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REVIEW

Methodological aspects of anti-human leukocyte antigen antibody analysis in solid organ transplantation

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Abstract

Donor human leukocyte antigen (HLA)-specific antibodies (DSA) play an important role in solid organ transplantation. Preexisting IgG isotype DSA are considered a risk factor for antibody mediated rejection, graft failure or graft loss. The post-transplant development of DSA depends on multiple factors including immunogenicity of mismatched antigens, HLA class Ⅱ typing of the recipient, cytokine gene polymorphisms, and cellular immunoregulatory mechanisms. *De novo* developed antibodies require special attention because not all DSA have equal clinical significance. Therefore, it is important for transplant clinicians and transplant immunologists to accurately characterize DSA. In this review, the contemporary immunological techniques for detection and characterization of anti-HLA antibodies and their pitfalls are described.

Key words: Human leukocyte antigen; Transplantation; Antibodies; Solid phase analysis; Flow cytometry

Core tip: In solid organ transplantations the graft outcomes critically depend on the degree of human leukocyte antigen (HLA) matching between the donor and recipient. Although the cellular component of the allogeneic immune response to the transplanted tissue plays a key role, the contribution of antibodies should not be underestimated. The detection of anti-HLA class Ⅰ and class Ⅱ antibodies is an important component of the initial work-up of a potential transplant candidate. The introduction of new highly sensitive technologies such as solid-phase based technologies has had a tremendous effect on organ allocation and immunomodulation strategies.

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INTRODUCTION

In most cases the development of alloantibodies against human leukocyte antigens (HLAs) is related to immunization *via* blood and/or blood product transfusions, pregnancy, and transplants. There are scattered reports in the literature indicating the production of HLA antibodies may be elicited by vaccinations and infections due to cross reactivity between viral/bacterial antigens and HLAs[1-4] or through the bystander effect[5-8]. Humoral or antibody-mediated immunity requires noncovalent contact between antigens and antibodies. The hyper variable regions of the light and heavy immunoglobulin chains are termed complementarity-determining regions and they are primarily involved in the interaction with antigens. Antibody effector functions are specified by the constant domains of the heavy chains. The most

important function of these domains is the activation of the complement cascade, which is triggered by conformational changes in the hinge area after antigen binding. Complement activation results in the destruction of the cell membrane.

In solid organ transplantations of the kidney, heart, lung, and pancreas graft outcomes critically depend on the degree of HLA matching between the donor and recipient[9-17]. The cellular components of the allogeneic immune response to the transplanted tissue play a key role in this matching and the contribution of antibodies should not be underestimated[18-22]. The detection of anti-HLA class I and class Ⅱ antibodies is an important component of the initial work-up of a potential transplant candidate (TC). The rationale for obtaining this information is related to clinical studies, which have universally demonstrated that pre-existing donor specific antibodies (DSAs) represent a significant risk factor for graft outcome[23-34]. The importance of the post-transplant monitoring of DSAs in kidney and cardiac transplants has been widely described[35-45]. The *de novo* development of DSAs strictly depends on the antigenicity and immunogenicity of mismatched HLAs. The substantial influence on antibody production involves other factors such as the HLA class Ⅱ type of the responder, immunosuppressive medications, cytokine and chemokine genomic polymorphisms, and the hormonal background of the recipient[11,45-50].

It is generally accepted that *de novo* developed DSAs represent a risk factor for graft failure even at low concentrations. The early detection of DSAs considerably reduces the incidence of antibody-mediated rejection (AMR) and transplant glomerulopathy[23,51-58]. A post-transplant antibody analysis is a part of the routine monitoring of recipients. The introduction of new highly sensitive technologies such as solid-phase based technologies has had a tremendous effect on the clinical approach to anti-HLA antibody analysis[56-58]. The purpose of this review is to familiarize the reader with the methodological aspects and pitfalls of anti-HLA antibody analysis in solid-organ TC. Solid phase (SP) techniques will be specifically addressed.

METHODS OF ANTIBODY IDENTIFICATION

Cell based assays

Complement-dependent cytotoxicity: The long-established NIH complement-dependent cytotoxicity (CDC) method and its modifications are still widely used[59-64]. This assay allows the identification of high concentrations of antibodies to HLAs. There are two main purposes for applying the CDC method. This method can be used to estimate the percent of reactive antibodies (PRA) when the recipient serum is incubated with a panel of HLA typed T- or B-lymphocytes. If the serum contains antibodies against a particular HLA then the addition of rabbit complement causes cell death that is visualized by staining and microscopic examination. This test is also used for the cross-matching (CM) or detection of complement binding antibodies against the HLAs of a particular donor. Various modifications of the NIH CDC method including extended incubation, additional washings, and the addition of secondary antihuman light kappa chain specific antibodies have been used to increase the sensitivity of the assay. Notably, the NIH CDC assay detects anti-HLA antibodies of the IgG and IgM isotypes. However, the IgM isotype has considerably less clinical significance[20,64-68]. Donors with HLA recipient antibodies detected by CDC should be avoided due to the high risk of hyperacute or delayed hyperacute rejection. Currently, this assay is primarily used to determine the efficacies of the desensitization or immunomodulation of recipients with high concentrations of anti-HLAs and identify recipients that are CDC-CM positive for their donors. Numerous reports have demonstrated that changing CDC-CM from positive to negative *via* durable DSA removal significantly reduces the risk of graft loss. The short-term graft survival in such recipients is not significantly different from recipients without CDC-positive DSA[69-75].

In the early 1970s, Patel *et al*[76] reported a considerable rate of graft failure in recipients who were CDC-CM negative with their donors. These observations indicated that the sensitivities of the CDC assay and its modifications were insufficient to detect low concentrations of DSA and was deleterious for the transplanted organ. Methods for increasing the sensitivity of antibody detection by flow cytometry (FC) based techniques were introduced more than 30 years ago[77].

