CVI Accepts, published online ahead of print on 23 February 2011
Clin. Vaccine Immunol. doi:10.1128/CVI.00265-10

DEVELOPMENT OF A MULTIPLEX BEAD-BASED ASSAY FOR HEPATITIS C DIAGNOSIS

Bruna P.F. Fonseca,*[1] Christiane F.S. Marques,[1] Lílian D. Nascimento,[1] Marcelle B. Mello,[1] Leila B.R. Silva,[1] Nara M. Rubim,[1] Leonardo Foti,[2,3] Edimilson D. Silva,[1] Antonio G.P. Ferreira,[1] Marco A. Krieger,[2,3].

Laboratório de Tecnologia Diagnóstica, Bio-Manguinhos - Fiocruz, Av. Brasil 4365, CEP 21045-900, Rio de Janeiro, RJ, Brasil[1]; Instituto Carlos Chagas - Fiocruz, R. Professor Algacyr Munhoz Mader, 3775, CEP 81350-010 Curitiba, PR, Brasil[2]; Instituto de Biologia Molecular do Paraná, R. Professor Algacyr Munhoz Mader, 3775, CEP 81350-010 Curitiba, PR, Brasil[3]

*Corresponding author. Mailing address: Laboratório de Tecnologia Diagnóstica, Bio-Manguinhos - Fiocruz, Av. Brasil 4365, CEP 21045-900, Rio de Janeiro, RJ, Brasil. Phone: +55 21 3882-7142. Fax: +55 21 3882-9399. E-mail: bruna.fonseca@bio.fiocruz.br

ABSTRACT

Hepatitis C (HCV) infection is a major burden to public health worldwide, affecting approximately 3% of the human population. Although HCV diagnosis is currently based on reliable tests, the field of medical diagnostics has a growing need for inexpensive, accurate, and quick high-throughput assays. By using the recombinant antigens HCV NS3, NS4, NS5 and Combined we describe a new bead-based multiplex test capable of detecting HCV infection in human serum samples. The first analysis, made in a singleplex format, showed that each antigen coupled to an individual bead set presented high responses for anti-HCV-positive reference serum pools and lower responses for the negative pools. Our next approach was to determine the sensitivity and specificity of each antigen by testing 93 HCV-positive and 93 negative sera. When assayed in singleplex, the NS3, NS4 and NS5 antigens presented lower sensitivity values (50,5%, 51,6% and 55,9%, respectively) when compared to the Combined antigen, which presented a sensitivity of 93,5%. All antigens presented 100% specificity. These antigens were then multiplexed in a 4-plex assay, which resulted in increased sensitivity and specificity values, performing with 100% sensitivity and 100% specificity. Predictive negative and positive values for the 4-plex assay were of 100%. Although preliminary, this 4-plex assay showed robust results that, aligned with its small sample volume requirements and also cost- and time-effectiveness, make it a reasonable alternative to tests currently used in HCV screening of potentially infected individuals.

INTRODUCTION

According to the World Health Organization, the hepatitis C virus (HCV) affects approximately 200 million people worldwide, almost 3% of the world population. HCV infection is characterized by a high propensity to progress to persistent infection leading to chronic liver disease which, in certain patients, may evolve into cirrhosis and hepatocellular carcinoma (10). International studies estimate that because the risk of HCV-related chronic liver disease is associated to the duration of infection, it is likely that HCV-related complications will increase in the upcoming decades, being quadrupled in 2015 (5). To curb this trend, health services need to improve the screening of infected individuals in order to treat them when liver disease is asymptomatic and not life-threatening.

Currently, routine diagnosis of HCV is based on detecting anti-HCV IgG antibodies in serum or plasma by enzyme immunoassay (EIA). Cloning of the HCV genome and sequence analysis has led to the development of a variety of antigens and synthetic peptides that have been successfully used in these immunoassays, improving the reliability of the test and increasing detection of anti-HCV earlier in the course of infection (1, 2, 6). In spite of that, false-positive results of EIAs are still prevalent especially among low-risk subjects, such as blood donors, or populations without liver-related diseases (4). This requires supplemental or confirmatory tests, potentially increasing the volume of sample needed, as well as the associated technologist and instrument time required for testing, and most of the times leading to unnecessary health-care costs and difficulties in diagnosis (3). These tests also have important impairments: low processing speed, high labor time, low throughput capacity, limited multiplex capability and high cost (18, 24).

