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Detection of Antibody to HCV and NS3 by using a Microsphere Immunoassay*

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Key Words:

- HCV Protiens
- Flow Cytometry
- Microsphere Immunoassay
- Biotinylation
- Optimization
- Microsphere Gating
- Microsphere Stability

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Using two dye levels of Thermo Scientific Cyto-Plex Avidin Microspheres (L5 and L10), two outside researchers developed a duplex-type assay for the detection of antibodies to the hepatitis C virus (HCV).

Two HCV proteins were produced, then biotinylated according to the protocol presented. The biotinylated antigens were captured onto avidin-coupled beads and subsequently analyzed by incubating the microsphere-bound antigens with patient samples. The microspheres were then analyzed using flow cytometry. This project was performed with support from Thermo Fisher Scientific scientists.

Detection of Antibody to HCV Capsid and NS3 by using a Microsphere Immunoassay

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1. INTRODUCTION

The Hepatitis C Virus (HCV) is an RNA Flavivirus. HCV is the primary cause of socalled non-A, non-B hepatitis and was discovered using a variety of molecular methods in the 1980s (Prince, Inchauspe et al). It is currently estimated that there are 3 million individuals in the US infected with HCV (Pearlman). Infection with HCV frequently leads to chronic infection with potentially life-threatening complications. HCV infection is the leading cause for the need for liver transplantation. Detection of infection with HCV is based upon the demonstration of antibody to HCV in the blood of the patient. The detection of infection with HCV can lead to intervention to reduce the likelihood of transmission of the virus and therapy to reduce the long-term consequences of HCV infection.

ABBREVIATIONS

HCV = Hepatitis C Virus
EIA = enzyme immunoassay
RIBA = recombinant immunoblot assay
NS3 = non-structural region 3
PMT = photomultiplier tube
FSC = forward light scatter
SSC = side (orthogonal) light scatter
FL1 = 1st fluorescence channel
FL2 = 2nd fluorescence channel
FL3 = 3rd fluorescence channel
FL4 = 4th fluorescence channel,
MESF = molecules of equivalent soluble fluorochrome
% CV = coefficient of variation

DETECTION OF INFECTION WITH HCV

Testing for infection with HCV is performed by using an EIA. Recent studies in managed care populations indicate that 0.7% of enrolled members are tested for antibody to HCV and 6.7% are positive (Shatin et al). In addition all blood, stem cell and organ donors are tested for antibody to HCV (>30 million tests per year). The current EIAs use a combination of antigens including the structural protein called capsid or core and a nonstructural protein called NS3. In the US there are 2 FDA approved assays (Abbott Laboratories and Ortho Diagnostics). Samples, which are reactive in the EIA, are usually reflexively tested using a supplement assay. The supplemental test currently available is the RIBA (Chiron Corp). The RIBA has the capsid and NS3 proteins placed onto different locations on a nitrocellulose strip, which allows the user to recognize the presence of antibody to either or both proteins. Samples can be reactive in the screening EIA but negative or indeterminate in the supplemental assay and studies suggest that approximately 8% of samples, which are reactive in the EIA, cannot be confirmed with the supplemental assay (Schroter et al). This frequency of samples, which are probably false positive reactions in the EIA, underscore the need for a sensitive supplemental assay. While the RIBA performs acceptably the results are qualitative (pos/neg) and the proteins used for the RIBA are the same proteins that are used for the production of the EIA. As such recombinant HCV proteins form a different isolate of HCV were used and the microsphere

immunoassay was developed to provide sensitive and semi-quantitative detection of antibody to HCV. Previously the use of a microsphere immunoassay demonstrated the improved detection and resolution of samples, which were reactive with the EIA but indeterminate with the RIBA (McHugh et al).

PRODUCTION OF HCV PROTEINS

HCV has not been cultured in vitro however the genome has been sequenced and a number of gene products have been cloned and expressed using recombinant DNA techniques (Inchauspe et al). Clones of cDNA from viral RNA obtained from the extensively studied HUTCH strain of the virus and the two most important gene products for antibody capture were expressed in E. coli as recombinant proteins and purified prior to use.

The capsid, or core, antigen of HCV was obtained as a full-length polypeptide (amino acid 1-120) and was expressed without any additional carrier protein. The protein was purified from induced and sedimented cultures by the rupture of the cells by using a combination of lysozyme treatment and ultrasonication followed by differential centrifugation steps, extraction of the ruptured cell paste using 6M urea and a combination of gel chromatography on Sephacryl HR-300 and ion exchange chromatography on CM-Sephacryl gels. The resulting protein produced a single band when analyzed by using SDS gel electrophoresis in 16% acrylamide gel and was stored in 4M urea-0.2M NaCl in 20 mM acetate buffer, pH 6 at –20°C until used.

