

CONFIRMATION OF MHC CLASS II ANTIBODY REACTIVITY IN SOLID PHASE BEAD
ASSAYS USING AN HLA EXPRESSING CELL LINE

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ABSTRACT

In order to assess compatibility between donors and recipients for organ transplantation, the recipient's anti-HLA antibody profile is compared to the donor's HLA typing. Determination of the recipient's anti-HLA antibody profile is done through use of a single antigen bead assay. Unfortunately, false positive reactivity within the assay is one of its major limitations. While not as dangerous as a false negative, a false positive result can greatly reduce a recipient's chances of receiving an organ. For example, a DRB4 false positive result in the MHC class II single antigen bead assay, a patient is considered non-compatible with 50% of the population.

One of the primary ways false reactivity is identified is through surrogate flow cytometric crossmatching, where a donor expressing the antigen of interest is incubated with the recipient's serum to see if a reaction occurs. While surrogate flow cytometric crossmatches are useful in determining the true reactivity of a recipient, finding an acceptable donor can often be difficult if the patient is highly sensitized. Therefore, it was hypothesized that a cell line expressing an MHC antigen of interest could be used in place of a surrogate donor.

This study focused on using T2 cell lines expressing either DRB4 or DQA1*05:01/DQB1*02:01, two common MHC class II false positive results within the single antigen bead assay, as surrogate donors for a flow cytometric crossmatch. Results showed that the T2 cell line could be used as a surrogate donor. When compared to clinical surrogate crossmatches, the T2 cell line surrogate flow cytometric crossmatches showed concordance rates of 95% and 92% for DRB4 and DQA1*05:01/DQB1*02:01 respectively.

In conclusion, it was determined that the T2 cell line could be used as a surrogate donor for ruling out false positive results in the single antigen bead assay. While further testing is still necessary, the success of this experiment opens the door for further investigation. It is likely that other MHC class II antigens could be transduced into the T2 cell line to rule out more false positive results, or another cell line could be used to allow MHC class I false positives to be ruled out. Overall, the success of this experiment shows promise for the future as new ways are found to improve patients' chances of receiving a transplant, and improve overall outcomes in the field of transplantation.

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INTRODUCTION

Transplantation

Solid organ and bone marrow transplants are life-saving procedures. When comparing donor-recipient pairs for compatibility, the primary focus is matching for HLA-Class I alleles A, B, and C, which are expressed on all nucleated cells, and HLA-Class II alleles DR, DQ, and DP which are expressed most prominently on antigen presenting cells, such as B-cells, dendritic cells and macrophages.¹ In an ideal world, the donor and recipient would be matched at every allele. However, finding a perfect match is incredibly unlikely, so the next best option is to match for as many alleles as possible.² In solid organ transplantation, any allelic mismatches between the recipient and the donor may be a target for rejection. In stem cell transplants, both rejection and graft vs host disease (GVHD) are concerns. In addition, anti-HLA antibodies can develop when individuals are exposed to MHC complexes on another person's cells. These sensitizing events include pregnancy, blood transfusions, or previous transplants. Ensuring the recipient does not have any pre-existing HLA antibodies to donor mismatched alleles is critically important as not doing so could lead to various negative outcomes such as graft loss, GVHD, or even death.

Antibody Screening and Single Antigen Bead Assays

One of the revolutionary assays in improving patient outcomes was the development of the solid-phase Single Antigen Bead (SAB) assay to detect specific anti-HLA antibodies that could cause graft rejection. For example, instead of indicating that the patient has Class I reactivity, the SAB assay shows that there are antibodies to HLA-A2, B5, Cw7, etc. Knowing the specific antibody profile of each recipient helps predict if a graft will be rejected or run a higher risk of failing, as the presence of an existing donor specific anti-HLA antibody (DSA) indicates an unsuitable donor.³ Additionally, if the patient has received a transplant, SAB assays can be utilized to monitor if the graft is still healthy, as a spike in DSA post-transplant can indicate that the graft might be rejecting. In our laboratory, DSA is considered present when the MFI of the corresponding bead is ≥ 2000 . It should be noted that while DSA is defined at 2000 MFI, the MFI may need to be higher in order to see adverse outcomes.

This information also allows scientists to use population statistics to create a Calculated Panel Reactive Antibody (cPRA) profile which helps determine what percentage of the population is an unsuitable donor for the recipient (Fig. 1).^{4,5} This means that patients with a higher cPRA,

will face more difficulty finding acceptable donors, as is often the case when a patient has undergone multiple sensitizing events.

The SAB assay does have limitations, including relatively common false positive results.⁶⁻⁹ The most common cause of false positive reactivity on the SAB assay is cross-reactivity between antibodies and denatured antigens on the Luminex beads that form as a byproduct of the bead-antigen hybridization process. Previous studies have shown that Immucor's LIFECODES SAB assay has false Class II reactivity with alleles HLA-DRB1*09:01, DRB3*01:01, DRB3*02:02, DRB3*03:01, DPB1*02:01, DPB1*20:01, DPB1*28:01.⁶ Additionally, studies have shown that One Lambda's SAB assay has shown false Class II reactivity to DRB1*01, DRB1*01:03, DRB1*04, DRB1*07, DRB1*08, DRB1*09, DRB1*10, DRB1*11, DRB1*12, DRB1*13, DRB1*14, DRB1*15, DRB1*16, DRB1*17, DRB1*18, DRB5, DRB3, DRB4, DQB1*02, DQB1*04, DQB1*05, DQB1*06, DPB1*20, DPB1*28, DQA1*01:01, DQA1*01:02, DQA1*03:01, DQA1*04:01, DQ8, DQ9, DPB1*01:01, and DQA1*02:01.^{6,9} When appearing as a false positive, these beads produce recognizable patterns that indicate false reactivity may be present, such as pan-reactivity of all the DRB1 beads, pan-reactivity to all the DQB1*03 (DQB1*07, DQB1*08, DQB1*09) beads, or the appearance of single alleles, such as the DRB4 beads. Of all of the antigens mentioned, DQ and DP allele combinations, such as DQA1*05:01/DQB1*02:01, DQA1*03:02/DQB1*03:02,

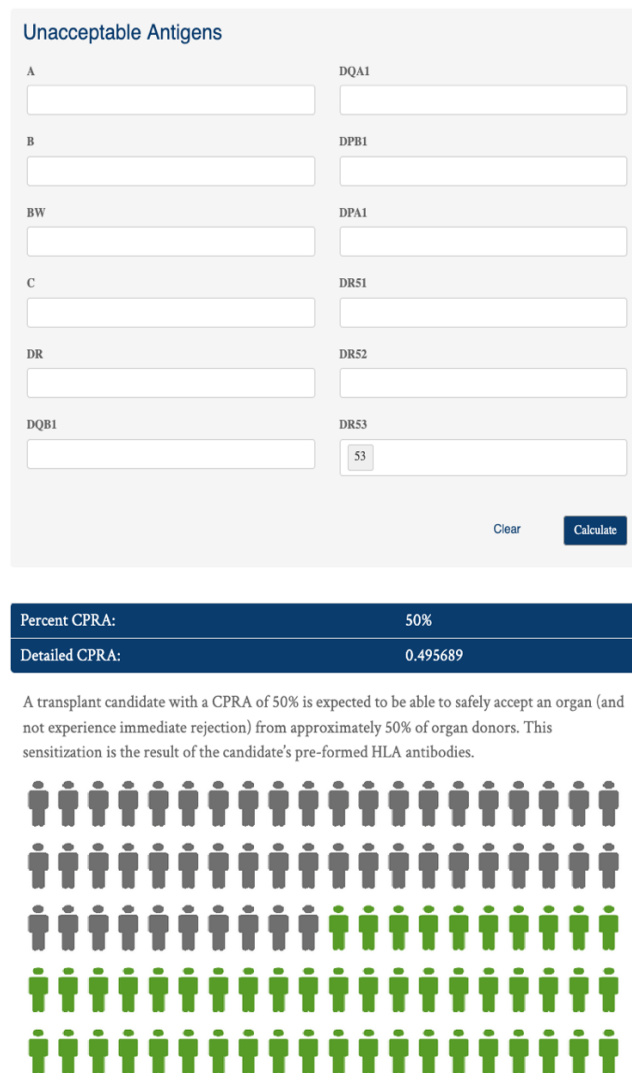


Figure 1: UNOS cPRA Calculator¹³ - The patient shows reactivity to DR53 (DRB4). Given that this antibody is defined just under 50% of the population is not considered to be an acceptable donor for the recipient.

