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Fast DNA and protein microarray tests for the diagnosis of hepatitis C virus infection on a single platform**

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Keywords:
DNA microarray; protein microarray; HCV; time-to-result; point-of-care; seroconversion panel; clinical sample

Abstract

Hepatitis C virus (HCV) is a major cause of chronic liver disease and liver cancer and remains a large health care burden to the world. In this study we developed a DNA microarray test to detect HCV RNA and a protein microarray to detect human anti-HCV antibodies on a single platform. A main focus of this study was to evaluate possibilities to reduce the assay time as short time-to-result (TTR) is a prerequisite for a point-of-care test. A significant reduction of hybridisation and washing times did not impair the assay performance. This was confirmed first using artificial targets and subsequently using clinical samples from an HCV serconversion panel derived from a HCV infected patient. We were able to reduce the time required for the detection of human anti-HCV antibodies to only 14 min achieving nanomolar sensitivity. The protein microarray exhibited an analytical sensitivity comparable to that of commercial systems. Similar results were obtained with the DNA microarray using a universal probe which covered all different HCV genotypes. The assay time after PCR could be reduced from 150 min to 16 min without loss of sensitivity. Taken together, these results constitute a significant step forward in the design of rapid, microarray-based diagnostics for human infectious disease and show that the protein microarray is currently the most favourable candidate to fill this role.

Introduction

HCV continues to be a global health problem. An estimate from the World Health Organization (WHO) from 1999 suggests that about 3 % of the world's population is infected with HCV causing more than 86000 deaths in Europe in 2002[1;2]. The increasing incidence of undiagnosed chronic societal infections, including those spread sexually (e.g. human immunodeficiency virus (HIV)) and those spread percutaneously (e.g. HCV), has resulted in a major financial burden on global health services and generated an urgent requirement for improved medical diagnostics[3;4]. Underlying characteristics of most current detection systems which require improvement are numerous. These devices can only be operated by a limited team of highly skilled personnel within hospital environments, specimens for testing require time-consuming transport to diagnostic laboratories and various tests specific for only one biomarker need to be performed before a positive diagnosis can be made. The fact that some patients fail to return for the results of these laboratory tests compounds this already difficult situation[5]. The development of automated 'point-of-care' diagnostic devices for application within local medical practices would allow for faster diagnosis, earlier treatment, reduced clinic visits and hence significantly aid in alleviating these problems[6].

For the diagnosis of HCV infections there are three HCV markers useful in the clinical practice, including total anti-HCV antibodies, HCV genotype and HCV RNA[7]. The microarray platform

developed in this work contains two of these three markers and has the capability to be extended to also detect the HCV genotype by introducing genotype specific DNA probes to the DNA microarray. Currently, initial diagnosis for HCV-infection comprises testing individuals for the serologic presence of specific antibodies to a range of immunogenic HCV epitopes (core, NS3, NS4 and NS5 proteins) using a third-generation enzyme immunoassay (EIA) and then assessing serum HCV RNA levels by nucleic acid test (NAT) before beginning therapy and subsequently during therapy[8]. Despite both EIA and NAT being highly sensitive techniques, they both have considerable shortcomings since they are only specific for one class of microbe and have a lengthy time-to-result (TTR), which ultimately culminates in a diagnosis end-point of days to weeks[9]. Indeed, even with experimental multiplex PCR diagnostics is used in order to increase diagnostic capacity, complications with the amplification of primer dimers and the difficulty of matching optimal primer annealing temperatures arise[10;11].

The advent of microarray technology has made possible the simultaneous analysis of thousands of distinct biomarkers on a single platform[12;13]. The principle behind this technology is the attachment of capture probes to a solid substrate (e.g. glass) and the subsequent hybridization of e.g. fluorescently-labeled targets to these capture probes. Microarray slides are subsequently scanned and any bound targets detected by fluorescence analysis[14-16]. It is a distinct advantage over conventional ELISA's which rely on physisorption for the attachment of capture probes that oligonucleotides (synthesized *ex situ* with a terminal functionalizing group (e.g. thiol or amino)) and proteins can be covalently attached to a chemically modified substrate (e.g. epoxysilane)[17-19]. Studies have already shown the utility of protein microarrays for the combined typing of whole blood and the simultaneous serodiagnosis of various infectious agents (e.g. HIV and HCV infection) in blood samples[20-23]. Overall, these devices have found extensive application in a variety of fields including expression profiling, diagnostics, drug discovery and DNA sequencing[24-28]. In addition to discriminating between multiple classes of microbe, by incorporating genotype and even sub-type-specific probes, 'theranostic' devices could be constructed for the tailored treatment of infected patients undergoing treatment[29].

Fundamental properties of microarrays that have highlighted their candidacy for implementation into 'point-of-care' diagnostics include: their high-throughput nature for the identification of diverse test analytes, small platform size for compatibility into microfluidic devices and small sample volume (e.g. patient blood serum) requirement. Several studies have already shown the basic application of microarray-based diagnostics for the screening of allergen-specific IgE antibodies, drugs and even for early diagnosis of prostate cancer[30-32]. Coupled with portable computers for data analysis, these devices are paving the way towards fully-comprehensive front-line diagnostics. There are still, however, important factors which preclude the introduction of microarray-based diagnostic devices

into mainstream healthcare, these include a lack of sample automation, lengthy TTR, the reproducibility of microarray manufacture and the initial cost of microarray production[33].

Here we describe the fabrication of DNA and protein microarrays directed against universal HCV determinants for the rapid detection of HCV infection in clinical samples. Significant advances in TTR have been made achievable with assay optimization, such that anti-HCV antibodies can now be identified from clinical samples within a timeframe of less than 20 minutes. Furthermore, both the DNA and protein microarray were validated by comparing the analytical sensitivity to commercial diagnostics for the serodiagnosis of HCV infection using a seroconversion panel taken from a HCV infected patient. The results of which have comprehensively shown our DNA and protein microarrays to be as sensitive as current commercial detection systems. All data considered, this study comprises a proof-of-principle for the application of microarray-based detection platforms for the rapid detection of human infectious disease.