The second recombinant antigen was derived from the non-structural portion of the viral genome (see clone 20, Table 9 PCT/US/91/0637) thus encompassing 105 amino acid codons of the HCV NS3 region. The cloned sequence was first inserted into a pUC18 vector and subsequently recovered as a SmaI-EcoRI fragment. This fragment was ligated into an expression vector, which had been engineered to contain, in the correct reading frame, a preceding sequence encoding 6 histidine residues as well as SmaI/EcoRI restriction sites to accommodate the insert. Thus, the NS3 protein was expressed with a hexahistidine tag at its N-terminus. The hexahistidine tag allows for more effective purification using an affinity column containing an iminodiacetic acid chelate saturated with Ni^{2+} ions (Nakagawa et al). The chelate binds strongly to the hexahistidine tag of the protein, which is subsequently eluted from the column using increasing concentration of an imidazole buffer. Prior to final purification of the NS3 antigen using the iminodiacetic acid chelate, the induced culture containing the expressed NS3 protein was sedimented as described above, the cell paste ruptured by using lysozyme and ultrasonication, subjected to another centrifugation step to remove debris, concentrated by ammonium sulfate precipitation and chromatographed on a Sephacryl S-300 column eluted with 20 mM Tris-0.2M NaCl, pH 8.6. The fractions containing the protein of interest (as determined by SDS gel electrophoretic analysis of individual fractions) were pooled and further purified by affinity chromatography as described above. The purified protein produced a single band on analysis by SDS gel electrophoresis in 16% acrylamide gel and was stored at –20°C in 50 mM carbonate buffer, pH 8.6-0.2M NaCl until used.

BIOTINYLATION OF HCV PROTEINS

Several experiments were performed in order to establish a protocol for effectively biotinylating the HCV polypeptides, using an EIA-type procedure to qualitatively assess properties of the biotin-protein conjugate under various reaction conditions. The trial preparation of biotinylated material was first captured onto Thermo Scientific avidin-coupled beads (Type L5 [NS3] and L10 [capsid] respectively) and subsequently analyzed by incubating the microsphere-bound antigens with:

1. A reference human serum, positive for antibodies to HCV, for 30 minutes
2. Washing 3X
3. Incubation with peroxidase labeled anti human IgG antibody for 30 minutes
4. Washing 3X
5. Addition of a peroxide-TMB substrate mix and incubating for 10 minutes
6. Stopping the color development after 10 minutes by acidification and,
7. Reading the absorbance at 450 nm

Based on preliminary studies, the protocol for biotinylating these antigens adopted was as follows:

Capsid

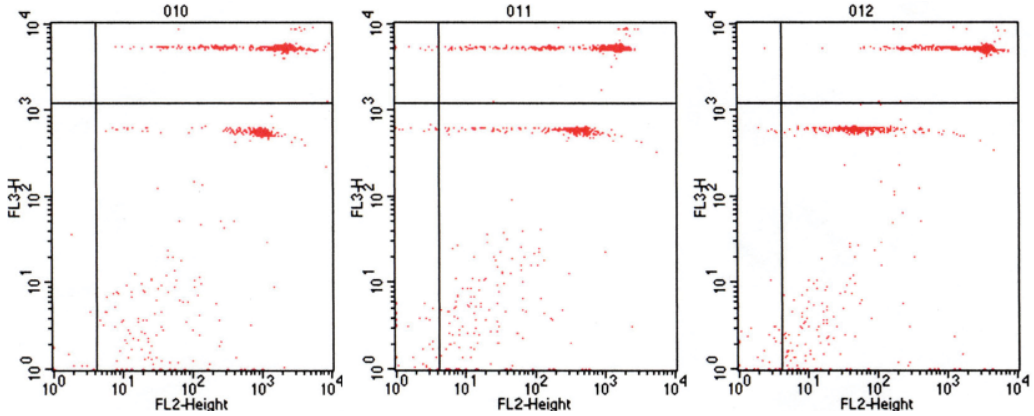
HCV capsid protein was dialyzed against 25 mM sodium carbonate-0.1M NaCl in 4M urea, pH 8.6 overnight and 0.2 mL (1.12 mg protein per mL) mixed with 8 uL of LHC Biotin in DMSO (2 umoles per mL, Sigma Chemical Co., St. Louis MO) and incubated for 5 hours at room temperature, then diluted to 1.2 mL and dialyzed against the same buffer for 48 hours with three intermittent buffer changes. The dialyzed material was used directly to capture the antigen to L10 microspheres as described below.

