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Comparison of different luminex single antigen bead kits for memory B cell-derived HLA antibody detection

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Detection of HLA-specific memory B cells can provide additional information on sensitization of alloantigen-exposed individuals and refine immunological risk assessment. We have recently developed an assay enabling profiling of memory B cell-derived HLA antibodies using luminex single antigen bead (SAB) assay. Here, we compared the performance of the SAB kits from two vendors for memory B cell-derived HLA antibody detection. IgG was isolated from culture supernatants of polyclonally activated B cells from alloantigen-exposed ($n = 7$) or nonexposed ($n = 5$) individuals, using our previously established method. Eluates containing isolated IgG from culture supernatants were tested for the presence of HLA antibodies using luminex SAB analysis from both One Lambda and Lifecodes (Immucor). In contrast to Lifecodes, high mean fluorescence intensity (MFI) signals were found for negative control beads in One Lambda (median MFI for class I:1730 and for class II:728), accompanied by high MFI values for self HLA-coated beads, especially for HLA-C. Despite high background in the One Lambda assays, 91% concordance for HLA class I and 85% concordance for HLA class II were found between the specificities detected using SAB kits from the two vendors. Our results show that HLA-specific memory B cells can be profiled using kits from both vendors. However, when analyzing One Lambda results one should be aware of the restrictions related to nonspecific binding particularly in HLA-C-coated beads, and pay attention to self HLA-coated beads in order to accurately identify the reactivities leading to the definition of the actual HLA antibody specificities.

KEYWORDS

bead-based assays, donor-specific antibody, humoral alloimmune response, memory B cells

1 | INTRODUCTION

Sensitization to HLA occurs as a result of exposure to allogeneic HLA through blood transfusions, pregnancies

or previous transplantations and can manifest itself as circulating serum antibodies and/or HLA-specific memory B cells. Current immunological risk assessment based on serum donor-specific HLA antibody (DSA) analysis

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does not reflect the possible presence of donor-reactive memory B cells.¹

Methods to detect HLA-specific memory B cells refine the immunological risk assessment in patients with a history of alloantigen exposure.² There are a number of methods for HLA-specific memory B cell detection.³ Among these, HLA antibody analysis in B cell culture supernatants is the only method allowing serum HLA antibody profiles to be directly compared with those of memory B cell-derived HLA antibodies.²

We have previously developed an HLA-specific memory B cell assay in which IgG isolated from culture supernatants (eluates) is utilized to define the presence and specificity of memory B cell-derived HLA antibodies using luminex single antigen bead (SAB) assay kits from Lifecodes.^{4,5} Using this method, we were able to increase IgG concentrations up to 100-fold in supernatants of alloantigen-exposed individuals, resulting in an 18% increase in detectability of HLA-specific B cell memory.⁴

Currently, two vendors provide kits for luminex SAB testing. In our previous research, we optimized the HLA-specific memory B cell assay using SAB kits from Lifecodes. Since both kits are widely used in clinical practice, we aimed to compare the performance of Lifecodes and One Lambda SAB kits for memory B cell-derived HLA antibody profiling.

2 | MATERIALS AND METHODS

2.1 | Study cohort

Peripheral blood samples from healthy individuals who had never been exposed to alloantigens and were proven to be serum HLA antibody negative ($n = 5$) and multiparous women ($n = 7$) were obtained with informed consent under guidelines issued by the medical ethics committee of Leiden University Medical Center (Leiden, the Netherlands). Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque density gradient centrifugation and kept frozen in liquid nitrogen until further use. All individuals in the study cohort were HLA typed by next-generation sequencing for HLA-A, B, C, DRB1, DRB3/4/5, DQB1, DQA1, DPB1, and DPA1 loci on the Illumina platform (Illumina, San Diego, CA), as previously described.⁴

2.2 | Culture supernatant preparation and HLA antibody testing

PBMC were polyclonally activated using 2.5 $\mu\text{g}/\text{ml}$ Toll-like receptor 7/8 agonist (resiquimod [R848]; Sigma-

Aldrich, St. Louis, MO) and 1000 IU/ml Interleukin-2 (IL-2) (Proleukin, Novartis, the Netherlands). Supernatants were collected at day 10 and processed using Protein G affinity purification followed by concentration using ultra-centrifugal filters (Amicon ProAffinity Concentration Kit; Millipore, Ireland), as previously described.⁴ All eluates were tested for HLA class I and II antibodies using luminex SAB kits from Lifecodes (LSA, Immucor Transplant Diagnostics, Stamford, CT) and One Lambda (LabScreen, One Lambda Inc., Canoga Park, CA). The LABScan 100 flow analyzer (One Lambda, Canoga Park, CA) was used for all data acquisition.