In the past decade, several technologies have emerged as diagnostic tools capable of improving diagnosis by using multiplex principles. The diagnostic process becomes faster, less expensive, and the hands-on time in laboratories decreases substantially since these platforms can be fully automated (17). One of the most promising multiplex techniques uses digital signal processing to classify small polystyrene beads. The beads are internally dyed with distinct proportions of red and near-infrared fluorophores and these proportions define an intrinsic fluorescence or spectral address for each bead population (12, 15). Each group of beads can be coupled to a specific capture molecule, including protein antigens, acting as solid supports for the detection of their respective antibodies. Since the beads can be distinguished by their spectral addresses, they can be combined to produce multiplex assays, thereby allowing the rapid screening of multiple antibodies using a small volume of plasma. The captured antibodies are detected and quantified following the addition of a fluorescently labeled reporter antibody whose emission is measured by a flow-based detector (12, 15).

Bead-based immunoassays allow quantitative and qualitative analysis of multiple targets with a unique combination of features, including rapid data acquisition, excellent sensitivity and specificity, and multiplexed analysis capability (19). This system is an open platform that allows the detection of several molecules, having applications for the screening of serum antibodies against a plethora of infectious agents (8, 9, 13, 14) and also in vaccine trials (21).

In this study, we developed a bead-based high-throughput immunoassay for determination of antibodies against HCV in patient serum samples using the antigenic properties of four recombinant proteins: NS3, NS4, NS5 and Combined. These proteins have been widely used on screening tests for HCV and represent the most relevant epitopes

for HCV diagnosis, being associated with both acute and chronic phases of the disease (20, 22). We describe its application as a rapid, less time consuming and less serum demanding assay, demonstrating that this platform is suitable for epidemiologic and also diagnostic applications in HCV management.

MATERIAL AND METHODS

Human Sera. A total of 186 serum samples were analyzed in the study: 93 positive and 93 negative sera for HCV, obtained from the Technology Institute for Immunobiologicals (Bio-Manguinhos)/FIOCRUZ. All serum samples were previously analyzed using conventional serologic tests (ELISA) and assigned a definitive serostatus (positive or negative for anti-HCV). Samples were tested with two commercially available HCV ELISA kits from different manufacturers and were classified as serologically "positive" or "negative" according to the instructions provided by each manufacturer. Of these 186 samples, 53 (23 positive and 30 negative) were obtained from the National Panel for Blood Screening Quality Control (AEQ), which was elaborated and tested by Bio-Manguinhos/FIOCRUZ and the National Institute for Health Quality Control (INCQS)/FIOCRUZ. From the AEQ samples, six serum pools were constructed: three "negative" pools consisting of 10 sera each, which had negative results in all assays; and three "positive" pools containing 10, 8 and 5 sera each, which were unequivocally positive on all tests. These samples were also individually used throughout the experiments.

Antigens. The recombinant antigens NS3, NS4, NS5 and Combined were purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel). The Combined antigen is a fusion protein comprised of nucleocapsid, NS3, NS4 and NS5 immunodominant epitopes.

Bead Coupling to HCV antigens. Coupling of recombinant antigens to paramagnetic carboxylated beads (Luminex Corp., TX, USA) was performed according to the manufacturer's instruction. Briefly, 10^6 beads were vortexed and sonicated to assure a

homogeneous distribution. The beads suspension was then washed twice with double-distilled water (dH2O) and suspended in 80 µl of activation buffer (100mM sodium phosphate, pH 6.2). Solutions (10 µl of each) of N-hydroxysulfosuccinimide (sulfo-NHS; Pierce, IL, USA) and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC; Pierce), both diluted to 50mg/ml in dH2O, were added to stabilize the reaction and activate the beads. After mixing, the beads were incubated for 20 min in the dark at room temperature. The activated beads were subsequently washed with coupling buffer (0.1M $NaHCO_3$ pH 8.0), after which 200 µl of antigen solution was added and incubated with shaking for 2 h. Antigen concentrations for coupling were experimentally determined previously (data not shown). After incubation, the beads were washed (PBS 1x, 1% BSA, 0.02% Tween 20, 0.05% sodium azide) and suspended in 500 µl of blocking/storage buffer (PBS 1x, 1% BSA, 0.02% Tween 20, 0.05% sodium azide). The beads were counted with a hemocytometer, adjusted to a concentration of 10^6 beads/ml with storage buffer, and stored protected from light at 2–8 °C.

