

# A Multiplex Microsphere-Based Immunoassay Increases the Sensitivity of SIV-Specific Antibody Detection in Serum Samples and Mucosal Specimens Collected from Rhesus Macaques Infected with SIVmac239

Rebecca L.R. Powell, Ian Ouellette, Ross W. Lindsay, Christopher L. Parks, C. Richter King, Adrian B. McDermott, and Gavin Morrow

## Abstract

Results from recent HIV-1 vaccine studies have indicated that high serum antibody (Ab) titers may not be necessary for Ab-mediated protection, and that Abs localized to mucosal sites might be critical for preventing infection. Enzyme-linked immunosorbent assay (ELISA) has been used for decades as the gold standard for Ab measurement, though recently, highly sensitive microsphere-based assays have become available, with potential utility for improved detection of Abs. In this study, we assessed the Bio-Plex<sup>®</sup> Suspension Array System for the detection of simian immunodeficiency virus (SIV)-specific Abs in rhesus macaques (RMs) chronically infected with SIV, whose serum or mucosal SIV-specific Ab titers were negative by ELISA. We developed a SIVmac239-specific 4-plex bead array for the simultaneous detection of Abs binding to Env, Gag, Pol, and Nef. The 4-plex assay was used to quantify SIV-specific serum IgG and rectal swab IgA titers from control (SIV-naive) and SIVmac239-infected RMs. The Bio-Plex assay specifically detected anti-SIV Abs in specimens from SIV-infected animals for all four analytes when compared to SIV-naive control samples ( $p \leq 0.04$ ). Furthermore, in 70% of Env and 79% of Gag ELISA-negative serum samples, specific Ab was detected using the Bio-Plex assay. Similarly, 71% of Env and 48% of Gag ELISA-negative rectal swab samples were identified as positive using the Bio-Plex assay. Importantly, assay specificity (i.e., probability of true positives) was comparable to ELISA (94%–100%). The results reported here indicate that microsphere-based methods provide a substantial improvement over ELISA for the detection of Ab responses, aid in detecting specific Abs when analyzing samples containing low levels of Abs, such as during the early stages of a vaccine trial, and may be valuable in attempts to link protective efficacy of vaccines with induced Ab responses.

**Key words:** HIV; immunology; microbiology; SIV

## Introduction

**R**ESULTS FROM THE RV144 HIV-1 VACCINE TRIAL recently demonstrated that the elicitation of specific antibodies (Abs) may be linked to the protective efficacy observed. The precise location, activity, and levels of such Abs remain to be determined; however, the overall titers of the protective antibodies may have been quite low.<sup>1,2</sup> Enzyme-linked immunosorbent assay (ELISA) titers were approximately 10% of those measured in the predecessor AIDSVAX trial, and roughly 1% of titers typically measured in HIV-1-infected subjects.<sup>3,4</sup> Furthermore, nonneutralizing Abs associated with protection were found to be V2-directed and belonged to the IgG3 subset, suggesting Ab-dependent cellular cytotox-

icity via Fc-receptor activities.<sup>5–9</sup> Although mucosal samples were unavailable for testing, these findings have led to the speculation that the active Abs induced in RV144 might have been localized primarily at mucosal sites of potential infection, which is a phenomenon shown to correlate with protection in animal models.<sup>10,11</sup> These data have highlighted the necessity for careful measurement of Ab specificity, type, and level in both serum and mucosal samples, which can present technical challenges, particularly if Ab titers are low.

Over the past decade, soluble microsphere (bead)-based arrays have become an increasingly popular alternative for quantifying Abs.<sup>12–21</sup> Such assays are advantageous as replacements for traditional ELISAs, because they can easily be multiplexed, reducing the sample volume required to

evaluate multiple Abs, provide soluble-phase binding of antigen–antibody pairs, and offer a vastly increased dynamic range compared to the ELISA optical density scale. One of the most popular microsphere-based assays relies on Luminex® xMAP® technology (Luminex Corp., Austin, TX). In this system, different microsphere populations are internally labeled with varying concentrations of fluorescent dyes, giving each microsphere type in the array a unique fluorescence signature that can be identified with an appropriate instrument, such as the Bio-Plex® 200 System plate reader (Bio-Rad, Hercules, CA). As a result, when proteins of interest are covalently linked to unique bead populations, up to 100 different analytes can theoretically be measured in a single Bio-Plex plate well. Such multiplexed assays are particularly useful when simultaneous measurement of numerous analytes in a given sample is desired, as is often the case with cytokines, for which numerous assays have been developed.<sup>15,16,19,20,22–29</sup> In addition, multiplexed bead-based immunoassays have been established and commercial kits are available for simultaneous measurement of specific Abs against various disease, atopic, autoimmune, and cancer-related antigens.<sup>17,21,28,30–34</sup> These assays have been employed to measure analytes in cultured cells or in various bodily fluids such as serum, semen, and cervical mucus from humans or numerous experimental animal models.

