Detection of antibodies to denatured Human Leucocyte Antigen molecules by Single Antigen Luminex

Short title: Antibodies to denatured HLA by Luminex

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Figures:2

Appendices: 3 tables and 2 figures

Abbreviations

Ab, antibody

ABMR, antibody mediated rejection

AM, manufacturer A

AT1R, angiotensin II type 1 receptor.

B2M, beta 2-microglobulin.

BM, manufacturer B.

CDC, complement-dependent cytotoxicity.

dHLA, denatured-HLA.

DSA, donor-specific antibodies.

EDTA, ethylenediaminetetraacetic acid.

FCXM , flow cytometry cross-match.

HLA, human leukocyte antigen.

MFI, mean fluorescence intensity.

MICA, MHC class I chain-related sequence A.

nHLA, native-HLA.

PBMCs, peripheral blood mononuclear cells

SA, single antigen.

SAB, single antigen bead.

SSP, sequence specific primer.

Abstract:

Introduction: The anti-HLA antibody detection has been improved in sensitivity and specificity with solid-phase antigen bead (SAB) assays based on Luminex. However, false positive results due to denatured HLA may arise after single antigen test. The aim of this study was to compare the performance of the two Luminex technology-based anti-HLA detection kits available in the market in revealing undesired anti-HLA antibody results.

Methods and materials: A prospective cohort was assessed for anti-HLA antibodies with single antigen A manufacturer (AM) kit and a comparison cohort with single antigen B manufacturer (BM) kit.

Results: A total of 11 out of 90 patients in a prospective cohort presented monospecific HLA-I antibodies with AM, and 5 out of 11 confirmed monospecific reaction with BM. Despite the confirmation of monospecific reaction with both manufacturers, 80% were assigned as denatured HLA reaction by specific crossmatch. Further comparative cohorts detected 4 out of 6 monospecific reactions with BM that were confirmed as possible denatured HLA reactions.

Conclusions: A positive SAB test should rule out a reaction against a denatured HLA molecule, thus avoidance of prolonged waitlist periods or misattribution of anti-HLA reactions after transplantation.

Keywords: HLA antigens / immunology; Antibodies / analysis; Flow cytometry / methods; Microspheres; Cytotoxicity Tests, Immunologic / methods; Histocompatibility testing / methods.

1. Introduction

Anti-HLA antibody (Ab) testing experienced a major breakthrough with the development of solid-phase assays based on Luminex technology, increasing the sensitivity and enabling the detection of low levels of HLA-specific Abs [1]. Despite this, several potential pitfalls should be taken into account regarding Luminex anti-HLA Ab detection [2]. Three different kinds of errors could appear. First, there are false positive results due to Ab binding to misfolded HLA proteins. Thus, denaturing of HLA antigens during the processes of purification and bead coating could lead to the generation of neo-epitopes that under physiological circumstances would be inaccessible for Abs. Second, lack of cut-off consensus between laboratories and wide intra- and inter-assay variability could also result in false positive results. Finally, the prozone effect is a widely accepted source of false negative results [3-7].

Two types of panels depending on the composition of their target antigen are available. The screening panel has two different bead groups, coated with either HLA class I or class II protein molecules obtained from multiple individual cell lines, and are used as a screening test for the presence or absence of anti HLA class I/II Abs. However, they

do not provide accurate information about Ab specificity. The single-antigen beads (SAB) panel has multiple beads, each one coated with a unique protein molecule representing a single cloned HLA antigen which enables the identification of any given antibody specificity, and provides the highest degree of anti-HLA Ab resolution. However, denatured beta 2-microglobulin (B2M)-free heavy chains and structurally different HLA-class-I molecules are present in a single bead which could lead to false positive results [8], whereas denatured HLA molecule cannot be ruled out in screening panels [9,10].

The denatured HLA (dHLA) reaction has been detected not only in sensitized transplant patients but also in unsensitized males [10]. The presence of denatured HLA reactions was detected in more than 30% of sensitized patients [11]. However there are few reports assessing the clinical impact of the presence of dHLA in the transplant setting [12,13,14].

