Received: 29 March 2022 | Accepted: 4 April 2022

DOI: 10.1111/tan.14781

REVIEW ARTICLE

HLA Immune Response Genetics WILEY

Clinical utility of serial serum dilutions for HLA antibody interpretation

Anat R. Tambur[1] | **Carrie Schinstock**[2]

[1]Fienberg School of Medicine, Department of Surgery, Comprehensive Transplant Center, Northwestern University, Chicago, Illinois, USA

[2]Division of Nephrology & Hypertension, Department of Internal Medicine, Mayo Clinic, Rochester, Minnesota, USA

Correspondence
Anat R. Tambur, Feinberg School of Medicine, Northwestern University, 300 E Superior Avenue, Tarry Building suite 11-711, Chicago, IL, USA.
Email: a-tambur@northwestern.edu

Luminex single antigen bead (SAB) testing has increased the sensitivity and specificity of accurately identifying HLA antibodies, in support of all organ transplantation. However, as described in manufacturers' recommendation, the output of the assay, using mean fluorescence intensity (MFI) units, is only semi-quantitative. Therefore, the ability to use MFI values to compare between different assays, to accurately guide clinical practice, or be used as an endpoint measure in clinical trials, is limited. To improve potential quantification, one must circumvent inherent limitations of SAB assays such as interference and saturation phenomena. In this review, we discuss how measurement of pre-transplant serum dilutions can be used to determine unacceptable antigens for wait-listing, determine the likelihood for successful HLA antibody reduction with desensitization, and compare degree of HLA (in)compatibility among various living donors. We also discuss how serum dilutions are optimal for measuring and comparing the efficacy of antibody depletion therapies for desensitization or antibody mediated rejection treatment post-transplant. Historically, one of the main criticisms for the use of serum dilutions and titer has been the potential labor and cost associated with additional testing. Here, we show how only one or two dilutions can add major value in most circumstances. In summary, the practical use of serum dilutions and titer determination are important methods that can be used before and after transplantation of all organs to quantify antibody accurately and reliably in routine practice and in clinical trials.

KEYWORDS
dilutions, HLA antibody, SAB, titer

1 | INTRODUCTION

The term high-titer antibody was re-introduced into the HLA literature about a decade ago. Originally, it was used based on high reactivity in complement-dependent

Abbreviations: ABMR, antibody mediated rejection; cPRA, calculated panel reactive antibody; DSA, donor specific antibody; IHC, immunohistochemistry; KPD, kidney paired donation; MFI, mean fluorescence intensity; SAB, single antigen bead; UA, unacceptable antigen.

serological assays as the only measure to express HLA antibody strength. The assay was performed using several dilutions of the patient's serum sample, the higher the dilution required to eliminate positive results, the higher the declared titer. Once Luminex-based single antigen bead (SAB) assays were introduced, the field had shifted to use mean fluorescence intensity (MFI) units to express relative antibody strength. Consequently, the transplant community began using the terms *high MFI value* and *high titer* antibody interchangeably, even though dilutions were rarely performed to substantiate the term "titer." Therefore, in most settings, describing HLA antibody as *high titer* may be inappropriate and inaccurate.

When used in appropriate settings, serum dilutions and titer determination can allow the user to overcome inherent limitations of the SAB assay and better quantify HLA antibody. We have previously reported on the use of serial dilution studies as an aid to appreciate inherent limitations of the SAB assay and have described how they can be applied to clinical practice. In this review we aim to summarize our experience and elaborate on the following topics:

1. Describe inherent limitations of the SAB Luminex assay that can be rectified using titration studies
2. Discuss specific clinical scenarios demonstrating when donor specific antibody (DSA) quantification is advantageous
3. Discuss a fiscally reasonable approach to introduce dilution studies in mainstream practice

1.1 | Serial dilutions and determination of antibody titer

Serial dilution of serum samples for the purpose of analyzing specific constituents has been common practice in immunology for decades. The scientific method is called titration, and it is designed to evaluate how many dilutions can occur before the tested substance is no longer detected in the serum. The result is documented as a titer, the last dilution in which the substance is still present; for example, 1:256. A common use of such tests includes checking for antibodies to infectious diseases, whether following exposure to the immunogen or as a response to vaccination. Examples include MMR, Influenzas, or tetanus.[1–3] Antibody titer is also measured routinely as part of diagnosing and monitoring patients with autoimmune diseases.[4,5] Some examples include systemic lupus erythematosus and thyroid autoimmunity. The blood bank is another entity that routinely performs antibody titer testing as part of routine transfusion medicine assessment, as well as part of evaluation for organ transplantation.[6]

1.2 | The "hook effect" and "prozone"

Antibody titer and dilutions are also significant when performing immunohistochemistry (IHC) and Western blot assays. An appropriate dilution of the antibody used as reagent is critical to maintain high quality of the staining, with minimal background/non-specific reactions. This is especially apparent in sandwich immunoassays where one (primary) antibody recognizes the specific target/antigen and a secondary antibody, recognizing the primary antibody, provides a measurable signal when it binds to complexes of primary-antibody/antigen. If the primary-antibody concentration is very high, it will not only bind to the specific antigen, but it can also saturate the free secondary antibody such that there is no sufficient secondary antibody to bind and report on primary-antibody/antigen complexes (Figure 1). This phenomenon, termed "Hook effect," is known to lead to falsely reporting low levels of the immune complexes. The Hook effect has been reported for multiple assays, especially with very high levels of the tested analyte, including prolactin, cancer markers, and so on.[7] The best way to eliminate the Hook effect is by performing serial dilutions. The term Hook effect has been conflated with the term "Prozone" which is relevant for agglutination or precipitation reactions. Specifically, "prozone" is meant to describe a "zone" in which reactions do not happen. Most always it reflects the presence of high concentration of the antibody (excess), but it may represent situations in which blocking antibodies or non-specific inhibitors are present in the serum and preventing detection of the antibody/antigen binding.

1.3 | Hook effect and Prozone phenomena in HLA antibody testing

The phenomena of "missing" (falsely low) antibody reactivity when using the luminex SAB assays for HLA antibodies evaluation was demonstrated by multiple investigators. Several theories were put forth to explain these observations. Kosmoliaptsis et al[8] showed that the presence of IgM antibodies in the tested sera can compromise the ability to detect IgG antibodies. The investigators demonstrated that for some patients, serum dilution of 1:50 or treatment with dethiothreitol (DTT) revealed increased strength of some of the HLA antibodies. They further concluded that to avoid masking of clinically relevant antibody responses, serum samples should be treated with (DTT) prior to testing on the luminex platform. Alternatively, Weinstock and colleagues[9,10] proposed that complement component 1 (C1) is responsible for this phenomenon by competitively displacing the detection (secondary) antibodies. These

