Immunomagnetic isolation of PBMCs from Cyto-Chex® BCT-stabilized whole blood

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Introduction

Streck Cyto-Chex Blood Collection Tubes (BCT) are used in the collection and stabilization of whole blood specimens intended for immunophenotyping white blood cells. Cyto-Chex BCT® has been FDA 510(k) cleared for consistent recovery of HIV-associated lymphocyte subsets for up to 14 days at room temperature blood storage while maintaining appropriate cellular morphology and surface antigen expression. EDTA BCT, however, are unable to maintain cell counts and cellular morphology in whole blood beyond 24 hours at room temperature[1-5]. Blood stabilization with Cyto-Chex BCT provides laboratories with the flexibility to store and/or ship samples for extended periods of time, thereby reducing the occurrence of errors due to blood cell degradation.

Ficoll, or density-gradient separation, is one of the most common methods for isolating Peripheral Blood Mononuclear Cells (PBMCs) from whole blood samples prior to flow cytometry analysis[6]. Although separation by Ficoll is readily accomplished with samples collected in EDTA BCT, a stabilization reagent in Cyto-Chex BCT is not compatible with this density-gradient separation. Herein lies an alternative PBMC isolation technique compatible for use with whole blood stabilized in Cyto-Chex BCT. Importantly, the recovered cell ratios obtained using this technique are comparable to those obtained with density-gradient separation from EDTA-collected whole blood.

The EasySep™ Direct Human PBMC Isolation Kit[7] (STEMCELL™ Technologies), is capable of separating PBMCs from whole blood, buffy coat, or bone marrow samples. Magnetic particles conjugated to antibodies are used to target and bind unwanted cell populations, such as red blood cells, granulocytes, and platelets. The magnetic particles are then captured by an EasySep magnet, allowing PBMCs to be poured off and collected in a separate tube. PBMCs collected by this method are immediately available for use in downstream applications such as flow cytometry, isolation of subpopulations for specific assays, nucleic acid extraction for DNA- or RNA-based analysis, or immunology research, among others[6,8]. Due to the diverse utility of PBMCs and the value of stabilizing whole blood samples for later use, the following study is intended to showcase the compatibility of Cyto-Chex BCT with the EasySep PBMC isolation technique with blood samples stored for up to 7 days at room temperature.

Materials and Methods

Whole blood was collected from five separate donors by venipuncture into EDTA and Cyto-Chex BCTs. EDTA-collected whole blood was processed via Ficoll density gradient separation approximately two hours after blood draw. Cyto-Chex BCT-stabilized whole blood was processed using the EasySep Direct Human PBMC Isolation Kit at approximately 24 hours, 5 days, and 7 days after blood draw (Table 1). Before and after PBMC isolation, samples were stained with CD45-FITC (BD Biosciences) for enumeration of leukocyte populations by flow cytometry. A minimum of 2×10^4 events per sample were acquired using a FACSCanto II™ (BD Biosciences) and analyzed using Kaluza Analysis Software (Beckman Coulter). Post-isolation T, B, and NK cell populations were characterized using BD Multitest™ CD3 FITC / CD8 PE / CD45 PerCP / CD4 APC and CD3 FITC / CD16+CD56 PE / CD45 PerCP / CD19 APC antibody cocktails with BD Trucount™ tubes (BD Biosciences). Samples were analyzed using BD FACSCanto™ Clinical IVD software for enumeration of lymphocyte subsets.

Tube	PBMC Isolation Method	Time Elapsed After Blood Draw
EDTA	Ficoll	2 hours
Cyto-Chex BCT	EasySep Kit	24 hours
Cyto-Chex BCT	EasySep Kit	5 days
Cyto-Chex BCT	EasySep Kit	7 days

Table 1. Isolation Method and Blood Storage Time of EDTA and Cyto-Chex BCT-Drawn Samples.