Flow cytometry methods

Although FC methods of analysis are more sensitive than the CDC method they are subject to the effects of non-HLA and autologous antibodies that complicate the interpretation of the results. This complication is particularly important in the case of B-lymphocytes because they express Fc receptors and various adhesion molecules on their surfaces that facilitate non-specific binding[67,78]. B-cell false positive FC CM results may erroneously preclude transplants that should have favorable outcomes. The non-specific antibody binding may be reduced by the incubation of the lymphocytes with pronase, enzyme destroying Fc receptors, and other members of the Ig superfamily[79-82]. FC cell-based assays can also be used for PRA analysis. In this case, pooled HLA-typed lymphocytes are incubated with the recipient serum, and the percentage of positively reactive cells is determined based on the median channel shift[83]. This method has limited applicability because further testing is required to determine the antibody specificity. Additionally, the absorption of anti-HLA class I is required when class Ⅱ PRA is analyzed.

SOLID PHASE ASSAYS

SP technologies use purified HLA class I and/or class

Ⅱ proteins that are attached to an artificial substrate or matrix. These assays offer significantly higher sensitivities and specificities than cellular methods[84-86].

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was the first solid-phase analysis developed for antibody screening and specificity determination[87]. In this assay, HLA molecules are bound to the wells of plastic plates and positive reactions are measured by the color signal intensity produced by enzymes conjugated to anti-human antibodies following the addition of substrate to the wells. Purified pooled HLA- Ⅰ and -Ⅱ molecules bound to the wells are used for antibody screening/detection and PRA analysis. To define the antibody specificity, HLA proteins isolated from one individual are used to coat each well of the plate.

FC

FC SP assays use microspheres that have been coated with soluble HLA proteins that are extracted from a single cell line for specificity analysis or mixed for PRA analysis (FlowPRA Specific, FlowPRA, One Lambda)[88]. After the addition of the fluorescence-conjugated anti-human secondary antibodies a light signal is generated that indicates a positive binding of the antibody to HLA molecule(s). As with ELISA, this method can be designed to detect antibodies of IgG and IgM isotypes depending on the specificity of the secondary antibodies[84,89,90].

Luminex

Luminex-based technology has revolutionized the approach to anti-HLA antibody analysis and resolved ambiguities associated with the interpretation of CDC and FC results. This highly sensitive methodology has become an integral component of clinical decision-making and pathological diagnosis of transplanted organ injury. Luminex technology also incorporates microparticles (beads) that have been conjugated to varying amounts of two dyes, which enable the identification of 100 sets of beads. HLA-specific alloantibodies are detected *via* the addition to a reaction mixture of secondary phycoerythrin (PE)-conjugated anti-human antibodies. Each group of beads can be identified by the amount of conjugated fluorochromes and it is possible to identify which HLAs have bound antibodies. The light signal produced by bound antibody is proportional to its concentration and is expressed as the mean fluorescence intensity (MFI). The original assay was introduced as a combination of beads that were coated with HLA proteins extracted from individual cells. More recently, Luminex technology introduced a modification of the assay that included beads coated with a single class Ⅰ or class Ⅱ HLA. This methodological approach significantly improved antibody specificity analyses, particularly in highly sensitized patients[88,91-95]. The considerably higher surface density of HLAs on the microbeads compared to that on lymphocytes makes the Luminex single-antigen (SA) methodology extremely specific and highly sensitive. As a result, investigators can detect very low concentrations of HLA-directed antibodies. In the last decade, numerous reports have addressed the methodological aspects, clinical relevance, and standardization of the Luminex SP SA assay for the detection of antibodies[53,54,95-100]. The results of these studies have significantly expanded our understanding of anti-HLA antibody biology and the mechanism of the interaction between antibodies with antigens. This increased understanding includes improved information on isotypes and subtypes of antibodies, their abilities to bind complement, and fine epitope specificity. Fine epitope specificity is particularly important for the prediction of graft rejection[93,101-103]. There are also many new questions such as how does the signal produced by the DSAs detected in the SA Luminex assay correlate with positive FC CM, what is the clinical relevance of minimally reactive DSA, and how can the bead saturation effect be identified and overcome. Additionally, there are many other questions to address.

In this review, I describe the pitfalls, caveats, and limitations of the Luminex SP SA assay based on seven years of experience and clinical outcomes/observations at our transplant center.

Luminex SA SP assay: Technical challenges

Correlation of DSA MFI values with the results of FC CM tests: It is generally accepted that preexisting DSA directed against HLA can cause allograft injury or loss. FC CM is the most sensitive immunological method and it allows for the detection of DSA in TC serum. Positive FC CM is associated with an elevated risk of AMR[12,16,17,21,32,104-107]. The accurate detection of the spectrum of anti-HLA antibodies is critical for organ allocation and the prediction of FC CM results. Comparisons of the antibody profiles in the sera of TCs with the HLA typing of the potential donors are called virtual cross matches (VCMs). VCMs are particularly important in cases of heart and lung allocation in which the cold ischemia time is limited. VCM has been widely used at our transplant center for several years. Although SP Luminex technology allows for the very specific detection of DSAs at relatively low concentrations, issues regarding how strong the DSAs must be to cause positive FC CM results have to be addressed. The results of a multicenter study performed by Reed *et al*[89] suggested optimal cutoffs from 1000 to 1500 MFIs for antibodies to the HLA-A, -B, -DRB1, and -DQB1 loci. DSAs with MFIs within the indicated range are considered weak, and those below this range are considered negative. The correlation analyses between MFI values of DSAs and the results of FC CM assays performed in our laboratory have demonstrated that MFIs ⩾ 2600 produced by anti-HLA Ⅰ antibodies (HLA-A and -B loci) most likely result in T cell-positive FC CM results (positive predictive value 97%). Furthermore, MFIs ⩾ 3100 produced by anti-HLA Ⅱ antibodies (HLA-DRB1 and -DQB1 loci) most likely generate B cell-positive FC CM results (positive predictive value

