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Development of a chemiluminescent DNA fibre optic genosensor to Hepatitis A Virus (HAV)

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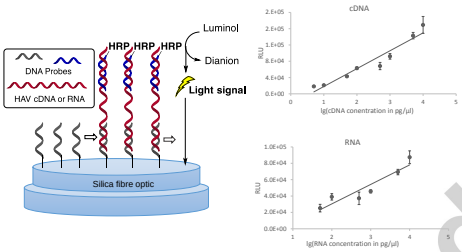
Abstract

Hepatitis A virus (HAV) infection has caused substantial morbidity and economic losses to human society, presenting a major public health problem in many parts of the world. Despite the capability for low-concentration detection, current PCR-based techniques are limited by the requirement of specialized lab equipment, trained personnel and a relatively large time-commitment. The need for a prompt in-field quantitative identification of HAV in real

samples has led us to develop a chemiluminescent fibre optic genosensor system. In this study, a two-probe sandwich-type hybridization process was implemented on the tip of a fibre optic with an area of 0.12 mm^2. After optimization of the probes and the working conditions, we showed that the biosensor was able to work for both cDNA and RNA with a relatively large signal/noise ratio and a good sensitivity. An excellent specificity was also confirmed by screening with a broad range of other pathogen samples. The nucleic acid probes method was validated by optimized PCR and qPCR, and may thus be used when field testing would be required.

Graphical abstract



Keywords:

Genosensor; DNA probe; Hepatitis A virus; Chemiluminescence; in-field detection; miniaturised device

1. Introduction

Hepatitis A virus (HAV) infection is responsible for around half of the total number of hepatitis infections diagnosed worldwide [1], presenting a major public health problem in many parts of the world [2]. HAV is a virus classified in the *Hepatovirus* genus within the *Picornaviridae* family [1]. It is a nonenveloped virus containing a positive-sense, single-stranded RNA genome of 7.5 kb which is polyadenylated at the 3′ end and has a polypeptide (VPg) attached to the 5′ end [3]. Studies show it has an estimated infectious dose of 10 - 100 particles [1]. HAV infection is a self-limiting disease [4, 5]. The signs of infection usually don't appear until a few weeks after exposures and in most cases, resolve within six months. The symptoms may include: fatigue, nausea, vomiting, abdominal discomfort, low-grade fever, joint pain and jaundice. The symptoms are typically more severe in older children and adults than in children less than six years old, in whom 70% of cases are asymptomatic [6, 7]. Although rare, cases of acute liver failure and death may happen, particularly in the elderly and in patients with pre-existing liver diseases [6, 8-12]. In the environment, HAV can survive for more than one month [13] and is reported to be resistant to many physical and chemical pressures [14-18]. This has enabled HAV to play a significant role in many outbreaks worldwide [1, 2, 6, 8, 10, 19-21] with 1.4 million new cases reported every year according to World Health Organization [5]. In most cases, HAV is transmitted via the fecal-oral route [6, 22], mainly through ingestion of contaminated water and/or food, particularly shellfish, fruits and salad [13].

In view of the morbidity and economic losses to human society, it is important to have a fast, sensitive and reproducible preventive method for timely detection of HAV in water and food samples. Traditional virus infectivity assays and immunoassays have been found to be unsuitable for HAV detection as they require long incubation times and the presence of virus

in the samples is generally of limited quantity [6, 22]. Instead, nucleic acid-based detection techniques have become the most popular methods for HAV detection due to their accuracy and sensitivity, such as with reverse transcriptase polymerase chain reaction (RT-PCR) [23], real-time quantitative PCR (RT-qPCR) [24-27] or nested real-time quantitative PCR [28-30].

To further improve the detection method in potential field-operations, which would be more effective for early warning, DNA fibre optic genosensors emerge as a promising candidate attributed to their various attractive advantages: (1) they offer the solid phase DNA probes, being highly specific, sensitive, cheap and easy to synthesize. Furthermore, in the proposed method, the specificity is further ensured by a two-probe system; (2) the unique features of fibre optics provide the possibility for further equipment miniaturization and offer the flexibility as a dispatch tool for most sites; (3) the absence for the need of PCR eliminates the usual inhibitive effect of polyphenol compounds to DNA polymerases reactions; (4) it is independent of thermal-cycler equipment and the operation is simpler hence not requiring well-trained lab personnel. The working principle of a fibre optic genosensor is illustrated in Figure 1 ((4) - (8)): a specific ssDNA probe (the capture probe or the 1st probe) is covalently immobilized onto the chemically functionalized surface ((1) - (3)) of the fibre tip, followed by a blocking step and thereafter a hybridization event with the complementary target DNA or RNA sequences. A second HRP-linked, ssDNA probe (the detection probe or the 2nd probe) which is complementary to a different region of the target sequence is then used for the generation of the chemiluminescent signal by an HRP-catalyzed oxidation reaction. Once the sandwich-type structure is assembled and exposed to the luminol/buffer mixture, a chemiluminescent signal is generated and immediately propagates through the fibre optic, which is finally collected by a photodetector connected to a computer. The previous similar approach to this technique was demonstrated by our group for *brettanomyces bruxellensis* detection [31], which demonstrated a significant difference between positive and negative

controls. However, there was still further room for reducing the lower detection limit (LDL) and of course applying the fibre optic technology to a wider range of pathogen detection. In the present work, specific probes were designed based on an HAV RNA sequence. The sensitivity and specificity of the samples were confirmed by a dot blotting method. The conditions for this genosensor system were optimized and the performance was evaluated for both RNA and cDNA targets. The cDNA samples were also validated by PCR and qPCR technique.

2. Experimental

2.1. Microorganisms

Samples of RNA extracted from pure cultures of HAV (HAV4, HAV5, HAV6) were used to produce cDNA by reverse transcription (RiboClone® cDNA Synthesis System, Promega) and the concentration was recorded by NanoPhotometer® (Implen, 7122 version 2.3.1). HAV cDNA and norovirus cDNA was then used as a positive sample and negative sample, respectively, to test the specificity and sensitivity of the primers and probes in the optimization of the design. DNA or RNA strands or proteins extracted from bacterial and viral samples, *Escherichia coli, Campylobacter lari DSM 11375, Pseudomonas aeruginosa, Aeromonas sobria, Listeria monocytogenes, Lactobacillus bulgaricus, Legionella pneumophila, Vibrio 14379, Lactococcus lactis, Morganella morganii, Proteus vulgaris, Bacillus subtilis, Staphylococcus aureus Pseudomonas putida*, Norovirus G1, Norovirus G2, Dengue virus and South Bay virus, were used as negative controls in probes' specificity tests.