NS3 Antigen

Hexahistidine-NS3 polypeptide was dialyzed against 25mM sodium carbonate-0.1M NaCl, pH 8.6 overnight and 0.2 mL (1.85 mg protein per mL) was mixed with 1 uL of LHC biotin in DMSO (2 uL per mL, Sigma) and incubated for 5 hours at room temperature, then diluted to 1.2 mL and dialyzed against the same buffer for 48 hours with three

intermittent buffer changes. The dialyzed material was used directly to capture the antigen as described below.

and a 100-uL aliquot of the coated L10 microspheres were added to a test tube (each tube contained a total of 0.2 mL). This is the microsphere prep used for the antibody assay.



Figures 1, 2 and 3 show the effect that different biotinylation conditions had on the detection of antibody to the HCV proteins. The same antibody positive serum sample was used in these 3 different biotinylation experiments. The optimum biotinylation was selected based upon these flow cytometric analyses.

ATTACHMENT OF BIOTINYLATED PROTEINS TO AVIDIN-MICROSPHERES AND PREPARATION FOR FLOW CYTOMETRIC ANALYSIS

In order to optimize the conditions of the assay, the effect of varying individual parameters was subjected to in-depth testing in order to determine the optimum assay conditions. These included determining assay performance as a function of antigen concentration and antigen capture time, total microsphere input, incubation times, antibody dilution and anti-human IgG conjugate concentration, in addition to the parameter settings on the flow cytometer and method of data analysis and presentation. The final assay protocol is as follows:

Optimized procedure for coating HCV protein and the detection of HCV specific antibody

1. For the NS3 antigen 15.6 uL of the L5 microspheres (0.6% suspension with approximately $2.9x10^8$ microspheres per mL) was mixed with 125 uL of the biotinylated NS3 polypeptide (300 ug of protein per mL) in a total of 1.25mL HCV sample diluent .
2. In a separate tube, 15.6uL of the L10 microspheres (0.6% suspension containing approximately $3x10^8$ microspheres) was mixed with 125 uL of the biotinylated HCV capsid antigen (185 ug protein per mL) in a total volume of 1.25 mL HCV sample diluent.
3. The microspheres and biotinylated HCV proteins were incubated for 60 minutes at room temperature after which the tubes were centrifuged.
4. The microspheres were washed three times with HCV wash buffer and resuspended in 1.25 mL HCV wash buffer.
5. A 100-uL aliquot of the coated L5 microspheres
6. These tubes were centrifuged and the supernatant was discarded.
7. A 100-uL aliquot of positive and negative sera (as determined by Abbott EIA testing) were added to a tube containing the HCV-coated microspheres.
8. The tubes were vortexed and incubated for 30 minutes at room temperature.
9. The tubes had 1 mL of HCV wash buffer added and were centrifuged, the wash step was performed 3 times.
10. To the resuspended microsphere pellet 0.1 mL of anti-human IgG-PE (Jackson Immunoresearch, West Grove PA) at a 1:180 dilution in HCV conjugate buffer was added.
11. The tubes were incubated for 30 minutes at room temperature.
12. The tubes were washed 3x as before with 1 mL HCV wash buffer at each step.
13. The microsphere pellet was suspended in 200 uL HCV wash buffer and then analyzed by flow cytometry.

SUMMARY OF MICROSPHERE ANTIBODY ASSAY PROCEDURE

Following initial analyses using an EIA type procedure as described above (done in March 2004), twelve individual sets of experiments encompassing, in each instance, 12- 24 separate tubes of microspheres sequentially incubated with (a) Antigen (b) Antibody and (c), anti-human IgG-PE were prepared and analyzed (done in April through June 2004). The final protocol for the assay is summarized below but described in greater detail above.

Assay conditions selected were:

1. A serum dilution of 1:100.
2. 30-minute serum incubation with the microspheres at room temperature.
3. A 1:180 dilution of the PE anti-human IgG.
4. A 30-minute incubation with the PE anti-human IgG at room temperature.

