RPR by Becton Dickinson) antigens. The relative sensitivity and specificity were as follows: treponemal: 92.2% sensitivity (n = 51), 93.6% specificity (n = 94); and non treponemal: 94.2% sensitivity (n = 52), 97.6% specificity (n = 96).

Another area of infectious disease testing to which Copalis has been applied is Epstein-Barr serology. In this case, three different antigens from the Epstein-Barr virus (EBV) were coated onto three different sizes of polystyrene latex microparticles. The antigens represented are the Epstein-Barr nuclear antigen, the viral capsid antigen, and the early antigen. Reduction in the size of any of the three peaks indicates the presence of antibodies against the corresponding antigen. Such testing may be useful in the differentiation of new infections, immune status, and reactivation of latent infections.

The systemic autoimmune diseases are characterized by the presence of antibodies against a number of different autoantigens (4). The simultaneous determination of these autoantibody specificities might be helpful in the differential diagnosis of diseases such as systemic lupus erythematosus, Sjogren syndrome, mixed connective tissue disease, and systemic scleroderma. The application of Copalis technology to the detection of autoantibodies is demonstrated by an assay for the simultaneous detection of antibodies to the autoantigens SSA (Ro) and SSB (La). For evaluation of the SSA Copalis assay, 23 ELISA-positive samples and 44 healthy blood-bank donors were run. Relative to ELISA, the sensitivity and specificity of the Copalis assay were 96% and 93%, respectively.

**LATEX-GOLD FORMAT**

Utilizing both forward-scatter and side-scatter parameters, this format is useful for the quantitative detection of analytes with greater sensitivity than that achieved using the latex-latex format. Antigen-mediated binding of colloidal gold to antibody-coated latex microparticles causes a broadening of the latex side-scatter histogram (2). The extent of this broadening can be measured and correlated to analyte concentration (see Fig. 1B).

The Latex-Gold format has been applied to the assessment of human fertility. Antibodies against different epitopes on luteinizing hormone (LH) were coated separately onto latex particles and onto colloidal gold. The same was done for follicle-stimulating hormone (FSH), using a different size of latex. When these reagents are combined and incubated with patient sample, the presence of either analyte leads to formation of a “sandwich” between the antibody on the gold and that on the latex. The coupling of gold and latex in turn causes a broadening of the LH and FSH side-scatter peaks, the magnitude of which is dependent on the concentration of the corresponding analyte.

Using this method for simultaneous measurement of LH and FSH, we obtained the following correlations with the Abbott IMx methods. For LH, Copalis = 0.16 + 0.83 (IMx); r = 0.95; and n = 64. For FSH, Copalis = 0.196 + 0.98 (IMx); r = 0.98; and n = 36.

**ADDITIONAL FEATURES**

Utilizing the forward- and side-scatter detection parameters, Copalis instrumentation is capable of differentiating and enumerating various cell populations and thus could function as a hematology analyzer, as well as an immunoassay analyzer. In addition, using electronic gating to mask the scattering signal from blood cells, Copalis II should have the capability of performing immunoassay analysis on whole-blood specimens.

**CONCLUSIONS**

Copalis is a versatile technology that may be applied to many areas of immunoassay, including infectious disease, autoimmunity, and endocrinology. A key feature of this technology is the ability to measure multiple analytes in the same reaction. This has now been demonstrated with multiplex assays for ToRC (toxoplasmosis, rubella, and cytomegalovirus), EBV (Epstein-Barr nuclear antigen, viral capsid antigen, and early antigen), syphilis (treponemal and non treponemal antigen), autoimmunity (SSA and SSB), and fertility (LH and FSH). Several other technologies for multianalyte detection have been proposed (5). For example, Fulton et al. (6) report a system that also utilizes latex particles as solid phase, with fluorometric detection on a flow cytometer. Advantages of Copalis over these other techniques include the homogeneous format (no washing steps) and detection by light scattering. These features allow Copalis instrumentation to be relatively simple and inexpensive.

**References**


**A Rapid, Sensitive, Multiplexed Assay for Detection of Viral Nucleic Acids Using the FlowMetrix System**, Perry L. Smith,1 Cindy R. Walker-Peach,2 R. Jerrold Fulton,1 and Dwight B. DuBois2 (1 Luminex Corporation, 12212 Technology Blvd., Austin, TX 78727 and 2Ceneteron Diagnostics, 2170 Woodward St., Austin, TX 78744; * address correspondence to this author at: Luminex Corporation, 1638 Osprey Dr., DeSoto, TX 75115; fax 972-224-9689, e-mail perrys@luminexcorp.com)

Sensitive assays for viral nucleic acids are important tools for the accurate diagnosis and treatment of viral diseases.
Unlike serological methods, viral load analysis provides quantitative information about viral replication. Studies have shown that viral load is a powerful predictor of disease progression in both human immunodeficiency virus (HIV) and hepatitis C virus (HCV) infections (1, 2). Additionally, monitoring viral load is a proven method for assessing the effects of antiviral treatments for both HIV and HCV infections (3–6).

