

# Evaluation of rapid optimized flow cytometry crossmatch (Halifaster) in living donor kidney transplantation

Luis Ramalhete<sup>1,2,3</sup>  | Rúben Araújo<sup>2</sup>  | Cristiana Teixeira<sup>1</sup> | Ana Teixeira<sup>1</sup> | Paula Almeida<sup>1</sup> | Isabel Silva<sup>1</sup> | Alice Lima<sup>1</sup>

<sup>1</sup>Blood and Transplantation Center of Lisbon, Instituto Português do Sangue e da Transplantação, Lisboa, Portugal

<sup>2</sup>NOVA Medical School, Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, Lisbon, Portugal

<sup>3</sup>iNOVA4Health – Advancing Precision Medicine, RG11: Reno-Vascular Diseases Group, NOVA Medical School, Faculdade de Ciências Médicas, Universidade NOVA de Lisboa, Lisbon, Portugal

## Correspondence

Luis Ramalhete, Blood and Transplantation Center of Lisbon, Instituto Português do Sangue e da Transplantação, Alameda das Linhas de Torres, n° 117, 1769-001 Lisboa, Portugal.  
 Email: [luis.m.ramalhete@edu.nms.unl.pt](mailto:luis.m.ramalhete@edu.nms.unl.pt)

Kidney transplantation is often the preferred treatment for end-stage renal disease. However, the presence of preformed donor-specific antibodies (DSA), including those against HLA, can lead to antibody-mediated rejection and significantly impact transplant outcomes. The Flow Cytometry Crossmatch (FCXM) is a crucial tool in kidney transplantation, as it also enables the measurement of low levels of anti-HLA DSA antibodies. However, current methodologies for detecting these antibodies, however, are time-consuming and require extensive reagents. In this study, we analyzed the performance of the Halifaster FCXM protocol in 133 consecutive living kidney donor pairs, correlating these results with single antigen-based anti-HLA DSA results. Anti-HLA DSA was identified in 31 patients (23.3%). Both T and B lymphocyte FCXM assays demonstrated high sensitivity and specificity in detecting anti-HLA DSA. Furthermore, a Tree model to determine the levels of anti-HLA DSA to produce a flow crossmatch positivity, was developed offering an accuracy of 93% and 90% for T and B lymphocytes, respectively. Both approaches point to a threshold of 1000–2000 MFI for T lymphocytes and 3000 MFI for B lymphocytes. Our findings indicate that the Halifaster protocol facilitates fast and efficient FCXM testing without compromising accuracy, marking a significant advancement in the field of kidney transplantation. The inclusion of HLA-specific antibody analysis underscores the protocol's comprehensive approach to improving transplant outcomes.

## KEY WORDS

donor specific antibodies, flow cytometry crossmatch, Halifaster protocol, kidney transplantation, living donor

## 1 | INTRODUCTION

Kidney transplantation is a life-saving treatment option for patients with end-stage renal disease.<sup>1</sup> The success of

this procedure depends on several factors, including the selection of a well-matched, compatible donor and effective immunosuppressive therapy to prevent rejection.<sup>2</sup> However, even with these precautions, the recipient's

**Abbreviations:** ABMR, antibody-mediated rejection; AUC, area under the ROC curve; CA, classification accuracy; DPBS, Dulbecco's phosphate-buffered saline; DSA, donor-specific antibodies; EDTA, ethylenediaminetetraacetic acid; FCXM, flow cytometry crossmatch; HLA, Human Leucocyte Antigens; LKD, living kidney donors; Ly, lymphocyte; MFI, mean fluorescent intensity; NGS, Next-Generation Sequencing; RT, room temperature; SAB, single antigen beads; vXCM, virtual crossmatch.

immune system may still recognize the transplanted kidney as foreign, leading to graft failure.

A significant cause of graft failure is antibody-mediated rejection (ABMR), which occurs when preformed or de novo donor-specific antibodies (DSAs) in the recipient's blood recognize antigens in the donor as targets.<sup>3</sup> Preformed DSA are antibodies already present in the recipient's blood prior to transplantation. These antibodies can develop due to prior exposure to foreign antigens, from previous transplant, blood transfusions, or pregnancy. Preformed DSAs can increase the risk of hyperacute rejection, a rapid and severe rejection reaction occurring within minutes to hours after transplantation.<sup>4</sup>

To evaluate the presence of DSAs, flow cytometry crossmatch (FCXM) plays a vital role in kidney transplantation, significantly influencing transplant success.<sup>5</sup> This technique measures the binding of DSAs in the recipient's blood to the donor's lymphocytes. It is both highly sensitive and specific, also allowing for the detection of low levels of DSAs that might otherwise go unnoticed in a classical complement-dependent cytotoxicity crossmatch. This helps identify potential immunological barriers to successful transplantation.<sup>6,7</sup> In the context of living donors, the importance of FCXM cannot be overstated, as it aids in selecting the most suitable donor, thereby improving transplant outcomes. Hence, FCXM is a crucial pre-transplantation test, that has revolutionized solid organ transplantation and has become essential in transplant patient risk stratification.

Since its introduction in the 1980s by Garovoy et al.,<sup>8</sup> FCXM, has evolved significantly. Numerous modifications have been made, such as the dual-color T and B-lymphocytes analysis Bray et al.<sup>9</sup>; and the use of pronase treatment of lymphocytes (which removes Fc gamma receptors and minimizes nonspecific IgG binding, reducing B lymphocyte background reactivity), as presented by Vaidya et al.<sup>10,11</sup> Furthermore, advancements in target cell isolation methods have been introduced by Liwski et al.<sup>12</sup>

Although the Flow Cytometry Crossmatch (FCXM) assay is a critical test for determining the presence of pre-formed antibodies, traditional FCXM assays can be time-consuming and may require extensive sample preparation, potentially delaying the transplant process. Recently, Liwski et al.<sup>13</sup> introduced two new protocols: the Halifax protocol and its faster variant, Halifaster. Both were developed to address some of the challenges associated with conventional assays, offering a more streamlined and efficient approach to FCXM testing. In their study, the authors compared the performance of the Halifax and Halifaster protocols with traditional FCXM assays. They observed that, while both new

protocols were faster than the traditional assays, with the Halifaster protocol delivering results in just 85 min, including cell isolation and preparation they did not compromise on sensitivity and specificity. In fact, both the Halifax and Halifaster protocols demonstrated a significant improvement over traditional FCXM assays, providing rapid and efficient testing while maintaining accuracy.

Clinical histocompatibility laboratories play a pivotal role in the organ transplantation process, as they are tasked with assessing compatibility between donors and recipients. A significant challenge faced by these laboratories is the variation in sensitivity among different techniques used for antibody screening and crossmatching. This variability can lead to discrepancies in results, complicating the accurate stratification of transplant risks within a reasonable timeframe.

In our study, we analyzed the performance of the Halifaster FCXM protocol in 133 consecutive living kidney donation pairs and correlated these results with DSA findings based on Single Antigen Bead (SAB) assays. In addition to these methodologies, our study also involved the implementation of a machine learning decision tree model to calculate the Mean Fluorescence Intensity (MFI) thresholds necessary for a positive flow cytometry crossmatch (FCXM). This advanced approach allowed us to refine the accuracy and predictive power of our FCXM analysis, contributing significantly to the risk stratification process in kidney transplantation. The primary objective of our study was to evaluate the effectiveness of the Halifaster FCXM protocol.