Recommended daily set up for the microsphere immunoassay

The analyses for the HCV microsphere assay were performed interchangeably on the FACScan and FACSCalibur instrument. The daily set up for this assay should include:

- Determination that the instrument is operating as expected.
- Setting standard instrument settings for the microsphere assay including the trigger/threshold and PMT settings.
- Fluorescence compensation settings.

Instrument settings used

FSC - log, with threshold at 360
SSC - lin
FL1 - (FITC, 530/30) - log, PMT voltage at 735, although FL1 was not used in analysis
FL2 - (PE, 575/25) - log, PMT voltage at 735
FL3 - (red dye, 670LP) - log, PMT voltage at 650
FL4 - was not used in analysis

The FL3 PMT voltage setting was selected to keep the L10 singlet population on scale while increasing the L5 signal to help reduce the compensation issues primarily seen with a strong PE signal (e.g., high antibody level). The daily settings gave FL3 signals that were within ≈25% of the target FL3 channel values (L5 target = 565 and L10 target = 5075; these target values are the mean fluorescence channel from a 4-decade log scale using log amplification of the signal detected in FL3).

An alternative threshold would be to trigger using the FL3 fluorescence although data comparing FSC with FL3 trigger indicates essentially the same results, which would be expected.

HCV Antibody data units

The mean fluorescence channel was used as the expression of antibody units for the HCV assay. Alternative expression units would be Pos/Neg, conversion to an "antibody unit" based upon the positive control (or another sample which was assigned an arbitrary value such as 1000 Ab units) or MESF.

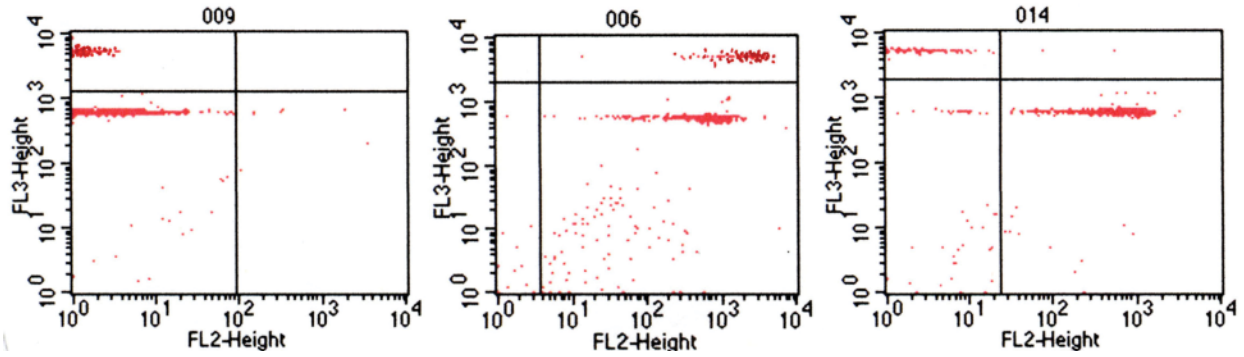
HCV antibody data presentation and analysis

The HCV antibody level to the NS3 (L5) protein and to the capsid (L10) protein was expressed as the mean FL2 channel. In addition the mean FL3 channel for the L5 and the L10 microspheres is shown.

The sample shown in Fig. 6 was strongly positive (>2.2 absorbance units) on the screening HCV 2.0 EIA (Abbott Laboratories) but by the microsphere assay lacked antibody to the capsid (L10) protein. Antibody reactivity to the capsid protein is almost universal in HCV infected individuals. The lack of antibody to capsid as evidenced by using the microsphere

	NS3 reactivity on L5 microspheres, data is FL2 Channel #			
	Mean	**SD**	**Min**	**Max**
Negative samples (n = 18)	28	14	7	60
Positive samples (n = 29)	779	381	287	2434
	Capsid reactivity on L10 microspheres, data is FL2 Channel #			
	Mean	**SD**	**Min**	**Max**
Negative samples (n = 18)	14	11	2	50
Positive samples (n = 29)	1980	1019	400	3540
	L5 microspheres, data is FL3 Channel #			
	Mean	**SD**	**Min**	**Max**
n = 52	565	42	426	609
	L10 microspheres, data is FL3 Channel #			
	Mean	**SD**	**Min**	**Max**
n = 52	5072	166	4793	5410

Table 1: Summary of the negative and positive samples analyzed



Figs 4, 5 and 6 show representative displays of an antibody negative sample, an antibody positive sample and a sample with NS3 (on L5) reactivity, which lacks reactivity to the capsid protein (on L10).

