

RESEARCH ARTICLE

Optimization of a Density Gradient Centrifugation Protocol for Isolation of Peripheral Blood Mononuclear Cells

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Objective: Peripheral blood mononuclear cells (PBMC) are extremely important in the body's immune response. Their isolation represents a major step in many immunological experiments. In this two phase study, we aimed to establish an optimum protocol for PBMC isolation by density-gradient centrifugation. **Methods:** During Phase-1, we compared two commercially available PBMC isolation protocols, Stemcell Technologies (ST) and Miltenyi Biotec (MB), in terms of PBMC recovery and purity. Twelve blood samples were assigned to each protocol. Each sample was divided in three subsamples of 1ml, 2ml and 3ml in order to assess the influence of blood sample volume on isolation performance. During Phase-2, a hybrid protocol was similarly tested, processing six blood samples. Additionally, we performed a flow cytometric analysis using an Annexin-V/Propidium-Iodide viability staining protocol. **Results:** Phase-1 results showed that, for all subsample volumes, ST had superior PBMC recovery (mean values: 56%, 80% and 87%, respectively) compared to MB (mean values: 39%, 54% and 43%, respectively). However, platelet removal was significantly higher for MB (mean value of 96.8%) than for ST (mean value of 75.2%). Regarding granulocyte/erythrocyte contamination, both protocols performed similarly, yielding high purity PBMC (mean values: 97.3% for ST and 95.8% for MB). During Phase-2, our hybrid protocol yielded comparable results to MB, with an average viability of 89.4% for lymphocytes and 16.9% for monocytes. **Conclusions:** ST yields higher cell recovery rates and MB excels at platelet removal, while the hybrid protocol is highly similar to MB. Both cell recovery and viability increase with blood sample volume.

Keywords: density gradient centrifugation, PBMC isolation, PBMC viability

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Introduction

Peripheral blood mononuclear cells (PBMC)

Peripheral blood mononuclear cells (PBMC) are extremely important in the body's immune response. PBMCs are comprised of lymphocytes, monocytes and dendritic cells. In humans, lymphocytes account for most of the PBMCs, followed by monocytes and only a small fraction of dendritic cells. Given their involvement in virtually any immune process, PBMC isolation is a key step in many immunological experiments. Once isolated, PBMCs can be used for a wide range of downstream applications such as studying autoimmune diseases [1-3], cancer research [4-10], developing new vaccines [11-13] and immunotherapies [6-10], drug discovery and testing [14-16] etc.

Due to their practical importance in the study of the functional immune system, PBMCs represent an area of continuous interest. Over time, several descriptive and comparative studies have approached the issue of PBMC preparation by studying isolation principles [17,18] and techniques, various isolation devices [18-23] and the effect of physical factors such as time [23-25], storage temperature [24, 26] and cryopreservation [22, 25, 27-29]. Commonly investigated parameters and performance indicators include cell recovery, cellular population composition, pu-

rity, viability, sterility, activation status and functionality. So far, not a single isolation technique proved to be superior in all aspects. In fact, it is the researcher's responsibility to choose the most suitable method that fits a project's design, financial resources and available laboratory equipment and personnel.

One of the most commonly used techniques for PBMC isolation is the density gradient centrifugation method. The separation principle takes advantage of the slight difference in density among blood cells. Briefly, whole blood is first diluted with phosphate buffer saline (PBS) and then carefully layered over the density gradient medium. Granulocytes and erythrocytes have a higher density than mononuclear cells and therefore sediment through the bottom layer during centrifugation. PBMCs form a distinct band at the medium-plasma interface and will be carefully retrieved by pipette. Additionally, the freshly isolated cells can be resuspended in PBS for further centrifugation, which will subsequently increase purity.

Aims and objectives

In this two phase study, our aim was to establish an optimum protocol for PBMC isolation by density gradient centrifugation. If successful, the protocol would then be implemented as a standard operating procedure (SOP) at the Cellular Immunology Laboratory of the Center for Advanced Medical and Pharmaceutical Research (CCAMF) of the University of Medicine and Pharmacy of Tîrgu

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Mureş (UMFTGM, Romania). During Phase 1, two commercially available protocols – Stemcell Technologies (ST) and Miltenyi Biotec (MB) – were compared in terms of PBMC recovery and purity. Although based on the same density gradient principle, the two protocols recommend different ratios between blood, PBS and gradient medium. Moreover, the specified centrifugal force (CF, i.e. x g) and centrifugation time (CT) as well as the number of cell washing steps are different. The schematic outline of Phase 1 and technical specifications are shown in Figure 1 and Table I, respectively. The symmetrical branched design of this phase is of great importance as it allows us to 1) compare performances between the two protocols; 2) assess the effect of blood sample volume on separation performance; 3) analyze the intraindividual variability for each of the 2 examiners; and 4) analyze the interindivdual variability by comparing all corresponding results between the 2 examiners. During Phase 2, a hybrid protocol (HY) was tested for the same performance indicators to which we added a flow cytometry assessment of viability.