Materials and methods

Microarray construction

A DNA capture probe of sequence: 5' – GGCAATTCCGGTGTACTCACCGGTTCCGCAGACCACTATGGCTCTCCCGGGAGGGGGGG – thiol - 3' was designed by performing an alignment of the conserved 5'-UTR of 113 HCV isolates, covering genotypes 1 to 6, using the Los Alamos HCV database (http://www.hcvdb.org/). Oligonucleotides were obtained from Metabion (Martinsried, Germany). A 5'-thiol HCMV negative control probe of sequence: 5' – CAAATACCGTGGGACGACACGCACCGGCAGTGCGCAGGCAGCGTCGGACACAACACGCT TACGGCCCTCAACACT – 3'was also used[34]. Thiol modified oligonucelotides were spotted in 1x Schott Nexterion spot buffer (20 µM) containing 5 mM Tris(2-carboxy-ethyl)phosphine hydrochloride (TCEP) to cleave the mercapto-ethyl protection group on Schott Nexterion Slides E (epoxy silane modified surface; Schott UK Ltd., Stafford, U.K.) with a Microgrid II spotter (Digilab, Huntingdon, UK) using 200 µm solid pins. After printing, microarrays were incubated in saturated NaCl humidity box for 1 hr at room temperature followed by incubation under dry conditions over night in order to facilitate probe immobilization. Thereafter, microarrays were washed for 5 min in 0.1% Triton X-100, 4 min in 1 mM HCl and 10 min in 100 mM KCl. Each wash step was carried out at room temperature and with stirring. Microarrays were rinsed three times for 1 min in deionized water at room temperature and with stirring. After washing, microarrays were blocked in 0.1 M Tris + 50 mM ethanolamine + 0.1% SDS; pH9, for 15 min at 50°C with stirring. Microarrays were rinsed as above and stored at room temperature until use.

Protein probes for antigen microarray printing were prepared with an optimized printing buffer comprised of 50 mM PBS + 10% glycerol. HCV NS4 (Fitzgerald Industries International, Massachusetts, U.S.A.) and core antigen (Virogen, Massachusetts, U.S.A.) printing buffer were prepared as 500 μg/mL probe solutions. *Arabidopsis* total plant protein negative control (AMS Biotechnology, Oxon, U.K.) and positive control mouse anti-HBV IgG or human serum IgG probes (Sigma-Aldrich, Dorset, U.K.), were prepared as 100 μg/mL probe solutions. Probes were printed in replicates of five. Antigen probes were printed and immobilized as for DNA microarrays. Thereafter, microarrays were blocked with phosphate buffer saline Tween-20 solution (PBST) + 1% (w/v) bovine serum albumine (BSA) for 1 hr at room temperature, rinsed with deionized water and centrifuged dry. Completed microarrays were stored under N_2 at 4°C until use.

Detection probes for indirect detection of unlabeled HCV cDNA

Six Cy3-conjugated oligonucleotide detection probes between 19 and 24 nucleotides in length (Metabion, Martinsried, Germany) were designed against distinct regions of the HCV cDNA amplified product based on published primer sequences of the HCV 5' UTR[35;36]. The universal primers which contained degeneracies at certain positions were modified to be specific for certain genotypes and adjusted to have a similar melting temperature These detection probes allowed for universal coverage of all six HCV genotypes and were of sequence: 5' – Cy3=CGTGACAGAAGTTCCTCACAGG (genotype 3), 5' – Cy3=GTGACAGTAGTTCCTCACAG (genotypes 1, 2, 4, 5 & 6), 5' – Cy3=TGCACGGTCTACGAGACCT (genotypes 1-6), 5' – Cy3=ACACTCTAACTAACGCCATGGCTA (genotypes 1, 2, 4, 5 & 6), 5' – Cy3=ACACTCCAACTAACGCCATGGCTA (genotype 3) and 5' – Cy3=AAGCACCCTATCAGGCAGT (genotypes 1-6).

Target preparation

For the detection of HCV cDNA, total RNA was extracted from human clinical samples at the Royal Infirmary of Edinburgh, Edinburgh, UK. Human clinical samples used for testing comprised five HCV RNA isolates from HCV-positive patients and one anti-HCV seroconversion panel (ZeptoMetrix HCV seroconversion panel HCV 9041, Donor No. 63625), obtained from the Royal Infirmary of Edinburgh, Midlothian, U.K. and ZeptoMetrix Corporation (New York, U.S.A), respectively. A one-step reverse transcription – PCR (RT-PCR) using a published set of primers against conserved HCV 5' UTR region (5'- ccc tgt gag gaa ctw ctg tct tca cgc; 5'- ggt gca cgg tct acg aga cct) were used to generate and amplify HCV cDNA of approximately 300 bp length (SuperScript,

Invitrogen, Paisley, U.K)[35]. Approximately 2 ng/µL of patient RNA or 2 µL extracted seroconversion panel RNA was mixed with 0.2 µM primers, 1 X Reaction Mix, 1 µL RT/Platinum® *Taq* Mix and water for a total reaction volume of 50 µL. RT-PCR was performed with a Techne TC-512 Thermal Cycler (Bibby Scientific Limited, Staffordshire, U.K.) using the following protocol: 50°C for 30 min, 94 °C for 2 min, 40 cycles of 94 °C for 15 s, 55 °C for 15 s and 68 °C for 1 min and a final extension at 68 °C for 10 min. Following RT-PCR, HCV cDNA was purified using a Qiagen PCR cleanup kit (Qiagen, West Sussex, U.K.) and subjected to PCR amplification by mixing 2 µL template with 0.4 µM primers, 1 x *Taq* buffer, 100 µM dNTPs, 1 mM $MgCl_2$ and water for a total reaction volume of 25 µL. PCR was performed with the thermal cycler as above using the following protocol: 95 °C for 15 min, 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 10 min. For labeling PCR, a ratio of 3:2 between Cy3-dCTP (40 µM; GE Healthcare, Buckinghamshire, U.K.) and dCTP (60 µM) were included in the reaction. After purification of the amplified PCR product as before, confirmation of 300 bp HCV cDNA was shown using a Bioanalyzer (Agilent Technologies, South Queensferry, U.K.) and the Cy3 incorporation rate and DNA concentration measured using a Nanodrop spectrophotometer (Labtech, East Sussex, U.K.). All HCV cDNA samples were stored at -20 °C. Various concentrations of target sample were prepared by dilution of HCV cDNA with 4 x sodium saline citrate solution (SSC) + 0.01% sodium dodecyl sulfate (SDS) including detection probes (2 µM) for the indirect detection of HCV cDNA. For the direct detection of HCV cDNA, targets between 100 pM and 1 pM were generated from a Cy3-labeled 40-mer oligonucleotide complementary to the HCV detection probe. The sequence of the artificial Cy3-labeled target was 5' – Cy3=CCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGG – 3' (Metabion, Martinsried, Germany). For the direct detection of HCV cDNA from the anti-HCV seroconversion panel, targets were prepared by diluting Cy3-labeled HCV cDNA 12.5-fold with hybridization buffer (4 x SSC + 0.01% SDS).

