Original Article

Evaluation of the Three-Colored Flow Cytometric Crossmatch Assay in Living-Related Kidney Transplantation

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Abstract :

Introduction: *Flow cytometric crossmatch (FCXM) is a cell-based assay for DSA detection. Validation and performance evaluation are essential.* ***Objective:*** *To optimize and validate the Siriraj three-colored FCXM protocol, and compare its performance with the two-colored FCXM protocol for living-related kidney transplantation.* ***Materials and Methods:*** *The three-colored FCXM were optimized and 30 donor lymphocyte samples were assayed with negative and positive control sera to determine cutoff values. Eighteen anti-HLA positive sera were assayed against donor lymphocytes with corresponding HLA antigens to determine accuracy. Twenty patients' sera and their donor lymphocytes were crossmatched using CDC, AHG-CDC, two-colored FCXM and three-colored FCXM protocols in parallel to compare performance.* ***Results:*** *The cutoff values were median channel fluorescence shift (MCS) more than 22.46 channels for T cell FCXM and more than 52.78 channels for B cell FCXM. All results of three-colored FCXM assays performed on 18 pairs of donor cells versus sera with DSA were concordant with positive virtual crossmatches. Chi-square analysis showed association between the results of three-colored FCXM protocols and the results of two-colored FCXM protocols (T cell FCXM $p < 0.05$ and B cell FCXM $p < 0.05$). The three-colored FCXM protocol required less number of cells for analysis (1×10^6 cells vs. 4×10^6 cells), less number of test tube (5 tubes vs. 8 tubes), and less total assay time (250 mins vs. 360 mins) compared with the two-colored FCXM protocol.* ***Conclusion:*** *The optimized three-colored protocol were equivalent to the two-colored protocol for FCXM interpretation but less time and labor consumption.*

Keywords : ● *Flow cytometric crossmatch* ● *Kidney transplantation* ● *Living-related*

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นิพนธ์ต้นฉบับ

การประเมินการทดสอบความเข้ากันได้ด้วยวิธีโฟลไซโตเมทรีสามสี สำหรับการปลูกถ่ายไตจากผู้บริจาคที่มีชีวิต

สุทธิศักดิ์ แจ่มใส กุลวรา กิตติสาเรศ ยุบลรัตน์ ธนเขตไพศาล สมพร งามถาวรวงศ์ และ กฤษดา โคกตาทอง
ภาควิชาเวชศาสตร์การธนาคารเลือด คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล

