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Optimization of peptide arrays for studying antibodies to hepatitis C virus continuous epitopes

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Abstract

Accurate and in-depth mapping of antibody responses is of great value in vaccine and antibody research. Using hepatitis C virus (HCV) as a model, we developed an affordable and high-throughput microarray-based assay for mapping antibody specificities to continuous antibody epitopes of HCV at high resolution. Important parameters in the chemistry for conjugating peptides/antigens to the array surface, the array layout, fluorophore choice and the methods for data analysis were investigated. Microscopic glass slide pre-coated with N-Hydroxysuccinimide (NHS)-ester (Slide H) was the preferred surface for conjugation of aminooxy-tagged peptides. This combination provides a simple chemical means to orient the peptides to the conjugation surface via an orthogonal covalent linkage at the N- or C-terminus of each peptide. The addition of polyvinyl alcohol to printing buffer gave uniform spot morphology, improved sensitivity and specificity of binding signals. Libraries of overlapping peptides covering the HCV E1 and E2 glycoprotein polypeptides (15-mer, 10 amino acids overlap) of 6 major HCV genotypes and the entire polypeptide sequence of the prototypic strain H77 were synthesized and printed in quadruplets in the assays. The utility of the peptide arrays were confirmed using HCV monoclonal antibodies (mAbs) specific to known continuous epitopes and immune sera of rabbits immunized with HCV antigens. The methods developed here can be easily adapted to studying antibody responses to antigens relevant in vaccine and autoimmune research.

Keywords

HCV; E1E2 glycoprotein; peptide array

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1. Introduction

Hepatitis C virus (HCV) infection is a major public health problem with an estimated 170 million infected people worldwide (Shepard et al., 2005). It is a leading cause of chronic liver disease, cirrhosis and hepatocellular carcinoma. Therapeutic antibodies and vaccines have been successfully developed to protect at-risk populations against many viral diseases, but, so far, have not been successful for HCV.

Early studies in chimpanzee using HCV envelope glycoproteins E1 and E2 demonstrated a correlation between antibody response and protection against virus challenge (Choo et al., 1994; Rosa et al., 1996). However, complete protection was only observed with homologous but not heterologous virus challenge. This highlights the need of eliciting cross-neutralizing antibodies in any candidate HCV vaccines to be broadly effective against this antigenically diverse virus. Recently, a phase I clinical trial of a HCV vaccine candidate composed of recombinant E1E2 formulated in MF59 adjuvant resulted in only weakly neutralizing antibody responses in humans (Ray et al., 2010; Meyer et al., 2011). Ray and coworkers performed antibody mapping using 4 biotinylated peptides in a standard ELISA format to study antibody responses to the E1 neutralizing epitope aa313-327 (Meunier et al., 2008), the E2 hypervariable region 1 (HVR1) aa384-411 important for antibody neutralization and escape (Weiner et al., 1992), the conserved E2 antigenic site aa412-419 (Kong et al., 2012a; Kong et al., 2012b; Potter et al., 2012) and the antigenic E2 aa434-446 region (Zhang et al., 2009). It has been suggested that antibodies to E2 aa434-446 can interfere with virus neutralization by antibodies to aa412-423 (Zhang et al., 2009), although neutralizing antibodies to E2 aa434-446 have recently been reported by multiple groups (Keck et al., 2012; Morin et al., 2012; Tarr et al., 2012; Deng et al., 2013). Clearly, better tools in the analysis of global antibody responses are needed to provide information for the improvement of HCV immunogens. Because of the polyclonal nature of antibody responses and the extreme genetic diversity of HCV field isolates with genomic sequences differing by up to 35% (Kuiken and Simmonds, 2009), existing analytic methods for monitoring antibody responses, e.g. ELISA, are difficult to address the viral diversity problem in assay design.

With the growing interest in anti-HCV antibodies for vaccine development, it would be highly desirable to develop sophisticated tools to improve the detail and throughput in mapping HCV antibody responses. Traditional methods are labor intensive, time consuming, requiring large amount of coating antigens in the μg/ml range and low in throughput. ELISA and other assays rely on the adsorption of macromolecules either by electrostatic forces or hydrophobic interactions. Several strategies have been developed to improve these assays, which include sandwich or indirect ELISA, competition ELISA, direct covalent attachment of peptides to modified plate surface (Niveleau et al., 1995), using a biotinylated peptide with a streptavidin modified plate (Selo et al., 1996), or using polystyrene-binding peptide tags (Kogot et al., 2012). However, the cost of these methods are prohibitive and highly labor-intensive if large number of features (>1,000) are to be studied.