Whole human blood was supplied with ethical approval by the Scottish National Blood Transfusion Service (SNBTS, Edinburgh, UK) and blood serum prepared by centrifugation at 4000 r.p.m. for 10 min at 4 °C. Human blood had been pre-screened to confirm the absence of HIV, HBV, HCV, syphilis and HTLV I/II. Whole human blood and blood serum were stored at 4°C. Clinical samples of human HCV infected sera were provided in the form of an anti-HCV seroconversion panel (HCV9041; ZeptoMetrix Corporation, New York, U.S.A.). Seroconversion panel members were stored at -20°C. Target samples were prepared by 1:10 serial dilutions of blood serum or plasma with 10 mM PBS + 0.05% Tween20 (PBST; Sigma-Aldrich, U.S.A.) + 1% BSA (Sigma Aldrich, U.K.). For mock experiments, 6.7 nM anti-HCV NS4 and core recombinant antibodies were spiked into 1:10 diluted human serum and further target samples generated by serial dilution in diluted serum.

Microarray hybridization/incubation

DNA target samples were denatured at 95° for 5 min and incubated on ice for 5 min. The "long" protocol consisted of target hybridization for 2 hr at 55°C with an Agilent (Agilent Technologies, South Queensferry, U.K.) 8 well gasket slide in an Agilent hybridization oven under agitation (rotation speed 4). After hybridization slides were washed for 10 min each in 2 x SSC + 0.1% SDS, 2 x SSC and then 0.2 x SSC, all wash steps at room temperature and with stirring. Microarray slides were centrifuged dry for 2 min at 1000 r.p.m. The "short" protocol consisted of target hybridization for 10 min at 55°C, one wash each for 2 min in 2 x SSC + 0.1% SDS, 2 x SSC and then 0.2 x SSC, all wash steps at room temperature and with stirring. Microarray slides were centrifuged dry for 2 min at 1000 r.p.m.

Protein target samples were incubated with the HCV antigen microarrays using Agilent 8 well gasket slides and hybiridization chambers in an Agilent hybridization oven (Agilent Technologies, South Queensferry, U.K.). The "long" protocol consisted of target incubation for 4 min at 37°C, two washes for 20 min in PBST and then two washes for 10 min in PBS, all wash steps at room temperature and with stirring. Microarrays were centrifuged dry as before and incubated with 60 µL sheep anti-mouse IgG-Cy3 conjugate (mock samples; Sigma Aldrich, Dorset, U.K.) or goat anti-human IgG-Cy3 conjugate (clinical samples; Sigma Aldrich, Dorset, U.K.) for 10 min at 37°C. Wash steps were then repeated, microarrays rinsed in water and centrifuged dry. The "short" protocol consisted of target incubation for 4 min at 37°C, one wash for 2 min in PBST and then one wash for 2 min in PBS, all wash steps at 37°C and with stirring. Microarrays were centrifuged dry and incubated with secondary detection antibody as before for 2 min at 37°C, wash steps repeated, rinsed in deionized water and centrifuged dry.

Image processing and analysis

Microarray slides were scanned with a Tecan LS Reloaded Scanner using an excitation wavelength of 532 nm and a 575 nm detection filter (Tecan UK Ltd., Reading, U.K.). Microarray spots were analyzed with QuantArray software (Perkin Elmer, Waltham, U.S.A.) and graphs constructed by presenting data as either mean fluorescence intensity or signal-to-noise ratios by defining a detection threshold (i.e. S/N > 1.0 was regarded as a positive signal) as shown below:

$$\text{Threshold} = \frac{\text{Mean probe intensity}}{(\text{Mean} + 3*\text{SD of mock}) + (\text{Mean } 3*\text{SD of negative control})}$$

where SD is standard deviation.

Results and Discussion

Microarray sample incubation and washing times

There is great hope that microarrays can be integrated into fully automated detection systems for diagnosing human infectious diseases, such as those caused by HCV and HIV. One of the foremost factors obstructing introduction of microarray-based diagnostics into point-of-care devices is the lengthy TTR associated with this technology. We developed a DNA microarray for HCV RNA detection and a protein microarray for human anti-HCV antibody detection on the same platform. Both thiol modified HCV specific DNA probes and HCV antigens were immobilized on epoxy-functionalized glass slides. The detection was based on fluorescence generated by Cyanine3 (Cy3) dyes introduced in the HCV cDNA amplicon during reverse transcription-PCR reaction and secondary anti-mouse and anti-human Cy3-labelled antibodies. In this study, we investigated the possibility of optimizing both protein and DNA microarrays in order to reach a TTR significantly shorter than that of current clinical diagnostics. We investigated possibilities to reduce target incubation and washing times and compared the analytical behaviour in terms of sensitivity and specificity of a "long" commonly used microarray protocol[15;37-39] with a significantly faster "short" protocol as shown in ***Fig. 1***.