บทคัดย่อ

บทนำ *การทดสอบความเข้ากันได้ด้วยวิธีโฟลไซโตเมทรีสามสีเป็นการทดสอบโดยใช้เซลล์เพื่อหาแอนติบอดีที่จำเพาะกับผู้บริจาคในผู้ป่วยเตรียมรับการปลูกถ่ายอวัยวะ การประเมินความถูกต้องและความสามารถในการใช้งานก่อนนำมาใช้ในงานประจำเป็นสิ่งที่จำเป็น* ***วัตถุประสงค์*** *เพื่อพัฒนาวิธีการในการทดสอบและประเมินความถูกต้องและความสามารถในการใช้งานของการทดสอบความเข้ากันได้ด้วยวิธีโฟลไซโตเมทรีสามสีเพื่อการปลูกถ่ายไตจากผู้บริจาคที่มีชีวิต* ***วัสดุและวิธีการ*** *หาค่าจุดตัดในการแปลผลโดยทดสอบซีรัมควบคุมผลลบและซีรัมควบคุมผลบวกกับเซลล์ของผู้บริจาคเลือดจำนวน 30 ราย ประเมินความถูกต้องโดยทดสอบซีรัมที่มีความจำเพาะกับแอนติเจนของผู้บริจาคเลือดจำนวน 18 ราย เปรียบเทียบความสามารถในการใช้งานของการทดสอบความเข้ากันได้ด้วยวิธีโฟลไซโตเมทรีโดยการย้อมทีและบีเซลล์สามสีและสองสีในผู้ป่วยที่จะได้รับการผ่าตัดปลูกถ่ายไตจากผู้บริจาคที่มีชีวิต* ***ผลการศึกษา*** *ในการทดสอบความเข้ากันได้ด้วยวิธีโฟลไซโตเมทรีสามสีพบว่าค่าจุดตัดในการแปลผลคือ ผลการทดสอบที่ค่ามัธยฐานของการติดสีที่เบี่ยงเบนไปมากกว่าค่าของซีรัมควบคุมผลลบ จำนวน 22.46 ช่องสำหรับการทดสอบทีเซลล์และมากกว่า 52.78 ช่องสำหรับการทดสอบบีเซลล์ การประเมินความถูกต้องพบว่าการทดสอบความเข้ากันได้ด้วยวิธีโฟลไซโตเมทรีสามสีให้ผลบวกกับซีรั่มที่มีความจำเพาะกับแอนติเจนของผู้บริจาคเลือดทั้ง 18 ราย ผลการทดสอบความเข้ากันได้ด้วยวิธีโฟลไซโตเมทรีสามสีสัมพันธ์ไปกับผลการทดสอบความเข้ากันได้ด้วยวิธีโฟลไซโตเมทรีสองสี (การทดสอบทีเซลล์ $p < 0.05$ และการทดสอบบีเซลล์ $p < 0.05$) โดยใช้เซลล์น้อยกว่า (1×10^6 เซลล์กับ 4×10^6 เซลล์) หลอดทดลองน้อยกว่า (5 หลอดกับ 8 หลอด) และเวลาน้อยกว่า (250 นาทีกับ 360 นาที)* ***สรุป*** *การทดสอบความเข้ากันได้ด้วยวิธีโฟลไซโตเมทรีสามสีที่พัฒนาขึ้นให้ผลการทดสอบสัมพันธ์ไปกับผลการทดสอบความเข้ากันได้ด้วยวิธีโฟลไซโตเมทรีสองสี โดยลดเวลาและแรงงานในการทำการทดสอบ*

คำสำคัญ : ● *Flow cytometric crossmatch* ● *Kidney transplantation* ● *Living-related*

วารสารโลหิตวิทยาและเวชศาสตร์บริการโลหิต 2561;28:239-48.

Introduction

Significance of compatibility test for detection of patient's antibodies against donor human leukocyte antigens (HLA) relative to kidney transplantation outcome was demonstrated since 1969.[1] Presence of preformed donor specific HLA antibodies (DSA) can result in hyper-acute rejection and acute antibody-mediated rejection.[1,2] Complement dependent cytotoxicity (CDC) crossmatch has been used as a standard pre-transplantation work up for more than 40 years. Positive CDC crossmatch indicates clinical significant DSA and is considered a contraindication to kidney transplantation.[3] Several methods were developed to improve sensitivity of DSA detection including addition of antihuman globulin to enhance CDC (AHG-CDC), flow cytometric crossmatch (FCXM), and solid-phase immunoassay (SPI).[3]

Flow cytometric crossmatch is a cell-based assay, performed by incubating donor lymphocytes with recipient serum, adding fluorochrome-conjugated antihuman globulin to detect donor reactive antibodies on T and B lymphocytes, and measuring detected DSA by flow cytometer. FCXM was stated to have more sensitivity than CDC since lower titer of DSA and non-complement binding DSA could be detected.[4-7] These antibodies could cause acute antibody-mediated rejection and early graft loss in several studies.[8-11] Many transplant centers use the FCXM assay as one of pre-transplantation work up process for kidney and other organ transplantations.[3] However, standardization of flow cytometric assays might be problematic due to variability among antiglobulin reagents, fluorochromes, and flow cytometers.[3,12] Validation of FCXM protocol and result threshold in respect of transplantation outcomes is recommended.[3,13-15]

