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ORIGINAL ARTICLE

Spherotech-EDTA combined serum treatment reduces background more effectively as compared to One Lambda Adsorb Out™ and LIFECODES Serum Cleaner in Luminex-based solid-phase immunoassays for HLA antibody detection

Maneesh Kumar Misra[1] | **Jerome G. Weidner**[1] | **Rebecca L. Upchurch**[1] | **Arianne M. Mankey**[2] | **Marcelo A. Fernandez-Viña**[2] | **Susana G. Marino**[1]

[1]Department of Pathology, The University of Chicago Medicine, Chicago, Illinois, 60637, USA

[2]Department of Pathology, Stanford University, Palo Alto, California, 94304, USA

Correspondence
Maneesh Kumar Misra, Department of Pathology, The University of Chicago Medicine, Chicago, IL 60637, USA.
Email: mmisra@bsd.uchicago.edu

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Spherotech (SPT) microparticles capture non-specific binding materials present in test serum, and EDTA removes the so called" prozone effect". This study presents a novel approach of combined SPT-EDTA serum treatment prior to Luminex HLA antibody testing to remove high background, and prozone effect in a single step process, and compared the efficacy of SPT-EDTA serum pre-treatment with AdsorbOut (ADS) and Serum Cleaner (SC) to reduce background in solid phase immunoassays (SPI). A total of 21 serum samples with a history of elevated negative control (NC) values ≥500, and 20 samples with normal NC values were included to assess the potential adverse effects. A problem of high background was noted in 25% of our samples in SPI. We observed 80% effectiveness in reducing NC values <500 with SPT-EDTA serum pre-treatment compared to 72%, and 67% for ADS and SC-treated sera, respectively. Interestingly, we found a strong correlation in antibody-binding levels between SPT versus ADS; and ADS versus SC-treated sera for both phenotype and single antigen bead assays ($p < 0.001$). No adverse effect was noted on NC, positive control (PC) values, PC/NC ratios in the upfront use of SPT-EDTA as compared to EDTA alone. Our data revealed that combined SPT-EDTA treated sera is more effective than ADS, and SC in reducing high background in SPI. Taken together, SPT-EDTA serum treatment prior to Luminex HLA Ab testing is cost-effective, our laboratory saves nearly 30% of the annual total cost for Ab testing and improved test turnaround time by two business days.

KEYWORDS
high background, HLA antibodies, prozone effect, solid phase immunoassays, Spherotech

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1 | INTRODUCTION

Luminex-based solid-phase immunoassays (SPI) have become the standard of care in pre-transplant screening and post-transplant monitoring of HLA antibodies in the field of HLA diagnostics and clinical allogeneic transplantation.[1,2] The accurate identification and characterization of HLA antibodies are crucial in preventing allograft rejection in solid organ transplantation, and engraftment failure in hematopoietic cell transplantation.[3,4] In Luminex-based SPI, purified HLA antigens of individual derived cell lines (Phenotype) or recombinant HLA proteins (Single Antigen) are coated on polystyrene microspheres (beads) as targets for binding of specific anti-HLA antibodies present in tested sera. After unbound serum components are washed away, a fluorescent-conjugated secondary antibody is utilized, which binds to the HLA antigens immobilized on the bead surface. Detection of fluorescent emission by the Luminex instrument is used in test interpretation. The fluorescent signal obtained is compared with the background signal from the same test serum, which reacts with negative control beads, and the signal obtained from a separate negative control serum. A resultant significant shift over background indicates a positive reaction with the target antigen. Luminex-based assays have high sensitivity and specificity and require small serum volumes[5]; however, there are limitations to the assay which can affect correct interpretation of the results.[6,7]

Two common problems that have been described in accurate identification and characterization of HLA antibodies in human test serum are the "prozone effect"[5,8] and non-specific binding (high background) in test serum.[6,9] The prozone effect also known as high dose hook phenomenon, is false-negative results in immunological reactions, due to an excess of either antigens or antibodies,[10] and by definition occurs in liquid phase and should be considered different from the inhibitory effects of the test. The inhibitory effect (so called "prozone effect"), a phenomenon characterized by the impairment in the formation of immune complexes, often interferes with the detection of HLA antibodies, yielding false-negative results. This inhibitory effect in SPI is observed when high titer HLA antibodies lead to complement activation and deposition of C1 complex on the test beads[11,12] or in the presence of IgM antibody[13] in test serum. The inhibitory effect can be mitigated using ethylene diamine tetra-acetic acid (EDTA), dithiothreitol (DTT) serum treatments or by testing serum dilutions. A previous report from our group has shown a superior effect of EDTA serum treatment in removing the so called prozone (inhibitory) effect over both DTT treatment and serum dilutions.[14]

In addition to the inhibitory effect, a subset of samples tested may display non-specific reactivity to the beads, including those without HLA antibody (NC bead) utilized in the assay. This elevation of mean fluorescence intensity (MFI) values hinders accurate identification and characterization of HLA antibodies[6,9] which complicates the workflow in the laboratory as additional testing may be required for result interpretation. These complications lead to increased cost of HLA antibody testing, extended test turnaround time (TAT), and delayed patient care.

