



## Review Article

# Enhancing Precision of the Single-antigen Bead (SAB) Assay: Considerations and Challenges

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### Abstract

Our capacity to recognize and manage allosensitized transplant recipients and optimize organ allocation has been greatly improved by the development of single-antigen bead tests for detecting human leukocyte antigen antibodies. The main drawbacks of this technology have been thoroughly discussed in the literature, covering problems like artifacts that result in nonspecific background, variability, lack of uniformity, and difficulties in data interpretation. Consequently, it is not always easy to understand single-antigen bead data. This review will discuss the interpretation of donor-specific antibody data while considering the associated technical limitations. To ensure the correct clinical application of this test and to enhance the quality of antibody data used to support published clinical research in the era of epitope-based computational matching algorithms, a detailed understanding of the single-antigen bead assay is necessary.

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### Introduction

In recent years, significant advancements in virtual cross-match technology have been achieved due to the utilization of single-antigen bead (SAB) assay for the detection of anti-human leukocyte antigen (HLA) antibodies.<sup>1,2</sup> Further, the SAB assay offers a sensitive and semi-quantitative approach for tracking donor-specific antibodies (DSAs) after transplant, which significantly enhances the understanding of how DSA development unfolds during humoral rejection and its associated significance.<sup>2,3</sup> At present, in most of the major transplant centers, the SAB assay plays a crucial role in the management of both pre- and post-transplant tests in identi-

fying DSAs. However, correct interpretation of its data poses a major challenge for testing laboratories due to its dependency on several factors that need to be thoroughly understood.<sup>3,4</sup>

Generally, the presence of anti-HLA antibodies is determined by assessing the mean fluorescence intensity (MFI) exhibited by beads coated with the particular HLA antigen.<sup>1,5</sup> The clinical significance of MFI in the SAB assay remains a subject of ongoing debate. While there is no consensus on a clinically significant MFI, most laboratories consider an MFI  $\leq$  1,000 acceptable for pre-transplantation assessment.<sup>6</sup>

Subsequent determination of the presence or absence of a DSA involves correlating the results of the SAB assay with the donor's high-resolution HLA typing. Furthermore, the recipient's HLA typing aids in excluding any autoantibodies, if present.<sup>7</sup> The successful interpretation of SAB testing is dependent upon various factors, including but not restricted to the patient's clinical background, cross-reactive groups, MFI, and comprehensive HLA coverage. While there have been notable advancements in this technique, it is important to acknowledge certain limitations, which include issues related to non-specific background signals, the absence of standardized procedures, and the complexities involved in data interpretation. These factors collectively contribute to inter-laboratory variations in the interpretation of SAB results.<sup>2-5,8</sup> In view of all these limitations, achieving precise interpretation of SAB data demands a substantial level of expertise. This review explores different approaches to enhance the precision of identifying DSAs in SAB results, taking into account the technical limitations associated with the process and the availability of epitope-based computational matching algorithms.

### Identification of related studies

The present review is a result of a comprehensive systematic web search across various databases, including PubMed, Google Scholar, Europe PMC, and Science Direct. We aimed to compile a comprehensive assessment of pertinent literature using search terms such as "single antigen bead assay," "SAB," "MFI in SAB analysis," and "interpretation of SAB" in various combinations. It is important to note that the literature cited in this review encompasses a global perspective and is not confined to any specific geographic region. We were primarily interested in various methods that can enhance the precision of detecting DSAs in SAB test results in clinical settings. [Table 1](#) represents some key milestones and developments in the history of the SAB assay. In this review, we have meticulously examined and consolidated essential

**Keywords:** Single-antigen bead assay; Donor-specific HLA antibodies; Mean fluorescence intensity; Virtual crossmatch; HLA; Organ transplant.

**Abbreviations:** DSA, donor-specific antibodies; HLA, human leukocyte antigen; MFI, mean fluorescence intensity; PIRCHE-II, Predicted Indirectly Recognizable HLA Epitopes; SAB, single-antigen bead.