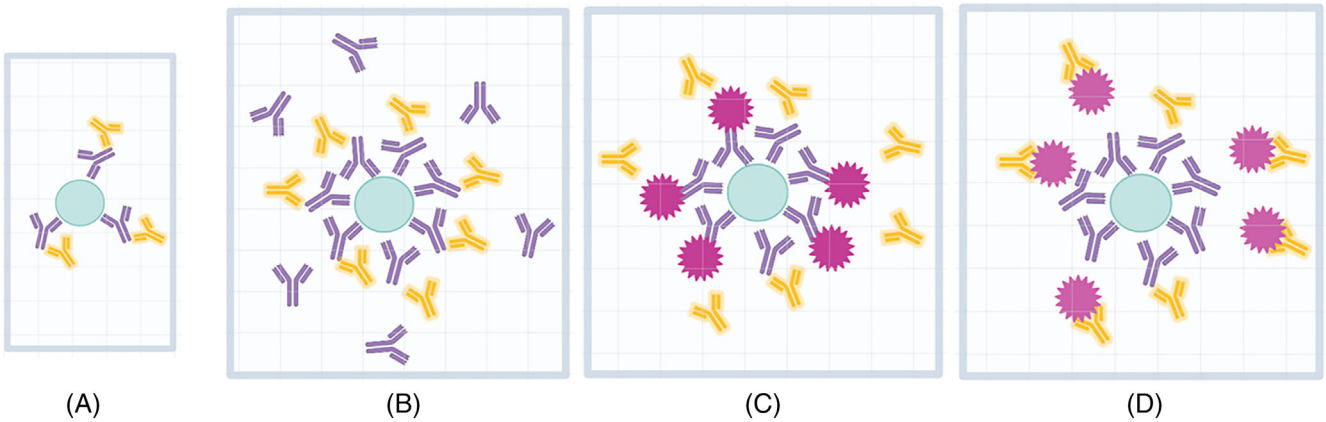

FIGURE 1 Single antigen bead assay: (A) A luminex bead, coated with multiple copies of a single HLA antigen, is represented by the circle, HLA antibodies (purple) bind to their cognate antigen, and the secondary antibody (yellow) binds to the HLA antigen, emitting fluorescence (not shown) and translated into mean fluorescence intensity—mean fluorescence intensity (MFI)—units. (B) A serum with high levels of HLA antibodies leads to saturation of the cognate antigen, with additional antibodies not having a target to bind to. The secondary antibody can bind only to antibodies attached to the beads (and hence, antigen) and thus the "floating" antibodies go undetected. (C) The Fc portion of the HLA antibody is bound to an "inhibitor" and therefore the secondary antibody does not find its target and no/low MFI values are recorded. (D) The secondary antibody binds to the "inhibitor" and therefore is not available to bind to the HLA antibodies leading to no/low MFI values.

investigators tested a handful of samples using multiple approaches ranging from serum dilution of 1:10 through pretreatment with EDTA, DTT, heat inactivation and the complement inhibitor C1INH. They concluded that addition of EDTA to serum of highly sensitized patients prior to testing is the easiest way to avoid false-negative results in SAB analysis.

Our laboratory has been performing *serial* serum dilutions (as opposed to a single dilution that is sometimes performed in other centers) for almost 20 years now. The original purpose was to have a reliable method to compare antibody strength for patients post transplantation, especially those undergoing desensitization protocols. Since our program used Alemtuzumab as induction agent, using cell-based crossmatch assays post transplantation (as was customary at the time) was not a viable option. Our serial dilution studies at the time were performed using the FlowPRA Single Antigen Class I and Class II antibody testing, a platform that remained underappreciated/underutilized because of the requirement of having access and expertise in operating a flow cytometer. The luminex SAB assays were introduced a few years later. They offered multiple advantages. A luminex instrument was significantly cheaper than a flow cytometer. It is easy to operate and does not require much experience as the run-parameters are locked. It also comes with automated analysis software expediting computerized resulting of the tests. Lastly, the testing reagents were multiplexed, having about 100 different analytes in one single assay, thus requiring very low volumes of the patients' sera (5–40 μl depending on the vendor) and lowering the cost of the test. As we transferred our serial dilution practices from the FlowPRA Single Antigen testing to the luminex platform we made several important observations.

1.4 | SAB testing using serial dilution

1.4.1 | Inhibition

Regardless of the cause of inhibition, it may have a differential effect on different antibody-specificities within a single test-tube.[11] It is not a trivial observation. An illustration is provided in Figure 2, showing serial dilution of a serum sample from a highly sensitized patient awaiting heart transplantation. Note that some antibodies (black arrow, Figure 2), are not affected by inhibition at all, namely the MFI values decease as the dilution increases, as expected. Other antibodies start with high MFI values that increase to higher values with dilution, and then decrease as the serum is diluted (green arrow, Figure 2). Lastly, some antibodies experience strong inhibition, starting from low MFI values in the neat sample that increases with dilutions up to a titer of 1:64 (brown arrow, Figure 2) or even 1:256 (purple arrow, Figure 2). Only after that relatively high dilution does the MFI value being to decrease. Note that this patient exhibits very strong levels of antibodies as some still maintain high MFI values, over 2000 units, even at a titer of 1:65,356. Importantly, laboratories that perform a 1:10 dilution (which is the single most common dilution performed) or even a 1:100 dilution will be missing the strength of many of these antibodies that are several folds higher. It is

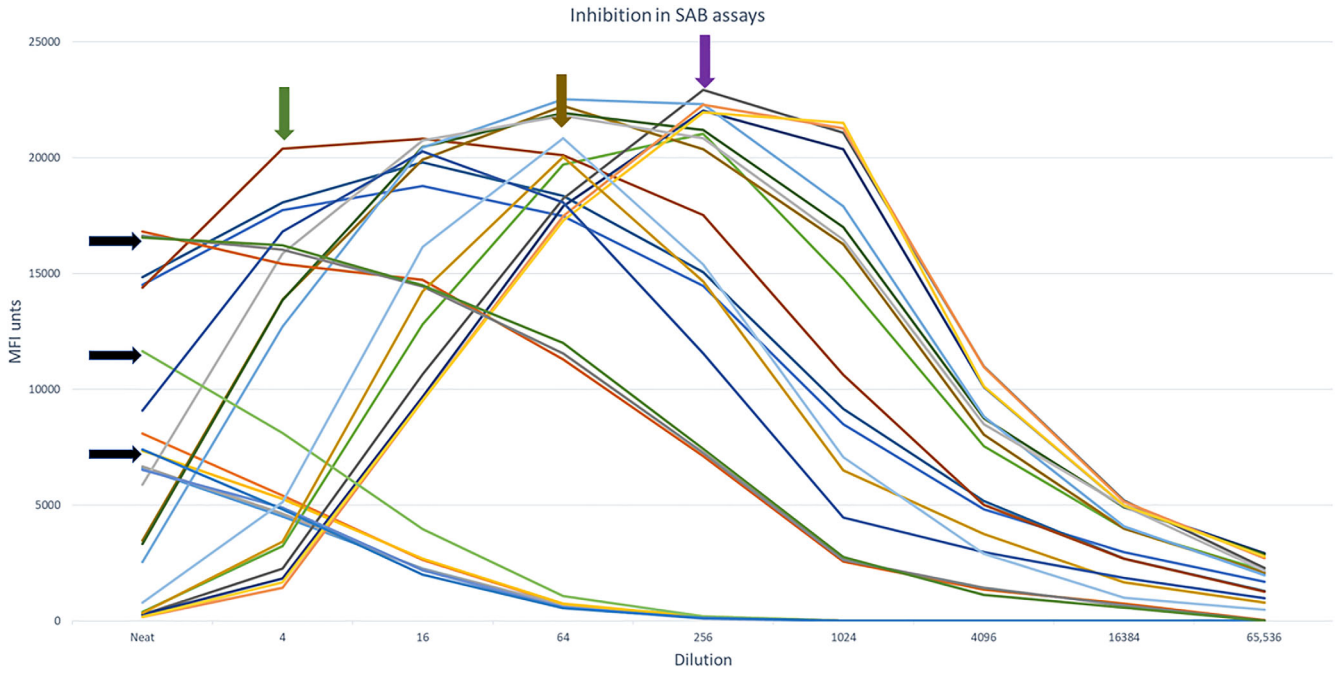

FIGURE 2 Inhibition of SAB assays: Serial dilution of a serum sample from a highly sensitized patient awaiting heart transplantation. Dilution is shown on the *X* axis. MFI units are shown on the *Y* axis. Note that some antibodies (black arrow), are not affected by inhibition at all and the MFI values decease as the dilution increases, as expected. Other antibodies start with high MFI values that increase to higher values with dilution, and then decrease as the serum is diluted (green arrow). Lastly, some antibodies experience strong inhibition, starting from low MFI values in the neat sample that increases with dilutions up to a titer of 1:64 (brown arrow) or even 1:256 (purple arrow). MFI, mean fluorescence intensity; SAB, single antigen bead.