2.2. Oligonucleotide sequences

Oligonucleotides for PCR and qPCR applications were designed (see 2.2.1) and probes for dot blot and biosensor applications were defined.

2.2.1. Primers for PCR and qPCR

Two sets of primers were designed by aligning sequences retrieved from the NCBI online repository with the ClustalW software, optimized using Primer3 [32] and tested with Primer-Blast tool. The primers for external amplification were: HAVEF-56 (5'-GGACTTGATACCTCACCGCC-3') and HAVER-684 (5'-CAAACACCACATAAGGCCCCA-3'). The primers for internal amplification were: HAVIF-274 (5' - GGGGTCAACTCCATGATTAGCA-3') and HAVIR-412 (5' - GGTTTCACCCGTAGCCTACC-3').

2.2.2. Probes for dot blot and fibre optic biosensors

Oligonucleotide probes for HAV were designed by aligning the selected regions of the HAV RNA sequence available from GenBank. The oligonucleotides sequences were obtained from Integrated DNA Technologies, Pte. Ltd. The first probe is a 24-base ssDNA sequence (5'-TCTTAACAACTCACCAATATCCGC-3'). For fibre optic biosensensing, the 5' end was modified with a -NH_2 group with a 6-carbon spacer. For dot blotting, the 5' end was labeled with digoxigenin. The 24-base second probe (5'-CCAATTTAGACTCCTACAGCTCCA-3'), was modified with a biotin at the 3' end. A 24-base ssDNA complementary to the first probe was synthesized as the positive control in the dot blotting test.

2.3. PCR and qPCR conditions

The first run PCR, involving primers HAVEF-56 and HAVER-684, was performed according to the following steps: 1 cycle pre-denaturation at 95 °C for 2 min followed by 30 cycles denaturation at 95 °C for 45 sec, annealing at 56 °C for 45 sec, extension at 72 °C for 45 sec and finally 1 cycle of extension at 72 °C for 7 min. The reaction mixture containing 1 µL

template was used in a final volume of 50 µL with 1X Colorless GoTaq® Reaction Buffer, 1.25 U GoTaq® G2 DNA Polymerase, 1 µL dNTP mix (10 mM), and 1 µL each primer (10 µM). Following PCR the product was analyzed in a 1% (w/v) agarose gel with a loading of 5µl of resulting synthesized DNA.

Several sets of 10-fold serial dilutions of the standardized cDNA were prepared to build the qPCR standard curves using the following reaction conditions: 1 cycle activation at 95 °C for 2 min, followed by 40 cycles denaturation at 95 °C for 15 sec, annealing/extension at 60 °C for 60 sec, and 1 cycle melt curve at 60-95 °C. The reaction mixture consisted of GoTaq® qPCR Master Mix 1X, 1 µL each of HAVIF-274 and HAVIR-412 primers at 10 µM and 10 µL DNA template from the first run.

2.4. Dot blotting assay

Synthetic ssDNA as the substrate was used to test the probes and to define their working concentrations. The target DNA sequences were denatured in a thermal cycler (Biorad, T100) at 95 ºC for 10 min, and 1 µl from each was spotted onto a positively charged nylon membrane (Sigma). RNA samples were directly spotted without heating. The spotted membrane was exposed to UV at 254 nm for 10 min to allow for nucleic acid cross-linking. The surface was pre-hybridated in pre-warmed DIG Easy Hyb™ (Roche) buffer with shaking. Then it was incubated overnight in 10 ml DIG Easy Hyb™ (Roche) buffer containing 10 µl denatured dig-probe (68ºC, 10min) at 35 ºC. The membrane was washed twice with SSC 2X with 0.1% (w/v) SDS for 5 min, twice with SSC 0.5X with 0.1% (w/v) SDS for 15 min, and once with 1X washing buffer (Roche) for 5 min. Then it was blocked using 10 ml maleic buffer 1X with 10% (v/v) blocking solution (Roche) before incubating the membrane in antibody solution (10 ml blocking buffer with 0.02% (v/v) Anti-Digoxigenin-AP (Roche)), each for 30 min under 35 ºC. The membrane was washed twice with washing buffer for 15

min under 35 ºC then neutralized with detection buffer 1X (Roche) for 5 min at 35 ºC. In the final colouring stage, the membrane was treated with 10 ml detection buffer with 2% (v/v) NBT/BCIP solution (Roche) and kept in a dark box. It was checked every 15 min for the development of coloured dots until no more dot(s) appeared.

2.5. Fibre optic genosensor assay

The preparation of the fibre optic assay (Figure 1) consists of fibre surface functionalization, capture probe immobilization, nucleic acids hybridization and signal generation. The detailed steps are described in the following sections.

2.5.1. Fibre surface functionalization.

The cutting, acid washing, hydroxyl groups activation and silanization steps were described in our previous optimized procedures [33] as shown in Figure 1 ((1) – (3)). The resultant fibre optic was thus functionalized with a –CHO layer on the cross-sectional surface.

2.5.2. Immobilization of -NH_2 modified capture probe.

The -NH2 modified capture probe was denatured at 95 ºC to avoid double-stranded complexing then dissolved in incubation buffer. Chemically modified fibre optic cores were then exposed to the probe solutions with concentrations ranging from 6 to 48 ng/µl in TBS solution (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH7.4) with or without 1% (w/v) bovine serum albumin at 4 ºC. Other buffers tested in this section were: PBS (Sigma, 10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4), HEPES (diluted from stock solution from ThermalFischer, 20 mM, pH 7.4), PBS + $NaBCNH_3$ (Sigma, 0.3 M), NaOAc (diluted from stock solution from Sigma, 30 mM, pH 5.2) + $NaBCNH_3$ and Borax (100 mM boric acid, 75 mM NaCl, 25 mM sodium tetraborate, pH 8.1).

2.5.3. Blocking

After overnight incubation, the fibre bundles were washed with TBST (0.1% (v/v) Tween 20 in TBS) five times and then incubated in blocking conditions for 1.5 hours at 40 ºC then washed five times again with TBST followed by incubation with 0.3 M $NaBCNH_3$ in TBS buffer for 40 min at 40 ºC. The different blocking conditions were: 1%, 3% and 5% (w/v) BSA, 5% (w/v) skim milk and 1 M Glycine in 0.1% (v/v) TBST.

2.5.4. Hybridization

The hybridization mixture was prepared by mixing 24 ng/µl detection probe and nucleic acid sequence in hybridization buffer and incubated with fibre optic tips under 35 ºC for 2 hours. The buffers tested were: Church buffer (0.5 M Na_2HPO_4, 0.5 M NaH_2PO_4, 1% (w/v) SDS and 10 mM EDTA, pH 7.5; all reagents mentioned above were supplied by Sigma.), Church + 1% (w/v) BSA, TBS pH 7.4, PBS pH 7.4, 0.1% (v/v) PBST pH 7.4, TBS pH 7.4 + 1% (w/v) SDS + 10mM EDTA, Borax + 1% (w/v) SDS + 10 mM EDTA.