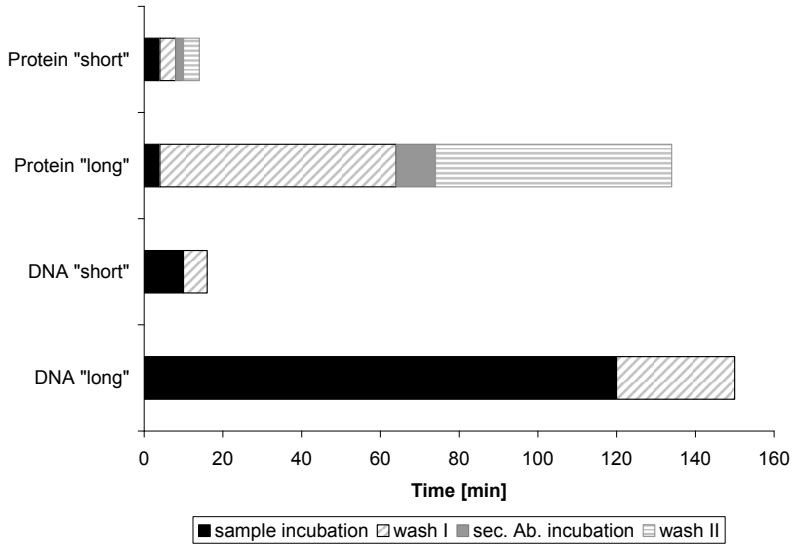
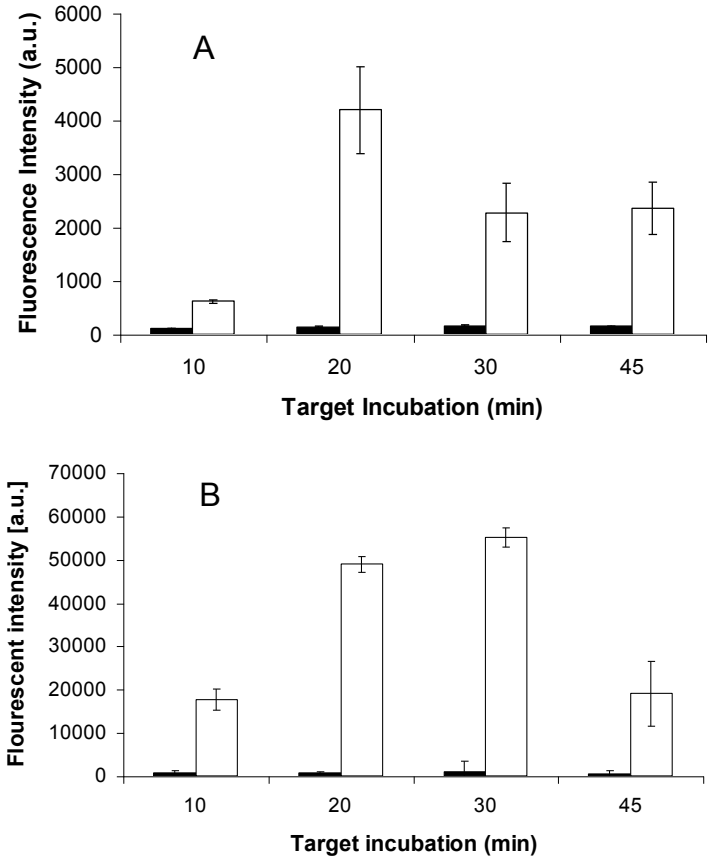


Fig. 1. Comparison of the time required for individual steps within protocols tested for both HCV RNA detection (DNA "short" and DNA "long") and anti-HCV antibody detection (protein "short and protein "long"). Note that the wash times represent the overall time required for a set of different wash steps and times required for DNA protocols do not include times for PCR. All protocols displayed include a final fluorescence scan (2 min) and data evaluation (2 min).

HCV DNA microarray

Artificial targets – In order to broadly detect all six genotypes of HCV, a universal oligonucleotide detection probe was designed based on a sequence alignment against the highly conserved 5' UTR of the HCV genome. An oligonucleotide probe length of 59 bases was chosen since it should offer a high level of analytical sensitivity towards target HCV cDNA. Longer probe lengths are routinely used for the design of diagnostic DNA microarrays as they have been shown to exhibit increased sensitivity for dilute target concentrations resulting in a larger dynamic range of detection[34]. As a starting point to optimise, the hybridization of an artificial 40-mer Cy3-labeled target complementary to the HCV probe the hybridization time was varied between 10 and 45 min and the results are shown in (← ***Fig. 2***). These results showed that artificial HCV target concentrations down to 10 pM were clearly distinguishable from background signal after a hybridization period of only 10 min. As expected, there was only very low level background signal from the HCMV negative control probes regardless of hybridization duration. Based on these results and on data from wash time experiments (data not shown), for clinical testing we used a "long" DNA microarray protocol of 150 min (2 hr hybridization) and a "short" protocol of 16 min (10 min hybridization) in order to evaluate the serodiagnostic potential of the DNA microarray.



← ***Fig. 2.*** Effect of the hybridization time on the direct detection of (A) 10 pM and (B) 100 pM artificial 40-mer Cy3-labeled HCV target. Following hybridization, microarrays were washed three times for 10 min. Mean negative control and HCV specific probe fluorescence intensities are shown by black and white bars, respectively. n = 5.

Validation with HCV RNA derived from HCV infected patient samples – Having successfully characterized the DNA microarray using artificial 40-mer Cy3-labeled targets, it was important to asses the clinical sensitivity of the microarray using full length HCV RNA derived from human clinical samples. Five clinical isolates of HCV RNA from HCV-infected patients were obtained from the Royal Infirmary of Edinburgh and confirmed to have viral loads between 4,000 and 6,500,000 IU/mL (correlating to approximately 10 aM and 10 fM RNA respectively). However this was found to be too low a concentration of RNA for microarray detection (data not shown) and so a one-step reverse transcription and PCR was employed to amplify the RNA generating approximately 200 nM HCV cDNA. As a preliminary experiment to deduce whether the 300 bp cDNA fragments could hybridize to the DNA microarray probes, unlabeled HCV cDNA was pre-hybridized with specific Cy3-labeled detection probes and subsequently hybridized with the DNA microarray. The six oligonucleotide detection probes of between 19 and 24 bases were designed to hybridize with non-overlapping regions of the amplified HCV cDNA product. Strong hybridization signals showed that the Cy3-labeled detection probes specifically hybridized to the 300 bp cDNA fragments and all five patient samples were found to be strongly positive (S/N > 1.0) indicating that the DNA microarray has a clinical sensitivity required for clinical diagnostics (see Fig. 3). This result was of particular importance because it demonstrated that the size and secondary structure of the larger PCR amplified product did not sterically hinder hybridization to the HCV capture probes.

