

a step **3228**. In particular, as an example, the radius of each reaction site is calculated as an average of found reaction sites, the raw signal is integrated over the reaction sites, then a per-pixel average (at the camera) is calculated as the reaction site signal. In a step **3230**, a normalization process is implemented in order to calculate normalized signals for the reaction sites using, for instance, a power normalization algorithm. In a step **3232**, signals at each antigen site is determined to calculate antigen signals. For instance, step **3232** may involve the identification of the antigen sites (in accordance with the cartridge configuration), then averaging the signals at the antigen sites. Additionally, a “look-up” table may be used as an error control, such as in the case of noisy signals, highly-fluctuating signal intensity, etc. Finally, the calculated antigen signals are compared against predetermined cut-offs so as to determine the disease status for the various antigens in a step **3234**. The image analysis step is ended in an end step **3240**, and the process proceeds to step **3156** of FIG. **31**.

[0167] As previously described, the specific steps of process **3154** as shown in FIG. **32** may be performed onboard reader instrument **100** or at peripheral device **1980** of FIG. **19**.

Clinical Results

[0168] With the combination of commercial positive controls, the UCSD HIV-1 samples, the CDPHE Syphilis samples, the UCSD Co-Infection samples, and the negative controls, a total of 251 different samples (serum and plasma) are presented in this clinical results section. It is emphasized that antibody reactivity against all antigens in FIGS. **33** and **34** is measured simultaneously for each sample.

[0169] HIV-1 Antibody Assay Results. A total of 224 samples in the collection had known HIV-1 seroreactivity status, with 82 HIV-1 Ab positive and 142 HIV-1 Ab negative. Antibody reactivity (signal as described above) against the two HIV antigens in the array (gp41 and p24) are linked to the known HIV Ab reactivity status and plotted in FIG. **33(a)**. Reaction site signal intensity is the background subtracted, normalized intensity, as discussed above. The horizontal solid bar in each box represents the median; upper and lower boundaries of the boxes are the 75th and 25th percentiles; and the upper and lower whisker bars are the 90th and 10th percentiles, respectively. The open circles represent samples with values above and below the 90th and 10th percentiles. The dashed lines are empirically derived cutoffs. FIG. **33(a)** shows antibody reactivity results for a total of 224 samples with known HIV-1 Ab reactivity status (82 positive and 142 negative).

[0170] Ideally, samples that are known HIV Ab negative should show little or no intensity on the HIV antigen sites. As expected, gp41 and p24 signal results are clustered near zero for these negative samples. We note that the gp41 sites do show some cross-reactivity, with normalized signals between 0 and 0.4 on this scale. For the HIV Ab positive samples, we see a distribution of intensities. A strong gp41 antibody response is expected in seroconverted individuals represented in this collection, and the FIG. **33(a)** gp41 results are consistent with this expectation. One known HIV Ab positive sample does show low signal on the gp41 site. This particular sample yielded robust signal on the p24 site. For the collection, reactivity against the p24 antigen is varied, as seen in FIG. **33(a)**.

[0171] The data plots of FIG. **33(a)** are used to establish empiric cutoff values that will be used for subsequent signal/

cutoff (“S/CO”) calculations. Cutoffs are set near the highest signal negative sample in the collection. Increasing the cutoff value will yield a more specific assay (fewer false positives). Decreasing the cutoff will yield a more sensitive assay (fewer false negatives). Given that the antibody reactivity status of the samples in this collection was known, individual antigen cutoffs were adjusted empirically to optimize “effective” sensitivity and specificity. We note that the results obtained in this study are based on a self-referential dataset, i.e., we are applying the cutoff to the same data used to generate the cutoff. We therefore do not report results in terms of sensitivity and specificity. On the scale shown in FIG. **33(a)**, the gp41 and p24 cutoffs are set at a value above the highest signal negative sample in the collection (cutoffs are 0.45 and 0.16, respectively). If it is assumed that a S/CO>1 for either antigen constitutes overall HIV-1 Ab reactivity for that sample, then the cutoffs as defined yield 100% agreement between the multiplex assay as described herein and the reference result.

[0172] Syphilis Assay Results. A total of 170 samples in the collection had known *T. pallidum* antibody reactivity status, including 68 treponemal positive and 102 treponemal negative samples. Results for the treponemal antigens p17 and p47 are provided in FIG. **33(b)**. FIG. **33(b)** shows antibody reactivity results for a total of 170 samples with known treponemal Ab reactivity status (68 positive and 102 negative). As described above, cutoffs were empirically determined and are indicated on the plots. Applying these cutoffs to the 170 samples, we report treponemal Ab reactivity on 67 of 68 known treponemal Ab reactive samples (one false negative). The assay correctly identifies 100 of 102 treponemal negatives (two false positives). Next steps will be to improve specific activity and minimize non-specific binding to the treponemal antigen sites.

[0173] Hepatitis C Assay Results. A total of 181 samples had known HCV antibody reactivity, including 60 HCV Ab positive and 121 HCV Ab negative. Results for the four recombinant antigens are provided in FIG. **34**, which shows the antibody reactivity in the exemplary assay for 181 clinical samples with known HCV antibody serostatus (60 positive and 121 negative). Reaction site signal intensity is the background subtracted, normalized intensity described in the text. Antibody reactivity against all antigens is measured simultaneously for each sample. The dashed lines are empirically derived cutoffs for the core and NS3 recombinant antigens. The synthetic NS4* and Multi** multiple-epitope antigens showed relatively high signals on the HCV Ab negative samples, so cutoffs for these two antigens were not used in subsequent analyses.

[0174] Continuing to refer to FIG. **34**, a large spread of antibody reactivity signals is observed, as expected. The core and NS3 antigens appear to provide the best overall performance in terms of distinguishing positive from negative. The two multiple epitope antigens (NS4* and Multi**) did not offer a benefit over the core and NS3 antigens in this assay. Using these results, cutoffs were set for the core and NS3 antigen as described above. With the selected cutoffs, the assay correctly identifies 59 of 60 positive and 120 of 121 negative HCV samples. FIG. **34** shows a large number of samples (positive and negative) near the cutoff values. Antigen selection and activity improvements are required to improve robustness of the HCV assay.

[0175] Whole Blood Assay Results. Because point-of-care is the target application of the present system, whole blood performance of the assay system is an important demonstra-