DQA1*03:02/DQB1*03:03, and DPA1*02:01/DPB1*01:01 along with the DRB4 alleles, DRB4*01:01 and DRB4*01:03, have been notably problematic.^{10,11}

False positives occur for both HLA Class I and Class II, and while not as dangerous as false negatives with regards to transplantation failure risk, false positive results may result in a qualified individual not receiving a transplant. This is of critical importance as one study by Kim et al., showed that overall false positivity rates for both Class I and Class II HLA antibodies in pre-transplant patients can be around 40% in women and 60% in men.¹⁰ This is further solidified by Sullivan et. al., who showed that 11% of males have some level of false reactivity to Class II antigens.¹² This is problematic as each additional positive antigen means that the typing must be avoided in the donor, limiting the number of opportunities for the patient to receive the transplant.

Being able to rule-out false positive results is critical to ensuring that highly sensitized patients have greater opportunities to match with a potential donor. False positive DRB4 antibodies are prolific and can rule out 50% of all possible donors unacceptable for transplant (Fig. 1).¹³ Additionally, while it is not possible to calculate the specific cPRA for patients showing allele-level reactivity to DQA1*05:01/DQB1*02:01, it can be estimated by calculating the cPRA for DR17 which is in strong linkage disequilibrium with that particular DQ2 allele. Therefore, DQA1*05:01/DQB1*02:01's estimated cPRA is 19%.¹³

One cause of false reactivity is the sensitivity of the beads themselves. Since only one antigen is present on each bead, some beads are more sensitive than donor cells; as the concentration of less expressed antigens would be relatively higher on the bead. One of the main counters to this increased sensitivity are screening assays. Screening assays use multiple antigens per bead, and can aid in identifying false positive results. Due to a different manufacturing process, the antigens on the surface of the screening beads are more similar to their native confirmation and expression on a cell. While screening beads do not provide details on specific antigens like the SAB assay, they can show if a patient has either Class I or Class II reactivity more generally. This makes the screening assay ideal in situations where the patient is only expressing false positive patterns in a particular class. However, if false positive anti-HLA antibodies are in the presence of true anti-HLA antibodies, the screening assay may not be helpful.¹¹ If the individual has other anti-HLA antibodies, the screening assay would still appear to be positive. Fortunately, the flow cytometric crossmatch (FXM) can be used to identify false results in these cases.

Flow Crossmatching in Transplantation

In our laboratory, the FXM is performed by using recipient blood, collected into red-top serum tubes and donor blood collected into blood tubes containing acid citrate dextrose (ACD) anticoagulant. The recipient serum is aliquoted for use later in the assay while lymphocytes are isolated from the donor's sample. The process for isolating donor lymphocytes begins by extracting the buffy layer from the donor whole blood sample and treating it with Rosettesep™ lymphocyte enrichment solution (Stemcell Technologies™). The blood is then diluted in a 1:1 ratio using Roswell Park Memorial Institute 1640 medium (RPMI). In the case of a deceased donor, lymph nodes and spleen can be used as a source of lymphocytes. The diluted blood or cells from the lymph nodes or spleen is layered over histopaque-1077 in a 2:1 ratio and centrifuged. This creates a density gradient where the lymphocytes will form a layer above the histopaque, red blood cells and granulocytes create the bottom layer, and plasma and RPMI is above the lymphocyte layer. The lymphocytes are collected, treated with red blood cell lysis solution, washed with plain RPMI, and diluted to a working concentration of 1.0×10^6 cells/mL. Cells are then treated with pronase to cleave Fc receptors. This reduces non-specific binding of the secondary antibody binding to Fc receptors found on B cells¹⁴. Pronase can cause false positive results with the T cells in the FCXM if the recipient is HIV positive.^{14,15} In these cases, non-pronased cells can be used in the FXM with the understanding that any excessive background signal on the B cells could make the results difficult to interpret.¹⁶

At this point the recipient sera are also treated with DTT to remove IgM as this class of antibody, typically responsible for autoantibodies, is not correlated to graft loss or rejection, and is known to cause false positive results.¹⁷ Additionally, some patients may be on antibody therapies such as rituximab, a monoclonal human/mouse anti-CD20 antibody, that may require that the serum be treated to reduce the likelihood of a false positive result due to it binding IgG during the cell staining process.¹⁶

Once the donor cells and recipient sera have both been prepared, the recipient serum is incubated with the donor cells. After a 30-minute incubation the cells are washed and incubated for another 20 minutes at 40° C, in the dark, with an antibody cocktail of goat-antihuman-CD3-PerCP-Cy5.5 (BD Biosciences) to detect T-cells, goat-antihuman-CD19-PE (BD Biosciences) to detect B cells, and goat-antihuman-IgG-APC (Jackson ImmunoResearch Labs) to detect anti-HLA

antibodies bound to either lymphocyte population. CD19 is used to identify B cells, instead of CD20, as pronase cleaves the CD20 in addition to the Fc receptor.¹⁶

The cells are analyzed using a flow cytometer. Forward-scatter (FSC) and side-scatter (SSC) are used to gate on the donor lymphocyte population. CD3 and CD19 vs. FSC is used to identify the T-cell and B-cell populations, respectively. The mean fluorescence intensity (MFI) of recipient IgG bound to the surface of the T and B cells is then measured. An increase in the MFI of IgG indicates the presence of DSA.

Surrogate Crossmatching to Confirm Discrepant Results

While the FXM is performed between patient serum and donor cells, it can also be used to identify SAB false-positives through a process known as surrogate flow-cytometric crossmatching (sFXM), where the recipient's serum is incubated with a surrogate donor's cells that expresses the allele in question. In this instance, the surrogate donor is an individual whose typing allows us to verify the patient's antibody reactivity while not necessarily actively undergoing testing to be a donor for that recipient. A positive result sFXM indicates true antibody reactivity. The donor is chosen by comparing their HLA typing against the suspected false positive HLA antibody. Careful consideration is taken to ensure that while the potential false positive antigen(s) are represented in the donor, antigens to any other anti-HLA antibodies in the recipient's SAB assay profile are avoided. However, this can be complicated when a patient has previously experienced one or more sensitizing events (previous transplant, pregnancy, or blood transfusion) and has multiple anti-HLA antibodies, making an acceptable surrogate donor difficult to find.^{18,19} Additionally, some antigens that are likely to react as a false positive, such as A80, are rare within the population making it difficult to find donors with this typing. When a surrogate donor needs to be found, we use a query to identify employees, or another donor undergoing testing, that express the HLA antigen required for the FXM. If one is found, a blood sample is collected and the FXM can be performed. Unfortunately, our population of potential surrogate donors is small and it is impossible to have a full library of potential HLA antigens at our disposal. Even if an allele is available, rare or common, there is still the likelihood that the recipient will have additional HLA antibodies to the other antigens that potential surrogate donor carries. Finding a cell source for testing the HLA antigen/antibody of concern, while limiting the possibility of other HLA antigens being expressed, is a present need.

The T2 Cell Line

The T2 cell line has the potential to be used in sFXM as an alternative to a traditional surrogate donor to rule out false class II reactivity. This cell line is easily transduced to express individual Class II antigens.²⁰ The T2 cell line is an ideal candidate as they do not express Class II antigens, and only have minimal expression of Class I antigens, HLA-A*02, -B*51, and -Cw1 due to a mutation that affects the processing and presentation of Class II alleles.^{21–24} Of the Class I antigens produced, A*02 is the highest expressed antigen with B*51 and Cw1 being weak to even undetectable on the surface of the cells.^{21,22}

This study focused on using HLA-Class II expressing T2 cell lines, in place of a sFXM lymphocytes, to emulate B cells in an sFXM and rule out potential false Class II reactivity within the SAB Class II assay. Specifically, this study focused on the DQ2(DQA1*05:01/DQB1*02:01) and the DR53 (DRB4*01:03) alleles. In addition to comparing the results of the T2 cell sFXM to traditional sFXMs, this study also sought to determine the limit of detection for the T2 cell sFXM.

METHODS

IRB Approval and Conflict of Interest

Approval for this study was received from Michigan State University's Biomedical and Health Institutional review board under the category of an Expedited 5 study. There is no conflict of interest around this study.

