeling and detection using a regular flatbed scanner (see **Scheme 1**).

<Insert Scheme 1> in color

3.2. Optimization of the assay

In a DNA microarray, a number of factors may affect the analytical signal, such as: length and specificity of the probes, annealing time, hybridization temperature, and buffer composition, among others. Therefore, several parameters were systematically optimized to improve the sensitivity of the assay as follow:

3.2.1. Probe immobilization

It is well known that nucleic acids can be covalently attached to nitrocellulose by means of UV irradiation.[36] In this experiment, the hybridization performance of the unlabeled capture probe and its 5'-amino-labeled version was evaluated as

shown in **Fig. 1**. Results after UV exposition showed that the sensitivity of the 5'-amino-labeled capture probe was higher compared with unlabeled analogous probe. Although the crosslinking mechanism is not yet fully understood, it has been proposed that a covalent bond is formed due to the generation of reactive functional groups within the bases of DNA that react with those located on the surface of the membrane.[37,38] The presence of an amino group coupled with a spacer at the 5'-end of the capture probe favors its anchoring onto the nitrocellulose and allows improved hybridization as well since the probe is not sterically hindered, thus enhancing the sensitivity of the assay.[39] This effect has also been reported in an aptamer lateral flow assay.[40]

<Insert Fig. 1>

3.2.2. Hybridization: buffer and flow rate

Buffer compositions can affect the hybridization by alteration of salt concentrations or addition of denaturants. In this study, two types of buffers were evaluated for hybridization: SSC and TBS. A slightly modified recipe reported by Carter *et al.*[14] was used in preliminary experiments. Briefly, this buffer consisted in SSC at several concentrations (*i.e.* 1X, 2X, 4X, 8X and 16X) supplemented with 0.1% SDS and 5% deionized formamide. When SSC was used as buffer, higher SBR were obtained for lower concentrations and 2X was found to be the optimal concentration (see **Fig. 2A**). Despite being one of the most common buffers for hybridization assays, this formulation still resulted in high background signal in our experiments (*i.e.* pinkish background), thus TBS was considered as an alternative. Tris buffer is commonly used in molecular biology to preserve nucleic acids structures and avoid their degradation. As shown in **Fig. 2B**, when comparing both buffers, a lower background signal was obtained using TBS compared to SSC 2X, resulting in a higher SBR values for TBS. This could be due to hybridization being favored at high stringency conditions for complementary sequences and that the presence of a non-ionic

surfactant (*i.e.* Tween-20) provided a better flow of gold nanoparticles avoiding unspecific adsorptions.[41,42]

Flow rate was controlled by a syringe pump and optimized to increase the sensitivity. Three different flow rates were tested: 1000, 150 and 45 µL/min that resulted in hybridization times of 30 s, 3 min and 10 min, respectively (see **Fig. 2C**). As was expected, a high flow rate did not allow a proper hybridization between the target and the capture probes showing lower hybridization signals. On the other hand, assays conducted at lower flow rates of 150 and 45 µL/min, showed higher signals, likely due to longer hybridization times of 3 and 10 min, respectively. The effect on the sensitivity produced by the different flow rates was noticeable at very low capture probe concentration. However, at higher capture probe concentrations, flow rates of 150 and 45 µL/min gave rise to similar signal intensities. Therefore, a flow rate of 150 µL/min was chosen for the hybridization step of the remaining experiments in order to avoid compromising the sensitivity of the assay.

<Insert Fig. 2>

3.2.3 Printing conditions

Hybridization performance depends also on the morphology of the spot, which can be affected by printing conditions such as: relative humidity, capture probe density, printing buffers, as well as the printing method.[43–45]

In typical DNA microarray printing processes on glass slides, the relative humidity recommended is around 50-65% in order to slow down the evaporation rate of the droplet.[37] However, in this work, the substrate used (unbacked nitrocellulose) tended to curl when exposed to high relative humidity conditions, causing difficulties when printing. Consequently, a dryer environment with relative humidity was needed, and levels ranging 35-40% were determined as optimal.

As shown in **Fig. 3A-B**, spot morphology can be strongly affected by relative humidity and the addition of reagents that delays the evaporation of the droplet.