Microarray technology has been widely used in the study of gene expression and regulatory profiles for over two decades (Schena et al., 1995) and has now been expanded to various

formats including protein, peptide, and glycan arrays (MacBeath, 2002; Reimer et al., 2002; Reineke et al., 2002; Blixt et al., 2004; Sun et al., 2004; Andresen and Bier, 2009; Pejchal et al., 2011; Walker et al., 2011; Zhu et al., 2012). We herein describe a novel low cost peptide array assay for mapping antibody specificities at high resolution. We investigated the use of an oxyamine linkage for peptide–surface chemistry for conjugation of analyte to surface. We demonstrate the utility of this method using HCV envelope glycoproteins as an experimental model.

2. Methods

2.1. Peptide preparation

A library of peptides, consisting of 15 amino acids in length with an offset of 5 amino acids, corresponding to the amino acid sequences of HCV E1 and E2 envelope glycoproteins representing 6 major HCV genotypes (genotype 1a, H77; 1b, UKN1b12.6; 2a, J6E3; 2b, UKN2B2.8; 3a, UKN3A1.28; 4, UKN4.11.1; 5, UKN5.15.7; and 6, UKN6.5.34), were custom synthesized in-house at 25-35 μg/well using a MultiPep RS automated peptide synthesizer. The entire H77 isolate polypeptide sequence was synthesized similarly as above for testing human samples. The peptides had a β-alanine C-terminus and a 2.5 polyethylene glycol (PEG) aminooxy N-terminus to ensure optimal epitope orthogonal attachment and presentation. The aminooxy group has greater reactivity kinetics to surface functional groups than lysine or other amine side chains at pH 8.

Synthesized peptides were suspended in 12.5 μl DMSO and 12.5 μl of ultra-pure water. Immediately prior to printing, suspended peptides were diluted 1:4 in a custom protein printing buffer [Saline Sodium Citrate (SSC) 300 mM sodium citrate, pH 8.0, containing 1 M sodium chloride supplemented with 0.1% Polyvinyl Alcohol (PVA) and 0.05% Tween 20], in a 384-well non-binding polystyrene assay plate. Two positive control peptides, hemagglutinin A (HA) (YPYDVPDYA) and FLAG tag (DYKDDDDK), were initially included in the print to guide proper grid placement and peptide ID alignment, and also served as controls for the assays. They were later replaced with a permanent fluorescent dye at 488 nm. The controls were used in the initial optimization of the array printing conditions.

2.2. Microarray printing

All peptide samples were printed in quadruplicatic at an approximate density of 1 ng/spot, on epoxy or N-hydroxysuccinimide ester (NHS-ester) derived glass slides (Slide E or H, Schott AG) using a Microgrid II (DigilabGlobal) microarray printing robot, equipped with solid steel (SMP4, TeleChem) microarray pins. Humidity was maintained at 50% during the print. Immediately prior to interrogating the arrays, slides were blocked for 1 h with ethanolamine buffer to quench any unreacted NHS-ester or epoxide amine residues on the slide. All slides were used within 2 months of printing and stored at -20°C (see Figure 1 for the attachment chemistry). The slides were stable for over 6 months in a -20°C fridge with no apparent loss of activity.

2.3. Immunolabeling with Alexa based detection system

Incubation area was circumscribed around the printed grids using a Peroxidase Anti-Peroxidase (PAP) hydrophobic marker pen (Research Products International Corp) and the subsequent steps done in a humidified chamber at room temperature on a rotator. Control monoclonal antibodies anti-HA and -FLAG were assayed at a concentration of 10 μg/ml whilst sera was diluted to 1:300 in PBS-TM and incubated for 1 h followed by three washes in PBS buffer. The arrays were then incubated for 1 h with goat antimouse IgG with Alexa-Fluor® tag (Invitrogen) or relevant secondary antibody. Arrays were washed three times in PBS-T, two times in PBS, and another two times in deionized water and centrifuged dry at 200 × *g* for 5 mins.

2.4. Analysis of array data

The processed slides were scanned using a ProScanArray HT (Perkin Elmer) microarray scanner and images saved as TIF files. The median feature and background pixel intensities for each antigen spot were determined by Imagene® 6.1 microarray analysis software (BioDiscovery). The fluorescence signal was digitalized and exported as comma-delimited text files into Excel for further analysis.