2.5.5. Avidin-HRP linkage

Avidin-HRP (Sigma) was dissolved in 0.1% (w/v) PBST with 1% (w/v) BSA in various concentrations: 1/500, 2/500, 5/500 and 12/500 (v/v) and incubated with fibre bundles for 1 hour under shaking at room temperature. Then the fibres were washed 5 times with 0.1% (v/v) PBST.

2.5.6. Chemiluminescent light measurement

The near-end of a single fibre optic was inserted into a lightproof tube with 120 µl of peroxide buffer. Then 120 µl luminol solution was added immediately before taking measurements by a photomultiplier tube (PMT) detector (Sensetech) in a confined black box (350 mm /250 mm/200 mm). 5 fibres were used for replication and the signal for each fibre was recorded as an average of 100 readings after the signal reached the maximum range. The light signal was interpreted as relative light intensities (RLU).

3. Results and discussions

3.1. PCR and *qPCR assay*

PCR and qPCR as conventional methods were conducted to validate the cDNA products from the reverse transcription from HAV RNA samples. They can also serve as further investigational lab tools for putative HAV contaminated sample as indicated by the in-field fibre biosensor system. Figure 2(A), shows the PCR products, where the three bands obtained represent the three strains of HAV analyzed (HAV4, HAV5 and HAV6). Each of the PCR product presents a single band of 500 bp, which is the desired length for the external primers, indicating the presence of the target cDNA sequences. As reported in Figure 2 (B), the qPCR reaction yielded amplification products at around 200 bp using the internal primers and the PCR products as template. The standard curve Figure 2 (C), demonstrated a significant linear relationship ($r^2 = 0.99$), indicating the reliability for quantitative HAV detection.

3.2. Dot-blotting assay

The results of the dot-blotting assay are reported in Figure 3. In the first row, blue dots corresponding to the sequence complementary to the first probe were visualized as positive controls, in which sequential dilutions were appreciated until 0.1 pg/µl. In the lower row, the visible dots indicate that the target sequences were present in the HAV RNA and the cDNA samples.

3.3. Fibre optic genosensor optimization

3.3.1. Capture probe concentration

The amount of immobilized ssDNA probe is one of the critical factors that affect the hybridization efficiency [34-36]. In this study, silanization chemistry was employed to functionalize the surface of the fibre tip and then the probe's working concentration was

optimized to achieve the best capture probe surface coverage. As illustrated in Figure 4, the best working concentration of the capture probe was found to be 12 ng/µl in borax buffer after 15 hours incubation at 4ºC, which gave the highest RLU value among the conditions tested.

The optimized concentration is around half (calculated as a molar concentration) of that in the previous study [31], which is 50 ng/µl for a 53 bp DNA probe. This is probably due to the different behaviors brought by the differences in ssDNA probes' lengths [35], and when using 24 bp strand, a relatively lower probe concentration is favoured. The effect of the addition of BSA was also explored in the test, no enhancement was observed for the immobilization in the range of probe concentrations tested.

3.3.2. Capture probe incubation buffer

Regarding the incubation buffer, a good candidate should maintain the stability of the DNA sequence and facilitate the immobilization. On the other hand, it should be inert to the –CHO tail layer on the fibre surface. The results using various buffers were shown in Figure 5, showing that the light responses differ notably in different buffers. Initially, TBS was used in the concentration test as a tris-based buffer, one of the most popular buffers used for many experiments involving DNA molecules [37, 38]. However, the main drawback is its competing reaction with ssDNA chains due to its presence of amine groups, which considerably decreases the immobilization efficiency. The inclusion of $NaBCNH_3$ was intended to improve the binding and avoid non-specific binding as the imine bond is reduced under relatively acidic conditions. However, it is possible that due to the harmful effect to the DNA molecule in the relatively low pH environment, the immobilization efficiency is not satisfying. The improved performance by the borax buffer may be possible due to the

stabilization effect by the formation of a DNA-borate complex [39], which leads to both the highest RLU value and lowest variation.

3.3.3. Blocking condition

Blocking is another crucial step in avoiding non-specific binding by exhausting the free -CHO groups on the fibre surface and washing away the unbound ssDNA chains [40]. BSA has been considered to be one of the most common blocking agents used for the latter purpose [41, 42]. As indicated in Figure 6, it was found that 3% (w/v) in TBS was the best blocking condition for this system compared to lower (1%) or higher (5%) BSA concentrations as well as skim milk and glycine solutions.

3.3.4. AV-HRP concentration

HRP serves as the catalyst in the chemiluminescence generating reaction and it was introduced in the two-probe system by the highly stable biotin-avidin interaction ($K_d = 10^{-15}$M [43]), the results in Figure 7 shows that an appropriate stoichiometric control gave a good signal to noise ratio.

3.3.5. Hybridization buffer

A few hybridization conditions have been examined in this step and information was collected in Figure 8. The borate-based buffer was inefficient in the hybridization step as indicated by the lowest response and signal/noise ratio, which may have been caused by the formation of a DNA-borate complex. On the other hand, this benefited the immobilization of the capture probe. Church buffer [44] turned out to be the best buffer condition for the hybridization. The addition of BSA in the Church buffer was observed to increase the viscosity of the hybridization mixture solution, both hindering the process and increasing the variation. The other TBS and PBST-based buffers gave satisfactory positive results, but the significant non-specific bindings presented a major drawback.

4. *Genosensor performance*

The performance of this genosensor was evaluated by the calibration and specificity test. From the calibration for both DNA and RNA detection, a few parameters including a lower detection limit (LDL) and linear range parameters were obtained. Finally, the specificity test was conducted by involving the DNA or RNA sequences from a variety of pathogens.

4.1. Calibration

The calibration experiment generally describes the performance of a detection system. The results are presented in Figure 9, which was obtained by collecting the light response of several dilutions of cDNA or RNA samples, from where, the lower detection limit (LDL) and linear range can be determined.

