



## Crossmatch Strategies in Renal Transplantation: A Practical Guide for the Practicing Clinician

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### Abstract

Immunogenetic profiling of transplant recipients and pre-transplant tissue crossmatch between potential donor and recipient is mandatory in modern-day renal transplantation. Pre-transplant crossmatch allows characterization of preformed donor specific antibodies in the recipient, allowing prognostication of the prospective transplant and minimizing potentially catastrophic antibody mediated allograft injury. With advancement in technology and better overall understanding of the donor-recipient immune interactions, different crossmatch strategies have evolved to detect different types of alloreactive antibodies in the recipient. This article takes a closer look at available crossmatch techniques and their clinical use in modern-day transplantation.

### Keywords

Histocompatibility testing, HLA antigens, Kidney transplantation, Antibodies, Virtual crossmatch

### Introduction

In his landmark paper, Terasaki, et al. (1969) demonstrated Hyperacute Rejection (HAR) resulting from pre-formed cytotoxic antibodies in the recipient against Class-I Human Leucocyte Antigen (HLA) in the allograft [1,2]. Since the discovery of these antibodies and understanding of their possible impact, routine tissue crossmatch became an integral component in renal transplantation. The purpose of a pre-transplant crossmatch is to identify such pre-existing Donor Specific Antibodies (DSA) in the recipient's serum that would potentially react with donor antigens. It gives an indication about possible immunological compatibility between the donor-recipient pair thus allowing to avert major complications such as HAR, Antibody Mediated Rejection (AMR) and graft loss [3,4].

Since its adoption as a critical step in pre-transplant work up, tissue crossmatch compatibility testing has advanced and evolved with new techniques and better overall understanding. This review looks at different crossmatch strategies to detect DSA in the transplant re-

ipient, which in turn allow risk stratification and equitable organ allocation while maintaining the best possible graft and patient outcomes.

### The Complement System in Transplant Immunity

The complement system is a group of proteins which was first identified as a support system complementing the actions of the body immune system in removing pathogenic organisms. Subsequent research revealed that the complement system has numerous other functions including augmentation of the innate and adaptive im-

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immune mechanisms. Since then, the complement system has also been identified as playing a pivotal role in transplant immunology. The complement proteins naturally exist in their precursor state. They can be activated by the classical, alternate or lectin pathways thereby forming a cascade of events that culminates in the formation of a Membrane Attack Complex (MAC). The MAC leads to immune mediated destruction of pathogens and ‘foreign’ antigens. This complement mediated activity is a key component of serum based ‘wet’ crossmatch testing in transplantation.

### The Complement Dependent Cytotoxicity Crossmatch (CDC-XM)

The CDC-XM was the first commonly used crossmatch technique adopted in routine practice. While evolving in technique to minimize its short comings, the CDC-XM remains an integral component of pre-transplant crossmatch among most transplant centers worldwide. The technique uses donor lymphocytes and recipient serum, which are incubated before addition of complement. It detects all complement fixing IgG, IgM antibodies of HLA and non-HLA origins as well as autoantibodies.

The CDC-XM relies on subjective visual assessment of complement mediated cell death caused by the MAC following antigen-antibody interaction. A fluorescent dye is added which penetrates the lysed cells, giving a distinct colour compared to viable cells on fluorescent microscopy. The test depends entirely on the complement fixing capability of antibodies and does not detect non-complement fixing antibodies. A CDC-XM+ result, is generally considered a contraindication to proceed with transplantation unless it can be conclusively established that the result was not caused by IgG HLA alloantibodies [5].

**Importance of a T/B cell positive CDC-XM:** The CDC-XM is performed separately on T and B cell lines. T lymphocytes carry only HLA class-I antigens. DSA against such HLA-I antigens are associated with significant risk of HAR and AMR [6]. Therefore, the T cell crossmatch is a crucial component in the CDC-XM.

B cells carry both HLA class-I and class-II antigens. Therefore, a positive B cell crossmatch means DSA against either HLA class-I, II or both. A B-cell positive, T-cell negative crossmatch, signifies either DSA against HLA class-II only or low titre DSA against HLA class-I undetectable by T cells. The significance of such an isolated B cell crossmatch is often inconclusive with false positive rates as high as 50% being reported in earlier studies [7]. The use of certain immune modulating medications such as alemtuzumab and rituximab can also lead to false positive B-cell crossmatch [7-9]. However, newer studies have emerged that highlighted the possible impaired