The main objective in anti-HLA Ab testing should be the detection of Abs against a native HLA molecule. Two manufacturers provide anti-HLA Ab tests based on Luminex technology. Several works have raised differences between them. Some authors have identified a greater proportion of denatured HLA-I antigens on the surface of beadsets in One lambda SAB kits, whereas Immucor SAB kits present most of its antigens in the native trimeric form composed of HLA-I heavy chain, B2M and peptide, as evidenced by monoclonal Ab binding [15]. The main goal of this study was to analyze

and compare the performance of the two Luminex technology-based anti-HLA detection kits on revealing unexpected anti-HLA antibody results.

2. Materials and Methods

2.1. Patient selection

The anti-HLA antibody test is performed within routine sampling in patients while listed and after solid organ transplant monitoring. The patients gave their written consent once included on the waiting list. The study was approved by the Local Ethics Committees and conducted in accordance with the Declaration of Helsinki.

2.1.1. Prospective cohort

A total of 90 serum samples with negative results after anti-HLA Ab screening assay (manufacturer A) were recruited for the study during one year in University Hospital Marqués de Valdecilla (Santander, Spain). Forty out of 90 (44.4%) sera came from solid organ transplant candidates newly included in the institution's waiting list whereas 50 (55.5%) sera came from solid organ transplant patients followed post-transplant within one year. A subsequent assessment of samples rendered negative after screening but positive on SAB assay results were selected for further analysis (i.e., acid treatment of the beads, CDC and FCXM as described below). Finally, 11 sera with confirmed negative screening results but positive monospecific anti-HLA class-I results were selected. Their main demographic and immunological parameters

are summarized in Appendix Table 1. The 11 samples were chosen in order to facilitate the selection of donor HLA-typed cells for testing each monospecific HLA reactive sera.

In order to elucidate if such findings may be differentially affecting the performance of the kits on detecting unexplained anti-HLA results on SAB assays, we reanalyzed them with B manufacturer's anti-HLA Ab kit.

2.1.2. Comparison cohort

Serum samples from 6 patients with confirmed negative anti-HLA screening and positive anti-HLA class I monospecific reaction in SAB assay (manufacturer B) not explained by previous sensitization events were selected in the Clinic Hospital (Barcelona, Spain) and reanalyzed using AM kit. The main demographic and immunological parameters of the patients for potential allosensitization are summarized in Appendix Table 2.

An overview of the study is detailed in Appendix Figure 1.

2.2. Anti-HLA antibody test

The presence of anti-HLA Abs was tested by using screening and SAB assays from the only two manufacturers available as described in the text (LABScreen Mixed, Cat LSM12, Lot: 020; LABScreen Single Antigen Class I, Cat LS1A04, Lot: 010; and LABScreen Single Antigen Class II, Cat LS2A01, Lot 012, One Lambda, Canoga Park, CA, United States) referred as A manufacturer; and (Lifescreen Deluxe, Cat LMX, Lot

3006878; LSA class I, Lot 3006346 and LSA class II, Lot 3006494, Immucor, Gateway Drive, GA, United States) referred as B manufacturer and following manufacturers' instructions; and analyzed on a Luminex platform (LabScan100).The cut-off value was set at 500 baseline MFI for screening and 1,500 baseline MFI for SAB test . Such a difference in the cut-off is intended to minimize false negative results in anti-HLA Ab screening and false positive results in SAB assays. We considered sera with values between 1,500 and 3,000 baseline MFI as weakly positive.

2.3. In vitro anti-HLA reactivity against denatured vs. native HLA-molecule

In order to decipher the reaction against denatured (dHLA) or native HLA-molecule (nHLA), in samples analyzed with AM kit rendering negative screening but positive SAB results, acid treatment of the SAB beadsets was performed as previously described [11]. Briefly, the beads were treated with 1 mL of 1% bovine serum albumin and 0.1M glycine buffer (pH=2.8) during 30 minutes, then washed twice with the wash buffer provided by manufacturer prior to sera incubation and subsequently followed the manufacturer´s instructions. The denatured beads were confirmed with anti-w6/32-phycoerythrin monoclonal antibody (Biolegend, San Diego, CA), a representative example of acid treatment of the beads is shown (Appendix Figure 2). Because there is no consensus for a cut off value of MFI after acid treatment, we decided to set at 1,500 MFI. Values between 1,500 and 3,000 MFI were considered as weakly positive. The MFI values pre and post acid treatment are described in Appendix Table 3.