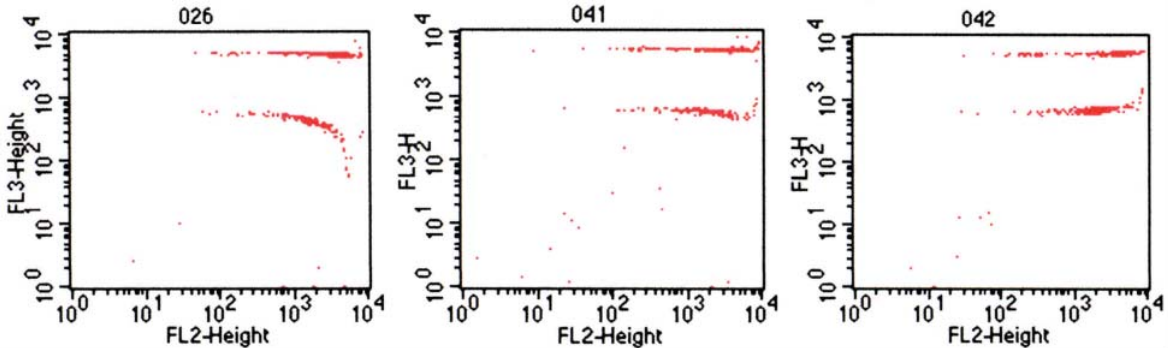
immunoassay suggests that this sample may represent a false positive EIA result. This sample was tested by using the RIBA immunoblot assay, which also did not show antibody presence to the capsid protein.

Fluorescence compensation issues with the L5 and L10 microspheres

The FL3 fluorescence of the L5 microspheres was significantly affected by the intensity of the PE signal collected in FL2. High analyate concentrations, representing elevated HCV specific antibody in this experiment, produces a strong PE (FL2) signal. This PE signal effects the detection of the FL3 signal of the L5 microspheres and to a lesser extent the FL3 signal of the L10 microspheres. The fluorescence compensation settings must be carefully evaluated at any PMT voltage used to reduce the possibility that the FL3 signal will be effected to such an extent that there will be "crossing-over" of one FL3 microsphere population into another FL3 microsphere population. Since this project only used L5 and L10 microspheres there was no apparent crossing-over due to the large difference in FL3 signals between these two microsphere populations.

With the HCV assay the optimal fluorescence compensation settings on the BD FACSCan and FACSCalibur were:

FL1 - %FL2 = 00.0
FL2 - %FL1 = 00.0
FL2 - %FL3 = 0.6



Figs. 7, 8 and 9 show the effect of different compensation settings on the L5 and L10 FL3 fluorescence. These figures show mixed L5 and L10 microspheres incubated with a sample, which has high levels of antibody to NS3 (L5) and to capsid (L10). The FL3-%FL2 settings were 15%, 10% and 5% in Figs. 7, 8 and 9 respectively.

FL3 - %FL2 = 5.0 - 10.0 (varied)
FL3 - %FL4 = N/A
FL4 - %FL3 = N/A

These were with the PMT voltage settings as described above in the section Instrument Settings Used.

In an effort to reduce the frequency of compensation problems due to a high HCV specific antibody level we coated the L10 microspheres with the capsid protein as antibody levels to capsid are generally higher than antibody levels to NS3. A number of assays were performed however with the capsid protein coated onto the L5 microspheres and with the NS3 protein coated onto the L10 microspheres. These experiments supported our supposition that there would be more frequent FL2 and FL3 compensation issues with the L5 microspheres if they were coated with the capsid protein.

MICROSPHERE GATING

The microsphere populations can be identified by the internal red dye the intensity of which is used to distinguish the different sub-populations (L5 from L10). Prior to the detection of the L5 and L10 microsphere populations debris and aggregated microspheres must be eliminated. The debris is best eliminated by using a threshold prior to acquisition such that small or non-fluorescent events are not collected whereas aggregates are best eliminated by using a gating strategy, post-acquisition. The threshold prior to acquisition can either be a FSC or a fluorescence threshold (FL3 for the red dye). Once the debris is eliminated the microspheres can be acquired.

Analysis of the microspheres can be performed without additional gating although this is unlikely to be practical.
The HCV antibody values used, mean FL2 signal, were not significantly different when these different threshold (FSC vs. FL3) settings or the different gating strategies were used (data not shown).