**Table 1:** Limitations of CDC-XM [49].

Limitations of CDC-XM
• Requires a constant supply of fresh viable donor lymphocytes
• Testing process is cumbersome especially in the emergency setting for deceased donor transplants
• Low sensitivity with false negative results; caused by low titre DSA, complement inactivation, etc.
• Low specificity with false positive results due to autoantibodies, immune complexes, etc.
• Non-complement fixing antibodies are not detected
• Lack of standardization regarding panel composition among different laboratories

**Table 2:** Interpretation of CDC-XM results [11].

T cell XM	B cell XM	Explanation
Negative	Negative	No significant DSA Very low titre DSA Non-complement fixing DSA
Positive	Positive	HLA antibodies Autoantibodies Non-HLA antibodies IgG IgM antibody Recent treatment with thymoglobulin/ alemtuzumab
Negative	Positive	DSA to HLA class II only Low titre HLA class-I DSA Treatment with rituximab

graft survival associated with low-titre B-cell crossmatch positivity as detected in flow-cytometry crossmatch [10].

**Interpretation of the CDC-XM results:** A CDC-XM+ result should always be investigated further as proceeding regardless has been shown to result in a higher risk of early graft loss [2]. It should be viewed in conjunction with other tests such as flow-cytometry and DSA screening, to identify immunologically significant alloantibodies. In the absence of corroborative positive results from flow-cytometry or DSA screening, the possibility of auto-antibodies (IgM) should be considered as a reason for CDC-XM+ result. Such IgM autoantibodies are considered harmless in the transplant setting [11,12]. Table 1 enlists the common limitations encountered in CDC-XM while Table 2 depicts the basic interpretation of CDC-XM results for transplantation.

If a flow-cytometry or DSA screening were negative in the presence of a CDC-XM+ result, an initial step would be to repeat the CDC-XM to exclude laboratory error. If still positive, further evaluation is mandatory to rule out autoantibodies. The first step in this process is to perform an auto-crossmatch, using recipient’s serum against own lymphocytes [11]. Most histocompatibility laboratories would perform an auto-crossmatch routinely. If positive, it indicates the presence of IgM autoantibodies, while not completely ruling out coexisting alloantibodies. Therefore, a second test is done concurrently, that involves repetition of CDC-XM after neutralizing IgM as discussed below.

**Improving specificity and sensitivity of CDC-XM:** Several modifications of the CDC-XM are used to neutralize the effect of IgM. One strategy is the use of Dithiothreitol (DTT), which inactivates IgM by cleaving their disulfide bonds [12]. This test is done with a control that uses buffered saline instead of DTT to give the same liquid dilution effect. Hence if the DTT treated CDC-XM becomes negative while the control remains positive, it signifies the presence of IgM autoantibodies and rules out alloantibodies, allowing the transplant to proceed.

- Removing auto-reactive antibodies: Reactivity from IgM HLA-specific antibody is indistinguishable from IgM autoreactive antibody. By pre-absorbing serum with autologous cells, autoreactive antibodies are removed prior to screening.
- Increasing incubation time and heating: Alternate methods of inactivating IgM include extended incubation times and heating the sera to 55 °C [13]. These modifications also result in inactivation of IgM while sparing the immunologically significant IgG-DSA.
- The low sensitivity of CDC-XM has resulted a significant incidence of early graft rejection despite a negative pre-transplant CDC-XM [14]. Several further modifications to the original CDC-XM were introduced to increase its sensitivity. Among them, two commonly used modifications are the additional wash step and addition of Anti-Human Globulin (AHG).
- Addition of wash steps: In Amos (3-wash) and Amos-modified (1-wash) modifications, additional wash steps are added to eliminate anti-complementary factors that prevent complement fixing. Such anti-complementary factors may result in false negative crossmatches [15]. The wash step modifications maximize DSA-complement interaction and results in higher sensitivity of the test.
- Anti-Human Globulin (AHG) augmentation: In this technique, a complement fixing Antihuman light chain is added to washed cells before addition of complement. Multiple AHG molecules get bound to individual DSA, amplifying its effect on complement activation and cell lysis. This identifies low titre antibodies as well as those that do not fix complement *in vitro*, improving overall sensitivity [16,17].

### The Flow-Cytometry Crossmatch (FCXM)

Until the early 1990s, a negative CDC-XM was considered a clear indication to proceed with a proposed transplant. However, subsequent introduction of Flow-Cytometry Crossmatch (FCXM) resulted in identification of clinically relevant DSA even with a negative CDC-XM [18,19].

FCXM also depends on donor lymphocytes being incubated with recipient serum. However, instead of add-

ing complement factors, a fluorescence-coated second antibody is added that acts against the IgG-DSA. This anti-IgG antibody binds to the donor Ag-DSA complex and allows detection through a flow-cytometer. The reading is semi-quantitative and more objective in that instead of a visual count of cell death, it uses channel shifts above the baseline [20].