2.4. Ex vivo anti-HLA reactivity

Cytotoxic (CDC) and flow cytometry cross match (FCXM) were performed when possible in samples with negative results for screening while positive SAB results using A manufacturer kit against reactive HLA typed cells. CDC was conducted as previously described[16]. For FCXM, peripheral blood mononuclear cells (PBMCs) isolated by Ficoll gradient were incubated with rat serum (Sigma Aldrich, St. Louis, MO) during 20 minutes to avoid unspecific binding (Cytometry A. 2016;89(11):1001-1009). Undiluted and 1/10 diluted serum was added to PBMCs and incubated during 30 minutes at room temperature. Anti-CD3 ECD (clone UCHT-1, Beckman Coulter, Brea, CA), CD19 PC5 (clone J3-119 Beckman Coulter, Brea, CA) and subsequently Fab´-IgG FITC (Immunostep, Salamanca, SPAIN) were added and samples were acquired using a Navios EX Flow Cytometer (Beckman Coulter, Brea, CA). Flow cytometry crossmatch for class-I antigens was considered positive in the CD3+ gate when the ratio of median fluorescence value serum / median fluorescence value negative control was > 1.5; for class-II antigens, the FCXM was considered positive in CD19+ gate when the ratio was > 2.0.

The HLA typing of the donors was performed for HLA *loci* A, B, DRB1, DQA1 and DQB1 by high resolution SSP (Life Technologies, Brown Deer, WI) to confirm reaction against nHLA.

2.5. Statistical analysis

Kolmogorov-Smirnoff test was used to reveal if the data of baseline MFI followed a normal distribution. The Mann Whitney test was used to compare median baseline

MFI. All tests were performed using GraphPad software version 7.0 (San Diego, CA). The R software and “ggplot2” package have been used for graphics.

3. Results

3.1. Single Antigen positive reactions against denatured or native HLA molecules

A total of 21 patients from a prospective cohort (23.3%) showed positive SAB results (both in HLA class-I and class-II specificities) with negative screening test result. For further analysis we focused only on the 11 patients (9.9%) with positive reactions only against HLA class-I for simplicity. Five out of 11 (45.4%) were transplanted patients, although none of the monospecific reactions were donor-specific. Of note, only two samples were from unsensitized males. A summary of potential sensitization of the patients is summarized in appendix Table 1. Upon acid treatment the HLA antigens attached to the beads were denatured; a negative result after SAB of acid-treated beads would suggest a native-HLA (nHLA) reaction. However, a total of 11/11 (100%) patients had positive reactions after bead-treatment (Figure 1), so denatured-HLA (dHLA) reaction could not be ruled out. Moreover, to confirm the reactivity of the anti-HLA Abs, both CDC and FCXM against a limited number of specific HLA-typed cells were performed. As expected, all the samples with potential anti-dHLA reactions that could be tested in crossmatch rendered both CDC and FCXM negative results, summarized in Tables 3 and 4.

3.2. Comparison of baseline MFI from screening vs single antigen test

The SAB test was performed with sera from a prospective cohort with a previous negative result on screening test. Once a positive result in HLA class-I was found, we calculated the mean baseline MFI of the bead groups where the positive HLA antigen was included (i.e. A*11:01 is present in beads #008, #014, #015 and #016 in the screening full panel of Lot 020 from One Lambda and in bead#CLI02, #CLI04, #CLI05, #CLI06 panel of Lot 3006878 from Immucor). The Figure 2 describes the MFI values obtained after screening and Single Antigen test of the sera.

3.3. Confirmation of non-concordant results with B manufacturer's kit

In order to clarify the monospecific reactions observed with A manufacturer (AM), 11 serum samples of patients with confirmed negative screening results and positive anti-HLA class I monospecific reactions with AM kit were selected for further analysis using B manufacturer's (BM) kit. Seven out of the 11 serum samples (58.3%) continued displaying negative screening but positive anti-HLA Class I SAB assay results, although a significant reduction of median (interquartile range) baseline MFI levels (AM vs BM) 3,877 (3,633-6,101) vs 1,405 (100-2,555), was observed ($p<0.0001$). In this comparative study the anti-HLA reactions of samples rendering negative crossmatch results were considered to be directed against denatured HLA antigens on the beads. The comparative results between the two manufacturers are summarized in Table 1.