4.1.1. LDL

The lower detection limit was set by Equation (1):

$$LDL = NEG + 3STD \quad (1)$$

Where it is the sum of the reading lacking the target sequence plus three times of the standard deviation. The LDL reading calculated from the equations are 17,954 for cDNA and 23,367 for RNA, which correspond to the nucleic acid concentrations of 5 pg/µl and 50 pg/µl, respectively. The performance in the LDL is much better than that in the dot blotting technique, as the PMT is more sensitive to the photons than the raw observation of the coloured dots. On the other hand, it seems much lower than that in some well-studied real-time PCR methods, the best of which is able to detect the concentration as slow as $1.1*10^{-4}$ pg/µl [45]. However, because the NanoPhotometer® reading of the cDNA sample stock concentration is the sum of a mixture of nucleic acids in different lengths, the actual nucleic acid concentration could be much lower that the reported value. In addition, as the

genosensor is a relatively new technique, there is a lot of room for improvement of the LDL of genosensor, such as optimization of the probe design, better probe immobilization methods, improved HRP linkage techniques and enhancement of light signals.

4.1.2. Linear range

The linear range, also known as the dynamic range, is the concentration range where the detection system displays a linear relationship between the signals and the dilutions. It was calculated based on the data extracted from its calibration and fit into Equation (2).

$$RLU = y_0 + D \lg\left(concentration\ in \frac{pg}{\mu l}\right) \quad (2)$$

The linear fit was shown in the top right corner in each diagram in Figure 9. It was found to be 5 pg/µl to 10 ng/µl for cDNA and 50 pg/µl to 10 ng/µl for RNA with r^2 values of 0.93 and 0.87, respectively. The sensitivity is described by the slope D, as it reflects the impact of the change of the concentration to the change of the light response, which is 43948 for cDNA and 23819 for RNA.

4.2. Specificity test

The specificity test was conducted by covering a wide range of other pathogens' DNA or RNA, the results being summarized in Figure 10. Both the genosensor and dot blotting method presented a good specificity as the response to all other noise samples were below the LDL or not visible on the membrane. The rest of the pathogens' samples mentioned in Section 2.1 all presented negative results in the dot blot test. This is mainly attributed to the specificity of the designed probes, further improved by the second probe in genosensor, carries less risk of potential cross-reactivity comparing to the immune system.

5. Conclusions

Nucleic acid-based detection techniques have become the most popular method for HAV detection. In this study, traditional PCR and qPCR methods were firstly developed to validate the HAV probe method and to serve as a further investigational tool for putative HAV contaminated samples. To get rid of the inhibitive effect of polyphenol compounds to DNA polymerases and to achieve possible faster field-detection of HAV virus contamination, a two-probe sandwich-type system on a fibre optic platform was proposed. We have shown a relatively large signal/noise ratio after the assay was optimized. The genosensor system works for both cDNA and RNA at a detection limit of 5 pg/µl and 50 pg/µl respectively. The linear ranges were found to be 5 pg/µl to 10 ng/µl for cDNA ($r^2 = 0.93$) and 50 pg/µl to 10 ng/µl for RNA ($r^2 = 0.87$), showing the reliability of the calibration. An excellent specificity was also confirmed by screening with a broad range of other pathogens' samples. The whole device is miniaturized in a portable confined black box (350 mm / 250 mm/ 200 mm). This preliminary research shows the potential for applying the two-probe genosensor concept as an in-field early warning system for the presence of HAV virus. It can work for the screening of suspicious samples or as a continuous monitoring tool in environmental water sources. Further development with focus on the lower detection limit could be approached from many directions including optimizing the probe design, probe immobilization methods, HRP linkage techniques and an enhancement of chemiluminescent signal.

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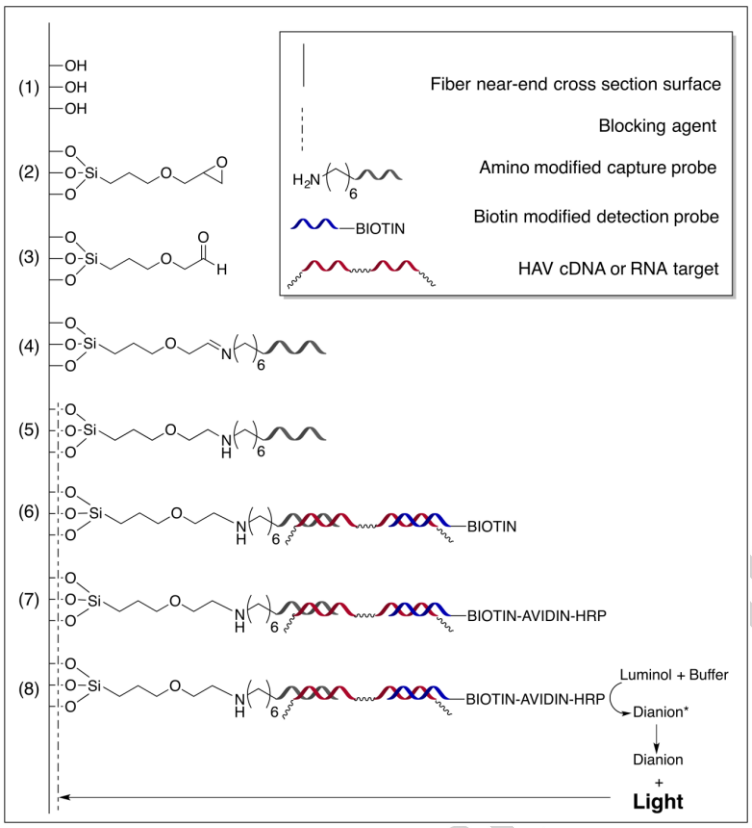


Figure 1. Schematic description of the protocol. The treatments were applied to the near-end surface of the cross-section of the fibre: (1) Acid and piranha wash to expose hydroxyl groups on the surface. (2) Silanization to introduce the epoxy groups. (3) Conversion of epoxy groups to aldehyde groups after a few chemical reactions. (4) Overnight incubation with capture probes modified with an amino group on the 5' end. (5) Blocking step followed by imine reduction reaction. (6) Hybridization with target nucleic acid sequences and detection probes modified with biotin at 3' end. (7) Linkage of HRP by biotin-avidin interaction. (8) Oxidation reaction of luminol catalyzed by HRP. The chemiluminescence signal was generated upon relaxation of the oxidation product and transmitted through the fibre optic to the photodetector.