The presence of microsphere aggregates can be confounding in a multiplex assay. A random sampling of 25 analyses over the time course of the study was selected. From these 25 determinations the average % of singlet microspheres for analysis was 48.5% with a range from 23% - 68%. The range in the percentage of singlet microspheres was not apparently related to the HCV protein (capsid and NS3 coated microspheres had similar aggregates), or to the patient sample used (antibody negative samples had similar aggregates as did antibody positive samples) or to the use of L5 or L10 alone or when in used in combination. It is unlikely that the antibody would cross-link the L5 and L10 microspheres, as the antigens used on the two

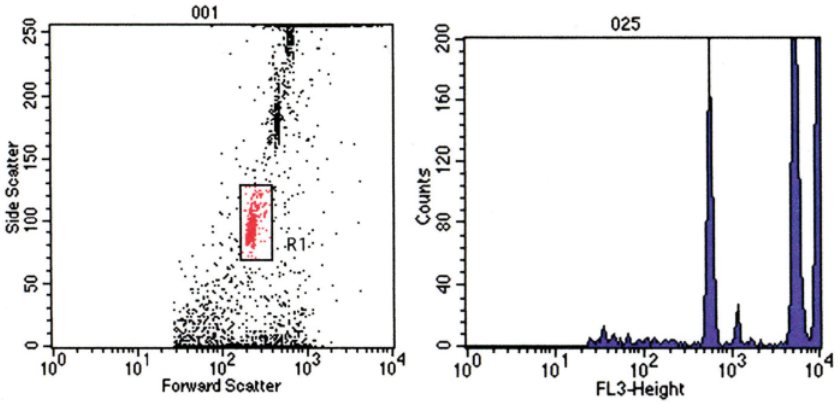
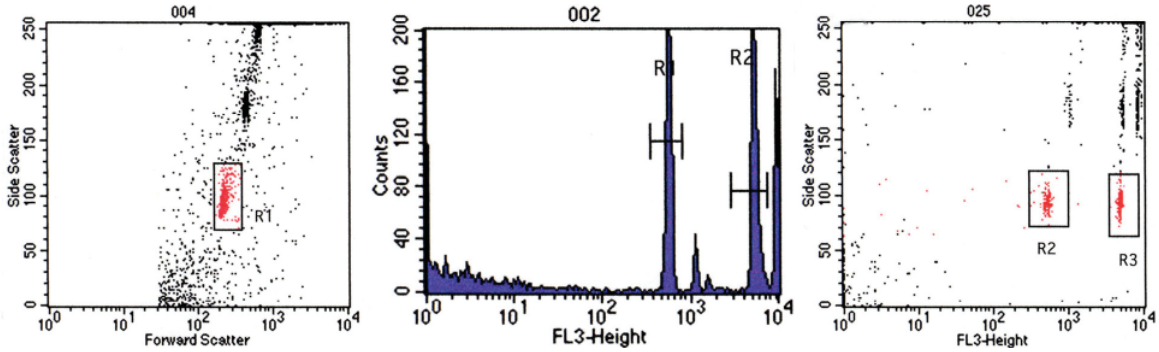


Fig. 10 shows a FSC threshold and Fig. 11 shows a FL3 threshold.

different microspheres are distinct. This suggests that the aggregates are forming due to charge interactions, which could be contributed to from the polymer, the avidin, the coated HCV protein and the serum sample.

HEAT STRESSED AVIDIN COATED MICROSPHERES

Aliquots of the L5 and L10 avidin microspheres originally provided to Pinnacle Biosystems, Inc. were held at 40°C for 21 days at Thermo Fisher Scientific Corp (3/12/04 through 4/2/04). A 300 uL aliquot of these heat stressed L5 and L10 microspheres were coated with the biotinylated HCV proteins in tandem with L5 and L10 microspheres that had been stored at 2-8°C. These L5 and L10 microspheres represented the control non-heat stressed microspheres.



The three likely gating strategies would be a gate using FSC/SSC as shown in Fig. 12, by using a FL3 (red) fluorescence gate as shown in Fig. 13, or by using a combination of a scatter and fluorescence gate such as a SSC/FL3 gate shown in Fig.14.