EDTA/Ficoll Density Gradient Separation Protocol:

Estimated time to complete: 50 minutes

1. Using a 15 mL conical tube, dilute 3 mL EDTA-collected whole blood with 3 mL PBS + 2.5% FBS.
2. Underlay diluted blood with Lymphoprep™ (STEMCELL Technologies) using a sterile serological pipette, taking care to maintain a clear separation between the Lymphoprep layer at the bottom and the diluted blood layer at the top of the conical tube.
3. Centrifuge at 800 g for 20 minutes at room temperature (RT) with no brake.
4. Use a new sterile pipette to collect the PBMC layer at the plasma/Lymphoprep interface without disturbing the erythrocyte pellet.
5. Transfer PBMCs to a 15 mL conical tube and wash once (centrifuge at 220 g for 5 minutes at RT with brake) with PBS + 2.5% FBS prior to processing for flow cytometry.

Cyto-Chex BCT/EasySep Direct Human PBMC Isolation Kit Protocol:

Estimated time to complete: 45 minutes

Note: The EasySep PBMC Isolation Kit IFU suggests adding EDTA to whole blood samples to avoid loss of monocytes. This is not recommended when isolating PBMCs stabilized in Cyto-Chex BCT.

1. Acceptable sample volume range is 1-6 mL.
2. In a 14 mL round-bottom tube, add desired volume of Cyto-Chex BCT-stabilized whole blood.
3. Add 50 uL EasySep Isolation Cocktail per mL of whole blood sample volume.
4. Incubate at RT for 5 minutes.
5. Dilute sample 1:1 with PBS + 2.5% FBS and vortex to mix.
6. Vortex EasySep RapidSpheres for 30 seconds and add 50 uL per mL original whole blood volume.
7. Immediately place sample into "The Big Easy" EasySep magnet and incubate at RT for 5 minutes.
8. Picking up the magnet and tube together, pour the cell suspension into a new 14 mL round-bottom tube.
9. Add EasySep RapidSpheres to the cell suspension, using the volume from Step "6".
10. Remove the previous tube from the magnet and place 14 mL round-bottom tube containing cell suspension into the magnet, incubating at RT for 5 minutes.
11. Picking up the magnet and tube together, pour the cell suspension into a new 14 mL round-bottom tube.
12. Immediately remove the previous tube from the magnet and place the next 14 mL round-bottom tube in the magnet (this step requires no addition of RapidSpheres™).
13. Incubate at RT for 5 minutes.
14. Picking up the magnet and tube together, pour the cell suspension into a final tube.
15. Isolated PBMCs are now ready to process for flow cytometry.

Results

Cyto-Chex BCT-stabilized whole blood samples separated with the EasySep PBMC Isolation Kit yield similar PBMC recovery results to EDTA-collected whole blood samples separated with the Ficoll density-gradient separation technique (Figure 1). After both isolation techniques < 3% of leukocytes recovered were granulocytes, leaving a larger proportion of monocytes and lymphocytes (PBMCs) in the samples as expected.