3.4. Monospecific reactions observed with B manufacturer- comparison cohort

Six serum samples from the comparison cohort were found to repeatedly show negative screening but positive monospecific anti-HLA Class I SAB assay results when analysed employing BM kit. One out of six (16.7%) confirmed such results by analysis using AM kit, while the other five rendered negative results in both screening and SAB assays with AM kit. The comparative results between the two manufacturers are summarized in Table 2.

4. Discussion

The anti-HLA Ab test is mandatory prior to any solid organ transplantation in order to quantify the level of allosensitization. The SAB-assay itself has several pitfalls and a proper monitoring of anti-HLA Abs should be performed while the patient is included on the waiting list. The guidelines indicates monitoring every 3 months in all solid organ waiting list patients [17]. Patients that have lost a previous transplant, women potentially sensitized due to pregnancy or abortions and transfused patients are potential candidates to identify reactions against HLA antigens. Our idea was to better identify this kind of patients by performing directly SAB assay as first option instead of anti-HLA Abs screening. However, an increased number of missassigned-HLA specificities could lead to unacceptable mismatches with potential donors and prolonged periods on waiting list. Moreover, testing anti-HLA Abs by SAB assays in transplant monitoring could assign a patient with donor-specific anti-HLA antibodies (DSA), when the

identified antibodies could actually be directed against dHLA, with uncertain impact on the allograft. The interference with complement and prozone effect could be avoided with serum EDTA-treatment.

Deciphering the reactivity of the anti-HLA Abs between denatured or native HLA would help in the identification of deleterious anti-HLA Abs, with different implications if on waiting list or post-transplant monitoring. In the first case, it would potentially increase the time on waiting list, whereas in transplanted patients, humoral rejection diagnosis requires a confirmatory biopsy with circulating DSA. Thus, the identification of the found antibodies as directed against denatured HLA would avoid treatment of non-relevant DSA. However, with clinical and/or histological findings, other means of humoral rejection mediated by non-HLA Abs (MICA, AT1R, endothelial cells, and others) should be ruled out [18-20] .

The presence of anti-HLA Abs in patients not exposed to any allosensitizing event due to cross-reaction with viral epitopes has been documented [21, 22]. However, the literature is mixed with recent reports identifying no cross reactivity with viral and allo antibodies [23].

For this reason, a proper assessment of the nature of these antibodies is of great importance for their clinical implication. Pre-transplant anti-dHLA DSA reactions have been associated with negative FCXM, low risk of acute antibody-mediated rejection (ABMR) [13] and better long-term graft survival [12]. It should be taken into account the different anti-HLA detection level of FCXM and SAB assay. In our study, no clinical

assessment was performed but recently kidney transplant recipients with anti-dHLA-Cw DSA showed negative FCXM reactions, lower ABMR rate and better graft survival [14]. The HLA-Cw is expressed at a lower frequency on cells used within FCXM, but is at a high density on the SAB assay. This could lead discrepant results. In our study, most of the monospecific reactions selected had more than 3000 MFI with a negative FCXM which supports the reaction against denatured HLA. However, a limitation of the work is that, in order to demonstrate reaction against dHLA, only acid treatment in prospective cohort was performed. Previously, several groups also confirmed the assessment with iBeads with good correlation with acid treatment [24].

In patients waiting for heart or lung transplantation, the definition of forbidden HLA antigens should be accurate due to virtual crossmatch and lack of prospective crossmatch performance. Cao S. *et al* have performed direct SAB in lung-waiting list patients and identified more anti-HLA antibodies compared with screening assays in samples rendering negative crossmatches [25]. These observations are consistent with our findings; however the screening and SAB assays have different source of HLA molecules coating the beads, the screening panel is manufactured from non-recombinant cell line whereas SAB panel with recombinant cell lines denaturing differently and present on the surface of the beads at different densities providing potential discrepant results.

Since there are two different manufacturers of SAB assay for anti-HLA antibody testing, several studies have compared the results provided by both of them, with no

substantial differences [26]. Some discordant results could be explained by different HLA-load constructs on the beads [15]. A recent study compared the performance and accuracy of the only two commercially available Luminex-based SAB assays in the ABMR setting. Authors concluded that both kits, despite significant differences in their sensitivity and specificity, had a good correlation [27].