**Advantages of FCXM:** FCXM has a better sensitivity and specificity compared to CDC-XM. It is more sensitive in that it does not depend on complement activity, thereby detecting both complement fixing as well as non-complement fixing DSA. The use of FCXM over AHG-enhanced CDC-XM has shown significantly reduced incidence of AMR and graft loss at 1 year [14,21]. A negative FCXM rules out the possibility of immunologically significant DSA. Furthermore, FCXM also has a higher specificity than CDC-XM as it detects only IgG and does not detect IgM antibodies. Overall, FCXM has shown better correlation with graft outcomes compared to CDC-XM. Numerous studies have shown improved short and long-term term outcomes with reduced rates of rejection and better graft survival with FCXM based transplantation [22,23].

**Limitations of FCXM:** Like the CDC-XM, FCXM cannot differentiate between for HLA and non-HLA antibodies. One method of further increasing its sensitivity and specificity is with the addition of enzyme pronase to digest the Fc receptors on donor lymphocytes [24]. Pronase treatment minimizes the binding of non-specific antibodies, thereby improving overall specificity. This becomes useful in interpretation of results where the CDC-XM was negative with a positive FCXM.

### Solid Phase Immunoassay (SPI)

SPI is a predictive assay based on commercial kits of purified recombinant HLA molecules coated on a microtitre plate; (ELISA) [23] or synthetic beads; (Luminex) [25,26]. SPI is specific for HLA antibodies and thereby eliminates the false positives in CDC-XM and FCXM caused by non-HLA antibodies and autoantibodies. ELISA test is more sensitive than CDC-XM while the Luminex is more sensitive than both the CDC-XM and FCXM.

The Luminex-SPI is now considered the benchmark in detecting immunologically significant DSA [27]. This consists of a series of polystyrene microsphere beads to which target HLA antigens are attached after purification. The relevant beads are labelled with differing ratios of fluorescent dyes giving them a unique fluorescent signal. Test sera is added where any DSA present in the sera would bind to appropriate HLA molecules on beads. The resulting antigen-antibody binding can be detected via laser based fluorescent imaging quantified as Mean Flu-

orescent Intensity (MFI). The assay can be taken one step further with the Single Antigen Bead (SAB) test, where the relevant beads are all coated with a single cloned antigen. The SAB test is the most precise and specific in detecting DSA against a specific antigen [28].

SPI is designed as a qualitative test to detect and differentiate specific HLA antibodies. Nevertheless, some studies have also shown that the resulting MFI values may correlate with CDC-XM results and clinical outcomes, thereby making it a semi-quantitative assessment of DSA [29,30]. However, the MFI values do not directly correlate with the antibody titres and can be affected by additional factors such as antigen-antibody avidity, antibody conformation and orientation in the beads [31].

**Advantages of SPI:** The advent of SPI revolutionized pre-transplant identification and characterization of DSA. SPI depends on commercially available kits and do not depend on the viability of donor lymphocytes. It has very high specificity, detecting specific anti-HLA antibodies and minimizes false positives due to non-HLA and auto-antibodies. It also has high sensitivity and is not restricted to complement fixing antibodies. In addition, the readings are semi-quantitative, based on optical density and can be partially automated to make it more objective.

**Limitations of SPI:** Patients on long-term haemodialysis tend to have elevated levels of IgM. High concentrations of IgM or Complement factors (C1) can competitively bind to and saturate antigen beads, preventing their binding to actual IgG-DSA [32]. This is called the 'prozone effect' or 'hook effect'. It results in a false negative luminex test, which becomes strongly positive in CDC-XM. Similar false negative results can occur with very high DSA levels, where the high dose antibodies agglutinate in suspension thereby failing to bind to the target antigen beads. In such circumstances, removing excess IgM by heat inactivation, EDTA or DTT treatment can assist in clinical interpretation. An alternative method is to use diluted recipient serum or serum after hypotonic dialysis that removes excess IgM. Alternatively, addition of C1 Inhibitor (C1INH) to neutralize C1 effect is also possible.

A given antigenic epitope may occasionally appear on multiple beads in the luminex kit. Hence the DSA bind to multiple beads and gets diluted rather than binding to a single bead, thereby giving a false negative signal. This effect is minimized by using the highly specific SAB assays. Furthermore, lack of standardization with regard to antigen concentration on different beads gives rise to confusion in interpretation of results. Different antigens may appear in different concentrations among different beads in the test that result in under or overestimation of DSA content.