Up to the present, no study has compared two density gradient protocols, namely Stemcell Technologies and Miltenyi Biotec. The present report addresses this gap and, to our knowledge, it is the first comparative analysis aiming to unravel the effect of CF, CT and number of washing steps on PBMC isolation performances.

Methods

Samples and study design

The study was approved by the UMFTGM Ethics Committee for Scientific Research (no.84/02.05.2017). The blood samples were collected from 12 healthy students who previously signed a written informed consent. The same student may have donated blood on multiple occasions.

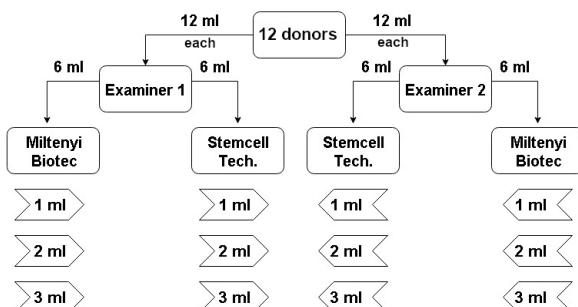


Fig. 1. Schematic outline of Phase-1. The symmetrical multi-branched design allows for assessment of intra- and interindividual variability for different sample volumes

Table I. Technical specifications for each protocol

	1st step		2nd step		3rd step		4th step	5th step
	B:PBS:H	CT	CF	CT	CF	CT	CF	
ST	1:1:1	20	800	10	250			
MB	1:3:1.7	30	400	10	300	10	200	bis
HY	ST					MB		Viability

P:PBS:H – blood:PBS:Histopaque ratio; CT – centrifugation time (min); CF – centrifugal force (xg)

About 18ml of blood was collected from each donor using two 9ml NH Sodium Heparin vacutainer tubes (Greiner Bio-One, catalog No.455051) from which the blood was processed within 1 hour and PBMC were separated within a maximum of 4 hours. The study was designed as a two phase laboratory experiment.

During Phase-1 of the study (see Figure 1), ST and MB were compared in terms of PBMC recovery and purity. Three distinct subsamples of blood (1ml, 2ml and 3ml) were assigned to each protocol in order to assess how sample volume influences the outcome of PBMC isolation. Furthermore, all 12 samples were processed by the same two examiners, 6 samples each, in a mirror-like manner as to cast light upon the effect of both intra- and interindividual variability on PBMC separation.

Based on the results from Phase-1, a new hybrid PBMC separation protocol was established and implemented in Phase-2 where another 6 samples were processed by only one examiner. Throughout the study, PBMC recovery (%), PBMC purity (%) and Platelet removal (%) were regarded as performance indicators. Additionally, a viability flow cytometric analysis was performed on all samples from Phase-2, using the FITC-conjugated early apoptosis marker Annexin-V and the late-apoptosis/necrosis marker Propidium Iodide (PI). Technical specifications of each protocol are detailed in Table I.

Phase 1 – Stemcell Technologies vs. Miltenyi Biotec

First, an automated complete blood count (CBC) was performed from each donor's blood sample using a SYSMEX XS-800i hematology analyzer. Each examiner was given 12ml of peripheral blood from their corresponding donor, 6ml to be processed according to ST and the other 6ml according to MB.

For ST, 6ml of blood was diluted (1:1) with Dulbecco's Phosphate Buffered Saline (DPBS; EuroClone, catalog No.ECB4053). Corresponding to a ratio of 2:1, volumes of 2ml, 4ml and 6ml from the diluted blood were carefully layered over 1ml, 2ml and 3ml, respectively, of Histopaque-1077 (Sigma-Aldrich, catalog No.10771) in 3 distinct 15ml conical centrifuge tubes (VWR, catalog No.89039-666). The gradients were centrifuged at 800xg for 20 min at room temperature (RT), without brake. The newly formed PBMC bands were removed using fine tip pipettes (Thermo Scientific Samco, catalog No.22-610-178) and transferred to 3 new 15ml tubes. The cells were then resuspended in DPBS up to a total volume of 14ml and centrifuged at 250xg for 10 min at RT, without brake.