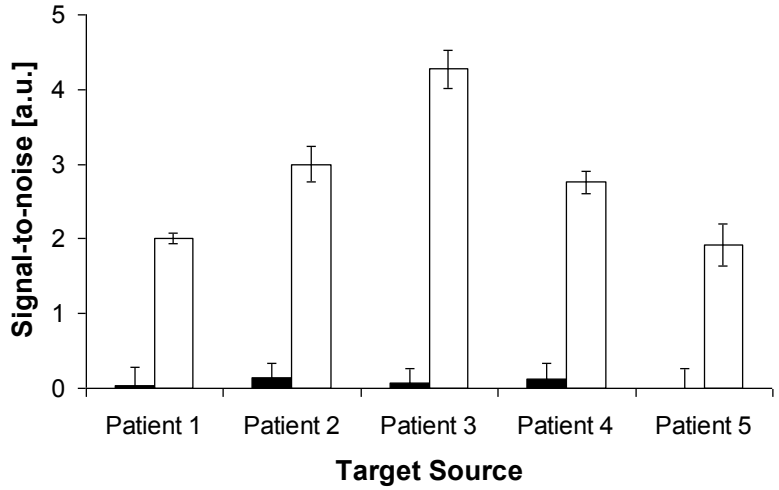


Fig. 3. Patient samples analysed with the HCV viral load DNA microarray using the "long" protocol with Cy3-labeled detection probes. Normalized hybridization signals obtained with Cy3-labeled detection probes for the indirect detection of HCV cDNA from HCV-positive clinical samples. Negative control probe and HCV detection probes are represented by black and white bars respectively. n = 25.

Following a successful demonstration of the serodiagnostic capability of the DNA microarray using clinical samples, an anti-HCV seroconversion panel was obtained for the purpose of addressing its analytical sensitivity compared to commercial diagnostics. In order to reduce the overall assay TTR, a second strategy was employed focusing on directly labeling the HCV cDNA with Cy3-dCTP during a second-round of PCR. Compared to using Cy3-labeled detection probes to indirectly detect HCV cDNA, this strategy offers the advantages of less sample handling, reduced operator-specific variation (critical for investigating small differences between panel members) and reduced assay time resulting from not having to prepare and add detection probes during target preparation. Target samples were prepared by diluting Cy3-labeled HCV cDNA from each panel member 12.5-fold with hybridization buffer before testing with the DNA microarray (***Fig. 4***).

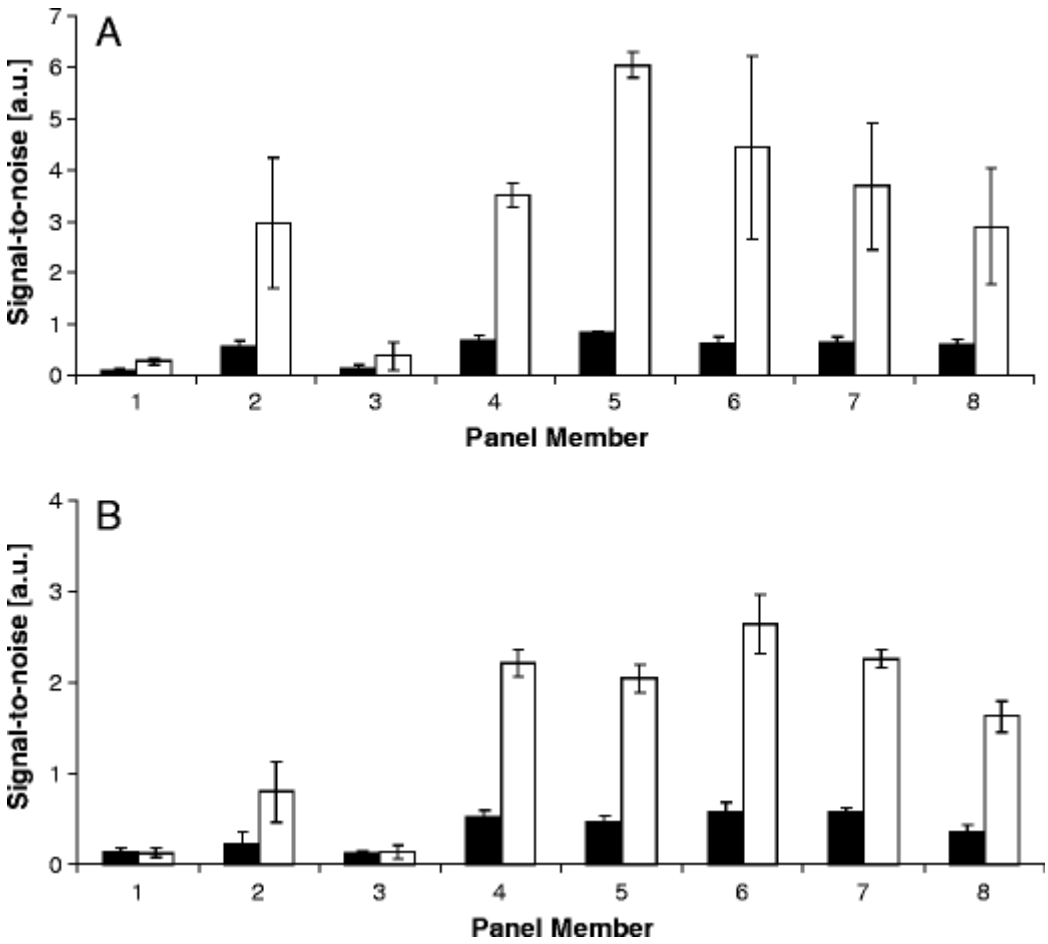


Fig. 4. Seroconversion panel samples analysed with the HCV viral load DNA microarray using Cy3-labeled cDNA PCR products. Normalized hybridization signals for the direct detection of Cy3-labeled HCV cDNA from a commercial anti-HCV seroconversion panel using the (A) "long" and (B) "short" protocol. Negative control and HCV detection probes are represented by black and white bars respectively. n = 25.