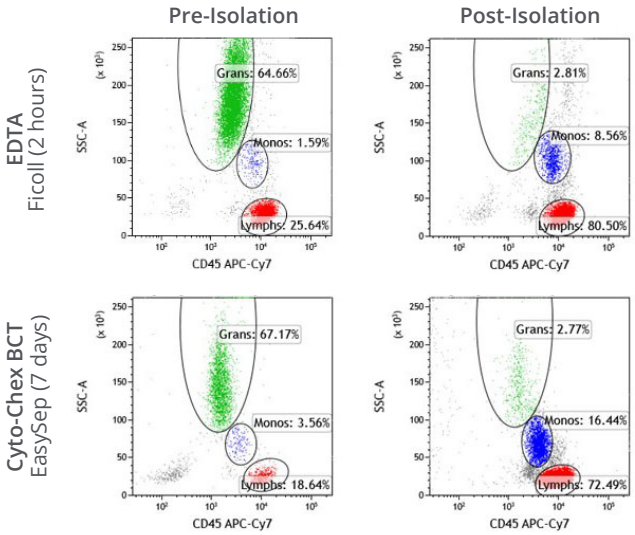

Figure 1. Lymphocyte subset recovery pre- and post- PBMC isolation. *Seven days post blood collection, EasySep Kit PBMC recovery from Cyto-Chex BCT-stabilized whole blood is comparable to Ficoll density-gradient PBMC recovery from EDTA-collected whole blood. Leukocyte populations in a single donor pre- (left) and post- (right) PBMC isolation for the Ficoll separation technique (top) and the EasySep PBMC Isolation kit (bottom).*

Efficient PBMC isolation can be achieved using the EasySep kit between 24 hours and 7 days after blood collection in Cyto-Chex BCT (Figure 2). At 24 hours, 5% of leukocytes recovered were granulocytes, and by 7 days < 3% granulocytes remained post-isolation. At each time point, the majority of remaining leukocytes post-isolation were monocytes and lymphocytes.

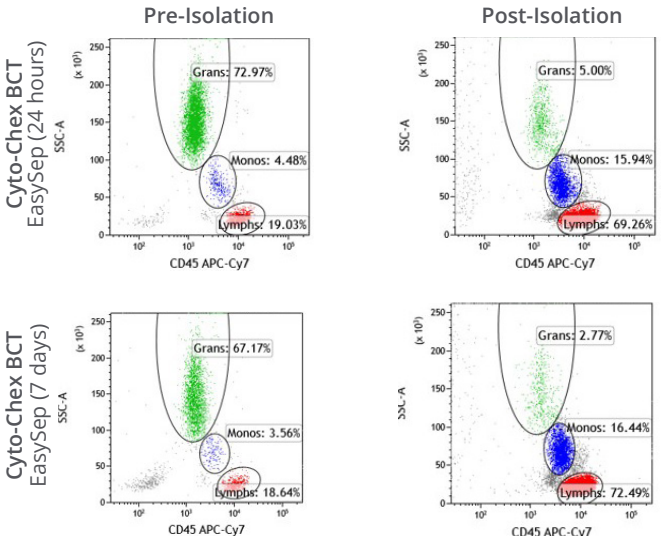

Figure 2. Easy-Sep PBMC isolation lymphocyte subset recovery as a function of time. *EasySep kit PBMC recovery from Cyto-Chex BCT-stabilized whole blood is appropriate as early as 24 hours, and as late as 7 days post blood collection. Leukocyte populations in a single donor pre- (left) and post- (right) PBMC isolation at 24 hours (top) and 7 days (bottom) after blood collection.*

Lymphocyte, monocyte, and granulocyte recoveries pre- and post-PBMC isolation demonstrated Cyto-Chex BCT-stabilized whole blood responded to EasySep PBMC isolation in the same manner as the gold-standard EDTA-collected Ficoll separation in terms of leukocyte population ratios (Figure 3). One-way ANOVA analysis with multiple comparisons was used to compare average post-isolation results between EDTA/Ficoll and Cyto-Chex BCT/EasySep separation. There was no significant difference ($p>0.05$) found between EDTA/Ficoll or Cyto-Chex BCT/EasySep PBMC isolation at any time point. Furthermore, no significant difference was observed between Cyto-Chex BCT/EasySep PBMC isolation at 24 hours compared to equivalent samples processed 7 days post blood draw. Taken together, this data suggests recoveries from Cyto-Chex BCT/EasySep separation between 24 hours and 7 days post blood draw are comparable to results from EDTA/Ficoll separation. Numerical results for average leukocyte population results are detailed in Table 2. Figure 4 illustrates the stability of T-cell, B-cell, and natural killer-cell antigen expression after PBMC isolation with the EasySep PBMC Isolation Kit, up to 7 days after blood collection in Cyto-Chex BCT.