Microsphere-based assays have demonstrated good correlation with ELISA data for most, but not all analytes. This correlation appears largely dependent on analyte concentration in the sample, with microsphere-based assays often detecting analytes present in low concentrations that ELISA does not, which is interpreted as a false positive when ELISA is used as the gold-standard.<sup>23–25,31,35,36</sup> However, this common observation might indicate that microsphere-based assays are superior for detecting low levels of analytes compared with ELISA. To determine if microsphere-based assays such as the Bio-Plex system provide increased sensitivity for detection and quantification of Abs in serum and mucosal samples, we used the Bio-Plex Suspension Array System to develop a 4-plex bead array to detect Abs specific for SIVmac239 Env, Gag, Pol, and Nef. This assay was used to reassess endpoint titers for serum IgG and rectal swab IgA from specimens collected from rhesus macaques (RMs) infected with SIVmac239, which exhibited negative ELISA titers despite detectable viral loads.

## Methods

### Samples

Serum and rectal swabs were obtained from Indian RMs as part of ongoing vaccine studies. Of the RMs from which rectal swabs were obtained, 24 had been vaccinated by the intratonsillar or intramuscular route using a 3× SIVmac239 *Env/Gag/Pol* DNA prime+SIVmac239-recombinant adenovirus boost regimen. All RMs, excluding the SIV-naive control animals, were challenged with low-dose SIVmac239 by the intrarectal route and confirmed to be infected by a positive SIVmac239 viral load. All samples assayed herein were obtained between 7 and 123 days postinfection. Sera were obtained in 2004–2005 or in 2012 and stored at –80°C. Rectal swabs were obtained in 2011–2012 and stored at –80°C. Viral loads were determined within 20 days of sampling and were found to be between 10<sup>3</sup> and 10<sup>8</sup> genome copy equivalents per milliliter.

### Rectal swabs

Rectal swabs were collected as previously described using Weck-Cel® Eye Spears (Beaver-Visitec, Waltham, MA).<sup>37</sup> Sample collection minimized bleeding and subsequent elution was performed according to the published protocol.<sup>37</sup> Prior to use, samples were tested for the presence of blood using Hemocult® Test Cards (Beckman Coulter, Brea, CA) according to the manufacturer's protocol using 20 µL of eluate. If blood was detected, samples were not used for this study.