## 2 | MATERIALS AND METHODS

### 2.1 | Study population

This retrospective study was conducted at *Centro de Sanguine e Transplantação de Lisboa—Área da Transplantação*, involving living kidney donor transplantation pairs. A total of 133 consecutive pairs with varying levels of anti-HLA antibodies were tested from January 2022 to December 2023. All sera were simultaneously tested using the FCXM Halifaster protocol and SAB assays, as described below.

### 2.2 | Sera preparation

Peripheral blood was collected in serum separation tubes and centrifuged at 1100 g for 10 min. Prior to use in FCXM, samples were centrifuged at 3500 g for 5 min, and all SAB assay samples were pre-treated with EDTA.

## 2.3 | Cell isolation

Nine milliliters of heparinized sodium whole venous blood were collected from 133 potential living kidney donors (LKD). The samples were centrifuged at 800 g for 10 min. The buffy coat was extracted for Total Lymphocyte Isolation, negatively sorted with immunomagnetic beads using an EasySep™ Direct Human Total Lymphocyte Isolation Kit (STEMCELL Technologies Inc., Vancouver, BC, Canada), following the manufacturer's recommendations. The cells were resuspended in 1 mL of Dulbecco's phosphate-buffered saline (DPBS) medium until use. Cell counts were performed in a Neubauer chamber, and viability was assessed using trypan blue.

## 2.4 | Cell treatment with pronase and DNase

Briefly, the total lymphocytes (Ly) from donors were centrifuged at 800 g for 4 min. After discarding the supernatant, the cells were resuspended in a pronase solution (2.35 U/mL, Sigma-Aldrich, St Louis, MO) at a ratio of 100 µL per  $1.0 \times 10^6$  cells and incubated at 37°C for 15 min. DNase (11,000 U/mL; Sigma-Aldrich) was then added at a ratio of 4 µL per 100 µL of pronase solution and incubated for an additional 2 min at 37°C. Samples were then washed twice with DPBS by centrifugation at 800 g for 4 and 2 min, respectively. Cells were counted and adjusted to a concentration of  $10 \times 10^6$  cells/mL with DPBS.

## 2.5 | Positive and negative controls

Three negative controls were obtained from non-immunized human males of blood group AB. Additionally, two positive controls at two different dilutions, a high positive and a low positive (near cut-off positivity), were obtained from pools consisting of more than 10 highly immunized patients (PRA > 80%). Prior to use, control samples were centrifuged at 10,000 RPM for 5 min.

## 2.6 | Halifaster FCXM protocol

The Halifaster FCXM protocol is summarized in Table 1. Washing steps were performed using DPBS with 2% Fetal Bovine Serum. Anti-CD3-PE (clone UCHT1, Ref: A07747) was purchased from Beckman Coulter (Brea, CA, USA), and anti-CD19-PerCP Cy 5.5 (clone SJ25C1, Ref: 332780) monoclonal antibodies were obtained from

TABLE 1 Halifaster flow cytometry crossmatch protocol.

Cells/Reagents	
Sample	Heparin peripheral blood
Isolation method	Magnetic negative selection (total lymphocyte)
Cell adjustment	$10 \times 10^6$ /mL
Cell volume per well	15 µL ( $0.15 \times 10^6$ )
Serum volume per well	30 µL
Serum incubation time/temperature	20 min/RT
Washing steps/total time	4/10 min total at 800 g
Incubation with specialty reagents per well	-
Vol. of anti-CD3—PE	1.5 µL
Vol. of anti-CD19—PerCP cy 5.5	1.5 µL
Vol. of anti-IgGhu-FITC	0.125 ML
Incubation time/temperature	5 min/RT
Washing steps/total time	2/6 min total at 800 g
Positive control ( $n = 2$ , high and low reactivity)	Pool hypersensitized patients
Negative control ( $n = 3$ )	Pool AB serum
Assay sample replicates	2

Abbreviations: IgGhu-FITC, anti-human IgG fluorescein; PE, phycoerythrin; PerCP cy 5.5, peridinin chlorophyll-Cy5.5; RT, room temperature.

BD Biosciences (Mississauga, ON, Canada). The Fluorescein (FITC) conjugated F(ab')2 fragment goat anti-human IgG, Fcγ specific polyclonal antibody (IgG-FITC, Ref: F0315), with a 1.0 mg/mL stock solution, was sourced from DaKo (Glostrup, Denmark).

The interpretation of the FCXM involves comparing the fluorescence intensity of donor cells mixed with the patient's serum to that of the same donor cells mixed with a known negative control serum. These fluorescence values are then mathematically converted into Relative Number Ratio (NR) values using the formula: NR = (Mean Channel Sample/Mean Channel Negative Control). A FCXM result is classified as negative if  $NR \leq 1.5$ , weakly positive if  $1.5 < NR \leq 1.8$ , and positive if  $NR > 1.8$ . The NR cut-off was based on the mean plus three times the standard deviation of all true negative FCXM results.

## 2.7 | Luminex single antigen beads assay

The evaluation of HLA class I and II antibodies were performed using LABScreen Single Antigen Beads, following

the manufacturer's instructions (One Lambda, West Hills, CA). Briefly, 20 µL of EDTA-pretreated serum was added to 5 µL of SAB in individual wells of a 96-well plate. The plate was then incubated in the dark under 400 RPM agitation for 30 min at room temperature (RT). Subsequently, it was washed three times with 200 µL of wash buffer (One Lambda) by centrifugation at 1300 g for 2 min each. After discarding the supernatant, 100 µL of phycoerythrin-conjugated human immunoglobulin G (IgG) was added to each well, followed by another incubation in the dark under 400 RPM agitation for 30 min at RT.

Post-incubation, the microwell plate was centrifuged at 1300 g for 2 min, the supernatant was removed, and the beads were washed twice with 200 µL of wash buffer. After the final removal of the supernatant, 100 µL of wash buffer was added. Bead acquisition and initial analysis were conducted using a Luminex FLEXMAP 3D instrument (Luminex Corp, Austin, TX). Final assignment and interpretation of anti-HLA antibodies were performed using HLA Fusion software version 4.6 (One Lambda, West Hills, CA), and the In our analysis, we specifically utilized normalized Mean Fluorescence Intensity (MFI) values, with all measurements taken being above 0 MFI to ensure accurate and consistent quantification results were MFI.

A positive virtual crossmatch (vXM) result was defined when an MFI of  $\geq 1000$  was detected for an HLA allele present in the donor's HLA typing. In cases where an HLA allele was not represented in the SAB assay panel, the closest allele in the panel was considered.

## 2.8 | Patients and donors HLA typing

Briefly, Next-Generation Sequencing (NGS) for patients and donors typing was performed using the AllType™ FASTplex™ NGS 11 Loci Amplification Kit (OneLambda, Thermo Fisher Scientific Inc.). Library preparation, template preparation, and chip loading were conducted on the Ion Chef™ system, followed by sequencing on the Ion GeneStudio™ S5 system (Thermo Fisher Scientific Inc., Waltham, MA, USA). Analysis was carried out using TypeStream™ Visual NGS Analysis Software (OneLambda, Thermo Fisher Scientific Inc.), referencing the IMGT/HLA 3.29.0 databases.

## 2.9 | Statistical analysis

Statistical analysis of FCXM performance was conducted using GraphPad Prism version 8.0.2 for Windows (GraphPad Software, San Diego, CA, USA, [www.graphpad.com](http://www.graphpad.com)).