Patient Serum Selection

To identify if the T2 cell sFXM could distinguish when a serum had true or false reactivity multiple types of patient samples were tested. Sera from patients with negative SAB assay results were used as a control (true negative (TN)). Sera containing known false positive antibody (KFP) reactivity and sera containing true positive (TP) antibody reactivity previously confirmed using an sFXM were also utilized. It should be noted that, while the DRB4 samples could be classified as just TP, the DQ2 study utilized two types of true positive samples as there are a total of five DQ2 beads in the SAB assay. The first type of TP samples in the DQ2 T2 sFXM were from patients showing reactivity to the specific DQA1*05:01/DQB1*02:01 bead (allele-specific). As finding patients specifically reactive to that single bead are rare, sera from patients reactive to all five DQ2 beads (pan-DQ2) were also tested to ensure TP samples could be tested. This was not necessary for the DRB4 experiments as a pan-DRa antibody was used as a positive control. Patients were excluded if anti-HLA-A*02, -B*51, and -Cw1 antibodies were detected in their Class I SAB assay.

For the DRB4 study, sera from five TN patients (no antibody reactivity), six KFP patients with proven false positive antibody reactivity, and seven TP patients with proven reactivity to DRB4 were tested. For the DQ2 study, sera from two TN patients without antibody reactivity, three pan-DQ2 patients, three allele-specific patients, and four KFP patients were tested.

Once all samples were selected, each one, along with their respective patient were assigned a number (I.e., Patient 1 = Sample 1, Patient 2 = Sample 2). However, for the first patient identified in the study, as will be discussed, multiple sera were tested. In this instance a second number was added to clarify the specific sample. (I.e., Patient 1, Sample 1 = Sample 1.1, Patient 1, Sample 2 = Sample 1.2). If a patient sample was used in both the DRB4 and DQ2 T2 sFXM, the same number was used in both experiments.

HLA Class II Expressing T2 Cells

The T2 cell lines used in this study were previously cloned in the lab to express either the DQB1*02 (DQA1*05:01/DQB1*02:01) or the DRB4*01:03 (DR53) molecule. To summarize, cDNA sequences were cloned into MSCV-IRES-GFP retroviral plasmids which were transfected into the retrovirus Phoenix 293T cells (provided by Dr. Andrew Fontenot).^{25,26} The retrovirus was used to transduce T2 cells resulting into their respective lines. Cells were stained and sorted for high expression of HLA-DR α or DQB1 and GFP expression (FACS Aria, BD Biosciences). After sorting, RNA was isolated from each cell line (Qiagen RNeasy, 74106) and the DRB1 and DQA1/DQB1 sequences verified by Sanger sequencing (Quintara Biosciences).²⁷ Cells lines were thawed and then expanded and maintained in Iscove's Complete T cell media (ICTM; IMDM-GlutaMAX supplemented with 10% FBS +1% thio-streptomycin + 1mM sodium pyruvate).

Cells were maintained at a concentration between 2.0×10^5 and 4.0×10^5 cells/mL with a viability greater than 90% using Trypan Blue. Cells were discarded after culturing for 3 weeks and new aliquots thawed to ensure high receptor expression.

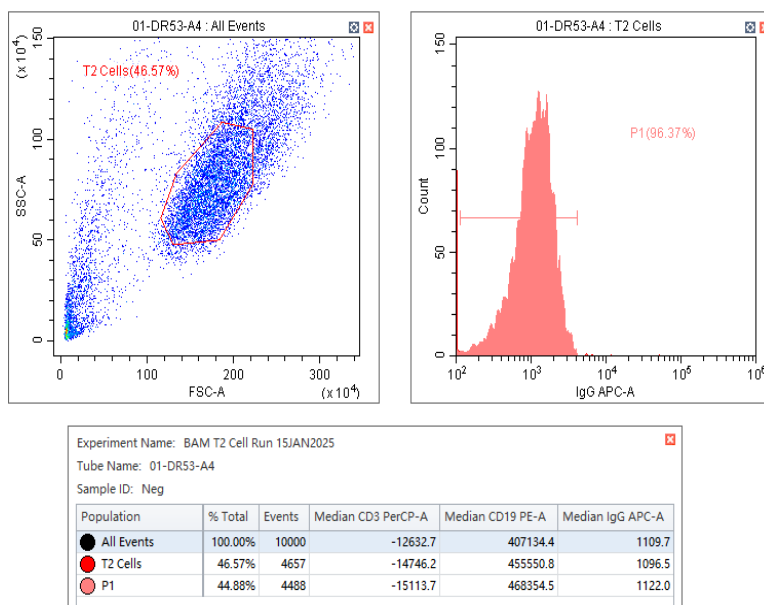


Figure 2: T2 cell sFXM Gating Strategy – Forward (FSC) and Side Scatter (SSC) were used to gate around the T2 cells. The median T2 cell IgG APC MFI was recorded.

T2 Cell Crossmatching

The testing of T2 cells followed an adapted flow crossmatch protocol. Briefly, T2 cells were collected, washed twice in plain RPMI, and incubated with a 1:1 dilution of pronase and RPMI for 30 minutes. Cells were then washed twice in RPMI enriched with 5% FBS (5% RPMI) and diluted to a working concentration of 1.0×10^6 cells/mL in 5% RPMI. 200uL of cells were added to each well of a 96-well plate and spun down. The supernatant was then removed using the flick method. The cells were incubated with 30uL of 5% RPMI and 20uL of DTT-treated serum

for 30 minutes. Cells were washed three times in phosphate-buffered saline solution (PBS) before staining them with a 1:90 dilution of goat-antihuman-IgG-APC (Jackson ImmunoResearch Labs) in PBS and incubated at 4°C in the dark, for 20 minutes. Cells were washed twice more in cold PBS before they were run through a flow cytometer (Cytotflex, Beckman Coulter). The red and blue laser were utilized to detect APC (excitation: 650nm, bp = 660/20nm) and SSC (bp = 488/4nm) respectively. Cell populations were gated using FSC and SSC, and the median IgG APC MFI was recorded (Fig. 2). All samples were tested in multiple runs in duplicate within each run.

Limit of Detection

Sera confirmed to contain true anti-HLA antibody (TP) in their respective assay and with a corresponding SAB assay result ≥ 5000 MFI were serially diluted with negative control sera then tested on the HLA-expressing T2 cells following the T2 cell XM protocol above.

Analysis

The MFIs of the anti-human IgG for each patient sample were normalized to the negative control using a compensated MFI (patient sample - negative control). Cutoffs for determining negative and positive results were established and compared to the result of the initial sFXM.

DISCUSSION – EXPERIMENT SET-UP

Setting up the T2 cell sFXM – Gathering Patient Samples

Identifying known false positive samples for the experiment.

In the case of both TP and KFP results, the patients' SAB assay needed to first be suspected of having false reactivity to the DQA1*05:01/DQB1*02:01, and/or both the DRB4*01:01 and DRB4*01:03 beads where the SAB assay MFI is greater than 2,000. Most often false results are suspected if the beads in question are the only positive results within the SAB assay, or if there are no other corresponding antibodies present. For example, in the case of DQA1*05:01/DQB1*02:01, anti-HLA antibodies may be detected to other class II antigens while no other DQ antigens are detected. If the result were “real” we would expect some reactivity amongst all DQ2 antigens, not just the one bead. It would also be suspected to be real if it appeared with DR17 as the genes encoding for DRB1*17 and DQA1*05:01/DQB1*02:01 are linked and inherited together. Therefore, if someone has been sensitized to one, it would be expected that they would have been sensitized to the other. For the DRB4 beads, results would be suspect if there are no anti-HLA antibodies detected to DRB1*04 or DRB1*07 as these genes are linked to DRB4. Results may also be suspected of false reactivity if there is a discrepancy between the SAB assay and another assay, such as an FXM or screening assay. Additionally, if there is no indication that the antibody should be present based on patient history i.e., the patient has no sensitization events, the results may be investigated further.

Sample Limitations – Changing SAB assay results causes difficulties in finding positive samples.