### Bead coupling

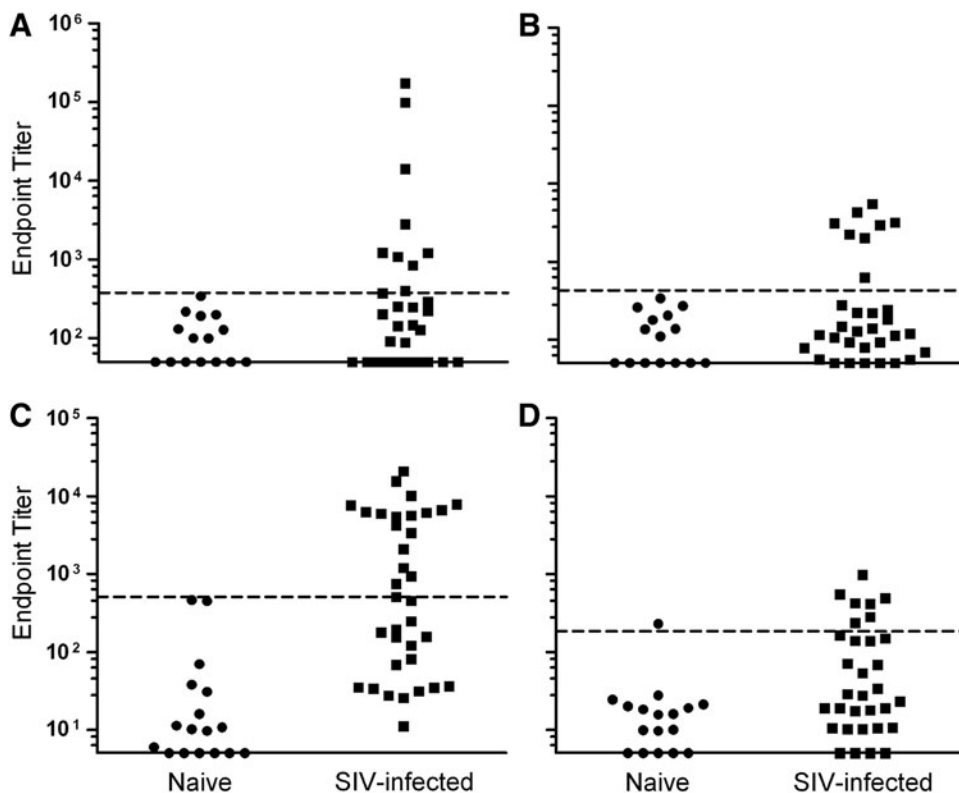
One milliliter of Bio-Plex COOH (carboxylated) beads were conjugated to SIV proteins according to the manufacturer's protocol using the Bio-Plex amine coupling kit (Bio-Rad). Beads were counted just prior to conjugation using a Vi-Cell Viability Analyzer (Beckman-Coulter) to ensure a consistent bead-to-protein ratio since bead loss occurs during the conjugation process.<sup>38</sup> Protein was added at a ratio of 25 µg of protein/5×10<sup>6</sup> beads for Envelope gp130 (produced in-house), Gag p55 (Protein Sciences Corp., Meriden, CT), and Pol (Immune Technology, New York, NY), or 75 µg/5×10<sup>6</sup> beads for Nef (Immune Technology). Final volume was adjusted to 5 mL in phosphate-buffered saline (PBS), and tubes were agitated for 2 h. Beads were then washed with 5 mL of PBS, resuspended in 2.5 mL of Stabliguard blocking buffer (SurModics, Eden Prairie, MN), and agitated for 30 min.<sup>39</sup> Beads were washed, resuspended in 400 µL of storage buffer, counted, aliquoted, and stored at –80°C.

### ELISA

Polystyrene, flat-bottom, high-binding, half-area plates (Corning, Kennebunk, ME) were coated overnight at 4°C by adding 17.5 ng of Env gp130, 125 ng of Gag p55 (IgG ELISA), 150 ng of Env gp130 or Gag p55 (IgA ELISA) per well. Plates were washed in PBS/0.02% Tween-20 and blocked for 1 h at 37°C with 120 µL of PBS/3% bovine serum albumin (BSA; IgG ELISA) or PBS/3% milk (IgA ELISA). Plates were washed, and serum or swab eluate was added at 1:100 or 1:10 diluted in PBS/1% BSA (IgG ELISA) or PBS/1% milk (IgA ELISA), respectively. Serum was diluted threefold across the plate, while swab eluate was diluted twofold. Plates were incubated and washed as above before the addition of biotin-conjugated goat antimouse IgG for serum (0.125 µg/mL in PBS/1% BSA; Rockland Immunochemicals, Gilbertsville, PA) or goat antimouse IgA for swabs (0.66 µg/mL for Env ELISA, 4 µg/mL for Gag ELISA, in PBS/1% milk; Rockland Immunochemicals). Plates were incubated and washed as before prior to the addition of streptavidin–horse radish peroxidase (1 µg/mL; Biolegend, San Diego, CA). After incubation and washing, TMB Substrate Reagent Set (3,3',5,5'-tetramethyl benzidine; Biolegend) was added according to the manufacturer's protocol. The reaction was stopped with 2 N H<sub>2</sub>SO<sub>4</sub> and plates were read at 450 nm using a VERSAmax ELISA reader (Molecular Devices, Sunnyvale, CA).

### Bio-Plex assay

Analysis of serum and swab eluate with Bio-Plex assays were performed using conditions suggested by the manufacturer (Bio-Rad). One hundred fifty microliters of assay buffer



**FIG. 1.** ELISA endpoint titers. (A) Env-specific and (B) Gag-specific IgG titers in serum obtained from 32 SIV-naive and SIV-infected rhesus macaques. (C) Env-specific and (D) Gag-specific IgA titers in eluates from rectal swabs obtained from 35 SIV-naive and SIV-infected rhesus macaques. Dotted lines indicate positive cutoff values. ELISA, enzyme-linked immunosorbent assay; SIV, simian immunodeficiency virus.