Bead-based immunoassay standard protocol. Serum samples were diluted in assay buffer (PBS 1x, 1% BSA, 0.02% Tween 20, 0.05% sodium azide) and the test was performed using standard procedures described by the manufacturer (Luminex Corp.). A total of 50 µl, containing approximately 2,000 coupled beads, was added to each well of a flat-bottom 96-well plate. For the multiplex assays, the same number of coupled beads was added to each well for each bead set. Diluted serum (50 µl) and beads were mixed and incubated for 30 minutes in the dark. The beads were then washed twice with 100 µl of wash buffer (PBS 1x, 1% BSA, 0.02% Tween 20, 0.05% sodium azide), and 100 µl goat anti-human IgG conjugated to phycoerythrin (Sigma-Aldrich, MO, USA) was added and

incubated for 30 minutes in the dark. The beads were washed twice with 100 µl of wash buffer and the beads reporter fluorescence, expressed as median fluorescence intensity (MFI), was determined with a LabScan 100 (One Lambda, CA, USA). All incubations were performed at 37ºC on a microplate shaker (set at 600 rpm) and the wash steps were performed on a Hydroflex plate washer with a magnetic plate support (Tecan, NC, USA).

Net MFI and cut-off determination. Samples were always assayed in duplicates and the MFI values were considered valid when the bead count reached a minimum of 100 beads per bead set per well. The net MFI values were obtained by subtracting the mean MFI of the duplicates of each sample from the mean of the MFI obtained from the background wells (no serum added). The cut-off value for each antigen was determined using the receiver operating characteristic (ROC) analysis, defined as the MFI value that gave the best combination of sensitivity and specificity. Samples were then classified as "positive" or "negative" according to the cut-off values specific for each antigen. For the multiplex assay, a sample was considered "positive" if it had one or more positive results from the individual antigens.

Statistical analysis. According to the cut-off values determined for each antigen, test performance was assessed. All samples (positive and negative) were initially assayed in duplicate. Upon repeat testing, specimens were classified as "positive" (reactive) or "negative" (non-reactive). The results of the second test confirmed the previous ones in 100% cases. Sensitivity was defined as the correct identification of anti-HCV-positive samples, using the following formula: sensitivity = true positive/(true positive + false negative) × 100%. Specificity was defined as the correct identification of anti-HCV-

negative samples, using the following formula: specificity = true negative/(true negative + false positive) × 100%. True positives and true negatives were defined as the numbers of anti-HCV-positive and -negative samples identified correctly by each test. False positives and false negatives were defined as the numbers of anti-HCV-negative or -positive samples identified incorrectly by each method. Other outcome measures were as follows: Positive predictive value = true positive/(true positive + false positive) × 100%. Negative predictive value = true negative/(true negative + false negative) × 100%.

RESULTS

Antigen performance in singleplex. As the performance of each antigen influences the assay final outcome, we initially assessed the efficiency of antigen coupling to the beads and identified the best sample dilution to be used in the assay using six serum sample pools derived from the AEQ panel (three positive for anti-HCV and three negative). Each HCV antigen was coupled to an individual bead set. Serial dilutions of pools ranging from 1:25-1:1,600 were prepared and assayed with the standard test protocol. The analyses of each antigen performance were made separately in order to assess the range of fluorescence generated by each individual bead set. All antigens presented high MFI values for the positive pools and lower values for the negative pools, demonstrating proper antigen coupling. In spite of the different behavior of the three positive pools, a 1:200 dilution gave optimal signal results for all antigens (Fig. 1). This dilution proved to be the best compromise for the multiplexed format where a common dilution is needed and, for that reason, it was used in all subsequent experiments. The test for the coupling efficiency showed that the highest coupling readings ranged from 2,000 to 12,000 MFI for the five different antigens, while backgrounds (no serum added) were lower than 100 MFI, proving that antigen coupling to the beads was successful (Fig. 1).