<Insert Fig. 3> in color

In early experiments, capture probes were printed with pure TBS buffer at low relative humidity levels, a “coffee-ring effect” was observed. In these ring-shaped spots, the signal intensity is higher at the edges due to the DNA accumulation, likely produced by the evaporation of the solvent, which makes the DNA molecules flow outwards with the fluid flow to compensate the evaporation losses.[46,47]

Therefore, two additives were tested in the printing buffer (TBS) for delaying the drop evaporation at low relative humidity: glycerol 20% and betaine 1.5 M (see **Fig. 3C**). Glycerol and betaine have been used for increasing the viscosity of the printing solutions in protein and DNA microarrays, thus reducing the evaporation rate of the droplet.[48]

Results showed that the addition of the mentioned additives, especially betaine, improved the morphology of the spot and increased the signal intensity (**Fig. 3A-B**). It has been reported that betaine reduce the gap between the stability of AT and GC base pairs during hybridization, leading to lower melting temperatures regardless of the GC content.[49] This effect jointly with the ability to increase the viscosity of the printing solution has been reported to produce highly homogenous DNA microspots in glass slides.[44] Therefore, for the remaining experiments, TBS supplemented with 1.5 M betaine, was used as printing buffer.

Capture probe concentrations were optimized to maximize the colorimetric signal intensity of the DNA-VFM assay. At high concentration, hybridization may be affected by steric hindrance and electrostatic forces of the immobilized probes, and consequently an incomplete hybridization could occur resulting in false positive signals. On the other hand, at low capture probe concentration, the signal strength would be reduced affecting the sensitivity and dynamic range of the assay, and the background might increase due to non-specific

adsorptions.[50] Therefore, based on the optimized conditions a calibration curve was done using several capture probe concentrations ranging from 1 to 80 µM, and serial dilutions of synthetic DNA target to establish the optimal capture probe concentration and achieve the lowest possible limit of detection.

As shown in **Fig**. **4B**, a saturated signal was observed at 40 µM of capture probe at different target concentration. Hence, this concentration was chosen for subsequent experiments (combined with the previously optimized parameters) to ensure the highest hybridization signal (see **Table S1**).

The colorimetric signals from the calibration experiment were fitted by means of non-linear regression (Four Parameter logistic equation, 4PL) using GraphPad Prism Software. The limit of detection (LOD) was defined as the mean value of the blank plus three times its standard deviation, giving a value of 4.4 nM in less than 8 minutes. Compared to other reports, the obtained LOD is comparable to LFAs using gold nanoparticles[51] or dye-encapsulated liposomes[52] as labels. Other researchers have presented lower LOD in LFA format including those with microarrays,[14] but some required the use of signal enhancement strategies.[53,54]

<Insert Fig. 4> in color

3.3. Detection of isothermally amplified bacterial DNA

3.3.1 N. meningitidis detection

The *ctrA* gene is restricted to *N. meningitidis* and is highly conserved in all meningococcal serotypes, making it useful for molecular diagnostics.[55] Variants of PCR[25,56,57] and loop-mediated isothermal amplification (LAMP),[58–61] have been reported for detection of *N. meningitidis*. Our choice for RPA was based on its ease of use and high performance at low operating temperatures, both suitable for future point-of-care applications. To the best of our knowledge, *N. meningitidis* detection by means of RPA assays has not been

previously reported. Different primer sets were designed and screened to amplify the *ctrA* gene of the target pathogen (data not shown) and the best primer pair amplified a segment of 146 bp of the most conserved region of the gene, under optimized RPA conditions as follows**.** Since RPA has been demonstrated to amplify DNA isothermally between 7-35 min giving similar performances to PCR,[29] we followed the standard protocol suggested by the manufacturer and optimized the amplification time for our experiments. Several assay times (5, 10, 20 and 30 min) were evaluated and samples were analyzed *via* agarose gel to compare the intensities of the bands, showing that 20 min is an optimal assay time to amplify DNA with minimal artifacts or false positive signals (see **Fig. S1**).

Once the dsDNA product was amplified and purified, ssDNA generation was needed for the DNA to hybridize to the microarray capture probes. Among the different methods to produce ssDNA, thermal denaturation is the most common because of its simplicity and low cost, albeit exhibiting low reproducibility. On the other hand, lambda-exonuclease despite of increasing costs and assay time, has been reported as a reliable method for generating ssDNA from purified dsDNA,[62] hence was selected for our experiments. Briefly, in presence of lambda exonuclease, the 5'-phosphorylated strand in the isothermal amplified dsDNA product was selectively digested by the enzyme, with the remaining 5'-biotinylated strand then available for hybridization to the probes attached on the membrane.