The major limitation of this study for both antigens, tested was DRB4 or DQA1*05:01/DQB1*02:01, was the lack of TP samples. Due to the nature of the SAB assay giving false positive results, the number of TP samples confirmed by sFXM was limited. The pool of overall TP patients was further reduced due to patients having non-compatible anti-Class I HLA antibodies. Furthermore, there were instances in which a potential sample was identified; however, due to the need for additional testing previously, there was no serum left. When investigating the patient history for other potential samples, the newer samples on the patient showed that their SAB assay was now negative. Thus, additional serum from a patient previously deemed TP may not have been able to be used in this study. The multiple limitations encountered made it difficult to

get a large enough sample size to combat any outlying data points, or find general trends within some of the data sets.

Identifying and explaining variable MFIs within the T2 cell sFXM

Originally samples were analyzed based on MFI cutoffs, as is done in our clinical sFXM assay. In the clinical FXM assay, a negative result for T-cells is an MFI less than 2,000, a negative result for pronased B-cells is less than 3,000 MFI, or less than 10,000 if the cells are not pronased. However, the second experiment testing the DRB4 T2 cell sFXM had increased background with a raw negative control MFI = 7,389 with the next highest negative control resulting with an MFI = 2,657. In this case, all the subsequent samples also had higher MFIs due to the increased background, with TN and KFP patients also having raw MFIs ranging from 5,414 to 13,797.

There are multiple potential explanations for the increased MFIs. The primary explanation is that the run was likely under-washed after the fluorochromes were added causing a higher background. This likely arose due to a change in how the XM was performed compared to the clinical method, using a 96-well plate instead of microcentrifuge tubes. The fact that the subsequent DRB4 and DQ2 XMs all had negative controls with MFIs < 2,000 as techniques improved, further substantiates this explanation.

It is also possible that the experiment run with increased background was under-pronased. As stated above, pronase is used on B-cells to cleave the Fc receptor and reduce overall background in the FXM assay. While other labs may titrate their pronase, our lab uses 750uL of a 1:1 ratio of RPMI to pronase for anywhere from 3.0×10^6 to 1.0×10^7 cells. The overall cell concentration pronased in this run was approximately 8.0×10^6 cells. Therefore, it is possible that due to the large number of cells, more pronase was needed, resulting in an XM with a higher background. This is corroborated by the fact that, in our laboratory, clinical non-pronased XMs can have a negative XM MFI up to 10,000.

Another variable that might have had some influence on the overall background of the cells is how the T2 cells are transfected to express the antigen of interest. When inserting the gene that encodes for the antigen of interest, green fluorescent protein (GFP) is utilized to verify the gene was successfully inserted. The presence of GFP likely caused increased background noise, thus causing a generalized increase in the resulting MFI values.

While the aforementioned possibilities explain how an increased MFI can occur, there are also variables that could cause either an increase or decrease in overall MFI due to the nature of the T2 cells themselves. These include, the age of the cells, cell concentration within culture, and cell viability, which may lead to variance in the overall MFI. To reduce the variance as much as possible it was determined that cells should be cultured for a minimum of three days before experimentation in order to ensure proper cell growth and development. Additionally, after the third repeat run using the DQA1*05:01/DQB1*02:01 cells failed, it was determined that the cells should not be used if they are older than three weeks. The failed run was later repeated with a new set of cells and yielded usable results.

Interpretation of the T2 cell sFXM

As stated previously, our laboratory uses raw MFI cutoffs of 2,000 (T-cell) and 3,000 (B-cell) in our clinical FXM to identify negative XM results. Most other transplant laboratories use a different method. Some labs use a compensated MFI (cMFI), where the negative control MFI is subtracted from the patient sample's MFI, to establish MFI cutoffs. Others may use channel-shifts where a ratio of the MFI intensity between a sample and the negative control is evaluated.

As such, all three options were evaluated for the T2 cell sFXM. Each patient sample was averaged together to get a raw MFI, then the cMFI and ratio were calculated (Fig. 3). It was determined that cMFI cutoffs would be used for the T2 cell sFXM to compensate for the increased background in the second DRB4 repeat. Results would be compared to the raw MFI results of the clinical assay as there is negligible background in those results, and the assay has been validated using raw MFI values. This allows the MFI values between the SAB assay, sFXM, and T2 cell sFXM to be compared, while keeping the data interpretation between the assays as similar as possible.

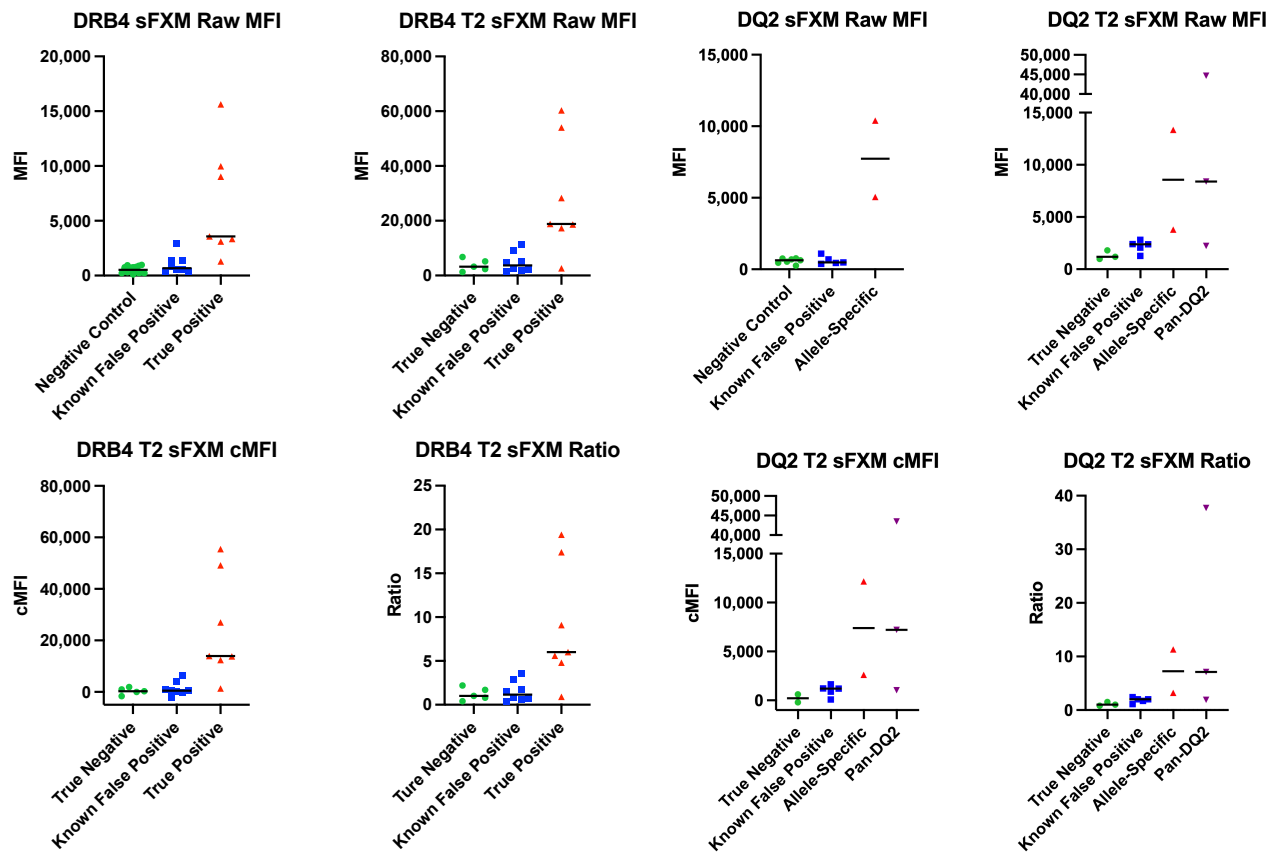


Figure 3: Data Interpretation options – Results of the T2 cell sFXMs were analyzed by raw MFI, cMFI, and ratios. The average for each patient was calculated and plotted. Results were then compared to the sFXM results.

RESULTS/DISCUSSION – THE DRB4 T2 CELL sFXM

DRB4 Transfected T2 Cells Can Be Used for Surrogate Crossmatching

Concordance between the surrogate flow cytometric crossmatch and the DRB4 T2 surrogate flow cytometric crossmatch is 95%.