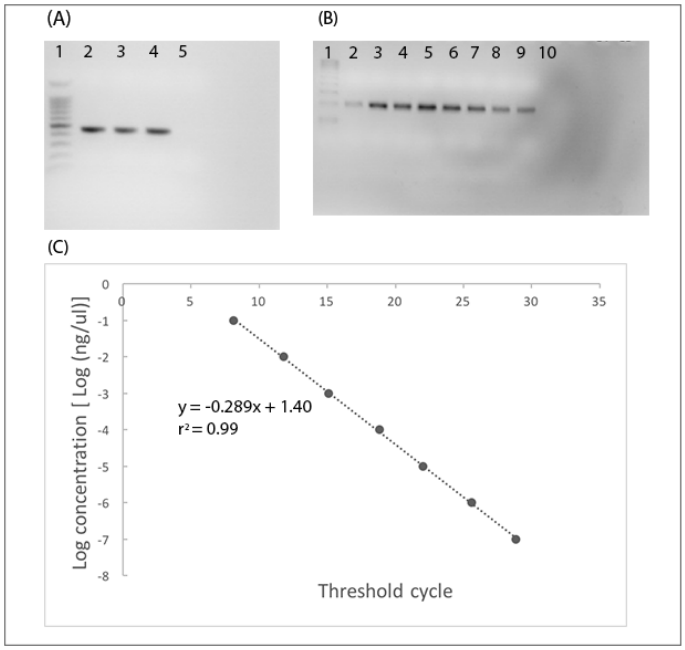


Figure 2. PCR and qPCR results. (A). Results of external PCR using primers HAVEF-56 and HAVER-684. Lane 1: Molecular weight marker 100 bp (Norgen, Canada, USA); Lane 2, 3, 4 are the amplicons of HAV 4, HAV 5 and HAV 6, respectively; Lane 5: blank. (B). Results of the internal qPCR using primers HAVIF-274 and HAVIR-412. Lane 1: DNA Ladder 100 bp (Norgen, Canada, USA); Lane 2: HAV5 1.0 ng/µL; Lane 3: 0.1 ng/µL; Lane 4: 0.01 ng/µL; Lane 5: 0.001 ng/µL; Lane 6: $1.0 * 10^{-4}$ ng/µL; Lane 7: $1.0 * 10^{-5}$ ng/µL; Lane 8: $1.0 * 10^{-6}$ ng/µL; Lane 9: $1.0 * 10^{-7}$ ng/µL; Lane 10: blank. (C). Standard curve from qPCR using 10-fold serial dilutions.

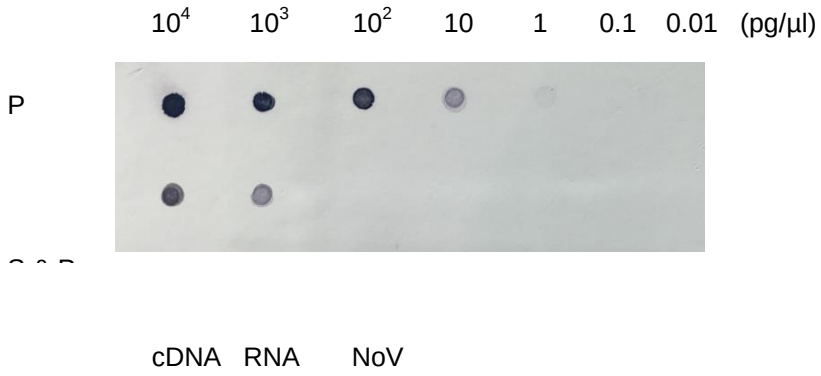


Figure 3. Optimized dot blot results showing: P (positive controls) the sensitivity of the capture probe to its complementary sequence as the positive control. The concentrations of the complementary sequence are labeled on the top; S & N (samples and negative control) , the responses to the reverse transcribed cDNA and RNA of HAV as samples, and the cDNA of Norovirus as negative control.

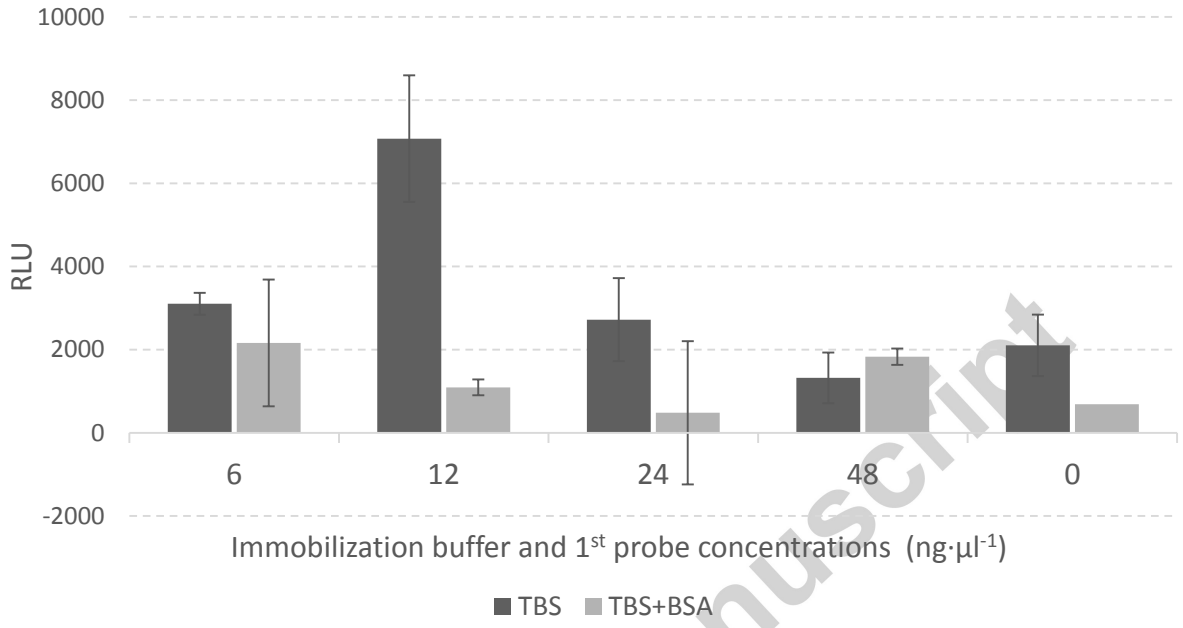


Figure 4. Optimization of the capture probe concentration during overnight incubation at 4 °C. The concentrations for testing are 6 ng/µl, 12 ng/µl, 24 ng/µl and 48 ng/µl. The effect of BSA in the incubation mixture was also included in this test.