Our array consisted of six capture probes (see **Table 1**), which were designed to exhibit affinities across the amplified sequence to increase the probability to detect the target (**Fig. S2**). Several bacterial DNA concentrations were amplified by RPA, visualized by gel electrophoresis (**Fig. S3**), and also analyzed in our vertical-flow setup as depicted in **Fig. 5A**. In this case, the blank signals were subtracted from the sample signals to obtain net intensity values that were plotted against the DNA concentration in the VFM assay (**Fig. 5B**).

<Insert Table 1>

<Insert Fig. 5> in color

Although the capture probe length has been reported as an important factor on the sensitivity of DNA microarrays,[63,64] this effect was not evaluated due to the small panel of probes retrieved by the probe designer software. Nonetheless, results revealed a large variability in performance of hybridization and we observed that shorter probes consisting of 22 bp (*i.e.* Probe 1 and Probe 5) resulted in poor or negligible signals at high DNA copy number, while probes ranging between 25 and 60 bp displayed higher intensities. The colorimetric signals of the capture probes were fitted in non-linear regression curves to obtain their LOD, which resulted in 993 copies/VFM_{assay} for Probe 1 (R^2=0.968), 467 copies/VFM_{assay} for Probe 2 (R^2=0.905), 297 copies/VFM_{assay} for Probe 3 (R^2=0.952), 38 copies/VFM_{assay} for Probe 4 (R^2=0.997), and 91 copies/VFM_{assay} for Probe 6 (R^2=0.982). The worst performing was Probe 5, obtaining a LOD of $2.1x10^6$ copies/VFM (R^2=0.951).

Another factor that could influence the extent of hybridization is the formation of secondary structures as this may impede the accessibility to the target. While the hybridization event in solution differs from the one on the solid-phase, probe folding predictions could give an idea of the possible structures formed at certain experimental conditions. For this reason, folding *in-silico* analyses were conducted to check if there could be any correlation to our findings (**Table S2**). It was expected that Probe 3 and Probe 5, which had higher negative free energy (ΔG) values exhibit poor performances since their secondary structures were more stables, but only Probe 5 showed negligible signals compared with Probe 3 (**Fig. S4**). While all capture probes displayed loops at different locations that might influence on the hybridization efficiency as reported by Ermini *et al.*,[65] only Probe 5 was predicted to form a closed structure along the whole sequence that could have impeded the formation of the hybrid. This could be due to the short length of the probe and the presence of G and C bases on the terminals contribute to its stability (*i.e.* high ΔG_{loop}). When comparing Probe 5 and Probe 3, the latter one has two loops (one of them with the same ΔG_{loop} value of the Probe

5) and the main difference between them is that Probe 3 has a larger length and there is still a free segment on the probe capable to hybridize with the target.

The efficiency of the hybridization for a given capture probe with its complementary target cannot be predicted based solely on individual parameters related with the probe itself, such as length, sequence and thermodynamic properties. On the contrary, we observed that these factors, including a proper probe design, work in synergy with those associated to printing conditions in order to get the maximal hybridization signal.

In this work we demonstrate the feasibility of amplifying meningococcal genomic DNA at 37ºC in just 20 minutes, obtaining limits of detection ranging between 38 and $2x10^6$ copies/VFM_{assay} (500 µL) by using capture probes with different hybridization features. Low-cost hybrid microfluidics devices have been shown to allow successful detection of up to 3 copies/reaction (25 µL) in single[61] and multiplexed[66] LAMP assays. Lee *et al.* reported a LAMP assay for the detection of *N. meningitidis* in clinical samples obtaining a LOD of 10 copies/reaction (25 µL) in 60 min., The reported LAMP assays were considerably more sensitive than conventional PCR.[60] In comparison, LAMP may be somewhat more sensitive compared with RPA for *N. meningitidis* detection. Although LAMP is a well-established and highly specific isothermal amplification technique, RPA shows good performances in shorter reaction times (around 20 min) and does not require additional primers sets or higher temperatures to amplify DNA.

3.3.2 Reproducibility and specificity of the DNA-VFM set up

To assess the reproducibility of the biosensor, the assays were repeated three times, performed both on the same, and on different days. The inter-assay coefficient of variation (CV) at the optimal probe concentration varied between 2.8 and 12.8% on the same day. Inter-assay CV on different days were higher, around 20% and possibly due to the unreliable printing and environmental conditions. This might be due to the fact that factors such as relative humidity and temperature if are not properly controlled could affect the sensitivity on

paper-based assays as previously reported.[51] Further improvements are being considered to obtain a better reproducibility between assays.