Singleplex specificity analysis. With the coupling efficiency confirmed and the sample dilution determined, our next step was to verify specific anti-HCV responses in patient samples. For that purpose, 93 positive and 93 negative sera for anti-HCV were individually assayed against all antigens. When the NS3, NS4 and NS5 antigens were assayed with individual samples in singleplex format, they were not able to clearly

differentiate the negative from the positive serum samples, whereas the Combined antigen succeeded in doing so (Fig. 2). This was not an unexpected result since the Combined antigen is a fusion protein composed of different epitopes that include all three antigens mentioned above, reinforcing the importance of combining distinct antigens for better detection.

Antigen performance in multiplex. To determine if MFIs were equivalent when antigen-coupled microspheres were used in a singleplex (i.e., a single antigen) or in a multiplex (i.e., equal amounts of four antigen-coupled microspheres used simultaneously), the same positive and negative sera used previously were screened (Fig. 2). All antigens in the multiplex assay showed similar results for positive and negative samples when compared to their singleplex performance (Fig. 2). Thus, multiplexing the four HCV antigens did not alter the results obtained when antigens were tested individually.

Performance evaluation for the singleplex and multiplex tests. To evaluate the accuracy parameters of the single and multiplex tests, the samples were classified as "positive" or "negative" according to the cut-off values specifically determined for each antigen. Table 1 shows the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for the singleplexes and for the multiplex test. All singleplexes, with the exception of the Combined singleplex, presented unsatisfactory results with poor sensitivity despite good specificity. Alternatively, the multiplex test showed excellent results, with 100% sensitivity, specificity, PPV and NPV.

DISCUSSION

Determination of anti-HCV antibodies is indispensable for the identification and screening of HCV infected individuals. In this study, we have described the development of a multiplex assay for the simultaneous identification of human antibodies against five HCV antigens, in a single serum dilution.

To confirm antigen coupling to the beads our first approach was to determine the antibody responses against each antigen individually using serially diluted positive and negative serum pools. Although different patterns were observed in each dilution curve of the positive pools, these results were not surprising. Since antibody responses to HCV antigens can be influenced by HCV genotype, antigenic variation or viremia, as well as MHC or TCR and immunoglobulin phenotypes, antibody sets may differ among infected individuals (7, 23). In fact, when the samples that comprise the pools were analyzed individually, we observed that they presented different responses to the four individual antigens evaluated and that their overall MFI signals were fully compatible with the heterogeneity in responsiveness observed among the pools (data not shown).

A major concern with multiplexing beads is antibody competition or blocking (11, 19). These occurrences are to be expected when antigens that share antibody epitopes are detected together in a multiplex. As the Combined antigen has epitopes that are also present in the NS3, NS4 and NS5 antigens, one could predict a signal decrease caused by competition of binding sites. However, the present work demonstrated for all antigens tested that multiplexing does not appear to alter the quality or sensitivity of the assays compared to the singleplex format. Another issue to be considered is the degree of interference and cross-reactivity between the different bead sets. While multiplex

immunoassays facilitate the analysis of various antibody responses simultaneously, the mixture of several antigens can lead to unspecific binding of antibodies to the wrong antigen (16, 24). Nevertheless, in this study, no significant difference was found when antigen-coupled beads were used alone or in combination, indicating that the antigen-antibody complexes formed were specific and stable.

The main criteria for an HCV screening assay are to attain the highest sensitivity possible in combination with excellent specificity. In our study, the sensitivity, specificity, predictive negative and predictive positive values of the multiplex test were 100%, higher than the NS3, NS4, NS5 and Combined singleplexes. Despite the small number of samples evaluated, the high values of specificity and sensitivity obtained are particularly important, given the potential use of the HCV multiplex test in blood screening and/or epidemiological surveillance programs. It is important to point out that although the use of the Combined antigen was essential to achieve good sensitivity in the multiplex assay, the identification of the positive samples that were not reactive to the Combined antigen was only possible because these samples were responsive to the other three antigens used (NS3, NS4 and/or NS5), clearly demonstrating the benefit and importance of using and combining these distinct antigens to improve detection.