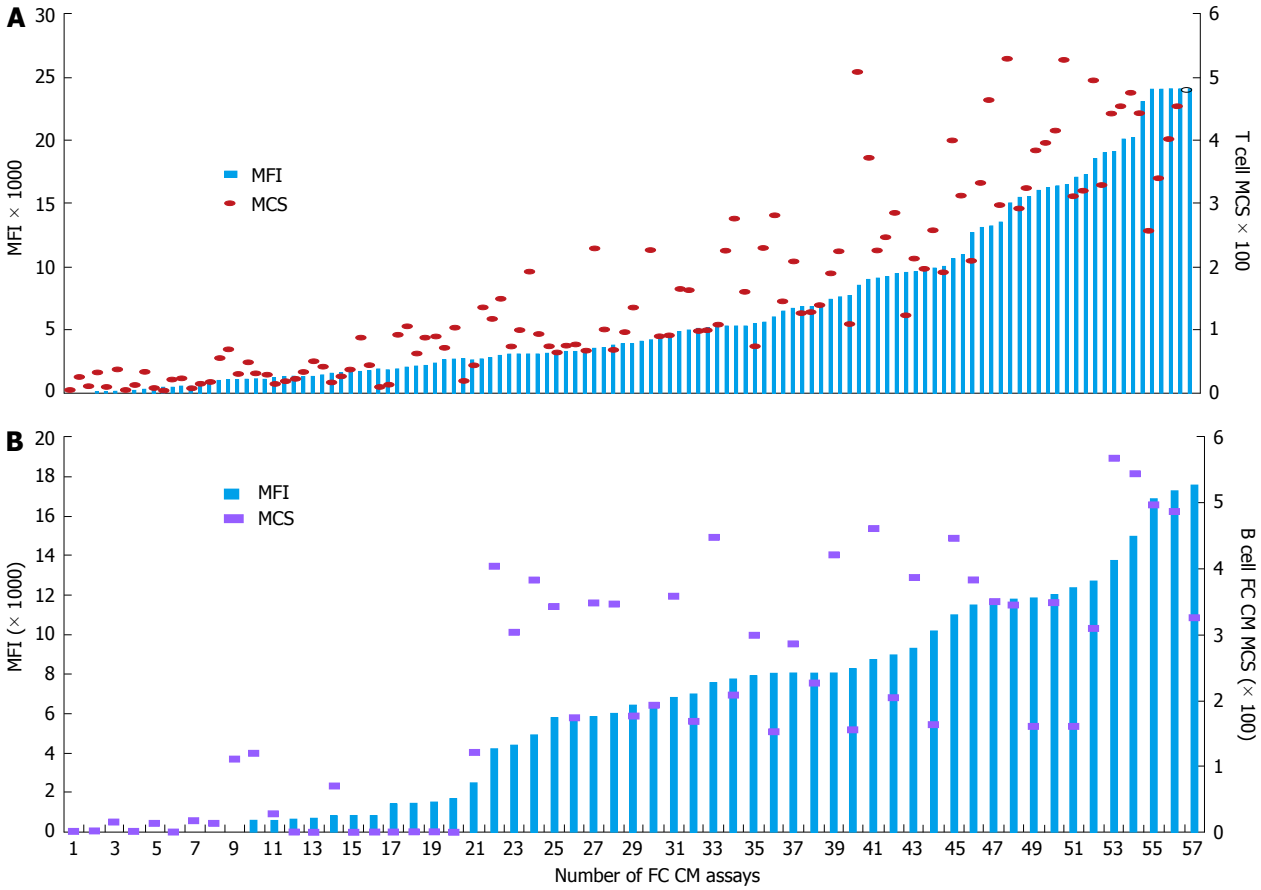

Figure 1 Concordance of mean fluorescence intensity values of monospecific anti-human leukocyte antigen class Ⅰ (A) and class Ⅱ (B) antibodies and results of flow cytometry cross match assay. Left Y axis indicates MFI values of antibodies, right Y axis indicates MCS for T- and B-cells FC CM assay, respectively. Blue bars indicate class Ⅰ and class Ⅱ MFI values, respectively; red circles and purple squares indicate MCS values of each FC CM test for T and B lymphocytes, respectively. MCS: Median channel shift; MFI: Mean fluorescence intensity; FC: Flow cytometry; CM: Cross match.

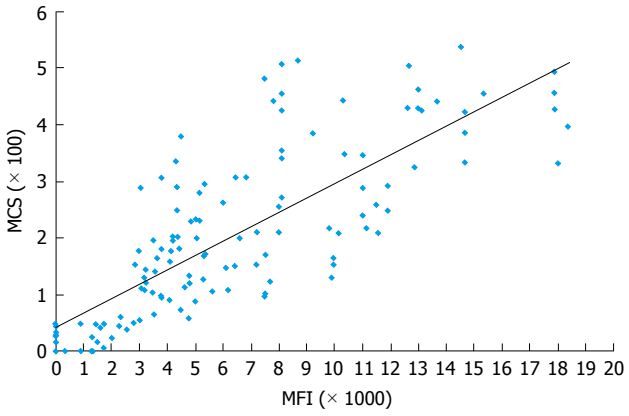

Figure 2 Correlation analysis between mean fluorescence intensity of anti-human leukocyte antigen class Ⅰ antibodies and T cell flow cytometry cross match median channel shift. Each blue diamond represents a single test. MCS: Median channel shift; MFI: Mean fluorescence intensity.

95%, R = 0.78)[93] (Figures 1 and 2). The positive cutoffs determined in this assay also predict positive FC CM results when the recipient has multi-specific DSAs.

Quantities of HLA proteins on the beads

The density of the HLA molecules on the beads is significantly higher than that on lymphocytes (5×10^4-10^5 molecules per cell) or endothelial cells. Therefore, even a minor admixture of anti-HLA antibodies or anti-idiotypic antibodies of the IgM isotype may cause false negative results. To overcome this obstacle, serum Dithiothreitol (DTT)-treatment is recommended[108-110].

Table 1 Donor specific antibodies solid phase single-antigen Luminex analysis in unmodified and Dithiothreitol treated serum

Serum treatment	HLA specificities		
	B8	B18	DR53
DDT treated	24777[1]	23500	23100
Untreated	550	1764	4852

[1]The numbers in the table indicate MFI values. HLA: Human leukocyte antigen; MFI: Mean fluorescence intensity; DDT: Dithiothreitol.