To simplify calculations, after discarding the supernatant, pellets were resuspended in DPBS up to the original volume of blood that was processed for each. Finally, a CBC was performed from each tube in order to determine the total number of cells and PBMC populations, while performance indicators were computed with respect to the initial whole blood CBC.

For MB, 6ml of blood was diluted (1:3) with DPBS. Corresponding to a ratio of 7:3, volumes of 4ml and 8ml from the diluted blood were carefully layered over 1.7ml and 3.4ml, respectively, of Histopaque-1077 in 2 distinct 15ml centrifuge tubes. The remaining 12ml of diluted blood was similarly layered over 5.1ml of Histopaque-1077, in a 50ml centrifuge tube (VWR, catalog No.89039-656). The gradients were centrifuged at 400xg for 30 min at RT, without brake. The newly formed PBMC bands were removed using fine tip pipettes and transferred to 3 new 15ml tubes. The cells were then resuspended in DPBS up to the volume of 14ml and centrifuged at 300xg for 10 min at RT, without brake. After discarding the supernatant, pellets were similarly washed two more times and centrifuged at 200xg for 10 min. As in the first phase, pellets were resuspended in DPBS up to the original volume of blood that was processed for each and a CBC was performed from each tube in order to calculate the performance indicators.

Phase 2 – Hybrid protocol and Viability

Following interpretation of data from Phase-1, a likely more effective hybrid (HY) PBMC isolation protocol was proposed. A series of 6 PBMC separations was performed by a single examiner using the same equipment mentioned above. After performing an initial CBC, 6ml of blood were processed following the complete ST protocol, as previously described. Additionally, to reduce platelet contamination, pellets from all three tubes were washed two more times according to MB protocol (200xg, 10 min, RT, without brake). After performing a final CBC from each tube, the samples were washed with DPBS and centrifuged at 400xg for 10 min at RT without brake and the supernatants were discarded. For the viability staining protocol, a FITC-Annexin-V Apoptosis Detection Kit I (BD Pharmigen, catalog No.556547) was used. Based on the final CBCs, calculations were made and pellets were resuspended in Binding Buffer at a concentration of 1×10^6 cells/ml. From each tube, 100 µL of solution (1×10^5 cells) was transferred to distinct 5ml tubes. FITC-Annexin-V and PI, 5µL of each, were added to all tubes and the cells were incubated for 15 min at RT in the dark. After the incubation, 400 µL of Binding Buffer was added to each tube and cells were analyzed within 30 minutes using FACSAria III flow cytometer with FACSDiva v8.0 Software (both Becton-Dickinson Biosciences). Based on cell morphology (FSC/SSC), PBMCs were gated into two main populations – lymphocytes and monocytes – and a secondary population including all other non-debris events (Figure 2a). The vi-

ability assessment (FITC/PI) was performed only on gated singlets (Figure 2b).

Statistical processing

In our study, the significance threshold was set at 0.05. All statistical processing was performed using Microsoft Excel, GraphPad Prism 6 or BD FacsDiva-v8.0. A Kolmogorov-Smirnov normality test was applied for each data set. Intraindividual analysis between data sets of different sample volumes was performed using the paired t-Test, while for interindividual analysis, the two-sample t-Test was applied. All significant differences were reported using two-tailed p-values.

Results

All numerical values from Phase-1 are presented in Table II. Each examiner's sets of values have been statistically analyzed and found to be normally distributed. No intraindividual variability was identified ($p>0.05$), regardless of what examiner/protocol/volume association was considered (data not shown).

