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REVIEW

Caveats of HLA antibody detection by solid-phase assays

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SUMMARY

Solid-phase assays for human leukocyte antigens (HLA) antibody detection have clearly revolutionized the field of HLA diagnostics and transplantation. The key advantages are a high sensitivity and specificity for detection of HLA antibodies compared with cell-based assays, as well as the potential for standardization. Solid-phase assays enabled the broad introduction of tools such as "virtual crossmatching" and "calculated panel reactive antibodies," which are essential components in many organ allocation systems, kidney-paired donation programs, and center-specific immunological risk stratification procedures. The most advanced solid-phase assays are the so-called single antigen beads (SAB). They are available now for more than 15 years, and the transplant community embraced their significant advantages. However, SAB analysis and interpretation is complex and many pitfalls have to be considered. In this review, we will discuss problems, limitations, and challenges using SAB. Furthermore, we express our wishes for improvements of SAB as well as their future use for immunological assessment and research purposes.

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Solid-phase assays: a revolution in HLA antibody diagnostics

Solid-phase assays for HLA antibody detection have clearly revolutionized the field of HLA diagnostics and transplantation. The key advantages are a high sensitivity and specificity for detection of HLA antibodies compared with cell-based assays, as well as the potential for standardization [1,2]. Solid-phase assays enabled the broad introduction of tools such as "virtual crossmatching" and "calculated panel reactive antibodies" (cPRA), which are essential components in many organ allocation systems, kidney-paired donation programs, and center-specific immunological risk stratification procedures [3–7]. In addition, a much more accurate assignment of donor-specific HLA antibodies (DSA) improved the diagnosis and classification of antibody-mediated rejection (ABMR) processes in various transplanted organs [8,9].

The most frequently used system for HLA antibody detection by solid-phase assays is the Luminex platform. Two vendors offer different products ranging from rather less expensive screening assays containing a collection of HLA molecules on their surface to more expensive single HLA antigen beads (SAB) to define the specificity of HLA antibodies. In this review, we will discuss problems, limitations, and challenges using SAB on the Luminex platform. Furthermore, we will express

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our wishes for the improvements of SAB as well as their future use for immunological assessment and research purposes.

Readout of the Luminex platform

Most commercial Luminex tests for serological approaches do not exploit the full potential of the xMAP® technology, yet the SAB assay that uses up to 100 differently coated beads does. This panel of HLA-coated SAB is incubated with the patient serum, followed by a second incubation with the detection antibody conjugate. Each SAB carrying an individual HLA protein is identified by the first laser of the Luminex instrument. The second laser excites the fluorochrome of the detection antibody. The emitted fluorescence thus reports the number of bound HLA antibodies. To define negative and positive results, a two-step procedure has to be applied.

In a first step, the signal must be technically adjusted for background fluorescence signals. This adjustment can be achieved either "classically" by subtracting the signal of the negative control bead and a negative serum, or "biologically" by subtracting the average signal of those SAB representing self-HLA of the recipient [10]. The remaining signal is reported as baseline subtracted trimmed (removal of upper and lower 5% extreme values) mean fluorescence intensity (MFI). It represents the "technical" cutoff. A second step determines which level of MFI indicates clinically relevant HLA antibody strength (i.e., predicting ABMR and graft survival). This "clinical" cutoff should be set in accordance with the collaborating transplant center and is discussed later.

Technical challenges and limitations

Due to the complexity of the SAB analysis, false-positive and false-negative results can occur and should be carefully evaluated (Table 1). Here, we will describe the most important technical challenges and current limitations in the SAB analysis potentially leading to false-positive and false-negative results.

Quantification

While the multiplex assay principle is suitable to qualitatively assess DSA according to their HLA specificity, their quantification is inappropriate due to the intrinsic characteristics of both SAB and DSA. First, precise quantification would require each SAB to be present in the same proportion and coated with the same quantity and quality of HLA molecules. Even though these bead properties were improved over time, conformity over such a high number of SAB will always remain challenging and a standard assessment for determining HLA molecule concentration on SAB is not established yet. Second, in the majority of cases DSA react to public epitopes present on several SAB [11]. Binding distribution of such DSA will subsequently diminish MFI on the SAB of interest (representing the HLA allele of the donor), and DSA strength will thus be underestimated.

