# Questioning the Added Value of Luminex Single Antigen Beads to Detect C1q Binding Donor HLA-Specific Antibodies

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Luminex single antigen bead (SAB) technology enables highly sensitive and rapid characterization of immunoglobulin G (IgG) human leukocyte antigen (HLA)-specific antibodies. It is widely used to determine antibody compatibility for renal transplantation and to aid the diagnosis of antibody-mediated rejection and response to therapy. HLA-specific antibodies may contribute to allograft rejection through a variety of mechanisms, including activation of the classical complement pathway, endothelial cell activation, and recruitment of Fc-dependent effector cells. Of these, complement-mediated cytotoxicity has long been associated with hyperacute rejection and, more recently, antibody-mediated rejection for which the deposition of the complement component C4d on peritubular capillaries is a diagnostic marker. In the standard SAB assay, patient serum is incubated with microbeads coated with purified HLA proteins. Human leukocyte antigen–specific IgG antibody binding is then detected using a fluorescent anti-IgG detection antibody. Increasing levels of donor HLA-specific antibodies detected by this assay predict inferior long-term graft outcome, but not all patients with IgG donor-specific HLA antibodies (DSA) have a poor graft outcome (*1, 2*). This observation is consistent with the notion that HLA antibodies with the same specificity may differ in their ability to cause graft injury, and this variability may be related to differences in complement fixing activity.

In an effort to improve the clinical utility of the IgG-SAB assay, a novel C1q-SAB Luminex assay was developed, which aims to detect only complement fixing HLA-specific antibodies. In this assay, heat-inactivated patient serum is incubated with HLA-coated microbeads, with the addition of purified human C1q. C1q is the first component of the classical pathway of complement activation and binds to the Fc region of complement fixing IgG isotypes, mainly IgG1 and IgG3. Bound C1q is then detected by the addition of fluorescent-conjugated anti-human C1q detection antibody (Fig. 1A, panel a). Several studies have suggested that in patients after renal transplantation with DSA detected by IgG-SAB, the use of the C1q-SAB assay provides improved prediction of those most at risk of graft failure (*3–5*). In the most comprehensive analysis to date, Loupy et al. (*4*) found that for patients where DSA were identified within the first year of renal transplantation, those with C1q-binding DSA showed a much worse graft survival than those with non–C1q-binding DSA (54% vs. 93% 5-year graft survival).

Although the presence of C1q-binding DSA are associated with inferior transplant outcome, it is by no means clear that this is a direct consequence of the ability of detected antibodies to activate complement. First, there are technical limitations to the C1q-SAB assay that have important consequences for interpretation of the test results. C1q is only able to bind when two IgG molecules are spaced 30 to 40 nm apart (*6*), which occurs readily when IgG cross-links target HLA glycoproteins that have mobility within the lipid bilayer of a cell membrane. In solid phase binding assays, if HLA target molecules immobilized on the surface of SAB meet this strict spatial requirement for C1q, high levels of complement binding IgG will give a strongly positive readout (Fig. 1A, panel a). However, high levels of DSA IgG isotypes that are complement binding may not be detected in the C1q-SAB assay if the density of HLA protein is low and there are high levels of denatured HLA protein unable to bind antibody that interfere with the spatial position of intact HLA (Fig. 1A, panel b). Complement fixing antibodies of potential clinical importance may also be present, but at levels below the threshold required for effective binding of C1q in the C1q-SAB assay (Fig. 1A, panel c). Second, the C1q-SAB assay may reveal high levels of C1q binding in a serum that apparently has only low levels of IgG detected in the standard IgG-SAB. However, this apparent anomaly can be explained by the presence of blocking factors (particularly C1q itself) present in undiluted patient serum that interfere with binding of the fluorescent anti-IgG detection antibody (Fig. 1B panel a) (*7, 8*). Pretreatment of test sera by heat inactivation or dithiothreitol to denature