### 2.9.1 | Tree model for MFI calculation of anti-HLA antibodies

This study employed a tree model to calculate the necessary MFI of anti-HLA DSA antibodies, detected using SAB to generate a positive flow cytometry crossmatch.

#### Data collection

Baseline MFI values for each anti-HLA DSA were collected and were used as the sum of alleles at a locus.

#### Tree model description

The tree model, specifically a decision tree algorithm, was utilized for this analysis. This model was selected for its ability to handle complex, hierarchical data structures, offering a clear visual representation of decision-making processes.

The construction of the model involved standard decision tree algorithms, facilitated by the Orange data mining software (University of Ljubljana, Ljubljana, Slovenia). MFI values of anti-HLA antibodies served as the primary features. The model was structured to split nodes based on clinically relevant thresholds of MFI values, following a predefined criterion that aimed at maximizing the distinction between different risk levels associated with the antibodies.

#### Analytical approach

The tree model was trained on a subset of the collected data, with 3-fold cross-validation employed to assess its accuracy and robustness. Prior to analysis, data preprocessing was conducted to normalize MFI values and handle missing data. Specific parameter settings for the tree model were adjusted to optimize performance, including depth of the tree and minimum samples per leaf node. The model's fit was assessed by the Area Under the Receiver Operating Characteristics Curve (AUC). Additionally, Classification Accuracy (CA), F-1 Score, Precision, and Recall were also considered.

## 3 | RESULTS AND DISCUSSION

Among the 133 patients, DSAs were identified in 31 individuals (23.3%) as presented in Table 2. Of these, 7 were directed against HLA-class I, 17 against HLA-class II, and 7 targeted both HLA classes. Within this court, 21 patients had a single DSA, while 10 had multiple (two or more) DSAs, with one case (a mother-to-son pair) exhibiting a maximum of 11 DSAs. In our study 3 T lymphocyte flow cytometry crossmatches, presented positive in the absence of anti-HLA DSA (Table 2), this was not the case in the B lymphocyte flow cytometry crossmatches. The

**TABLE 2** List of patients with positive donor specific anti-HLA antibodies ( $MFI > 1000$ ), with either positive or negative flow cytometry crossmatch for T and B lymphocytes, with MFI values for DSA and ratio for flow cytometry crossmatch.

**List of patients with positive donor specific anti-HLA antibodies ( $MFI > 1000$ ), with either positive or negative flow cytometry crossmatch for T and B lymphocytes**

Patient <i>(n)</i>	DSAs MFI max-min	DSAs class I (n)	DSAs class II Id	MFI max- min class I	DSAs class II Id (n)	DSAs class II Id	MFI max- min class II		FCXM_	
							FCXM_LyT ratio	FCXM_LyT ratio	LyT	LyB
1	3	6523-2708	1	A*31	6523-6523	2	DRB1*08, DQB1*04	2995-2708	Positive	3.22
2	1	2417-2417	0	-	-	1	DQB1*04	2417-2417	Negative	0.96
3	1	1795-1795	0	-	-	1	DQB1*02	1795-1795	Negative	0.91
4	1	1067-1067	1	B*51	1067-1067	0	-	-	Positive	1.74
5	2	1712-1053	2	B*08, C*03 (Cw10)	1712-1053	0	-	-	Positive	2.46
6	1	21,287	1	A*02	21,287-	0	-	-	Positive	30.96
		-21,287			21,287					Positive
7	4	22,847	1	C*04	1562-1562	3	DRB1*07, DRB1*15, DQB1*06	22,847- 1654	Positive	2.86
8	1	25,606	1	A*02	25,606-	0	-	-	Positive	38.45
		-25,606			25,606					Positive
9	1	1100-1100	0	-	-	1	DRB1*10	1100-1100	Negative	0.99
10	7	8126-1003	3	A*02, A*25, B*51	6227-4172	4	DRB1*11, DQB1*03 (DQ7), DPB1*04, DRB3	8126-1003	Positive	5.28
11	11	21,294- 1494	5	A*23, A*24, B*50, C*05, C*06	14,067- 7093	6	DRB1*04, DRB1*11, DQB1*03 (DQ7), DQB1*03 (DQ8), DPB1*02, DRB3	21,294- 1494	Positive	25.15
12	1	12,069- 12,069	0	-	-	1	DQB1*03 (DQ7)	12,069- 12,069	Negative	1.11
13	3	2331-1069	1	C*04	2331-2331	2	DQB1*06, DPB1*10	1197-1069	Negative	0.97
14	1	12,646- 12,646	0	-	-	1	DQB1*03 (DQ7)	12,646- 12,646	Negative	1.04
15	1	2284-2284	0	-	-	1	DPB1*01	2284-2284	Negative	1.02
16	3	3225-11224	0	-	-	3	DRB1*04, DRB1*13, DQB1*06	3225-11224	Negative	0.96
17	1	1152-1152	0	-	-	1	DRB3	1152-1152	Negative	1.07
									Positive	1.55

(Continues)

TABLE 2 (Continued)

**List of patients with positive donor specific anti-HLA antibodies (MFI > 1000), with either positive or negative flow cytometry crossmatch for T and B lymphocytes**

Patient <b>(n)</b>	DSAs				DSAs class I Id (n)	DSAs class II Id	DSAs class II n	MFI max- min	MFI max- min	MFI max- min	FCXM_	
	DSAs	MFI max-min	DSAs class I Id	DSAs class II Id								
18	1	1369-1369	0	-	-	1	DRB5	1369-1369	Negative	0.97	Negative	0.78
19	1	1222-1222	1	B*08	1222-1222	0	-	-	Positive	1.74	Negative	1.28
20	1	1370-1370	1	A*74	1370-1370	0	-	-	Negative	1.03	Negative	1.43
21	4	1963-1036	2	B*58, C*03 (Cw10)	1963-1154	2	DPB1*03, DPB1*05	1784-1036	Positive	2.48	Positive	1.99
22	1	1045-1045	0	-	-	1	DRB4	1045-1045	Negative	0.93	Negative	0.61
23	4	2677-1092	0	-	-	4	DQB1*02, DPB1*06, DPB1*02, DPB1*04	2677-1092	Negative	0.99	Negative	1.14
24	7	16,784- 1102	3	B*38, B*52, C*12	16,784- 10,154	4	DRB1*04, DRB1*04, DPB1*03, DPB1*05	14,522- 1102	Positive	28.14	Positive	19.18
25	1	1035-1035	1	C*07	1035-1035	0	-	-	Negative	1.05	Negative	0.93
26	1	1269-1269	0	-	-	1	DRB1*04	1269-1269	Negative	1.06	Negative	0.88
27	1	2044-2044	0	-	-	1	DRB4	2044-2044	Negative	0.93	Negative	0.79
28	1	2020-2020	0	-	-	1	DQB1*02	2020-2020	Negative	0.99	Negative	1.13
29	1	1591-1591	0	-	-	1	DRB3	1591-1591	Negative	0.97	Negative	0.99
30	1	1068-1068	0	-	-	1	DRB1*13	1068-1068	Negative	0.87	Negative	0.98
31	1	1157-1157	0	-	-	1	DRB3	1157-1157	Negative	1.07	Positive	1.55
<b>DSA negative (MFI &lt; 1000) flow cytometry positive</b>												
A	-	989-0	0	-	82-0	0	-	989-0	Positive	2.09	Negative	1.19
B	-	243-0	0	-	62-0	0	-	243-0	Positive	1.53	Negative	1.3
C	-	269-0	0	-	269-0	0	-	55-0	Positive	1.64	Negative	0.82

Note: List of false positive flow cytometry crossmatch DSA negative (MFI &lt; 1000).

reasons for false positive FCXM (in the absence of DSA) are not so easily identifiable. Nevertheless, 1 patient was ABO incompatible (patient B), these findings have also been described in ABO-incompatible kidney transplantation, in which anti-blood type IgG antibodies can bind to ABO antigens on T lymphocytes.<sup>14</sup> This binding is detectable in FCXM as anti-donor IgG antibodies, leading to false-positive results. Out of the 12 ABO-incompatible only one other presented positivity, patient 7 but in this case were able to identify anti-HLA DSAs. Other factors that may be involved might be the presence of non-HLA antibodies.<sup>15</sup> At our center, we have a very low false positive FCXM (2.3%).

