TECHNICAL BULLETIN

Cell Separation Solutions for the Flow Cytometric Crossmatch Assay

Case Study: Development and Validation of a Rapid Optimized Flow Cytometric Crossmatch Assay

To reduce turnaround time and standardize the FCMX assay, Dr. Robert Liwski developed the Halifaster Protocol. This Technical Bulletin discusses how this rapid, optimized FCXM assay protocol reduces the overall time to complete the assay, in part, by using a faster cell isolation technology.

"It is about time."

Dr. Robert Liwski, MD, PhD, commenting on the need for faster and standardized protocols for the FCXM assay.

Dr. Robert Liwski is the Medical Director of the HLA Laboratory at the Queen Elizabeth II Health Sciences Centre and Professor of Pathology at Dalhousie University in Halifax, Canada. He is a clinician scientist with interests in transplantation immunology, ischemia reperfusion injury and optimization of diagnostic testing in transplantation.

Background

To facilitate solid organ transplantation, the flow cytometric crossmatch (FCXM) assay is used as part of a pre-transplant risk assessment to determine whether the organ recipient has donor specific antibodies (DSA).[1] The FCXM assay can be divided in two parts:

1. Lymphocyte enrichment followed by pronase and DNAse treatment[2]
2. Crossmatch assay followed by antibody detection using flow cytometry.

Performing the FCXM assay is time consuming and the various steps in the protocol, including the donor lymphocyte enrichment step, are not performed consistently across laboratories. This lack of standardization may lead to inconsistent results. In order to optimize the standard FCXM assay, Dr. Robert Liwski from Dalhousie University in Halifax, developed, optimized and validated two FCXM procedures, the Halifax and Halifaster FCXM protocols.[3,4] The different parameters that were optimized included the cell isolation method, assay platform, cell number, serum volume, and incubation times. In this Technical Bulletin we focus on the different cell isolation methods followed by these protocols.

In the standard and Halifax FCXM protocols, lymphocytes are enriched from the donor's blood using density gradient centrifugation, which can be a very laborious and lengthy procedure. The lymphocyte purity obtained using density gradient centrifugation varies between 15-90%, depending on the donor and sample quality.[5] In the Halifaster FCXM protocol lymphocytes are isolated directly from whole blood without density gradient centrifugation or red cell lysis by using EasySep™ Direct lymphocyte enrichment technology. This lymphocyte isolation method is fast and the typical total lymphocyte purity is 95.8 ± 2.2% (not gated on CD45). Since lymphocyte purity varies between the different lymphocyte enrichment methods, Dr. Liwski assessed the impact of donor lymphocyte purity on FCXM results.[4,6]

Here we describe the following:

1. Lymphocyte purity and its impact on the detection of DSA and FCXM results.
2. Comparison of the FCXM results and the total time to perform the assay using the three different protocols.

Methods

The FCXM assays were performed following the standard, Halifax and Halifaster protocols as previously described.[3,4] In the standard and Halifax protocols, cells were isolated from whole blood by density gradient centrifugation using the density gradient medium Lympholyte® (~45 minutes/sample). In the Halifaster protocol, cells were isolated directly from whole blood with the EasySep™ Direct Human Total Lymphocyte Isolation Kit Catalog #19655 (~25 minutes/sample) following instructions on the product information sheet. The results of the FCXM assays following the different protocols were compared.

In addition, the impact of lymphocyte purity on FCXM results was assessed.[6] Briefly, lymphocytes (Ly), neutrophils (Nu), and monocytes (Mo) were isolated from 5 volunteer donors using the corresponding EasySep™ Direct kits (Catalog #19655, #19666, and #19669, respectively). Whole leukocyte (WL) preparations were obtained by adding Ly, Nu, and Mo cells in equal proportions (1/3 of each). The results of the FCXM assays using WL (low lymphocyte purity) and Ly (high lymphocyte purity) preparations were compared.