COATED MICROSPHERE STABILITY

This study did not evaluate the stability of the microspheres once coated with the biotinylated HCV proteins. For the commercial development of an assay such as this or for routine research use it would be of interest to determine how long the coated microspheres could be stored prior to being used in the specific assay. However once the HCV coated microspheres were reacted with human antibody and with the goat anti-human IgG PE these reagents were stable for at least 24 hours when stored at 2-8°C. In 2 of the experiments performed the microspheres were analyzed on the day of the assay, stored at 2-8°C for 24 hours and reanalyzed. There was no significant difference in the FL2 mean signal, the FL3 signal, the % of aggregates or the % CV of the FL2 peak in the 24 hour old microspheres vs. the freshly prepared microspheres. No time points longer than 24 hours were evaluated. The biotinylated HCV proteins were stable over the time course of this project (6 months) when stored at 2-8°C.

These data indicate there is probably no significant difference between the performance of the heat stressed microspheres as compared to the non-heat stressed microspheres. A serum sample-containing antibody to NS3 and capsid was used at a 1:200 dilution (a higher dilution providing less antibody was selected to help identify deceased ability to capture antigen and antibody) and the assay was performed in triplicate.

CONCLUSIONS

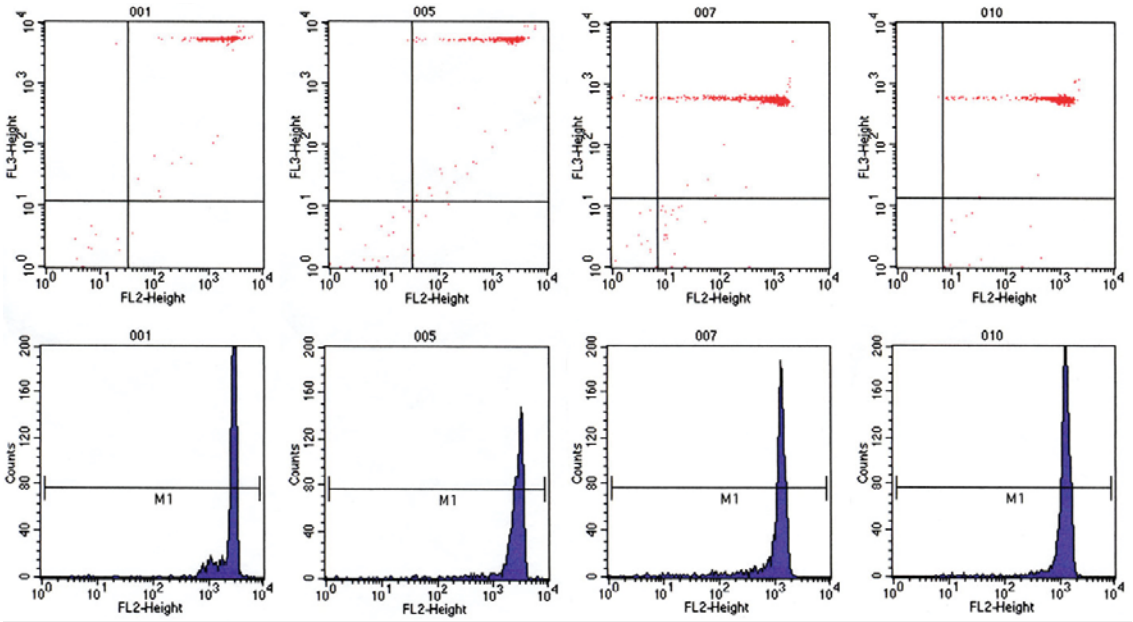
The microsphere immunoassay for the detection of antibody to HCV worked well using the L5 and the L10 microspheres. The FL3 fluorescence of the L5 microspheres was stable over the time course of this project (6 months) and the flow cytometer setting for FL3 could be set to locate the L5 and L10 microspheres reproducibly. The PE reporter molecule fluorescence signal in FL2 had an effect on the FL3 signal but using the appropriate fluorescence compensation settings could mitigate this effect. There was significant aggregates of microspheres in this assay, which could not be eliminated by vortexing of the sample. Aggregates in this type of microsphere assay are commonly seen but can present challenging

FL2 mean L5 Control Microspheres	FL2 mean L10 Control Microspheres	FL2 mean L5 Heat Stressed	FL2 mean L10 Heat Stressed
1088	2543	1144	2723
947	2219	1043	2495
1006	2031	801	2746
FL3 mean L5 Control Microspheres	**FL3 mean L5 Control Microspheres**	**FL3 mean L5 Heat Stressed**	**FL3 mean L5 Heat Stressed**
559	5410	563	5225
585	5297	559	5238
577	5383	573	5249

Table 2: Listing the mean FL2 and FL3 signal from the heat stressed microspheres as compared to the control microspheres when used in the HCV antibody assay.

analysis issues. The semi-quantitative nature of this assay is of value in the detection of antibody to different HCV proteins. The assay format allowed for the simultaneous detection and semi-quantitation of antibody to the NS3 and capsid protein from HCV. This represents an improvement over the currently available supplemental assay for the serodiagnosis of HCV infection. As evidence of this a random sample believed to be positive for antibody to HCV lacked antibody to the capsid protein coated onto the L10 microspheres. The lack of this antibody is highly suggestive that the screening results were falsely positive.