When analyzing the FCXM results using the Halifaster protocol, the majority of pairs studied were FCXM negative for both B and T lymphocytes (Ly), accounting for 115 pairs (86.5%). The remaining pairs showed positivity, with 5 pairs (3.8%) positive for T Ly, 3 pairs (3%) positive for B Ly, and 9 pairs (6.8%) positive for both T and B Ly. Meanwhile, examining data from both SAB assays and FCXM in an aggregate manner—considering vXM results (DSAs  $\geq$  or <1000 MFI) and physical cross-match results—it was observed that for T Ly FCXM, 116 patients were negative in both FCXM and vXM, while 11 were positive in both. In terms of false positive and negative results, 3 cases were FCXM positive in the absence of DSAs (false positives for FCXM), and 3 cases were a false negative for FCXM, found in a patient with a DSAs against *HLA-C\*04*, *HLA-A\*74*, and *HLA-C\*07* with MFIs of 2231, 1370, and 1035, respectively. This resulted in a sensitivity of 78.57% and a specificity of 97.48% for T Ly FCXM, as detailed in Table 3. Similarly, for B Ly FCXM results, 120 patients were FCXM negative and 13 were FCXM positive. None of the patients presented a false positive result (vXM positive and DSA negative), however, 18 patients exhibited false negative results with DSAs MFI ranging from 1045 to 3225. Interesting to notice that in 2 cases (1.5% of all FCXM) in spite of B Ly FCXM negative results the crossmatches were positive for T Ly FCXM, this is particular interesting as HLA-class I antigens are found on both T lymphocytes and B

lymphocytes. In both cases the anti-HLA DSA was directed against locus HLA-B with and MFI of 1067 and 1222, these findings are similar to those found by other groups.<sup>16</sup> The other 16 cases were majority directed against a single DSA (13 out of 16). All of these presented low MFI and were directed against HLA-DRB1 ( $n = 3$ , range 1068–1269), HLA-DQB1 ( $n = 3$ , range 1795–2417), HLA-DRB3/4/5 ( $n = 4$ , range 1045–2044), HLA-DPB1 ( $n = 1$ , MFI = 2284), and anti HLA-class I ( $n = 2$ , range 1035–1370). These results might be partial justified by either lower HLA expression on donor cells when compared to SAB. Sometimes, donor cells express HLA antigens at low levels, which may differ from those represented on testing beads, or either by the fact that these DSAs present a low affinity and avidity, even when donor HLA is expressed, resulting in a negative flow cytometry crossmatch.<sup>17</sup> Other possible cause for some of these cases can also be the detection of antibodies to denatured HLA molecules in the SAB assay.<sup>18</sup> Despite all of these, the overall performance of B Ly FCXM assay with DSA MFI higher than 1000 was in terms of sensitivity and specificity was 41.94% and 100.00%, respectively. Interestingly, these results are consistent with those reported by other groups using the Halifax protocol.<sup>19,20</sup>

However, there is some inconsistency in research regarding their expression levels: some studies indicate that these antigens are more prominently expressed on B lymphocytes compared to T lymphocytes. Therefore, in the presence of HLA-class I antibodies, a T+B+ Flow Cytometry Crossmatch (FCXM) result is more likely than a T+B− result. Conversely, in the presence of only HLA-class II antibodies, a T-B+ FCXM is expected. This is because HLA-class II antigens are present on B lymphocytes but are typically not found on resting T lymphocytes.

### 3.1 | What MFI makes a crossmatch positive?

The determination of appropriate DSA MFI cut-off threshold values has been a subject of extensive research.

TABLE 3 Halifaster FCXM assay performance for T and B lymphocyte based on a DSA positivity of 1000 MFI.

Statistic	T Ly FCXM assay performance DSA > 1000 MFI (HLA-class I)		B Ly FCXM assay performance DSA > 1000 MFI (HLA-class I + II)	
	Value	95% CI	Value	95% CI
Sensitivity	78.57%	49.20%–95.34%	41.94%	24.55%–60.92%
Specificity	97.48%	92.81%–99.48%	100.00%	96.45%–100.00%
Positive predictive value	78.57%	53.72%–92.05%	100.00%	75.29%–100.00%
Negative predictive value	97.45%	93.41%–99.06%	85.00%	80.77%–88.43%
Accuracy	95.49%	90.44%–98.33%	86.47%	79.46%–91.78%

Abbreviations: CI, Confidence interval; DSA, Donor specific antibody, Ly, Lymphocyte; MFI, Mean fluorescence intensity.

These thresholds are crucial in predicting whether a vXM will yield results comparable to a physical crossmatch. A low cut-off may lead to false-positive crossmatch results, whereas a high cut-off could result in false-negative outcomes.

Therefore, it is vital to consider the DSA MFI cut-off thresholds carefully when interpreting flow cytometry crossmatch results. With this consideration, we further investigated the impact of these thresholds on the sensitivity and specificity of the Halifaster FCXM protocol. To do so we implemented two approaches. The first approach, in a more conventional statistical manner evaluates sensitivity, specificity, and accuracy at various cut-off levels. The goal is to find the optimal balance between sensitivity, (true positive rate, measures the ability of a test to correctly identify patients with DSA), and specificity (true negative rate, measures the test's ability to correctly identify patients who do not have DSAs), maximizing the overall accuracy of the test.

The second approach was performed by implementation of a machine learning decision tree model to calculate the MFI thresholds necessary for a positive flow cytometry crossmatch (FCXM). This novel approach was designed to address the gap in utilizing MFI values of DSAs detected by SAB to predict physical FCXM positivity. This represents a pioneering step in applying unsupervised modeling on anti-HLA DSA.

### 3.1.1 | The impact of DSAs MFI cut-off in the sensitivity and specificity of FCXM

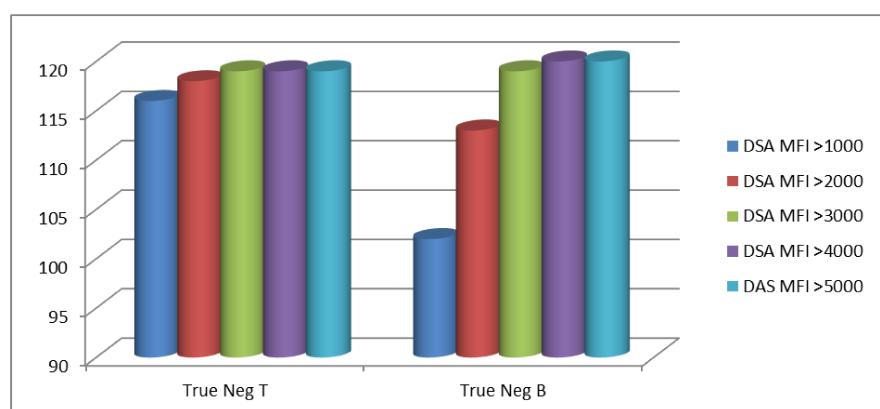
As shown in Figure 1, varying MFI cut-offs appear to have minimal impact on the true negative rates for T Ly FCXM. In fact, there is a slight increase in the number of true negatives ( $n = 2$ , DSA directed against HLA-A and HLA-C) as the DSA MFI cutoff value increases from  $>1000$  to  $>2000$ , suggesting that raising the threshold slightly reduces the number of false positives. However,

from a cutoff of  $>2000$  onwards, the number of true negatives plateaus at 119, indicating that increasing the cutoff beyond this point does not significantly change the number of true negative results.