We have observed strong positive T cell FC CM results and weakly reactive DSAs to HLA-B8 by SA Luminex. Subsequent DTT treatment of the serum resolved this discrepancy and revealed DSAs to the antigen with MFI values of 24000 (Table 1).

Denatured HLA and cryptic epitopes

Discrepancies between negative FC CM results and strongly reactive DSAs are observed when biochemical

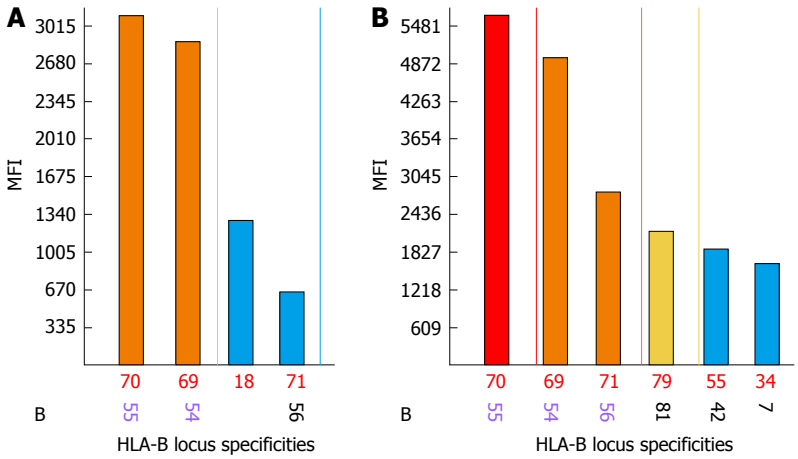

Figure 3 High affinity antibodies against denatured human leukocyte antigen-B55 and human leukocyte antigen-B54 proteins. A: Acid untreated sample; B: Acid treated sample; Y axis indicates MFI values of the antibodies; X axis indicates HLA-B locus specific antibodies. Colored bars represent MFI values of different intensity: Blue 500-2000, yellow 2001-3000, brown 3001-5000, and red > 5000. HLA: Human leukocyte antigen; MFI: Mean fluorescence intensity.

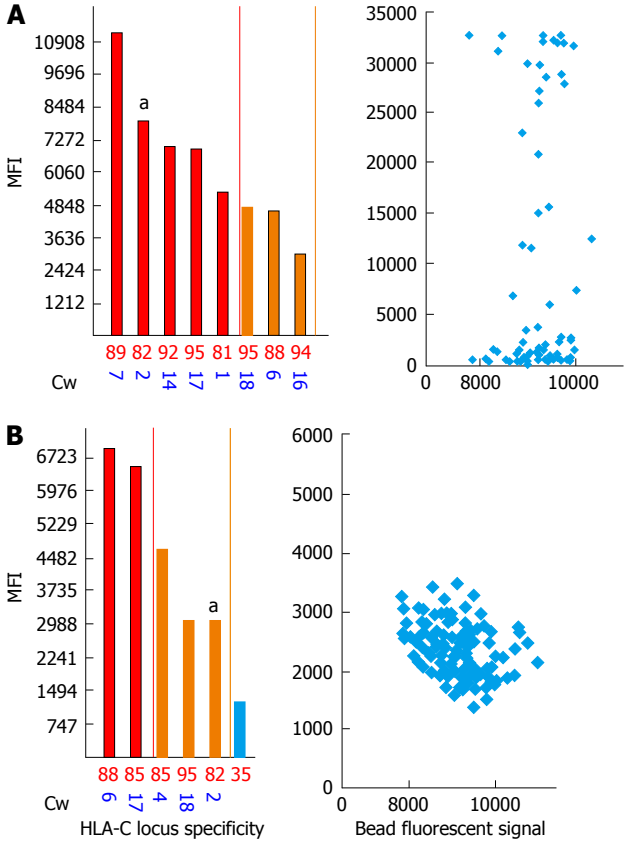

Figure 4 Uneven bead (bead #82) distribution results in falsely positive reactivity of antibodies to human leukocyte antigen-C2. [a]Anti-HLA-C2 antibodies MFI values in original (A) and repeated (B) samples, respectively. Left panels in figure A and B represent HLA-C locus specific antibody analysis, whereas right panels represent beads distribution on the basis of their fluorescence and MFI values produced by bound antibodies. Colored bars represent MFI values of different intensity: Blue 500-2000, yellow 2001-3000, brown 3001-5000, and red > 5000. Blue diamonds represent beads coated with HLA-C2 proteins. HLA: Human leukocyte antigen; MFI: Mean fluorescence intensity.

modification (denaturing) of HLA proteins during the bead conjugation process occurs. Denatured HLAs have higher affinities for DSAs and when present in large amounts they produce strong false positive signals. An acid treatment is used to exclude antibodies against denatured HLAs. Antibodies against denatured HLA-B55 and HLA-B54 proteins are likely present when no differences or increased MFI values are observed between treated and untreated beads[110-113] (Figure 3). The acid treatment procedure is ineffective when analyzing anti-class Ⅱ antibodies. In these types of situations, Luminex screening tests or Luminex class Ⅱ phenotypic bead assays are performed.

Uneven bead distributions on SA Luminex dot blot histograms

Strong DSAs to HLA-C2 in the recipient serum have been shown to yield negative FC CM results[114] (Figure 4). To investigate this discrepancy, we analyzed the bead counts and bead distributions on the SA dot blot histograms of the aforementioned bead sets. Figure 4A shows the MFI values of 13100 for the anti-HLA-C2 antibodies and an uneven bead distribution. As shown in the figure, a majority of the beads are located within the negative MFI range and only a few have MFI values of approximately 30000. These findings indicate that the MFIs observed on SA antibody panels represent an average number between the lowest and highest values. A repeat of the assay confirmed the absence of DSAs, and the SA dot blot C2 histogram formed single clusters of beads located in the negative area (Figure 4B).