(PBS, 0.5% casein, 0.1% BSA, 0.02% Tween-20, 0.05% sodium azide, pH 7.4) was added to each well of a Multiscreen HTS, HV clear plate (Millipore, Billerica, MA) before the plates were incubated for 10 min with shaking. All subsequent sample or assay dilutions were carried out in assay buffer. Samples were thawed and centrifuged at 20,000 *g* for 1 min to pellet any debris. Plates were drained by vacuum pressure before 1:50 diluted serum samples or 1:10 diluted swab samples were added. Serum samples were titrated threefold across the plate, while swab samples were titrated twofold across the plate. An equal volume of conjugated bead set for all four SIV proteins (50 beads/ $\mu$ L; 1250 beads/well) was added to diluted samples before the plate was incubated at room temperature for 1 h with agitation. Plates were washed twice by vacuum with 150  $\mu$ L assay buffer before the addition of goat antimouse IgG (0.07  $\mu$ g/mL) or IgA (1  $\mu$ g/mL) biotin-conjugated secondary antibody (Rockland Immunochemicals) and were incubated at room temperature for 1 h with agitation. Plates were washed twice by vacuum with 150  $\mu$ L assay buffer before incubation with 50  $\mu$ L of Phycolink Streptavidin-R-Phycoerythrin (1  $\mu$ g/mL, Thermo Scientific, Waltham, MA) at room temperature for 1 h with agitation. Plates were finally washed twice in casein-free assay buffer before resuspension in 80  $\mu$ L of casein-free assay buffer with agitation at room temperature for 5 min. Plates were read using a Bio-Plex 200 System plate reader (Bio-Rad).

#### Data analysis

Background was subtracted before data points were fit to four-parameter curves using Prism version 5.03 (GraphPad Software, Inc.). Endpoint titers were interpolated from the curves using the median RLU (Bio-Plex) or optical density

(ELISA) of the SIV-naive samples at the lowest sample dilution (1:100 for serum, 1:10 or 1:20 for rectal swabs) as a cutoff value. Statistical analyses were carried out with GraphPad Prism software, using a two-tailed unpaired *t*-test. Titers were considered positive for a given assay if they were greater than the mean of the control sample endpoint titers + 3 standard deviations (positive cutoff value).<sup>40</sup> Specificity of the ELISA and Bio-Plex assays were calculated as (number of true negatives [i.e., SIV-naive samples]) / (number of true negatives + number of false positives).

## Results

### Determination of serum and swab anti-SIV IgG and IgA endpoint titers by ELISA

Thirty-two serum samples from SIVmac239-infected RMs with detectable viral load and 15 serum samples obtained from SIV-naive control RMs were tested for Env- and Gag-specific IgG titers by ELISA. It was found that the naive control sera exhibited a mean Env-specific ELISA endpoint titer of 117, thus samples exhibiting endpoint titers greater than 379 were considered positive for Env-specific IgG (Fig. 1A, dotted line). Among the sera obtained from SIV-infected RMs, 9 of 32 samples were found to be positive for anti-Env IgG by ELISA, with endpoint titers ranging from 402 to 172,809, while the remaining 23 titers fell below the assigned cutoff value, exhibiting titers of 50–376 (Fig. 1A). Furthermore, naive control sera exhibited a mean Gag-specific ELISA endpoint titer of 131, resulting in a positive cutoff of 423. Eight SIV-infected samples exhibited positive Gag-specific titers ranging from 625 to 5444, while the remaining negative sample titers ranged from 50 to 274 (Fig. 1B).

