Journal of Associated Medical Sciences 2026; 59(2): 1-8

JAMS
Journal of Associated Medical Sciences

Scopus Indexed & Thai-Journal Citation Index Centre (TCI)

Journal of Associated Medical Sciences

Journal homepage: https://www.tci-thaijo.org/index.php/bulletinAMS/index

Cut-off determination of HLA crossmatching by flow cytometry

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ARTICLE INFO

Article history:
Received 26 May 2025
Accepted as revised 16 December 2025
Available online 8 January 2026

Keywords:
HLA crossmatching, flow cytometry, cut-off, kidney transplantation.

ABSTRACT

Background: Microlymphocytotoxicity test is a gold standard for HLA crossmatching but provides low sensitivity. Emerging flow cytometry crossmatching (FCXM) provides more sensitivity, however, suitable cut-off values for FCXM must still be established for crossmatching result interpretation.

Objectives: This study aimed to determine the appropriate cut-off value for FCXM in kidney transplant patients and donors.

Materials and methods: A total of 30 sample pairs with known HLA typing and known antibody specificity were analyzed. Median channel fluorescence (MCF) and median channel fluorescence shift (MFS) were used to determine cut-off value using three different approaches and % accuracy was determined.

Results: The result showed that the cut-off values from first approach using lowest MCF of positive control and highest MCF of negative control were 1655.99 MCF for T-cells (yielding 100% accuracy) and 43668.06 MCF for B-cells (yielding 36.67% accuracy). The cut-off values from second approach which calculated from standard deviation (SD) of negative control MCF showed SD of T-cells MCF as 203.81 (100% accuracy) and SD for B-cells MCF as 11109.30 (60% accuracy). The cut-off values from third approach derived from the lowest MFS of the expected positive results were 1753.50 for T-cells (100% accuracy) and 593.50 for B-cells (73.33% accuracy).

Conclusion: The cut-off values from the third approach exhibited the highest accuracy. However, FCXM still had false positives and false negatives, underscoring the necessity for continuous refinement and adjustment of cut-off values based on the data accumulated within the laboratory to be used further for routine laboratory practice.

Introduction

For patients with end-stage organ failure, transplantation has emerged as a crucial therapeutic strategy that offers a chance for survival and an enhanced quality of life. However, immunological compatibility between the donor and the recipient has a significant impact on the outcome of a successful transplant, which is not exclusively determined by the surgical procedure.[1] Human leukocyte antigen system located on the short arm of chromosome no. 6 is a key component of the immune response and being one of the most important factors affecting transplant outcome. Two major classes of *HLA* gene system which

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doi: 10.12982/JAMS.2026.031
E-ISSN: 2539-6056

are class I include HLA-A, HLA-B, HLA-C and class II including HLA-DR, HLA-DQ, HLA-DP are responsible for antigen presentation. The class I molecules primarily present endogenous peptides to cytotoxic T cells, while class II molecules present exogenous peptides to helper T cells. When donor and recipient HLA molecules are not compatible, the immune system may recognize and react, which could result in immune-mediated graft rejection and transplant failure.[2] Not only HLA matching is important in selecting compatible donors for recipients, but preventing HLA-mediated rejection is also paramount for successful transplantation outcomes. Central to this process is HLA compatibility testing or HLA crossmatching which is the detection of pre-existing HLA antibodies in patient serum which can prevent the risk of hyperacute rejection from donor specific antibody (DSA).[1]

A serological method, microlymphocytotoxicity testing or complement-dependent cytotoxicity crossmatch (CDCXM), has been traditionally utilized in HLA crossmatching to detect these antibodies against donor HLA antigens. Although it is the gold standard method for HLA compatibility testing, its limitations including false negative results due to low sensitivity and the inability to detect low levels of antibodies and false positive results from autoantibodies could limit the successful transplantation outcome and may still cause graft rejection.[2,3] Studies have shown that patients with negative CDCXM but positive flow cytometry crossmatch (FCXM) results have an increased risk of organ rejection.[4] As a result, flow cytometry crossmatching, which offers improved sensitivity and precision in identifying donor-specific antibodies at lower levels, has been widely adopted to mitigate this risk.[2,5]