2.5. Animal immunization

Two rabbits were immunized with a peptide whose sequence corresponds to the HCV1 mAb epitope HCV amino acids 412-423 (QLINTNGSWHIN) three times, three weeks apart using complete and incomplete Freund's adjuvant (Invitrogen). Bleeds were taken one week pre- and post-immunization and the sera pooled. The sera was interrogated in the array to see specific immune responses to the epitope and as well as the quality of immune responses in terms of cross-binding with other HCV genotypes in addition to the homologous H77 sequence (Kuiken et al., 2006) used in the immunization regiment.

2.6. Human sera analysis

The antibody responses in HCV-positive human sera and a normal donor serum were compared using the peptide arrays. The infected sera are a mixture of five HCV-positive human sera known to be HCV neutralizing. The samples were diluted 1:300 in PBS-TM buffer and tested on the peptide array consisting of E1 to E2 regions (Fig 6A) and the entire H77 polypeptide sequence from Core to NS5 (Fig 6B).

3. Results and Discussion

Peptide array performance is affected by various environmental conditions including degradation and denaturation of the peptides during synthesis, printing and storage, it is important to fully optimize and validate the experimental conditions for arrays to be useful as high throughput analytical assays. Additional experimental considerations are the print surfaces, peptide chemistry, print buffers, print conditions, array format, slide storage, assay format, detection system, image capture and data analysis. Orthogonal covalently attached peptides in a microarray format offers potential improvement to traditional immunoassay formats, e.g. ELISA.

A peptide array consisting of 15mer peptides, 10 amino acids overlap of the entire E1E2 region of the HCV glycoprotein covering the 6 major HCV genotypes (Simmonds et al., 2005; Kuiken and Simmonds, 2009) was developed. The peptides were synthesized from C- to N-terminus. For all peptides, there was a beta-alanine at both C- and N-terminus to protect peptides from exopeptidase degradation (Galati et al., 2003). The N-terminal β-alanine was linked to a 2.5 PEG linker enabling the peptides to be extended away from the conjugation surface thus maximizing accessibility and presentation of the antibody epitopes. Finally, an aminooxy group was added to the PEG linker. The unique properties of aminooxy group present an opportunity for chemo selective site-specific immobilization of peptides (Adamczyk et al., 2001). Conjugation of the peptides to the NHS-activated slide was performed at pH 8. The pK_a of aminooxy groups is in the range of 5–6, while primary amines have a pK_a >9. Thus at pH 8, aminooxy, but not amine functional groups in the epitopes (lysine or arginine), will be present at a nucleophilic protonated state and be used preferably in the conjugation reaction (Lees et al., 2006). Based on such solution chemistry, the kinetics of an aminooxy group will out-compete secondary amines on the peptide thus ensuring specific orientation of HCV peptides.

Using this amine conjugation chemistry, the optimal surface for peptide immobilization was investigated. NHS-ester (Slide H) and Epoxide (Slide E) surfaces were evaluated and the NHS slide was found to produce better resolution with lower limit of detection and background compared to Slide E (Figure 2). This method allows a more stable and orthogonal attachment of the peptides compared to the standard method in peptide array synthesis onto nitrocellulose medium or coating peptides directly onto plastic-based ELISA microwells (Cretich et al., 2006). Attempts to use biotin conjugated peptides with streptavidin coated slides were unsuccessful due to low signals (data not shown).

Printing buffer was also optimized for efficient spotting. Several solvent and detergent buffers were investigated to test their effect on spot morphology (Figure 3). Addition of PVA to the buffer (3× SSC + 0.1% PVA and 0.005% Tween-20) not only increases limit of detection, but also improves spot morphology and signal strength. In fact, addition of other commonly used additives in printing buffer, glycerol or PEG, seemed to reduce attachment/immobilization. The results are consistent with Wu *et al.* (Wu and Grainger, 2006) that among the various hydroxylated additives they investigated, PVA produced the most regular spot morphologies. The printing buffer also helps stabilize the droplet and ensures sufficient time for the attachment chemistry between peptides and surface to occur. Consequently, peptides were printed in PVA containing buffer in subsequent experiments as it gave the best spot morphology and uniformity.