Specificity of the selected *N. meningitidis* capture probes was investigated against a conserved and amplified sequence of the protein D gene (*hpd*) restricted to *H. influenzae*. Here, genomic DNA from both bacteria were amplified separately under optimized conditions and tested in the DNA-VFM as shown in **Fig. S5**. Results showed that only specific primers for *N. meningitidis* were capable to amplify their respective genomic DNA, showing higher colorimetric signal intensities. On the other hand, *hpd* amplified sequence did not hybridize to the *ctrA* probes printed on the array, indicating the high specificity of these capture probes towards *N. meningitidis*. When *ctrA* primers were used to amplify *hpd* gene, only Probes 1 and 4 displayed negligible signal intensities; while Probes 2, 3 and 6, which exhibit a high sequence identity (see **Fig. S2**) showed cross-hybridization. The same trend was also found in the blank for *N. meningitidis* detection by vertical-flow DNA microarray (see **Fig. 5A**).

Finally, while sensitivity and variability measures could likely improve with further optimization, the presented assay could offer a useful alternative for detection of *N. meningitidis* at clinical relevant levels because of its multiplexed capability in a paper-based format for its use in low-income countries. Future work in our group will focus on investigating improved RPA conditions and signal enhancement strategies for increasing the sensitivity, as well as extending this approach to detect multiple targets.

4. Conclusions

A multiplexed colorimetric DNA vertical-flow paper-based microarray was successfully developed and applied for detecting bacterial DNA. The presented biosensor was based on hybridization of isothermally amplified DNA to an oligonucleotide microarray printed on a low-cost paper-based substrate appropriate for point-of-care testing. Different parameters such as: probe immobilization, hybridization and printing conditions were optimized obtaining a

LOD of 4.4 nM of synthetic DNA. The multiplexed system was successfully applied for detection of *N. meningitidis*, by using six capture probes at different locations of the amplified sequence, allowing to detect down to 38 copies/VFM_{assay}, and using RPA as an isothermal alternative method to conventional PCR.

This approach could contribute to a new generation of multiplexed field friendly biosensors for improving the diagnostic precision for infectious diseases and their applicability in low-resources settings.

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Appendix A. Supporting Information

DNA sequences and optimized conditions used in this work, as well as agarose gel images, capture probe specificity, folded structures of capture probes and their thermodynamic details are listed on the supplementary material.

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Figure and table captions

Scheme 1. DNA-vertical-flow microarray procedure

Figure 1. Effect of labeling capture probe immobilization under UV exposition at 1 nM of synthetic target concentration.

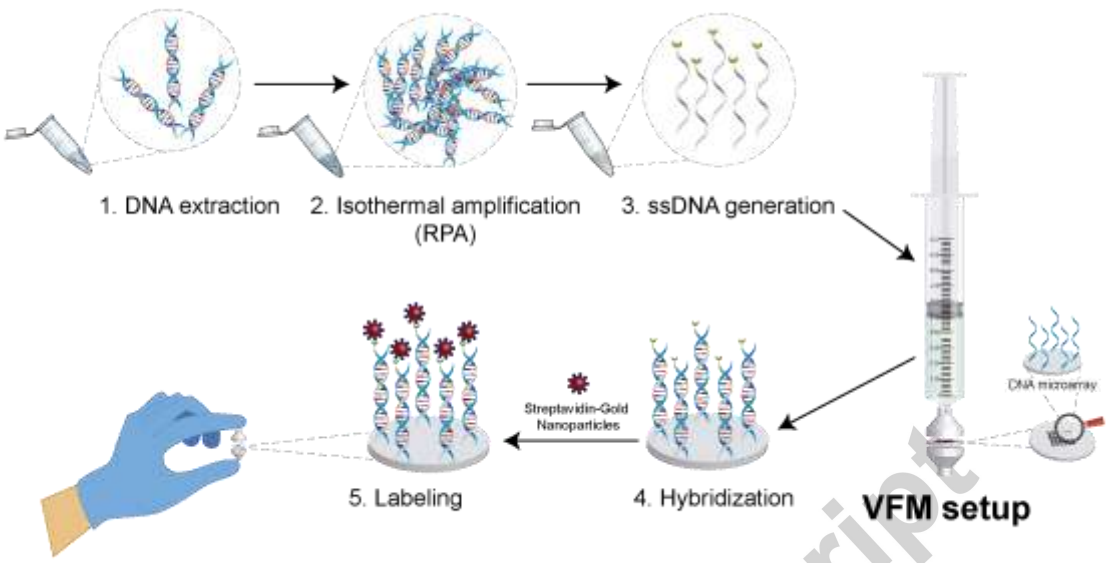
Figure 2. Hybridization buffers: (A) SSC concentration; (B) comparison between buffers; and (C) flow rate TBS buffer.