of interest to highlight that the dilution patterns are shared by groups of antibodies that as we have previously proposed, likely demonstrate recognition of similar epitope targets.[12]

A salient observation is that EDTA treatment does not always eliminate the full impact of the inhibition when antibody titers are high, as demonstrated in Figure 3. This means that MFI values, even when EDTA treatment have been applied, are not always an indication to antibody strength (this is usually true when MFI values are higher than a certain threshold; >10–12,000 MFI units in our hands). In the example shown—two groups of beads have similar neat MFI values. In the one group (brown box, Figure 3), antibodies titer out at about 1:1024. In the other group (purple box, Figure 3), antibodies responses become negative only at a titer of 1:16,384. These differences can have clinical ramifications, for example in the treatment of ABMR, as will be illustrated below.

1.4.2 | Saturation

The quantity/titer of HLA antibodies that an individual may develop can be greatly underappreciated. Two components of the SAB assay may affect the ability to determine the level of antibodies in the tested sera. (i) The detection (secondary) antibody, which can be modified by the end user, and is often added in excess amounts; and (ii) the HLA target antigens coating the beads, which have finite amount, cannot be modified by the end user, and thus can be a limiting factor. Once the HLA antigen targets are saturated, additional HLA antibodies, in the tested sera, will remain masked because the signal from the detection antibody is captured only once a sandwich (HLA antigen—tested antibody—detection antibody) is formed.[13]

An example of saturation is present in Figure 4. This is one representation of HLA class II antibody-signature from a patient awaiting kidney re-transplantation; first graft loss was because of antibody mediate rejection (ABMR). Note that many of the antibodies remain with MFI values >5000 even at dilutions of 2^{14} (1:16,384) and some show reactivity even at a titer of 1:65,356. It is important to observe that the MFI values on the undiluted sample do not provide any indication of the differential strength of the antibodies (there is not a big difference between the neat MFI of antibodies with titers of <1:1024 and those that reach >1:65,356). While observing such high levels of antibodies is not common,

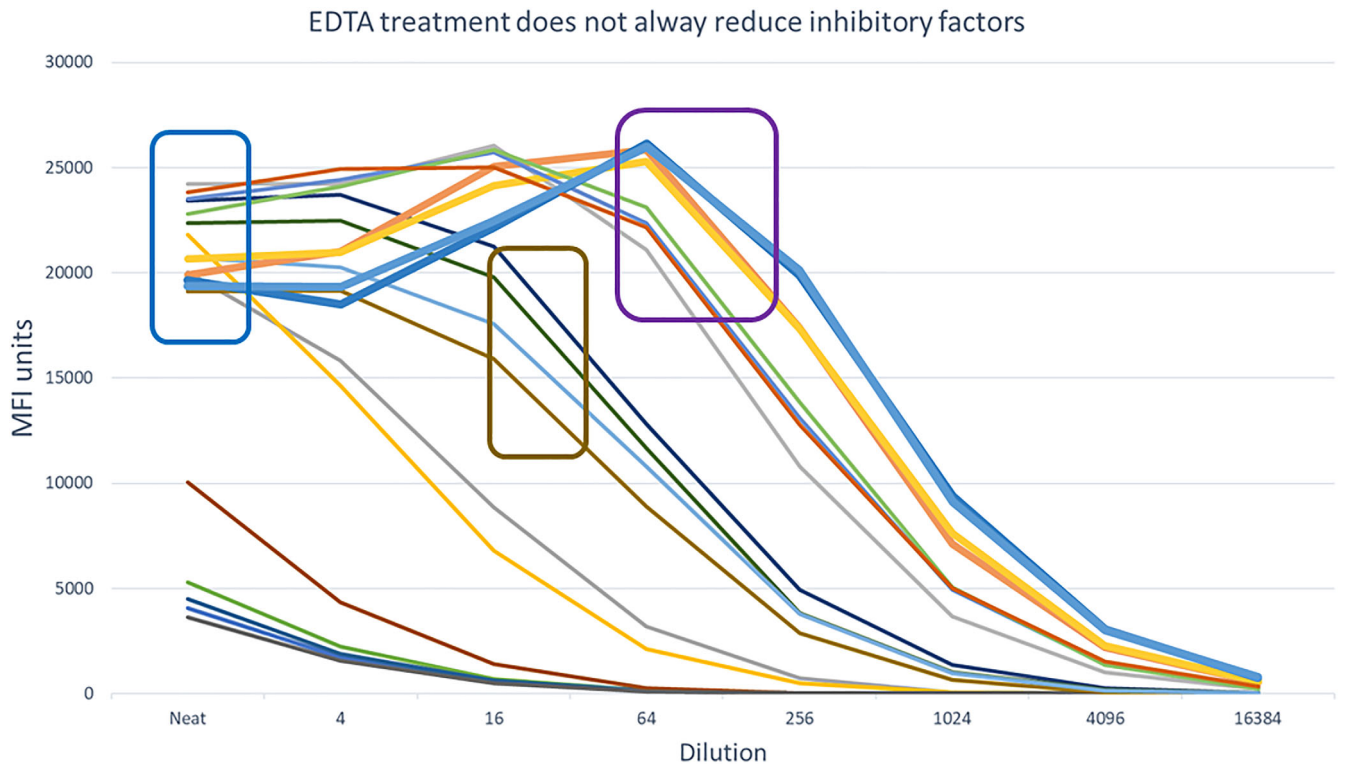

FIGURE 3 EDTA treatment does not always reduce inhibitory factors: Serial dilution of a serum sample from a kidney transplant recipient at times of ABMR diagnosis. Dilution is shown on the *X* axis. MFI units are shown on the *Y* axis. Many antibodies show similar, very high, MFI values—with MFIs ranging between 19,000 and 24,500 units (blue box). One group of antibodies are not as strong (brown box), demonstrating complete elimination at a titer of 1:1024. The other group of antibodies (purple box) are much stronger, becoming negative only at a titer of 1:16,384. ABMR, antibody mediated rejection; MFI, mean fluorescence intensity

it is also not a rare event. Performing only a 1:10 dilution, or even a 1:100 dilution, will not provide sufficient information on the strength of such antibodies.