A print of 941 unique peptides in quadruple was designed based on the total length of HCV E1E2 glycoprotein primary amino acid sequence of the 6 major HCV genotypes and J6E3 plus H77 isolates. If the same number of features were done using a standard ELISA 96 well plate format, for same number of interactions totaling 3764 measurements, a total of 62 ELISA plates would be required at a cost of five times more than a NHS slide. The peptides were printed at 1 ng/spot in the peptide array, resulting in >100-fold reductions of necessary antigens, as an average of 250 ng of peptides are required for each microwell in ELISA. The experimental time for running peptide array usually takes 6 hours whilst running an ELISA

with 40 plates require much more labor and it can takes as much as 3 days of a full-time technical staff. In addition, the peptide array has automated data capturing and processing making it less tedious than ELISA. Once printed the slides are stable for at least 6 months if stored at -20°C, without any significant loss in activity. Limitations of using peptide microarrays include the restriction to mimicking continuous epitopes and utility diminished in instances of complex three-dimensional antibody interactions.

To validate the above strategy, the specificity and sensitivity of the peptide array were investigated using HCV specific monoclonal antibodies and sera from rabbits immunized with KLH-conjugated with the HCV E2 peptide aa412-423. Various control monoclonals were interrogated to investigate the specificity and performance of the peptide arrays. Positive controls included mAb HCV1 (Broering et al., 2009), specific to the highly conserved E2 linear epitope aa 412-423 (Kong et al., 2012b), mAb A4 (Dubuisson et al., 1994), specific to the E1 region aa 197 to 207 (SSGLYHVTNDC) (extensively used as an anti E1 glycoprotein antibody control in HCV studies)(Triyatni et al., 2002) and the negative control anti-HIV mAb b6. Figure 4 shows that the assay is highly specific to mAb HCV1 which binds to a highly conserved E2 region, hence recognizing the corresponding epitopes in all viral genotypes tested. We were also able to show that mAb A4 binds to genotypes 1a (H77), 4 (UKN4.11.1), 5 (UKN5.15.7) and 6 (UKN6.5.34). This epitope sequence is not conserved across the genotypes. The integrity of the control monoclonal antibodies and peptides was also confirmed by ELISA. These data validate that the peptide array can be used to characterize antibody responses to diverse HCV genotypes with high specificity.

The HCV peptide array assay can be used for probing immune responses to linear epitopes in immunization and for general antibody profiling experiments. The arrays were further validated using antisera from rabbits immunized 3 times with a 12-mer cyclic peptide conjugated to Keyhole limpet hemocyanin (KLH) carrier protein in Freund's complete/ incomplete adjuvant system and demonstrated its specificity to detect antibodies to E2 aa412-423 from different HCV genotypes (Figure 5). The antisera did not react with other HCV linear epitopes non-specifically nor did the pre-immune sera.

To demonstrate potential usage of the peptide arrays, HCV-positive patient sera were assessed (Figure 6). Five HCV-neutralizing sera from chronic patients were mixed in equal volume and evaluated using the E1E2 peptide array above and another array containing the entire HCV polypeptide (Core to NS5) of the genotype 1a prototypic strain H77. In this preliminary assessment, the HCV-immune sera specifically detected peptides around the E2 region 680-695 from all 8 isolates of the 6 major genotypes (Figure 6A). This region is part of the membrane proximal external region that is targeted by one of the broadly neutralizing mAb AR4A (Giang et al., 2012). In comparison to antibody responses to E1E2, antibody responses to the entire polypeptide (Figure 6B) showed high reactivity towards the Core, NS4A/B and NS5A regions. The uninfected serum generally had low reactivity towards the HCV peptides but non-specific background reactivity could be observed in the E1, E2, NS3 and NS5B regions, particularly to the N-teriminus of E1 and NS5B of the H77 isolate. Interestingly, NS5B is typically excluded from HCV diagnostic assays as a detecting antigen because of its significant homology to other RNA viruses (Maertens et al., 1999). Further experiments are underway to determine peptides in the arrays that are prone to generate non-

specific signals using a large panel of sera from normal blood donors. The results will identify the assay limitations for studying polyclonal antibodies, and help optimize the specificity and sensitivity of the assays.

Together these data support a robust, effective and rapid method to dissect immune responses to linear HCV epitopes in great details. The arrays can also be adapted for alanine scanning mutagenesis analysis for mapping epitope to single amino acid level. The method presented has broad applicability and adaptability as similar strategy can be used for other HCV structural and non-structural proteins and also in HIV, FLU and other infectious diseases studies.