Our results show that most of confirmed mono-specific HLA class-I reactions from unsensitized patients by AM and BM render both CDC and FCXM negative results that point to denatured-HLA reactions and could be due to similar way of HLA antigen-load on beads in their manufacture.

In the present study, all mono-specific reactions detected by SAB, irrespectively of baseline MFI levels, render negative CDC and FCXM suggesting dHLA reactions. Although it should be taken into account that the different sensitivity of the techniques could justify some discordant results between SAB test and crossmatch [28].

Upon these results, we propose that a positive monospecific reaction detected by SAB in a patient without history of sensitizing events should be defined as native HLA, after a negative reaction with acid treatment of the beads, CDC and/or FCXM against donor cells with the type of interest, and, besides the sample should be studied with the kit of the other manufacturer, which could have logistic problems in laboratory organization.

The main limitation of this study is the scarce number of clinical cases to discuss after transplantation; though the study was driven, in part, from a technical laboratory point

of view. The results were performed in a single mixed beads lot, however, the confirmation in different bead lots would improve the robustness of our results. Another limitation was the lack of CDC and FCXM for all anti-HLA reactivities detected, most of them due to their low frequency in our population (A*34: 0-1.5%, B*73: 0-2% and A*80: 0-2% of European Caucasians) [29]. Moreover, the identification of Abs against low frequency HLA could suggest false positive reactions.

To better rely on anti-HLA Abs tests the efforts tests, efforts should identify potential biases in anti-HLA Ab results, leading to the fine tuning of anti-HLA Ab profiles of the patients which could be of special importance in those who are highly sensitized.

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

Tables:

Table 1. Comparative results of samples from the prospective cohort.

Serum #	Class-I Bead	A_M	B_M	Reaction	Crossmatch CDC/FCXM	Assignment
1	B7	**3,633**	3,326	T_R	NEGATIVE	Denatured
2	A23	**3,877**	2,555	T_R	NEGATIVE	-
3	B17	**6,100**	1,321	F_{RA}	NEGATIVE	Denatured
4	A34	**3,861**	<100	F_{RA}	NA	-
5	B73	**13,574**	3,199	T_R	NA	-
6	A11	**5,645**	<100	F_{RA}	NEGATIVE	Denatured
7	Cw7	**3,572**	1,583	T_R	NEGATIVE	Denatured
8	A80	**10,139**	1,405	F_{RA}	NA	-
9	Cw9	**5,190**	<100	F_{RA}	NA	-
10	B37	**2,050**	1,617	T_R	NA	-
11	A34	**3,821**	<100	F_{RA}	NA	-

AM: Manufacturer A; BM: Manufacturer B; NA: Not available; TR: true reaction (confirmed in both manufacturers); FRA: false reaction A manufacturer.

Table 2. Comparative results of samples from comparison cohort.

Serum #	Class-I Bead	A_M	B_M	Reaction	Crossmatch CDC/FCXM	Assignment
12	A23/A24	206	**3,690**	F_{RB}	NEGATIVE	Denatured
13	A23/A24	66	**1,787**	F_{RB}	NEGATIVE	Denatured
14	A23/A24	396	**7,478**	F_{RB}	NEGATIVE	Denatured
15	A23/A24	397	**3,091**	F_{RB}	NEGATIVE	Denatured
16	B57/B58	92	**2,005**	F_{RB}	NA	-
17	B57/B58	5,961	**3,055**	T_R	NA	-