Erroneously, MFI units are commonly associated with the concentration of HLA antibodies. It is important to realize that MFI reflects just a proportion of all HLA antibodies present in the serum, namely the rate of antibodies capable of binding to SAB in a stable manner under the applied assay conditions (i.e., short incubation time). The assessed proportion is, therefore, not only triggered by the antibodies' concentration but also by their affinity, a characteristic that greatly differs between monoclonal HLA antibodies, as accurately shown by surface plasmon resonance [12]. Furthermore, the measured MFI represents only the fraction of isotypes that are targeted by the applied detection antibody conjugate. Indeed, coexistence of IgM and IgA DSA has been described [13–18]. In an own evaluation of 26 renal allograft recipients having only one HLA class I IgG DSA, we found coexisting IgM DSA in 31% and IgA DSA in 33%, respectively (unpublished data). Independent of their clinical relevance, these antibodies—not monitored by the standard anti-IgG_{pan}—may have an influence on MFI, as they are competing for binding on SAB and thus may decrease IgG-derived MFI [18,19]. Moreover, the final level of MFI is influenced by concentration, affinity, and incubation time of the used detection antibody conjugate [20].

Interference of serum components

The serum matrix of the probe is a major interfering factor and also the most uncontrollable one. Indeed, multiple endogenous molecules that potentially cross-react with assay components or disturb the outcome of immuno-assays have been described [21]. Since SAB assays are using undiluted serum—in contrast to the majority of solid-phase immuno-assays—high quantities of albumin, irrelevant immunoglobulins, haptoglobin, transferrin, antitrypsin, and fibrinogen can interfere in two ways: (i) by masking the binding of specific HLA antibodies and (ii) by nonspecific binding especially to

Table 1. Main reasons for false-negative and false-positive reactions in the IgG SAB assay.

False-negative	False-positive
• "Dilution" of the MFI signal across multiple beads sharing the same epitope • Serum matrix masks binding of HLA antibodies • High amount of bound HLA antibodies leading to accumulation of complement components, which interfere with binding of the detection antibody (i.e., complement interference) • Massive excess of HLA antibodies leading to steric hindrance of binding (i.e., hook or prozone effect) • IgA and/or IgM antibodies competing for binding sites of IgG antibodies • Rabbit anti-HLA antibodies present in medical products (e.g., polyclonal anti-thymocyte globulin) competing for binding sites of human HLA antibodies • IgG antibodies with low concentration or affinity unable to sufficiently bind during the 30 min incubation time	• Exposure of neo-epitopes • Unspecific binding of serum matrix components • HLA antibodies present in medical products (e.g., polyclonal anti-thymocyte globulin)

the negative control bead [22]. The latter effect can be partially removed by Adsorb Out™ beads [23].

By far the most significant disturbing interference, however, is caused by complement components, which accumulate after binding of high amounts of complement activating HLA-specific IgM/IgG1/IgG3 on SAB. Indeed, if enough Fc domains of adjacently bound antibodies are in close proximity, C1q can initiate the complement cascade [24–26]. The complement component C3 was identified as the main cause for steric hindrance of binding of the antibody detection conjugate, resulting in low MFI [27]. The formation of the C1 complex can be inhibited by pretreatment of the blood sample with heat, dithiothreitol (DTT), or ethylenediaminetetraacetic acid (EDTA) [28,29]. Using plasma samples or adding EDTA to the patient serum sample has meanwhile become the preferred option of many HLA laboratories to prevent this so-called "complement interference" [30], which often is still erroneously termed "prozone effect" as pointed out by Berth [31]. Some sera do—uniquely or additionally—exhibit a prozone or hook effect, which describes the phenomenon of a low signal because of an oversaturation with the investigated molecules in an immuno-assay (here HLA antibodies). It is important to note that dilution diminishes both complement interference and prozone effect.

Interference of medicinal drugs

Polyclonal anti-thymocyte globulin preparations (Gravalon, Neovii, and Thymoglobuline, Sanofi-Aventis) contain rabbit anti-human HLA antibodies, which can partly be detected by the anti-human IgG [32,33]. Since Thymoglobuline is produced by immunization with human thymocytes of several individuals, multiple HLA class I and II specificities can be present. In the case of Gravalon (immunization with a single Jurkat cell line expressing HLA-A3, A32, B7, B35), the specificities are better defined, and thus, false-positive results are easier to identify [34]. In addition, these rabbit HLA antibodies compete with human HLA antibodies for binding to the SAB and can therefore diminish the MFI signal.