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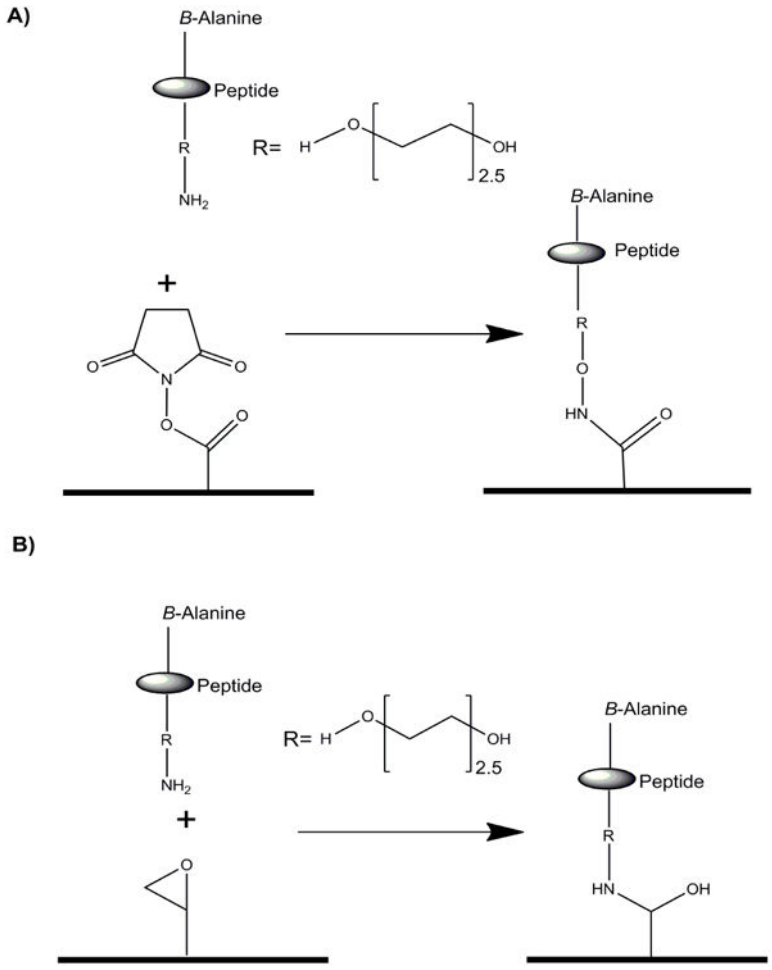

Figure 1.
Peptide chemistry for Slide H (A) and E (B).

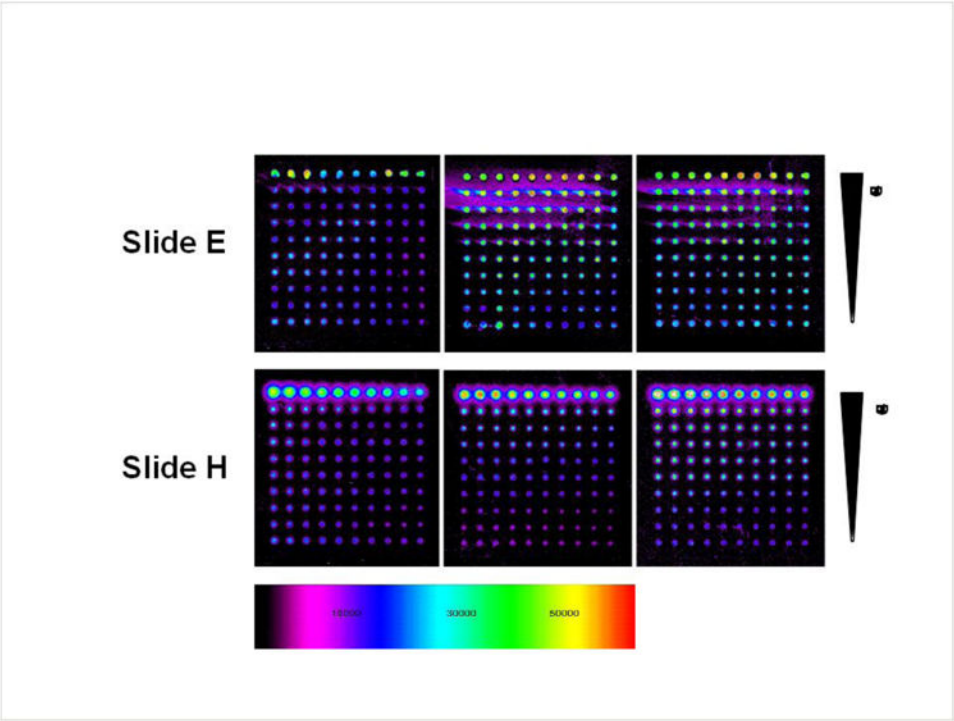

Figure 2.