FCXM provides a more sensitive and accurate result for identifying antibody-mediated rejection that might be missed by the CDC approach. Despite its benefits, to guarantee its best application in organ e.g. kidney transplantation, exact cut-off values for FCXM must still be established. These parameters aid in reducing false positives, ensuring donor-recipient compatibility, and striking a balance between sensitivity and specificity. However, a significant challenge remains in determining the optimal cut-off value for defining positive and negative FCXM results which can vary among laboratories.[6] The lack of standardized cut-off values across laboratories necessitates the establishment of laboratory-specific thresholds based on robust data analysis. Therefore, this study aimed to determine the appropriate cut-off value for HLA crossmatching by flow cytometry in kidney transplant patients and donors, to be used further for routine laboratory practice in the HLA laboratory. The findings of this study will provide a basis for optimizing the pre-transplant assessment of kidney transplant candidates, thereby improving patient outcomes.

Materials and methods

Preparation of lymphocyte cells

Flow cytometry crossmatching (FCXM) experiments were performed using cell preparations from 29 donor cell samples and 7 recipient sera, resulting in a total of 30 FCXMs reactions. All samples of this study were the residual blood samples from HLA routine laboratory, Blood Bank Section, Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University, Thailand. Donor lymphocytes were isolated from residual kidney donor blood samples after routinely tested with microlymphocytotoxicity test. Acid Citrate Dextrose (ACD) anticoagulated whole blood was used in a volume of 50 mL. Blood was divided into two equal portions and 5 mL of 5% dextran was added to facilitate red blood cell agglutination. The samples were then incubated at 37 °C with a 45-degree tilt. Lymphocytes were separated using Ficoll-Hypaque solution with a density of 1.077 (Robbins Scientific Corporation, Norway) in a volume of 10 mL. The samples were centrifuged at 2000 rpm for 20 minutes to obtain a lymphocyte ring layer. This layer was carefully extracted and diluted to 50 mL with normal saline solution. The cells were washed three times by centrifugation at 1400 rpm for 10 minutes and 1000 rpm for 10 minutes. The resulting cell pellet was resuspended in 2 mL of RPMI 1640 medium. Cell concentration was determined using a hemocytometer after mixing the cell suspension with 0.2% Trypan blue dye in a 1:1 ratio. Based on the cell count, the appropriate volume of cold flow wash buffer was calculated to achieve a final cell concentration of 10,000 cells/µL. After centrifugation and removal of the RPMI 1640 medium, the calculated volume of cold flow wash buffer was added to the cell pellet.

Preparation of patient serum

Seven of patient sera were prepared from residual blood samples collected after routine serological testing in the HLA laboratory, Blood Bank Section, Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University, Thailand. The blood samples were centrifuged at 1000 rpm for 10 minutes to separate red blood cells. One milliliter of serum was then collected and transferred into a 1.5 mL microcentrifuge tube. All patient sera were characterized using LABScreen™ PRA Class I (LS1PRA) and LABScreen™ PRA Class II (LS2PRA) (Luminex®, One Lambda, USA), which reliably detect the presence of anti-HLA antibodies at the antigen group level. Positive control sera were pooled serum collected from 4-6 sensitized patients. These sera were heat-inactivated at 58 °C for 30 minutes to inactivate complement and then characterized by anti-HLA antibody screening using routine lymphocytotoxicity test (LCT). The positive control sera demonstrated greater than 75% panel reactive antibody (PRA) with whole lymphocytes of 14

random whole blood donors. Negative control sera were pooled from 4-6 male, blood group AB, apheresis healthy donors without a history of transfusion. These sera underwent the same heat inactivation procedure and were confirmed negative by LCT using cells from 10 different donors. Both positive and negative control sera were not tested by Luminex PRA assay but tested by LCT only as they were routinely used in LCT crossmatching. Their consistency in this assay was validated.

Flow cytometric crossmatch (FCXM) procedure

Flow cytometric crossmatch (FCXM) procedure followed Chamsai *et al.*,[7] 20 µL of prepared cells were pipetted into test tubes and mixed with 25 µL of patient serum. The mixture was incubated at room temperature for 30 minutes. Following incubation, the cells were washed three times with cold flow wash buffer, centrifuging at 500 ×g at 4 °C for 10 minutes per wash. Subsequently, the following antibodies were added: 5 µL of anti-CD19-PE, 5 µL of anti-CD3-PC5, and 10 µL of anti-IgG-FITC (diluted 1:100 with cold flow wash buffer). The samples were then incubated in the dark at 4 °C for 20 minutes. After incubation, the cells were washed twice with cold flow wash buffer, centrifuging at 500 ×g at 4 °C for 10 minutes per wash. The reaction was stopped by adding 250 µL of 1% cold paraformaldehyde. Finally, the samples were analyzed using a Flow cytometer (Beckman Coulter, DxFLEX) with CytExpert for DxFLEX software.