After evaluating patient antibody histories, a total of 5 TN samples, 6 KFP, and 9 TP samples were selected for the DRB4 study. All samples were run in duplicate within two or three runs resulting in a total of 86 points of data. Of the 20 patient samples used in this experiment, one was deemed non-concordant. It should be noted that three samples used in this experiment were not the original sample that confirmed the patient's reactivity as TP. Therefore, sFXMs were ran on these samples to confirm reactivity. Sample 1.3 and sample 7 showed known false positive reactivity making them concordant with the T2 cell sFXM.

Sample 5 was non-concordant. Understanding this discrepancy requires more nuance as investigation into the patient's history showed that two sFXMs were performed on this sample. One resulted as negative and the other as positive. As such the patient was clinically deemed true positive to err on the side of caution. Overall, the concordance rate is 95% (Table 1).

Table 1: DRB4 Crossmatch Concordance

Patient/ Sample			
Number	sFXM Result	T2 sFXM Result	Concordant
1.1	TP	Positive	Y
1.2	TP*	Positive	Y
1.3	KFP*	Negative	Y
2†	TP	Positive	Y
3	TP	Positive	Y
4	TP	Positive	Y
5‡	TP	Negative	N
6	TP	Positive	Y
7	KFP*	Negative	Y
8	KFP	Negative	Y
9	KFP	Negative	Y
10	KFP	Negative	Y
11	KFP	Negative	Y
12	KFP	Negative	Y
13	KFP	Negative	Y
14	TN	Negative	Y
15	TN	Negative	Y
16	TN	Negative	Y
17	TN	Negative	Y
18	TN	Negative	Y

Patients and their corresponding concordance between their sFXM and T2 sFXM testing for DRB4 reactivity are listed. Note that Patient 1.1 - Patient 1.3 are all samples from the same patient collected at different times.

* Sample is not the same as the sample used for the initial sFXM. A new sFXM was performed on these samples to confirm reactivity.

† Samples had a negative sFXM, however they were deemed as true positive based on a B-cell shift.

‡ Sample had 2 sFXMs completed on the same sample within one week of each other. One was negative the other was positive. Sample was to be treated as true positive.

DRB4 T2 Crossmatch Statistics – Determining the cutoffs between negative, known false positive, and true positive results.

Of the concordant DRB4 samples, the mean T2 cell sFXM cMFI of each group was 341 for TN, 1,115 for KFP, and 25,229 for TP (Table 2). Sample means for each group were compared to each other using the Kruskal-Wallis test due to the limited number of overall patients in this study. Analysis showed that the TN and KFP data sets were similar ($p > 0.9999$), the TN and TP sets were statistically different ($p = 0.0001$), and the KFP and TP sets were statistically different ($p < 0.0001$). (Table 3).

Table 2: DRB4 T2 cell sFXM Descriptive Statistics

	True Negative	Known False Positive	True Positive
Minimum	-2,119	-3,351	5,868
Maximum	2,765	11,670	81,223
Mean	341	1,115	25,229
Std. Deviation	1,294	3,134	20,196

The cMFIs of concordant DRB4 T2 cell sFXM were analyzed to find the absolute minimum, absolute maximum, mean, and standard deviation.

Table 3: DRB4 T2 cell sFXM Analysis of Variance

	Significant?	Adjusted P Value
True Negative vs. Known False Positive	No	>0.9999
True Negative vs. True Positive	Yes	<0.0001
Known False Positive vs. True Positive	Yes	<0.0001

A Kruskal-Wallis test was performed to compare the mean values of each group in the T2 cell sFXM.

It was found that all TP patients had an average T2 cell sFXM cMFI greater than 10,000 while all TN and KFP patients had average cMFIs less than 7,000 (Table 4). This lines up nicely with the clinical results for non-pronase B-cell FXM where $MFI < 10,000$ is interpreted as negative. Therefore, it was concluded that the cutoff between a positive and negative result is

10,000 cMFI. It should be noted that repeated experiments may lower this cut-off as improved techniques may reduce the overall background.

Table 4: DRB4 SAB Assay, sFXM, and T2 cell sFXM Results

	Patient/Sample Number	SAB Assay MFI	Avg. sFXM MFI	Avg. T2 sFXM cMFI
Controls	Negative	-	545	3,111
	Positive	-	-	23,779
True Positive	1.1	19,864	9,019	55,458
	1.2	7,131	3,342	13,781
	2	5,838	1,299	12,401
	3	5,318	3,116	13,973
	4	17,361	9,980	49,163
	5*	4,730	3,570	1,314
	6	22,834	15,626	26,980
Known False Positive	1.3	12,014	2,886	-90
	8	8,965	1,415	4,110
	9	10,343	541	6,346
	10	2,611	348	-2,235
	11	7,653	802	431
	12	5,056	526	882
	13	3,706	336	-38
True Negative	7	3,734	1,389	508
	14	0	-	1,891
	15	2	-	266
	16	0	-	-1,615
	17	15	-	1,017
	18	0	-	-16

Each patient's SAB assay MFI, sFXM MFI, and average T2 cell sFXM cMFI have been listed.

*Sample is non-concordant.

Dilution Study – The limit of detection in the DRB4 T2 crossmatch is similar to the surrogate crossmatch.

Two patient sera were chosen for serial dilutions to determine the limit of detection. Both samples were diluted with negative control serum until the expected SAB assay MFI was < 2,000, as the antigen is no longer considered defined once the MFI is below that cutoff. Sample 6, was diluted from neat (T2 cell sFXM cMFI = 26,980) to 1:16 (T2 cell sFXM cMFI = -454). Sample 3, was titrated from the neat sample (T2 cell sFXM cMFI = 11,816) to 1:8 (T2 cell sFXM cMFI = -1376). For both patients, once the SAB assay MFI reached approximately 5,000, the T2 cell sFXM cMFI was below 10,000 (Fig. 4).

Therefore, it can be concluded that the limit of detection in the SAB assay for DRB4 is an MFI = 5,000 to yield consistent a positive result in the T2 cell sFXM assay. This aligns with what would be expected in an sFXM where antibodies usually are not detectable when they are less than 5,000

MFI on the SAB assay (Table 4). Understanding this limit not only provides guidance on the requirements a sample would need to qualify for this assay if it were to be used clinically, but also provides explanations for the non-concordant sample.

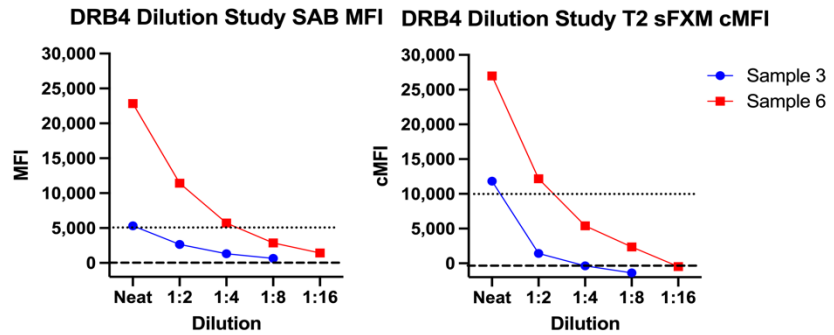


Figure 4: DRB4 Dilution Study – Patient sample were serially diluted in 1:2 ratios. The corresponding SAB MFI and T2 cell sFXM cMFI were then recorded under their respective dilution.

Troubleshooting inconsistencies within the assay.

Due to the number of samples utilized in this experiment, the clinical protocol was adapted so XMs were performed directly in a 96-well plate; whereas the clinical assay utilizes microcentrifuge tubes and then transfers samples to a 96-well plate. Since cells were added directly in the plate instead of the microcentrifuge tubes, new techniques such as the flick method for removing the supernatant, and vortexing the 96-well plate to resuspend cells were utilized. Inconsistencies in the flick method and vortexing of the plate likely meant the cells were not being fully washed, or not being fully resuspended, leading to increased MFI values in the second DRB4 run as excessive fluorochromes remained in the samples as they were loaded onto the flow cytometer. As techniques improved, more consistent data was available for future runs in both the second half of the DRB4 study, and the DQ2 study.

Troubleshooting discrepant samples.

Explaining Conflicting Results Due to the Limit of Detection.

Sample 5 had the only non-concordant result in the DRB4 experiment. Investigation into the patient's history provides multiple explanations as to why the discrepancy may be present.