The multiplex test described herein has numerous advantages for the simultaneous measurement of antibodies to multiple HCV antigens. The total time required for performing this assay was of two hours, virtually the same as required for two commercially available kits (Abbott HCV EIA 3.0 and Ortho HCV ELISA 3.0). The estimated reaction cost of the multiplex assay was 2-4 times lower than that of available commercial methods in Brazil. In order to estimate the costs of the multiplex assay, we employed the approximate costs of beads, antigens, reagents and disposables used during

the assay procedure. Test costs estimation was based on currently available commercial reagent prices in Brazil at the time the study was conducted. Equipment, human resources and other indirect costs were not considered for comparison and calculation. The cost reduction associated with this new assay would make its use possible in several health services in Brazil, allowing the establishment of the real prevalence of HCV in the country.

Further improvements to the assay could be gained by incorporating additional features to include new targets of interest, such as antigens/antibodies specific to other hepatitis viruses, like HBV for example. This would add further power to the assay by allowing the simultaneous detection of host antibodies and pathogen-specific antigens. In addition, the HCV-multiplex assay has great potential of being used as both a screening and confirmatory test. As the reactivity of all four antigens can be individualized, it is possible that samples could be both screened and confirmed with this one test by establishing RIBA-like criteria for the individual antigens. In such a situation, possibly a superoxide dismutase or other marker bead should be included to rule out interferences. This would decrease even more the costs associated with HCV diagnosis, improving treatment and surveillance strategies.

We believe that a more thorough study of the multiplex assay's performance, including the analysis of samples from patients with a resolved infection versus samples from patients with an active infection, and also comparing it to supplemental serological testing currently used for HCV diagnosis would be important to establish the true potential of this multiplex test. Although further work will be required to establish the use of the HCV-multiplex assay as a diagnostic tool, the test described herein is sensitive, rapid, and shows excellent specificity. Therefore, the assay has the potential to become a viable alternative to the standard tests and should simplify screening of HCV infection.

REFERENCES

1. **Abdel-Hamid, M., M. El-Daly, S. El-Kafrawy, N. Mikhail, G. T. Strickland, and A. D. Fix.** 2002. Comparison of Second- and Third-Generation Enzyme Immunoassays for Detecting Antibodies to Hepatitis C Virus. J. Clin. Microbiol. **40**:1656-1659.

2. **Alter, H. J.** 1992. New kit on the block: evaluation of second-generation assays for detection of antibody to the hepatitis C virus. Hepatology. **15**:350-353.

3. **Ansari, M. H. K. and M. D. Omrani.** 2006. Evaluation of diagnostic value of Elisa method (EIA) & PCR in diagnosis of hepatitis C virus in hemodialysis patients. Hepatitis Monthly. **6**:19–23.

4. **Araújo, E. S., J. S. Mendonça, A. A. Barone, F. L. Gonçales Jr, M. S. Ferreira, R. Focaccia, and J. M. Pawlotsky.** 2007. Consensus of the Brazilian Society of Infectious Diseases on the management and treatment of hepatitis C. Braz. J. Infect. Dis. **11**:446-450.

5. **Armstrong, G., M. J. Alter, G. M. Mcquillan, and H. S. Margolis.** 2000. The Past Incidence of Hepatitis C Virus Infection: Implications for the Future Burden of Chronic Liver Disease in the United States. Hepatology. **31**:777-782.

6. **Baath, L., A. Widell, and E. Nordenfelt.** 1992. A comparison between one first generation and three second generation anti-HCV ELISAs: an investigation in high- and low-risk subjects in correlation with recombinant immunoblot assay and polymerase chain reaction. J. Virol. Methods. **40**:287-296.

7. **Beld, M., M. Penning, M. van Putten, V. Lukashov, A. van den Hoek, M. McMorrow, and J. Goudsmit.** 1999. Quantitative antibody responses to structural (Core) and nonstructural (NS3, NS4, and NS5) hepatitis C virus proteins among seroconverting injecting drug users: impact of epitope variation and relationship to detection of HCV RNA in blood. Hepatology. **29**:1288-1298.

8. **Bellisario, R, R. J. Colinas, and K. A. Pass.** 2001. Simultaneous measurement of antibodies to three HIV-1 antigens in newborn dried blood-spot specimens using a multiplexed microsphere-based immunoassay. Early Hum. Dev. **64**:21-25.