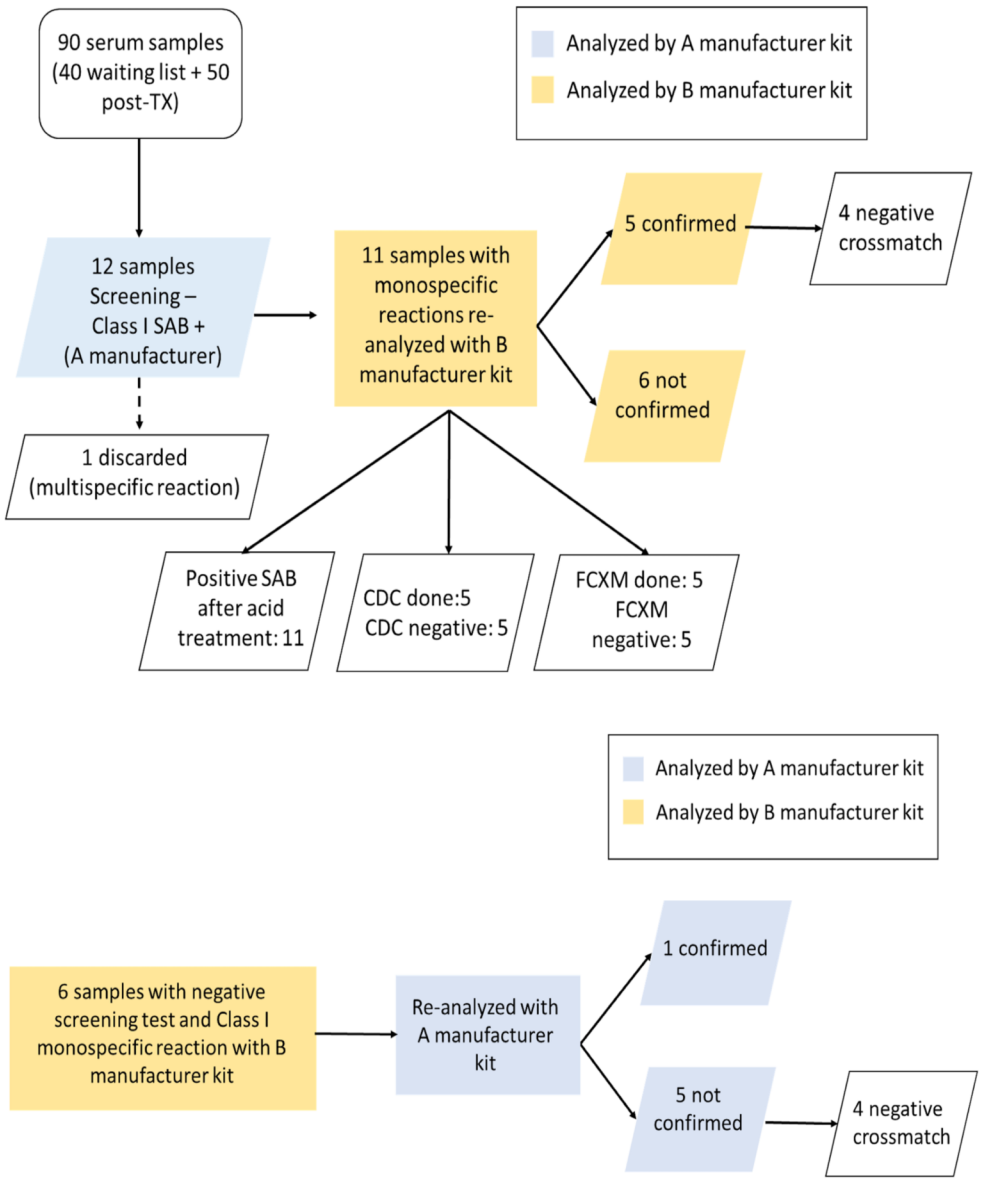
The monospecific reaction against A23/A24 and B57/B58 refers to A9 and B17 reactions. The mean fluorescence intensity (MFI) values are the mean of MFI from the different alleles in each single antigen panel. AM: Manufacturer A; BM: Manufacturer B; NA: Not available; TR: true reaction (confirmed in both manufacturers); FRB: false reaction B manufacturer.