Intravenous immunoglobulin potentially contains human HLA antibodies of any specificity [35]. However, as this product consists of IgG from thousands of plasma donors, HLA-specific antibodies are very likely diluted below the limit of SAB detection [33,36]. It has been observed that high concentrations of intravenous immunoglobulin can significantly increase the background fluorescence making interpretation of the SAB assay impossible (personal communication with Howard Gebel, Emory University, Atlanta, USA).

Denatured HLA molecules

Positive SAB results may be caused by so-called "natural" antibodies and do not necessarily refer to an HLA antibody specificity being present *in vivo*. In 2008, Morales *et al.* [37] found repeatedly detectable HLA antibodies in sera of nonsensitized men. While these specificities were mostly directed against rare alleles of the general population, similar observations have also been made for more frequently existing specificities [38,39]. In the meantime, it became clear that SAB can carry denatured HLA antigens exposing cryptic, usually nonaccessible epitopes. These neo-epitopes originate from the SAB production process leading to some denaturation of HLA class I molecules, lacking β2

microglobulin and/or the peptide in the binding groove. Furthermore, the transmembrane region and the cytoplasmatic tail of HLA molecules can be exposed on SAB, while these structures are embedded into the plasma membrane *in vivo*. Figure 1 schematically visualizes potential neo-epitopes on SAB class I. Although less data are available, HLA class II molecules, composed of two noncovalently associated polypeptides (α-chain and β-chain), can be denatured as well.

The clinical significance of these "natural" antibodies is a matter of debate and studies revealed conflicting results, even though the clinical impact might be rather limited [40–42]. Interestingly, one study reported that antibodies against denatured HLA antigens may also be able to bind to intact HLA molecules [43].

To reliably evaluate SAB test results, it is of critical importance to know the used bead sets. Since 2015, our laboratory monitors each new SAB lot with continuously obtained sera from nonsensitized males in order to identify "problematic" beads. The top 10 of these "problematic" beads from the three last lots for classes I and II are listed in Table 2. Some specific patterns such as DP1/DP5/DR53 and DR1/DR4/DR16/DQ7/DP19 were repeatedly observed. Notably, denatured HLA on SAB can be confirmed by parallel testing of suspected sera with acid-treated SAB [40].

Beads versus cells as HLA antibody targets

It is important to be aware of some fundamental differences between the physiological presence of HLA on the surface of surrogate cells (e.g., on T and B cells) and their rather artificial coating on beads. HLA molecules embedded in the lipid bilayer of the plasma membrane are mobile, highly accessible and tend to build clusters [44]. The precise organization of HLA antigens on SAB has not been explored, but they are fixed, and might be evenly distributed but arranged rather irregularly over the entire bead surface. Another striking difference between SAB and cells concerns the HLA molecule density. T and especially B cells show quite variable allelic HLA expression, while the HLA density on SAB is constant and two- to threefold higher (own estimation), which can lead to discordant results between SAB and crossmatch tests [45–48]. For example, if a patient serum with high amounts/affinities of DSA is incubated with donor cells having a low HLA expression, the complement-dependent cytotoxicity (CDC) crossmatch remains negative [48].