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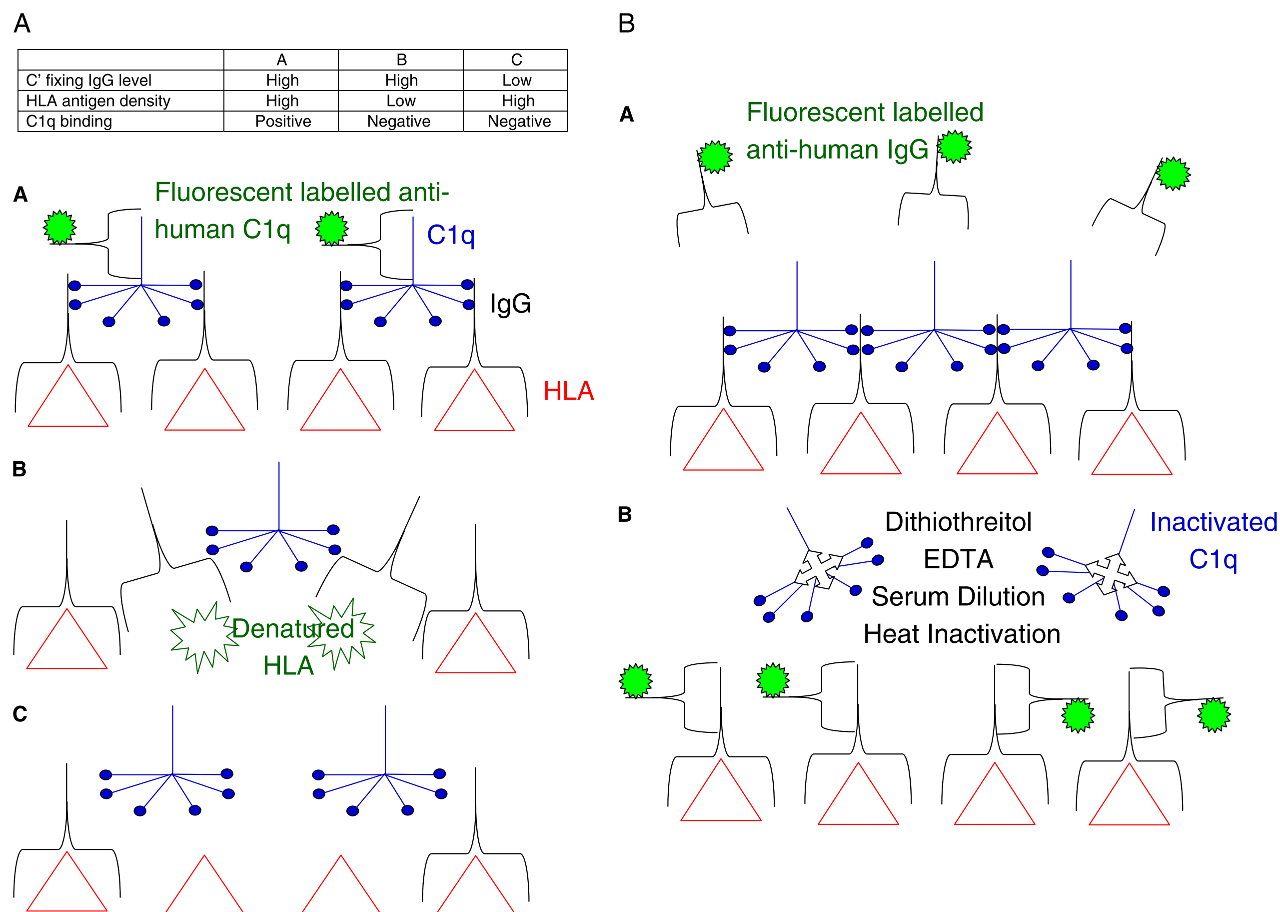

| | A | B | C |
|---|---|---|---|
| C' fixing IgG level | High | High | Low |
| HLA antigen density | High | Low | High |
| C1q binding | Positive | Negative | Negative |



**FIGURE 1.** Technical considerations for the detection of HLA-specific antibodies in the standard and C1q-modified Luminex single antigen bead assays. A, Detection of C1q-SAB assay is dependent on appropriate spatial orientation of intact, immobilized HLA molecules and high levels of HLA-specific, complement-binding, IgG antibody (panel 1A). If the density of HLA protein on SAB is low and there are high levels of denatured HLA protein that interfere with the spatial position of intact HLA, high levels of HLA-specific IgG isotypes that are complement-binding may not be detected in the C1q-SAB assay (panel 1B). Similarly, low levels of HLA-specific, complement-binding, IgG antibodies may be below the threshold required for effective binding of C1q in the C1q-SAB assay (panel 1C). B, Despite high levels of C1q-binding detected in the modified C1q-SAB assay, endogenous serum C1q interference preventing binding of fluorescent labeled anti-human IgG to IgG HLA-specific antibodies may lead to misleading low assessment of IgG binding in the standard SAB assay (prozone effect, panel A). Endogenous serum C1q inactivation or denaturation in the presence of DTT/EDTA/heat inactivation enables detection of true levels of HLA-specific IgG binding in the standard SAB assay (panel B). SAB, single antigen bead; HLA, human leukocyte antigen; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

C1q, the addition of ethylenediaminetetraacetic acid to inactivate C1q binding or serum titration to dilute out C1q while retaining saturating levels of IgG are all effective strategies for overcoming this artefact and one of these should be adopted routinely to avoid underestimating the levels of HLA-specific IgG (*9*).

In this edition of Transplantation, Schaub et al. (*10*) report an analysis of sera obtained from sensitized patients immediately before renal transplantation where the results obtained in the IgG-SAB assay were compared with those from the C1q-SAB assay. Although no attempt was made to negate the potential confounding effect of putative blocking factors in the test sera, a close relationship was observed between the levels of antibody detected by IgG-SAB and those detected in the C1q-SAB assay. High level IgG (median fluorescence intensity>14,154) predicted C1q positivity with 92% sensitivity and 96% specificity. In addition to the standard pan-IgG-SAB assay, Schaub et al. also determined the IgG isotype of antibodies detected and noted that the great majority of alloantibodies detected by the pan-IgG-SAB assay were of complement fixing isotypes (IgG1 and IgG3) which helps explain the strong correlation between the two assays. Interestingly, however, some sera that gave a negative result in the C1q-SAB assay were still found to contain high levels of HLA-specific IgG1 and IgG3 in the IgG-SAB assay. Given these observations, and the technical limitations of the C1q-SAB assay outlined above, it seems reasonable to question whether the C1q-SAB assay provides useful clinical information over and above that provided by the standard IgG-SAB assay, particularly given the substantial additional cost involved.

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