Non-specific antibody reactivity

One of the limitations of SA SP Luminex technology detailed in the literature is non-specific reactivity[90,106]. The main reasons for high background levels may be related to antibodies reacting to latex, autoimmune disease(s), and some medications (IVIG)[75,115-117]. Non-specific antibody binding complicates antibody analysis due to the appearance of multiple false positive antibodies and may affect organ allocation and the interpretation of VCM

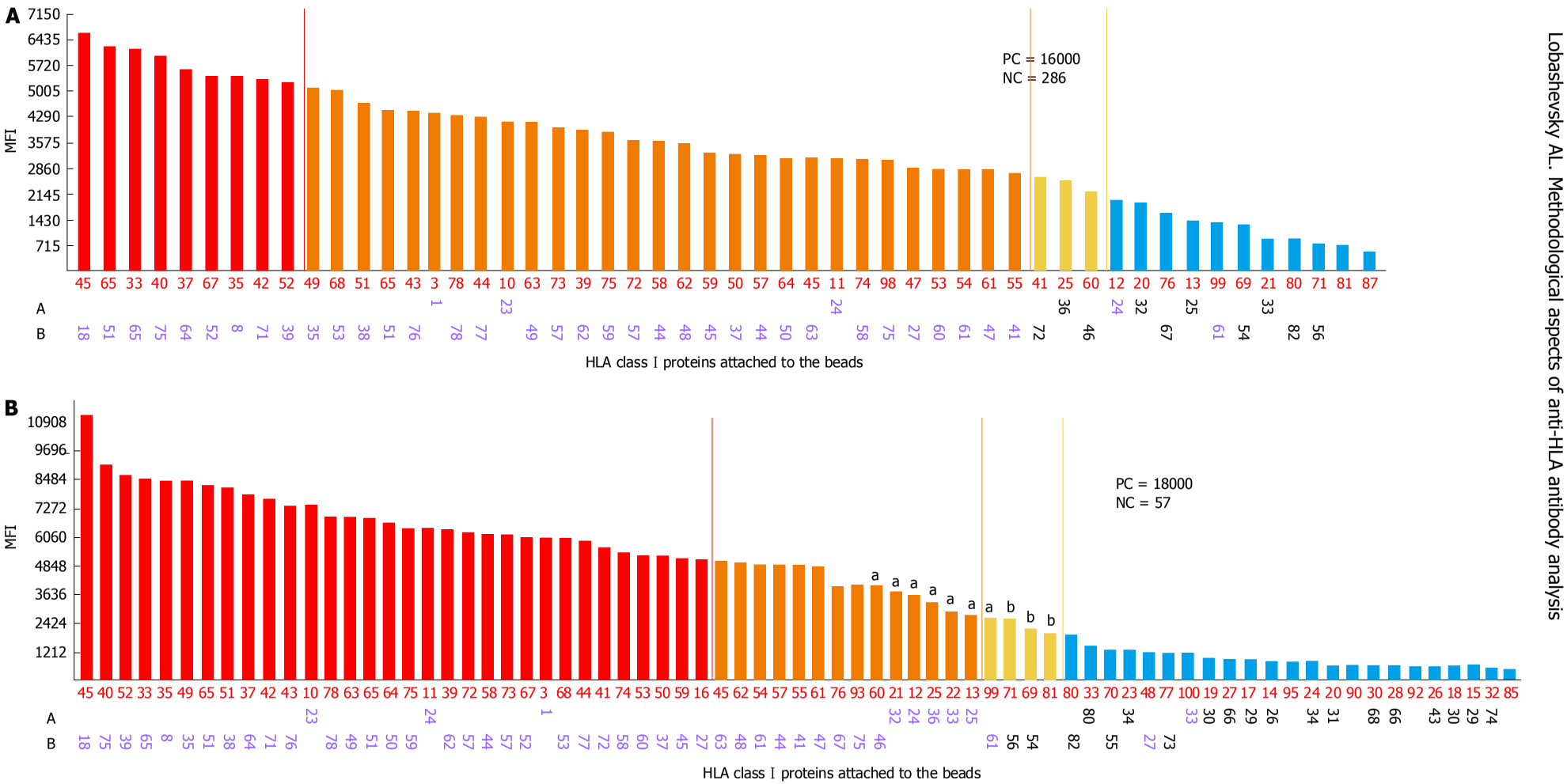

Figure 5 Anti-Class I antibodies detected in unabsorbed (A) and absorbed (B) serum samples. The numbers on the right indicate control values before and after absorption; [a]Additional antibody specificities with MFI ⩾ 2600; [b]Additional antibody specificities with MFI = 1500-2600; HLA: Human leukocyte antigen; MFI: Mean fluorescence intensity; NC: Negative control MFI values; PC: Positive control MFI values; Y axis indicates MFI values; X axis indicates bead number and HLA-A and -B loci specificities. Colored bars represent MFI values of different intensity: Blue 500-2000, yellow 2001-3000, brown 3001-5000, and red > 5000.

results. The reagent Adsorb Out™ is manufactured by One Lambda and consists of microparticles designed for serum pre-incubation. This product reduces or removes strong background signals due to non-specific binding. Figures 5 and 6 demonstrate the additional seven anti-Class I and two anti-Class Ⅱ antibody specificities that are associated with increasing the MFI (⩾ 2600 for anti-Class I , and ⩾ 3100 for Class Ⅱ) and decreasing the negative control MFI values from 286 to 57 and from 230 to 60 for class I and class Ⅱ antibodies, respectively. Additionally, the number of weakly reactive anti-Class I (MFI = 1500-2600) and anti-Class Ⅱ (MFI = 1500-3100) antibodies also increased from nine to eleven and from two to three, respectively. Accounting for the MFI values of the antibodies to self-HLA also improves the TC antibody profiles. The high MFI values for the