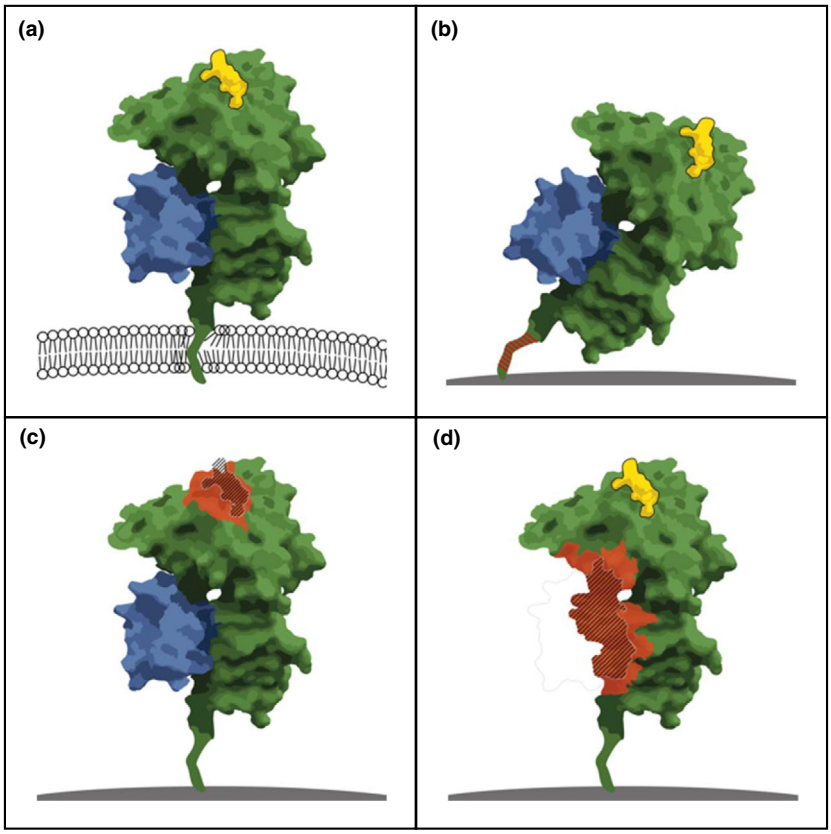

Figure 1 Potential neo-epitopes of SAB class I. Potential neo-epitopes are indicated by dashed lines and a hypothetical surrounding area in red color. (a) HLA class I molecule (α-chain in green, β2 microglobulin in blue, peptide in yellow) in its natural configuration embedded into the plasma membrane. (b) HLA molecule coated on single antigen beads exposing neo-epitopes in the tail structure. (c) Neo-epitopes in the area of the peptide binding groove at the α1 and α2 domains after loss of the peptide during the production process. (d) Neo-epitopes in the area of the α3 domain after loss of β2 microglobulin during the production process.

Table 2. Top 10 of positive SAB results in nonsensitized men.

Class I								
Lot 9 123 analyses from 95 nonsensitized men			Lot 10 133 analyses from 115 nonsensitized men			Lot 11 135 analyses from 111 nonsensitized men		
Antigen (allele)	% Positive	Mean MFI (max)	Antigen (allele)	% Positive	Mean MFI (max)	Antigen (allele)	% Positive	Mean MFI (max)
B76 (B*15:12)	11.4	1824 (6726)	B76 (B*15:12)	14.3	1708 (6454)	B76 (B*15:12)	9.6	1327 (3010)
B8 (B*08:01)	8.9	1547 (3995)	Cw17 (C*17:01)	8.3	1629 (5945)	Cw12 (C*12:03)	7.4	2981 (8733)
Cw17 (C*17:01)	8.9	1568 (3295)	A66 (A*66:02)	7.5	1557 (3432)	Cw17 (C*17:01)	7.4	1846 (4480)
Cw4 (C*04:01)	8.1	1054 (2476)	B37 (B*37:01)	6.8	2413 (6499)	B63 (B*15:16)	6.7	1932 (4947)
A34 (A*34:01)	7.3	668 (829)	B57 (B*57:03)	6.8	1756 (4410)	Cw4 (C*04:01)	6.7	1339 (4854)
A66 (A*66:02)	7.3	1170 (1902)	B73 (B*73:01)	6.0	1160 (2201)	A80 (A*80:01)	5.2	1343 (3067)
B46 (B*46:01)	7.3	968 (3094)	Cw12 (C*12:03)	6.0	1661 (3490)	B44 (B*44:02)	5.2	1443 (3500)
B57 (B*57:01)	7.3	1308 (5460)	B75 (B*15:11)	5.3	1115 (2457)	B45 (B*45:01)	5.2	2276 (4950)
B73 (B*73:01)	6.5	1064 (1933)	Cw4 (C*04:01)	5.3	1026 (1581)	A11 (A*11:02)	4.4	2107 (3362)
A11(A*11:02)	6.5	2543 (6659)	A25 (A*25:01)	4.5	1607 (5903)	B8 (B*08:01)	4.4	1983 (5074)
Class II								
Lot 11 181 analyses from 118 nonsensitized men			Lot 12 220 analyses from 147 nonsensitized men			Lot 13 39 analyses from 38 nonsensitized men		
Antigen (allele)	% Positive	Mean MFI (max)	Antigen (allele)	% Positive	Mean MFI (max)	Antigen (allele)	% Positive	Mean MFI (max)
DP1 (B*01:01/A*01:03)	12.2	1719 (4286)	DP1 (B*01:01/A*02:01)	18.6	1253 (4701)	DP1 (B*01:01/A*02:01)	15.4	1944 (4587)
DP5 (B*05:01/A*02:02)	11.6	1364 (3398)	DP5 (B*05:01/A*02:02)	16.8	1285 (4660)	DR53 (DRB4*01:01)	15.4	855 (1662)
DR4 (DRB1*04:04)	11.0	1047 (2856)	DP1 (B*01:01/A*01:03)	15.0	1257 (4269)	DP5 (B*05:01/A*02:02)	12.8	1212 (2748)
DP1 (B*01:01/A*02:01)	7.7	1238 (2589)	DP19 (B*19:01/A*01:03)	15.0	915 (2202)	DR4 (DRB1*04:01)	12.8	917 (1578)
DP11 (B*11:01/A*02:02)	7.7	1026 (1750)	DR16 (DRB1*16:02)	13.2	1120 (2482)	DP19 (B*19:01/A*01:03)	10.3	801 (1221)
DR16 (DRB1*16:02)	7.7	1078 (2155)	DR4 (DRB1*04:04)	12.3	913 (2031)	DP3 (B*03:01/A*01:03)	7.7	724 (927)
DR16 (DRB1*16:01)	7.2	1376 (2903)	DQ4 (B*04:01/A*02:01)	10.5	1090 (3771)	DQ7 (B*03:01/A*05:03)	7.7	787 (926)
DQ6 (B*06:03/A*01:03)	6.6	1334 (2894)	DP14 (B*14:01/A*02:01)	9.1	1291 (3625)	DQ7 (B*03:01/A*05:05)	7.7	862 (1109)
DR18 (DRB1*03:02)	6.1	958 (1837)	DQ7 (B*03:01/A*05:03)	9.1	3271 (9998)	DR103 (DRB1*01:03)	7.7	795 (909)
DQ2 (B*02:01/A*05:01)	5.5	1756 (7551)	DR53 (DRB4*01:01)	9.1	1070 (2671)	DR4 (DRB1*04:02)	7.7	1033 (2026)