Presence of denatured antigen on the bead surface can lead to presentation of numerous non-native HLA epitopes due to altered protein conformation. Strategies to tackle this include treating the beads with acid to fully denature the beads' protein repertoire and use of two manufacturer's kits.

A positive Luminex result occurring in the presence of a negative CDC-XM may be due to very low titre DSA and is of doubtful significance. There can also be significant inter-laboratory variation in reporting positive results thereby confounding its interpretation [20]. Therefore, a consensus between the cross-match laboratory and the clinical team is imperative in determining the significant 'cut off' values for SPI.

**The C1q assay:** The C1q assay is a newer modification to the Luminex based SAB-test, designed to overcome some of the existing shortcomings. The CDC-XM has the inherent weakness of low sensitivity and inability to detect low titre DSA which may become clinically significant. While the Luminex SAB technology overcomes this weakness, it may detect both complement dependent and non-complement dependent antibodies. The clinical significance of such DSA detected on Luminex SAB in the presence of a negative CDC-XM remains inconclusive. This resulted in the search for a test that is both sensitive to detect low titre DSA and specific to identify complement fixing ability that is considered crucial in antibody mediated graft injury.

C1q is the first component protein of the complement pathway. The C1q assay is designed to selectively identify only the DSA that bind C1q and thereby activate the complement pathway, with the same degree of sensitivity as the original SAB test but with enhanced specificity for complement fixing [33,34]. Initial experience with the assay have shown that C1q binding DSA are more predictive of allograft injury compared to those that do not bind C1q [35]. However, technical interferences in the testing as well as significant additional costs involved have limited its use in routine practice.

**Epitope matching:** During antigen-antibody binding, the DSA binds to a limited area of the HLA molecule comprising a 15-25 amino acid sequence. This specific binding area of the HLA is called an 'epitope'. While some of these epitopes are unique to a single HLA (private epitopes), others are shared among numerous antigens (public epitopes) [32]. These public epitopes result in numerous cross-reactions during HLA testing that result in false positive results. Using epitopes as the focus in HLA typing allows more precision compared to the traditional HLA molecule based matching. Epitope based matching has been shown to be more predictive of crossmatch results and subsequent graft outcome compared to HLA molecule matching [36].

Epitope based matching can be done both in the laboratory (*in-vitro*) and from a computer based system (*in-silico*). The commonest *in-vitro* epitope based matching system is the highly specific SAB system [32]. In contrast, several computer-based algorithms have been designed for *in-silico* epitope based matching. The most widely used such algorithm is the 'HLA Matchmaker algorithm' [37,38]. This is a fully computer and web-based algorithm that allows donor and recipient matching based on epitopes and has been used especially in relation to transplanting highly sensitized recipients.

### The Virtual Crossmatch (VXM)

One of the biggest technological advances in the field of transplantation in the recent past has been the shift from 'wet' crossmatch to Virtual Crossmatch (VXM) based on Luminex assays. Luminex-SAB assay has enabled accurate screening of prospective recipients for DSA without an actual 'wet' crossmatch based on donor and recipient serum sampling. The antigens against which these DSA are detected are referred to as *Unacceptable Antigens* (UA), which can trigger HAR. The VXM compares the recipient's UA against the HLA screening of the potential donor, by a virtual matching process rather than an actual laboratory 'wet' crossmatch [26,39]. Having a Luminex based accurate immunological profile of the potential recipient has allowed an 'in silico' computer based VXM to be performed, improving the overall transplant ability in sensitized patients [40]. It has also eliminated the need for mandatory pre-transplant physical crossmatch and improved organ allocation efficiency based on such pre-identified UA.

**Interpretation of VXM results:** A negative VXM in a patient with no known sensitization history can often be considered adequate to proceed with a deceased donor transplant without an actual 'wet' crossmatch, thereby avoiding unnecessary delays in laboratory and minimizing cold ischaemia times [41]. However, in patients with a history of possible sensitization due to pregnancy, previous transplant or blood transfusion, a FCXM is mandatory before transplantation. A negative VXM in the presence of a FCXM+ result may signify the presence of 'new' DSA and needs further evaluation before transplant. Similarly, the implications of a positive VXM should always be viewed in conjunction with FCXM results. A positive VXM with a negative FCXM, could be due to low titre antibodies of doubtful clinical significance and is often considered safe to proceed with transplantation [42].