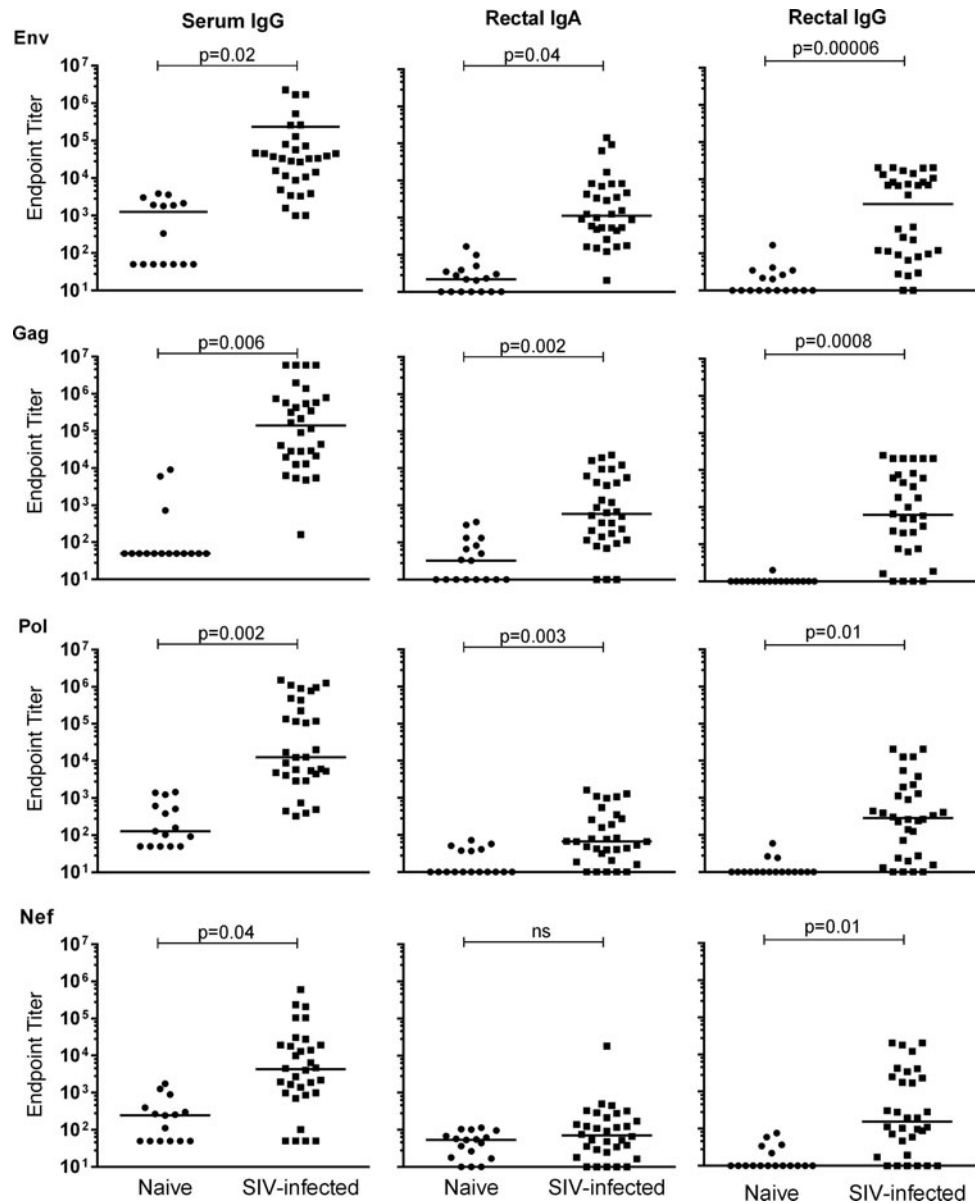


FIG. 2. Bio-Plex detection of anti-SIV specific antibodies in rhesus macaque samples. Lines indicate mean endpoint values.

Eluates from rectal swabs obtained from 35 infected RMs and 17 SIV-naive RMs were similarly analyzed by IgA ELISA. Naive control samples exhibited a mean Env-specific endpoint titer of 67 resulting in a cutoff value being assigned at 505. Accordingly, 18 of 35 SIV-infected samples were scored positive for Env-specific IgA, with titers ranging from 506 to 20,600 (Fig. 1C). Negative samples exhibited titers of 11–449. Additionally, naive control samples exhibited a mean Gag-specific endpoint titer of 26, which corresponded to a positive cutoff value at 184. Only 7 of 32 samples were found to be positive, with titers ranging from 233 to 969, while negative titers ranged from 5 to 163 (Fig. 1D).