From November 2015 to April 2019, three different lots of LabScreen™ SA beads class I/II (OneLambda) were used. Beads with an MFI > 500 were considered positive.

In this context, it is important to discriminate between binding and functional assays. Binding assays such as the SAB or the flow crossmatch are very sensitive and can detect low amounts of HLA antibodies. By contrast, functional assays such as the C1qScreen™ test or the CDC crossmatch will only turn positive, if a high density of HLA antibodies is reached on the SAB or cell surface enabling C1q binding. Figure 2 visualizes the impact of the HLA molecule density and the amount/affinity of HLA antibodies on the most commonly used assays.

Critical issues for data interpretation

Once acquired, SAB data interpretation is challenging and requires careful contextual evaluation including the patient's HLA typing and sensitization history, the donor's HLA typing, as well as the awareness of technical caveats as discussed above [49].

An important aspect in interpretation of SAB results is the antibody reactivity pattern, which was recognized as cross-reactive groups already several decades ago [50,51]. Over the last years, the understanding of shared antigenic determinants among HLA molecules, so-called epitopes, has emerged and resulted in the availability of a tool (HLA Matchmaker) that aids in identifying those patterns (reviewed in [52]). Beyond commonly used MFI cutoffs, an antibody reactivity pattern belonging to an epitope being clearly present in a recipient's serum should be considered as relevant, even in case of a weak or borderline signal and especially if the epitope has been a mismatch in a previous transplantation.

With the increasing use of SAB, it has become clear that HLA antibodies target not only public epitopes, but also private epitopes present on one or just a few HLA alleles. This has important implications for DSA assignment. If HLA antibodies detected by SAB are present, the donor has to be typed for the corresponding HLA antigen/alleles to facilitate an accurate DSA assignment. This might include typing for DQA1 and DPA1, as well as high-resolution typing, if allele-specific HLA antibodies with potential donor specificity are present.

The current SAB products consist of close to 100 different HLA molecules for class I and II. However, more than 20 000 HLA alleles have been reported, and around 600 have a frequency of >1:100 000 persons in a predominantly Caucasian population from Germany [53]. The question arises, whether the SAB products can ever cover this enormous polymorphism of the HLA molecules. Luckily, the HLA antibodies target epitopes and there are very likely fewer HLA epitopes than HLA alleles. The HLA Matchmaker version 2 has a repertoire of 132 eplets for class I (derived from 561 alleles) and 279 eplets for class II (derived from 290 alleles). The current SAB panels cover 98.5% of these eplets (LabScreen™, OneLambda). Therefore, the eplet/epitope coverage is very high, but there is still a small chance to miss some HLA antibodies. Ethnicity-adapted SAB panels might further increase the overall eplet/epitope coverage.

Clinical impact of pretransplant DSA defined by SAB

The MFI cutoff for a clinically relevant positive SAB result is widely debated [54]. We believe that it should be defined together with the transplant center in the context of an overall risk stratification concept. It is beyond the scope of this review to provide a detailed assessment of all available data for all organs.