Determination of cut-off values for HLA crossmatching using flow cytometry

Three different approaches were used to calculate the cut-off values for HLA crossmatching to determine the best suitable thresholds. Across all three analytical approaches, cut-offs were determined using results from 30 independent FCXM reactions. The first approach involved recording the lowest mean channel fluorescence (MCF) value of the positive control and the highest MCF value of the negative control for both T and B cells and selected the most appropriate cut-off based on the observed range.[8] The second approach employed the mean and standard deviation (SD) obtained from the negative control's MCF for both T and B cells.[7,9] The third approach analyzed using the median channel fluorescence shift (MFS) to determine the lowest MFS value that reliably indicated a positive result and highest MFS value that showed negative result in both T and B cells.[10] All the cut-off values calculated from these three methods were compared to HLA class I and II typing of donor lymphocytes using polymerase chain reaction-sequence specific oligonucleotides (PCR-SSO) (LABType™, One Lambda, USA) with antibody specificity in patient sera to determine the possibility of positive or negative crossmatching result. Results obtained from three cut-off methodologies were subsequently calculated for the percentage of accuracy as the sum of true positives (TP) and true negatives (TN), divided by the total number of samples tested. The most appropriate cut-off values for flow cytometry crossmatch (FCXM) were then identified.

Results

HLA typing and antibody matching were performed on 30 cell-serum combinations, with 20 and 10 intended to be true positive and true negative for T-cells, respectively. Similarly, for B-cells, 21 were intended to be true positive, and 9 were intended to be true negative.

In the first approach, the lowest MCF value of the positive control for T-cells was 37817.28 with 40% accuracy, while the highest MCF value of the negative control was 1655.99 with 100% accuracy. For B-cells, the lowest MCF value of the positive control was 59115.80 with 40% accuracy, whereas the highest MCF value of the negative control was 43668.06 with 36.67% accuracy (Figure 1). The second approach showed that the T-cells negative control serum had a mean MCF+SD of 1103.59+203.81. The B-cells negative control serum had a mean MCF+SD of 12027.66+11109.30. The accuracy of the T-cells means MCF+SD and mean MCF+2SD were 100%. Meanwhile, the accuracy of B-cells means MCS+SD and mean MCF+2SD were 60% and 56.67%, respectively (Figure 2). In the third approach, the lowest MFS value that reliably indicated a T-cells positive result was 1753.50 with 100% accuracy, while the highest MFS value for a T-cells negative result was 991.50 with 100% accuracy. For B-cells, the lowest MFS value that reliably indicated a positive result was 593.50 with 73.33% accuracy. Whereas the highest MFS value for a B-cells negative result was 30996.64 with 40% accuracy (Figure 3). All results are represented in Table 1, which compares the cut-off values for T and B cells FCXM.