Slides E and H in triplicate were interrogated with a mouse anti-hemmaglutinin (HA) antibody titrated from 10 μg/ml by 2-fold dilution down to 0.0195 μg/ml. AlexaFluor488 conjugated anti-mouse-IgG (10 μg/ml) was used to develop the array. Slide H was the preferred surface for further assay development.

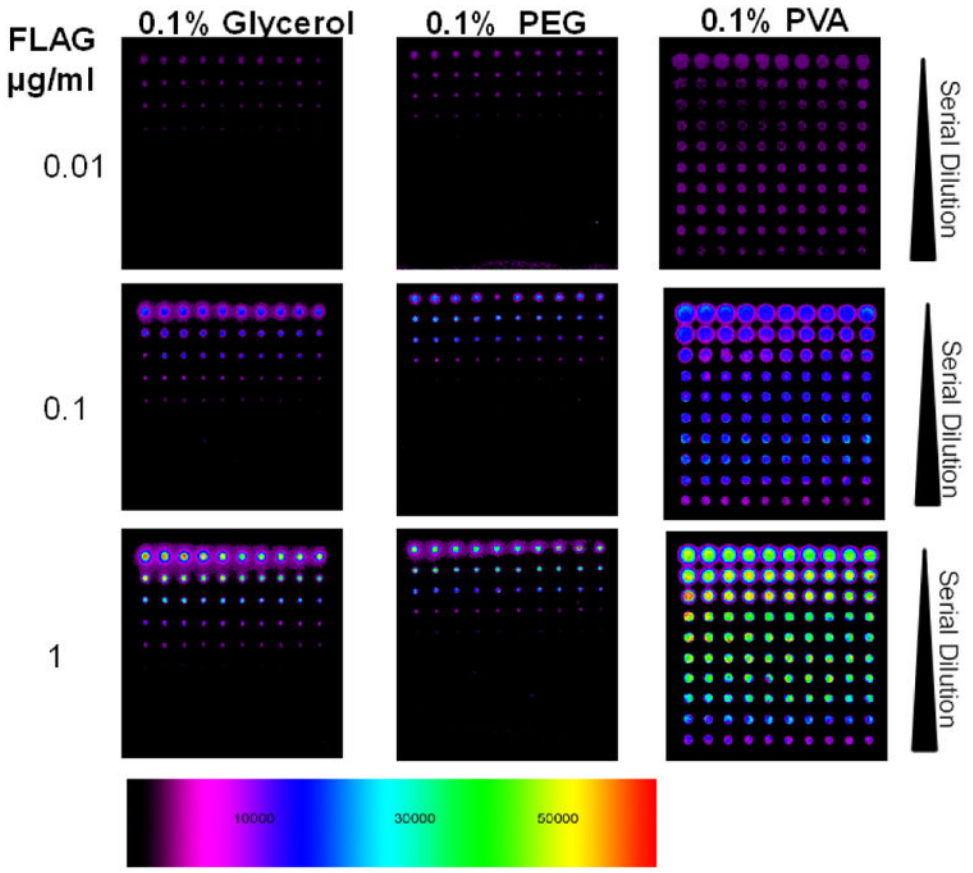

Figure 3.

Print buffer Optimization. Three buffer systems; Glycerol, PEG and PVA were investigated and FLAG peptides were printed at different coating concentrations. The buffer was titrated down with the first row being 10 µg/ml of FLAG peptide in 25% H_2O, 25% DMSO and 50% Buffer (Glycerol, PEG or PVA), followed by two-fold serial dilution of peptides in buffer.