A problem with high background arises in Luminex-based SPI when test sera have elevated NC bead values (indicative of high background) as this may mask a positive reaction with a specific antigen. There are two commercially available reagents used across many HLA laboratories to reduce or remove the serum factors that non-specifically bind to the beads used in the assay namely; LIFECODES Serum Cleaner (Immucor Transplant Diagnostics, Inc.)[15] and Adsorb Out™ (One Lambda).[9]

The novelty of this study is to describe a new reagent namely, Spherotech (SPT) polystyrene microparticles (Spherotech, Inc.) to reduce the background in Luminex SPI. SPT microparticles are prepared by conventional emulsion polymerization with styrene as the monomer and potassium persulfate or benzoyl peroxide as polymerization initiator.[16] These microparticles are treated with blocking solution without any specific antigen coating. The SPT microparticles are intended to reduce non-specific binding of material in human sera to Luminex beads by capturing IgG non-specifically as seen with reference sera known to demonstrate high levels of non-specific binding. A combined suspension of SPT microparticles and EDTA serum treatment is used prior to SPI in Luminex IgG Ab testing to prevent prozone effect and high background in a single step process to avoid the need for additional testing, and thus reducing the cost of testing, maintain test TAT, and to improve patient care Further, to the best of our knowledge, no study has been conducted to compare the effectiveness of commercial available reagents used frequently for serum pre-treatment, namely Serum Cleaner (SC), Adsorb Out™ (ADS) and SPT, to remove the background in Luminex SPI in HLA laboratories. The present study was conducted to fulfill this gap. Therefore, the aim was to compare the efficacy of SPT-EDTA combined serum pre-treatment with ADS and SC serum pre-treatments to reduce the background in Luminex SPI to accurately detect HLA alloantibodies in patient sera.

2 | MATERIALS AND METHODS

2.1 | Samples

A total of 53 serum samples collected from transplant patients for clinical purposes were included in this study.

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We defined a test serum with high background when NC bead values were elevated ≥500. Samples included consists of 21 serum samples (nine without detectable HLA antibodies, four with HLA class I antibodies, five with HLA class II antibodies, and three with both HLA class I and class II antibodies) with a history of high background to determine the effectiveness of the reagents in removing the background. An additional 20 serum samples (five without detectable HLA antibodies, five either with class I antibodies or class II antibodies, and five with both class I and class II antibodies) were included without elevated background to assess the potential adverse effects with the use of SPT-EDTA combined serum pre-treatment. Subsequently, 83 tests (46 LABScreen PRA class I and class II, 18 LABScreen Single Antigen class I, and 19 LABScreen Single Antigen class II) were performed for this study. Further, in order to show the significance of EDTA serum treatment to remove the inhibitory effects in SPI, we included IgG SAB, and C1q data generated on 12 serums with and without EDTA treatment, and observed the superior effect, and better correlation in mean fluorescence intensity (MFI) between IgG SAB and C1q assays ($R = 0.37$ vs. 0.32, $p = 2.2e{-}16$) with EDTA serum treatment as compared to no serum treatment (Figure S1).

2.2 | Preparation of EDTA spiked Spherotech beads suspension, and Luminex HLA antibody testing

A suspension of EDTA spiked SPT beads was prepared by mixing 10 mL of SPT microparticles in 600 μL of UltraPure 0.5 M EDTA, pH 8.0. The serum pre-treatment was performed to remove the background by mixing one part of EDTA spiked SPT beads with five parts of serum for IgG detection of both HLA class I and class II antibodies. After a 10 min incubation at room temperature with gentle shaking on the plate shaker, samples were centrifuged at maximum speed of 15,000 rpm for 10 min, allowing the beads to form a pellet, and transferred into a new labeled working tube for Luminex HLA antibody testing. Serum samples were analyzed using the standard protocol for Luminex antibody detection according to the manufacturer's instructions. Data was acquired on the LABScan 200, and results were analyzed using HLA FUSION software (One Lambda). Data were then compared to previous data generated from ADS and SC treated sera.

2.3 | Statistics

Qualitative data were expressed as frequency and percentage. The strength of the association between HLA-specific antibody binding levels, mean fluorescent intensity (MFI) was examined using Spearman's rank correlation coefficient (rs) in LABScreen assays with SC, ADS, and SPT-EDTA combined serum pre-treatment.

3 | RESULTS

3.1 | Characteristic of a high NC bead, and effect of SC, ADS, and SPT-EDTA combined serum treatment on NC value

Characteristic of a high NC bead, and a comparison of effectiveness for SC, and ADS, and SPT-EDTA combined serum treatment on reducing the background (NC value) in HLA class I and II antibody SPI is presented in Table 1. SPT-EDTA combined treatment was considered effective over SC, and ADS serum treatment if similar or better performance is achieved in reducing the NC values as compared to untreated serum, and ability to reduce NC below 500 without adverse effects to the PC bead value or PC/NC ratio.