In contrast, for B Ly FCXM, the true negative rates show a more pronounced increase with each threshold, moving from 102 at a cutoff of  $>1000$  to a plateau of 120 at a cutoff of  $>4000$ . This suggests that True Neg B Ly FCXM might have a higher rate of false positives at lower cutoff values, which is improved by increasing the DSA MFI threshold.

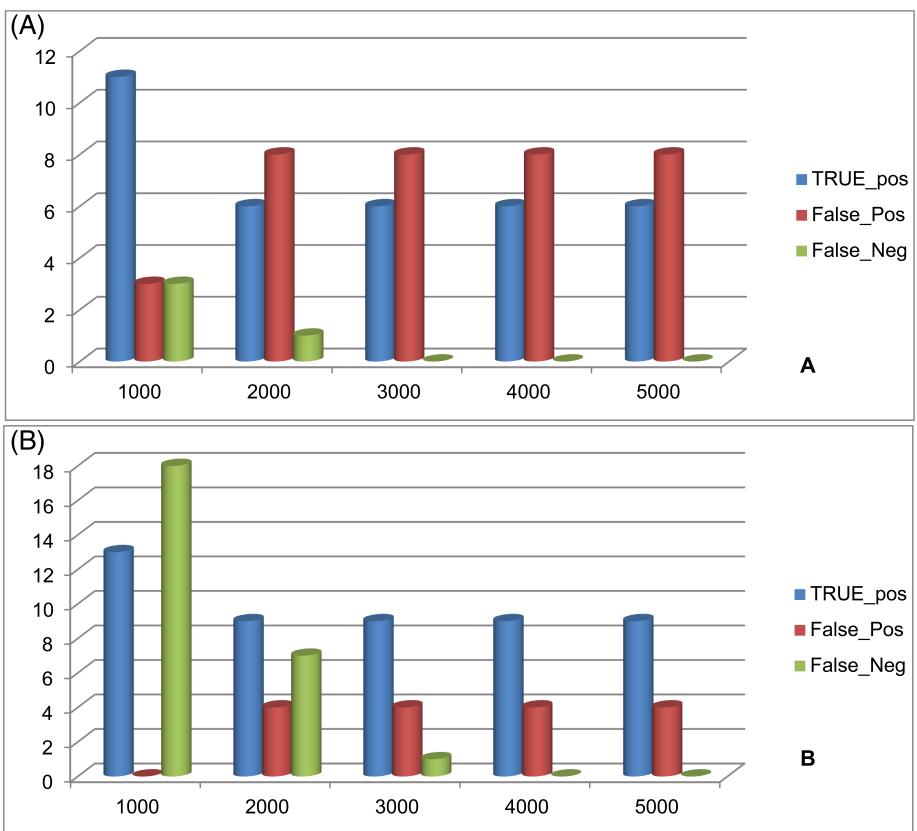
This variation might be due to the fact that a B Ly FCXM can be positive due to both class I and class II antibodies. The expression of these HLA molecules varies depending on the loci, which means that the *in vivo* effect of an antibody is more likely influenced by these variations in surface density than by *in vitro* SAB assays. For instance, HLA-A and HLA-B are expressed at similar levels, but HLA-C at about 15 times lower.<sup>21</sup>

In terms of true positive outcomes for T Ly FCXM (as illustrated in Figure 2A), increasing the MFI threshold by 1000 led to a 54.5% reduction in true positives rate (from 11 at 1000 MFI to 6 at 2000 MFI), however this increment resulted in an increase of false positive (from 3 at 1000 MFI to 8 at 2000 MFI). In contrast, in the case of B Ly FCXM (Figure 2B), which is influenced by both anti-HLA DSA directed against HLA class I and class II molecules, shows a notable trend in sensitivity and specificity across various DSA MFI cutoffs. At the lowest threshold of 1000, we observe a high number of false negatives ( $n = 18$ ), indicating that the test is not sensitive enough to detect all anti-HLA DSAs. However, the true positive rate is at their highest ( $n = 13$ ), suggesting that while some DSAs are detected at this level, many are missed. As we increase the DSA MFI cutoff to 2000, there is a marked reduction in false negatives to 7, improving the test's sensitivity to detect anti-HLA antibodies. Simultaneously, the true positive count decreases slightly to 9, indicating a refinement in identifying DSAs. This level



**FIGURE 1** Analysis of true negative results in flow cytometry crossmatch based on varying mean fluorescent intensity (MFI) cut-offs for donor-specific antibodies (DSAs).

**FIGURE 2** Analysis of true positive, false positive, and false negative results in flow cytometry crossmatch based on various mean fluorescent intensity (MFI) cut-offs for donor-specific antibodies (DSAs). (A) T lymphocyte (T Ly) FCXM; (B) B lymphocyte (B Ly) FCXM.



seems to be a significant improvement in the balance between avoiding false negatives and maintaining true positive identifications.

Further increases in the cutoff to 3000, 4000, and 5000 yield an elimination of false negatives, indicating that at these thresholds, the test is highly sensitive and does not miss any DSA. Interestingly, the true positive count stabilizes at 9, which could suggest that these are the robust detections of anti-HLA DSAs across class I and II. However, the emergence and persistence of false positives ( $n = 4$ ) from a cutoff of 2000 and above suggest a decrease in specificity.

When analyzing Table 4, in conjunction with data from Table 3, reveals that as the MFI cut-off value for DSAs increases, so does the sensitivity of both T Ly and B Ly FCXM assays. This indicates that as DSA levels rise, the assays become more effective at detecting these antibodies. Notably, the specificity of both assays remains consistently high across all cut-off values, suggesting that false positives are particularly rare, especially for B Ly FCXM.

Regarding the positive predictive value (PPV), an increase in the cut-off value leads to a decrease in the PPV of both assays. This implies a higher likelihood of a positive FCXM result occurring in the absence of DSAs. For both T and B Ly FCXM, the DSA cut-off value that optimally aligns with PPV is greater than 1000 MFI.

In terms of overall accuracy, both assays exhibit relatively high levels, though some variation is observed within the 95% confidence intervals. The T Ly FCXM assay demonstrates higher accuracy at lower DSA MFI cut-off values, whereas the B Ly FCXM assay shows greater accuracy at higher cut-off values, particularly around 3000–4000 MFI.

### 3.1.2 | Determining DSAs MFI thresholds with AI in flow cytometry crossmatch FCXM

The application of AI-driven approaches in kidney transplantation has been hypothesized by several researchers. These approaches range from predicting early kidney transplant rejection in antibody-incompatible transplants to applying unsupervised modeling on anti-HLA immune responses using Luminex platforms.<sup>22,23</sup> To our knowledge, there has been no machine learning implementation to date that utilizes MFI values of DSAs detected by SAB to predict physical FCXM positivity.