On a population level, pretransplant DSA defined by SAB are widely accepted as a risk factor for ABMR and inferior allograft survival for most solid organs [55–57]. However, on an individual patient level, the clinical impact of pretransplant DSA is highly variable ranging from uneventful courses to early ABMR with allograft loss [58]. There are many factors that influence the pathogenicity of DSA, which are still largely unexplored (e.g., epitope specificity of the DSA, magnitude and durability of the memory response, density of the antigen expression in the transplanted organ, regulation of effector functions) [59–62]. Indeed, many DSA might be rather unique for a given donor–recipient constellation, which makes it challenging to define universal risk factors with a high predictive value.

Modifications of the generic SAB assay

In an attempt to better predict the risk associated with DSA, the generic SAB assays have been modified. One approach is to assess the capability of DSA to bind C1q or C3d on SAB (reviewed in [63]). Notably, about 98% of DSA contain either IgG1 or IgG3 subclasses and hence have the intrinsic capability to bind complement [64]. The major determinant of C1q or C3d binding on SAB is the amount of DSA, which can be approximated by the MFI value [20,64]. It is not surprising that most studies found a very strong correlation of the MFI value and C1q or C3d binding [65–67]. While some studies including a recent meta-analysis found that C1q or C3d

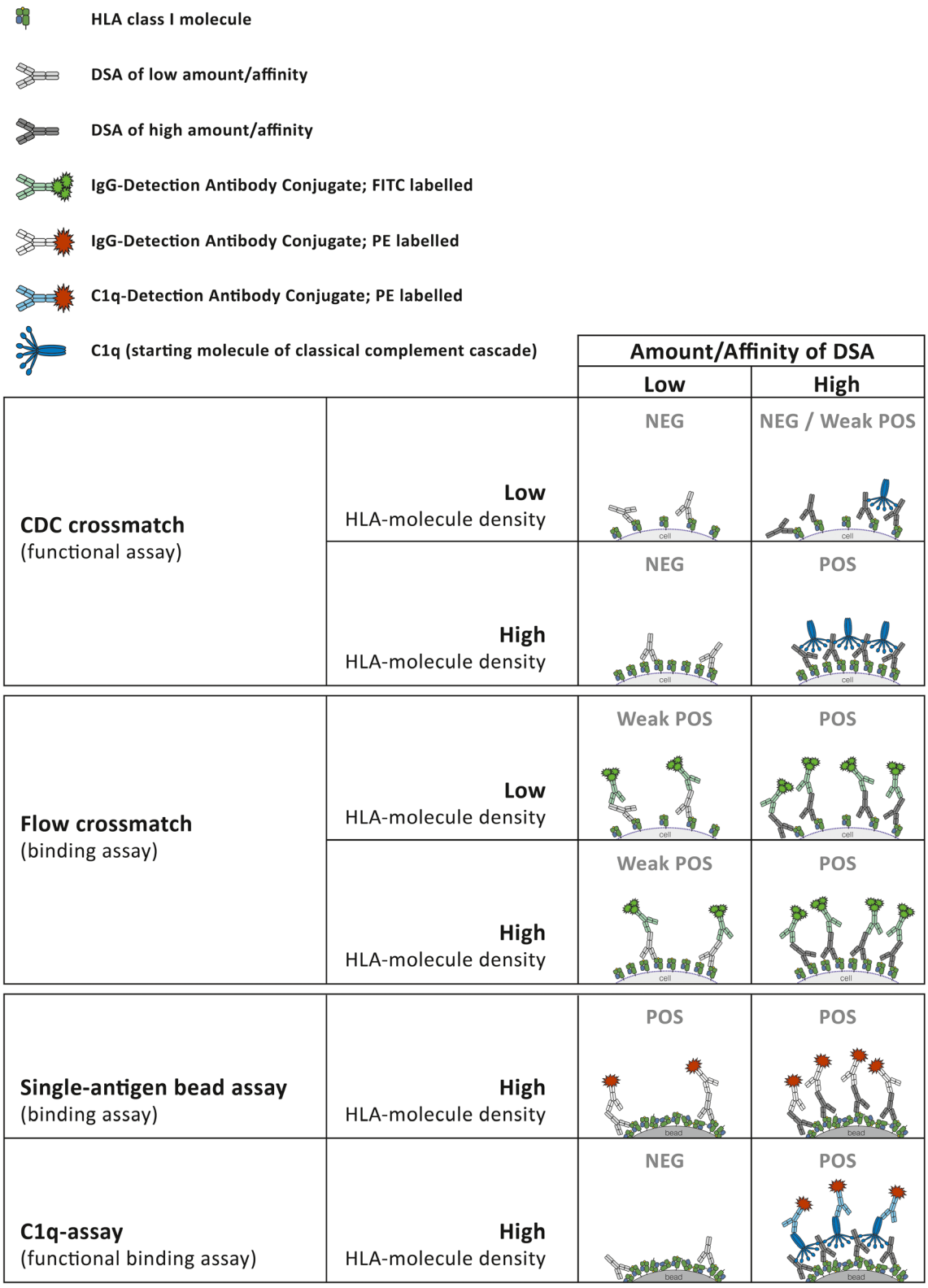

Figure 2 Impact of HLA molecule density and amount/affinity of HLA antibodies on test results. Based on the assay principle, the assays can be separated into binding assays (single antigen beads, flow crossmatch) and functional assays (C1q assay and CDC crossmatch). The anticipated results for all assays are given depending on the HLA molecule density and the amount/affinity of DSA. Overall, low amount/affinity DSA are still detectable in binding assays irrespective of the HLA molecule density. Functional assays require high amount/affinity DSA and a high HLA molecule density to become positive. Notably, single antigen beads have by default a high HLA molecule density.

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binding assays add some prediction beyond the MFI value, other studies did not [65,68–70].