In addition to CDC crossmatch, flow cytometric crossmatch was introduced to Faculty of Medicine Siriraj Hospital, Mahidol University since 2001 for pre-transplantation work up process in living related kidney transplantation.[7,16] Formerly, the two-colored-FCXM protocol used by Siriraj Hospital was deliberately validated and found to be 8-32 times more sensitive than CDC methods.[7] The two-colored FCXM protocol required two separate assays, for T cell FCXM using fluorochrome-conjugated antibody anti-CD3-PE and anti-IgG FITC and for B cell FCXM using fluorochrome-conjugated antibody anti-CD19-PE and anti-IgG FITC. This was time-consuming and labor-intensive. At present, flow cytometers with multi-color detectors were introduced and the three-colored FCXM protocol has been used by the majority of laboratories. In the three-colored FCXM protocol, T cell FCXM and B cell FCXM are performed simultaneously in a single assay by using three different fluorochromes conjugated with antibodies including anti-CD3-PC5, anti-CD19-PE and anti-IgG-FITC.[12,14-15] Application of the three-colored FCXM protocol for pre-transplantation work up can reduce work load and time consumed. As mention above, the new FCXM protocol needs to be prudently optimized and validated because of variability in reagents and flow cytometers. In this study, we optimized, validated the Siriraj three-colored FCXM protocol, and compared the performance with the two-colored FCXM protocol.

Materials and Methods

1. Samples

A total of 32 ACD blood samples were obtained from healthy blood donors; 2 samples were used for the three-colored FCXM protocol optimization, and 30 samples were assayed with negative and positive control sera to determine cutoff values and acceptable range of control sera. Moreover, 18 samples from blood donors and platelet donors who previously typed for HLA class I and class II antigens were assayed with 18 known anti-HLA positive sera to determine accuracy. Twenty pairs of patients' sera and their kidney donor ACD blood samples were obtained from patients who were planned to receive living related kidney transplantation at the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand during the February 2017 to July 2017. The three-colored FCXM were performed in parallel with CDC, AHG-CDC, two-colored FCXM on

these 20 pairs of patients' sera and donor lymphocytes to evaluate performance of the protocol. The protocol for this study was approved by the Siriraj Institutional Review Board (SIRB), COA no. 271/2015.

2. Reagents

To detect T lymphocytes, B lymphocytes, and donor specific antibodies, fluorochrome-conjugated antibodies were anti-CD3-PC5 (CD3-PC5, Immunotech SAS Beckman Coulter, France), anti-CD19-PE (CD19-PE, Immunotech SAS Beckman Coulter, France), anti-IgG-FITC (IgG-FITC, Fluorescein (FITC) AffiniPure $F(ab')_2$ fragment goat anti-Human IgG Fcγ specific, Jackson ImmunoReasearch, West Grove, PA), respectively. Flow wash buffer composed of IsoFlow sheath fluid (Beckman Coulter, CA), 0.1% v/v sodium azide, and 1% v/v fetal calf serum. Negative control serum was the in-house Siriraj negative control serum, which was prepared from non-transfused group AB male donor serum which was negative for HLA antibodies by CDC. Positive control serum was the in-house Siriraj positive control serum, which was a pool of patients' sera with high panel reactive activity (100% PRA) tested by CDC.

3. Three-colored flow cytometric crossmatch protocol

Total lymphocytes were isolated from donor's ACD blood by the standard Ficoll-Hypaque density gradient centrifugation technique, as previously described.[7] Five tubes of donor lymphocytes were assayed against blank control (flow wash buffer), negative control and positive control sera, and duplicates of patient's serum. Concisely, whole lymphocytes (2.0-2.5 x 10^5 cells) were resuspended in 20 μL of flow wash buffer and were incubated with 25 μL of test or control sera in Falcon tubes at room temperature for 30 minutes and washed three times with cold flow wash buffer. Then, 5 μL of CD3-PC5, 5 μL of CD19-PE and 10 μL of IgG-FITC were added to cells, incubated in the dark at 4°C for 20 minutes, and washed twice with cold flow wash buffer. The cells were resuspended in 250 μL of cold 1% paraformaldehyde in flow wash buffer and ready for acquisition. Flow cytometry acquisition was performed on Navios flow cytometer (Beckman Coulter, Miami, FL) and analyzed on Navios software v1.3 (Beckman Coulter, Miami, FL). A minimum of 1,000 B cell events was required for analysis. FITC, PE and PC5 fluorescences were measured in FL1, FL2 and FL4 channels, respectively. T and B lymphocytes were gates using dot plot of side scatter (SS) versus forward scatter (FS), FL4 (CD3-PC5), and FL 2 (CD19-PE). A crossmatch result was determined as median channel fluorescence (MCF) of IgG FITC histogram. The FCXM assay results are presented as median channel fluorescence shift (MCS), which is a number of channels shift of MCF of tested serum compared with MCF of negative control serum. A cutoff value for positive FCXM assay in this study was defined as more than 2 SD shifted to the right. CDC, CDC-AHG and two-colored FCXM protocols were performed in parallel, as previously described.[7]