To address this gap, we implemented a simple decision tree model to calculate the MFI thresholds necessary for a positive FCXM. For T lymphocyte (Ly) FCXM, we calculated the summations of HLA-A, HLA-B, HLA-C, and HLA class I DSAs. Similarly, for B Ly FCXM, in addition to the aforementioned DSAs, we also included HLA-

**TABLE 4** Performance metrics of the Halifaster FCXM assay for T and B lymphocytes at varying donor-specific antibody (DSA) positivity cut-off values.

Statistic	T Ly FCXM assay performance		B Ly FCXM assay performance	
	DSA > 2000 MFI (HLA-class I)	DSA > 2000 MFI (HLA-class I + II)	DSA > 2000 MFI (HLA-class I)	DSA > 2000 MFI (HLA-class I + II)
Sensitivity	85.71%	42.13%–99.64%	56.25%	29.88%–80.25%
Specificity	93.65%	87.97%–97.22%	96.58%	91.48%–99.06%
Positive predictive value	42.86%	26.44%–61.02%	69.23%	43.91%–86.61%
Negative predictive value	99.16%	95.05%–99.86%	94.17%	90.25%–96.57%
Accuracy	93.23%	87.54%–96.86%	91.73%	85.68%–95.80%
DSA > 3000 MFI (HLA-class I)		DSA > 3000 MFI (HLA-class I + II)		
Sensitivity	100.00%	54.07%–100.00%	90.00%	55.50%–99.75%
Specificity	93.70%	87.97%–97.24%	96.75%	91.88%–99.11%
Positive predictive value	42.86%	27.72%–59.46%	69.23%	45.64%–85.78%
Negative predictive value	100.00%	96.95%–100.00%	99.17%	94.88%–99.87%
Accuracy	93.98%	88.49%–97.37%	96.24%	91.44%–98.77%
DSA > 4000 MFI (HLA-class I)		DSA > 4000 MFI (HLA-class I + II)		
Sensitivity	100.00%	54.07%–100.00%	100.00%	66.37%–100.00%
Specificity	93.70%	87.97%–97.24%	96.77%	91.95%–99.11%
Positive predictive value	42.86%	27.72%–59.46%	69.23%	46.18%–85.51%
Negative predictive value	100.00%	96.95%–100.00%	100.00%	96.97%–100.00%
Accuracy	93.98%	88.49%–97.37%	96.99%	92.48%–99.17%
DSA > 5000 MFI (HLA-class I)		DSA > 5000 MFI (HLA-class I + II)		
Sensitivity	100.00%	54.07%–100.00%	100.00%	66.37%–100.00%
Specificity	93.70%	87.97%–97.24%	96.77%	91.95%–99.11%
Positive predictive value	42.86%	27.72%–59.46%	69.23%	46.18%–85.51%
Negative predictive value	100.00%	96.95%–100.00%	100.00%	96.97%–100.00%
Accuracy	93.98%	88.49%–97.37%	96.99%	92.48%–99.17%

Abbreviations: CI, confidence interval; DSA, donor specific antibody; Ly, lymphocyte; MFI, mean fluorescence intensity.

DRB1, HLA-DQB1, HLA-DPB1, HLA-DRB3/4/5, HLA class II DSAs and in this case as B Ly FCXM is influenced by both anti-HLA DSAs directed against HLA class I and class II molecules the results of T Ly FCXM were included.

Despite the relatively small number of patients enrolled and the limited number of patients presenting with DSAs (31 in total), we were able to generate two models with very good performance for both T Ly FCXM and B Ly FCXM. These models exhibited an AUC of 0.83 and 0.81, respectively (Figures 3 and 4). This indicates that the models have reasonably good discriminatory power to distinguish between positive and negative samples. Furthermore, in terms of CA, both models achieved a value of >0.92, meaning they could correctly classify more than 92% of the samples in the test set.

In the specific case of T Ly FCXM, Figure 3, and analyzing the sum of MFI DSAs against HLA-A, HLA-B,

HLA-C, and class I. The tree model demonstrated a remarkable accuracy in classifying true negatives and positives under specific conditions. For instance, when the sum of all class I DSAs is equal to or lower than 1047 MFI, the model can correctly classify 97.4% of true negatives. This indicates a high level of precision in identifying cases where no significant DSAs are present. Conversely, when the sum of class I DSAs exceeds 2893 MFI, all T Ly FCXM cases are identified as positive, suggesting a robust ability to detect significant levels of DSAs.

The performance of the model is further underscored by its AUC value of 0.83, which is a strong indicator of its capability to differentiate between positive and negative cases effectively. The model's CA is another point of strength, scoring an impressive 0.93. This high level of accuracy ensures that the vast majority of test results are correct. Moreover, the F1 Score, standing at 0.96, is

particularly noteworthy. This metric, striking a balance between precision (0.97) and recall (0.95), suggests a harmonious accuracy in the model's predictions. In medical diagnostics, this balance is essential; it means the model is not only reliable in identifying true positives but also in capturing the majority of actual positive cases. The high precision minimizes the risk of false positives, while

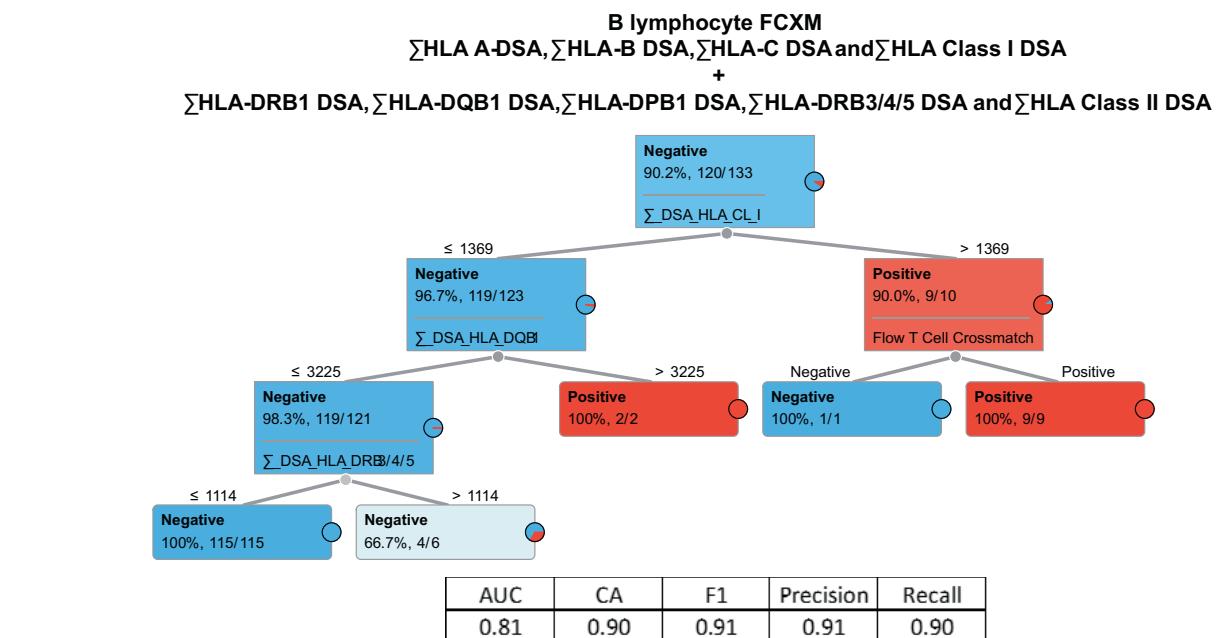
the high recall ensures that most positive cases are correctly identified, both of which are crucial for accurate diagnosis and appropriate treatment.