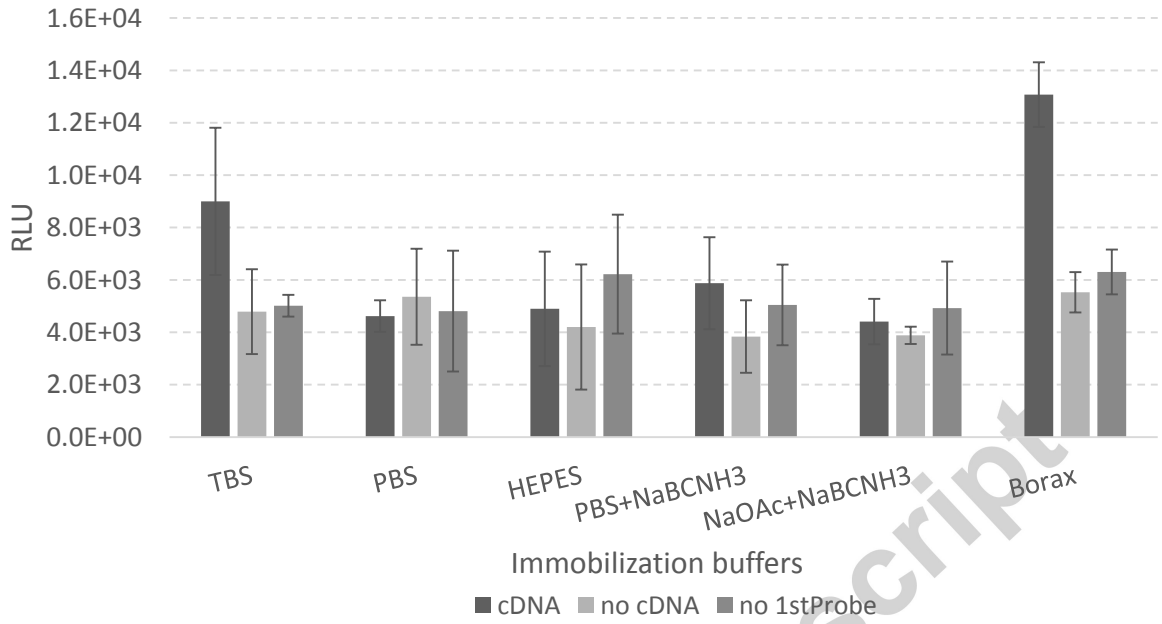


Figure 5. Optimization of the immobilization buffer during overnight incubation at 4 °C: TBS solution (25 mM Tris, 150 mM NaCl, 2 mM KCl, pH7.4); PBS (10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4), HEPES (20 mM, pH 7.4), PBS + $NaBCNH_3$ (0.3 M), NaOAc (30 mM, pH 5.2) + $NaBCNH_3$ (0.3M), Borax (100 mM boric acid, 25 mM sodium tetraborate, 75 mM NaCl, pH 8.1).

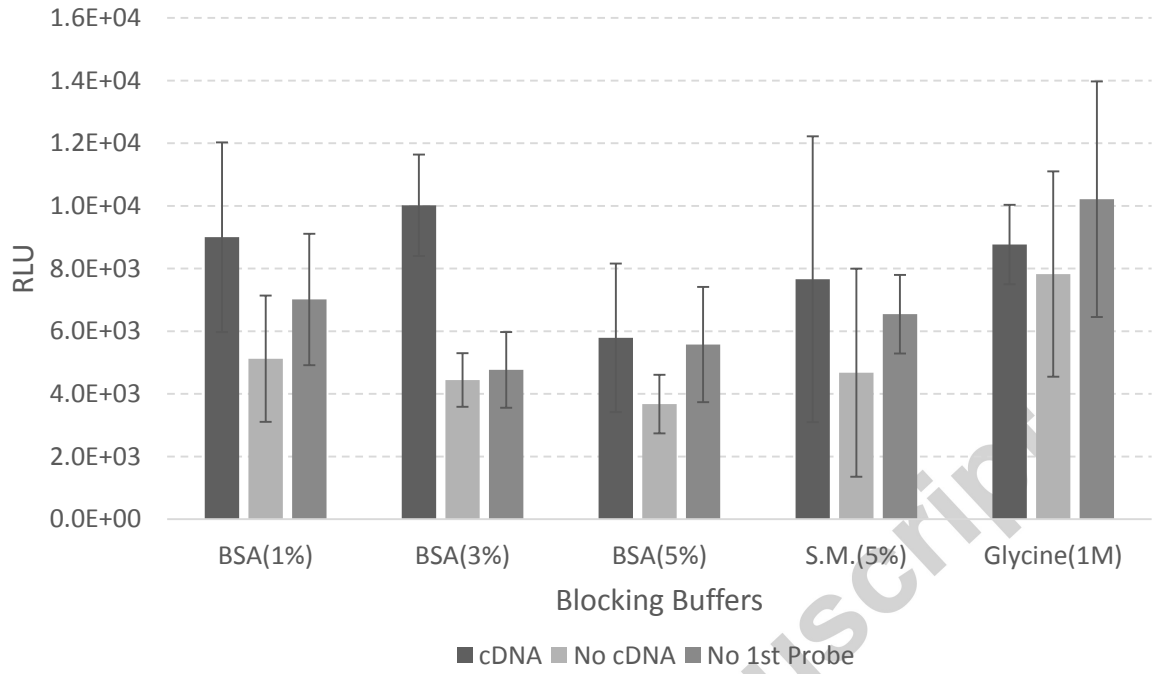


Figure 6. Selection of the blocking conditions after the immobilization of 1st Probe. The blocking agents were dissolved in TBST (0.1% v/v) buffer and the incubation was at 40 °C for 1.5 hours.

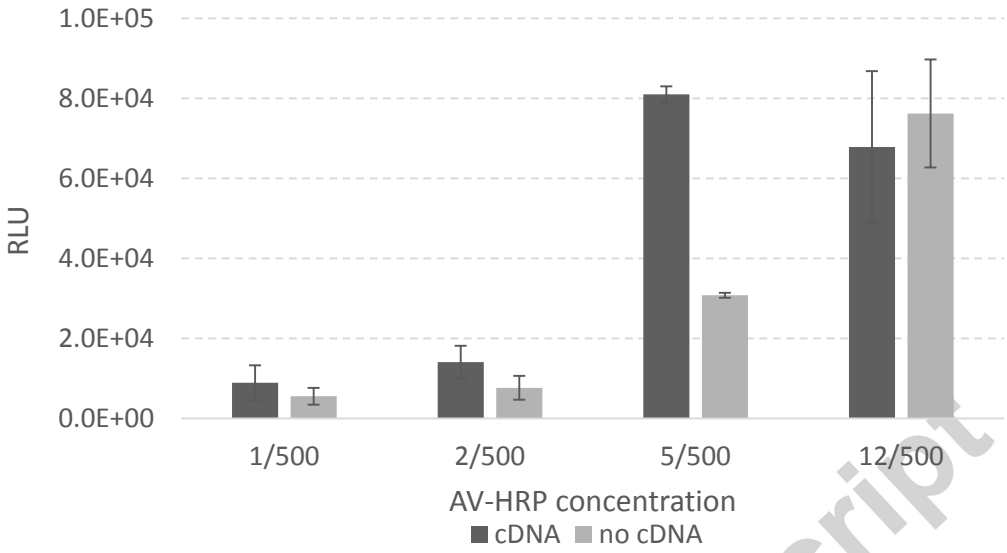


Figure 7. Effect of the avidin-HRP concentration on the light response. The labeled concentrations represent the ratio of the volume of the avidin-HRP to the volume of the incubation buffer. The light response from the group without cDNA targets was used as the reference.