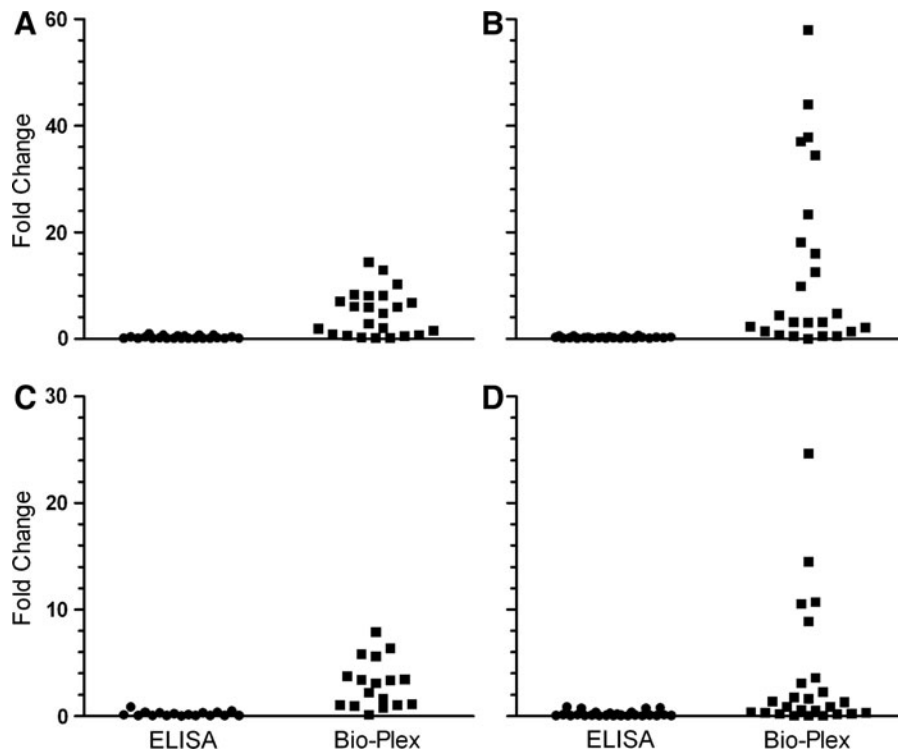
#### Determination of serum and swab anti-SIV IgG and IgA endpoint titers by Bio-Plex assay

In order to compare the Bio-Plex data with those obtained by ELISA, cutoffs for endpoint titer determination and positive endpoint cutoffs were calculated by the same methods for both assays. As well, both assays employed the same antigens

and biotinylated secondary Abs. Serum samples were tested for SIV-specific IgG titers, while rectal swab eluates were tested for SIV-specific IgG and IgA. Overall, the novel Bio-Plex assay was found to specifically detect the analytes tested as judged by the significantly higher antigen-specific Ab titers in serum samples obtained from SIV-infected RMs compared to the naive control samples for all four antigens in the Bio-Plex assay (Fig. 2A–D). Similarly, rectal swab eluates obtained from SIV-infected RMs exhibited significantly higher antigen-specific IgG titers compared to naive control samples for all four analytes and significantly higher Env, Gag, and Pol-specific IgA titers; however, Nef-specific IgA titers were not significantly different between the two groups (Fig. 2E–L).

#### Bio-Plex assay sensitivity improves detection of positive samples compared to ELISA

Many of the samples from infected animals that had exhibited negative Env- or Gag-specific endpoint titers by ELISA were found to be positive using the Bio-Plex assay.



**FIG. 3.** Samples found to be negative for SIV-specific antibody (Ab) titers by ELISA exhibit detectable titers using the Bio-Plex assay. Fold change in endpoint titer compared with positive cutoff value for each ELISA-negative sample is shown. (A) Env-specific and (B) Gag-specific serum IgG assays. (C) Env-specific and (D) Gag-specific rectal swab eluate IgA assays.

Overall, the Bio-Plex assay found 25 and 27 of 32 infected serum samples to be positive for Env- and Gag-specific Abs, respectively. Of the 23 serum samples negative for Env-specific IgG by ELISA, 16 samples (70%) were found to be positive by the Bio-Plex assay, exhibiting titers 1.6- to 14-fold higher than the Bio-Plex endpoint cutoff value (5544; Fig. 3A). Similarly, 18 of the serum samples (79%) found negative for Gag-specific titers by ELISA exhibited detectable titers by Bio-Plex, which were 1.4- to 58-fold higher than the cutoff value (9181; Fig. 3B). Env- and Gag-specific rectal swab IgA titers measured by Bio-Plex were also compared to ELISA data. Overall, the Bio-Plex assay found 30 and 18 of 35 infected rectal swab samples to be positive for Env- and Gag-specific IgA, respectively. Of the 17 samples found to be negative in the Env-specific IgA ELISA, 12 (71%) of these samples were found to be positive by Bio-Plex, exhibiting titers 1.05- to 7.9-fold higher than the cutoff value (157; Fig. 3C). Of the 25 samples found negative by ELISA for Gag-specific IgA, 12 samples (48%) were found to be positive by Bio-Plex, exhibiting titers 1.3- to 25-fold higher than the cutoff value (386; Fig. 3D).