The result of VXM is also dependent on the time of DSA screening and can vary depending on which sample of serum was used for testing. This is relevant for patients with a history of sensitizing events as the DSA profile can

change with time. Although the most recent serum sample may not detect DSA from a past exposure, the effect of memory cells can lead to significant AMR following transplantation. Such patients where previous samples had detected DSA but have a negative current DSA, have demonstrated increased rates of post-transplant graft loss, possibly due to memory cell effect [43]. Hence the VXM results should consider all previous stored serum DSA results where available [44]. Tests performed immediately before the transplant are the most reliable and represent the most recent DSA status. If a 'historical' DSA result was used for the VXM, any 'new' antibodies from recent sensitizing events would be missed giving rise to a false negative VXM.

**Difference between PRA/CPRA:** Testing recipient sera against a panel of donor lymphocytes from a sample of donors representing the potential local donor population, reacting antibodies in the recipient can be detected. These cross reacting antibodies are termed Panel Reactive Antibodies (PRA) and a %PRA score can be calculated. A recipient's %PRA indicates the probability of having a positive crossmatch against a given donor from that population and thereby the chances of receiving a crossmatch negative kidney [45].

PRA scoring is performed by testing recipient's serum against a sample donor pool consisting of approximately 100 blood donors. As the %PRA predicts the likelihood of getting a positive crossmatch, a high %PRA is an indication of increased difficulty in getting a crossmatch negative graft. However, it does not specify which donor antigens are unacceptable. Furthermore, PRA values from different centers, using kits from different vendors had a wide variation due to lack of standardization. This resulted in a wide variation among different donor panels which were not truly representative of the local donor population. This variation in results limits the clinical applicability of PRA results.

The calculated Panel Reactive Antibodies (cPRA) is a more accurate and specific measure of UA for a given recipient [46]. The cPRA, is a computerized calculation based on profiling of actual kidney donors (10000 in UK, 12000 in US between 2003-2005), which is more representative of the actual potential organ donor population. Once the recipient's UA are entered, the formula calculates the cPRA based on how many donors carry those UA. Kidneys from donors who carry the UA will not be offered during allocation, thereby minimizing the chance of an actual positive crossmatch after allocation. Therefore, even with a high cPRA, once a kidney is actually offered, the chances of a negative crossmatch and proceeding with transplant is high compared to a patient with a high %PRA who is offered a kidney [47]. Since cPRA uses the same computerized formula, variability

**Table 3:** Comparison of PRA with CPRA.

PRA	CPRA
Indicator of general non-specific reactivity between recipient and potential sample of donors	Calculates specific unacceptable antigens in the wide donor database
Measures class-I and class-II antibodies separately	Class-I and class-II both calculated together
High PRA indicates high probability of positive crossmatch with a donor offer	Even with a high CPRA, high probability of a negative crossmatch once the organ is offered
Variation in PRA based on laboratory and time of testing	No variation as it is based on a uniform database

among centers is eliminated and adds consistency to the organ allocation policy. **Table 3** compares the utility and characteristics of PRA and cPRA.

While significant forward strides have been made in organ allocation with cPRA, limitations do exist. One such limitation is the potential positive crossmatch that may occur due to the presence of antibodies against HLA-Cw, DQ alpha chain and DP antigens. While the exact immunological significance of these antigens and their relevant antibodies is still not conclusive, non-inclusion of these antigens in the deceased donor screening and thereby cPRA calculation, may result in subsequent positive crossmatch after allocation [46].

### Crossmatch in ABO and HLA Incompatible Transplant

Traditionally, ABO blood group incompatibility had been considered an absolute contra-indication to proceed with transplantation owing to the unacceptably high risk of HAR and graft loss. However, with the increasing demand for transplantation and shortage of compatible donors, advances have been made in successful transplantation across the blood group and HLA barrier. This is achieved by a process of desensitization that remove the blood group iso-haemagglutinins or anti-HLA DSA to the point of achieving a negative pre-transplant crossmatch. Although carefully planned desensitization protocols have offered hope in immunologically incompatible situations previously considered insurmountable, escalated costs, limited experience and paucity of long-term follow up data has prevented more widespread use of this strategy [48].

### Conclusions

Current solid phase crossmatch techniques such as Luminex SAB are much more specific and sensitive than CDC-XM, FCXM and ELISA assays. No single crossmatch technique is good enough in isolation when faced with a clinical challenge of managing a complex prospective recipient. In order to avoid exclusion of a good donor and to be successful in transplanting such a patient, an immunological assessment of a prospective recipient requires close cooperation between transplant immunologists, surgeons and nephrologists. This would involve interpretation of clinical background and the plethora of laboratory tests that includes various types of crossmatch and cPRA. We should

be aware of inherent strengths and limitations of the whole panel of investigations in order to make clinically meaningful informed decisions pertaining to transplantation.

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### Conflicts of Interest

None.

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