Another approach is to assess the IgG subclass composition of DSA enabled by IgG subclass-specific secondary antibodies (reviewed in ref. [71]). The basic assumption is that strong complement binding IgG subclasses (i.e., IgG1 and IgG3) are more detrimental than weak or noncomplement binding IgG subclasses (IgG2 and IgG4). Unfortunately, the biology is much more complex [71]. High amount of IgG2 and/or IgG4 can block the effect of IgG1 and/or IgG3 subclasses if the antibodies target the same epitope. By contrast, these subclasses can act synergistically if they target different epitopes on the same HLA molecule [20,72,73]. It has also been shown that an expansion of DSA to IgG2/IgG4 subclasses rather indicates an advanced immune response [74]. A clear picture how to best use IgG subclass assays has not emerged yet [71].

The vast majority of studies used secondary antibodies specific for IgG isotypes. As mentioned previously, it is known that HLA antibodies can also contain IgA and IgM isotypes. Due to the predominance of IgG isotypes, it is difficult to decipher the precise contribution of other isotypes in the process of organ rejection. Generally spoken, it seems that the presence of IgA isotypes reflects a broader and more mature immune response, while IgM isotypes could indicate an ongoing recruitment of naive B cells [14,17,75].

As already discussed above, quantification of DSA by means of the MFI value can be misleading. Titration studies are the best method to capture the true DSA strength (reviewed in [76]). Alternatively, the results of flow or CDC crossmatches are used in many transplant centers to define thresholds as transplantation barriers [77].

Overall, it is still a matter of debate whether the modified SAB assays (C1q/C3d-binding; IgG subclasses; IgA/IgM; titration studies) enhance risk prediction beyond the generic SAB assay in a clinically significant way. It is conceivable that they provide very important information in specific cases, but their general application might not be necessary and they are currently expensive as well as labor-intensive.

Screening for HLA antibodies

The development of a pre- and post-transplant HLA antibody screening protocol can be deemed a transplant center-specific process that should take various factors into account, such as the local performance of the assay, own clinical experiences, consensus guidelines, and cost-related issues [78,79].

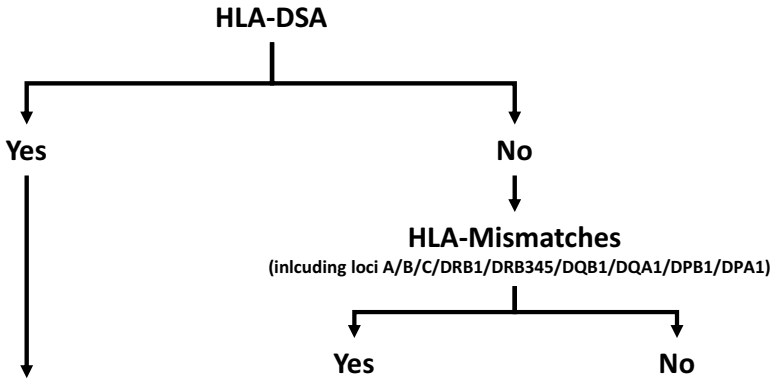
For patients on the waiting list, a regular screening strategy with additional testing following immunizing events or clinical decisions such as withdrawal/reduction of immunosuppression or allograft nephrectomy in previously transplanted patients should be established. To the best of our knowledge, changes in SAB-defined HLA antibody profiles in waitlisted kidney allograft recipients without interfering sensitizing events have not been studied. In our experience, HLA antibody profiles in such patients are quite stable, allowing to reduce the screening frequency to once a year, if a well-functioning reporting system for sensitizing events is in place [5,80].