In summary, the T Ly FCXM model exhibits a high degree of accuracy and reliability in HLA testing, particularly when assessing DSAs against HLA-A, HLA-B, HLA-C, and class I. The model's strengths in precision, recall, and overall classification accuracy make it a valuable tool in medical settings, especially in contexts like organ transplantation where accurate HLA matching is critical. The slight area for improvement lies in its specificity, which, while reasonable, suggests room for refinement to further reduce the rate of false positives.

Of notice that of the 3 T Ly FCXM negative results in the presence of DSAs, 2 are directed against HLA-C locus. Compared to other HLA molecules, particularly HLA-A and -B, HLA-C antigens have a lower expression level, which may explain the less frequent and weaker anti-HLA-C antibody responses.<sup>24,25</sup> However, it is important to note that HLA-C antigens are still capable of inducing an immune response. Preformed anti-HLA-C DSAs are clinically relevant, as they can bind to donor cells and activate the complement pathway, leading to poorer graft outcomes in kidney transplants.<sup>26</sup>

In the case of B Ly FCXM, the tree model also identified MFI cut-offs, as shown in Figure 4, the threshold value of 1369 was identified for  $\sum$  HLA class I DSAs MFI. If the value is less than or equal to 1369, it is likely to be classified as Negative (90.2%, 120 out of 133 cases). If the value is greater than 1369, it is likely to be classified

**FIGURE 3** Decision tree model for evaluating T lymphocyte Halifaster flow cytometry crossmatch protocol performance based on donor-specific antibodies (DSAs) mean fluorescent intensity (MFI) values with a 3-fold cross validation. AUC, area under the ROC curve; CA, classification accuracy.



**FIGURE 4** Decision tree model for evaluating B lymphocyte Halifaster flow cytometry crossmatch protocol performance based on donor-specific antibodies (DSAs) mean fluorescent intensity (MFI) values.

as Positive (90.0%, 9 out of 10 cases). The tree then branches further based on additional thresholds 3225 and 1114 for HLA-DQB1 DSA and HLA-DRB3/4/5 DSA, respectively. For instance, if the first parameter is less than or equal to 1369, the decision tree looks at the second parameter (HLA-DQB1 DSA). If this second parameter is less than or equal to 3225, the classification is Negative (98.3%, with 119 out of 121 cases). However, if it is greater than 3225, it is Positive (100%, with 2 out of 2 cases). For those cases where the first parameter is less than or equal to 1369 and the second parameter (HLA-DQB1 DSA) is less than or equal to 3225, the tree makes another decision based on a third parameter (HLA-DRB3/4/5 DSA). If this third parameter is less than or equal to 1114, the classification is Negative (100%, with 115 out of 115 cases). If the parameter is greater than 1114, the classification is still Negative, but with a lower confidence (66.7%, with 4 out of 6 cases).

On the other branch, where  $\sum$  HLA class I DSAs MFI is greater than 1369, and the Flow T lymphocyte Crossmatch is positive (9 out of 9 cases), the B Ly FCXM is positive.

Notably, in the B Ly FCXM tree model, the exclusion of specific HLA DSA locus from the analysis did not yield significant benefits (data not showed). This observation may be attributed to the fact that HLA expression varies both across different HLA loci and in terms of cellular expression.<sup>27</sup>

In summary, although the models demonstrate promising AUC and CA, it is crucial to consider the balance of classes in the test set, and the risk of overfitting training data. Class imbalance and high rates of false negatives may suggest that further analysis is essential to accurately determine the overall effectiveness of these models. Such analysis would help ensure that the models are robust and reliable for practical application in predicting FCXM outcomes.

## 4 | CONCLUSIONS

In conclusion, this study provides a comprehensive and insightful analysis of the Halifaster flow cytometry cross-match protocol. Traditional FCXM assays, while essential for detecting pre-formed antibodies, can be time-consuming and require significant sample preparation, potentially delaying the transplant process. Our analysis, encompassing 133 consecutive living kidney donor-recipient pairs, correlating the results with DSA findings based on SAB assays. Both T Ly and B Ly FCXM assays demonstrated high sensitivity and specificity in detecting DSAs, maintaining consistently high specificity and Negative Predictive Value (NPV) across all cut-off values. It successfully demonstrates how integrating the Halifaster

FCXM results with DSA findings from Single Antigen Bead (SAB) assays can enhance the precision of transplant risk stratification, bridging a crucial gap in clinical decision-making. While our findings are promising for living donor transplants, further research is needed to confirm if these results can be extrapolated to postmortem kidney transplants. Remarkably, the study also ventures into the realm of AI, applying decision tree models for determining DSA MFI thresholds, exhibiting remarkable accuracy in classifying true negatives and positives. It is important to note the limitations of this study, including its small sample size, with only 31 patients identified as having HLA DSAs. This limitation restricts the generalizability of the findings to larger populations. Additionally, the sensitivity and specificity of T Ly FCXM may vary based on the specific protocol used and the characteristics of the patient population. Overall, the results indicate that the Halifaster protocol is an effective and efficient method for FCXM testing in clinical transplantation settings, particularly suitable for identifying lower MFI preformed DSAs.

## AUTHOR CONTRIBUTIONS

LR, RA, and AL conceptualized the draft. LR, RA, and AL contributed equally to writing and reviewing of the original draft through interpretation of the literature. IS, CT, and AT reviewed and edited the original draft. All authors have read and agreed to the published version of the manuscript.

## CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

## DATA AVAILABILITY STATEMENT

The participants of this study did not give written consent for their data to be shared publicly, so due to the legal context the research supporting data is not available.

## ETHICS STATEMENT

Not applicable, all data was provided without any identifier or group of identifiers which would allow attribution of private information to an individual.

## ORCID

Luis Ramalhete  <https://orcid.org/0000-0002-8911-3380>  
Rúben Araújo  <https://orcid.org/0000-0002-9369-6486>

## REFERENCES

1. Abecassis M, Bartlett ST, Collins AJ, et al. Kidney transplantation as primary therapy for end-stage renal disease: a National Kidney Foundation/Kidney Disease Outcomes Quality Initiative