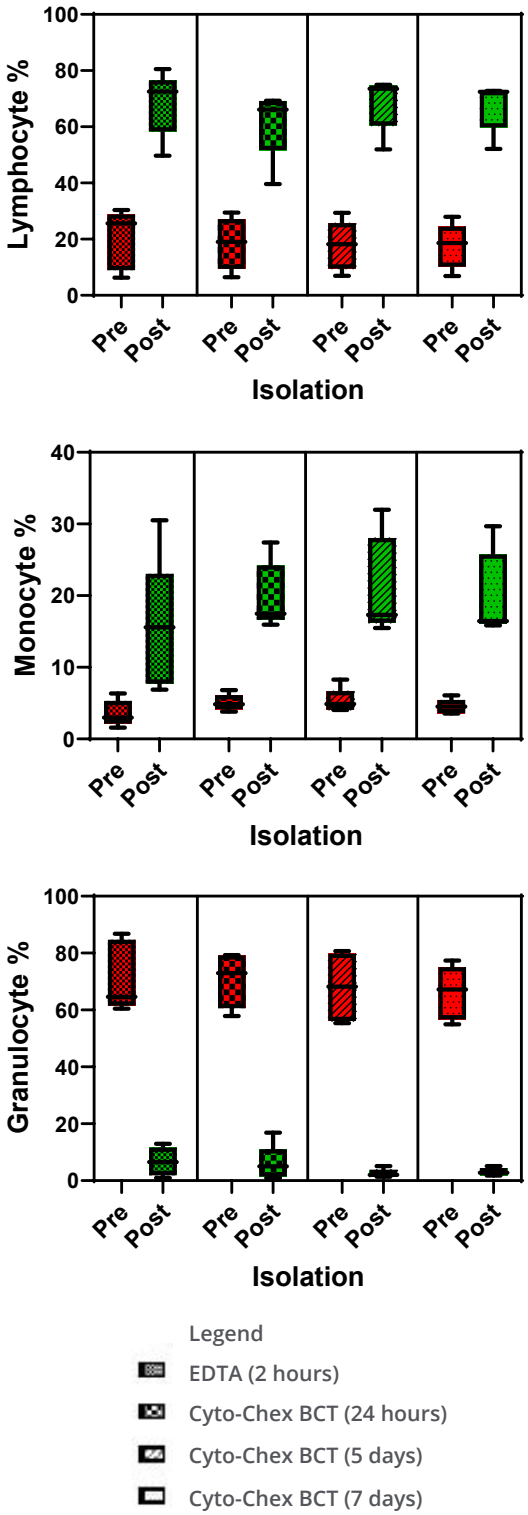

Figure 3. Efficient lymphocyte and monocyte isolation from Cyto-Chex BCT-stabilized blood as a function of blood storage time. *Leukocyte recovery is shown pre- (red) and post- (green) PBMC isolation for each method and isolation time point. Granulocyte recovery was reduced in all samples after PBMC isolation whereas monocyte and lymphocyte recoveries increased.*

	Tube	Method	Time After Draw	Lymph%	Mono%	Gran%
Pre-Isolation	EDTA	Ficoll	2 hours	20.3	3.6	71.4
	Cyto-Chex BCT	EasySep Kit	24 hours	18.4	5.1	70.6
	Cyto-Chex BCT	EasySep Kit	5 days	17.7	5.3	68.0
	Cyto-Chex BCT	EasySep Kit	7 days	17.6	4.5	66.1
Post-Isolation	EDTA	Ficoll	2 hours	68.5	15.4	6.7
	Cyto-Chex BCT	EasySep Kit	24 hours	61.6	19.9	6.0
	Cyto-Chex BCT	EasySep Kit	5 days	68.8	21.2	2.6
	Cyto-Chex BCT	EasySep Kit	7 days	67.5	20.0	3.1