Recently, the need for simultaneous viral load determinations of different viruses within a single individual has been established. Numerous studies indicate synergistic effects of co-infection with multiple viruses. For example, higher HCV titers have been reported in individuals co-infected with HIV (7). Likewise, herpes simplex virus (HSV) infections can be particularly severe in individuals with HIV-compromised immune systems (8).

This report describes a novel method for the detection and quantitation of viral nucleic acids in a rapid, multiplexed format. This method is based on fluorescent detection using the FlowMetrix analysis system. The FlowMetrix system is a computer-enhanced flow cytometer that uses microspheres dyed with multiple fluorescent colors (9). The microspheres are 5.5 μm in diameter and are composed of polystyrene and methacrylate to provide a surface carboxylate functionality. During manufacturing, two fluorophores (red and orange) are combined within the microspheres in a unique ratio. Currently, there are 64 different ratios of red and orange fluorescence, which identify 64 distinctly colored sets of microspheres. Differently colored microsphere sets can be individually coupled via the surface carboxylate moiety to a specific oligonucleotide probe for a unique nucleic acid sequence. Once individually coupled, the microsphere sets can be mixed, and the mixture can be used to probe complex solutions containing multiple sequences in a single hybridization.

In the current study, capture oligonucleotide probes specific for HIV, HCV, HSV, and their respective internal amplification control sequences were synthesized with a 5′ amino substitution and a 15-atom spacer between the reactive group and the hybridizing length (Oligo’s Etc.) (9–12). The six 5′-amino-substituted oligonucleotides were coupled individually to six differently colored microsphere sets using a carbodiimide (EDC) coupling method (9). After coupling, the six microsphere sets were mixed to form a multiplexed set.

HIV, HCV, and HSV nucleic acid sequences were amplified as described previously (10–12) and included a 5′ biotinylated primer in each case. For each viral amplification, a unique internal amplification control was also added. Control templates were engineered such that both viral and control sequences were amplified by the same primer set (10–12). After PCR amplification, the PCR products were denatured at 100 °C for 10 min. The denatured PCR products were added to an equal volume of 2× hybridization buffer containing 8000 of each of the six microsphere subsets (1× hybridization buffer contained 2.25 mol/L tetramethyl ammonium chloride, 0.75 g/L sodium dodecyl sulfate, 37.5 mmol/L Tris, pH 8.0, and 1.5 mmol/L EDTA, pH 8.0). This mixture was hybridized at 37 °C for 1 h. After hybridization, 2 μL of a 10-g/L solution of Alexa-488® (green fluorescence)-Streptavidin (Molecular Probes) was added. Samples were incubated at room temperature for another 30 min, then diluted with 200 μL of 1× hybridization buffer, and analyzed with the FlowMetrix system.

The FlowMetrix system consists of a Beckton Dickinson FACScan flow cytometer configured with proprietary hardware and software that controls all functions of the cytometer as well as providing real-time data acquisition and analysis for the multiplexed assay. During the analysis on the cytometer, the system categorizes each microsphere according to its red-orange fluorescent color set and determines the average green fluorescence for each set of microspheres, providing a quantification of that specific hybridized PCR product.

Results from the multiplexed assay are shown in Fig. 1. To determine specificity, increasing amounts of each PCR product were individually hybridized to the entire multiplexed set of six oligonucleotide-coupled microspheres. All reactions were performed in duplicate, and the mean values were plotted on semilog plots. These results show that the hybridization of each PCR product to its complementary microsphere set was highly specific and quantitative over a dynamic range of up to 3 logs. Only at extremely high amounts of PCR product (>10^10 copies) was there any

Fig. 1. Concentration response profile for hybridization of viral sequences (left) and controls (right).

Titrations curves of serial dilutions of HIV (▲), HIV-control (∆), HSV (●), HSV-control (○), HCV (■), and HCV-control (□) amplicons in a multiplexed assay are shown. All six microspheres are included in each reaction. As the concentration of each PCR product increases, there is a corresponding increase in the mean fluorescence intensity (green) of only the microspheres bearing the respective complementary capture probe.
detectable cross-hybridization. The internal amplification controls used in this study were designed to be used as calibrators by which the number of input viral template copies could be calculated. A known quantity of the control template was added to the test sample, and a single pair of primers was used to amplify both the viral and control sequences in a single PCR reaction. Under specific amplification conditions, the amount of control PCR product will be proportional to the amount of viral PCR product. The results presented here demonstrate the ability to quantitatively and specifically detect each PCR product in a multiplexed reaction. Preliminary work with clinical HIV samples suggests that multiplexed quantitation is possible.

In conclusion, these results indicate that the FlowMetrix assay system is rapid, sensitive, and specific. The system can be applied to the simultaneous detection of both RNA and DNA viruses, such as human immunodeficiency, hepatitis C, and herpes simplex viruses that are common in patients who are at risk for multiple infections. The flexibility of this system allows inclusion of new sequences, such as genetic variants or additional viruses, by simply adding appropriate microsphere sets to the multiplexed mixture.

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References