Case Study
Development and Validation of a Rapid Optimized Flow Cytometry Crossmatch (FCXM) Assay

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). The FCXM assay can be divided in two parts:
1. Lymphocyte enrichment followed by pronase and DNAse treatment
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. 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. In the Halifaster FCXM protocol lymphocytes are isolated directly from whole blood without density gradient centrifugation or red cell lysis 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.

Methods
The FCXM assays were performed following the standard, Halifax and Halifaster protocols as previously described. 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 (see schematic on page 4). The results of the FCXM assays following the different protocols were compared.

In addition, the impact of lymphocyte purity on FCXM results was assessed. 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 bars represents the mean ± SD from 24 mL whole blood (n = 20 donors).

Figure 2. Use of Highly Enriched Lymphocytes (Ly) Isolated with EasySep™ Direct Improves DSA Detection and Reduces Variability of FCXM Results Compared to Whole Leukocyte (WL) 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 proportions. 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 (MCF shifts) 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).
Cell Separation Solutions for Flow Cytometry Crossmatch (FCXM)

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

Summary

- The FCXM assays performed with highly enriched lymphocytes isolated with EasySep™ Direct, improved detection of DSA and reduced the variability of FCXM results.
- The Halifax FCXM protocol, which adopts the EasySep™ Direct technology for lymphocyte enrichment, reduced the overall time to complete the FCXM assay to less than 2 hours without compromising quality or sensitivity, in part by reducing the lymphocyte isolation step to less than 30 minutes.

References


Data kindly provided by Dr. Robert Liwski, HLA Laboratory, Department of Pathology, Dalhousie University, Halifax, Canada. Robert.Liwski@nshealth.ca
EasySep™ Direct
Cell Isolation Directly from Whole Blood

EasySep™ Direct isolates untouched and highly purified cells directly from whole blood without density gradient centrifugation, sedimentation or red blood cell (RBC) lysis. Unwanted cells and RBCs are depleted immunomagnetically leaving untouched, highly purified target cells that are immediately ready for the FCXM assay. Cell isolation with EasySep™ Direct is gentle, fast and efficient; individual samples of 0.5–30 mL can be processed manually in as little as 20 minutes.

For increased sample throughput and minimize sample handling errors, EasySep Direct™ cell isolation can be fully automated with RoboSep™ instruments. All cell labeling and magnetic isolation steps are performed by the instrument and the isolated cells are immediately ready for use in the FCXM assay.

Advantages of Using 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.

Typical EasySep™ Direct Protocol

1. Add EasySep™ Direct Isolation Cocktail and RapidSpheres™ to whole blood and incubate for 5 minutes*
2. Place tube in EasySep™ magnet for 5 minutes*
3. Pour off desired fraction into new tube, add EasySep™ Direct RapidSpheres™ to enriched cells and incubate for 5 minutes*
4. Place tube in EasySep™ magnet for 5 minutes*
5. Pour off highly purified and untouched cells into new tube.

*Times are typical for EasySep™ Direct kits. Times for each kit will vary depending on the exact isolation protocol.

Cells are ready for any downstream application.

Product Listing

Select EasySep™ Direct Products

<table>
<thead>
<tr>
<th>CELL TYPE</th>
<th>EASYSEP™</th>
<th>ROBOSEP™</th>
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<tbody>
<tr>
<td>Total Lymphocytes</td>
<td>19655</td>
<td>19655RF</td>
</tr>
<tr>
<td>T Cells</td>
<td>19671</td>
<td>19671RF</td>
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<tr>
<td>B Cell</td>
<td>19684</td>
<td>19684RF</td>
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Recommended Antibodies for Flow Cytometry Crossmatch Analysis

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<tr>
<th>ANTIGEN</th>
<th>CLONE</th>
<th>CATALOG #</th>
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<tr>
<td>Anti-Human CD3</td>
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</tr>
<tr>
<td>Anti-Human CD45</td>
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<td>60018</td>
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1. This kit carries the CE mark and is available as a Class I in vitro diagnostic device (IVD) in the European Union, Canada and Australia. To learn more about the regulatory status of this product in your specific region, contact info@stemcell.com.

VIDEO
Introduction to EasySep™ Direct
www.EasySepDirect.com

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