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**Table 1. Representation of key milestones and developments in the history of the single-antigen bead assay**

Year	Milestone
1992	Introduction of Luminex technology for bead-based immunoassays, a key component of SAB
1994	Initial use of Luminex technology for detecting anti-HLA antibodies in transplant recipients
2003	Commercialization of the Luminex-based SAB assay by One Lambda, Inc
2004	Commercial availability of Luminex-based assays
2005	Development of SAB assay for other non-HLA antigens, such as MICA
2006	Widely adopted for monitoring transplant rejection
2008	Expansion of the use of SAB assay to monitor antibodies in solid organ and stem cell transplantation
2015	Advancements in software and data analysis for SAB assay, allowing for improved data interpretation and reporting
2020	Ongoing development and refinement of SAB assay to improve sensitivity and specificity for a wide range of antigens and applications

HLA, human leukocyte antigen; MICA, major-histocompatibility complex class I-related chain A; SAB, single-antigen bead.

information crucial for comprehending SAB assays, their limitations, and the intricacies of data interpretation.

### SAB assay

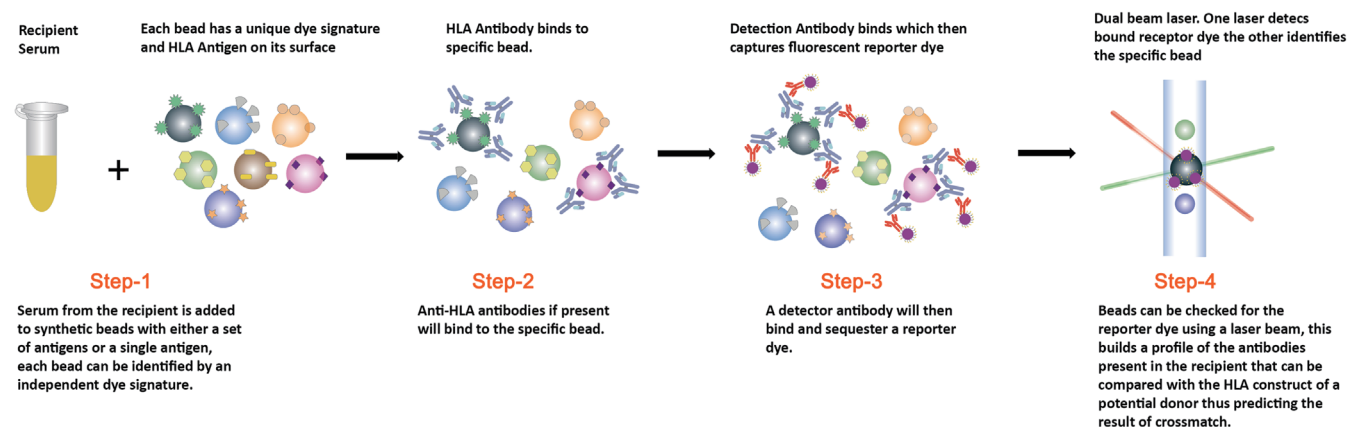
The existing SAB assay can simultaneously distinguish among as many as 100 different microparticles, commonly referred to as beads. These class I and II beads are designed to detect IgG antibodies to HLA class I and II glycoproteins. These are composed of different Luminex beads to which purified recombinant class I and II HLA glycoproteins are conjugated.<sup>2,3</sup> A portion of these beads is mixed with a small volume of a test serum sample for incubation. Following incubation, the sensitized beads undergo a washing process to eliminate any unbound antibodies. Subsequently, an anti-human IgG antibody, conjugated to phycoerythrin, is introduced. After another round of incubation, the test sample is diluted and then assessed using the Luminex instrument, as shown in Figure 1. The signal intensity emitted by each bead is compared to that of negative control sera and beads included in the bead preparation to determine whether each bead is positive or negative for the bound alloantibody.<sup>2,7</sup>

### Major limitations in SAB assay performance and interpretation

The SAB assay, a crucial laboratory test, has played a revolutionary role in HLA antibody testing. However, there are some

limitations associated with this assay. One such limitation is the “prozone effect,” also known as the “hook effect” or “inhibition,” primarily caused by complement interference. The prozone effect can be alleviated by diluting the serum, which eliminates inhibitory factors and allows the reporter antibody to regain its ability to bind to the antigen-antibody complex attached to the beads.<sup>9,10</sup> Various interventions can be employed to minimize this form of inhibition. Ethylenediaminetetraacetic acid, dithiothreitol, and heat treatment are commonly utilized techniques in HLA laboratories to effectively mitigate this inhibition.<sup>11,12</sup> Moreover, when dealing with the potential prozone effect, which can lead to erroneous results in antibody testing, the initial strategy often revolves around diluting the serum, not only to mitigate the prozone effect but also to ensure a more accurate evaluation of the patient’s immunological compatibility for transplantation, ultimately enhancing the safety and success of the procedure.<sup>9</sup>