The effectiveness of SC, ADS, and SPT-EDTA combined serum treatment to reduce the NC values below 500 was then calculated by using the following formula: (Samples with reduced NC / Sample tested) x 100, resulting in 80% effectiveness rate for SPT-EDTA combined treatment as compared to 67% and 76% for SC and ADS treatment respectively (Table 2).

3.2 | Accuracy of SC, ADS, and SPT-EDTA combined serum treatment

Accuracy was established by comparing MFI values and specificities obtained using ADS with results achieved from SC, and SPT-EDTA combined treatment with obtained results using ADS treatment, and results are presented in Table 3. Accuracy for specificities was established by comparing the antibody specificity assignments and determining concordance for each specificity obtained using the following formula: (#of concordant specificities/#of specificities tested) * 100. The concordance was measured by the ability to obtain identical specificities from the previous testing; in some samples, additional specificities may have been included due to slight variances in MFI values after the effective removal of the background.

Accuracy based on specificity assignment was 98% and 77% for HLA class I and class II antibodies respectively on both Phenotype ID and single antigen bead (SAB) assays for ADS serum treatment as compared to Serum Cleaner (Table 3A). While, accuracy based on

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TABLE 1 Characteristic of sample with high NC bead value and a comparison of effectiveness for Serum Cleaner, AdsorbOut™, and SPHERO™/EDTA combined serum treatment.

Sample	Assay	No treatment	Serum Cleaner	Reduce NC (Y/N)	AdsorbOut™	Reduce NC (Y/N)	SPHERO™/EDTA	Reduce NC (Y/N)
1	ID	NC: 1724.27	NC: 1214.1	Y	NC: 956.72	Y	NC: 106	Y
		PC: 6770.17	PC: 12525.96		PC: 11839.81		PC: 10377	
		PC/NC: 3.93	PC/NC: 10.32		PC/NC: 12.38		PC/NC: 98.03	
2	ID	NC: 3404.34	NC: 29.78	Y	NC: 26.42	Y	NC: 44	Y
		PC: 7758.72	PC: 9768.83		PC: 11927.58		PC: 10087	
		PC/NC: 2.28	PC/NC: 328.03		PC/NC: 451.46		PC/NC: 228.93	
3	ID	NC: 889.11	NC: 1557.25	N	NC: 1549.7	N	NC: 157	Y
		PC: 7068.05	PC: 13478.82		PC: 13571.4		PC: 12695	
		PC/NC: 7.95	PC/NC: 8.66		PC/NC: 8.79		PC/NC: 80.89	
4	ID	NC: 966.44	NC: 18.24	Y	NC: 16.94	Y	NC: 44	Y
		PC: 7137.71	PC: 11499.56		PC: 11063.71		PC: 11113	
		PC/NC: 7.39	PC/NC: 630.46		PC/NC: 653.11		PC/NC: 251.661	
5	ID	NC: 2016.69	NC: 47.89	Y	NC: 29.55	Y	NC: 141	Y
		PC: 7779.52	PC: 14077.53		PC: 13784.56		PC: 15033	
		PC/NC: 3.86	PC/NC: 293.96		PC/NC: 466.48		PC/NC: 106.329	
6	ID	NC: 2840.57	NC: 22.11	Y	NC: 28.97	Y	NC: 49	Y
		PC: 8964.91	PC: 9378.17		PC: 12099.31		PC: 10532	
		PC/NC: 3.16	PC/NC: 424.16		PC/NC: 417.65		PC/NC: 215.2	
	SA1	NC: 507.98	NC: 10.8	Y	NC: 21.76	Y	NC: 31	Y
		PC: 9197.62	PC: 9260.97		PC: 11512.07		PC: 11943	
		PC/NC: 18.11	PC/NC: 857.5		PC/NC: 529.05		PC/NC: 390.03	
7	ID	NC: 946.33	NC: 49.04	Y	NC: 32.83	Y	NC: 40	Y
		PC: 6367.28	PC: 11845.5		PC: 11876.54		PC: 10005	
		PC/NC: 7	PC/NC: 241.55		PC/NC: 631.76		PC/NC: 251.13	
	SA1	NC: 522.4	NC: 13.11	Y	NC: 13.04	Y	NC: 64	Y
		PC: 6097.83	PC: 10991.84		PC: 11407.88		PC: 14027	
		PC/NC: 11.67	PC/NC: 838.43		PC/NC: 874.84		PC/NC: 217.71	
8	ID	NC: 1403.33	NC: 687.28	Y	NC: 839.7	Y	NC: 378	Y
		PC: 10003.24	PC: 16511.68		PC: 17584.02		PC: 19011	
		PC/NC: 7.13	PC/NC: 24.03		PC/NC: 20.94		PC/NC: 50.3552	
9	ID	NC: 755.9	NC: 1505.11	N	NC: 1123.94	N	NC: 290	Y
		PC: 12394.32	PC: 16778.58		PC: 16507.44		PC: 20479	
		PC/NC: 16.4	PC/NC: 11.15		PC/NC: 14.69		PC/NC: 70.5432	
10	ID	NC:1275.48	NC: 3596.87	N	NC: 3124.44	N	NC: 2113	N
		PC:6046.54	PC: 12727.23		PC: 13762.12		PC: 15726	
		PC/NC:4.74	PC/NC: 3.54		PC/NC: 4.41		PC/NC: 7.44	
11	ID	NC: 4214.82	NC: 206.04	Y	NC: 185.39	Y	NC: 63	Y
		PC: 11980.3	PC: 10143.9		PC: 10428.34		PC: 12569	
		PC/NC: 2.84	PC/NC: 49.23		PC/NC: 56.25		PC/NC: 197.96	
12	ID	NC: 3842.76	NC: 395.04	Y	NC: 161.74	Y	NC: 684	Y
		PC: 8013.92	PC: 12998.82		PC: 13135.74		PC: 15077	
		PC/NC: 2.09	PC/NC: 32.91		PC/NC: 81.22		PC/NC: 22.04	

