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Rapid optimized flow cytometric crossmatch (FCXM) assays: The Halifax and Halifaster protocols

Robert S. Liwski[a,*], Anna L. Greenshields[a], David M. Conrad[a], Cathi Murphey[b], Robert A. Bray[c], Jorge Neumann[d], Howard M. Gebel[c]

[a] Department of Pathology, Dalhousie University, Halifax, Nova Scotia B3H 1V8, Canada
[b] Southwest Immunodiagnostics Inc., San Antonio, TX 78229, USA
[c] Department of Pathology, Emory University Hospital, Atlanta, GA 30322, USA
[d] Laboratory of Transplantation Immunology, Santa Casa Hospital, Porto Alegre, Brazil

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ABSTRACT

The flow cytometric crossmatch (FCXM) assay, which detects the presence of donor specific HLA antibodies in patient sera, is a cornerstone of HLA compatibility testing. Since relatively long FCXM assay turnaround times may contribute to transplant delays and increased graft ischemia time, we developed and validated two modified crossmatch procedures, namely the Halifax and Halifaster FCXM protocols. These protocols reduce FCXM assay time > 60% and simplify their set-up without compromising quality or sensitivity. Optimization of the FCXM (the Halifax protocol) includes a 96-well tray platform, reduced wash times, increased serum to cell suspension volume ratio, shortened incubations and higher incubation temperature. The Halifaster protocol is a further modification, employing methods that improve lymphocyte purity compared to density gradient centrifugation (96 ± 2.63% vs 69 ± 19.06%), reduce cell isolation time (by ~40%) and conserve FCXM assay reagents. Importantly, linear regression analysis of the median channel fluorescence shift (MCFS) values revealed excellent concordance (R^2 of 0.98–0.99) among all three FCXM protocols (standard vs Halifax vs Halifaster). Finally, a retrospective review of 2013 crossmatches performed using the Halifax protocol demonstrated excellent correlation with the virtual crossmatch (95.7% and 96.8% specificity and sensitivity, respectively) regarding the identification of donor specific antibodies (HLA-A/B/DR) assigned based on the single antigen bead (SAB) assay testing with a 2000 mean fluorescence intensity (MFI) cutoff. Implementation of the Halifax or Halifaster protocols will expedite pre-transplantation work-up and improve patient care.

1. Introduction

The flow cytometric crossmatch (FCXM) assay is used by the majority of histocompatibility laboratories in North America as part of pre-transplant risk assessment to detect donor specific anti-HLA antibodies [1–4]. The FCXM assay is significantly more sensitive than cytotoxic crossmatch assays [5,6] allowing better detection of low level HLA antibodies, thereby improving assessment of pre-transplant immunological risk [7–9].

The standard three color FCXM protocol currently used by the majority of laboratories was described > 25 years ago [2,10] and can be divided into two parts. The first part consists of donor lymphocyte isolation [10] and treatment (e.g., pronase; DNase) to reduce Fc receptor expression and remove dead and dying cells [11]. The second part is the crossmatch assay (set up, testing and acquisition) per se [10]. The entire protocol is time intensive, typically requiring four to five hours to complete, and can lead to significant transplant delays and compromise organ quality [12,13].

In this study, we developed an expeditious FCXM procedure without compromising quality or sensitivity of the current assay. In the first part of the study, we investigated the impact of several assay parameters on FCXM results. Subsequently, these parameters were modified resulting in a rapid optimized FCXM procedure (henceforth referred to as the Halifax protocol), which is completed within 40 min (a 60% decrease

Abbreviations: 3SD, 3 standard deviations; CDC, complement-dependent cytotoxicity; DSA, donor-specific HLA antibodies; FCXM, flow cytometry crossmatch; FWB, flow wash buffer; HLA, human leukocyte antigen; MCF, median channel fluorescence; MCFS, median channel fluorescence shift; MFI, mean fluorescence intensity; NC, negative control; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; PC, positive control; RT, room temperature; SAB, single antigen bead; SD, standard deviation; SFCXM, standard flow cytometry crossmatch; SWID, Southwest Immunodiagnostics Inc.; TAT, turnaround time; VXM, virtual crossmatch; XM, crossmatch

* Corresponding author at: Dr. D.J. Mackenzie Building, HLA Typing Laboratory, 5788 University Avenue, Room 625, Halifax, Nova Scotia B3H 1V8, Canada.
E-mail address: robert.liwski@nshealth.ca (R.S. Liwski).

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compared to standard protocol) and maintains excellent assay sensitivity. Next, we performed a retrospective analysis of 2013 crossmatches performed using the Halifax FCXM and correlated the results to a virtual crossmatch (VXM) based on the single antigen bead (SAB) LABScreen assay testing. Finally, we incorporated the EasySep Direct lymphocyte isolation technique [14] to improve lymphocyte purity (> 90%), isolation time, and further optimize the FCXM assay (Halifaster protocol).

2. Materials and methods

2.1. Flow cytometric crossmatch assay reagents

Flow wash buffer (FWB) composed of phosphate buffered saline (PBS; Life Technologies Inc., Burlington ON, Canada) with 2% (v/v) fetal calf serum (Life Technologies Inc.) was used for all FCXM washes. Anti-CD3-PerCP (clone SK7) and anti-CD19-PE (clone SJ25C1) monoclonal antibodies were purchased from BD Biosciences (Mississauga, ON, Canada). Fluorescein (FITC) conjugated $F(ab')_2$ fragment goat anti-human IgG, Fcγ specific polyclonal antibody (IgG-FITC), 1.0 mg/mL stock solution, was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA).