Results

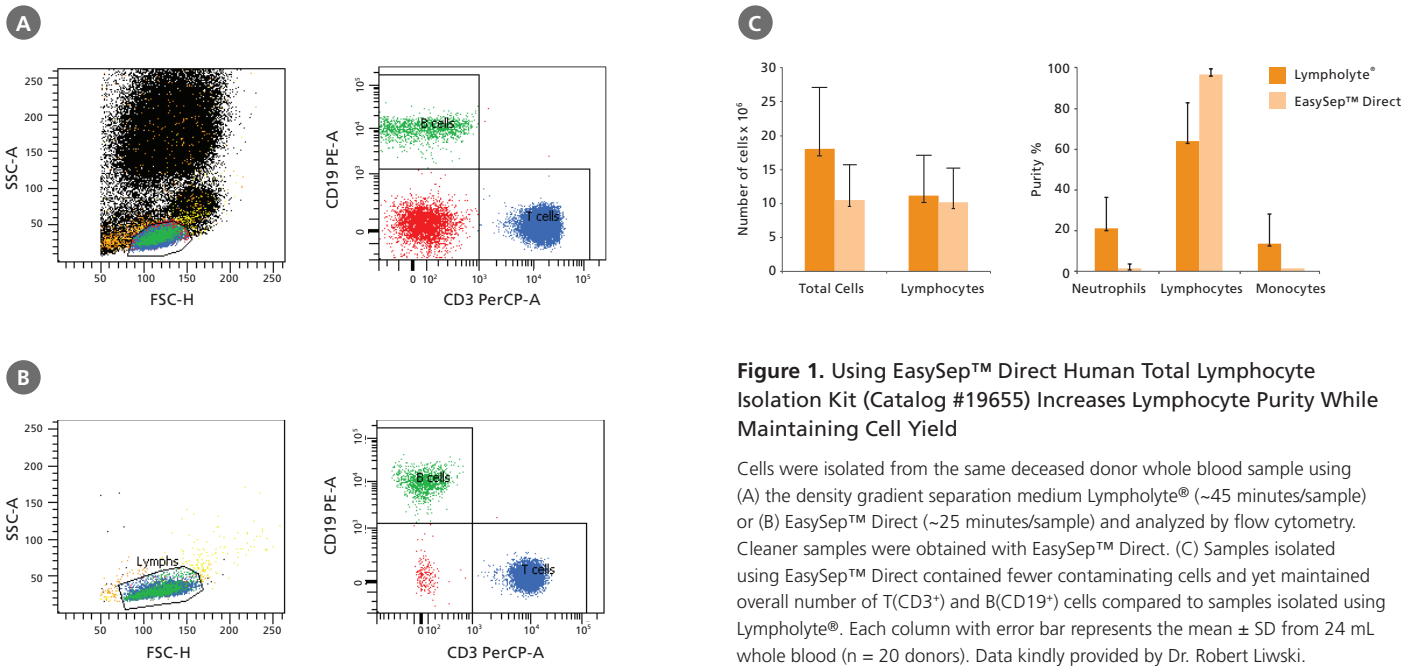

Figure 1. Using EasySep™ Direct Human Total Lymphocyte Isolation Kit (Catalog #19655) Increases Lymphocyte Purity While Maintaining Cell Yield

Cells were isolated from the same deceased donor whole blood sample using (A) the density gradient separation medium Lympholyte® (~45 minutes/sample) or (B) EasySep™ Direct (~25 minutes/sample) and analyzed by flow cytometry. Cleaner samples were obtained with EasySep™ Direct. (C) Samples isolated using EasySep™ Direct contained fewer contaminating cells and yet maintained overall number of T(CD3+) and B(CD19+) cells compared to samples isolated using Lympholyte®. Each column with error bar represents the mean ± SD from 24 mL whole blood (n = 20 donors). Data kindly provided by Dr. Robert Liwski.