4. Statistical analysis

Pearson correlation coefficients and chi-square analyses were performed using SPSS Statistics Version 18.0 (SPSS, Inc., Chicago, IL, USA). A p-value less than 0.05 was considered as being statistically significant.

Results

Three-colored FCXM assay optimization

The MCF of negative and positive control sera in three-colored FCXM assay performed by using IgG FITC from dilution 1:50 to 1:200 was summarized in Figure 1. The dilution at 1:100 was selected because it provided the most different value between MCF of negative control serum and MCF of positive control serum in B cell FCXM. The protocol for gating sub-population of lymphocytes, T lymphocytes (positive for CD3-PC5) and B lymphocytes (positive for CD19-PE), was established on the flow cytometer, as shown in Figure 2.

Negative control serum and cutoff for three-colored FCXM assay

The mean MCF ± SD of negative control serum in T cell three-colored FCXM was 164.17 ± 11.23. The mean MCF ± SD of negative control serum in B cell three-

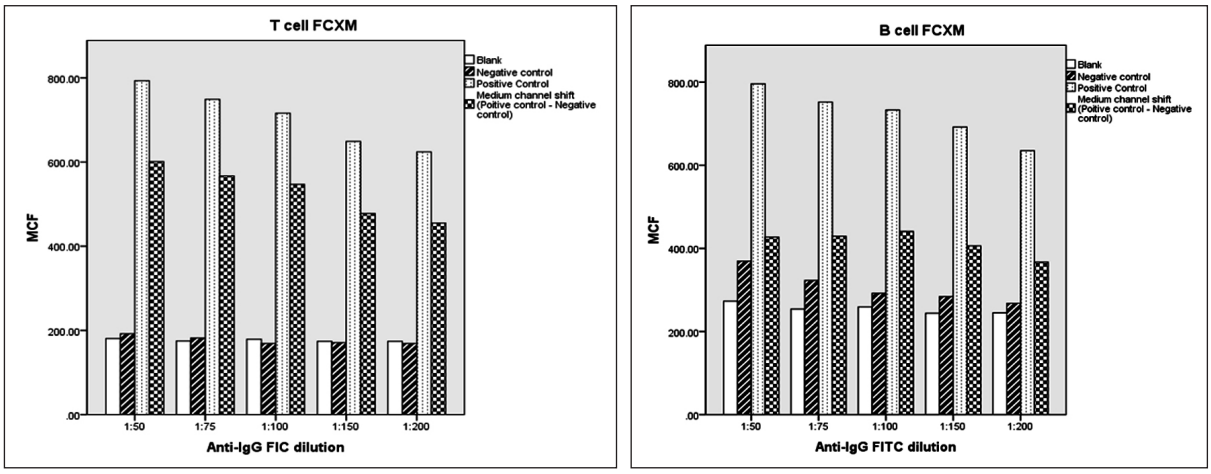


Figure 1 Three-colored flow cytometric crossmatches were performed with different dilution of anti-IgG FITC, T cell FCXM and B cell FCXM. The dilution at 1:100 provided the most different value between MCF of negative control serum and MCF of positive control serum in B cell FCXM.