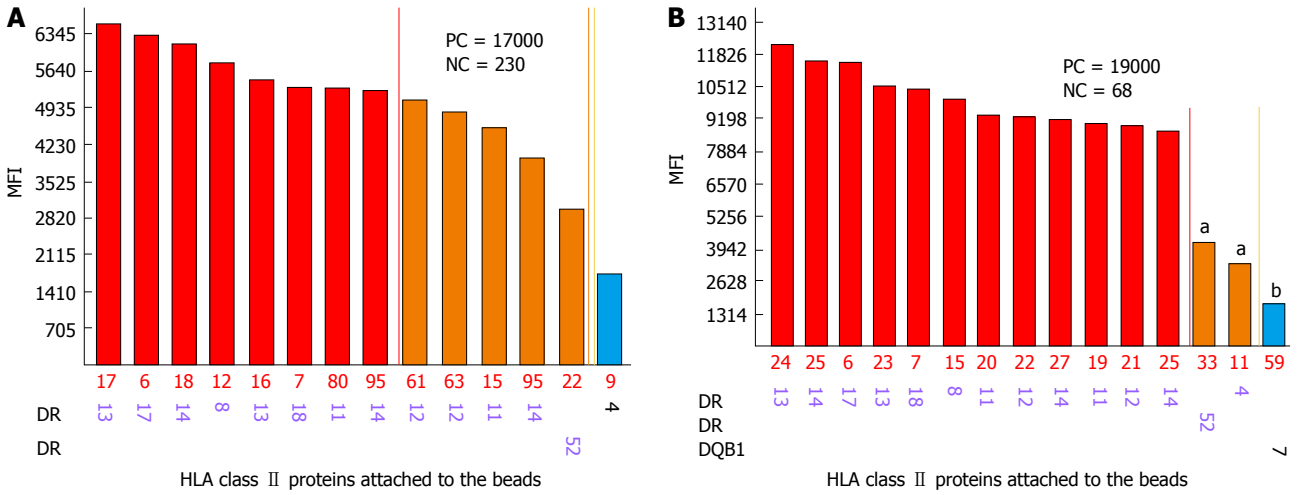

Figure 6 Anti-Class II antibodies detected in unabsorbed (A) and absorbed (B) serum samples. The numbers on the right indicate control values before and after absorption; [a]Additional antibody specificities with MFI ⩾ 3100; [b]Additional antibody specificities with MFI = 1500-3100; MFI: Mean fluorescence intensity; NC: Negative control MFI values; PC: Positive control MFI values; Y axis indicates MFI value, X axis indicates bead number and HLA-A and -B loci specificities. Colored bars represent MFI values of different intensity: Blue 2001-3000, brown 3001-5000, and red > 5000.

negative control (MFI > 100) usually indicate high background and require the test to be repeated. However, if serum samples taken at different time points consistently exhibit elevated non-specific reactivity after adsorption then antibody analysis can be performed appropriately[118]. An unusual anti-class Ⅱ antibody profile was observed in one kidney TC. The serum of this patient contained pan-reactive anti-DRB1 antibodies including self-specificities (Figure 7; the self-antigens are circled in red). A subsequent autologous FC CM assay was B cell positive and Luminex SP screening PRA analysis did not detect any antibodies. The results of these tests led to the conclusion that the anti-DRB1 antibodies in the serum of this TC serum were clinically irrelevant.

Bead oversaturation

The sera from patients may contain strong high-titer antibodies that can cause a prozoning or oversaturation effect. Figure 8 shows the oversaturation of SA beads with antibodies to HLA-I. In our experience this phenomenon is suspected when the MFI values exceed 20000. As shown in Figure 8, the antibodies against A2, A69, B51, and B52 exhibited oversaturation that disappeared upon the dilution of the serum. This prozoning effect has been reported by others with the SA Luminex bead assay[119-123]. Dilution of the serum to exclude the oversaturation of antibodies is critical when the antibody analysis is performed on the sera of highly sensitized TCs who have been subjected to immunomodulation/desensitization.

component 1q SA SP binding assay

The classical pathway of complement activation following antibody/HLA interactions is usually associated with graft cell damage and poor outcomes. Over the last decade it has been demonstrated that some of the DSAs detected with SA SP analysis but not with CDC (FC pos/CDC neg) can activate the complement system[124-128]. Numerous studies have demonstrated that inferior graft outcomes, graft loss, and C4d deposition are observed more frequently among recipients with DSAs that bind complement component 1 (C1q)[129-135]. The recently developed C1q SA SP assay (C1qScreen™) represents a reliable tool for distinguishing the binding IgG antibody from the complement-fixing antibodies of the IgG and IgM isotypes. The complement-fixing antibodies (C1q+) are detected using external C1q and anti-C1q antibodies conjugated to PE. The fluorescence intensity of the signal is proportional to the amount of bound C1q and is measured by MFI. The presence of C1q+ DSA is more strongly correlated with graft failure than the presence of antibodies that do not bind complement[126,136-139]. However, the absence of complement fixing antibodies has recently been reported in recipients with documented AMR. This result may indicate a low sensitivity of the C1q assay. The elegant studies of R. Liwski have demonstrated a good relationship between the anti-human globulin (AHG)-C1qScreen™ assay and CDC-AHG reactivity[128,129]. This highly sensitive modification of the original C1qScreen™ protocol would certainly be useful for risk assessment.

SA SP Luminex-based methodology and structural analysis of HLA epitopes

Each HLA protein represents a linear sequence of amino acid residues (AAR) or triplets, and the degree of mismatch is assessed as the number of triplets that are not shared between the donor and the recipient. There are two important points regarding this approach. First, only the AARs accessible to antibodies that reside in α-helical coils and β-loops are considered. In contrast, the triplets that are located in the β-pleated floor and beneath the α-chains are not available for antibody binding and are often not critical for antibody production because they are not immunogenic[140-143]. Second, alloantibodies can be