For Lifecodes SAB test, 7.5 μl eluate was mixed with 30 μl class I or II beads and tested by a previously described protocol using 75% of reagents.⁶ Data were analyzed using MATCHIT! antibody software version 1.3.1 (Immucor). Results were expressed as raw or background-corrected mean fluorescence intensity (MFI). Bead-specific cut-off based on raw MFI/lowest ranked antigen (LRA) (MFI/LRA) in combination with raw MFI >750 was utilized to assign positive beads.

For the One Lambda SAB test, 20 μl eluate was mixed with 4 μl class I or II beads and further tested as recommended by the manufacturer. For selected cases, SAB assays were performed after treatment of eluates with 1% and 5% bovine serum albumin (BSA) or Adsorbout reagent (One Lambda) or serum cleaner (Lifecodes). Data were analyzed using HLA FUSION antibody software version 3.4.18 (One Lambda). Results were expressed as raw or baseline (normalized) MFI or normalized background (NBG) ratios. Score 8 beads according to baseline MFI values were considered as positive.

3 | RESULTS

3.1 | HLA-C-coated beads in One Lambda are the highest ranked beads in alloantigen nonexposed individuals

Upon testing the eluates using luminex SAB kits from Lifecodes, median MFI values for class I and class II negative control beads in all samples were found to be 138 (range: 57–247) and 158 (range: 73–254), respectively (Figure 1(A)). Self HLA-coated beads had a median MFI of 0 (range: 0–623) for class I and a median of 38 (0–713) for class II (Figure 1(B,C)). Using One Lambda SAB kits on the same samples, we found a median MFI of 1730 (range: 101–4926) for class I and 728 (range: 94–2596) for class II negative control beads (Figure 1(D)). In addition, baseline MFI values for self HLA-coated class I and class II beads were found to reach 5289 and 1668, respectively. Among HLA class I beads, those with highest MFI were

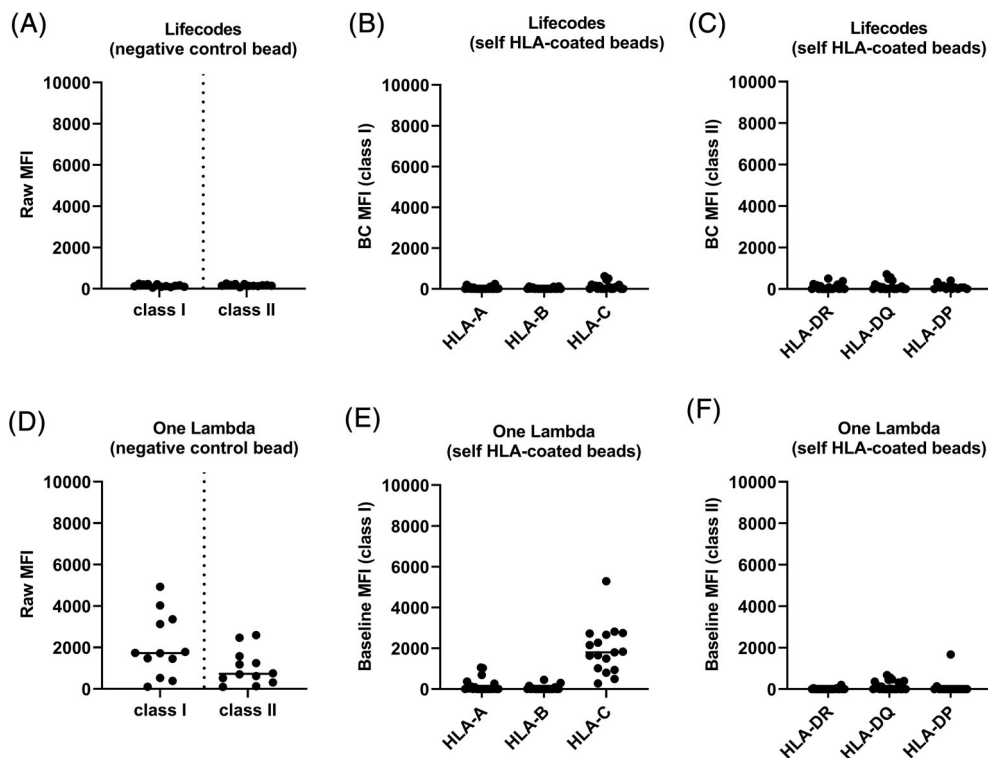


FIGURE 1 MFI values for negative control beads and self HLA-coated beads of the study cohort (n = 12) eluates tested using Lifecodes and One Lambda single antigen bead kits. BC, background corrected; MFI, mean fluorescence intensity