9. **Binnicker, M. J., D. J. Jespersen, J. A. Harring, L. O. Rollins, and E. M. Beito.** 2008. Evaluation of a multiplex flow immunoassay for detection of epstein-barr virus-specific antibodies. Clin. Vaccine Immunol. **15**:1410-1413.

10. **Boyer, N., and P. Marcellin.** 2000. Pathogenesis, diagnosis and management of hepatitis C. J. Hepatol. **32**:S98-Sl12.

11. **Carson, R. T., and D. A. Vignali.** 1999. Simultaneous quantitation of 15 cytokines using a multiplexed flow cytometric assay. J. Immunol. Methods. **227**:41-52.

12. **Dunbar, S. A.** 2006. Applications of Luminex xMAP technology for rapid, high-throughput multiplexed nucleic acid detection. Clin. Chim. Acta. **363**:71– 82.

13. **Foti, L., B. P. Fonseca, L. D. Nascimento, C. F. Marques, E. D. da Silva, C. A. Duarte, C. M. Probst, S. Goldenberg, A. G. Pinto, and M. A. Krieger.** 2009. Viability study of a multiplex diagnostic platform for Chagas disease. Mem. Inst. Oswaldo Cruz. **104S1**:136-141.

14. **Fouda, G. G., R. F. Leke, C. Long, P. Druilhe, A. Zhou, D. W. Taylor, and A. H. Johnson.** 2006. Multiplex assay for simultaneous measurement of antibodies to multiple Plasmodium falciparum antigens. Clin. Vaccine Immunol. **13**:1307-1313.

15. **Fulton, R. J., R. L. McDade, P. L. Smith, L. J. Kienker, and J. R. Kettman Jr.** 1997. Advanced multiplexed analysis with the FlowMetrix system. Clin. Chem. **43**:1749– 1756.

16. **Gouri, L., P. Balmer, H. Joseph, M. Dawson, and R. Borrow.** 2004. Development and Evaluation of a Tetraplex Flow Cytometric Assay for Quantitation of Serum Antibodies to Neisseria meningitidis Serogroups A, C, Y, and W-135 Clin. Diagn. Lab. Immunol. **11**: 272-279.

17. **Jani, I. V., G. Janossy, D. W. Brown, and F. Mandy.** 2002. Multiplexed immunoassays by flow cytometry for diagnosis and surveillance of infectious diseases in resource-poor settings. Lancet Infect. Dis. **2**: 243-250.

18. **Joos, T. O., D. Stoll, and M. F. Templin.** 2002. Miniaturized multiplexed immunoassays. Curr. Opin. Chem. Biol. **6**:76–80.

19. **Kellar, K. L., and M. A. Iannone.** 2002. Multiplexed microsphere-based flow cytometric assays. Exp. Hematol. **30**:1227– 1237.