1.5 | Advantages of HLA antibody dilutions in clinical practice

HLA antibody testing using the Luminex SAB platform provides essential information that is critical for clinical management of transplant patients. However, the SAB test has important limitations as discussed above. The relevance of these limitations and the need for additional testing depends on the clinical situation. Here we describe how the use of serum dilutions can be used in various common clinical scenarios (Table 1).

1.5.1 | Determining unacceptable antigens for highly sensitized patients awaiting transplantation

Pretransplant HLA-SAB antibody testing is performed to determine the baseline sensitization status of transplant candidates. If the patient is placed on the deceased donor transplant waiting list, this information is used to assign donor HLA antigens that are deemed unacceptable antigens (UAs) by the listing transplant center. This information is then translated into a unified sensitization metric, calculated panel reactive antibody (cPRA), which is the percent of donors toward which your candidate is likely to have antibodies. Importantly, determination of UAs is center, organ, and often patient specific depending on the acceptable immune-risk threshold. For example, if your candidate is highly sensitized toward HLA targets, the MFI threshold used to determine an UA may be higher to increase the access to organs. The risk threshold may also be higher for heart and lung candidates because of the lack of living donor options and the fact that heart and lung transplants are lifesaving.

Completely avoiding DSA is preferable in most cases, if possible. For these patients, the MFI threshold used to determine which HLA antigens to avoid is typically low (MFI values of 500–3000). Performing one serum dilution and testing for HLA antibodies may provide value even when these low MFI thresholds are used to prevent the under-detection of a high level of HLA antibody masked because of inhibition.[14,15] When the goal is to completely

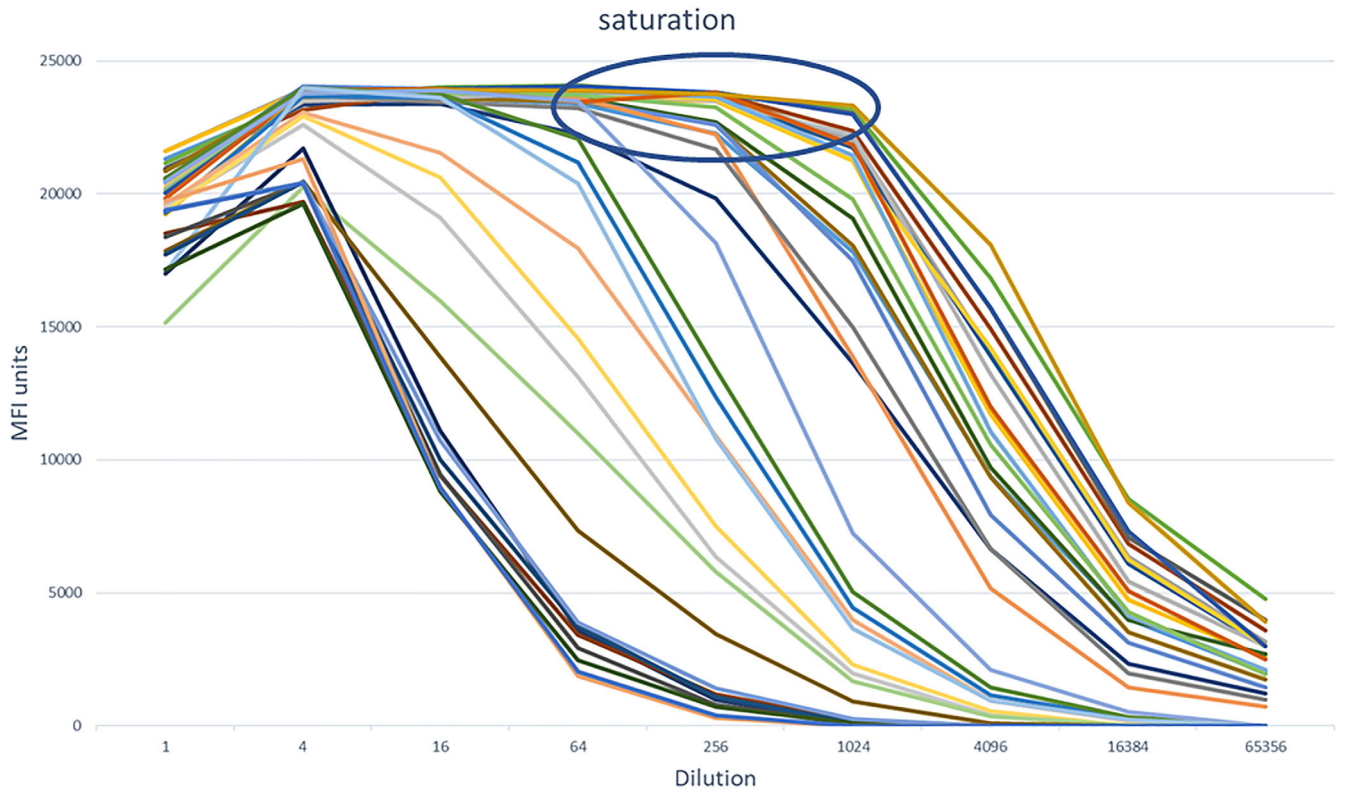

FIGURE 4 Saturation in SAB assays: example of significant saturation in a serum sample from a patient awaiting a third kidney transplantation. Dilution is shown on the *X* axis. MFI units are shown on the *Y* axis. More than half of the antibodies saturate their cognate antigens—with MFI values reaching near 25,000 units of MFI and remaining in this range despite multiple dilutions (circled in blue). Those antibodies are not likely to respond to any of the currently available therapies. Note that there is some inhibition (MFI values at 1:4 dilution is higher than neat sample despite EDTA treatment). MFI, mean fluorescence intensity; SAB, single antigen bead

TABLE 1 Advantages of HLA antibody dilutions in clinical practice

- Determining unacceptable antigens for transplant wait-listing
- Determining the likelihood for successful HLA antibody reduction with PP/IVIg
- Comparing the HLA incompatibility of various living donors
- Comparing the efficacy of desensitization for clinical trials
- Measuring the efficacy of antibody depletion therapy post-transplant

avoid HLA antibody, multiple dilutions are rarely needed.

When the goal of pretransplant testing is to risk stratify the HLA antigens toward which the patient has preformed antibodies, serial serum dilution provides further value. Depending on the candidate's baseline sensitization and/or urgency for transplant, the unacceptable antigens can be adjusted with more confidence when serial dilutions are performed. For example in a sensitized heart transplant candidate with a cPRA of 90% stable on the waiting list, you might initially avoid all HLA antigens to which there are antibodies with a titer >1:8. If no suitable donor is found quickly, you may choose to increase the threshold and only avoid antigens to which HLA antibodies are present at a titer >1:32 to increase the candidates access to organs based on your experience with managing alloantibody pre and/or post-transplant with desensitization.

1.5.2 | Determining candidacy for desensitization protocols when potential living kidney donor is available

While the need for desensitization has decreased in kidney transplantation with the advent of kidney paired donation (KPD) programs and allocation systems prioritizing sensitized candidates, there remains a need for desensitization particularly for kidney transplant candidates with a high cPRA (99.9%).[16] These candidates are unlikely to find an HLA compatible living donor even with KPD. If desensitization is considered, serum dilution and titration studies can be a tool to determine whether

TABLE 2 Example for utility of dilution in comparing HLA incompatibility of various potential living donors

	DSA specificity	MFI	DSA titer
Donor A	*DRB1*11:04*	16,817	1:256
Donor B	*DRB1*13:03*	16,111	1:32

Abbreviations: DSA, donor specific antibody; MFI, mean fluorescence intensity.

desensitization is likely to be successful in removing enough HLA antibody to avoid hyperacute rejection and plan the desensitization strategy.