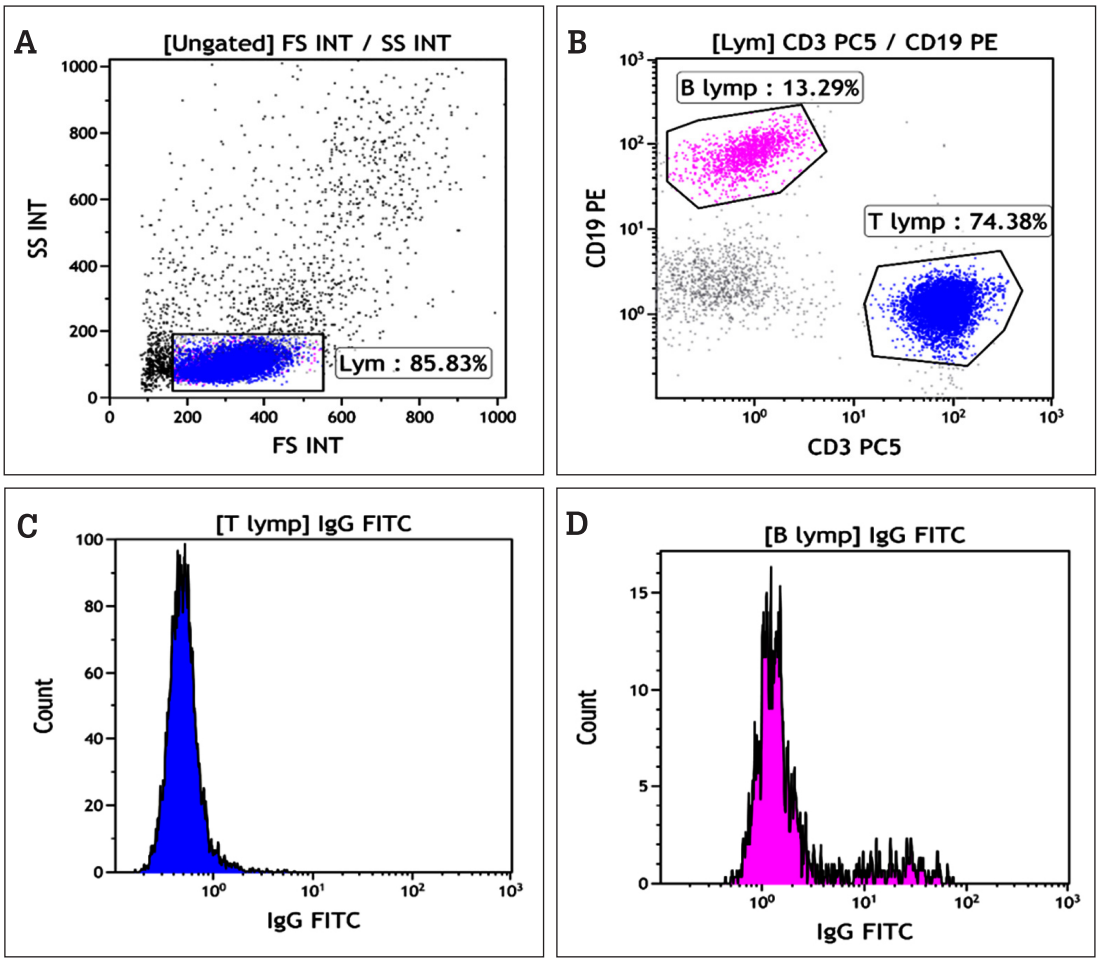


Figure 2 The protocol for gating sub-population of lymphocytes is shown in side scatter vs forward scatter dot plots and CD19-PE vs CD3-PC5 dot plots (A and B). Results of T cell FCXM and B cell FCXM were determined as mean channel fluorescence of IgG-FITC histogram (C and D).

Table 1 Results of three-colored FCXM: negative control serum tested against 30 donors

Parameter	T cell FCXM	B cell FCXM
Blank control MCF, mean MCF (SD)	167.97 (11.25)	238.50 (23.84)
Negative control MCF, mean MCF (SD)	164.17 (11.23)	275.50 (26.39)
Positive control MCF, mean MCF (SD)	407.17 (28.17)	478.57 (43.71)
2 SD cutoff value, MCS	22.46	52.78

FCXM = flow cytometric crossmacth; MCF = median channel fluorescence; MCS = median channel fluorescence shift

colored FCXM was 275.50 ± 26.39 (Table 1). The FCXM results are presented as median channel fluorescence shift (MCS), which is a number of channels shift of MCF of tested serum compared with MCF of negative control serum. A cutoff value for positive FCXM assay in this study was defined as more than 2 SD shifted to the right. Therefore, the cutoff values were MCS more than 22.46 channels for T cell three-colored FCXM and more than 52.78 channels for B cell three-colored FCXM (Table 1).