The variation of normalized hybridization signals observed between panel members can be explained by the oscillating levels of HCV RNA present in the starting material of the individual panel members (Table S-1). As the anti-HCV seroconversion panel represents a time-course of plasma samples taken at arbitrary time-points before and after the appearance of anti-HCV antibodies, the levels of HCV RNA also varies between panel members reflecting the immune response against the virus. In agreement with supplier data, the first panel member was found to be negative for the presence of HCV RNA when using the "short" and "long" hybridization protocol (***Fig. 4*** and supplementary Fig. S-2). Of the eight panel members tested, 6 out of 8 were found to give strongly positive hybridization signals consistent with the supplier data. Although panel member 3 gave a negative signal (S/N = 0.38) which is in contrast to supplier data (reported viral load: 63.72 MEq/mL), it was found to correspond to the absence of HCV cDNA after the RT-PCR step in this study (Fig. S-1). Therefore, the most likely explanation for the absence of HCV cDNA and hence negative hybridization signal is that there was a problem with the initial extraction of RNA from panel member 3. As a result of this, panel member 3 was regarded as an artifact of an unsuccessful pre-hybridization sample handling step and was not considered for assessment of the microarray's analytical sensitivity. Nevertheless, this highlights one of the major disadvantages of using a DNA-based device in comparison to protein assays for disease diagnosis. The requirement for additional handling steps (e.g. RNA extraction) has the potential to introduce contaminants, damage preciously low-yield nucleic acid and significantly increase the overall assay TTR. When the DNA microarray was tested with the "short" hybridization protocol representing an assay time of 16 min after PCR, the overall hybridization signals were found to decrease whilst the general trend across the anti-HCV seroconversion panel remained the same (***Fig. 4*** B & Fig. S-2 B). Although panel members 4 to 8 were still successfully serodiagnosed as being positive (albeit less strongly), panel member 2 was now marginally negative (S/N = 0.79). Nevertheless, these results showed that all panel members except panel member 3, which was unsuccessfully processed at the RNA extraction stage, were correctly serodiagnosed in concordance with commercial assays with an assay time of 150 min after PCR and six out of seven panel member samples were identified correctly with the "short" protocol comprising an assay time of 16 min after PCR. Thus, we have shown the utility of DNA microarrays for the serodiagnosis of HCV infection within a significantly reduced timeframe, e.g. compared to a recently published HCV genotyping DNA microarray where the PCR products were hybridized for 1h at 68 °C[40].

Improvement in DNA microarray sensitivity could realistically be achievable with sample pre-treatment integrated at steps prior to target hybridization[41-43]. In parallel to this, integrated systems for automated sample pre-treatment would be advantageous for the avoidance of external contaminants during manual handling and minimization of operator-specific variation. PCR microfluidic devices currently under development for the amplification of nucleic acid templates within biological samples, would clearly play a central role in such standardization and also in

reducing the length of time required for sample preparation before hybridization[44;45]. In addition to the serodiagnosis of HCV infection, the diagnostic resolution of our DNA microarray could be extended for discriminating between HCV genotypes by adding additional capture probes to the DNA microarray. This would be of great assistance to physicians as genotype determination is used clinically as an indicator of expected patient response to ribavirin therapy[46].

Anti-HCV antibody protein microarray

Artificial targets – A second line of investigation focused on the development of an HCV antigen microarray for the detection of human anti-HCV antibodies. Although the generation of protein microarrays is fundamentally both more expensive and technically difficult than DNA microarrays, they offer the distinct advantage of enabling protein biomarker identification (e.g. viral antigens, host antibodies and cytokines) which can often be carried out more quickly than nucleic acid analysis due to fewer processing steps. To demonstrate the specificity of the HCV core and NS4 antigens and to investigate the effect of wash duration on the dynamic range of sensitivity, a calibration curve based analysis of the dilution of recombinant anti-HCV core antibodies was performed (**Error! Reference source not found.**).