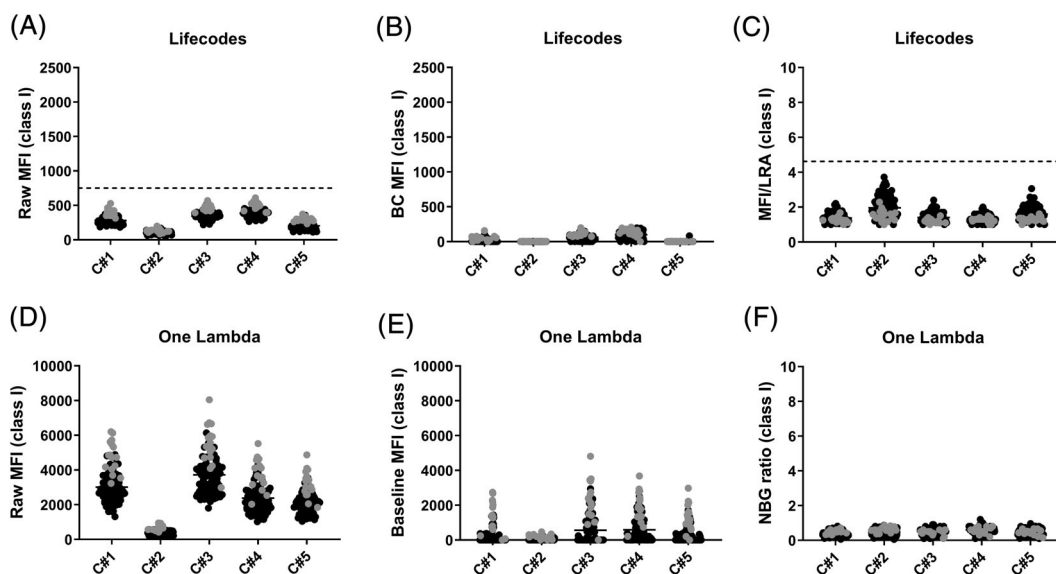


FIGURE 2 Bead reactivity pattern of all Lifecodes and One Lambda class I beads in eluates from alloantigen nonexposed individuals (n = 5). Dashed lines are set at MFI of 750 and MFI/LRA ratio of 4.62 (highest bead-specific cut-off). Gray dots indicate HLA-C-coated beads. NBG, normalized background

particularly self HLA-C-coated beads (median: 1801) (Figure 1(E,F)).

Next, the overall reactivity patterns of HLA class I beads in eluates from nonimmunized individuals were analyzed. Raw and background corrected MFI values for all beads of Lifecodes were below 570 and 207, respectively (Figure 2(A,B)), and no positive bead was assigned

by the MATCHIT! software based on MFI/LRA ratio and raw MFI >750 cut-off (Figure 2(C)).

On the contrary, when these eluates were tested with One Lambda, raw and baseline MFI values reached up to 8045 and 4815, respectively (Figure 2(D,E)). Beads in all eluates from alloantigen nonexposed individuals showed a similar reactivity pattern with C*04:01, C*07:02,

TABLE 1 Memory B cell-derived HLA antibody profiles as detected by SAB kits from two different vendors in samples from alloantigen exposed individuals