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TABLE 1 (Continued)

Sample	Assay	No treatment	Serum Cleaner	Reduce NC (Y/N)	AdsorbOut™	Reduce NC (Y/N)	SPHERO™/EDTA	Reduce NC (Y/N)
13	ID	NC: 6921.12	NC: 68.86	Y	NC: 36.36	Y	NC: 154	Y
		PC: 14708.84	PC: 10245.82		PC: 8688.05		PC: 12740	
		PC/NC: 2.13	PC/NC: 148.79		PC/NC: 238.95		PC/NC: 82.9851	
14	ID	NC: 3060.91	NT	NT	NC: 1041.60	Y	NC: 645	Y
		PC: 10673.62			PC: 10167.89		PC: 11817	
		PC/NC: 3.49			PC/NC: 9.76		PC/NC: 18.3354	
	SA2	NC: 2226.93			NC: 430.59	Y	NC: 235	Y
		PC: 13448.85			PC: 11561.72		PC: 14631	
		PC/NC: 6.04			PC/NC: 26.85		PC/NC: 62.3305	
15	ID	NC: 36.83	NT	NT	NC: 109.19	N	NC: 65	N
		PC: 7399.45			PC: 8270.39		PC: 11249	
		PC/NC: 200.91			PC/NC: 75.74		PC/NC: 173.376	
16	ID	NC: 88.73	NT	NT	NC: 71.62	Y	NC: 99	N
		PC: 7344.26			PC: 10533.07		PC: 11663	
		PC/NC: 82.77			PC/NC: 147.27		PC/NC: 118.075	
17	ID	NC: 54.5	NT	NT	NC: 54.43	Y	NC: 79	N
		PC: 10143.91			PC: 9968.56		PC: 11054	
		PC/NC: 186.13			PC/NC: 176.65		PC/NC: 139.98	
	SA2	NC: 127.8	NT	NT	NC: 135.22	N	NC: 85	Y
		PC: 10143.94			PC: 9908.16		PC: 12414	
		PC/NC: 69.61			PC/NC: 217.45		PC/NC: 146.3	
18	SA1	NC: 123.31	NT	NT	NC: 135.22	N	NC: 279	N
		PC: 5094.49			PC: 9864.16		PC: 11013	
		PC/NC: 41.314			PC/NC: 72.949		PC/NC: 39.4542	
19	ID	NC: 1255.88	NT	NT	NC: 96.59	Y	NC: 71	Y
		PC: 4997.60			PC: 9151.79		PC: 10520	
		PC/NC: 3.98			PC/NC: 94.75		PC/NC: 147.647	
20	ID	NC: 965	NT	NT	NC: 810	Y	NC: 564	Y
		PC: 6281			PC: 9676		PC: 11113	
		PC/NC: 6.51208			PC/NC: 11.94		PC/NC: 19.7131	
21	ID	NC: 2841	NT	NT	NC: 279	Y	NC: 859	Y
		PC: 6022			PC: 8784		PC: 10693	
		PC/NC: 2.12			PC/NC: 31.45		PC/NC: 12.45	

Note: NT, not tested because Serum Cleaner method was retired from our lab in 2018. Dark orange color is No treatment or when treatment failed to reduce NC value below 500. Light blue, Light green and Light orange color indicates that SC, ADS, and SPT-EDTA combined serum treatment respectively worked to reduce NC below 500.

specificity assignment in SPT-EDTA combined serum treatment as compared to ADS revealed 96% for HLA class I phenotype ID and SA1, and 91% on class II phenotype ID and 89% on SA2 assays (Table 3B). Taken together SPT-EDTA combined treated sera suggested overall higher accuracy in detection of HLA class I and II antibodies across the Phenotype ID and SAB assays as compared to SC Versus ADS (Table 3).