Pinelli et al showed that transplant candidates with DSAs of titer ≥1:1024 at baseline were unlikely to achieve sufficient DSA reduction with PP/IVIg alone. Among transplant candidates with lower baseline HLA antibody levels, the dilution studies provided additional valuable information guiding the number of PP/IVIg treatment cycles required to achieve the desired HLA antibody level.[17] The investigators found that reduction in HLA antibody was linear during the first few cycles of PP/IVIg, but additional treatment cycles had low yield. The ability to estimate the number of PP/IVIg treatments needed to decrease HLA antibody to a certain level can be particularly useful for pretransplant planning (e.g., determining date of surgery).

1.5.3 | Comparing the HLA incompatibility of various living donors

Serial dilution and titration studies can also be used to compare the HLA incompatibility of various donors by accurately comparing the *quantity* of DSA the recipient has to each potential donor. This can be a particularly useful approach among candidates in KPD programs when there are multiple potential living donors. In the example illustrated in Table 2, the transplant candidate has two potential living donors toward which the candidate has DSA, with a similar MFI and presumed similar risk at first glance. However, there are clear differences in the DSA titers. The ability to sufficiently remove DSA with PP/IVIG to prevent hyperacute rejection would likely be best for donor B.

1.5.4 | Comparing the efficacy of desensitization for clinical trials

Patients with cPRA >99.5% are inflicted by very long waiting time for deceased donor offers. While desensitization of these patients carries high cost because of the required resources and high risk of morbidity, the alternative is the increased risk of remaining on dialysis and the mortality associated with it.[18] For patients awaiting heart transplantation, the risks are even higher. New therapeutic modalities that will enable effective desensitization on the waitlist are currently pursued by several pharmaceutical companies for multi-center trials. The identification of appropriate endpoints to compare the efficacy of various therapeutic regimens has historically been a problem for desensitization clinical trials, particularly when candidates without a living donor are included.[19] Endpoints such as time to transplant are biased, and it is challenging to evaluate complex data when antibodies toward multiple HLA antigen specificities need to be analyzed. The inherent limitations of using MFI only worsen this problem.

To address this gap, we aimed to evaluate the utility and reliability of using cPRA combined with titers as an endpoint for desensitization trials. The overall goal was to find an uncomplicated reliable endpoint that was directly related to a patient's likelihood of receiving a transplant that could be conveniently applied to all organ groups. To do this, we studied serum samples from 20 highly sensitized transplant candidates (cPRA >99.9) and determined the corresponding cPRA at multiple serum dilutions.[20] This approach allowed us to visualize the decreasing cPRA per every dilution (simulating the cPRA that will be achieved by successful desensitization). One of the striking observations was that 30% (6/20) of patients reached complete reduction of the cPRA by diluting the serum 1:1024, but 25% (5/20) patients still had cPRA >60% even when diluting the serum 1:16,384. While this data is particularly informative for the design of clinical trials, it could be applied to clinical practice. For example, P20 and P2 are likely to respond very differently to PP/IVIg (Figure 5).

These results highlight a dimension of HLA sensitization that was not previously assessed. We have shown that despite having the same cPRA (99.9% at baseline) on undiluted serum sample, our cohort exhibited significant heterogeneity with regards to HLA antibody strength. Thus, the major advantage of combining cPRA calculation with serum dilution is the ability to incorporate two different metrics of sensitization (1) the breadth of sensitization and (2) the quantity of antibodies.[21] This key information can be used to identify patients presumed to be most likely to respond to desensitization at baseline (e.g., identify patients to include in desensitization trials) and the reduction in cPRA per dilution could be used as an endpoint to compare the efficacy of various therapies. An additional advantage of this measure is that it is sensitive enough to identify therapeutic efficacy that may not have been apparent with other endpoints. Therapeutic

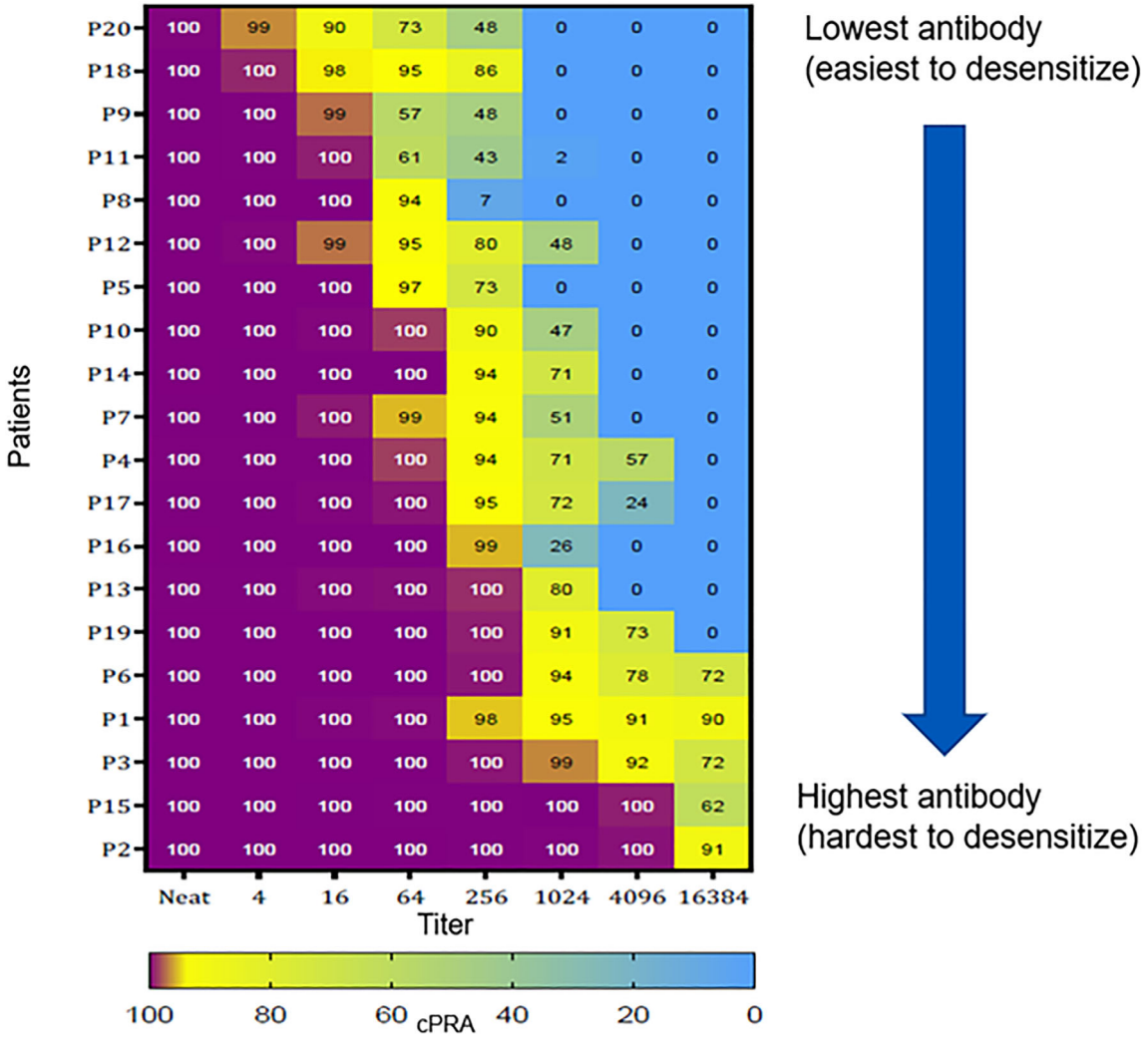

FIGURE 5 Calculating cPRA per serial dilutions: Dilution is shown on the *X* axis. Different patients are presented on the *Y* axis. Numbers represent calculated cPRA per specific dilution. Patient P20 shows rapid deceased in cPRA as the serum is diluted, demonstrating relatively low amounts of antibodies despite wide breath of sensitization. Patient P2 remains cPRA of 100% even in a 1:4096 dilution demonstrating not only wide breadth of sensitization but also high amounts of each of these antibodies—not likely to respond efficiently to desensitization attempts.

strategies with even a small effect show promise and could be added to additional therapy for future trials.