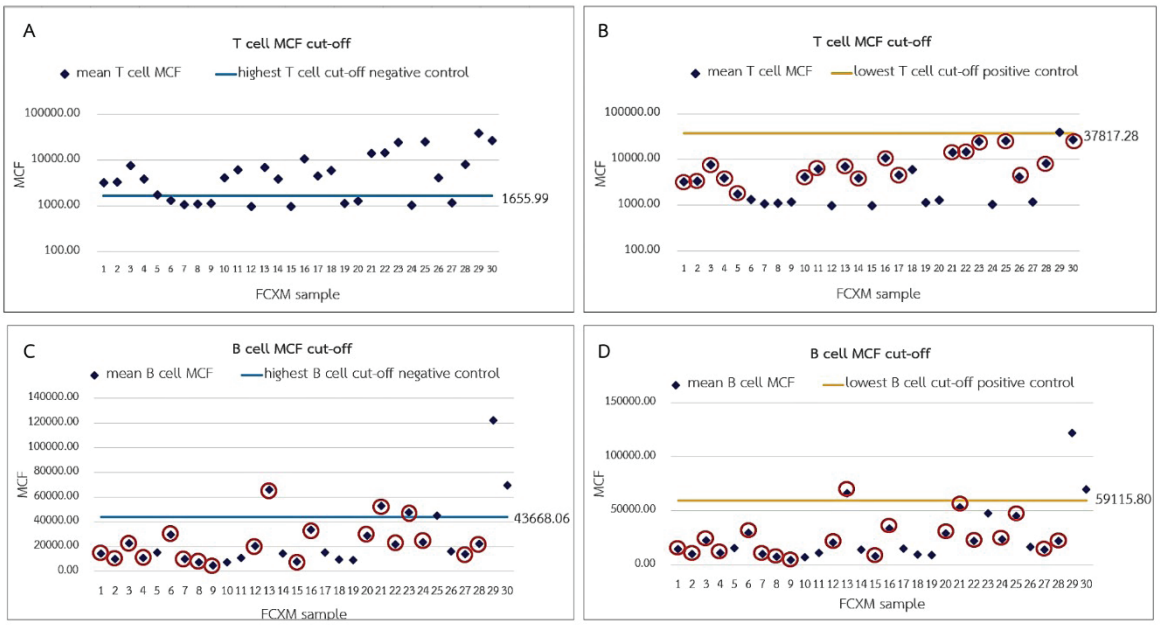

Figure 1. *Results from the first approach illustrating mean MCF across 30 FCXMs compared to each cut-off value. Red circles indicate discordant results between FCXM and expected outcomes. A: highest MCF T-cell negative control as cut-off, B: lowest MCF T-cell positive control as cut-off, C: highest MCF B-cell negative control as cut-off, D: lowest MCF B-cell positive control as cut-off.*

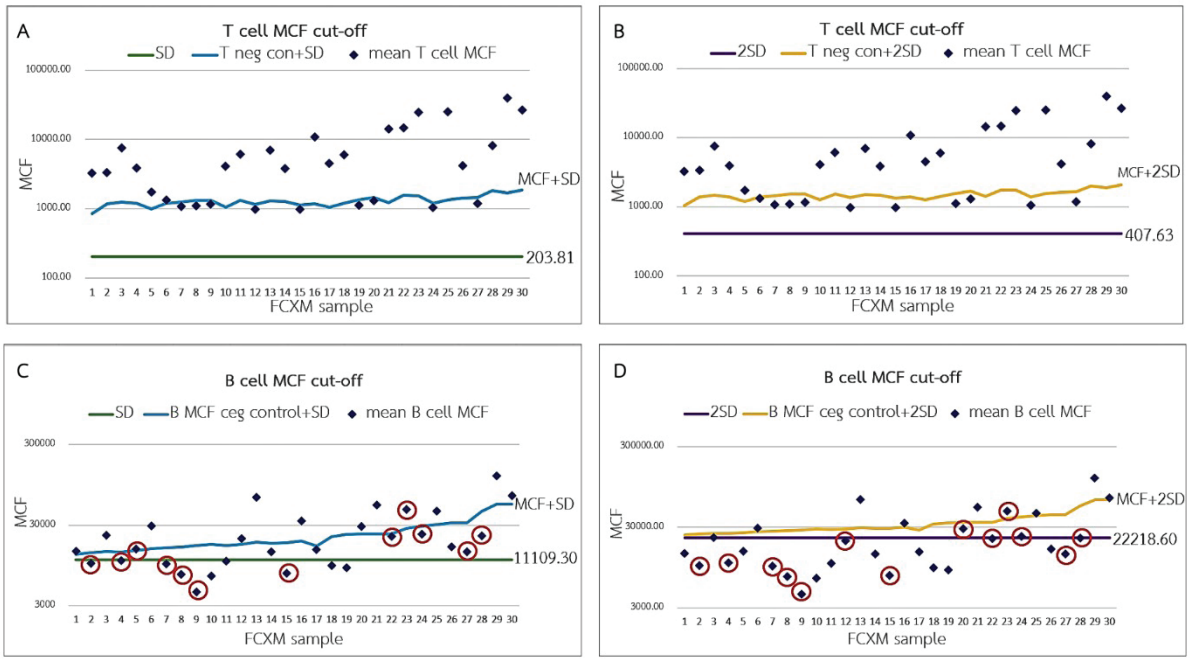

Figure 2. *Results from the second approach using statistical cut-offs based on mean MCF and standard deviations (SD) of negative control sera in 30 FCXMs. Red circles represent discrepant outcomes. A: Mean MCF+1 SD cut-off for T-cells, B: Mean MCF+2 SD cut-off for T-cells, C: Mean MCF+1 SD cut-off for B-cells, D: Mean MCF+2 SD cut-off for B-cells.*