Figure 3. Printing buffers assays: (A) with and without additives; (B) 3D surface plot for capture probe in different buffers, and (C) comparative bar graphic of printing buffer.

Figure 4. Capture probe and target concentration: (A) Schematic figure and picture of DNA-VFM at 100 nM of target; (B) capture probe optimization; and (C) calibration curve for target at optimal capture probe concentration, 40 µM.

Figure 5. *N. meningitidis* detection by vertical-flow DNA microarray. (A) Images of the DNA-VFM and (B) Performances of capture probes 1 to 6, both figures with varying concentrations of RPA amplified bacterial DNA

Table 1. Sequences for DNA-VFM optimization and detection of *Neisseria meningitidis*



Scheme 1

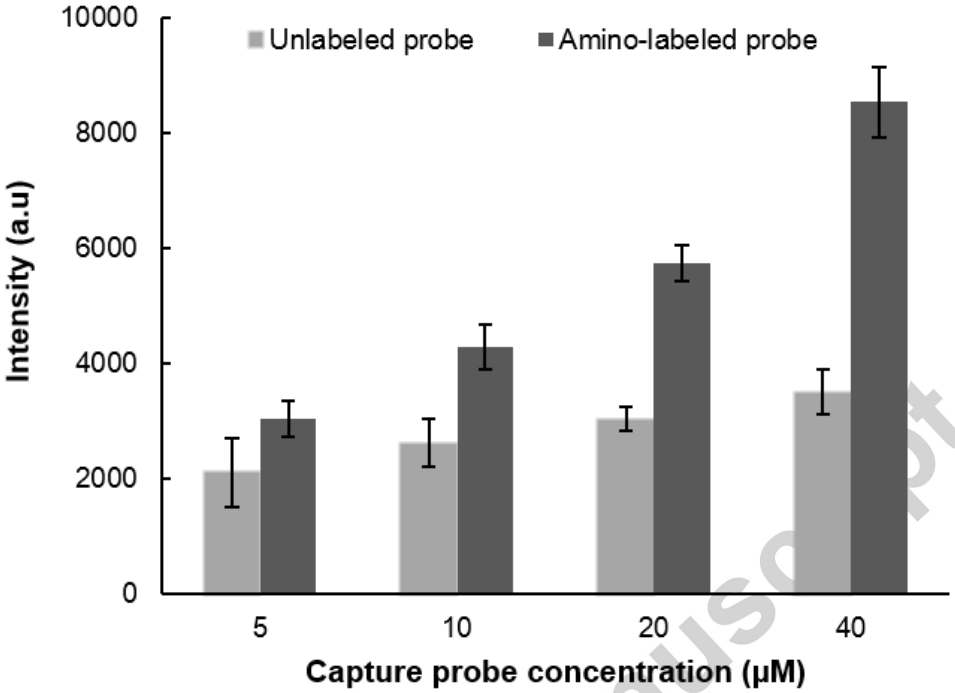


Fig 1.