1.5.5 | Measuring HLA antibody post-transplant

The presence of HLA antibody is one of the three key conditions used to fulfill the criteria for both active and chronic active ABMR, and many treatments for ABMR are focused on antibody depletion. Therefore, an accurate measure of antibody quantity has a fundamental role in post-transplant monitoring in both practice and clinical trial design. In the pretransplant setting, all HLA antibodies have relevance because they have an impact on organ allocation and access. In contrast, the major focus post-transplant is HLA antibody directed toward the donor DSA, which can simplify antibody measurement.

Reliably knowing whether a DSA is present or absent has major relevance for post-transplant monitoring because the presence of DSA, whether preexisting or de novo, is associated with reduced allograft survival.[22] Among transplant recipients with known preexisting antibody with or without a positive crossmatch, frequent DSA monitoring is essential to promptly identify increases in DSA (approximately postoperative days 3–7, when immunologic memory responses begin to occur). Serum dilutions are an essential part of this early monitoring as it can detect trends in antibody presence more reliably than changes in MFI.

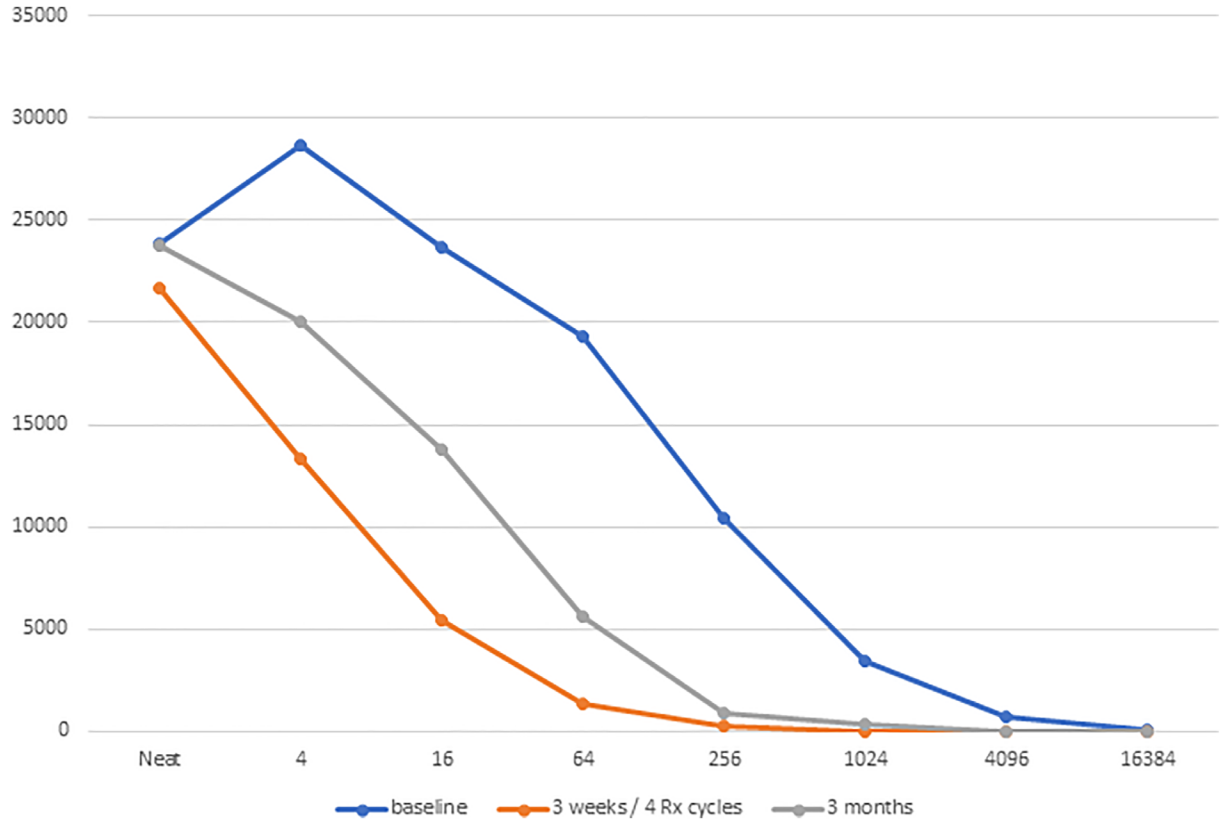

FIGURE 6 Added value of dilution studies in evaluating ABMR treatment efficacy: A 28-year-old female received a living related donor kidney transplant without complications but developed allograft dysfunction at 18 months post-transplant. At that time, de novo DSA toward HLA *DQA1*01:03/DQB1*06:03* was detected, and her allograft biopsy showed a mixed T cell and ABMR with peritubular capillaritis, glomerulitis, and C4d positivity (blue line—baseline). Treatment was initiated with anti-thymocyte globulin, solumedrol, and PP/IVIG. After a total of four cycles of PP/IVIG, she received rituximab. DSA levels were reexamined at 3 weeks (orange line) and 3 months (gray line) after treatment. All three serum samples show similar MFI in their neat/undiluted test. However, baseline DSA titer is 1:4096, reducing to 1:256 at the end of treatment cycles, in sample tested at 3 weeks. DSA rebounded to 1:1024 titer at the 3 months sample. ABMR, antibody mediated rejection; DSA, donor specific antibody

Serum dilution can also have value for de novo DSA detection. If a biopsy shows histologic features suggestive of ABMR with microvascular inflammation but it appears that DSA may not be present because of an MFI below the usual positive threshold, performing at least 1 serum dilution is recommended to ensure that the MFI is not falsely low.

While the performance of one serum dilution may provide value if there is a question whether DSA is present, testing multiple serum dilutions or knowing the antibody titer provides value when planning ABMR treatment in clinical practice. In fact, we believe that it should be required in therapeutic clinical trials to quantify HLA antibodies accurately and reliably, considering the semi-quantitative nature of the MFI. Before treatment, the antibody titer provides information about the antibody burden at baseline. If treatment is initiated, prospective DSA titer information reveals the changes in antibody quantity that is not apparent when examining the MFI alone.