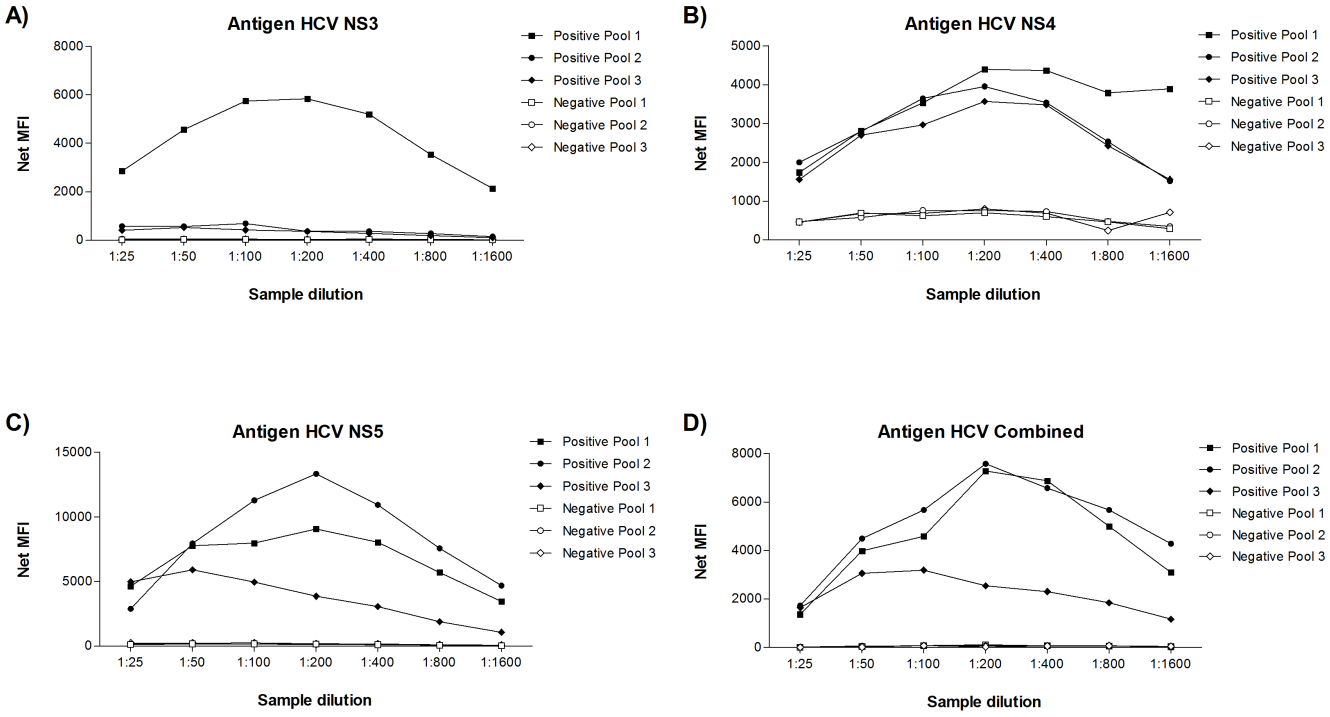
20. **Pawlotsky, J. M.** 2002. Use and interpretation of virological tests for hepatitis C. Hepatology. **36**:S65–S73.

21. **Raedler, M. D., S. Heyne, E. Wagner, S. K. Shalkowski, S. Secore, A. S. Anderson, J. Cook, L. Cope, T. McNeely, M. Retzlaff, J. Shanter, L. J. Rubinstein, T. Green, N. Kartsonis, and M. T. Esser.** 2009. Serologic assay to quantify human immunoglobulin G antibodies to the Staphylococcus aureus iron surface determinant B antigen. Clin. Vaccine Immunol. **16**:739-748.

22. **Sillanpää, M., K. Melén, P. Porkka, R. Fagerlund, K. Nevalainen, M. Lappalainen, and I. Julkunen.** 2009. Hepatitis C virus core, NS3, NS4B and NS5A are the major immunogenic proteins in humoral immunity in chronic HCV infection. Virol. J. **6**:84-95.

23. **Thimme, R., C. Neumann-Haefelin, T. Boettler, and H. E. Blum.** 2008. Adaptive immune responses to hepatitis C virus: from viral immunobiology to a vaccine. Biol. Chem. **389**:457-67.

95 24. **de Voer, R. M., F. R. M. van der Klis, C. W. A. M. Engels, G. T. Rijkers, E. A. Sanders, and G. A. M. Berbers**. 2008. Development of a Fluorescent-Bead-Based Multiplex Immunoassay To Determine Immunoglobulin G Subclass Responses to Neisseria meningitidis Serogroup A and C Polysaccharides; Clin. Vaccine Immunol. **15**:1188–1193.