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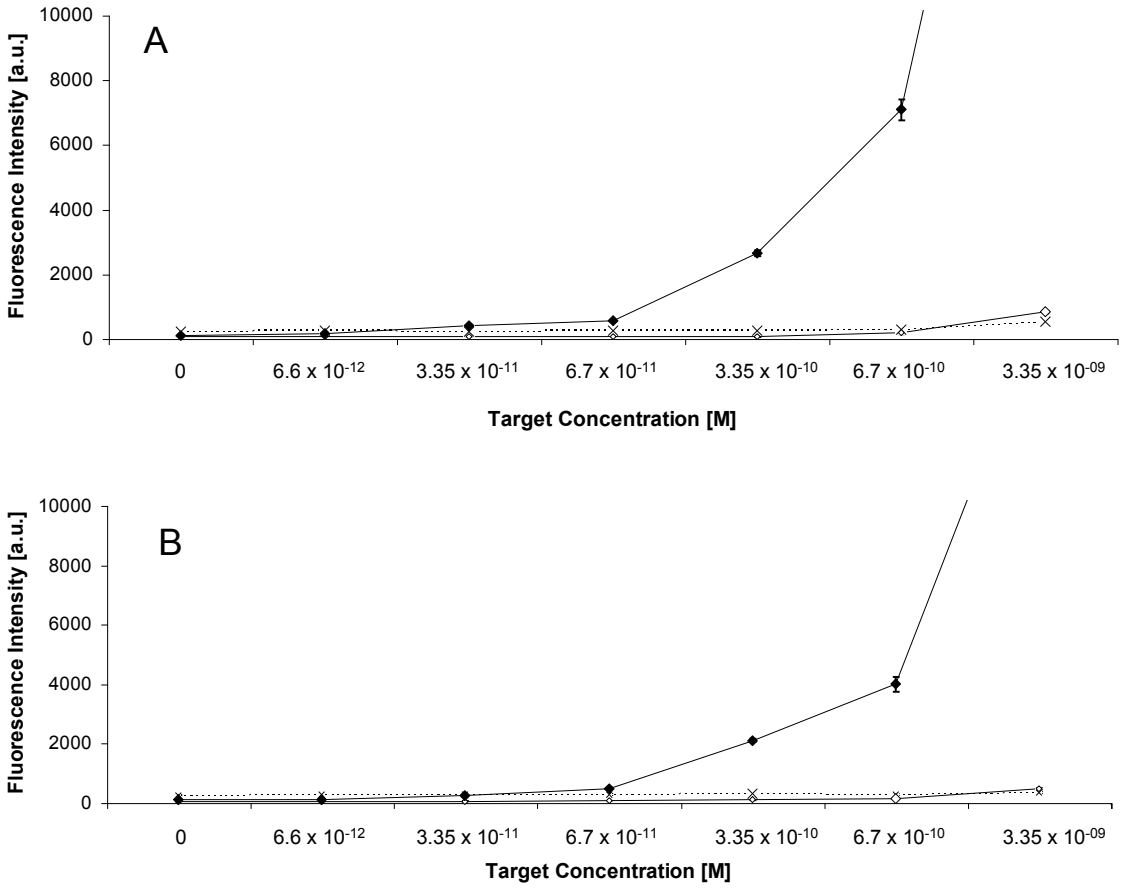

Fig. 5. Calibration plot for anti-HCV core antibody detection in 1:10 diluted human serum for the (A) "long" and (B) "short" protocol. HCV core, NS4 and *Arabidopsis* total plant protein negative control probes are represented by black diamonds, white diamonds and crosses respectively. n = 5.

A shows that anti-HCV core antibodies bound specifically to the immobilized core antigen with a limit of detection (LOD) of 6.7 x 10^{-11} M (10 ng/mL) in 1:10 diluted human serum with minimal cross reactivity. The LOD was the same for both protocols. For anti-HCV NS4 antibodies similar calibration curves resulting in LODs of 3.35 x 10^{-10} M (50 ng/mL) and 6.7 x 10^{-11} M (10 ng/mL) for anti-HCV NS4 antibodies were observed when using the "long" and "short" protocols, respectively (see also supplementary data Fig. S-3). Although these detection limits are in accordance with most other protein detection technologies, well documented methods of signal amplification such as tyramide deposition and rolling circle amplification would be expected to further increase the analytical sensitivity (but also the time to result) of the HCV antigen microarray[47].

Validation with HCV infected patient samples – A final experiment was designed to evaluate the clinical application and analytical sensitivity of the HCV antigen microarray with the same anti-HCV seroconversion panel as used for assessment of the DNA microarray. In a blinded experiment, target samples consisting of 1:10 diluted anti-HCV seroconversion plasma samples were incubated on the HCV antigen microarray (***Fig. 6***).