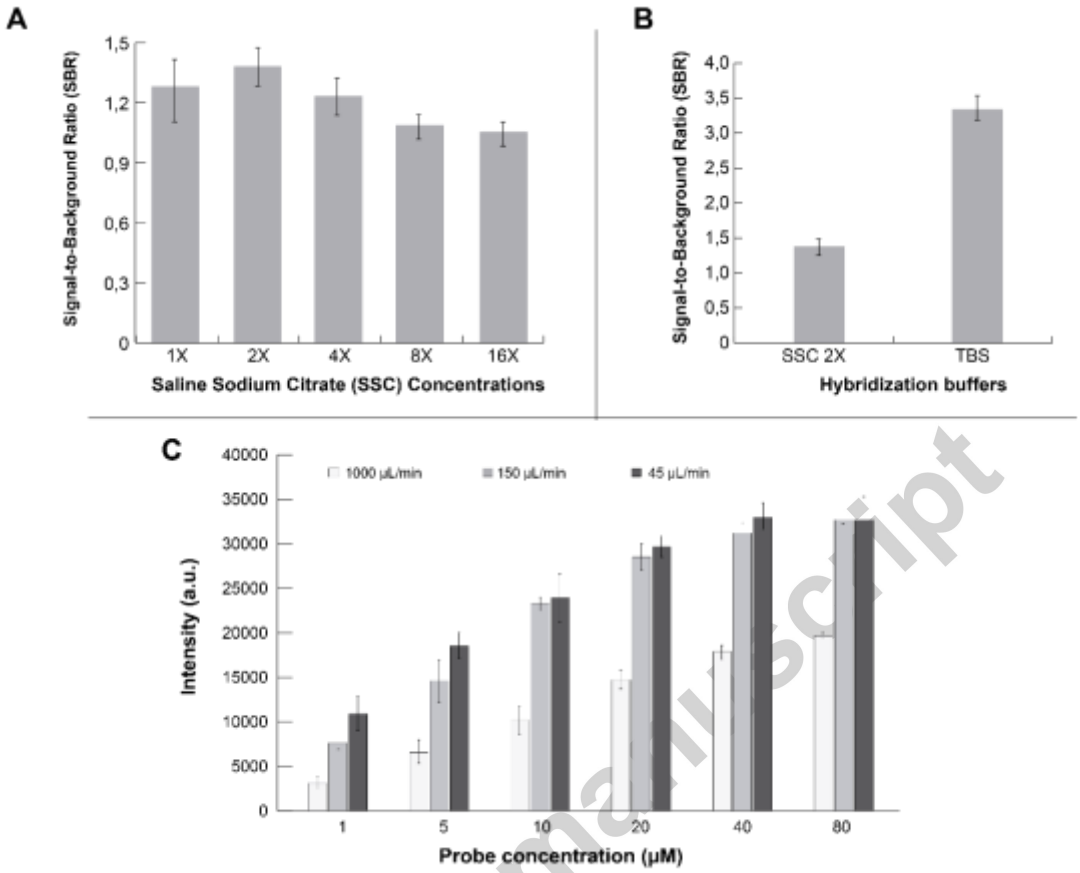


Fig. 2

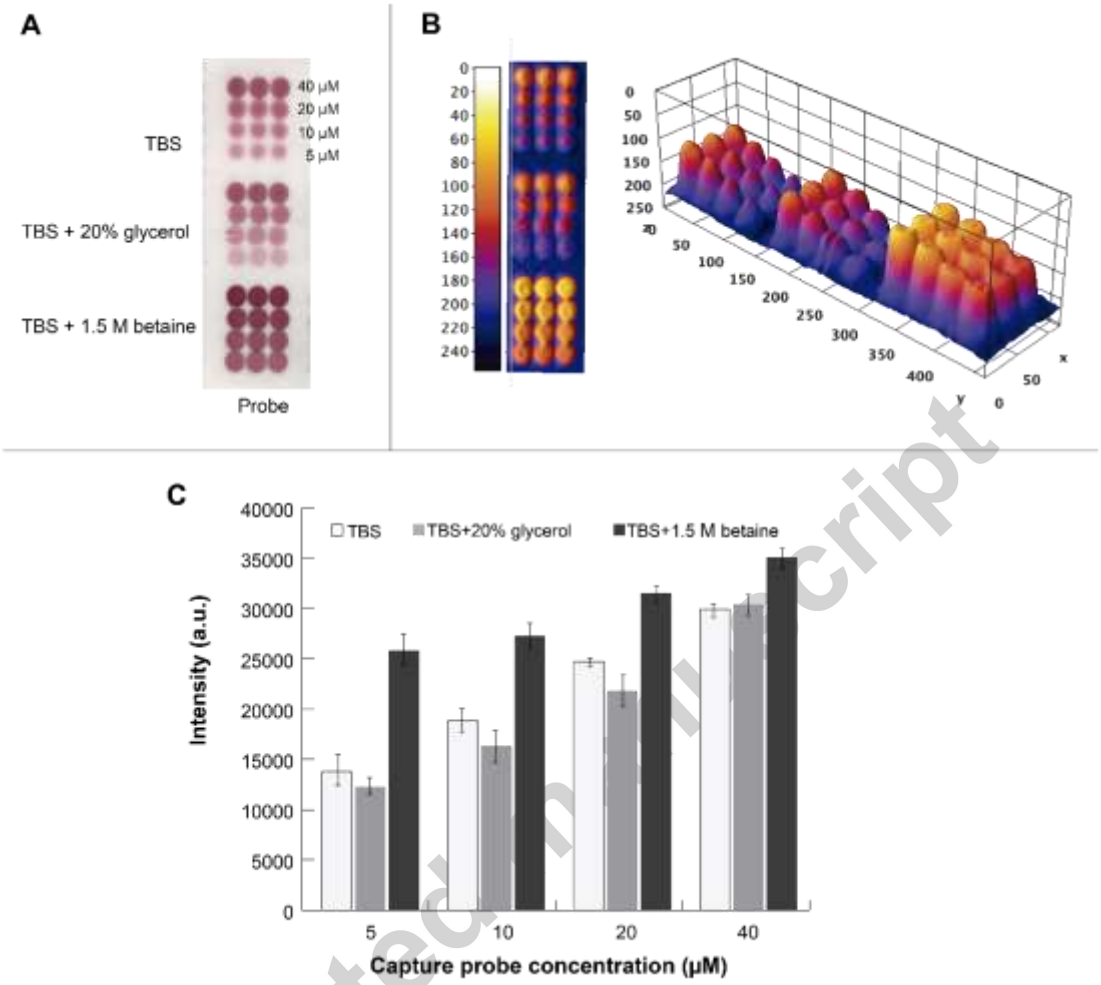


Fig. 3

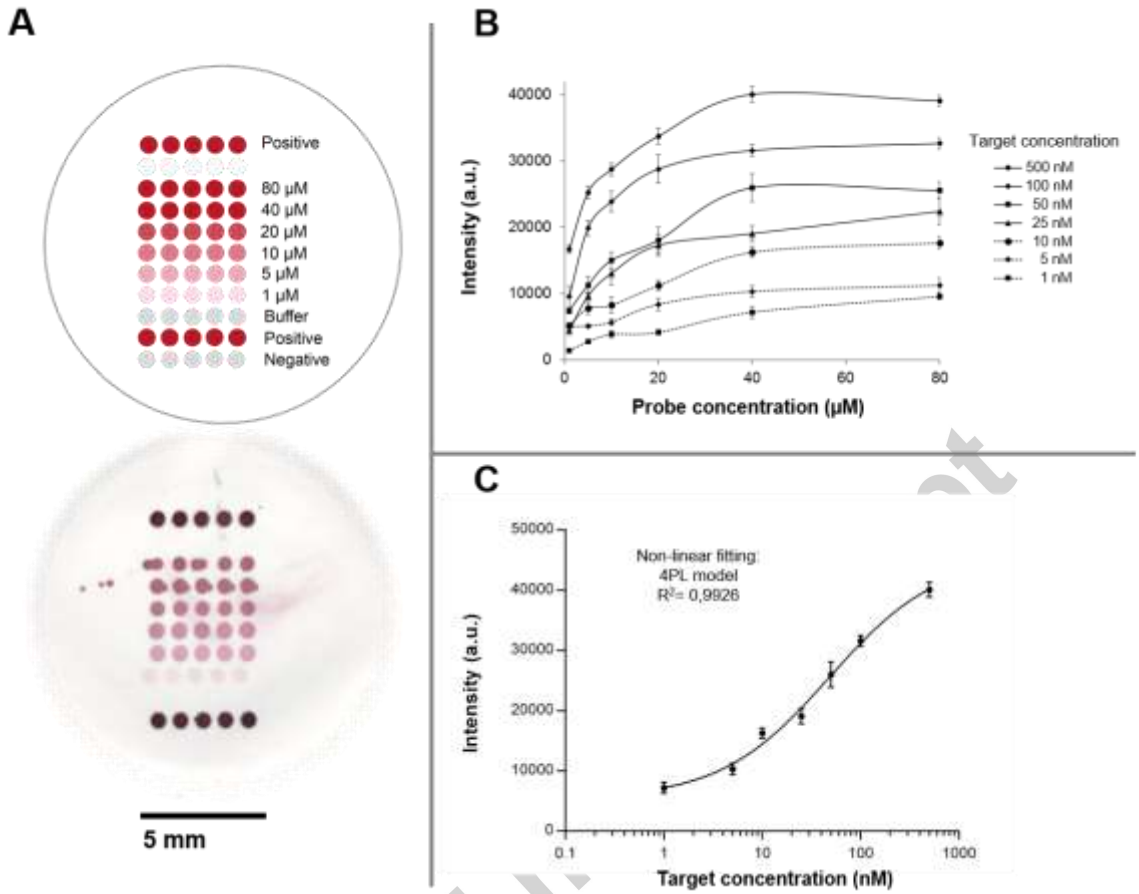


Fig. 4

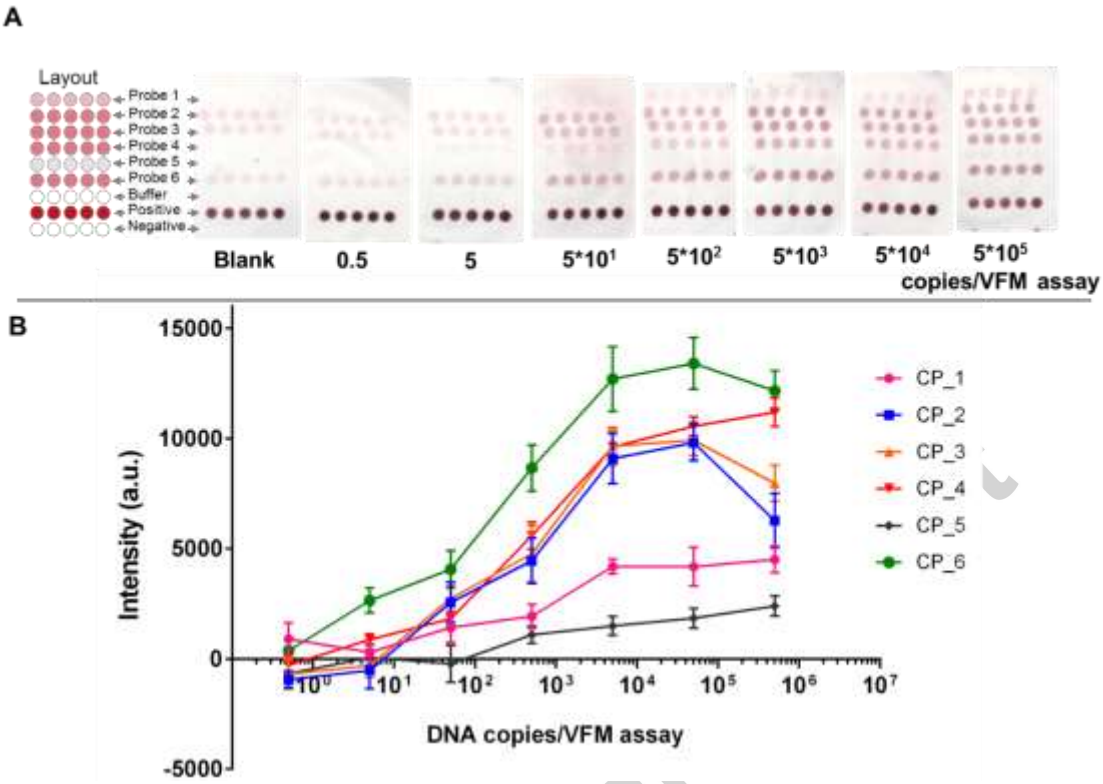


Fig. 5

Table 1.

Name	*Sequence (5' to 3')*	*Modification (5'-)*	*Length (bp)*
Synthetic Capture Probe	gcatctttacgcacggtgtaaggatgcacttccacattatat	Amino-C6	42
Synthetic Target	acaatataatgtggaagtgcatccttacaccgtgcgtaaagatgc actacccgcgtttttcacagacgtaaatcaaatgtatgatgcctta	Biotin-TEG	91