Table 2. Comparison of Cyto-Chex BCT-stabilized Easy-Sep PBMC isolation and the gold standard demonstrates no statistically significant differences. *Numerical representation of leukocyte population recoveries for each method, pre- and post-isolation. No significant difference was observed between samples. (n=5, p>0.05)*

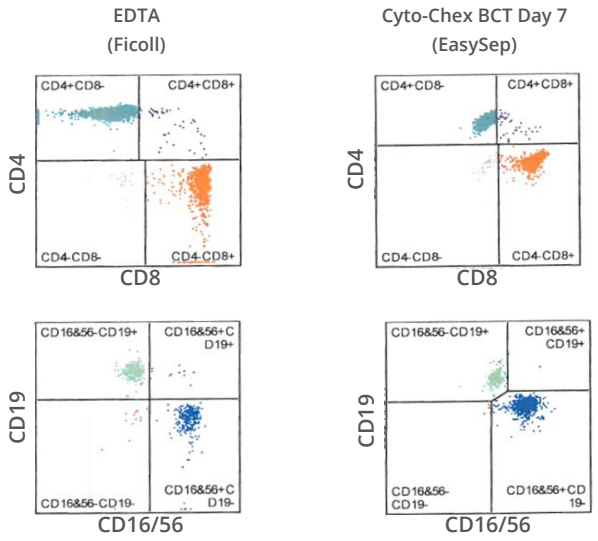

Figure 4. Lymphocyte subset stability post Cyto-Chex BCT/EasySep PBMC isolation. *Cell counts, percentages, and antigen expression were maintained for T cells, B cells, and natural killer cells after Cyto-Chex BCT/EasySep PBMC isolation, up to 7 days post blood collection. CD4/CD8 and CD16&56/CD19 plots are shown after EDTA-collected whole blood Ficoll isolation (left) or EasySep PBMC isolation 7 days post blood collection in Cyto-Chex BCT (right).*

Conclusions

Magnetic separation with the EasySep Direct Human PBMC Isolation Kit from STEMCELL Technologies successfully isolates PBMCs stabilized by Cyto-Chex BCT up to 7 days post blood collection. Statistical analysis using ANOVA indicates there are no significant differences between results obtained from the EasySep immunomagnetic separation method using Cyto-Chex BCT-stabilized whole blood and those obtained from Ficoll density-gradient separation using EDTA-collected whole blood (the gold standard for PBMC isolation).

Separation by EDTA/Ficoll is time-dependent, leaving users less than 24 hours from time of draw to perform the PBMC isolation. Cyto-Chex BCT/EasySep eliminates this rate-limiting step and allows for PBMC isolation up to 7 days post blood draw, offering the versatility to ship and process samples as scheduling permits with no appreciable loss to antigen integrity. However, all techniques illustrated within this text will necessitate in-house validation to demonstrate compatibility of the Cyto-Chex BCT/EasySep PBMC isolation method with the intended users downstream applications. Other similar magnetic separation kits are available on the market, such as the MACSprep™ kit from Miltenyi Biotec, although the results of other methods have not been outlined herein.

Conventional anticoagulants such as EDTA, while compatible with common PBMC isolation techniques such as density-gradient separation, are less adept at maintaining cellular characteristics and cell counts for more than 24 hours and are sensitive to storage and/or shipping conditions such as prolonged exposure to room temperature[1-5]. However, as this study demonstrates, Cyto-Chex BCT maintains PBMC characteristics including cell size, complexity, and surface antigen expression until isolation may be performed up to 7 days room temperature storage post blood collection. Cells isolated by such means are valuable for their utility in a variety of downstream applications, such as isolation of subpopulations for cell-specific assays, nucleic acid extraction for DNA- or RNA-based analysis, immunological research, and flow cytometry analysis[6,8].

In conclusion, isolation of PBMCs from Cyto-Chex BCT-stabilized whole blood is effectively achieved with the use of EasySep Direct Human PBMC Isolation Kit up to 7 days post blood collection.

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