Case	HLA class	Mismatched HLA (partner and/or child)	HLA antibody profiles by Lifecodes*	HLA antibody profiles by One Lambda**
C#6	I	B*40:01, C*03:04	<u>B*07:03</u> , B*13:02, B*14:01, B*15 (:01;:02;:03;:12;:13;:18), B*18, B*35 (:01;:08), B*37, B*38, B*39, B*40 , B*41, B*44, B*45, B*47, B*48, B*49, B*50, B*51, B*52, B*53, B*59, B*78	B*13 (:01;:02), B*14:01, B*15 (:01;:02;:03;:10;:11;:12;:13), B*18, B*35, B*37, B*38, B*39, B*40 (:01;:02;:06), B*41, B*44, B*45, B*47, B*48, B*49, B*50, B*51 (:01;:02), B*52, B*53, B*59, B*78
	II	n.a	Negative	Negative
C#7	I	A*01:01, A*68:01, B*51:01, C*15:02	B*51:01 , B*78:01	B*51:01
	II	DRB1*04:04, DQB1*03:02	Negative	Negative
C#8	I	B*53:01, C*04:01	Negative	C*07:02 , C*04:01 , C*06:02 , C*02:02
	II	DRB1*13:01, DQB1*06:04	DRB1*03, DRB1*08:01 , DRB1*08:02, 11, 13 , 14	DRB1*03, 11, 13 , 14
C#9	I	A*32, B*40:01, C*03:04	A*25, A*26 , A*29, A*30, A*31, A*32 , A*33, A*34, A*36, A*43, A*66:01 , A*66:02, A*74, B*07 (:02;:03), B*13, B*27, B*40 , B*41, B*45, B*47, B*48, B*49 , B*50 , B*81	A*25, A*29, A*30, A*31, A*32 , A*33, A*34 (:01;:02), A*36, A*43, A*66:02, A*74, B*07, B*13 (:01;:02), B*27, B*40 (:01;:02;:06), B*41, B*45, B*47, B*48, B*81, C*04:01 , C*07:02
	II	DRB1*04:04:01, DQB1*03:02	DRB1*04 , DRB1*07:01 , DRB1*09:01 , 11, DRB4, DQB1*03 (:01;:02;:03), DPB1*02, 03, 04:02, 06, 09, 14, 17, 18, 28	DRB1*04 , 11, DRB4, DQB1*03 (:01;:02;:03), DPB1*02, 03, 04:02, 06, 09, <u>10</u> , 14, 17, 18, <u>20</u> , 28
C#10	I	A*02:01, B*15:01, C*03:04	A*02 , A*23, A*24, A*25, A*26, A*66:01, A*68, A*69, B*15 , B*18, B*35, B*37, B*38, B*39, B*41, B*44, B*45, B*46, B*49, B*50, B*51, B*52, B*53, B*55, B*56, B*57, B*58, B*59 , B*78, C*03	A*02 , A*23, A*24, A*25, A*66:01, A*68, A*69, B*15 , B*18, B*35, B*37, B*38, B*39, B*41, B*44, B*45, B*46, B*48 , B*49, B*50, B*51, B*52, B*53, B*55, B*56, B*57, B*58, B*78, C*03
	II	n.a	DRB1*01, 04 (:01;:03;:04;:05), 10, 11, DQB1*02:01-DQA1*02:01 , DQB1*02:01-DQA1*05:01, DQB1*02:02-DQA1*02:01 , DQB1*02:02-DQA1*03:02 , DQB1*02:02-DQA1*05:01 , DQB1*05 DPB1*02, 03, 04:02, 06, 09, 14, 17, 18, 28	DRB1*01, 04 (:01;:03;:04;:05), 10, 11 DQB1*02:01-DQA1*03:01 , DQB1*02:01-DQA1*04:01 , DQB1*02:01-DQA1*05:01 , DQB1*05 DPB1*02, 03, 04:02, 06, 09, <u>10</u> , 14, 17, 18, <u>20</u> , 28
C#11	I	B*07:02, C*07:02	A*66:02 , B*07 (:02;:03), B*14, B*27, B*39, B*40, B42, B*47 , B*48, B*54, B*55, B*56, B*67, B*73, B*81, B*82:02	B*07 , B*14, B*27, B*39, B*40, B*42, B*48, B*54, B*55, B*56, B*67, B*73, B*81, B*82:01, C*14:02 , C*01:02
	II	n.a	DRB1*15, 16	DRB1*15, 16
C#12	I	A1, A3, B7, B27, Cw2	A*01 , A*03 , A*11, A*23, A*24, A*25, A*26, A*32, A*33, A*34, A*36, A*43, A*66, A*68, A*69, A*80, B*07 (:02;:03), B*08, B*13:02, B*14, B*15 (:03;:12;:13;:16;:18), B*18, B*27 , B*37, B*38, B*39, B*40, B*41, B42, B*44, B*45, B*46, B*47, B*48, B*49, B*51, B*52, B*53, B*54, B*57, B*58, B*59, B*67 , B*73, B*81, B*82:02	A*01 , A*03 , A*11, A*23, A*24, A*25, A*26, A*32, A*33, A*34 (:01;:02), A*36, A*43, A*66, A*68, A*69, A*80 B*07 , B*08, B*13 (:01;:02), B*14, B*15 (:03;:12;:13;:16), B*18, B*27 , B*37, B*38, B*39, B*40 (:01;:02;:06), B*41, B*42, B*44, B*45, B*46, B*47, B*48,