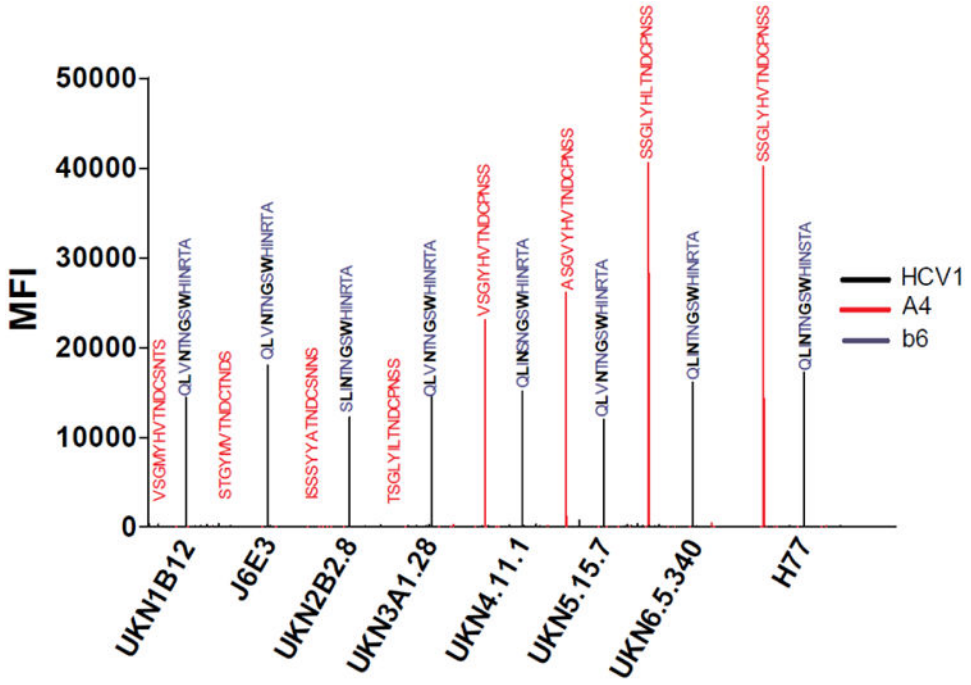

Figure 4.

A plot of Mean Relative Fluorescence Intensity (MFI) vs Genotype for mAb b6 (negative control, no detectable signal observed), A4 (anti-E1) and HCV1 (anti-E2). For mAb HCV1 epitope critical residues L413, N415, G418 and W420 are shown in black bold (Kong et al., 2012b) An HCV peptide print consisted of the E1E2 glycoprotein overlapping peptide sequences for the 6 major HCV genotypes and J6E3 plus H77 isolates. The signals were obtained after development with Alexa-Fluor tag conjugated anti-IgG antibodies. The processed slides were scanned using a ProScanArray HT microarray scanner and images saved as TIF files. Fluorescence signal was digitalized and exported as comma-delimited text files. The median feature and background pixel intensities for each antigen spot were determined by Imagene 6.1 microarray analysis software.

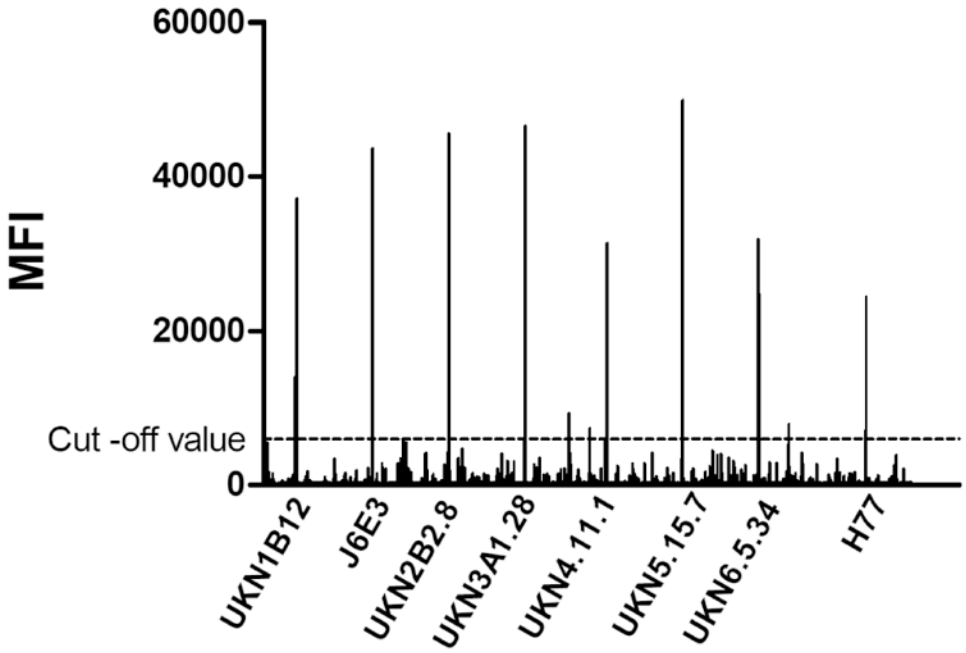

Figure 5.