Compared to pretransplant, there is even more controversy within the transplant community on when and how frequently a transplant recipient should be screened post-transplantation. In general, screening of DSA by SAB in the setting of allograft dysfunction with biopsy-proven microvascular inflammation or C4d deposition suggesting ABMR can be regarded as advisable at any time point, even though absence of DSA detection presumably because of absorption by the graft may occur [81]. As demonstrated by Sis *et al.* [82], DSA may even be absent in the context of ABMR with (severe) microvascular inflammation. This should not preclude its diagnosis and has also been acknowledged in the Banff 2017 meeting report [83]. Routine screening in the absence of allograft dysfunction is, however, more debatable and cannot, in terms of cost-benefit considerations, be commonly recommended within the first year post-transplant. Wiebe *et al.* [84] have nicely shown that the frequency of *de novo* DSA in the first year is only 2%, but steadily increases over the subsequent years at a rate of about 2%/year.

But how to deal with the detection of *de novo* DSA in a transplant recipient with stable allograft function beyond the first year post-transplant? Unfortunately, in case of evidence of allograft injury in a biopsy following *de novo* DSA detection, therapeutic options are currently very limited as neither the proteasome inhibitor bortezomib nor a combination of intravenous immunoglobulin and rituximab was effective in two randomized-controlled trials [85–87]. Detection of *de novo* DSA might, nevertheless, still be helpful to identify patient having insufficient immunosuppression (e.g., nonadherence or physician-induced minimization) and to tailor immunosuppression on an individual basis [88].

The future of SAB

Single antigen beads have been used in research and clinics for more than 15 years. They must be considered



Primary type of immune response	Memory immune response against HLA	*De novo* immune response against HLA	No immune response against HLA
Current risk assignment	High risk	Standard risk	Low risk
Key question for better risk stratification	• Magnitude/durability of memory immune response? • Epitope target of DSA?	• Immunogenicity of mismatched HLA-epitopes?	

Figure 3 General risk stratification based on presence/absence of donor-specific HLA antibodies and HLA compatibility.

a very important milestone in transplant diagnostics. The HLA and transplant community embraced their significant advantages, learnt to deal with their limitations, and developed skills for more accurate interpretation.

How can SAB be further improved and how can they contribute to a better immunological risk stratification? Immunological risk stratification is mainly based on the presence/absence of DSA and the number of HLA mismatches (Figure 3). If DSA are present, a memory immune response can be expected, but its magnitude and durability is currently not predictable. Furthermore, the targeted epitopes of the DSA might be an important determinant for the clinical impact, but this is also often unknown. In case of a *de novo* immune response, the load of mismatched epitopes—and even more their individual immunogenicity—might be the driving force for rejection. Novel "memory" assays and sophisticated software algorithms (i.e., HLA Matchmaker, PIRCHE II) have been developed to address these gaps [89–95]. All these tools and their further improvement rely heavily on SAB analyses. Detailed epitope analyses of DSA will often require some absorption/elution studies, which can be facilitated by individual SAB that are not compiled in a full SAB panel (so-called "singles") [11,96]. "Singles" were once on the market, but are currently not commercially available anymore. In addition, "singles" are also a valuable tool to study the affinity/avidity as well as the quantity of DSA.

Our wish list for future SAB products includes (i) SAB carrying only a minimal amount or no denatured HLA molecules, (ii) renewed availability of "singles", (iii) ethnicity packages covering the most frequent alleles and DQB1-DQA1/DPB1-DPA1 dimers for a given ethnic group, and (iv) a cocktail of secondary antibodies allowing for simultaneous detection of different immunoglobulin isotypes and IgG subclasses.

The HLA community will need to improve standardization of SAB across different providers and different HLA laboratories. Clinicians and researchers are called to fill the above-mentioned knowledge gaps in a common effort. May the SAB be with us!

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Conflicts of interest

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