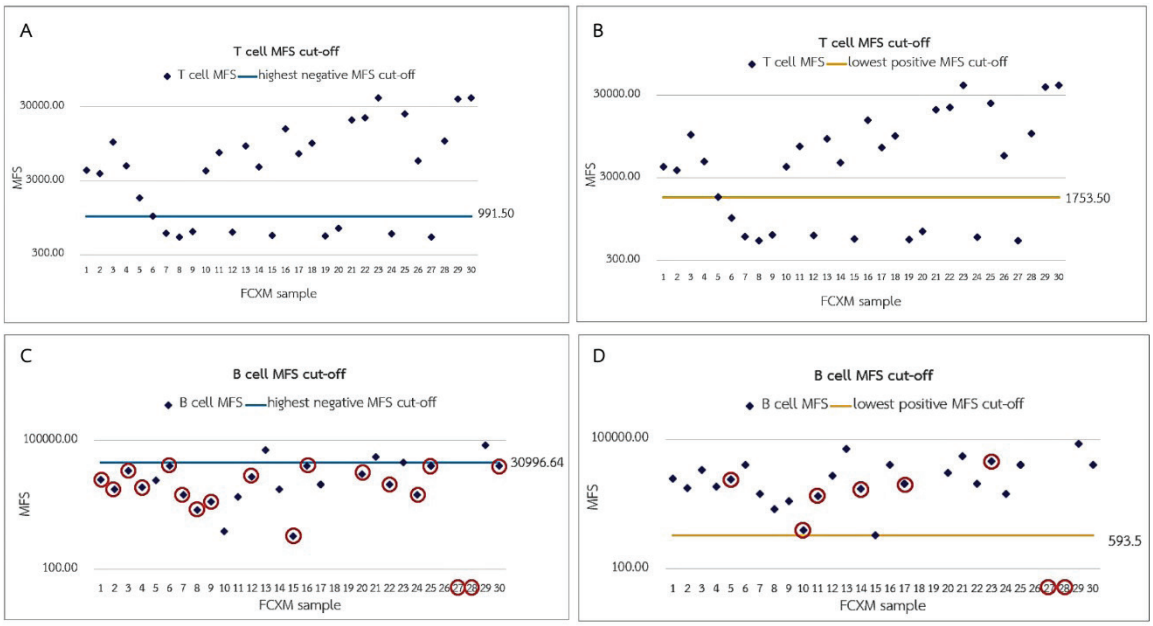

Figure 3. *Results from the third approach illustrating MFS across 30 FCXMs compared to defined cut-off values. Red circles indicate discordant results between observed and expected crossmatch outcomes. A: highest MFS value for T-cell predicting a negative result used as a cut-off, B: lowest MFS value for T-cell predicting a positive result used as a cut-off, C: highest MFS value for B-cell predicting a negative result used as a cut-off, D: lowest MFS value for B-cell predicting a positive result used as a cut-off.*

Table 1. Comparison of the cut-off values for T- and B-cell FCXM.

Parameter	Cut-off	T-cell FCXM				B-cell FCXM			
		Cut-off	% Accuracy	FP	FN	Cut-off	% Accuracy	FP	FN
1st Approach	Lowest MCF positive control	37817.28	40.00	0/10	18/20	59115.80	40.00	0/9	19/21
	Highest MCF negative control	1655.99	100.00	0/10	0/20	43668.06	36.67	1/9	18/21
2nd Approach	SD cut-off value, MCS	203.81	100.00	0/10	0/20	11109.30	60.00	2/9	10/21
	2SD cut-off value, MCS	407.63	100.00	0/10	0/20	22218.60	56.67	1/9	12/21
3rd Approach	Lowest MFS positive	1753.50	100.00	0/10	0/20	593.50	73.33	6/9	2/21
	Highest MFS negative	991.50	100.00	0/10	0/20	30996.64	40.00	0/9	18/21

Note: FP: false positive, FN: false negative.