The value of performing serum dilutions is demonstrated in the following case. A 28-year-old female received a living related donor kidney transplant without complications but developed allograft dysfunction at 18 months post-transplant. At that time, de novo DSA toward HLA *DQA1*01:03/DQB1*06:03* was detected, and her allograft biopsy showed a mixed T cell and ABMR with peritubular capillaritis, glomerulitis, and C4d positivity. Treatment was initiated with anti-thymocyte globulin, solumedrol, and PP/IVIG. After a total of four cycles of PP/IVIG, she received rituximab. DSA levels were reexamined at 3 weeks and 3 months after treatment. As shown in Figure 6, the DSA MFI appeared to be unchanged from baseline, 3 weeks, and 3 months after treatment. Based on that information, one could conclude that the antibody depletion therapies used were futile and failed to reduce antibody quantity. However, the performance of serial dilutions revealed a clear reduction in DSA quantity, which may have had a meaningful clinical impact. Based on this

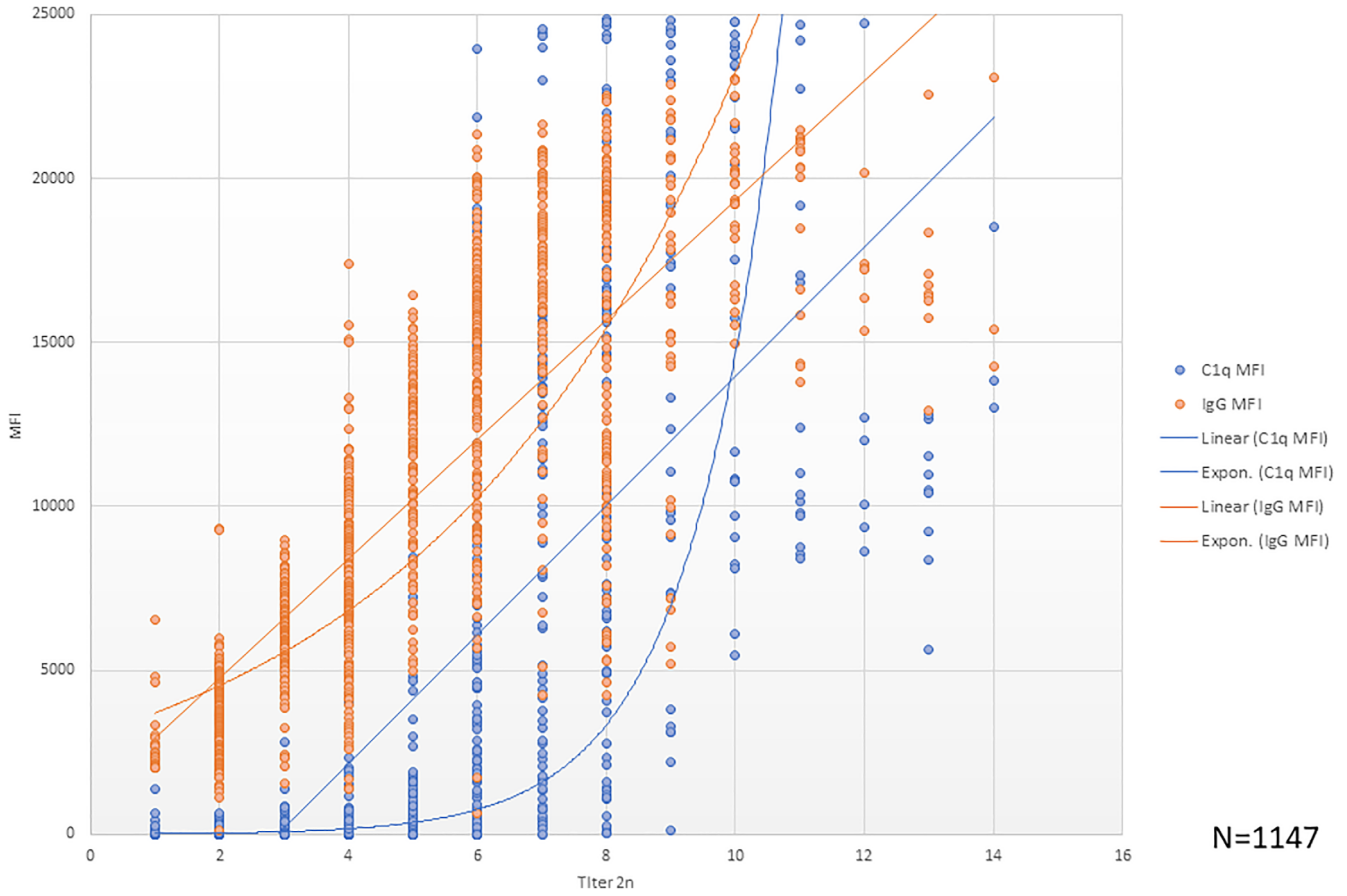

FIGURE 7 Comparison between IgG-MFI, C1q-MFI and dilution studies: SAB assay results for 1147 positive antibodies are compared, demonstrating that he relationships between IgG-MFI (orange) and final dilution (titer) is, for the most part, linear. On the other hand, the relationships between C1q-MFI (blue) and final dilution (titer) is mostly logarithmic, with a slow rise in MFI values for the C1q assay at the lower dilutions, increasing logarithmically as the dilution increases above a titer of $2^{7/8}$ (1:128–1:256). MFI, mean fluorescence intensity; SAB, single antigen bead

information, we conclude that the routine performance of serial dilutions to quantify DSA must be an integral part of evaluating ABMR treatment for clinical trials in addition to following allograft function and evaluating changes in histologic features. Not only will this practice aid in our understanding of the effect of antibody reduction on the progression of ABMR, but it will also allow us to compare the relative efficacy of various antibody depleting strategies.

1.6 | A practical approach to performing serum dilutions

We have previously shown that HLA antibodies with titers of 1:32–1:64 strongly correlate with C1q positivity in SAB assays.[11,13] In other words, >95% of antibodies with these titers were also bound by C1q reagent in in-vitro assays. This makes a lot of sense as each C1q molecule must be bound by IgG portion of 5–6 antibodies to be activated, thus, complement activation requires high concentration of antibodies.[23] Consequently, antibodies with titers ≥1:32 or 1:64 show positivity in CDC crossmatch assays. Flow cytometry crossmatch assays show positivity with antibody titers that are often 2–3 logs lower. Beyond that point, MFI values for the routine (IgG) SAB assay increase linearly, up to saturation while that of the C1q assay increase logarithmically, again demonstrating the different ratio of antibodies that is required for test positivity (Figure 7).

For listing of UA, as addressed above, and especially if other measures are used to minimize inhibition, complete dilution studies (determination of titer) do not contribute much significant information. For the purpose of desensitization using PP/IVIg, for a living donor kidney transplantation—in our experience the likelihood of successfully desensitizing a patient awaiting living kidney transplantation with DSA >1:1024 (using PP/IVIg) is dismal. We therefore recommend doubling dilutions up to a titer of 1:256 (or 1:1024 is increased risk is acceptable). No practical information will be gained beyond that

point. Knowing the exact titer up to this level can provide guidance as to the number of PP/IVIg cycles needed for successful desensitization. For desensitization of patients on the deceased donor waiting list, sufficient information may be gathered by testing SAB assays on neat serum, one dilution around the C1q/CDC positivity (i.e., 1:32/64) and one dilution near bead saturation (1:256/1:512). For the purpose of measuring efficacy of ABMR treatment—similar approach may be taken, with the potential addition of one higher dilution (1:1024/1:2048) as many of the patients are diagnosed with high level antibodies. Knowing the strength of antibody at baseline may encourage continuation of treatment as it will enable better documentation of efficacy.