First, Sample 5's average DRB4 SAB assay MFI was 4,730, which has shown to be at the limit of detection in the sFXM. Therefore, it is likely that the antibodies present were not strong enough to elicit a positive result. Additionally, we found that two sFXMs were run using the same sample, one week apart, using two different donors to evaluate for the presence of DRB4 antibodies. These sFXMs showed conflicting results where one sFXM showed a negative result, and the other was just over the positive threshold. While difficult to identify an exact reason for the discrepancies within the sFXMs, it is possible that one donor had better expression of DRB4 than the other leading to differing results. Therefore, it is not completely unexpected that the patient's sample would be negative on the T2 cell sFXM.

Examining Patient Reactivity Over Time.

Among the positive samples tested, three of them were from the same patient, Patient 1 (Samples 1.1 - 1.3), who was originally deemed to be a TP using the sFXM. Sample 1.1 was collected in the beginning of October 2023, while Sample 1.2 was collected at the end of October 2023. Sample 1.3, collected in February of 2024, was the patient's most recent sample at the time of the experiment and was the first sample to be tested using the DRB4 T2 cells sFXM. In the initial test, sample 1.3, had an average SAB assay MFI of 12,013. However, when tested using the DRB4 T2 cell sFXM, the resulting cMFI was -90. Upon further investigation it was decided that the original sFXM sample, Sample 1.1, as well as the sample that was collected directly after, Sample 1.2, should be run alongside sample 1.3. When tested using the T2 cell sFXM, the results were cMFIs were 55,458 for sample 1.1 and 13,781 for sample 1.2 making both samples concordant with the expected positive sFXM result.

After establishing that samples 1.1 and 1.2 were concordant but sample 1.3 was not, an updated sFXM using donor cells was performed to confirm reactivity. The updated sFXM showed that Patient 1.3 was indeed negative making the T2 cell sFXM concordant.

One other pattern for Patient 1 still required investigation. Although both the surrogate and T2 cell sFXMs showed a decrease in reactivity over time, the SAB assay MFI increased between samples 1.2 and 1.3 (Fig. 5). Investigation into the patient’s history showed Sample 1.1 was ordered as a 6-hour STAT post-transplant sample needing evaluation for DSA. The need for STAT testing is indicative of a possible rejection event. The rejection event could have caused an increase in antibody production, causing an increase in the SAB assay MFI. This is confirmed by the TP sFXM. Following the initial sample, the patient’s overall antibody load was reduced with immunosuppressive treatment leading to subsequent negative sFXMs. However, non-specific binding to misfolded and partial proteins in the SAB assay continued due to general antibodies found in the patient’s serum. It should be noted that some monoclonal antibody-based therapies, such as rituximab, may also cause some level of MFI increase in the SAB assay but are not always the main cause, as false reactivity has also been seen in pre-transplant patients not undergoing immunosuppressive therapy.

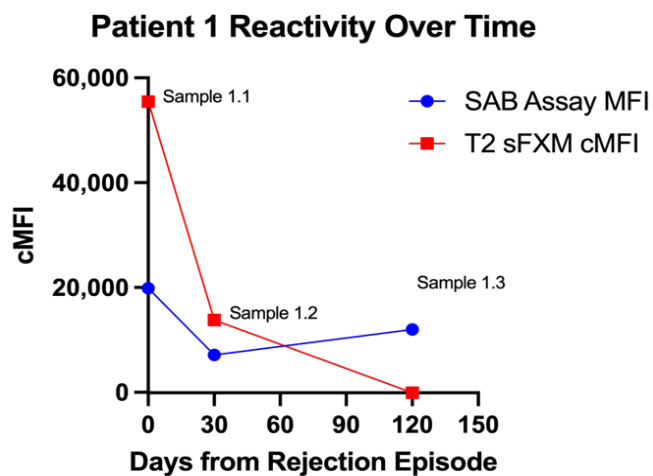


Figure 5: Patient 1 Reactivity Over Time – Patient 1’s PRA reactivity, and corresponding T2 cell sFXMs were plotted following a rejection episode.

It is possible that even if there was some “true” reactivity was present in Patient 1’s SAB assay samples, the real MFI was “masked” by additional non-specific binding, making the MFI appear more elevated. For example, while the Sample 1.1’s average SAB assay MFI was 19,864, non-specific binding may be responsible for a significant portion. If the non-specific binding were removed, it is possible that the average MFI would be closer to 10,000. While still positive and detectable by both the traditional and T2 cell sFXMs, the lower MFI would mean that treatment would likely reduce the antibody load to an MFI close to 5,000 in subsequent samples. Therefore, while the subsequent samples still appear positive in the SAB assay, masking by non-specific binding is likely the cause.

RESULTS/DISCUSSION – THE DQ2 T2 CELL sFXM

DQA1*05:01/DQB1*02:01 Transfected T2 cells Can Be Used in Surrogate Crossmatching

Concordance between the surrogate flow cytometric crossmatch and the DQ2 T2 surrogate flow cytometric crossmatch is 92%.

Two TN samples, three pan DQ2 reactive, three DQA1*05:01/DQB1*02:01 (allele-specific) positive, and four KFP samples were selected for the study. All samples were run in duplicate on two or three runs depending on the available serum, resulting in a total of 58 points of data. Of the 12 patient samples included in this experiment, one serum sample from the allele-specific group was different than the original serum used to determine that the patient was a TP because all the original sera were used for prior clinical testing. When a negative result emerged on the T2 cell sFXM, an sFXM was able to be performed for this newer sample. The result of the updated sFXM was negative making the sample concordant. The overall concordance rate for this experiment was 92% with one non-concordant result, Sample 20 (Table 5.) Unfortunately, an sFXM could not be performed on Sample 20, due to the patient's high reactivity to MHC class-I antigens.

Table 5: DQ2 Crossmatch Concordance

Patient/Sample Number	sFXM Result	T2 sFXM Result	Concordant
19	N/A Pan-DQ2	Positive	Y
20	N/A Pan-DQ2	Negative	N
21	N/A Pan-DQ2	Positive	Y
3	TP	Positive	Y
22	TP	Positive	Y
7	KFP*	Negative	Y
23	KFP	Negative	Y
24	KFP	Negative	Y
25	KFP	Negative	Y
26	KFP	Negative	Y
16	TN	Negative	Y
18	TN	Negative	Y

Patients and their corresponding concordance between their sFXM and T2 sFXM testing for DRB4 reactivity are listed.

* Sample is not the same as the sample used for the initial sFXM. A new sFXM was performed on these samples to confirm reactivity.

DQ2 T2 Crossmatch Statistics - Determining the cutoffs between true negative, known false positive, and true positive results.

Of the concordant DQ2 samples, the mean T2 cell sFXM cMFI were 391 for TN, 1,008 for KFP, 8,018 for allele-specific, and 29,289 for pan-DQ2 (Table 6). The means of each group were analyzed using a Kruskal-Wallis test due to the limited sample size. The following groups were statistically similar to one another: TN vs. KFP ($p = 0.8408$), pan DQ2 vs. Allele Specific ($p > 0.9999$). TN vs. Allele-Specific ($p = 0.0002$), TN vs. pan-DQ2 ($p < 0.0001$), KFP vs. Allele-Specific ($p = 0.0016$), and KFP vs. pan-DQ2 ($p < 0.0001$) were statistically different. (Table 7).

Table 6: DQ2 T2 cell sFXM Descriptive Statistics

	True Negative	Known False Positive	Allele-Specific	Pan-DQ2
Minimum	-344	-804	1,606	4,727
Maximum	3,506	2,992	18,814	74,904
Mean	391	1,008	8,018	29,289
Std. Deviation	1,244	820	7,255	25,444

The cMFIs of concordant DQ2 T2 cell sFXM were analyzed to find the absolute minimum, absolute maximum, mean, and standard deviation.

Table 7: DQ2 T2 cell sFXM Analysis of Variance

	Significant?	Adjusted P Value
True Negative vs. Known False Positive	No	0.8408
True Negative vs. Allele-Specific	Yes	0.0002
True Negative vs. Pan-DQ2	Yes	<0.0001
Known False Positive vs. Allele-Specific	Yes	0.0016
Known False Positive vs. Pan-DQ2	Yes	<0.0001
Allele-Specific vs. Pan-DQ2	No	>0.9999

A Kruskal-Wallis test was performed to compare the mean values of each group in the T2 cell sFXM.