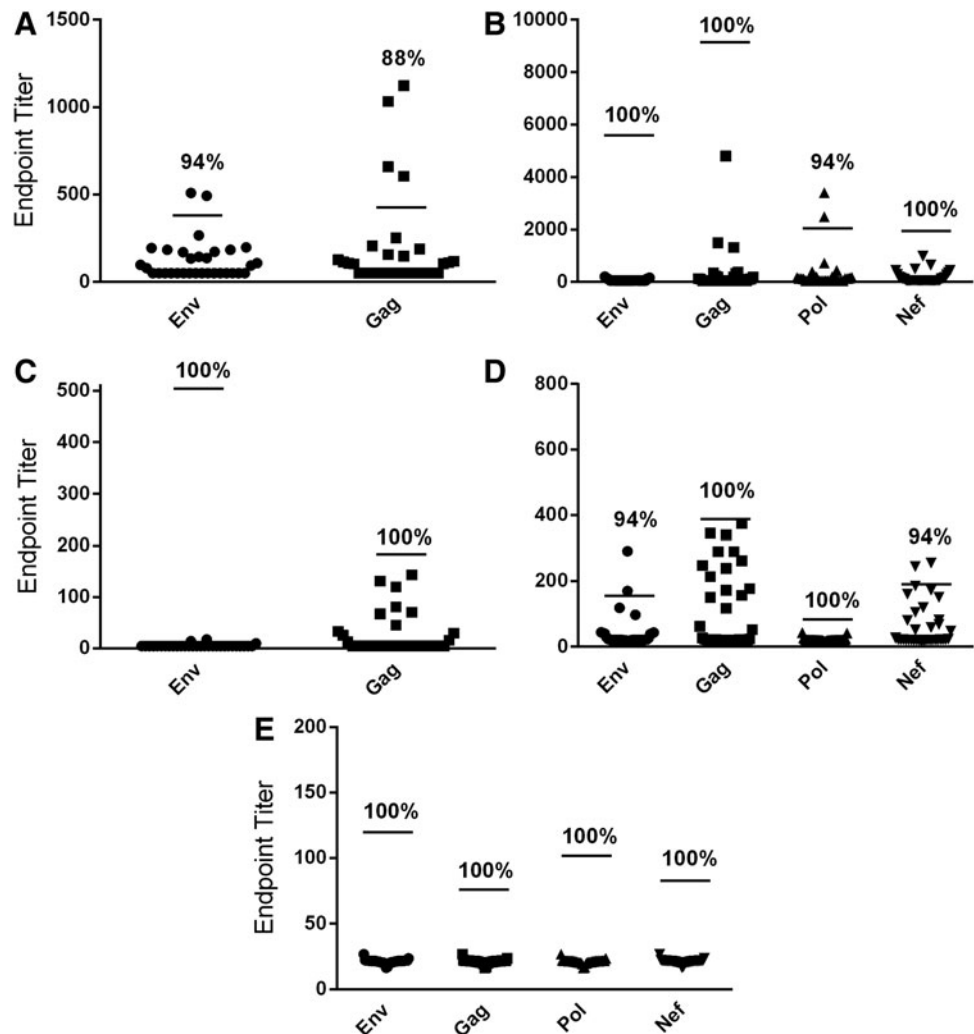
Importantly, the specificity of the Bio-Plex assay (i.e., the proportion of viral load-negative samples predicted to test negative for SIV-specific Abs) was assessed to ensure that this assay was truly superior to ELISA for SIV-specific Ab detection and was not increasing the false-positive rate. Thirty-two additional SIV-naive serum and rectal swab samples were tested, and positive endpoint cutoff values were applied as already described. It was found that the serum ELISA exhibited 94% and 88% specificity for the Env and Gag IgG assays, respectively, while the serum Bio-Plex assay exhibited 100% specificity for the Env, Gag, and Nef IgG assays, and 94% specificity for the Pol IgG assay (Fig. 4A, B). Furthermore, it was found that the rectal swab ELISA exhibited

100% specificity for both the Env and Gag IgA assays, while the Bio-Plex rectal swab assay exhibited 100% specificity for the Env, Gag, Pol, and Nef IgG and Gag and Pol IgA assays, while the Env and Nef IgA assays exhibited 94% specificity (Fig. 4C–E).