Discussion

Flow cytometric crossmatch (FCXM) plays a critical role in preventing acute antibody-mediated rejection. The establishment of appropriate laboratory-specific cut-off values is essential to minimize false positive and false negative results. In this study, three different methodologies were evaluated for determining FCXM cut-off values using median channel fluorescence (MCF) and median channel fluorescence shift (MFS) obtained from 30 donor-recipient pairs. These approaches were compared to assess their accuracy based on the results of HLA typing and LABScreen™ PRA Class I and II for antibody typing to identify the most suitable cut-off values for routine use. The selected three cut-off methodologies were based on previously described approaches in the literature. The first approach, based on the lowest MCF value of the positive control and the highest MCF value of the negative control, was adopted from a prior undergraduate term paper conducted in our laboratory.[8] This method was selected because it demonstrated the highest level of agreement between flow cytometric crossmatch and complement-dependent cytotoxicity crossmatch results in that study. However, low accuracy with 80% specificity in T-cells and 60% specificity in B-cells was observed, therefore, alternative approaches for calculating the cut-off values were considered to achieve improved performance. The second approach with using the mean and SD of the negative control's MCF applied in this study is consistent with the methodology previously reported in Chamsai *et al.*[7] and Chulalongkorn University thesis.[9] The third approach with using median fluorescence shift (MFS) was consistent with published in de Moraes *et al.* [10]

From the results in this study, the first approach resulted in 18 false negatives out of 20 for T-cells. For B-cells, the lowest MCF value of the positive control led to 19 false negatives out of 21. Additionally, the highest MCF value of the negative control produced 1 false positive out of 9 and 18 false negatives out of 21 for B-cells, while no false positive and negative was observed for T-cells (Table 1). Comparison of sample MCF with the cut-off revealed a high rate of false negative results, likely due to the elevated background in both positive and negative control sera. Therefore, this cut-off value is not suitable for reliable use. The cut-off value determined by the second approach demonstrated a 100% match for T-cells. However, for B-cells, using MCF negative control+SD resulted in 2 false positives out of 9 and 10 false negatives out of 21, whereas MCF negative control+2SD yielded 1 false positive out of 9 and 12 false negatives out of 21 (Table 1). Comparing sample MCF with these cut-off values suggests that MCF negative control+SD is the preferable choice for this method, as it provides higher overall accuracy. All false-negative results had MCF values lower than the cut-off due to the high and variable background of the negative control. These false negative results may have occurred for several reasons, including poor washing technique leading to incomplete removal of serum immunoglobulin, poor lymphocyte purity, high background in the negative control serum, and a low serum-to-target cell ratio.[2,11,12] The third approach achieved a 100% match for T-cells. For B-cells, the lowest MFS value that reliably indicated a positive result resulted in 6 false positives out of 9 and 2 false negatives out of 21. In contrast, the highest MFS value for a negative B-cell result led to 18 false negatives out of 21 (Table 1). By comparing sample MFS with the cut-off and selecting the value with the highest accuracy, the results support using the lowest MFS that indicates a positive result as the cut-off. These two false negative results may have been influenced by a low antibody titer in the serum and low expression of loci on donor cells.[2,11] The low-expression loci present on these two donor cells included HLA-DR51, DR52, and DR53.[13,14] However, the selected cut-off value also resulted in false positive reactions for B-cells FCXM likely reflects the challenges in B-cells crossmatching sensitivity and specificity. B-cells express Fc receptors that can bind nonspecifically to immunoglobulins, which can lead to elevated background fluorescence and false positive results.[15] Additionally, the lowest MFS positive cut-off may be too low or less stringent, making it more prone to detecting weak or nonspecific binding signals that do not represent true donor-specific antibody reactions. Also, the presence of therapeutic antibodies (such as Rituximab) in the recipient's serum, autoantibodies, and the low background of the negative control serum can be the causes.[2,11,12]