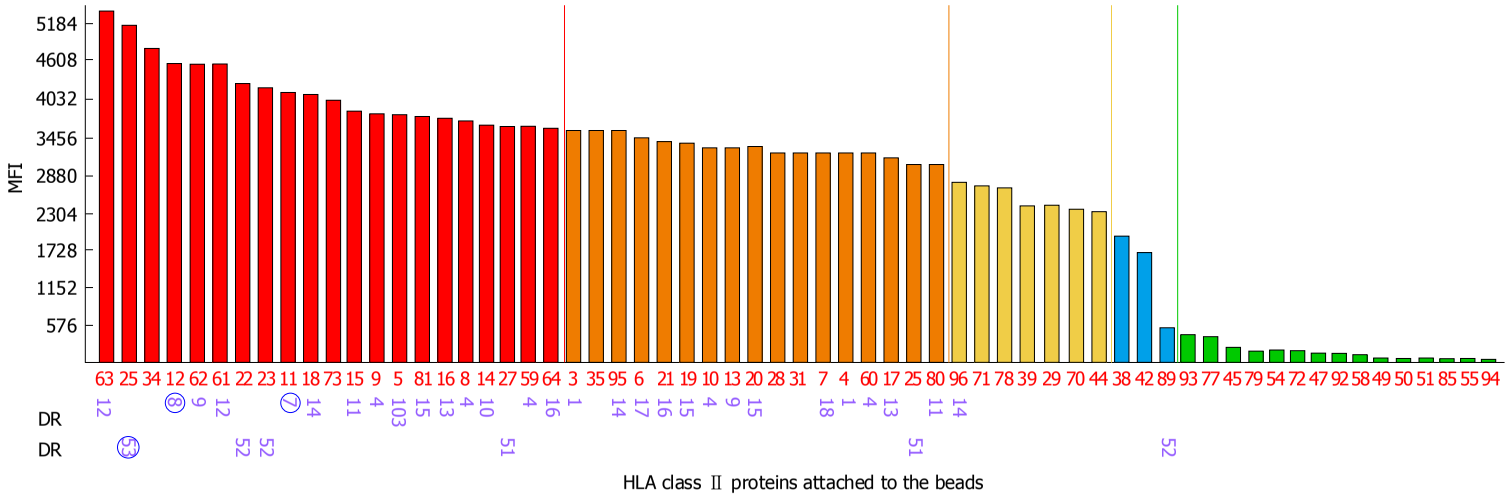

Figure 7 Pan-reactive anti-human leukocyte antigen-DRB1 antibodies detected in kidney transplant candidate serum. Y axis indicates MFI values, X axis indicate beads number and HLA-DR specificities; HLA-DR specificities in the blue circles are self. Colored bars represent MFI values of different intensity: Green < 500; blue 501-2000; yellow 2001-3000; brown 3001-3500; red > 3500. HLA: Human leukocyte antigen; MFI: Mean fluorescence intensity.

Table 2 Epitope analysis of human leukocyte antigen recipient developed antibodies to "antigens"

HLA	MFI	Reactive eplets/pair of eplets	Number of mismatched eplets	Mismatched eplets	Comments
B*07:02[a]	1689	44re	5	44re[b], 65qia, 70iaq, 113hd, 177dk	44re + self 69at
B*45:01[a]	1433	167es	1	167es[b]	
B*44:03	1398	167es	5	76ent, 79rt, 94ii, 167es, 199v	
B*82:01	1077	44re, 167es	4	44re, 65qia, 70iaq, 167es	
B*44:02	841	167es	5	76ent, 79rt, 94ii, 167es, 199v	
C*07:02	657	44re, 167es	5	44re, 65qia, 70iaq, 94ii, 167es	
B*81:01	1222	44re	4	44re, 65qia, 70iaq, 254ta	44re + self 69at
B*27:08	1130	44re	3	44re, 65qia, 71ka	44re + self 69at
B*56:01	1059	44re	3	44re, 65qia, 71ka	44re + self 69at
B*55:01	837	44re	3	44re, 65qia, 71ka	44re + self 69at
B*42:01	761	44re	3	44re, 65qia, 71ka	44re + self 69at
B*67:01	681	44re	4	44re, 65qia, 70iaq, 158t	44re + self 69at
B*27:05	607	44re	5	44re, 65qia, 71ka, 76 edt, 79rt	44re + self 69at
B*73:01	327	44re	6	44re, 65qia, 71ka, 103 m, 267qe, 275kp	44re + self 69at
C*06:02[a]	379	44re, 167es	5	44re, 65qia, 70iaq, 94ii, 167es	
B*08:01	2046	44re	1	44r	44re + 69tn
B*15:12 (B76)	1294	ND[c]	3	45rma, 113hd, 163lg	Unexplained

[a]Alleles in bold font are mismatches with the 1st donor; [b]Reactive eplets; [c]Not determined. HLA: Human leukocyte antigen; MFI: Mean fluorescence intensity.

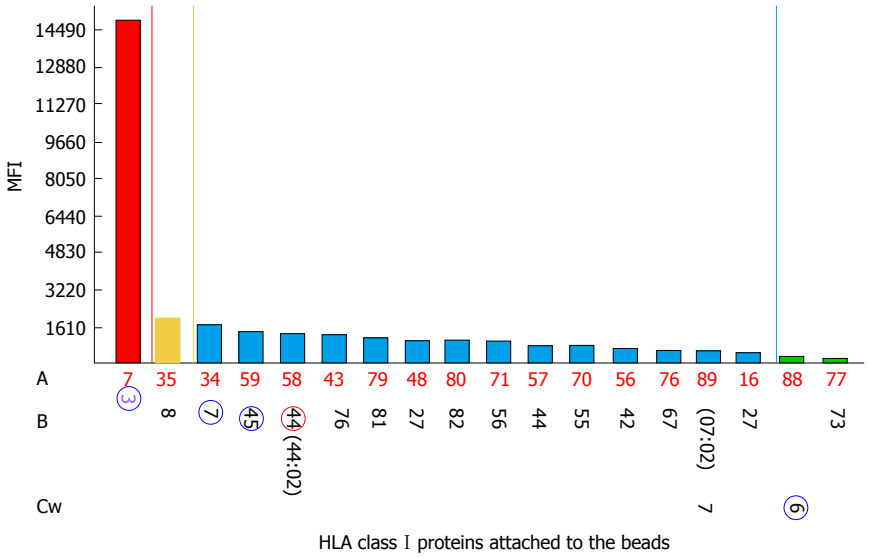

Figure 9 Anti-Class I antibodies detected in the serum of kidney transplant candidate C. The numbers in blue circles indicate HLA specificities mismatched with the 1st donor. The number in red circle indicates HLA specificity mismatched with the current donor; the numbers in parenthesis indicate allelic assignment of HLA. Y axis indicates MFI values, X axis indicates bead number and HLA-A and -B loci specificities. Colored bars represent MFI values of different intensity: Green < 500; blue 501-1600; brown 1601-2500; red > 2500. HLA: Human leukocyte antigen; MFI: Mean fluorescence intensity.