2.2. Control sera

A pool of 10 non-sensitized patient sera and 20 highly-sensitized patient sera served as the negative control (NC) and positive control (PC), respectively, for all FCXM optimization and validation experiments performed by the Halifax HLA laboratory.

2.3. Virtual crossmatching

A positive virtual crossmatch (VXM) was defined as having at least one HLA DSA (HLA-A/B/C/DR/DQ) with ≥1000 mean fluorescence intensity (MFI) identified by the LABScreen SAB luminex assay (OneLambda, Canoga Park, CA). A negative VXM was assigned when all HLA DSAs had MFI values < 1000. All patient sera were treated either with 6.0 mM EDTA (Halifax and SWID HLA laboratories) [15,16] or heat (56 °C; Santa Casa HLA Laboratory) prior to SAB testing to prevent complement mediated interference.

2.4. Donor cells

Cells from volunteer donors were used for all FCXM optimization experiments. Donor cells for the Halifax FCXM and Halifaster FCXM protocol validation experiments were obtained from whole blood samples collected from volunteers as well as live and deceased donors. For the Halifax protocol evaluation studies performed by the Santa Casa HLA laboratory cells were isolated from deceased donor spleen or lymph node samples.

2.5. Cell isolation

2.5.1. Peripheral blood mononuclear cell enrichment

Peripheral blood mononuclear cells (PBMC) were enriched by density gradient centrifugation. Briefly, 12 mL of acid dextrose citrate anti-coagulated donor blood was mixed 1:1 with PBS. This blood mixture was layered over 18 mL of Lympholyte-H density medium (Cedarlane, Burlington, Ontario, Canada) in a 50 mL Falcon tube and centrifuged at 400 × *g* for 30 min with no brake. The mononuclear cell layer was transferred into a fresh 15 mL Falcon tube and washed three times in 10 mL PBS at 400 × *g* for 10 min.

2.5.2. Lymphocyte isolation from peripheral blood samples

Lymphocyte purification from acid dextrose citrate anti-coagulated donor whole blood was performed by immunomagnetic bead negative selection method using the EasySep™ Direct Human Total Lymphocyte Isolation Kit (EasySep™ Direct; STEMCELL Technologies Inc., Vancouver, BC, Canada) according to the manufacturer's instructions. After isolation, lymphocytes were washed twice with PBS at 400 × *g* for 5 min.

2.6. Cell treatment with pronase and DNase

Following isolation, all donor cells (PBMC or lymphocytes) were treated with pronase (4.7 kuntz units/mL; Sigma-Aldrich, St Louis, MO) for 15 min, followed by DNase (11,000 u/mL; Sigma-Aldrich) for 2 min at 37 °C. The cells were then washed twice in 1 mL PBS at 400 × *g* for 1 min, and resuspended in 1 mL of FWB. Total cell number and cell purity was determined using Sysmex XN10 analyser (Sysmex Canada Inc. Mississauga, ON). The cell concentration was adjusted to either 8.3×10^6 cells/mL for the standard FCXM or 1.0×10^7 cells/mL for both the Halifax and Halifaster protocols.

2.7. Flow cytometry crossmatch protocols

2.7.1. Standard FCXM, tube method

The standard FCXM (SFCXM), tube method (Table 1), was performed as previously described [10]. Briefly, 30 µL of test or control sera and 30 µL of PBMC (2.5×10^5 cells) were added to 5 mL polystyrene Falcon tubes, mixed by vortexing, and incubated for 30 min at 4 °C. The cells were washed three times with 1 mL FWB at 500 × *g* for

Table 1
Comparison of the standard, Halifax, and Halifaster FCXM protocols.

Parameter	Standard FCXM Tube Method	Standard FCXM Tray Method	Halifax FCXM Protocol	Halifaster FCXM Protocol
Assay platform	5-mL, 12 × 75 mm tubes	96-well tray	96-well tray	96-well tray
Serum (µL)	30	30	50	30
Cell isolation method, cell preparation	Lympholyte-H, PBMC	Lympholyte-H, PBMC	Lympholyte-H, PBMC	EasySep™ Direct, Lymphocytes
Pronase/DNase treatment time (min)	15/2	15/2	15/2	15/2
Cell preparation time (min)	90	90	90	55
Cell suspension (µL)	30	30	25	15
Cell number	2.5×10^5	2.5×10^5	2.5×10^5	1.5×10^5
First incubation (min)	30	30	20	20
Washes (1st set)	3 × 5 min at 500 × *g*	3 × 1 min at 500 × *g*	3 × 1 min at 500 × *g*	3 × 1 min at 500 × *g*
Wash buffer (µL)	1000	200	200	200
Antibody cocktail (µL)	100	100	100	50
PBS/CD3/CD19/IgG-FITC	89.75/5/5/0.25	89.75/5/5/0.25	94.75/3/2/0.25	46.88/2/1/0.125
Second incubation (min)	30	30	10	5
Washes (2nd set)	2 × 5 min at 500 × *g*	2 × 1 min at 500 × *g*	2 × 1 min at 500 × *g*	2 × 1 min at 500 × *g*
Final suspension µL)	500	500	400	400
FCXM assay time (min)	85	65	35	30
Total time, including cell preparation (min)	175	155	125	85