To determine accuracy based on MFI, we compared MFI values for each bead in the LABScreen phenotype beads and SAB assays for SC, ADS and SPT-EDTA combined treated sera, and results are presented in Figures 1 and 2. An average MFI variance of ≤30% (and > 30% when there was a reasonable cause) was considered acceptable. Acceptable results, with expected variation, were obtained, and when average variance

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TABLE 2 Assessment of Serum Cleaner, AdsorbOut™, and SPHERO™/EDTA combined serum treatment on effectiveness to reduce NC values below 500.

	Serum Cleaner	AdsorbOut™	SPHERO™/EDTA
Samples with effective treatment to reduce NC below 500	10	18	20
Sample tested	15	25	25
% Effectiveness	67%	72%	80%

TABLE 3 LABScreen Accuracy for Serum Cleaner versus AdsorbOut™, and AdsorbOut™ versus SPHERO™/EDTA serum treatment.

	ID CI	ID CII	SA1	SA2
A. Serum Cleaner versus AdsorbOut™				
Number of concordant specificities	59	17	64	17
Number of specificities tested[a]	60	22	65	22
% Accuracy	98%	77%	98%	77%
B. AdsorbOut™ versus SPHERO™/EDTA				
Number of concordant specificities	79	64	76	70
Number of specificities tested[b]	82	70	79	79
% Accuracy	96%	91%	96%	89%

[a]Specificities assigned across both treatments that is, LIFECODES Serum Cleaner and One Lambda AdsorbOut™.
[b]Specificities assigned across both treatments that is, AdsorbOut™ and SPHERO™/EDTA.

exceed 30%, the data was manually inspected. Taken together upon the manual inspection of the data when average variance exceeds 30%, it was noted that the SPT-EDTA combined treatment has been able to reduce the background effectively by unmasking the MFI's, and hence, clustering the positive specificities on the top of the antigen panel (assay). Therefore, we have been able to detect additional specificities which, could not have been assigned on ADS treatment, and/or SC treatment.

We observed a strong correlation in antibody-binding levels between SC, and ADS-treated sera for Phenotype ID beads HLA class I ($R = 0.97$, $p < 2.2\text{e}{-16}$), and class II ($R = 0.98$, $p < 2.2\text{e}{-16}$), and SAB assays (SA1: $R = 0.94$, $p < 2.2\text{e}{-16}$; and SA2: $R = 0.96$, $p < 2.2\text{e}{-16}$). Similarly, a strong correlation in antibody-binding levels were also noted between ADS and SPT-EDTA combined sera for Phenotype ID beads HLA class I ($R = 0.84$, $p < 2.2\text{e}{-16}$), and class II ($R = 0.93$, $p < 2.2\text{e}{-16}$), and SAB assays (SA1: $R = 0.91$, $p < 2.2\text{e}{-16}$; and SA2: $R = 0.93$, $p < 2.2\text{e}{-16}$).

3.3 | Test for interference in SPI with SPT-EDTA serum treatment

We observed a higher effectiveness and accuracy in SPT-EDTA combined treated sera as compared to ADS, and SC, and therefore, the next step was to determine whether use of SPT-EDTA treated sera upfront had any inhibitory (adverse) effect as compared to serum treated with EDTA only. Inhibitory effects of using SPT-EDTA combined treatment as compared to EDTA alone were determined by re-testing a subset of 20 samples based on two criteria. First, based on the absence of elevation in NC bead values without any inhibitory (adverse) effects to the PC bead value or PC/NC ratio (Table S1), and second no reduction in MFI values of each positive beads with SPT-EDTA combined treatment upfront as compared to EDTA alone treatment.

Interestingly, we found no inhibitory effect on NC, PC bead values, PC/NC ratio in upfront use of SPT-EDTA combined treatment as compared to EDTA alone (Table S1). As a measurement of inhibitory effect on MFI values, the percent (%) reduction in MFI beads was calculated by using the formula:

$$\begin{aligned}\%\text{MFI reduced beads} &= (\text{total number of MFI reduced positive beads}/\\ &\quad \text{total number of tested beads on ID or SAB assays}) \times 100.\end{aligned}$$

MFI values for each positive bead in the LABScreen Phenotype ID and SAB assays were compared. A percentage reduction in MFI of less than or equal to 30% was considered acceptable for positive beads on tested ID and SAB panel. However, if the percentage of MFI reduction