“Bead saturation” is another challenge observed in SAB assays when the quantity of antibodies surpasses the available binding sites (antigens) presented by the microbeads. This results in reaching the maximum possible MFI. Serum samples containing higher antibody levels than the antigen level on the bead will consistently display elevated MFI values across various dilutions until the serum is diluted sufficiently to reduce the antibody concentration below that of the antigen. However, when MFI values reach saturation levels, it becomes exceedingly challenging to accurately assess antibody levels unless titration studies are incorporated.<sup>13</sup>



**Fig. 1. Schematic representation of the process of single-antigen bead assay.** HLA, human leukocyte antigen.

“Shared epitopes” is another constraint in SAB testing, which can lead to inaccurately low MFI values. This occurs when an antibody identifies an epitope that exists on multiple HLA antigens (on beads) within the assay. Consequently, the antibody becomes distributed across the epitope-positive antigen beads, leading to diminished fluorescent signals upon the addition of the reporter antibody. In contrast, if an antibody targets a unique, private epitope exclusively present on a single HLA (antigen bead), it would exclusively bind to that specific bead, yielding a much more robust fluorescent signal.<sup>14,15</sup> As antigens consist of many epitopes, understanding this concept is crucial when interpreting test results. Furthermore, it is important to understand that the SAB assay evaluates how antibodies interact with antigens, rather than directly quantifying antibody levels.

“Denatured antigen” is another issue found in SAB assays, as per the published literature. Normally, HLA antigens appear on the cell surface as complexes made up of multiple molecules. However, when it comes to the beads used in SAB assay, HLA class I antigens can take on various forms, including certain variations that differ from the native state. Furthermore, these variations often involve misfolded molecules referred to as denatured HLA molecules. The existence of these structural variations within the bead may impede the accurate assessment of anti-HLA antibodies.<sup>15–17</sup> Numerous strategies have been suggested to address this challenge, such as the application of a pretreatment reagent that captures nonspecific antibodies and the utilization of acid treatment to differentiate specific antibodies targeting denatured class I HLA. The adoption of acid treatment in combination with iBead has proven highly effective in mitigating issues associated with antibodies against denatured class I HLAs.<sup>18–20</sup>

### MFI values and data interpretation in SAB assay

The interpretation of SAB results mainly relies on qualitative assessments presented as MFI. Converting these measurements into precise quantitative antibody titers is not straightforward; instead, they are depicted as correlations, such as those drawn from cell-based assays and a patient’s immunologic history. It is crucial to note that MFI values are influenced by various factors such as antibody alignment, density, and concentration.

A study conducted by Schinstock *et al.*<sup>3</sup> considered an MFI of less than 2,000 as negative at their kidney transplant center. They also noted that at most kidney transplant centers, an MFI lower than 1,000 is considered negative in patients who have not experienced sensitizing events. In another study, common clinical significance cutoffs for MFI were set at 3,000 for class 1 DSAs and 5,000 for class 2 DSAs.<sup>6</sup> The reported thresholds for clinically significant MFI vary widely across different studies, ranging from 1,000 to 10,000, depending on the specific antigens.<sup>3,6,13,21–25</sup> Thus, establishing a universally applicable MFI threshold for consistent use across various institutions and laboratories poses an incredibly complex challenge.<sup>2,3,26</sup>

The categorization of SAB assay results as either negative or positive, based on MFI cutoffs, is dependent on the transplant center’s risk tolerance with respect to antibody-mediated rejection and the specific clinical circumstances. Moreover, the MFI threshold varies in accordance with the particular organ being evaluated for transplantation. Furthermore, laboratory variability contributes to variations in SAB results. This variability can stem from several sources, including differences in the SAB product itself, which can differ based on the manufacturer, batch, or lot, as well as internal factors within the laboratory, such as differences in personnel, reagents, equipment,

and conditions. Therefore, it is essential to consider these factors when interpreting any SAB report.<sup>27,28</sup>

### HLA epitopes

HLA-specific antibodies are designed to target specific epitopes, which are smaller fragments (typically 15–25 amino acid residues) found on HLA molecules. These epitopes are composed of short sequences (triplets/eplets) made of variable amino acid residues located in positions accessible to antibodies. Epitopes are categorized into two categories: private and public, influencing the specificity and cross-reactivity of HLA antibodies. Private epitopes are unique to individual antigens, while public epitopes are shared among multiple antigens, leading to cross-reactivity in HLA testing.<sup>29–31</sup>