Figure legends:

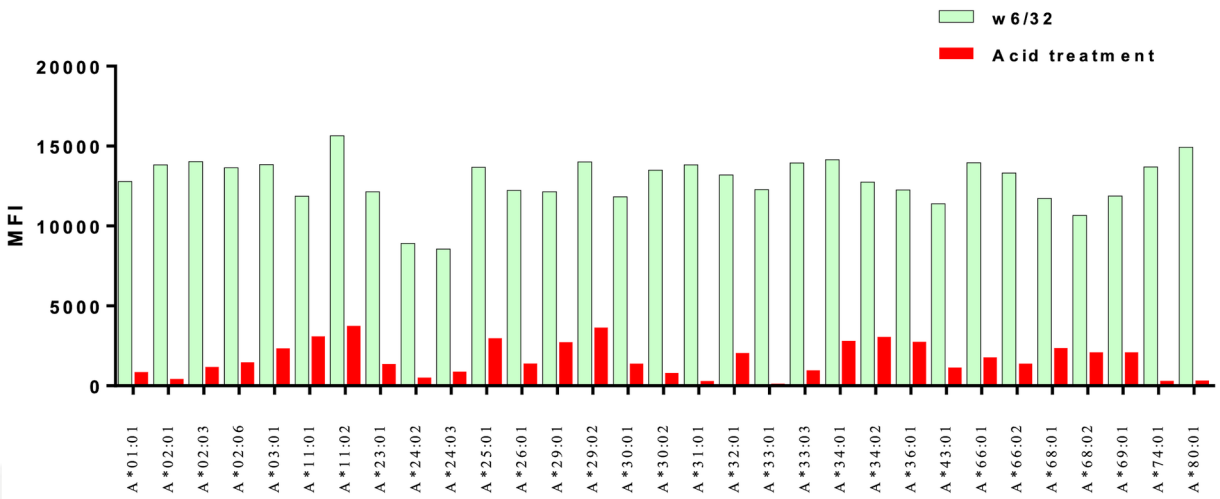
Figure1: Mean Fluorescence Intensity values in single antigen bead and acid-treated bead assays. The mean fluorescence intensity (MFI) values after single antigen bead (SAB) assay detecting mono-specific reactions in each patient are shown (A). After acid treatment of the beads as described in Material and Methods section, an increase in MFI values is observed (B). The cut off value in SAB assay was set at 3000 MFI (black line) and in acid-treated bead assay was set at 1500 MFI (dotted line). The arrows in each patient indicate the MFI value of each mono-specific reacion after acid treatment of the beads.

Figure 2: Comparison of median MFI for SAB Class I (white circles) and screening (black squares) test for anti-HLAantibodies in serum with mono-specific reactions.

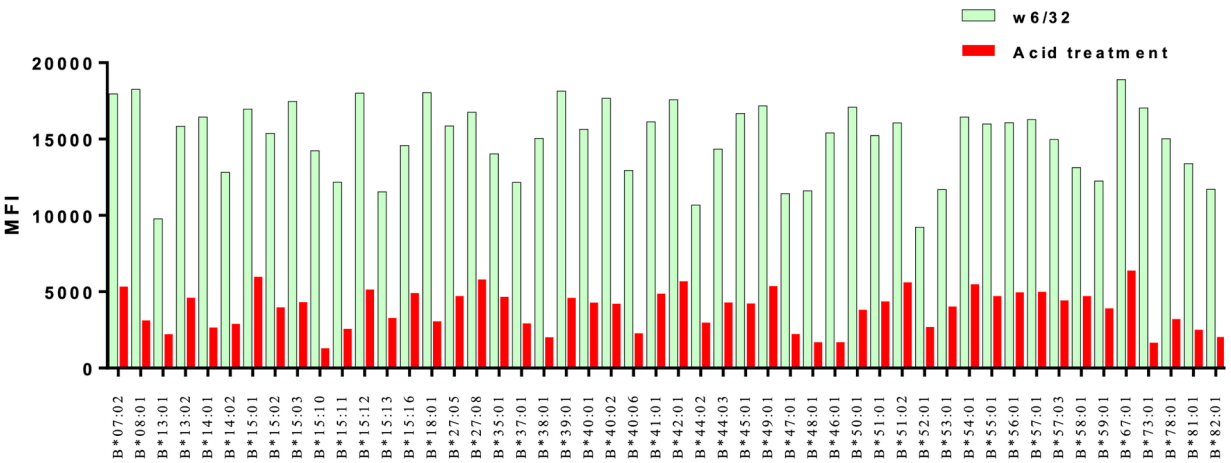
Appendix Figure 1. Overview of the study.