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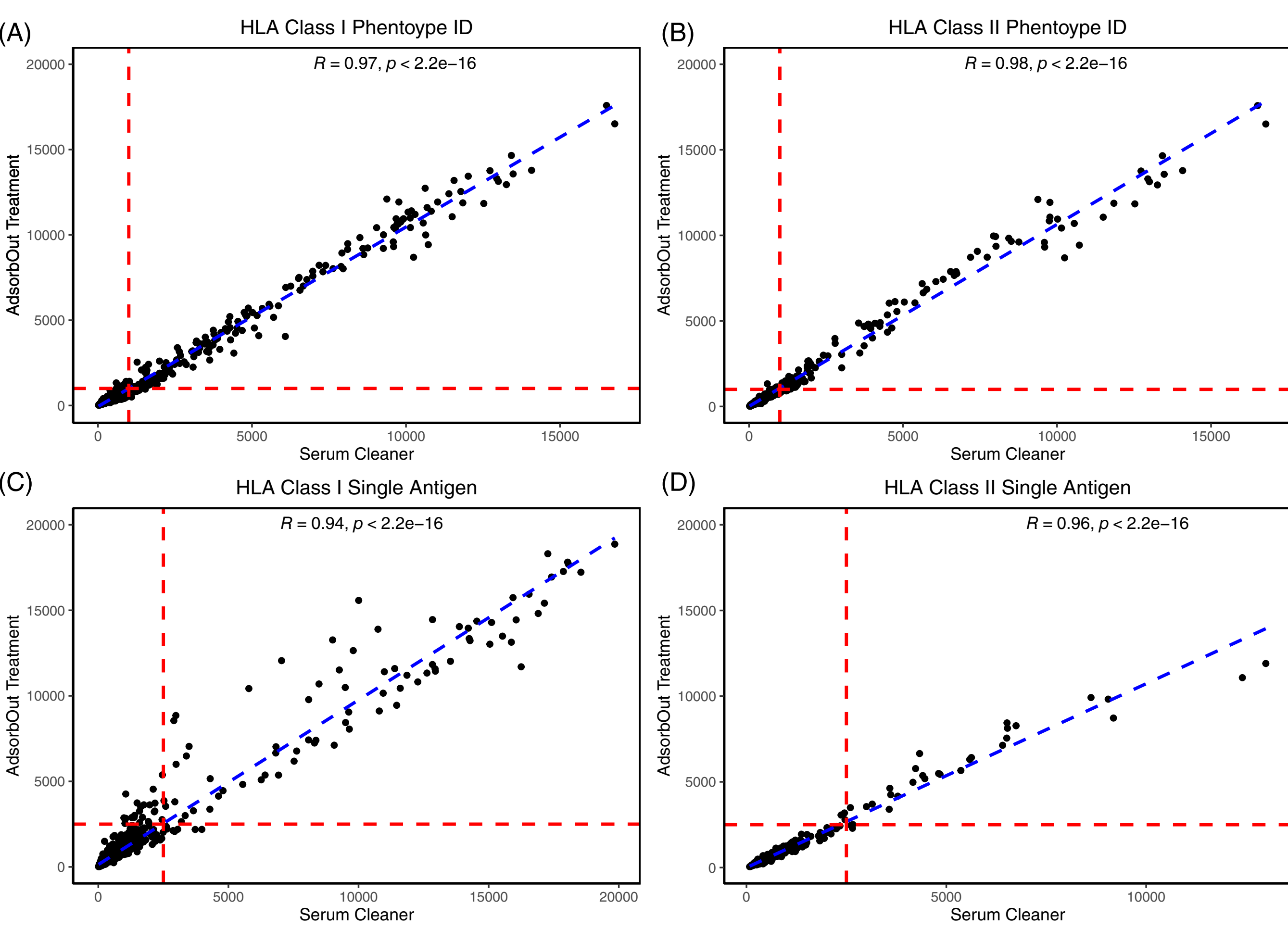

FIGURE 1 Mean fluorescent intensity (MFI) comparison of LABScreen phenotype and single antigen beads (SAB) assays between Adsorb Out™ (ADS) and Serum Cleaner (SC). (A) MFI of ADS treated sera for class I phenotype beads was plotted against SC. (B) MFI of ADS treated sera for class II phenotype was plotted against SC. (C) MFI of ADS treated sera for SA1 was plotted against SC. (D) MFI of ADS treated sera for SA2 was plotted against SC. The horizontal and vertical lines inside the graph indicate our MFI threshold of 1000 on phenotype, and 2500 on SAB assays to define a strong antibody, and to predict a positive flow cytometry crossmatch.

of positive beads exceeded 30%, the data was considered acceptable if there were no changes in specificity or strength, unless there was a possible explanation. We found no reduction in MFI values for positive beads on tested LABScreen Phenotype ID and SAB panel on the 20 tested sera in the LABScreen Phenotype ID and SAB assays. However, for one serum (sample ID AE11) the MFI reduction of 2% (two beads) for class I SAB assay exceeds the pre-set limits of 30% of variance, however, upon the further investigation, we noted no change in PRA and strength of assigned specificities for class I and II antibodies in this sample.