Validation of optimized three-colored FCXM assay

All results of three-colored FCXM assays performed on 18 pairs of donor cells versus sera with DSA were concordant with positive virtual crossmatches. Sixteen sera were positive both in T cell FCXM and B cell FCXM, eleven of which contained only anti-HLA class I, one of which contained only anti-HLA class II, and four of which contained both anti-HLA class I and anti-HLA class II. Two sera were positive only in B cell FCXM. These sera contained anti-HLA class II including anti-DR17 (13,530 MFI) in one serum and anti-DR9 (6,500 MFI) in one serum. In 14 sera, donor specific antibody MFI was previously identified, ranging from 2,565 to 21,151 MFI for anti-HLA class I and ranging 6,500 to 21,724 MFI for anti-HLA class II. There was strong correlation between the MCS of T cell FCXMs and the MFI of anti-HLA class I (R^2 = 0.80, $p < 0.05$; Figure 3A) and moderate correlation between the MCS of B cell FCXMs and the MFI of anti-HLA class II (R^2 = 0.60, $p = 0.04$; Figure 3B).

Performance evaluation of three-colored FCXM assay

A total of 20 HLA crossmatches for living related kidney transplantation were performed in parallel using CDC, AHG-CDC, two-colored FCXM, and three-colored FCXM protocols. For T cell FCXM, all results of three-colored FCXM were concordant with the crossmatch results of CDC, CDC-AHG and two-colored FCXM (Figure 4A). For B cell FCXM, 16 negative and 2 positive results, were concordant among all crossmatch protocols. However, there were two discordant results, one patient was positive in both FCXM protocols but negative in CDC and CDC-AHG protocols and one patient was positive in only in two-colored FCXM (Figure 4B). The patient' s serum that was positive only in B cell two-colored FCXM with donor's lymphocytes and autologous lymphocytes. Chi-square analysis showed association between the results of three-colored FCXM protocols and the results of two-colored FCXM protocols (T cell FCXM $p < 0.05$ and B cell FCXM $p < 0.05$; Figure 4C, D). The three-colored FCXM protocol required less number of cells for analysis (1×10^6 cells vs. 4×10^6 cells), less number of test tube (5 tubes vs. 8 tubes), and less total assay time (250 mins vs. 360 mins) compared with the two-colored FCXM protocol (Table 2).