The assay time was 90 minutes to coat the microspheres with the HCV proteins, and 90 minutes to perform the antibody assay. The flow cytometric analysis time was approximately 15 seconds per tube. If the microspheres could be coated with the HCV protein and stored a significant time savings would be realized.



Figs. 15, 16, 17 and 18 show a FL2/FL3 dot plot and a single parameter FL2 histogram of the control microspheres versus the heat stressed microspheres. Figs. 15 and 17 show the control microspheres reactivity with capsid and NS3 respectively whereas Figs. 16 and 18 show the same reactivity seen when using the heat stressed microspheres.

RECOMMENDATIONS FOR FUTURE ASSAY

Evaluate blocking, washing and resuspension buffers with the goal of reducing microsphere aggregates. It may be that blocking agents such as ethanolamine or Balanine with reduction to secondary amines can help to reduce charges, which may be contributing to the aggregates of the microspheres (Karlin et al).

In our study we detected a significant level of HCV protein bound to the avidin coated microspheres in the absence of biotinylation of the HCV protein. This may be due to the charge associated with the HCV proteins used but will no doubt be evident to varying degrees in the design and evaluation of other assays. As such a review of the detailed evaluation of NeutrAvidin binding and of the subsequent labeling with biotin is worthwhile to ensure that the avidin-coated microspheres are performing optimally (Vermette et al).

Use anti-human IgG coated microspheres as an internal control for both sample addition and reagent addition. L7 avidin-microspheres were supplied and will be used in a follow-up HCV study as an internal control as previously shown (McHugh et al).

Evaluate the fluorescence compensation issues by using biotin-PE and other possible reporter dyes (e.g., biotin-Alexa dyes, biotin-TexasRed) at high concentrations

to mimic strong analyte levels on mixtures of the FL3 microsphere populations.

Develop recommended fluorescence compensation protocols for prospective users when the user is employing multiple populations of the FL3 dyed microspheres.

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**The Cyto-Plex Advidin Microspheres have been discontinued by Thermo Fisher Scientific. Please review our product catalogs and website for current availability of our complete line of Cyto-Plex products. Updated January 2010*

Figure in paper	Figure is used to show what	Run #	File #	Display
1	Effect of biotinylation on reactivity of coated HCV protein using a positive serum sample	6	10	FL2/FL3 dot-plot with L5 & L10
2	"	6	11	"
3	"	6	12	"
4	Antibody negative sample reactivity with L5 and L10 microspheres	9	9	FL2/FL3 dot-plot with L5 & L10
5	Antibody positive sample reactivity with L5 and L10 microspheres	10	6	"
6	Antibody positive sample reactivity with L5 and L10 microspheres (false positive?	9	14	"
7	Compensation effects with FL3 -%FL2 = 15% using a positive serum sample	5	26	FL2/FL3 dot-plot with L5 & L10
8	Compensation effects with FL3 -%FL2 = 10% using a positive serum sample	5	41	"
9	Compensation effects with FL3 - %FL2 = 5% using a positive serum sample	5	42	"
10	FSC threshold used to eliminate small debris	11	1	FSC/SSC dot-plot
11	FL3 threshold used to eliminate nonfluorescent events	11	25	FL3 histogram with L5 & L10
12	FSC/SSC gating strategy	11	4	FSC/SSC dot-plot
13	FL3 gating strategy	11	2	FL3 histogram with L5 & L10
14	FL3/SSC gating strategy	5	25	FL3/SSC dot-plot with L5 & L10
15	Antibody positive sample reacted with control L10 microspheres	8	1	FL2/FL3 dot-plot and FL2 histogram with L10
16	Antibody positive sample reacted with heat stressed L10 microspheres	8	4	"
17	Antibody positive sample reacted with control L5 microspheres	8	7	FL2/FL3 dot-plot and FL2 histogram with L5
18	Antibody positive sample reacted with heat stressed L5 microspheres	8	10	"

In addition to our office, Thermo Fisher Scientific maintains a network of representative organizations throughout the world.

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