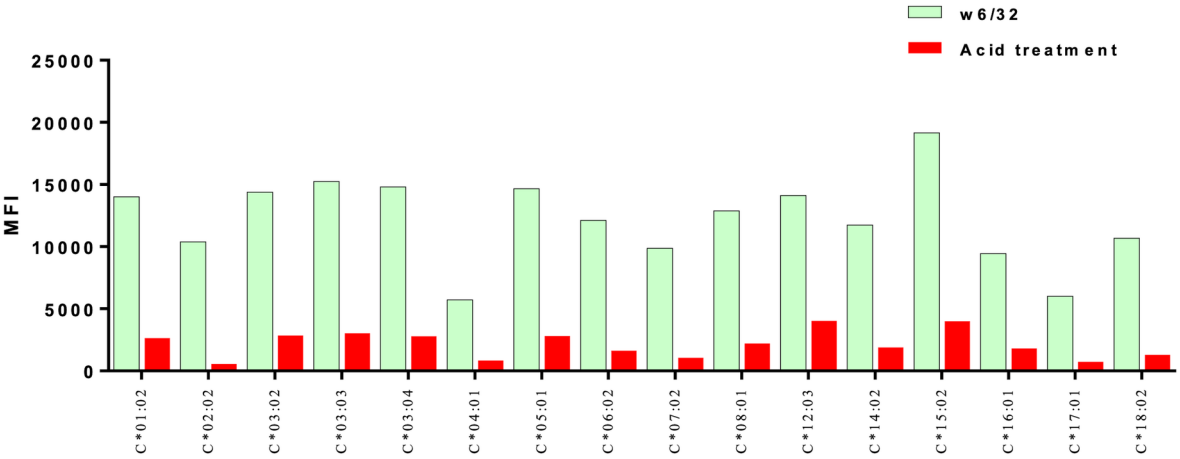
Appendix Figure 2A



Appendix Figure 2B



Appendix Figure 2C



Appendix Table1. Demographic and clinical parameters of prospective cohort.

Patient	Class-I Bead	Age	Gender	Transplantation	Retransplant (No/Yes)	Transfusions (No/Yes)	Serology (EBV/CMV/VZV)	Donor HLA	Time from transplantation (months)
1	B7	68	Male	Kidney	No	Yes	+/+/+	A2, A23, B18, B57	10
2	A23	60	Female	Kidney	No	Yes	+/+/+	A2, A3, B7, B12	309
3	B17	52	Female	No	No	No	+/+/+		
4	A34	30	Female	No	No	No	+/+/+		
5	B73	34	Female	No	No	No	+/+/+		
6	A11	54	Male	Kidney	No	Yes	+/+/+	A2, A32, B7, B64	5
7	Cw7	48	Male	Heart	No	Yes	+/+/+	A2, A26, B38,B40,	14
8	A80	30	Male	Kidney	No	No	+/-/+	A1, A23, B49, B57	48
9	Cw9	55	Male	No	No	No	+/+/+		
10	B37	68	Male	No	No	Yes	+/+/+		
11	A34	56	Male	No	No	No	+/-/+		

Abbreviations: SD, Standard deviation; EBV, Epstein-Barr Virus; CMV, Cytomegalovirus; VZV, Varicella Zoster Virus.

Appendix Table 2. Demographic and clinical parameters of comparison cohort.

Patient	Class-I Bead	Age	Gender	Transplantation	Retransplant (No/Yes)	Donor HLA
1	A23/24	56	Female	Kidney	No	A2, A25, B18, B27
2	A23/24	62	Female	Kidney	No	A11, A30, B14, B55
3	A23/24	68	Female	No	No	
4	A23/24	53	Female	No	No	
5	B57/58	52	Female	Kidney	Yes	A24, A26, B44, B52
6	B57/58	74	Female	No	No	

Appendix table 3. Description of MFI value after SAB and acid-treated bead assays in monospecific reactions**.**

Serum #	Class-I Bead	Non-treated (MFI)	Acid treated (MFI)
1	B7	3,633	8,729
2	A23	3,877	1,541
3	B17	6,100	4,093
4	A34	3,861	2,799
5	B73	13,574	8,127
6	A11	5,645	3,769
7	Cw7	3,572	25,296
8	A80	10,139	23,835
9	Cw9	5,190	11,032
10	B37	2,050	9,464
11	A34	3,821	1,320

MFI, mean fluorescence intensity
SAB, single antigen bead assay