Epitopes can further be classified based on their structure, as follows: linear (also known as sequential or continuous) vs. conformational (discontinuous). Linear epitopes consist of a continuous sequence of amino acids and can be recognized by antibodies and T-cell receptors based on the specific amino acid sequence. They are relatively easy to predict and can be mimicked using short peptide sequences for diagnostic or therapeutic purposes. Meanwhile, conformational epitopes involve the assembly of critical amino acid residues through protein folding to facilitate interaction. Conformational epitopes are often recognized by antibodies that can bind to the folded, native structure of the antigen. Disruption of the protein’s conformation can render the epitope inaccessible. These epitopes are typically more complex and harder to predict than linear epitopes.<sup>32–34</sup>

The functional epitope occupies a compact space within a range of 3 to 3.5 Angstroms. Within this compact region lies the essential core responsible for interacting with the complementarity-determining regions of the antibody. This core is composed of triplet amino acid configurations known as eplets and plays a pivotal role in the epitope-antibody interaction.<sup>35,36</sup>

The eplet, a component involved in antibody specificity, plays a key role in how antibodies react to HLA molecules. Exposure to foreign HLA molecules can lead to the development of antibodies that recognize multiple other HLA molecules that share similar functional epitopes. This raises questions about whether HLA mismatches at the eplet or epitope level are a more accurate measure of differences between donors and recipients. Comparing eplet repertoires between donors and recipients can identify specific discrepancies, aiding in the calculation of eplet mismatches for each HLA molecule or genetic locus. Recent research has shown that kidneys with disparities in HLA-A or -B but compatibility at the triplet level have similar graft survival rates to fully matched kidneys at the A and B loci, suggesting that triplet compatibility may be a better predictor of graft outcomes. Researchers are exploring the concept of an “eplet load” threshold to categorize individuals based on eplet mismatches for more effective immunosuppressive strategies. However, not all mismatches are equally significant, and highly immunogenic epitope mismatches are more critical than higher eplet mismatch loads. The goal is to predict which mismatches are tolerable for the immune system and which should be avoided due to their potential to trigger an immune response.<sup>37–39</sup>

### Epitope-based matching algorithms

Two computational techniques, HLAMatchmaker and Predicted Indirectly Recognizable HLA Epitopes (PIRCHE-II), have integrated the epitope-centered HLA matching principle into their algorithms for identifying the ideal donor for a recipient.

**Table 2. Representation of a key information comparison between HLAMatchmaker and PIRCHE-II**

Characteristic	HLAMatchmaker	PIRCHE-II
Objective	Used for finding permissible HLA mismatches in organ transplantation to reduce the risk of organ rejection.	Used to predict and quantify the number of non-inherited maternal and non-inherited paternal HLA class I epitopes presented by a patient's HLA molecules.
Main function	Kidney transplantation and other solid organ transplants.	Kidney transplantation, bone marrow transplantation, and autoimmune diseases.
Target HLA molecules	Focuses on donor-recipient compatibility for HLA class I and class II antigens.	Primarily focused on HLA class I epitopes (HLA-A, -B, and -C).
Input data	Requires HLA typing data for both the donor and recipient.	Requires HLA typing data for the recipient and a list of potential donors.
Output	Provides a list of permissible HLA mismatches based on the analysis of HLA epitopes.	Generates a PIRCHE-II score, which quantifies the number of epitopes mismatched between the recipient and potential donors.
Calculation	Analyzes the compatibility of HLA antigens at the epitope level using a computational algorithm.	Predicts the number of mismatched epitopes by comparing the recipient's HLA alleles to the potential donor's HLA alleles.
Clinical use	Used to select the most compatible donors in organ transplantation to improve transplant outcomes.	Used to estimate the immunogenicity of a potential donor in the context of kidney and bone marrow transplantation.

HLA, human leukocyte antigen; PIRCHE-II, Predicted Indirectly Recognizable HLA Epitopes.

### HLAMatchmaker

HLAMatchmaker operates as a structural algorithm that views HLA alleles as unique sequences of molecular structures, identifiable by important HLA antibodies relevant to transplantation.<sup>40,41</sup> This method encompasses Microsoft Excel-based tools to conduct structural matching of HLA class I and II molecules at the eplet level, while also assessing antibody reactivity patterns linked to precisely defined structural epitopes. Notably, HLAMatchmaker stands out as the sole algorithm grounded in experimentally verified epitopes authenticated by eplets. Additionally, it is regularly updated, with the most recent update being in July 2020.