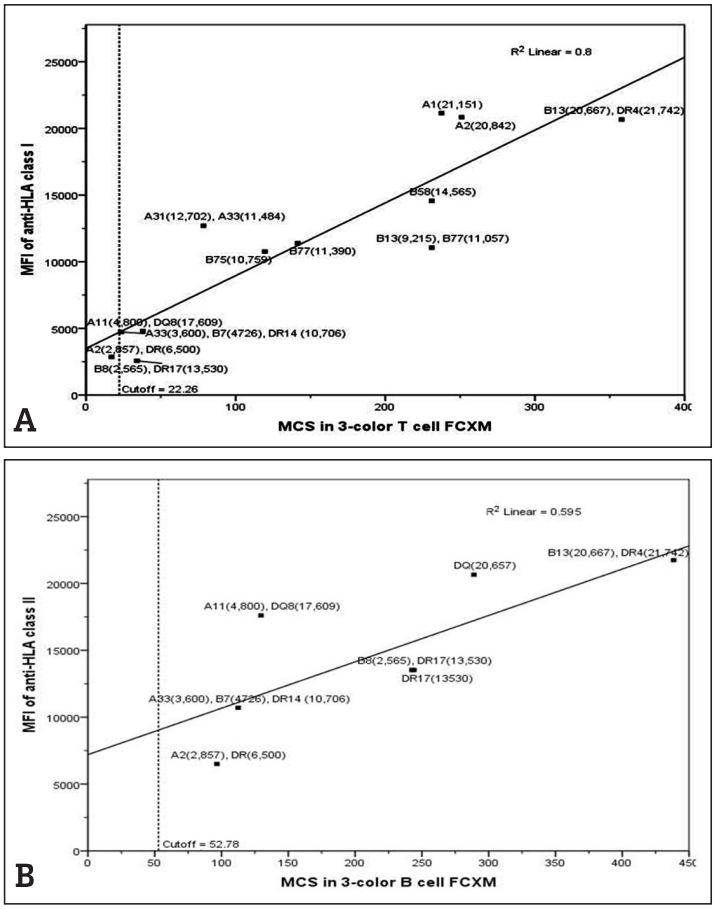
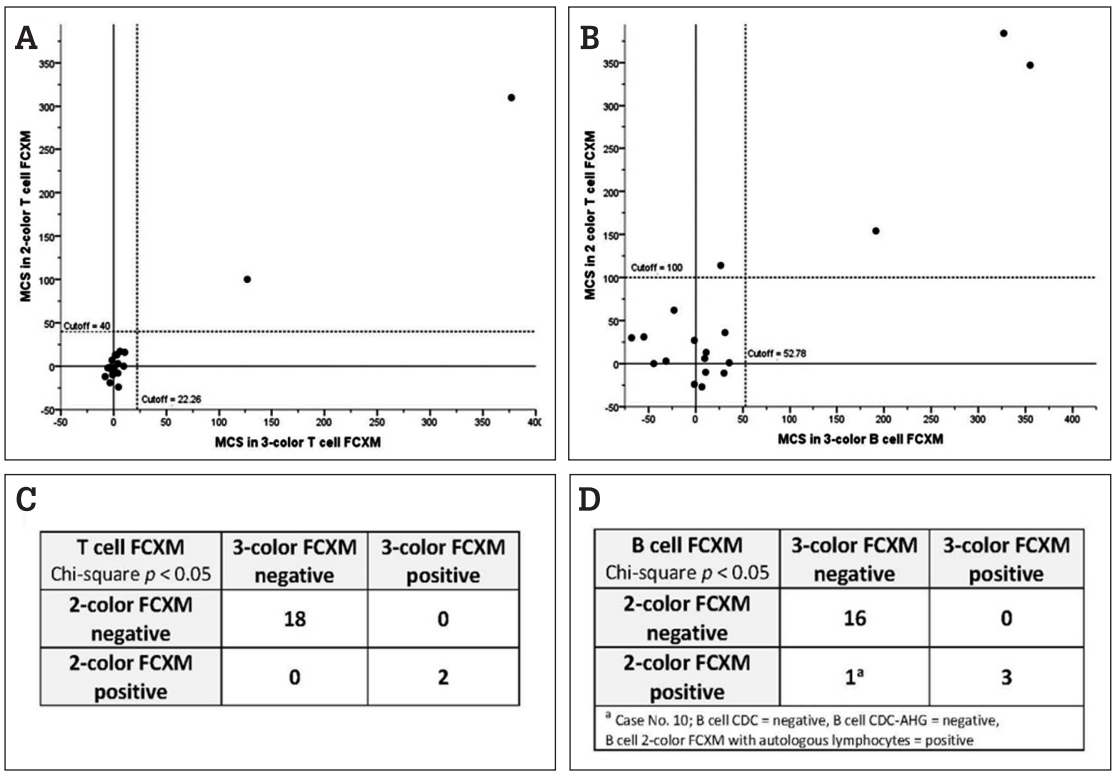


Figure 3 The MCS correlations of T cell FCXMs with the MFI of anti-HLA class I (A) and the MCS of B cell FCXMs with the MFI of anti-HLA class II (B).



C

T cell FCXM Chi-square $p < 0.05$	3-color FCXM negative	3-color FCXM positive
2-color FCXM negative	18	0
2-color FCXM positive	0	2

D

B cell FCXM Chi-square $p < 0.05$	3-color FCXM negative	3-color FCXM positive
2-color FCXM negative	16	0
2-color FCXM positive	1[a]	3

[a] Case No. 10; B cell CDC = negative, B cell CDC-AHG = negative, B cell 2-color FCXM with autologous lymphocytes = positive

Figure 4 T cell FCXM and B cell FCXM were performed in parallel using two colored FCXM protocol and three-colored FCXM protocol (A and B). Chi-square analysis of FCXM interpretation is presented (C and D).