It was found that all TP patients, regardless whether they were pan-DQ2 or allele-specific, had an average T2 sFXM cMFI greater than 2,000 while all TN and KFP patients had an average cMFI less than 2,000 (Table 8). These values correlate with our clinical assays using pronase that shows B-cell FXM interpretation where less than 3,000 MFI is negative. The accepted cutoff between a positive and negative result is 2,000 cMFI. It should be noted that the lower cutoff value

of the DQ2 T2 cell sFXM compared to the DRB4 T2 cell sFXM is likely due to improved techniques reducing the overall background.

Determining the Limit of Detection for the DQ2 T2 Crossmatch.

Table 8: DQ2 SAB Assay, sFXM, and T2 cell sFXM results

	Patient/Sample Number	SAB MFI	Avg. sFXM MFI	Avg. T2 sFXM cMFI
Control	Neg	-	587	0
Pan-DQ2	19	14,110	-	43,507
	20*	7,593	-	1,044
	21	16,260	-	7,205
Allele-Specific	3	9,588	10,395	12,168
	22	6,061	5,059	2,614
	7	3,876	1,099	93
Known False Positive	23	4,279	481	1,203
	24	9,945	690	1,636
	25	8,973	380	1,214
	26	2,096	444	883
True Negative	16	0	-	-199
	18	0	-	624

Each patient's SAB assay MFI, sFXM MFI, and T2 cell sFXM cMFI have been listed.

*Patient is non-concordant.

Dilution Study – Inconclusive and irreproducible data resulted in the limit of detection being determined by other means.

Two samples were chosen to perform serial dilutions on to determine the limit of detection for the DQ2 T2 sFXM. Both samples were diluted with negative control serum until the expected SAB assay MFI was < 2,000. The allele-specific patient serum, Sample 3, and pan-DQ2 serum Sample 19, were diluted from neat to 1:8 with negative control serum. Sample 3 was initially titrated from the neat (T2 cell sFXM cMFI = 9,588) to 1:8 (T2 cell sFXM cMFI = 1,198). This result however, was not repeatable in subsequent runs. Sample 29's reactivity did not decrease with titration; the cMFI of all dilutions were consistently above 43,000. (Fig. 6). Overall, the experiment was inconclusive as the experiment were not reproducible.

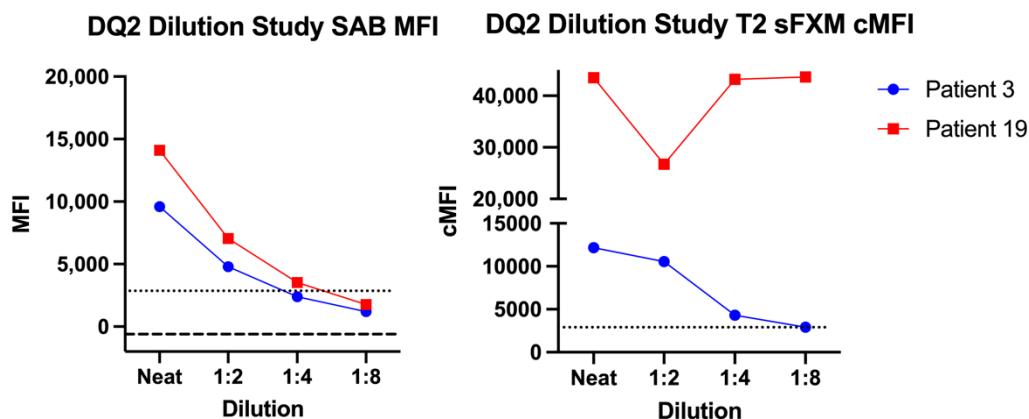


Figure 6: DQ2 Dilution Study – Patient samples were serially diluted in 1:2 ratios. The corresponding expected SAB MFI and T2 cell sFXM cMFI were then recorded under their respective dilution. The sample shown for Sample 3 is the one repeat that showed dilution.

Although the dilution experiment did not yield consistent results, there were other ways the limit of detection was able to be estimated. In evaluating each patient's SAB MFI, it was determined that of all concordant TP patients, the lowest SAB MFI was 6,061 (Table 8). This is similar to what we would expect in the clinical assay where the cutoff for detection is an SAB MFI of approximately 5,000.

Examining the inconsistent results from the dilution study.

The dilution studies performed using the DQ2 T2 cells did not produce repeatable results to determine the limit of detection. Results from Sample 19 did not yield reduced T2 cell sFXM cMFIs upon dilution. Therefore, the patient's sample would have needed to be diluted lower in order to see a reduction in the T2 cell sFXM cMFI. (Fig. 11). This is likely due to the patient's pan-DQ2 reactivity, which should have been accounted for when determining how far to dilute the sample.

While one of Sample 3's dilutions showed potential in determining a limit of detection, the age of the T2 cells may have also contributed to inconsistent results. While the repeat showed a decrease in the cMFI, the whole set of data was discarded as overall results for all patients on the run were inconsistent. When the cMFI titration was repeated, the serum was on its final freeze thaw cycle. This caused less reactivity in the undiluted sample, which was mirrored by the lack of reduction in the cMFIs of the diluted samples.

It should be noted that Sample 3 was used in both the DRB4 and DQ2 experiments. This meant that this serum had more freeze/thaw cycles compared to any other patient. Our laboratory has a limit of five freeze/thaw cycles per sample as more cycles can affect antibody integrity. Sample 3's DQ2 repeat sample was on its final freeze thaw cycle when the final repeat was performed, which may have contributed to the inconsistent results.

Troubleshooting Discrepant Samples.

Identifying outlying data points.

The third repeat for Sample 18 in the DQ2 study was identified as an outlier. The average cMFI of the final repeat was 2,302 just over the cutoff of a true positive sample at 2,000. This did not agree with previous results. The T2 cell sFXM repeats for this sample had cMFIs of -243 and -187 and the SAB assay MFI was 0. Additionally, this sample was used as a negative control in the DRB4 study, showing a maximum cMFI of 249. The outlying cMFI result of 2,302 was most likely due to ineffective washing. Including this data point did not change the overall result for the patient, nor did it have an impact on any statistical analysis of the patient groups.

Explaining conflicting results in a Pan-DQ2 patient.

Sample 20 had a negative T2 cell sFXM with an overall average cMFI of 1,044. In examining the patient's SAB assay, all five DQ2 beads were above the estimated limit of detection, with the DQA1*05:01/DQB1*02:01 bead having an MFI of 7,593. No prior sFXM had been performed on this patient because they were pan-DQ2 reactive. Further investigation showed the patient was highly sensitized to MHC Class I antigens, and no donors with a compatible class I typing could be found to allow for an sFXM. This inability to verify the non-concurrent sample shows just how difficult it can be to confirm suspected false positive results in a highly sensitized patient.

CONCLUSION

Experiment success and limitations both show the need for an assay that can be used to rule out known false positives

In conclusion, these data successfully demonstrate that HLA-expressing T2 cell lines can be used as surrogate donor cells to confirm false positivity in the SAB assay. The concordance rate for both T2 cell sFXMs was greater than 90%. The specific issues with sample and assay limitations were identified. First, for both DRB4 and DQA1*05:01/DQB1*02:01, our results suggest that the patient's SAB assay MFI needs to be $\geq 5,000$ to reliably result in a positive T2 cell sFXM assay. Additional dilution experiments are needed to confirm this limit of detection. This is especially true in the DQ2 T2 cell sFXM where the limit of detection is an estimate.

Second, the cMFI cutoff for determining a result as TP or KFP in the T2 cell sFXM assay mirrors the clinical FXM. For the DRB4 T2 cell sFXM, the cutoff of 10,000 is the same as the clinical non-pronase B-cell FXM. For the DQ2 T2 cell sFXM, the cMFI cutoff between TN and FP results is 2,000 MFI, which is lower than the clinical pronase B-cell FXM at 3,000 MFI. As the parent cell lines are the same, with repeat testing and improved techniques, it is likely that background will be reduced in the DRB4 assay making the cutoff similar to the DQ2 T2 cell sFXM. Regardless, both T2 cell sFXM had similar results as the clinical assay, demonstrating the potential for future clinical use.

Further testing, especially for the DQA1*05:01/DQB1*02:01 antigen, is complicated by finding patients with true positive allele-specific antibodies. When these patients are found, the reactivity is often weak. In fact, the difficulty in finding TP patients for this study exemplifies the need for the cultured cell assay described here that is specifically dedicated to ruling out false positive results. This was demonstrated when the expected pan-DQ2 patient was suspected to have some level of false reactivity.