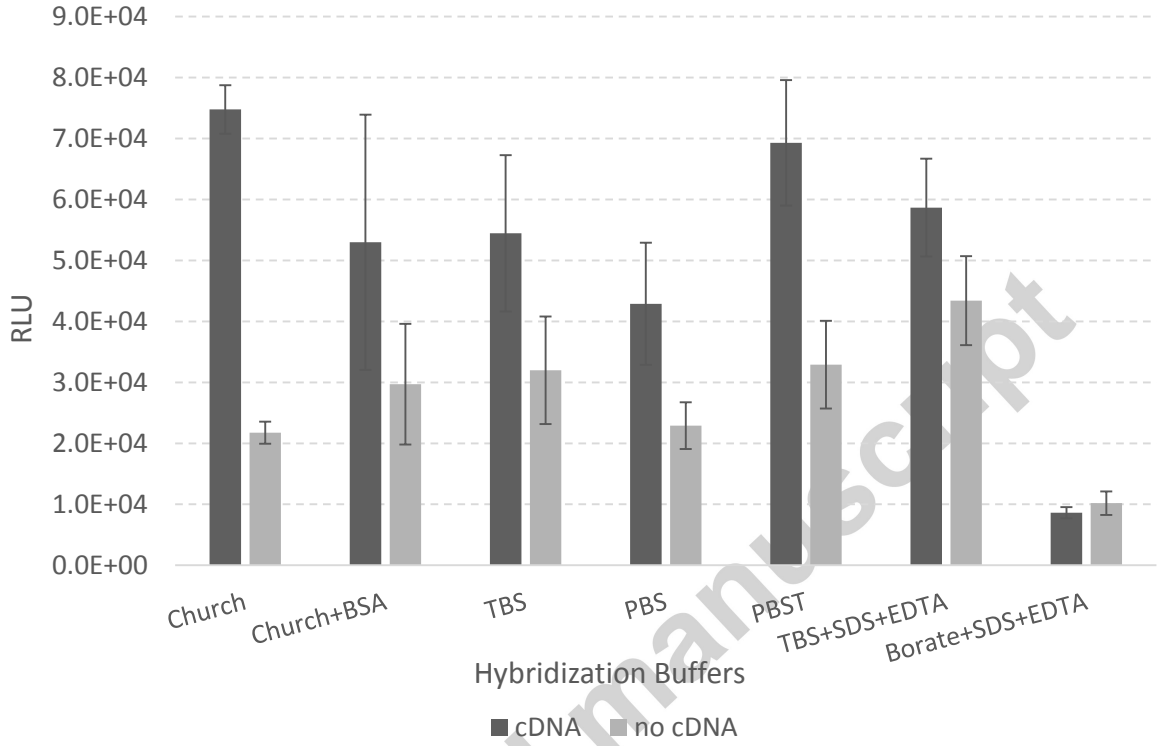
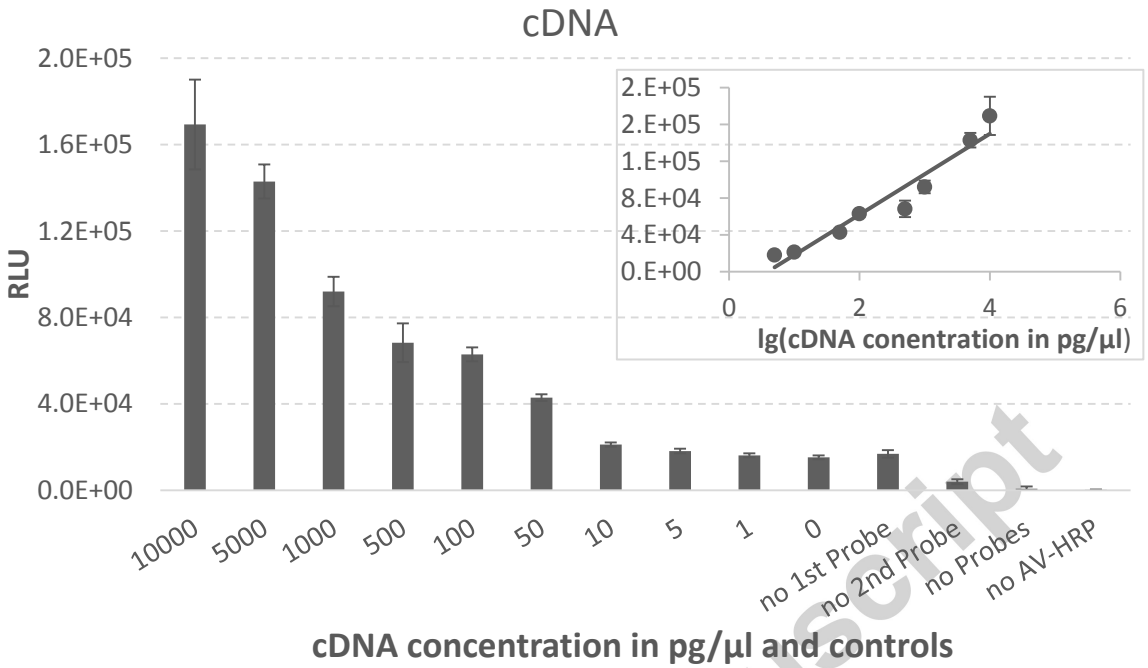
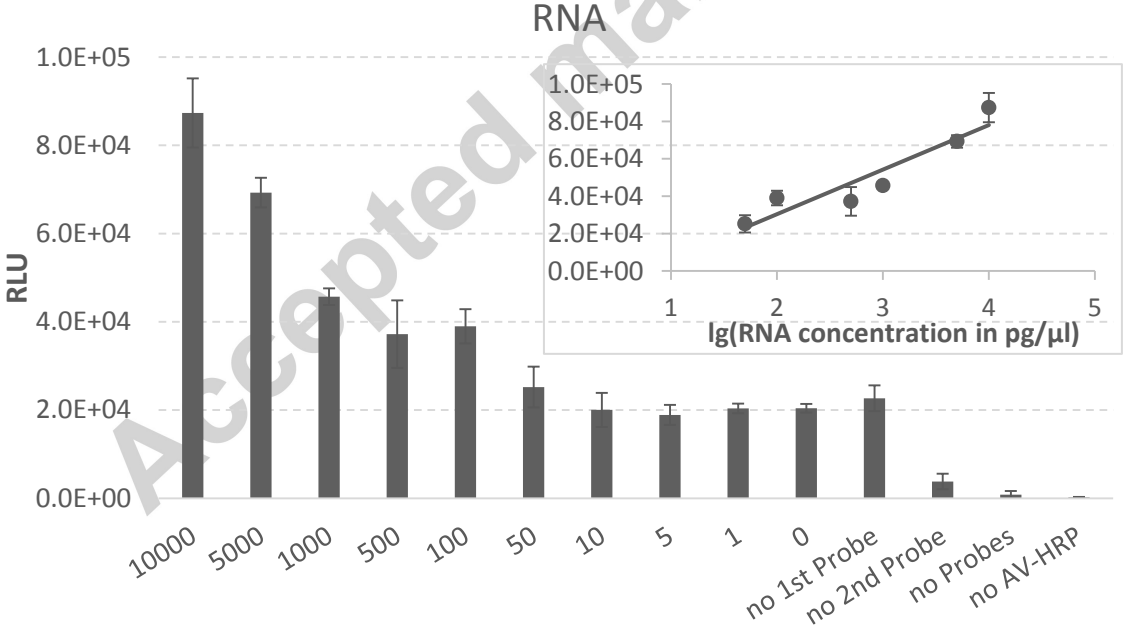