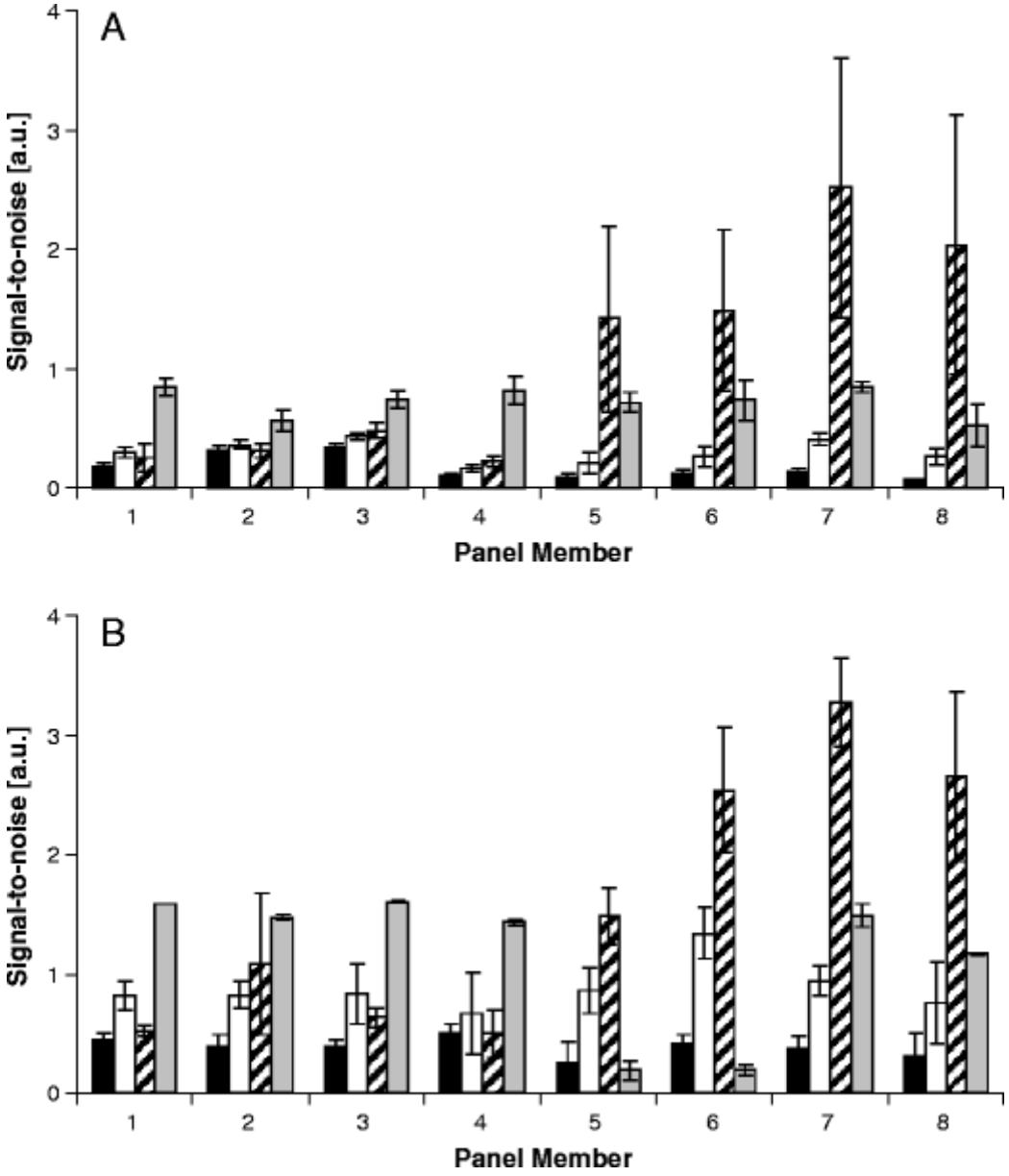

Fig. 6. Normalized hybridization signals for the detection of anti-HCV antibodies in a commercial anti-HCV seroconversion panel using the (A) "long" and (B) "short" protocol. Negative control, NS4, core and positive control probes are represented by black, white, dashed and grey bars respectively. n = 25.

As can be seen in ***Fig. 6***, the HCV core antigen enabled the successful serodiagnosis of panel members in contrast to the NS4 antigen. The normalized hybridization signal from HCV NS4 probes remained constant across each of the panel members, showing that they did not correlate with increasing antibody titres as described in the supplier data (see supplementary data Table S-1). This emphasizes the requirement of validation experiments with clinical samples. The applicability of using the presence or absence of anti-HCV core antibodies as a basis for serodiagnosis is supported by previous findings suggesting that anti-HCV core antibodies are amongst the first virus-specific antibodies to appear after acute infection[48]. As the HCV core antigen is thought to be the most abundant protein in the virion with highly conserved immunogenic epitopes, this antigen probably serves as the primary target for the selection of anti-HCV antibodies *in vivo*[49]. Under these conditions, all panel members were correctly identified as being either seropositive or seronegative in concordance with commercial assays when using the "long" protocol, representing a TTR of 134 min (***Fig. 6***A). The progressive increase in anti-HCV core antibody titre appears to be a temporal effect and fits with increasing antibody production during a typical immune response. When using the "short" assay protocol representing a TTR of 14 min, all of the panel members were again correctly serodiagnosed, with panel member 2 being classified as very weakly seropositive (S/N = 1.08; ***Fig. 6***B). This classification is still consistent with supplier data in which some commercial assays indicate a seropositive/seronegative classification for panel member 2 (e.g. Roche/Boehringer Mannheim Cobas Core Anti-HCV EIA; Table S-1). As expected, the normalized hybridization signals from the positive control and negative control probes remained at a relatively constant level across all panel members when using both the "long" and "short" protocols (***Fig. 6***A & B).

Implementation of the current DNA and HCV antigen microarrays into a microfluidic platform for 'point-of-care' diagnostics would be expected to further reduce the TTR of these assays. In such platforms, hybridization/incubation speeds are accelerated owing to the reduced distance target molecules must diffuse before reaching the microarray probes[50-53].

Conclusion

The goal of the current study was to determine whether it is possible to use microarray technology to detect HCV RNA and human anti-HCV antibodies in clinical samples within a timeframe required for 'point-of-care' diagnostics. A DNA microarray comprising arrays of a universal HCV oligonucleotide detection probe which covers all six genotypes was designed based on a sequence alignment of the 5'-UTR of 113 HCV isolates. Initial microarray characterization showed artificial HCV target could be detected with picomolar sensitivity with only a 10 minute hybridization step. The capability of the DNA microarray to analyse real clinical samples was tested with RNA isolates from HCV-positive

patients. These experiments showed that all patient samples were correctly identified in concordance with real-time PCR performed at the Royal Infirmary of Edinburgh. All samples of a commercial HCV seroconversion panel test were identified correctly with the "long" protocol. Applying the "short" protocol six out of seven serconversion samples were identified correctly. Only the first sample which was identified positive with the commercial CHIRON HCV RNA test (Novartis, Basel, Switzerland) was slightly below the cut-off using the "short" protocol. These data suggest that our DNA microarray has sufficient clinical sensitivity for the correct discrimination of seropositive and seronegative patients.

A second stream of development included designing an HCV antigen microarray for the detection of anti-HCV antibodies in clinical samples. Calibration curves from optimization experiments showed the LOD for anti-HCV core and NS4 antibodies to be 6.7×10^{-11} M (10 ng/mL) and 3.35×10^{-10} M (50 ng/mL) in 1:10 diluted serum respectively, with a TTR of only 14 minutes. The test performance was validated with an HCV seroconversion panel. Seroconversion panels are the 'Gold Standard' for the assessment of novel detection systems and are used to decide whether to introduce them into the clinical setting. A blinded experiment showed that the HCV antigen microarray using the HCV core protein as probe correctly serodiagnosed all panel members and exhibited an analytical sensitivity comparable to that of commercial systems with a TTR of only 14 minutes. It is abundantly clear that at the present, protein microarray technology offers the most realistic opportunity for developing a rapid, microarray-based, 'point-of-care' diagnostic platform. This is because the length of time required for sample processing (e.g. RT-PCR) currently prohibits the use of DNA microarrays for time-sensitive applications such as disease diagnosis and the extra handling steps associated with sample pre-treatment (e.g. nucleic acid extraction), can potentially contaminate/damage precious low yield template. Prevention of operator-specific variation and improvements to the TTR and analytical sensitivity of DNA microarrays could be achievable with automated modules for sample preparation. In conclusion, the results presented in this study constitute a significant step forward in reducing assay time for microarray-based diagnostics and provides a proof-of-concept for the integration of protein and DNA microarrays into 'point-of-care' diagnostics for the serodiagnosis of human infectious diseases.

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