(Continues)

TABLE 1 (Continued)

Case	HLA class	Mismatched HLA (partner and/or child)	HLA antibody profiles by Lifecodes*	HLA antibody profiles by One Lambda**
II	n.a		DRB1*01:01, DRB1*01:02, DRB1*01:03 , DRB1*04, DRB1*07, 10, 15, 16, DRB4 , DRB5 DPB1*04:01-DPA1*02:02, <u>DPB1*28:01-DPA1*02:02</u>	B*49, B*51, B*52, B*53, B*54, B*57 (:01,:03), B*58, B*59, B*81, B*82:01 DRB1*01:01, DRB1*01:02, DRB1*04, 14:02, 15, 16

Note: Antibodies directed to a mismatched HLA of an immunizing event are written in bold and italic. Specificities (within the common beads) that were found positive by only one vendor's kits are marked with red. Beads exclusive for one vendor are underlined.

Abbreviation: n.a, not available.

*Bead positivity based on raw MFI >750 and bead-specific MFI/LRA cut-off by Lifecodes MATCHIT! antibody analysis software.

**Bead positivity based on score 8 according to baseline (normalized) MFI values by One Lambda HLA FUSION antibody analysis software.

C*14:02, C*06:02 being the highest scored (score 8) specificities (Figure S1). Furthermore, all individuals in this group had HLA-C*07:01 and/or HLA-C*07:02 as the self HLA-C, suggesting a false positivity for these HLA-C-coated beads. Therefore, antibody analysis based solely on normalized MFI values did not aid in an accurate assessment of the results whereas utilization of NBG ratios for antibody assignment correlated well with the (expected) absence of memory B cell-derived HLA antibodies in these eluates (Figure 2(F)).

In an attempt to solve the issue of possible non-specific binding when using One Lambda SAB kits, eluates were treated with 1% or 5% BSA, Adsorb Out (One Lambda) or serum cleaner (Lifecodes), however this did not lead to a significant decrease in the reactivity of negative control beads in all samples tested (data not shown). With the aim to determine whether nonspecific binding in One Lambda SAB assays was a consequence of culture supernatant processing, we generated an eluate from a “blank” sample containing no PBMC but culture medium with polyclonal activation cocktail. As shown in Figure S2, no reactivity was detected in either negative control bead values or HLA-coated beads, indicating that supernatant processing steps were not causing non-specific reactions in One Lambda SAB assays.

3.2 | Positive HLA antibody assignments in eluates of alloantigen-exposed individuals

Having found unexpectedly high MFI values for negative control and some HLA-coated beads in eluates of nonimmunized individuals when using One Lambda SAB kits, we wondered whether this would complicate the analysis of samples with positive reactions. To test

this, we analyzed memory B cell-derived HLA antibody profiles of seven alloantigen-exposed individuals using SAB kits from both vendors. Table 1 shows the immunization history and the HLA antibody profiles detected using both Lifecodes and One Lambda SAB kits. Samples were analyzed using our positive assignment criteria for both vendors, disregarding the high negative control bead values in One Lambda SAB assay. Using this approach, Lifecodes classified six samples positive for HLA class I antibodies whereas all seven samples were positive according to One Lambda. For class II, both vendors classified the same five samples as positive.

There was a complete overlap for the positive class I and class II antibody specificities directed at the mismatched HLAs of the sensitizing events in all samples, with the exception of HLA-C*04:01 false positivity in sample C#8 (Table 1). Overall, among common beads, 179 HLA class I beads were defined as positive of which 162 (91%) overlapped between the two vendors. Of the 17 discrepant results, nine beads were positive by only Lifecodes and eight beads were positive by only One Lambda (Table S1).

Virtually all of the HLA class I beads positive by Lifecodes only (8/9) were score 6 in One Lambda which in this analysis was considered as a negative reaction. These beads covered a variety of HLA-A and -B specificities. On the contrary, seven out of eight beads “positive by One Lambda only” were HLA-C-coated beads corresponding to a median raw MFI of 194 in Lifecodes. Interestingly, these HLA-C-coated beads not only had low NBG ratios (median: 0.89; range: 0.46–1.27) but also were directed against self HLA-C*07:02 in sample C#8 and self HLA-C*04:01 in sample C#9, raising questions about the accuracy of these positive assignments. Given that the overall bead reactivity pattern of this sample (C#8) resembled those of nonimmunized samples

(as exemplified in Figure S3) and that highest ranked bead was a self-HLA bead, we considered these HLA-C reactivities as false positive.