Table 2 Comparison of the two-colored FCXM and three-colored FCXM protocols.

Parameter	Two-colored FCXM	Three-colored FCXM
Number of test tubes	8	5
Cell number	0.5-1.0 x 10^6, x8	0.2-0.25 x 10^6, x5
Cell suspension (uL)	30	20
Antibody reagent (uL)		
CD3-PC5	5, x8	5, x5
CD19-PE	5, x8	5, x5
IgG FITC	10, x8	10, x5
Flow wash buffer (mL)	40	25
Cell preparation time (min)	180	120
Flow preparation time (min)	120	90
Flow cytometry assay time (min)	60	40
Total assay time (min)	360	250

Discussion

Flow cytometry cell-based assay for transplantation compatibility testing was introduced since 1983 by Garavoy, *et al*.[4] Several studies demonstrated relevance of positive-FCXM results with early graft loss; especially, positive T cell FCXM in the absence of positive CDC crossmatch was well established of clinical relevance.[6,8-9] In spite of the fact that positive B-cell FCXM are controversial for significance on transplant outcomes, potential risk assessment responsible for positive B-cell FCXM has been postulated such as presence or absence of autoantibodies, DSA specificity including anti-HLA class I and anti-DR, IgG subclass including IgG2 and IgG4.[10-11,17] Standardization of FCXM protocols can also cause controversy in clinical significance of positive B-cell FCXM. Dual-color analysis for T cell and B cell reactivities was introduced since 1989. It was presented to have superior for the discrimination of T cell from B cell reactivities and has been recommended as a standard technique in the 2013 consensus guideline on the testing in transplantation.[3,5] In Siriraj hospital, kidney transplantation was conducted since 1973. The median graft survival of 326 living-related kidney transplantation before 2010 was 13.2 years.[18] The two-colored FCXM protocols has been served for the pre-transplantation anti-HLA detection in living-related kidney transplantation for 17 years.[7,16] To implement the new three-colored FCXM protocol to increase sensitivity and clinical relevance and to improve patient care, the protocol is needed to be standardized.

In this study, we obtained the optimized protocol and cutoff values, which can be implemented for routine service. Validation data showed good accuracy with virtual crossmatch and the MCS of FCXM assays had significant correlation with anti-HLA MFI. Performance evaluation demonstrated comparable crossmatch interpretation despite less time and labor consumed. It was found that two known sera contained anti-HLA DR and two patients were positive only for B-cell FCXM, which reminded us the clinical significance of positive B-cell FCXM. However, one discordance between two-colored FCXM and three-colored FCXM results was found by positive results only for two-colored B cell FCXM but negative for three-colored B cell FCXM. The discordant serum was further investigated and found to be positive to autologous lymphocytes, which might be non-specific positive B cell FCXM. Further investigation for autoantibody is applied to the three-colored FCXM protocol when either T cell or B cell FCXM are positive. All results will be reported to nephrologists for their

decision to go on DSA detection on solid-phase immunoassay, desensitization or discontinue transplantation. Additional study about result threshold with respect of clinical risks and outcomes should be conducted after the implementation.

Even though the solid-phase immunoassays (SPI) could provide higher sensitivity than both CDC and FCXM and convey semiquantitative results, leading to major changes in management of pre-transplantation and post-transplantation antibody monitoring for kidney transplantation. The cell-based assays, CDC and FCXM, remain the mainstay of compatibility testing for kidney transplantation.[3] The consensus guideline on testing and clinical management issues associated with HLA and non-HLA antibodies in transplantation recommended to correlate the level of antibody detected by SPI with cell-based assay and use both SPI and cell-based assay. In our circumstance, the cost of SPI was a lot more than cell-based assays. Therefore, FCXM assay is reasonable for pre-transplantation DSA investigation in terms of increase sensitivity of compatibility test and case selection for the most benefit from the high-cost SPI test.

Conclusion

In conclusion, the three-colored FCXM protocol in Siriraj hospital was optimized, validated and evaluated for its performance. This protocol found to be equivalent to the two-colored protocol for FCXM interpretation but less time and labor consumption.

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