Forward Primer (*N. meningitidis*)	gtcaggataaatggattgctcaaggtta	Biotin-TEG	28
Reverse Primer (*N. meningitidis*)	cgcattcgacacatacaatacatcttta	Phosphate	28
Probe 1	cgcatcagccatattcacacga	Amino-C6	22
Probe 2	cgacacatacaatacatctttattcttcacaggaaagcgc		40
Probe 3	cgacacatacaatacatctttattcttcacaggaaagcgctgcata gaaaatagcgaatg		60
Probe 4	accgttggaatctctgcctcactgc		25
Probe 5	cgctgcatagaaaatagcgaat		22
Probe 6	cattcgacacatacaatacatctttattcttcac		34
Negative	tgtatttgtcttcgatgaggcccgt	Amino-C6	25
Positive		Biotin-TEG	
Forward Primer (*H. influenzae*)	acaatataatgtggaagtgcatccttac	Biotin-TEG	28
Reverse Primer (*H. influenzae*)	taaggcatcatacatttgatttacgtctgtg	Phosphate	31

Highlights

- A paper-based vertical-flow microarray for bacterial DNA detection is presented
- A limit of detection of 4.4 nM of synthetic DNA was achieved
- The array-based approach was applied for *Neisseria meningitidis* detection
- The best probe allowed detection of 38 copies/vertical-flow assay