2 | CONCLUSION

In summary, the performance of serum dilutions has many practical applications pre- and post-transplant for all organ groups. The approach to using serum dilutions should be personalized to meet the needs of the patient and the situation. SAB testing on as little as 1–2 dilutions can provide adequate information in clinical practice for desensitization, waitlist management, and ABMR treatment. However, we strongly advocate that serial dilutions and/or actual determination of antibody titer should be standard protocol in clinical trials for desensitization or ABMR treatment to quantify HLA antibodies accurately and reliably. These practices in combination with accurate HLA typing to correctly identify DSA specificity are key aspects to further advance our understanding of the relevance of DSA quantity on ABMR outcomes and refine ABMR treatment approaches.

ACKNOWLEDGMENT

ART is a Paul I. Terasaki Scholar.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

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

ORCID

Anat R. Tambur https://orcid.org/0000-0002-0320-9825

REFERENCES

1. Schauer U, Stemberg F, Rieger CH, et al. Levels of antibodies specific to tetanus toxoid, Haemophilus influenzae type b, and pneumococcal capsular polysaccharide in healthy children and adults. *Clin Diagn Lab Immunol*. 2003;10(2):202-207.
2. Seagle EE, Bednarczyk RA, Hill T, et al. Measles, mumps, and rubella antibody patterns of persistence and rate of decline following the second dose of the MMR vaccine. *Vaccine*. 2018; 36(6):818-826.
3. Hammarlund E, Thomas A, Poore EA, et al. Durability of vaccine-induced immunity against tetanus and diphtheria toxins: a cross-sectional analysis. *Clin Infect Dis*. 2016;62(9): 1111-1118.
4. Arbuckle MR, McClain MT, Rubertone MV, et al. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med*. 2003;349(16):1526-1533.
5. Frohlich E, Wahl R. Thyroid autoimmunity: role of anti-thyroid antibodies in thyroid and extra-thyroidal diseases. *Front Immunol*. 2017;8:521.
6. Sprogoe U, Yazer MH, Rasmussen MH, Antonsen B, Bistrup C, Assing K. Minimal variation in anti-A and -B titers among healthy volunteers over time: implications for the use of out-of-group blood components. *J Trauma Acute Care Surg*. 2017; 82(6S):S87-S90.
7. https://www.sciencedirect.com/topics/immunology-and-microbiology/hook-effect.
8. Kosmoliaptsis V, Bradley JA, Peacock S, Chaudhry AN, Taylor CJ. Detection of immunoglobulin G human leukocyte antigen-specific alloantibodies in renal transplant patients using single-antigen-beads is compromised by the presence of immunoglobulin M human leukocyte antigen-specific alloantibodies. *Transplantation*. 2009;87(6):813-820.
9. Weinstock C, Schnaidt M. The complement-mediated prozone effect in the Luminex single-antigen bead assay and its impact on HLA antibody determination in patient sera. *Int J Immunogenet*. 2013;40(3):171-177.
10. Schnaidt M, Weinstock C, Jurisic M, Schmid-Horch B, Ender A, Wernet D. HLA antibody specification using single-antigen beads—a technical solution for the Prozone effect. *Transplantation*. 2011;92(5):510-515.
11. Tambur AR, Herrera ND, Haarberg KM, et al. Assessing antibody strength: comparison of MFI, C1q, and titer information. *Am J Transplant*. 2015;15(9):2421-2430.
12. Tambur AR. Hiding in plain sight-a new look at HLA epitopes: a case report. *Am J Transplant*. 2016;16(11):3286-3291.
13. Tambur AR, Wiebe C. HLA diagnostics: evaluating DSA strength by titration. *Transplantation*. 2018;102(1S):S23-S30.
14. Tambur AR, Campbell P, Claas FH, et al. Sensitization in transplantation: assessment of risk (STAR) 2017 Working Group Meeting Report. *Am J Transplant*. 2018;18(7):1604-1614.
15. Tambur AR, Campbell P, Chong AS, et al. Sensitization in transplantation: assessment of risk (STAR) 2019 working group meeting report. *Am J Transplant*. 2020;20(10):2652-2668.
16. Schinstock CA, Smith BH, Montgomery RA, et al. Managing highly sensitized renal transplant candidates in the era of kidney paired donation and the new kidney allocation system: is there still a role for desensitization? *Clin Transplant*. 2019; 33(12):e13751.
17. Pinelli DF, Zachary AA, Friedewald JJ, et al. Prognostic tools to assess candidacy for and efficacy of antibody-removal therapy. *Am J Transplant*. 2019;19(2):381-390.
18. Montgomery RA, Lonze BE, King KE, et al. Desensitization in HLA-incompatible kidney recipients and survival. *N Engl J Med*. 2011;365(4):318-326.

19. Schinstock C, Tambur A, Stegall M. Current approaches to desensitization in solid organ transplantation. *Front Immunol*. 2021;12:686271.
20. Tambur AR, Schinstock C, Maguire C, Lowe D, Smith B, Stegall M. Estimating alloantibody levels in highly sensitized renal allograft candidates: using serial dilutions to demonstrate a treatment effect in clinical trials. *Am J Transplant*. 2021; 21(3):1278-1284.
21. Maguire CH, Schinstock CA, Tambur AR. Measuring human leukocyte antigen alloantibodies: beyond a binary decision. *Curr Opin Organ Transplant*. 2020;25(6):529-535.
22. Aubert O, Loupy A, Hidalgo L, et al. Antibody-mediated rejection due to preexisting versus de novo donor-specific antibodies in kidney allograft recipients. *J Am Soc Nephrol*. 2017;28(6): 1912-1923.
23. Diebolder CA, Beurskens FJ, de Jong RN, et al. Complement is activated by IgG hexamers assembled at the cell surface. *Science*. 2014;343(6176):1260-1263.

AUTHOR BIOGRAPHIES

Anat R. Tambur is a research professor in the Department of Surgery, Comprehensive Transplant Center at the Fienberg School of Medicine, Northwestern University, Chicago, Illinois. She also serves as the Chair of the Sensitization in Transplantation: Assessment of Risk (STAR) working group through ASHI and the AST. Her clinical and research expertise is in HLA antibodies, with particular interest in HLA-DQ. She is involved in guiding approaches for quantifying and monitoring HLA antibodies and participate as a consultant and core laboratory director for multiple desensitization trials.

Carrie Schinstock MD is an associate professor in the Division of Nephrology and Hypertension at Mayo Clinic in Rochester, MN where she is the Medical Director of the Kidney Transplant program and Convergence Chair for the Mayo Clinic Kidney Transplant programs in Rochester, Phoenix, and Jacksonville. She also serves as a chair on the Banff Antibody Mediated Rejection Working Group and participates in the STAR working group through AST. Her clinical and research expertise is in antibody mediated rejection and kidney paired donation. She has particular expertise in getting patients transplanted with high levels of alloantibody toward their donor including HLA incompatible transplantation and desensitization strategies. She also has interest in therapeutic clinical trials for patients with chronic antibody mediated rejection.

How to cite this article: Tambur AR, Schinstock C. Clinical utility of serial serum dilutions for HLA antibody interpretation. *HLA*. 2022;100(5):457-468. doi:10.1111/tan.14781