- (NKF/KDOQI™) conference. *Clin J Am Soc Nephrol.* 2008;3(2):471-480. doi:[10.2215/CJN.05021107](https://doi.org/10.2215/CJN.05021107)
2. Oweira H, Ramouz A, Ghamarnejad O, et al. Risk factors of rejection in renal transplant recipients: a narrative review. *J Clin Med.* 2022;11(5):1392. doi:[10.3390/jcm11051392](https://doi.org/10.3390/jcm11051392)
  3. Aubert O, Loupy A, Hidalgo L, et al. Antibody-mediated rejection due to preexisting versus de novo donor-specific antibodies in kidney allograft recipients. *J Am Soc Nephrol.* 2017;28(6):1912-1923. doi:[10.1681/ASN.2016070797](https://doi.org/10.1681/ASN.2016070797)
  4. Yeung MY. Pre-formed DSA and kidney allograft outcomes. *Braz J Nephrol.* 2020;42(2):136-137. doi:[10.1590/2175-8239-jbn-2020-0043](https://doi.org/10.1590/2175-8239-jbn-2020-0043)
  5. Graff J, The R. Role of the Crossmatch in kidney transplantation: past, present and future. *J Nephrol Therapeut.* 2012;01(S4):139-148. doi:[10.4172/2161-0959.S4-002](https://doi.org/10.4172/2161-0959.S4-002)
  6. Kumar A, Kosi E, Halawa A. An update on crossmatch techniques in transplantation. *J Kidney.* 2017;3(4):1-5.
  7. Peräsaari JP, Jaatinen T, Merenmies J. Donor-specific HLA antibodies in predicting crossmatch outcome: comparison of three different laboratory techniques. *Transpl Immunol.* 2018;46:23-28. doi:[10.1016/j.trim.2017.11.002](https://doi.org/10.1016/j.trim.2017.11.002)
  8. Garovoy MR, Rheinschmidt MA, Bigos M, et al. Flow cytometry analysis: a high technology cross-match technique facilitating transplantation. *Transplant Proc.* 1983;1939-1944.
  9. Bray RA, Lebeck LK, Gebel HM. The flow cytometric cross-match. Dual-color analysis of T cell and B cell reactivities. *Transplantation.* 1989;48(5):834-840.
  10. Vaidya S, Cooper TY, Avandsalehi J, et al. Improved flow cytometric detection of HLA alloantibodies using pronase. *Transplantation.* 2001;71(3):422-428. doi:[10.1097/00007890-200102150-00015](https://doi.org/10.1097/00007890-200102150-00015)
  11. Vaidya S, Cooper T, Stewart D, Gugliuzza K, Daller J, Bray R. Pronase improves detection of HLA antibodies in flow cross-matches. *Transplant Proc.* 2001;33(1-2):473-474. doi:[10.1016/S0041-1345\(00\)02098-4](https://doi.org/10.1016/S0041-1345(00)02098-4)
  12. Liwski R, Adams G, Peladeau G, Heinstein K. P099. The impact of lymphocyte purity on flow cytometry crossmatch (FCXM) assay. It's not purely theoretical. *Hum Immunol.* 2016;2016(77):110-111. doi:[10.1016/j.humimm.2016.07.164](https://doi.org/10.1016/j.humimm.2016.07.164)
  13. Liwski RS, Greenshields AL, Conrad DM, et al. Rapid optimized flow cytometric crossmatch (FCXM) assays: the Halifax and Halifaster protocols. *Hum Immunol.* 2018;79(1):28-38. doi:[10.1016/j.humimm.2017.10.020](https://doi.org/10.1016/j.humimm.2017.10.020)
  14. Lindemann M, Lenz V, Nyadu B, et al. Effect of ABO incompatibility on T-cell flow cytometry cross-match results prior to living donor kidney transplantation. *Cytometry B Clin Cytom.* 2018;94(4):623-630. doi:[10.1002/cyto.b.21496](https://doi.org/10.1002/cyto.b.21496)
  15. Tait BD, Süssal C, Gebel HM, et al. Consensus guidelines on the testing and clinical management issues associated with HLA and non-HLA antibodies in transplantation. *Transplantation.* 2013;95(1):19-47. doi:[10.1097/TP.0b013e31827a19cc](https://doi.org/10.1097/TP.0b013e31827a19cc)
  16. Puttheti P, Sharma VK, Friedlander R, et al. T-cell-positive, B-cell-negative flow cytometry crossmatch: frequency, HLA locus specificity, and mechanisms among 3073 clinical flow cytometry crossmatch tests. *Exp Clin Transplant.* 2022;20(2):180-189. doi:[10.6002/ect.2021.0334](https://doi.org/10.6002/ect.2021.0334)
  17. Schinstock CA, Gandhi MJ, Stegall MD. Interpreting anti-HLA antibody testing data. *Transplantation.* 2016;100(8):1619-1628. doi:[10.1097/TP.0000000000001203](https://doi.org/10.1097/TP.0000000000001203)
  18. Gutiérrez-Larrañaga M, Riesco L, Guiral S, et al. Detection of antibodies to denatured human leucocyte antigen molecules by single antigen Luminex. *HLA.* 2021;97(1):52-59. doi:[10.1111/tan.14098](https://doi.org/10.1111/tan.14098)
  19. Yoo J, Lee S, Lee HW, et al. Assessment of rapid optimized 96-well tray flow cytometric crossmatch (Halifax-FCXM) with Luminex single antigen test. *Hum Immunol.* 2021;82(4):302-308. doi:[10.1016/j.humimm.2021.02.003](https://doi.org/10.1016/j.humimm.2021.02.003)
  20. Hiho SJ, Levvey B, Carroll R, et al. The clinical utility and thresholds of virtual and Halifaster flow crossmatches in lung transplantation. *HLA.* 2022;99(6):580-589. doi:[10.1111/tan.14613](https://doi.org/10.1111/tan.14613)
  21. Carey BS, Poultton KV, Poles A. Factors affecting HLA expression: a review. *Int J Immunogenet.* 2019;46(5):307-320. doi:[10.1111/iji.12443](https://doi.org/10.1111/iji.12443)
  22. Shaikhina T, Lowe D, Daga S, Briggs D, Higgins R, Khovanova N. Decision tree and random forest models for outcome prediction in antibody incompatible kidney transplantation. *Biomed Sign Process Control.* 2019;52:456-462. doi:[10.1016/j.bspc.2017.01.012](https://doi.org/10.1016/j.bspc.2017.01.012)
  23. Vittoraki AG, Fylaktou A, Tarassi K, et al. Hidden patterns of anti-HLA class I alloreactivity revealed through machine learning. *Front Immunol.* 2021;12(July):1-16. doi:[10.3389/fimmu.2021.670956](https://doi.org/10.3389/fimmu.2021.670956)
  24. Montgomery MC, Liu C, Petrarolla R, Weimer ET. Using nanopore whole-transcriptome sequencing for human leukocyte antigen genotyping and correlating donor human leukocyte antigen expression with flow cytometric crossmatch results. *J Mol Diagn.* 2020;22(1):101-110. doi:[10.1016/j.jmoldx.2019.09.005](https://doi.org/10.1016/j.jmoldx.2019.09.005)
  25. Lucas DP, Vega RM, Jackson AM. OR2 variable expression of HLA-C impacts T versus B cell crossmatch outcomes. *Hum Immunol.* 2016;2016(77):2. doi:[10.1016/j.humimm.2016.07.014](https://doi.org/10.1016/j.humimm.2016.07.014)
  26. Visentin J, Couzi L, Taupin J. Clinical relevance of donor-specific antibodies directed at HLA-C: a long road to acceptance. *HLA.* 2021;97(1):3-14. doi:[10.1111/tan.14106](https://doi.org/10.1111/tan.14106)
  27. Bishara A, Nelken D, Brautbar C. Differential expression of HLA class-I antigens on B and T lymphocytes obtained from human lymphoid tissues. *Immunobiology.* 1988;177(1):76-81. doi:[10.1016/S0171-2985\(88\)80093-7](https://doi.org/10.1016/S0171-2985(88)80093-7)

**How to cite this article:** Ramalhete L, Araújo R, Teixeira C, et al. Evaluation of rapid optimized flow cytometry crossmatch (Halifaster) in living donor kidney transplantation. *HLA.* 2024;103(2):e15391. doi:[10.1111/tan.15391](https://doi.org/10.1111/tan.15391)