In summary, the appropriate cut-off values are the lowest MFS indicating a positive result, set at 1753.50 for T-cells and 593.50 for B-cells. There should be some exceptions, though, because of false negative results, and samples must be collected continuously so that a clinically significant cut-off value can be found based on the data gathered in each lab.[6,16] The implementation of these cut-off values in a clinical setting should be carefully evaluated in collaboration with physicians. Given its direct impact on transplant rejection and patient outcomes, the importance of setting accurate cut-off values in FCXM has grown. Inappropriate threshold settings can either increase the risk of graft loss by failing to detect clinically relevant donor-specific antibodies or unnecessarily exclude potential donors by producing false positive results. Previous study has shown that recipients with positive FCXM results, particularly for HLA class I, including those with low-level antibodies, are more likely to increase risk of acute rejection and decrease graft survival rate.[4] The correlation between DSA and FCXM can vary significantly due to the difference in the cut-off value used which FCMX failed to detect DSA in samples with lower MFI (less than 2,000).[4] Accordingly, continual adjustment and validation of these cut-off values in the clinical context are essential to enhance transplant success and improve long-term graft function. However, as FCMX found to be 8-32 times more sensitive than CDC and 4-16 times more sensitive than AHG-CDC,[17] it was reported a high rate of false positive FCXM results as high as 35.9%.[4]

Therefore, appropriate conditions should be carefully evaluated to minimize false positive and false negative results. Although our FCXM protocol is based on the three-colored assay reported by Chamsai *et al.*,[7] additional optimizations, for example, the serum-cell ratio and titrated the fluorochrome-conjugated antibodies should be made to improve assay specificity and efficiency when different flow cytometer machine and settings are applied. Notably, the Halifax and Halifaster protocols employ optimized lymphocyte isolation techniques, shortened incubation times, and incorporate pronase treatment to reduce nonspecific binding in B-cells FCXM, increasing accuracy and decreasing false positive rates.[12,18-20] Additionally, some laboratories recommend using $F(ab')_2$ fragments IgG as secondary antibodies to further improve specificity.[20] False negative results can be reduced by using an alternative negative control serum, enhancing the washing technique, and adjusting the serum-to-cell volume ratio.[12] One limitation of this study is the source and selection of negative control sera. In the current study, negative control sera were pooled from 4-6 male, AB blood type, apheresis healthy donors without a history of transfusion. These sera underwent the heat inactivation procedure and were confirmed negative by LCT using cells from 10 different donors. As we used both positive and negative control in routine LCT crossmatching, we used the same control in FCXM. We realized that at least 30 different negative control sera is an ideal approach to minimize background and improve specificity. Due to limitations in sample availability, we

were unable to recruit enough of such negative control sera, which may affect the robustness of the negative control group. This limitation is acknowledged and should be addressed in future studies, where we plan to collect a larger number of negative control sera from AB blood type, non-sensitized donors confirmed negative for HLA antibodies. Notably, negative control sera should be confirmed negative, at minimum by solid phase antibody screening. Additionally, a comparative analysis of MFI values between donor-derived and patient-derived negative sera should be performed to better define baseline fluorescence and improve specificity in FCXM cut-off determination. Similarly, the positive control sera should be also confirmed broad HLA specificity and exhibit a high anti-HLA IgG titer with specific MFI range from solid phase immunoassay.

One more issue that might affect false positive and false negative in our study is selection of the positive samples. Although single antigen bead (SAB) assay provide higher resolution allele-specific antibody identification, they were not available during our study. Our study utilized LABScreen™ PRA Class I and PRA Class II for antibody detection in positive sera. These panel assays are proven methods for screening for class I, class II, or both anti-HLA antibodies. Future research would further improve antibody specificity assessment and cut-off accuracy by incorporating SAB assay and external quality assessment.

With some limitations of FCXM, alternatively, some laboratories have adopted virtual crossmatching, an immunological compatibility assessment that compares a patient's alloantibody profile with a donor's histocompatibility antigens.[2] This approach enhances antibody detection sensitivity and enables a virtual evaluation of compatibility. Additionally, it serves as a tool for deceased donor allocation, aiming to balance the likelihood of transplantation with the risk of a positive crossmatch result.[21]

Conclusion

The established cut-off values, representing the lowest MFS indicating a positive result, are 1753.50 for T-cells and 593.50 for B-cells. Ongoing sample collection and protocol optimization are essential to determine clinically significant cut-off values tailored to each laboratory's data especially applied flow cytometer machine and settings are different to the reference protocol. Implementing these cut-off values in clinical practice should be carefully evaluated in collaboration with physicians.

Conflict of interest

The authors declare no conflict of interest.

Ethical approval

This study was approved by the Research Ethics Committee, Faculty of Medicine, Chiang Mai University, Thailand (approval code: NONE-2567-0674; date of approval: 7 February 2025).

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