4 | DISCUSSION

To the best of our knowledge, this is the first study that has examined the efficacy of different serum pre-treatment methods prior to HLA antibody testing using Luminex based SPI to mitigate inhibitory effect (so called prozone effect) and non-specific reactivity. In this study, we observed 80% effectiveness in reducing the NC bead values below 500 with SPT-EDTA combined treatment compared to 67% and 76% for SC and ADS treated sera, respectively. Our results are consistent with an earlier report that shows the ADS treatment in reducing NC bead values of sera.[9]

Previous studies have shown that patient sera contain inhibitory substances, which may interfere with HLA IgG-specific antibody binding, and potentially mask the positive reaction and thus, cause hindrance in the accurate determination of clinically significant allo-antibodies.[8,13] An earlier report from our group has already demonstrated a superior effect of EDTA serum treatment in removing complement mediated inhibitory effect over both DTT treatment and serum dilutions.[14] Therefore,

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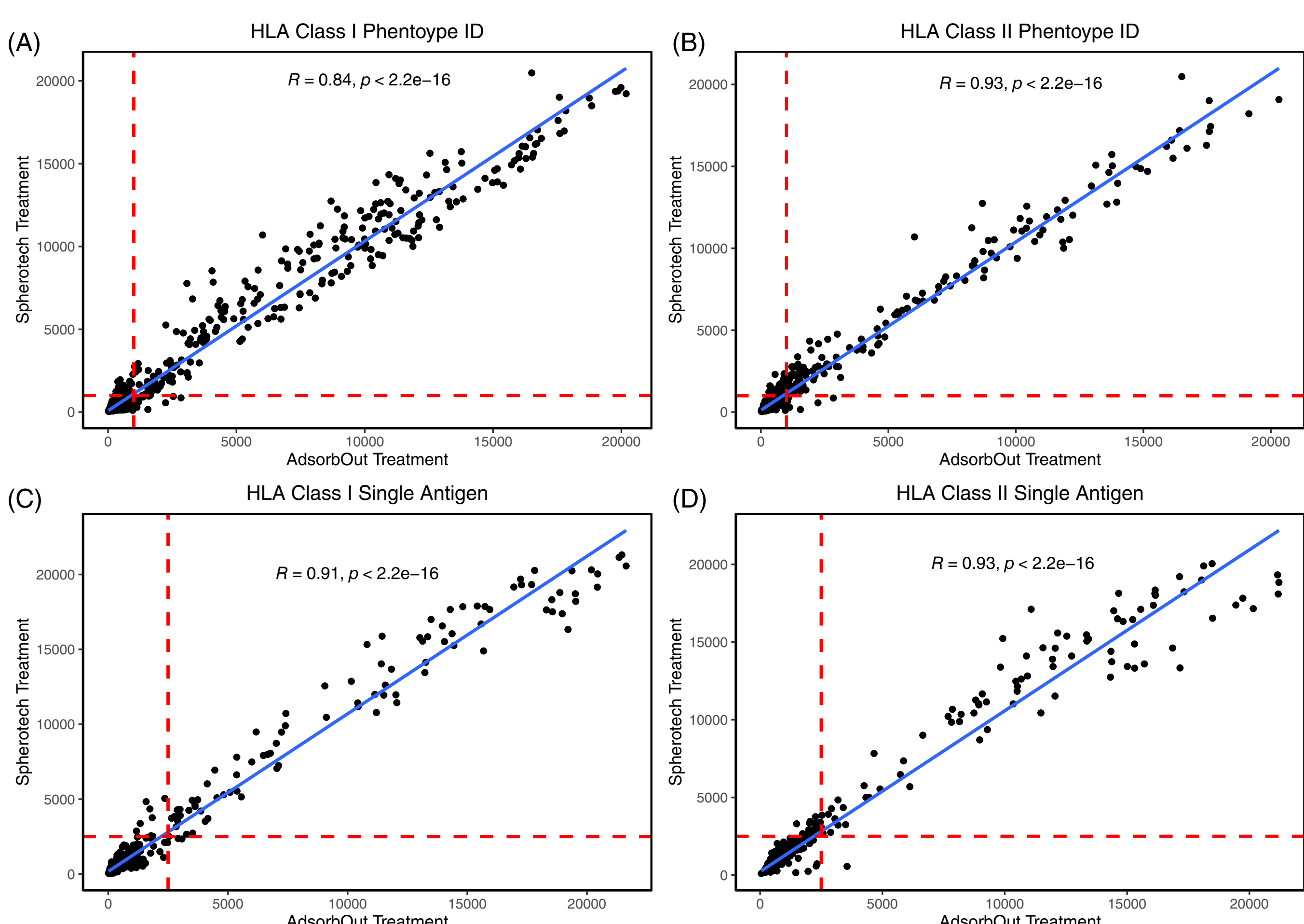

FIGURE 2 Mean fluorescent intensity (MFI) comparison of LABScreen phenotype beads and SAB assays between Spherotech-EDTA combined and Adsorb Out™ (ADS) treated sera. (A) MFI of SPT-EDTA combined treated sera for class I phenotype beads was plotted against ADS. (B) MFI of SPT-EDTA combined treated sera for class II phenotype was plotted against ADS. (C) MFI of SPT-EDTA combined treated sera for SA1 was plotted against ADS. (D) MFI of SPT-EDTA combined treated sera for SA2 was plotted against ADS. The horizontal and vertical lines inside the graph indicate our MFI threshold of 1000 on Phenotype ID, and 2500 on SAB assays to define a strong antibody, and to predict a positive flow cytometry crossmatch.

assessment of complement mediated inhibitory effect in the SPT-EDTA study was considered redundant, and out of scope of this study, since, EDTA is already present in SPT-EDTA suspension prepared to treat sera prior to Luminex IgG Ab testing. Interestingly, we found an overall higher accuracy in detecting HLA class I and II antibodies across the Phenotype ID and SAB assays compared to SC Versus ADS treated sera (Table 3). In addition, a strong correlation in antibody-binding levels was noted between ADS and SPT-EDTA combined sera and ADS and SC treated sera for both HLA phenotype beads and SAB assays (Figures 1 and 2).