For class II, a total of 74 beads were positive and 63 (85%) of these overlapped between the two vendors. Among the 11 discrepant specificities which were detected positive only by Lifecodes, ten were the highest ranked beads following “score 8” in One Lambda, suggesting a possible positive assignment, if a less stringent cut-off was used for One Lambda. Interestingly, the majority of these lower score specificities accumulated in sample C#12 which had the highest negative control bead MFI value of 2474.

4 | DISCUSSION

An easily applicable and sensitive HLA-specific memory B cell assay has a great potential in aiding clinical decision-making since serum antibody analysis does not provide information on the presence of HLA-specific memory B cells. We have recently developed a method enabling detection of memory B cell-derived HLA antibodies in culture supernatants at a comparable sensitivity to serum HLA antibody testing using Lifecodes SAB kits.⁴ Since there are two vendors of luminex SAB kits we assessed and compared the performance of SAB kits from both vendors for memory B cell-derived HLA antibody detection in eluates.

Overall, testing eluates with One Lambda SAB kits resulted in high MFI values in the negative control beads in the majority of samples and this was more pronounced for class I. When ignoring the negative control bead values, an almost complete overlap for the positive class I and class II antibody specificities directed at the mismatched HLAs of the sensitizing events in all samples was found.

In alloantigen nonexposed individuals, HLA-C-coated beads in One Lambda SAB assay were the most prominent beads with raw MFI values reaching up to 8045. High reactivity in self HLA-C-coated beads, low NBG ratios in combination with the fact that there is no alloimmunization history in these individuals led to the conclusion of false positivity in these beads. The presence of high reactivity in HLA-C-coated beads can be attributed to denatured HLA molecules displayed on these beads.^{7,8} Treatment of One Lambda class I beads with BSA, serum cleaner or adsorb out changed neither the negative control bead MFI values completely nor the nonspecific reactivity of HLA-coated beads.

Interestingly, in samples from HLA-immunized individuals, HLA-C antibodies were not the leading specificities and did not seem to be interfering with the analysis.

In one sample that was negative by Lifecodes (C#8), HLA-C-coated beads were again the highest ranked beads by One Lambda assay. Presence of HLA-C*04:01 mismatch in the immunization history of C#8 in addition to HLA-C*04:01 bead being the second leading bead in One Lambda assay with a baseline MFI of 1506 complicated the analysis of this sample. However, since the highest ranked bead in this sample was a self HLA-C*07:02 with an MFI 1652, we concluded all these HLA-C reactivities with lower MFI values in this sample as false positive. When false positive HLA-C beads were excluded, the overlap between two vendors' positive assignments for class I increased from 91% to 94%.

For HLA class II, despite less pronounced background MFI values for One Lambda, the consensus rate for positive assignments was only 85%. This relatively lower consensus rate was because of the discrepancies accumulated in a single sample (C#12).

If this sample was removed from the analysis, the consensus rate for HLA class II would be 91%.

Noteworthy, when we analyzed the luminex SAB data of paired serum samples from study individuals, we did not observe high negative control bead MFI values, nor dominant false positive binding to HLA-C coated beads when One Lambda assay was used (data not shown).

Our results indicate a vendor-associated nonspecific reactivity when eluates are tested using One Lambda SAB kits for HLA antibody detection. Nevertheless, using the same straightforward assignment criteria for every sample while ignoring the high negative control beads, an overlap of 91% and 85% could be achieved for class I and class II positive beads, respectively. This consensus rate may be improved by further modification of the analysis strategy on an individual sample basis, as is performed for serum analysis in daily practice. Nonetheless, one should be cautious about the high negative control bead values and nonspecific reactivity in particularly HLA-class I-coated beads when using One Lambda SAB assays in eluates from polyclonally activated B cells. Regardless, when analyzed with caution, SAB kits from both vendors can be used to profile memory B cell-derived HLA antibodies in eluates generated using our established protocol.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

GEK designed the study, analyzed the data, wrote the manuscript, YdV and KB performed the experiments, DR and FHJC interpreted the results, SH interpreted the results and revised the manuscript.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

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

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