## Discussion

Numerous fluorescent bead-based immunoassays have been developed over the past decade, and this technology is quickly becoming commonplace. These assays are beneficial because they provide a simple platform with which ELISA-type assays can be multiplexed, allowing for rapid, simultaneous detection of numerous analytes in bodily fluids. Furthermore, these assays offer a greatly increased dynamic range and lower detection limit compared with ELISA and require minimal sample volume. Numerous studies have been performed comparing data obtained using a microsphere-based assay with that obtained by ELISA. In general, these assays have shown fair correlation with each other and with traditional ELISA, though this has not been found to be uniformly true, especially with analytes at low concentrations.<sup>23–25,35</sup> Previous studies that have determined the sensitivity and specificity of Luminex-based assays have typically used ELISA data as the gold standard, often finding comparable or improved sensitivity but reduced specificity (i.e., an increased number of false positives compared with ELISA).<sup>24,35,40</sup> Importantly, it has been suggested that this apparent increase in the false-positive rate might be attributable to the ability of microsphere-based assays to detect a lower level of analyte as compared with ELISA.<sup>31,40</sup> Using specimens from RMs shown to be positive or negative for infection by analysis of virus loads, we determined whether the increased rate of positives found by Bio-Plex correlated with

**FIG. 4.** The Bio-Plex assay exhibits comparable specificity to ELISA. Thirty-two additional SIV-naive rhesus macaque serum samples and rectal swabs were analyzed using the same endpoint titer and positive cutoff values as determined for the initial sample set. **(A)** Specificity of serum IgG ELISA and **(B)** Bio-Plex assay. **(C)** Specificity of rectal swab eluate IgA ELISA and **(D)** Bio-Plex assay. **(E)** Specificity of rectal swab eluate IgG Bio-Plex assay. Lines indicate positive cutoff values. Percent specificities are shown.



infection or was truly a false positive. In our survey of RMs shown to be infected but exhibiting negative ELISA titers, the Bio-Plex assay detected positive endpoint titers in 48%–79% of these animals. This result is in agreement with previous studies of microsphere assays for detection of human leukocyte antigen-specific Ab (HLA-Ab) in organ transplant recipients.<sup>41–43</sup> These studies have found that such assays are far more sensitive than the conventional HLA-Ab assay for the detection of HLA-reactive Abs, whose presence was associated with worsened transplant outcomes. As with the present study, the utility of the microsphere-based assay in these studies was determined not by comparison to a typical gold standard Ab assay, but to more relevant disease markers.

Our results suggest that in the case of SIV and potentially HIV, ELISA cannot be used as a gold standard to link the presence of specific Abs with infection. Importantly, in our comparison of these assays, endpoint and positive cutoff values were calculated by the same method, and the same antigen–Ab pairs and reagents were used in both assay platforms where possible, in order to achieve a fair comparison of data.<sup>23–25,40</sup> Numerous factors explain the superior sensitivity of the Bio-Plex assay. Because the SIV antigens were covalently bound to the microspheres, higher assay avidity and lower background signal were achieved as compared with ELISA, in which antigen is noncovalently coated to plate

wells. Covalent binding also minimizes antigen loss during washing steps, and due to the significantly reduced surface area, washes are likely more efficient than in an ELISA, further reducing the background signal. Additionally, the ~3-log surface area reduction compared to an ELISA most likely contributes significantly to a reduction in nonspecific binding.<sup>27,44–46</sup>

In conclusion, these data demonstrate that microsphere-based platforms are advantageous beyond their multiplexing capacity, offering vastly increased sensitivity for specific Ab detection in bodily fluids compared with ELISA. Multiplexed, microsphere-based assays that differentiate low levels from negative levels of Abs may be critical to the interpretation of vaccine trial data, especially when samples are difficult to obtain. The results reported here also suggest the potential to use these methods for the prompt diagnosis of disease and seroconversion to various pathogens.<sup>43,47,48</sup>

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#### Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

*Rebecca L.R. Powell, PhD*

*International AIDS Vaccine Initiative*

*AIDS Vaccine Design & Development Laboratory*

*Brooklyn Army Terminal*

*140 58th Street, Bldg. A, Suite 8J*

*Brooklyn, NY 11220*

*E-mail: rpowell@iavi.org*