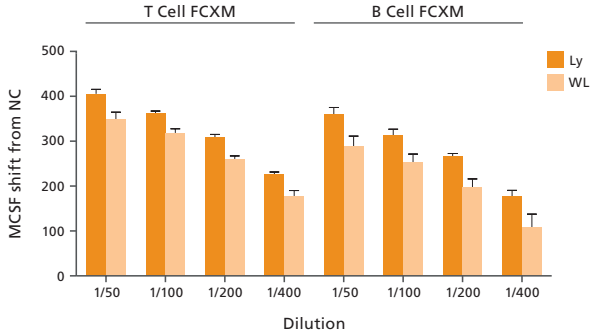

Figure 2. Use of Highly Enriched Lymphocytes Isolated with EasySep™ Direct Improves DSA Detection Compared to Whole Leukocyte Cell Preparations

Lymphocytes (Ly), neutrophils (Nu), and monocytes (Mo) were isolated from volunteer donors (n=5) using EasySep™ Direct Catalog #19655, #19666, and #19669, respectively. Whole leukocyte (WL) preparations were obtained by adding Ly, Nu, and Mo cells in equal propotions. WL (low lymphocyte purity) and Ly (high lymphocyte purity) preparations were treated with pronase and then used to perform the FCXM assay against negative control sera or several dilutions of positive control sera. The median channel fluorescence shifts (MCFS) were generated by using the negative control sera samples as a baseline. The MCF shifts between WL and Ly were then compared. Each column with error bars represents the mean ± SEM (n = 5 donors). Data kindly provided by Dr. Robert Liwski.

"Implementation of the EasySep™ Direct cell purification had a very positive impact on our cell isolation and FCXM assay set-up time. Furthermore, we observed an improvement in events acquisition as well as time and ease of gating and analysis based on the improved lymphocyte purity."

Liwski RS et al. (2018)

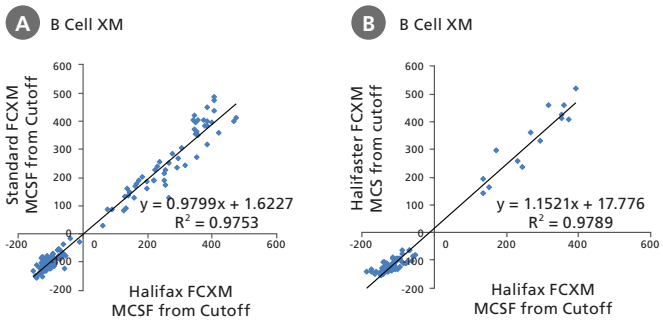

Figure 3. Isolation of Lymphocytes from Whole Blood Using EasySep™ Direct Does Not Compromise Sensitivity of the FCXM Assay

B cell FCXM assays were performed in parallel using cells isolated with EasySep™ Direct (Halifaster Protocol) or with the density gradient separation medium Lympholyte® (standard and Halifax Protocol). FCXM results were compared between (A) standard and Halifax and (B) Halifaster and Halifax FCXM protocols. Linear regression analysis of median channel fluorescence shifts (MCFS) showed an excellent correlation for the B cell and T cell (not shown here) FCXM assays between the two different isolation protocols. Data are expressed as MCSF from the cutoff level defined as the mean + three standard deviations. Data kindly provided by Dr. Robert Liwski.

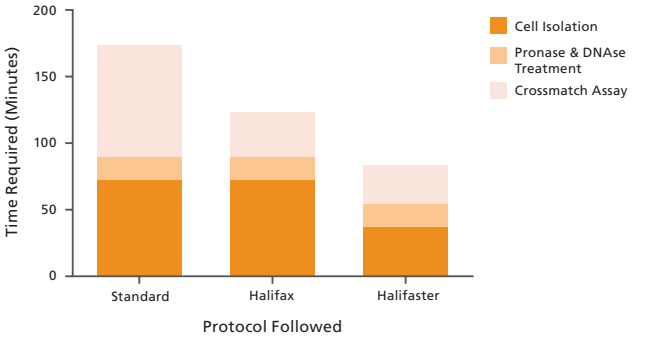

Figure 4. Following the Halifaster FCXM Protocol Reduces the Overall Time to Complete the FCXM Assay to Less than 2 Hours

The cell preparation part for the FCXM assay consists of the lymphocyte isolation step followed by treatment with pronase and DNAse. The Halifaster FCXM protocol reduced the time required for the cell preparation by almost 40% (from 90 to 55 minutes). This was achieved by using EasySep™ Direct technology for the lymphocyte isolation step. This technology is significantly faster than the density gradient centrifugation method used by the standard and Halifax FCXM protocols. The approximate total times it takes to complete the FCXM assay, (including cell preparation) following the standard, Halifax and Halifaster FCXM protocols is 175 minutes, 125 minutes and 85 minutes, respectively. Data kindly provided by Dr. Robert Liwski.