Our internal records show that previously determined TP antibody reactivity may become negative with time, as in the case of Patient 1. In some cases, the SAB assay also becomes negative, while other times, like in the case of Patient 1, the SAB assay may remain positive. This demonstrates the SAB assay's often-inconsistent nature, and how other potential immunological factors such as memory causing an increase in antibodies, common epitopes and cross-reactivity can make TN results positive and mask weak positive results as being stronger than they are.

Impact of false positives on patient care

Identifying and ruling-out false positive results ensures that patients have as many opportunities as possible to receive a transplant. While sFXMs are an excellent option to identify these results, it can be difficult to find surrogate donors, especially in the case of highly sensitized patients. In these cases, false positive patterns may be suspected, but are unable to be ruled out. The T2 cell sFXM shows proof that a cell line can be used to rule out false positive patterns, not only for class II anti-HLA antibodies, but likely class I as well, improving the chances for transplant recipients match with a potential donor.

Another area in which the T2 cell sFXM could be beneficial is in cord-blood transplants. In this type of transplant, donor samples for XM testing are unavailable. In theory, the T2 cell could be used to verify antibody reactivity, much in the same way XMs are used in solid organ transplantation.

The T2 cell sFXM shows promise for the future

These experiments show that a cell line can be used as an sFXM donor, suggesting that other class II antigens could be transfected into the T2 cell line. Additionally, transfecting MHC Class I antigens into the cell line K562 could be used to rule out class I reactivity. As manufacturers create/change lots and antigens on their SAB assays, new false positive patterns emerge. Anecdotally, as of the writing of this paper, our lab has moved to a new lot of our class II SAB assay. While this change removed a common false positive pan-DRB1 reactive pattern, the false positive DRB4, and DQA1*05:01/DQB1*02:01 patterns remain, and new ones specific to DRB1*01:02 and DPB1*14:01 have emerged. While the DRB1*01:02 does not have a significant impact on a patient's cPRA at 0%, DPB1*14:01 increases a patient's cPRAs to 3%.¹³

As technology currently stands, it is unlikely that all false reactive patterns will be removed from SAB assays. While this study only focuses on two common false positive reaction patterns, T2 cells or other cell lines could be transfected to express new antigens of interest. Further investigation into using cell lines as potential sFXM donors can help make up for the current limitations within the SAB assay and sFXM assay and would contribute to improved transplant success.

REFERENCES

1. Mehra, N. & Chopra, G. Importance of Donor Selection in Renal Transplantation. *Med J Armed Forces India* **50**, 205–210 (1994).
2. Heidt, S., Haasnoot, G. W., van der Linden-van Oevelen, M. J. H. & Claas, F. H. J. Highly Sensitized Patients Are Well Served by Receiving a Compatible Organ Offer Based on Acceptable Mismatches. *Front Immunol* **12**, 687254 (2021).
3. Lachmann, N., Todorova, K., Schulze, H. & Schönemann, C. Luminex® and Its Applications for Solid Organ Transplantation, Hematopoietic Stem Cell Transplantation, and Transfusion. *Transfus Med Hemother* **40**, 182–189 (2013).
4. Lan, J. H. *et al.* Pretransplant Calculated Panel Reactive Antibody in the Absence of Donor-Specific Antibody and Kidney Allograft Survival. *Clin J Am Soc Nephrol* **16**, 275–283 (2021).
5. Cecka, J. M., Kucheryavaya, A. Y., Reinsmoen, N. L. & Leffell, M. S. Calculated PRA: Initial Results Show Benefits for Sensitized Patients and a Reduction in Positive Crossmatches. *American Journal of Transplantation* **11**, 719–724 (2011).
6. Grenzi, P. C., de Marco, R., Silva, R. Z. R., Campos, É. F. & Gerbase-DeLima, M. Antibodies against denatured HLA class II molecules detected in luminex-single antigen assay. *Human Immunology* **74**, 1300–1303 (2013).
7. Ravindranath, M. H. *et al.* Antibodies to cryptic epitopes on HLA class I and class II heavy chains bound to single antigen beads: Clinically relevant? *Transpl Immunol* **69**, 101482 (2021).
8. In, J. W., Rho, E. Y., Shin, S., Park, K. U. & Song, E. Y. False-Positive Reactions Against HLA Class II Molecules Detected in Luminex Single-Antigen Bead Assays. *Ann Lab Med* **34**, 408–410 (2014).
9. Park, B. G., Park, Y., Kim, B. S., Kim, Y.-S. & Kim, H.-S. False Positive Class II HLA Antibody Reaction Due to Antibodies Against Denatured HLA Might Differ Between Assays: One Lambda vs. Immucor. *Ann Lab Med* **40**, 424–427 (2020).
10. Kim, B., Kim, S., Park, Y. & Kim, H.-S. False-positive reactivity of anti-human leukocyte antigen antibodies detected using the single-antigen bead assay. *Human Immunology* **82**, 409–413 (2021).

11. Sullivan, H. C., Krummey, S. M., Gebel, H. M. & Bray, R. A. The utility of second single antigen bead assay: Clearing the water or stirring up mud? *Human Immunology* **81**, 663–670 (2020).
12. Morales-Buenrostro, L. E. *et al.* ‘Natural’ human leukocyte antigen antibodies found in nonalloimmunized healthy males. *Transplantation* **86**, 1111–1115 (2008).
13. CPRA calculator - OPTN. <https://optn.transplant.hrsa.gov/data/allocation-calculators/cpra-calculator/>.
14. Brown, N. K., Meade, J. R., Wang, J. & Marino, S. R. Reanalysis of the role of pronase treatment of B cells in the flow cytometric crossmatch assay: Fc receptor is not the primary target. *Human Immunology* **78**, 704–709 (2017).
15. Goulder, P. J. R. & Walker, B. D. HIV and HLA Class I: an evolving relationship. *Immunity* **37**, 426–440 (2012).
16. Stephens, D. CytoFLEX XM-T & B Cell Crossmatch Staining. (2022).
17. Stastny, P. *et al.* Role of immunoglobulin (Ig)-G and IgM antibodies against donor human leukocyte antigens in organ transplant recipients. *Human Immunology* **70**, 600–604 (2009).
18. Pandey, P. *et al.* Effects of different sensitization events on HLA alloimmunization in renal transplant cases; a retrospective observation in 1066 cases. *Transplant Immunology* **75**, 101680 (2022).
19. Kim, I. K., Vo, A. & Jordan, S. C. Transplantation in highly HLA-sensitized patients: challenges and solutions. *TRRM* **6**, 99–107 (2014).
20. Riberdy, J. M. & Cresswell, P. The antigen-processing mutant T2 suggests a role for MHC-linked genes in class II antigen presentation. *The Journal of Immunology* **148**, 2586–2590 (1992).
21. Lorente, E., García, R. & López, D. Allele-dependent Processing Pathways Generate the Endogenous Human Leukocyte Antigen (HLA) Class I Peptide Repertoire in Transporters Associated with Antigen Processing (TAP)-deficient Cells. *J Biol Chem* **286**, 38054–38059 (2011).
22. Salter, R. D. & Cresswell, P. Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid. *EMBO J* **5**, 943–949 (1986).

23. Young, N. T., Mulder, A., Cerundolo, V., Claas, F. H. & Welsh, K. I. Expression of HLA class I antigens in transporter associated with antigen processing (TAP)-deficient mutant cell lines. *Tissue Antigens* **52**, 368–373 (1998).
24. Wei, M. L. & Cresswell, P. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. *Nature* **356**, 443–446 (1992).
25. Scott-Browne, J. P. *et al.* Germline-encoded recognition of diverse glycolipids by natural killer T cells. *Nat Immunol* **8**, 1105–1113 (2007).
26. Bowerman, N. A., Falta, M. T., Mack, D. G., Kappler, J. W. & Fontenot, A. P. Mutagenesis of beryllium-specific TCRs suggests an unusual binding topology for antigen recognition. *J Immunol* **187**, 3694–3703 (2011).
27. Roark, C. L., Anderson, K. M., Aubrey, M. T., Rosloniec, E. F. & Freed, B. M. Arthritogenic peptide binding to DRB1*01 alleles correlates with susceptibility to rheumatoid arthritis. *J Autoimmun* **72**, 25–32 (2016).