Peptide array analysis of anti-HCV antibody responses in rabbits immunized with KLH-conjugated peptide corresponding to HCV aa412-422 sequence. The array displayed clear sera specificity for the immunizing peptide (H77) and the corresponding peptides in the other HCV genotypes. The signal cut-off was defined as 10% of the maximum signal in the assay. The peaks are identical to the HCV1 epitopes shown in Figure 4. The array was incubated with rabbit sera diluted at 1:300, followed by an Alexa-Fluor 633 conjugated anti-rabbit IgG secondary antibody. Data was processed as described in Figure 4. Pre-immune sera did not produce any significant signals above the background cut-off value 5000 MFI (Data not shown).

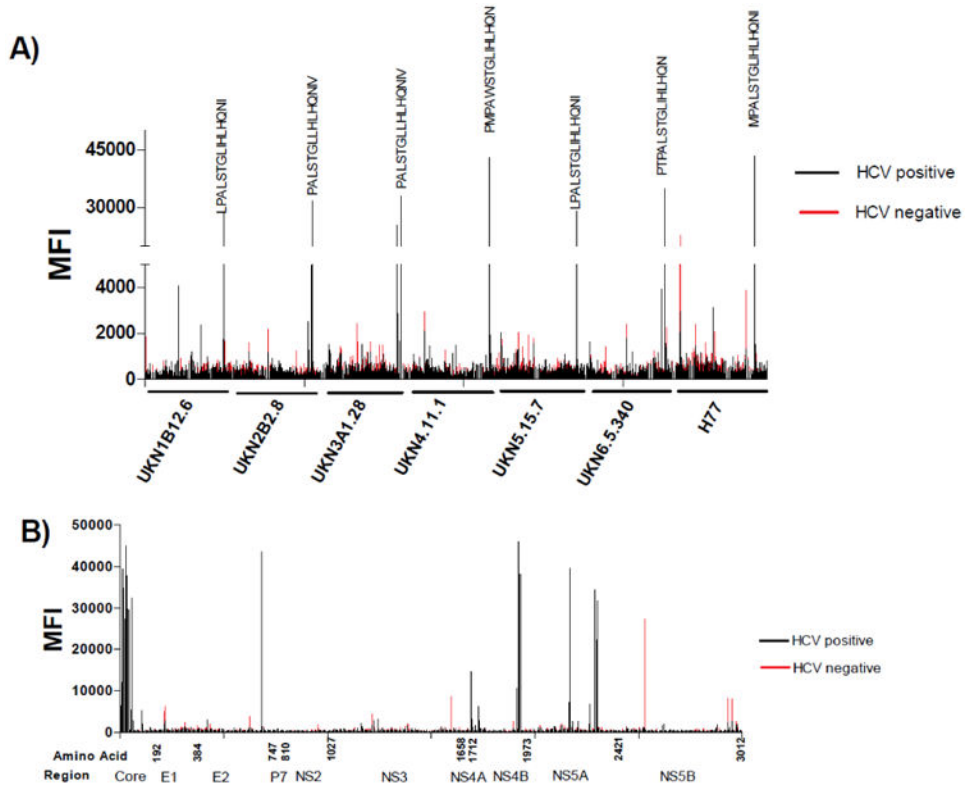

Figure 6.

Peptide array analysis of anti-HCV antibody responses in sera of infected patients and normal blood donor. **A)** Responses of normal and infected sera to the E1 and E2 regions of 6 major HCV genotypes. **B)** A snap shot of antibody responses to the entire polypeptide sequence of the prototypic genotype 1a isolate H77. The arrays were incubated with human sera samples diluted at 1:300, followed by an Alexa-Fluor 633 conjugated anti-human IgG secondary antibody. Data was processed as described in Figure 4.