

syphilis infection, many of these samples have positive reactivities for *T. gondii*, CMV, Epstein-Barr virus, and various human herpes viruses.

[0151] Negative Control Sera. Human serum controls were commercially-sourced, and were vendor-certified as HIV, HCV, and RPR negative. *T. pallidum* antibody reactivity was not provided by the vendor for this collection. In the event of a positive *T. pallidum* antibody result using the present system and method, reference testing was performed on those specific samples. Reference tests for *T. pallidum* included TPPA (Fujirebio, Malvern, Pa.), a syphilis RDT (SD Bioline, Korea), and Treponemal Enzyme Linked Immunosorbent Assay ("ELISA", Trep-Sure, Phoenix Biotech, Ontario, Calif.). *T. pallidum* reference testing was not performed on samples that were negative by RPR and negative using the present system.

[0152] Whole Blood Samples. Because one of the important uses of the present system and method will be in point-of-care settings, it is important to evaluate the performance of the system on whole blood samples. Whole blood was sourced under an IRB-approved protocol from HIV-positive donors at the UCSF Antiviral Research Center ("AVRC"). Venipuncture samples were collected in Ethylenediaminetetraacetic Acid ("EDTA") blood collection tubes (Lavender Cap BD Vacutainer®) and shipped overnight to the site where the assays were run within two hours of receipt of the samples (i.e., within 24 hours of draw). After whole blood samples were processed, the tubes were centrifuged and the plasma fraction was also assayed.

[0153] As shown in the examples, the HIV-1 assay have 100% agreement with known seroreactivity on a collection of 82 HIV Ab-positive and 142 HIV Ab-negative samples, including multiple samples with HCV and syphilis co-infection. The treponemal-specific syphilis assay correctly identifies 67 of 68 *T. pallidum* Ab positive and 100 of 102 *T. pallidum* Ab negative samples. The HCV assay correctly identifies 59 of 60 HCV Ab-positive and 120 of 121 HCV Ab-negative samples. Multiplexed assay performance on whole blood samples is also demonstrated.

[0154] Assay Cartridge and Instrument. The system described in the examples here combined single-use disposable assay cartridges with a reader instrument. Fluorescence assays were illuminated and imaged using a multi-mode planar waveguide technology. Various types of planar waveguides have been used in biosensor and immunoassay applications for decades, and are the subject of several technical reviews. Briefly, a light source (typically a laser) was directed into a waveguide substrate where it propagated by total internal reflection ("TIR") at the interface between the high index of refraction waveguide (glass or plastic) and the surrounding medium (air or aqueous solution). The present system uses a planar waveguide system as disclosed, for example, in aforementioned U.S. patent application Ser. No. 12/617,535.

[0155] Assay Procedure. Samples are processed in cartridges on the bench top at ambient temperature, which in this study was approximately 20 to 25° C. Since the assay procedure may be performed independently of the reader instrument, sample cartridges may be batch processed, with up to 30 run in parallel, for example. A tilt rack, such as that discussed below with respect to FIGS. 47-49, may be used during the assay to facilitate the batch processing, if desired. Sample volumes for the disposable cartridge are 6 microliters of serum or plasma or 12 microliters of whole blood, making

the cartridge compatible with finger stick capillary samples. The larger volume for the whole blood samples is believed to be required to compensate for the cell volume.

[0156] Results presented here are based on the following sample processing procedure. A 6 microliter aliquot of serum or plasma is diluted in 194 microliters of a diluent (PBS, 0.5% casein, 0.05% Tween20). 175 microliters of this diluted sample mixture is then loaded into the cartridge input port by transfer pipette. Passive flow through the cartridge during a 15 minute incubation occurs independently of any user interaction. 175 microliters of wash buffer (PBS, 0.1% Tween20) is then added to the input port and allowed to flow through the cartridge for 3 minutes, followed by 175 microliters of dye-conjugated anti-human IgG in a second diluent (PBS, 1 mg/mL BSA and 0.05% Tween20) and allowed to incubate for 10 minutes. The total per cartridge assay time, in the present example, is approximately 28 minutes. While the fluorescence signal generated at the assay region of the cartridge may change over time, the cartridge may be read on the reader instrument any time within an hour of sample processing without affecting the final test result. Read time and data processing in the reader instrument is approximately 30 seconds per cartridge.

[0157] Custom image processing software has been developed for reaction site finding, intra-site fluorescence signal intensity measurement and normalization. After results reporting, the cartridge is removed from the reader instrument and disposed as biohazard waste, and the next processed cartridge may be inserted into the reader instrument. The combination of parallel cartridge processing, large read window, and rapid analysis allows more than 100 samples per work shift to be processed.

Results: Multiplex Assay of HIV, Syphilis and HCV on the Same Cartridge

[0158] FIGS. 28-29 show an exemplary array layout and representative images for the multiplex HIV/Syphilis/HCV assay.

[0159] FIG. 28 provides an exemplary layout of the HIV-1/Syphilis (*T. pallidum*)/HCV array map along with images from a representative set of clinical samples. In-array control features include printed human IgG, anti-human IgG, and print buffer controls. These features should yield fluorescence signal in an acceptable range for a test to be considered valid, ensuring that sample and fluorescently labeled detect antibody were added to the cartridge and that the cartridge was properly illuminated. In particular, the 30 feature array (2 rows by 15 columns) of the present example includes pathogen-specific printed antigens as well as multiple in-assay controls. Dye-labeled bovine serum albumin ("BSA") sites serve as corner markers (C1) for imaging. These sites are not used in the analysis in this embodiment. Printed human IgG (C2) serves as a procedural control for the dye-labeled detect antibody. Printed anti-human IgG (C3) serves as a sample control. Print buffer sites serve as negative controls, and monitor for any unusual non-specific binding. Excessive fluorescence signal on the negative control spotsites yields an invalid test result.

[0160] The human IgG and anti-human IgG control sites were designed to give fluorescence signal comparable to a typical seropositive sample. Obviously, total human IgG and the goat anti-human IgG fluorescent conjugate were both in large excess relative to the specific antibodies reacting with