Causes for high background levels might be related to antibodies reacting to polystyrene beads used in SPI Luminex IgG Ab tests. Multiple serum factors could be associated with increased background, including disease type such as autoimmune conditions, current drug/immunosuppressive therapy, and the presence of hyperlipidemia. Still, the majority of them are unknown,[17] and an interesting study indicates the role of autoantibodies in patient's sera responsible for elevated NC bead values.[18] These serum factors alone or in conjunction may produce the interference causing the high NC bead values. Reva et al. used the ADS to remove elevated NC bead values.[19] They found that the ADS decreased NC bead values to a normal range in 57% of sera,[19] and another interesting report found ADS serum treatment resolved to reduce NC bead values to a normal range in 62.2% of sera.[9] These results further confirm our findings that ADS serum treatment succeeds in decreasing the NC bead values to a normal range in 76% of tested sera.

Solid-phase immunoassays with the advent of Luminex technologies, marked a significant advancement in histocompatibility testing and allowed for the ability to provide

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increased sensitivity and specificity in the characterization of anti-HLA antibodies.[5,20,21] This advancement in SPI had a significant impact on improved and precise monitoring of DSA strength, even in broadly reactive sera, and of sera containing lymphocyte reactive, non-HLA specific antibodies, and the ability to predict the outcome of physical crossmatch (virtual crossmatch). The virtual crossmatch (VXM) has increased the accessibility of transplants to highly sensitized patients[21,22] and broadened the geographical area from where organs with permissible cold ischemia times can be recovered.[23] However, a key to achieving this requires accurate identification and characterization of anti-HLA antibodies in patient sera.[5,24] Naturally occurring serum factors may produce high background which can greatly affect the outcome solid phase test results, leading to inaccurate interpretation of the tests results.[8]

In the present study, we observed high background in 25% of our patient populations requiring repeat testing, SPT-EDTA combined serum treatment used before SPI in Luminex HLA Ab testing in our laboratory reduces the overall cost of Luminex HLA antibody testing; with the implementation of this serum pre-treatment process the number of repeat tests has decreased dramatically in our laboratory; we estimated that the reduction in repeat tests saves approximately 30% of the annual total cost for antibody testing, improves the TAT by 2 days, and enhances patient care. The improvement in TAT by two business days should be considered of high significance specifically for patient that requires STAT anti-HLA antibody results for the management of care in situations such as monitoring of post-transplant patient with suspected antibody mediated rejections, matched platelet work up cases, and solid organ transplant patients requiring urgent listing to UNOS waitlist.

The major advantages of SPT-EDTA serum pretreatment use prior to Luminex antibody testing is its impact on annual cost savings in the laboratory and its impact on patient care and TAT. We assessed our workflow without SPT-EDTA serum pre-treatment and found that approximately 25% of our sera tested annually displayed high background resulting in test repeats. SPT-EDTA combined serum treatment used before SPI in Luminex HLA Ab testing is cost-effective by reducing repeats; our laboratory saves nearly 30% of the annual total cost for antibody testing, improved the TAT by 2 days, and enhanced patient care.

No systematic studies have been designed and performed to assess the efficacy of different available serum pre-treatment methods to reduce the background. This may highlight the significance of our study in terms of systematic assessment of efficacy of different commercially available reagents used for serum treatment in SPI to reduce the background, since the outcome of this study may encourage the HLA laboratories to adopt this method that shows a clear benefit in terms of cost-saving, improved TAT, and better patient care.

In conclusion, our study shows that combined SPT-EDTA treated sera is more effective than other commercially available reagents namely, One Lambda ADS, and LIFECODE SC, in reducing the high background observed in SPI. Taken together, SPT-EDTA combined serum treatment prior to Luminex SPI in HLA antibody testing is a cost effective and most efficient approach to reduce the background, and remove the prozone effect in a single step process.

AUTHOR CONTRIBUTIONS

This study was designed by Maneesh K. Misra and Susana G. Marino. The data were collected by Maneesh K. Misra, Jerome G. Weidner, Rebecca L. Upchurch, and Arianne M. Mankey. Arianne M. Mankey, and Marcelo A. Fernandez-Viña provided the initial study protocol. Maneesh K. Misra analyzed the data and wrote the manuscript. All coauthors read and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Maneesh Kumar Misra https://orcid.org/0000-0002-3757-3326
Susana G. Marino https://orcid.org/0000-0003-4369-018X

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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