Summary

- The Halifaster protocol incorporates EasySep™ Direct for the isolation of lymphocytes. This resulted in fewer contaminating cells compared to using the density gradient centrifugation method with Lympholyte®.
- Performing FCXM assays with highly enriched lymphocytes isolated with EasySep™ Direct improved detection of DSA and reduced the variability of FCXM results.
- The Halifaster FCXM protocol reduced the overall time to complete the FCXM assay to less than 2 hours without compromising quality or sensitivity. This was in part by reducing the lymphocyte isolation step to less than 30 minutes.

Cell Isolation for the Flow Cytometric Crossmatch Assay

EasySep™ Direct isolates untouched and highly purified cells directly from whole blood without density gradient centrifugation, sedimentation or red blood cell (RBC) lysis. Highly purified cells are immediately ready for the FCXM assay. Cell isolation with EasySep™ Direct is gentle, fast, and efficient and works well on older blood samples. Individual samples of 0.5–30 mL can be processed manually in as little as 20 minutes.

For increased sample throughput and minimized sample handling errors, EasySep Direct™ cell isolation can be fully automated with RoboSep™ instruments.

Why Use EasySep™ Direct for the FCXM Assay?

- Isolate highly purified total lymphocytes, T cells or B cells directly from whole blood without lysis or centrifugation.
- Speed up your FCXM assay without compromising assay sensitivity.
- Automate cell isolations and minimize sample handling with RoboSep™ instruments.

References

1. Rebibou JM et al. (2004) T-cell flow-cytometry crossmatch and long-term renal graft survival. Clin Transplant 18(5): 558–563.
2. Vaidya S et al. (2001) Improved flow cytometric detection of HLA alloantibodies using pronase: potential implications in renal transplantation. Transplantation 71(3): 422–8.
3. Liwski RS et al. (2015) Development and Validation of a Rapid Optimized Flow Cytometry Crossmatch (FCXM) Assay, the Halifax and Halifaster FCXM Protocols. ASHI Quarterly (Third Quarter 2015): 19–25.
4. Liwski RS et al. (2018) Rapid optimized flow cytometric crossmatch (FCXM) assays: The Halifax and Halifaster protocols. Hum Immunol 79(1): 28–38.
5. Hamrick C & Lebeck L. (2000) Flow cytometric T and B cell crossmatching. ASHI laboratory manual, 4th Ed. Philadelphia: American Society for Histocompatibility and Immunogenetics: 41–45.
6. Liwski RS et al. (2016) P099 The impact of lymphocyte purity on flow cytometry crossmatch (FCXM) assay. It's not purely theoretical. Hum Immunol 77, Supple: 110–111.

All data presented in this case study was kindly provided by Dr. Robert Liwski, HLA Laboratory, Department of Pathology, Dalhousie University, Halifax, Canada. Robert.Liwski@nshealth.ca

Product Listing

Select EasySep™ Direct Products

Cell Type	Catalog #	
	EasySep™	RoboSep™
Total Lymphocytes	19655	19655RF
T Cells	19671 89671	19671RF 89671RF
B Cell	19684 89684	19684RF 89684RF

Recommended Antibodies for Flow Cytometric Crossmatch Analysis

Antigen	Clone	Catalog #
Anti-Human CD3	UCHT1	60011
	SK7	60127
Anti-Human CD19	HIB19	60005
Anti-Human CD45	HI30	60018

VIDEO
Introduction to EasySep™ Direct
www.EasySepDirect.com