Table 3 Human leukocyte antigen Class I typing results of the recipient and previous and current donors

	A locus		B locus		C locus	
Recipient	23:01	66:01	41:01	49:01	07:01	17:01
1st donor (immunizer)	03:01[a]	26:01	07:02	45:01	06:02	07:01
Current donor	23:01	24:01	44:02	49:01	03:03	07:01

[a]Mismatched alleles are given in bold font.

produced only against non-self-mismatched triplets.

Furthermore, AAR triplet analysis (HLA Matchmaker computer algorithm) can explain or predict the development of post-transplant antibodies in kidney allograft recipients[46,50]. Subsequent analyses of patients' antibodies and HLA-specific monoclonal antibodies have revealed that each HLA consists of structurally defined "eplets" that represent epitopes comprised of the AARs within a 3 Å-5 Å radius of the surface of the molecule[48,10,122]. An example of such analysis is presented in Figure 9, Figure 10 and Table 2. The HLA typing results of the recipient, previous donors, and current donors are given in Table 3. The pre-transplant evaluation of the second kidney TC revealed multiple weakly reactive (MFI cutoff ⩾ 2600) antibodies including DSA B*44:02 (Figure 9) and strongly positive T cell FC CM results (ΔMCS = 129, positive cutoff = 50) (the autologous T cell FC CM results appeared to be negative). A HLAMatchmaker analysis of the HLA-B locus-specific antibodies determined that the antibody reactivity was restricted to two epitopes/eplets 44re and 167es on the immunizing HLA-B*45:01antigen. FC CM and SA bead assays revealed that the antigens targeted by the recipient antibodies shared the eplet pair 44re69at. Eplets that were mismatched with the immunizer were 44re and 167es, while 69at was shared by both

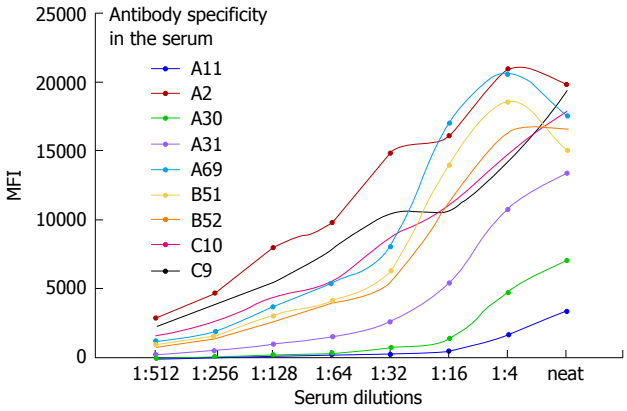

Figure 8 Phenomenon of class I bead oversaturation with antibodies. Y axis indicates MFI values, X axis indicates serum dilution. Ten percent fetal bovine serum in roswell park memorial institute medium was used as diluents. MFI: Mean fluorescence intensity.

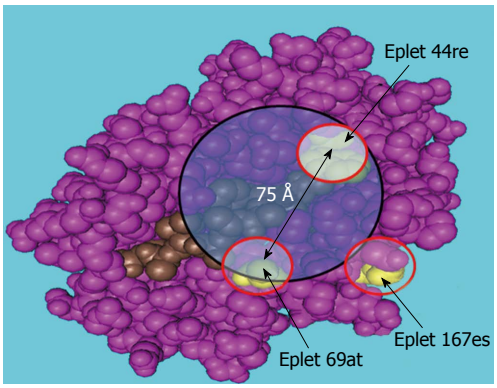

Figure 10 Three dimensional map of mismatched and shared eplets. Alpha chain domains are in purple; peptide in the antigen presenting groove is in brown color; eplets are in yellow; black transparent circle specifies the patch comprising two eplets.

the donor's B7 and the patient's A23 and A66 (Figure 10).

The identification of immunogenic epitopes significantly affects the prediction of post-transplant alloantibody specificities, donor selection, graft outcome, and organ allocation. The recent studies of Zeevi *et al*[142] and Duquesnoy *et al*[143] used monoclonal antibodies to demonstrate the anti-HLA antibody complement-fixing abilities strictly depend on the configuration of the critical contact eplet(s)[140,141]. The results of their studies indicated that complete complement cascade activation is determined by the energy produced from the antibody-HLA interaction. The amount of this energy should be sufficient to induce conformational changes of the constant region of the antibody to elicit C1q binding and subsequent component activation. The authors hypothesized that the binding energies of the SA C1q-negative antibodies are insufficient to induce conformational changes in the constant region. However, in the cases of the C1q+ and CDC+ antibodies this energy is sufficient to trigger complete complement activation and cell membrane damage[141,143].

CONCLUSION

The identification of anti-HLA antibodies in TC serum is a major task of HLA laboratories and transplant physicians and is important for graft failure risk assessment and donor selection. Furthermore, antibody detection is critical in highly sensitized TCs who have been subjected to desensitization (immunomodulation). SA SP analysis is a highly sensitive and highly specific method of antibody characterization that enables the detection of low concentrations of antibodies and their fine HLA specificities. However, this assay is not free from limitations including large variation in the numbers of HLA molecules per bead and the effects of manual (*i.e.*, technologist-to-technologist) factors on assay variance. In this review, I attempted to share multiple years of experience performing SA SP assays for pre- and post-transplant antibody analyses in my laboratory and address some pitfalls and caveats of this assay. Evaluations of the clinical significance of anti-HLA antibodies should undeniably include their concentrations, isotypes, ability to fix the complement, and fine epitope specificity.

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