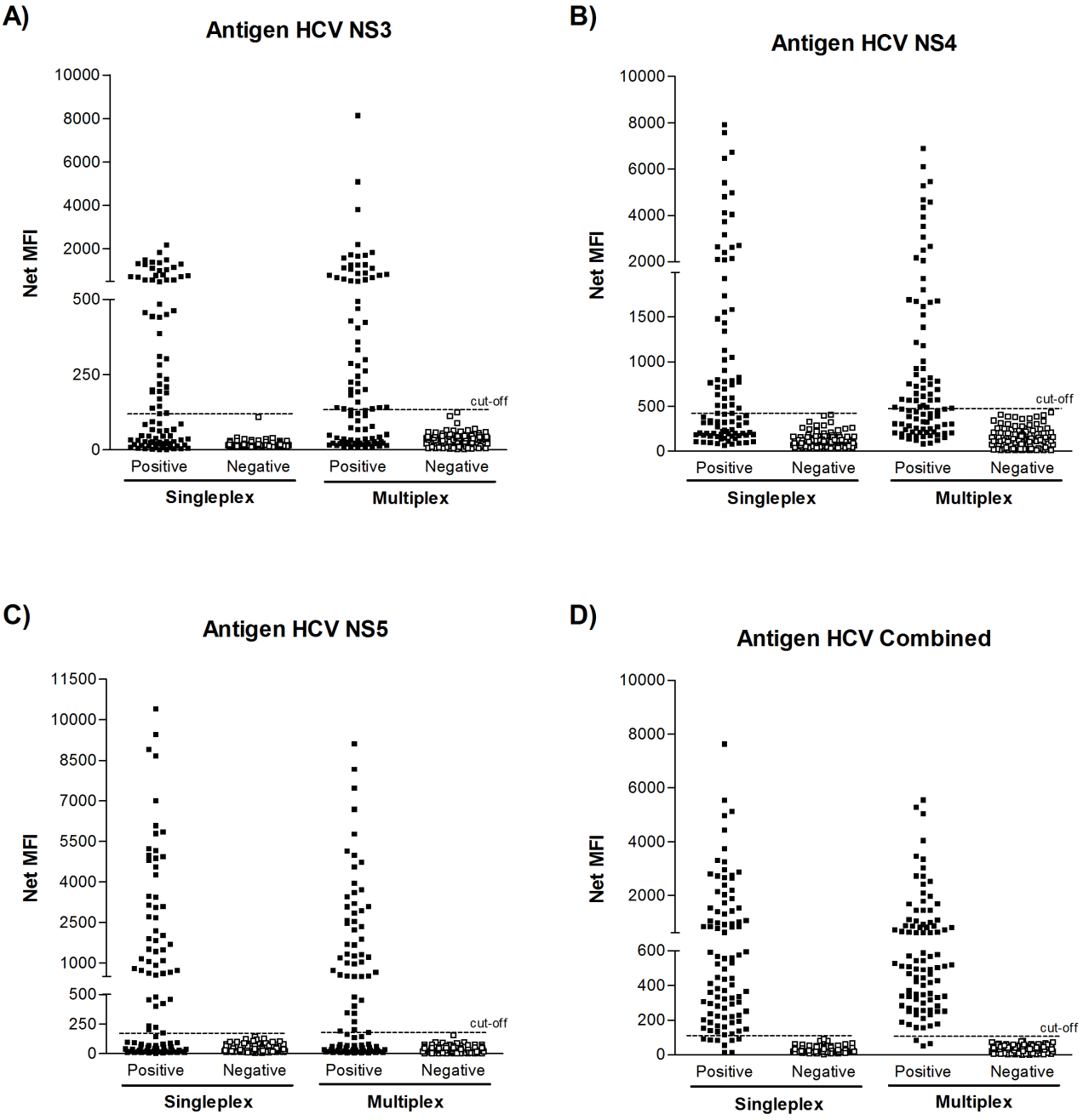


FIGURE CAPTIONS

Figure 1: Efficiency of HCV antigens coupling to microspheres. Net MFI of antigen detection for the antigens (A) NS3, (B) NS4, (C) NS5 and (D) Combined is shown in serially diluted samples of HCV positive pools 1, 2 and 3 and negative pools 1, 2 and 3. All samples were assayed in duplicate.

Figure 2: Comparison of the performance of the singleplex and multiplex assays. Net MFI obtained by the 1:200 dilution of 93 HCV positive serum samples and 93 negative sera evaluated in singleplex and multiplex against the antigens NS3, NS4, NS5 and Combined. For the multiplex assays, the same number of coupled microspheres was added to each well for each microsphere set. A sample was considered "positive" if it had one or more positive results from the individual antigens. All samples were assayed in duplicate.

TABLES

Table 1: Accuracy parameters of the singleplex and multiplex test formats.

Antigens	Accuracy Parameters (%)			
	Sensitivity	**Specificity**	**PPV[1]**	**PNV[2]**
NS3	50,5	100	100	66,9
NS4	51,6	100	100	67,4
NS5	55,9	100	100	69,4
Combined	93,5	100	100	93,9
Multiplex	100	100	100	100

[1]PPV: Predictive Positive Value; [2]PNV: Predictive Negative Value.