Figure 8. Optimization of hybridization buffer: Church (0.5 M Na_2HPO_4, 0.5 M NaH_2PO_4, 1% (w/v) SDS and 10 mM EDTA, pH 7.5), Church + 1% (w/v) BSA, TBS pH 7.4, PBS pH 7.4, 0.1% (v/v) PBST pH 7.4, TBS pH 7.4 + 1% (w/v) SDS + 10 mM EDTA, Borax as in immobilization step + 1% (w/v) SDS + 10 mM EDTA.



cDNA concentration in pg/µl and controls



RNA oncentration in pg/µl and controls

Figure 9. Light responses to nucleic acid dilutions and controls. The linear range can be visualized on the top right corner, by plotting the RLU values against the lg values of nucleic acid concentrations in pg/µl. The linear range was found to be 5 pg/µl to 10 ng/µl for cDNA (r^2 = 0.93) and 50 pg/µl to 10 ng/µl for RNA (r^2 = 0.87). The sensitivity of the biosensor was determined by the slope of the linear range as it describes the power to discriminate the difference between the two signals with the change of the analyte concentrations, as represented by its form $\frac{y_A - y_B}{x_A - x_B}$. It was found to be 43948 for cDNA and 23819 for RNA.

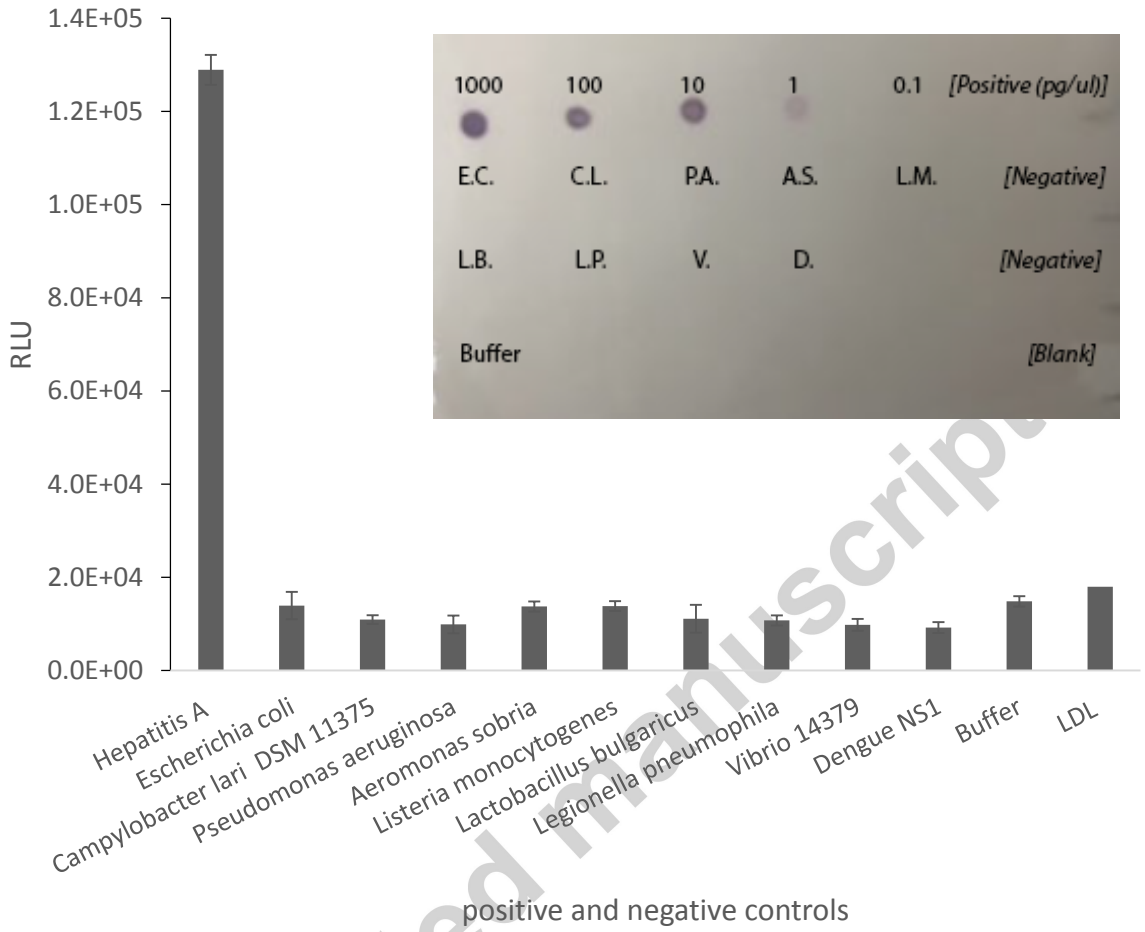


Figure 10. Specificity test covering a series of other pathogens' cDNA or proteins, the column chart shows the results from fibre optic genosensor, where the dashed line represents the lower detection limit. The picture on the top right corner shows the results from the dot blotting experiment, the aberrations are corresponding to the names of the pathogens shown in the x-axis labels of the chart. LDL stands for lower detection limit as calculated from Section 4.1.1.

Highlights:

- A set of nucleic acid probes were designed based on the hepatitis A virus (HAV) RNA sequence. They were found to work for both HAV cDNA and RNA with excellent specificity.
- The probes were immobilized on the tip of fibre optic and fitted into a portable device, which is possible for in-filed detection.
- The conditions to generate highest chemiluminescent signal/noise ratio in this technique were explored.
- The DNA probes were verified by qPCR.