HLAMatchmaker serves two primary purposes. First, it evaluates the suitability of eplet-based mismatches for patients with sensitivities who are being evaluated for transplantation or require platelet transfusions that need to be HLA-compatible. Second, it determines the eplet load, which represents the level of permissibility for HLA mismatches, to reduce allosensitization and enhance transplant success in patients who do not have prior sensitivities.<sup>42,43</sup>

The HLA Eplet Mismatch Calculator is a valuable resource accessible through the HLA Eplet Registry (<https://www.epregistry.com.br/>). This tool helps to assess compatibility between donors and patients in organ and tissue transplantation. Utilizing high-resolution HLA typing information, this calculator allows users to input detailed HLA typing data for both donors and patients.<sup>44</sup>

The tool then generates comprehensive analyses of single and overall eplet mismatches for both HLA class I and II antigens. This means that it can be used to evaluate eplet mismatches, a crucial consideration in organ and tissue transplantation, which can significantly influence the success and outcomes of these procedures. It serves as an invaluable aid in optimizing donor selection, potentially improving transplant outcomes.

### PIRCHE-II

The PIRCHE-II algorithm determines differences in HLA-derived T-helper epitopes between donors and recipients to

estimate the risk associated with transplant outcomes. For these predictions, the PIRCHE-II algorithm uses an algorithm designated as NetMHCIIpan.<sup>45</sup> PIRCHE-II scores, which measure the likelihood of an immune response between donor and recipient HLA mismatches, are linked to the risk of developing *de novo* DSAs and long-term kidney allograft survival.<sup>46,47</sup> To the best of our knowledge, HLAMatchmaker and PIRCHE-II are the two prominent tools used in the field of immunogenetics and transplantation to assess HLA compatibility. The key information comparing HLAMatchmaker and PIRCHE-II are presented in Table 2.

Assessing HLA antibodies requires a thorough investigation of SAB data in the context of complementary testing and the patient's history of sensitization.<sup>48</sup> It is crucial to understand that the acceptable level of immunological risk can differ depending on the type of graft and the specific transplantation protocol in place. Therefore, the testing process and the resulting interpretation should be customized not only to each patient-donor pair but also to the unique transplantation programs provided by the histocompatibility laboratory. The field of transplantation immunology is advancing with the development of new tests such as modified solid phase immunoassay, the characterization of memory B cells, the use of microarray technology to detect RNA-level biomarkers of rejection, and the utilization of epitope-based computational matching algorithms. These innovations are considerably enhancing our understanding of allograft rejection.<sup>49</sup> The combined use of HLAMatchmaker and PIRCHE-II enriches the interpretation of SAB data. By harnessing the power of epitope matching and T cell epitope prediction, transplant professionals can make more informed decisions, ultimately improving the outcomes of organ transplantation and reducing the risks associated with alloimmune responses.

### Conclusions

Evaluating HLA antibodies involves a comprehensive analysis of SAB data, considering complementary assays and the patient's sensitization history. It is essential to acknowledge that the acceptable level of immunological risk varies depending on the graft type and the specific transplantation



protocol being followed. As a result, both the testing procedure and its interpretation should be tailored to fit each patient-donor pair, as well as the unique requirements of the various transplantation programs facilitated by the histocompatibility laboratories. The advancements in this field are marked by the emergence of next-generation SAB assays and an increasing emphasis on epitope-based analysis, further emphasizing its importance.

The SAB assay plays a crucial role in organ transplantation by evaluating a patient's immune response after a transplant. This involves measuring antibodies, especially HLA antibodies, which are important in organ transplants. SAB technology is expected to improve with time, making it more precise in detecting HLA antibodies, reducing the potential for diagnostic errors and improving risk assessment during transplantation procedures.

In the future, clinicians will be able to personalize treatment by studying a patient's specific HLA antibodies, reducing complications and improving long-term treatment success. SAB technology will also become more automated and efficient. It will be used with other diagnostic tools and in research to better understand the immune system's response to transplants.

Overall, the future of SAB technology in organ transplantation looks promising. With increased precision, improved risk assessment, and the ability to provide more personalized care, SAB will enhance the success and longevity of organ transplants while reducing the risk of organ rejection.

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### Conflict of interest

The authors have no conflicts of interest to declare related to the publication of this manuscript.

### Author contributions

Data collection, technical assessment, and manuscript writing (VCM); and conception of the presented idea, supervision, and research findings (VR). All authors have made a significant contribution to this study and have approved the final manuscript.

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