A quick analysis of Table II reveals highly similar values between E1 and E2 which suggests low interindividual variability, also confirmed by stastical analysis ($p>0.05$). One exception is the MB-3ml lot where PBMC recovery is significantly higher for E1 ($p=0.005$). Given the observed low interindividual variability, an average between E1 and E2 was calculated and the results were also presented in Table II. Although (%) is a better performance indicator, we have also calculated the PBMC recovery in “million cells per ml of blood” which is a more practical manner of reporting cell recovery. Average PBMC recovery values for ST's 1ml, 2ml and 3ml sample lots are 1.44, 2.04 and 2.25 million cells per ml of blood, respectively. For MB, the corresponding values are 1.17, 1.59 and 1.28 million cells per ml of blood, respectively. It is also obvious that higher sample volumes yielded higher recovery rates, an observation that was also supported by statistical analysis (p values not shown), the only exception being again the MB-3ml lot. PLT removal was calculated by comparing the initial and final CBCs, using the following formula: PLT removal = (initial PLT – final PLT)/initial PLT. By comparing ST and MB in terms of PLT removal, it is evident that MB has an unquestionably higher PLT removal rate ($p<0.0001$ for all three sample volumes), with an average of 6x less contaminant PLTs for MB samples than for ST. For MB, there are no differences between blood sample lots regarding PLT contamination while for ST, the 1ml lot was significantly less contaminated than its 2ml and 3ml counterparts ($p=0.004$ and $p=0.0006$, respectively). It should be specified that, for both protocols, erythrocyte contamination was very low or absent (data not shown), while granulocyte contamination is represented by the “PBMC purity” performance indicator described in Table II. In general, contaminating granulocytes were mainly comprised of neutrophils with scarce eosinophils and basophils (data now shown). Regarding

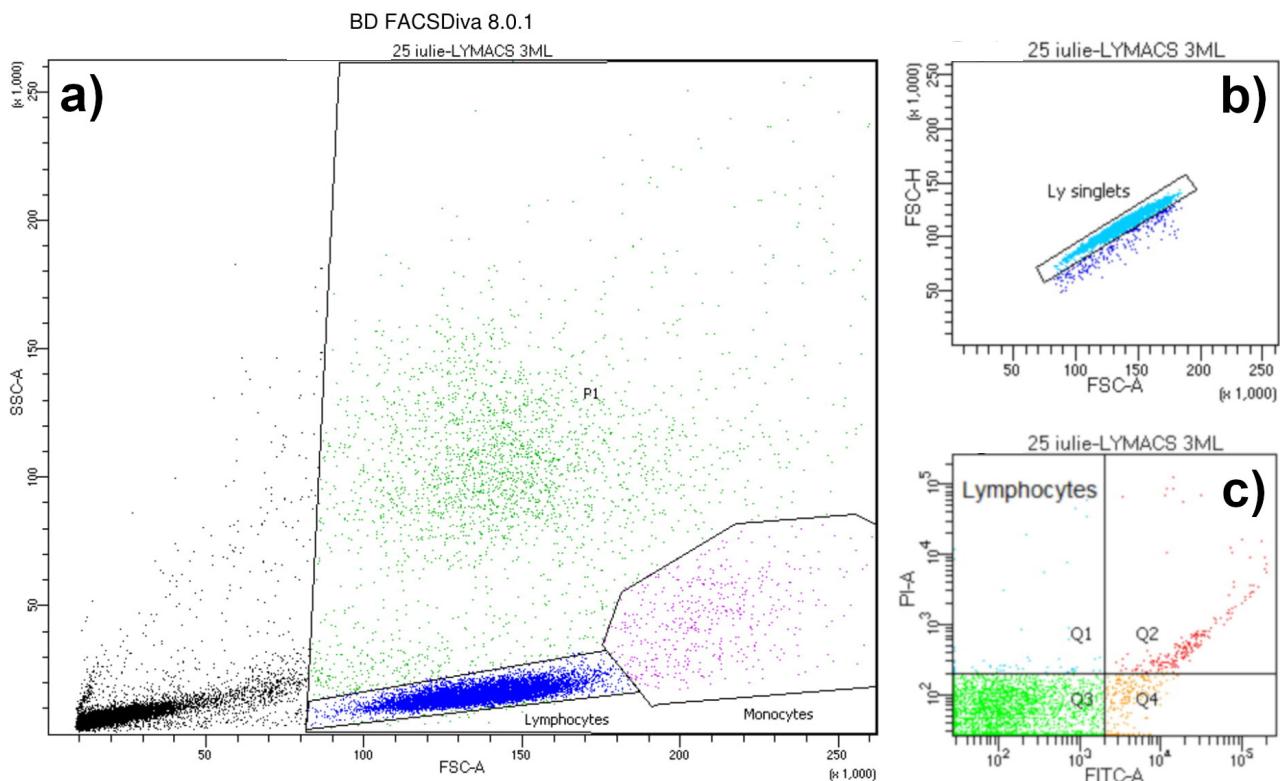


Fig. 2. An illustrative partial flow cytometry report showing a) gating of PBMCs into distinct populations based on morphological parameters (FSC/SSC); b) doublet discrimination and c) assessment of viability by FITC-Annexin-V and PI staining positivity

Table II. Average numerical values from Phase-1

	1ml	PBMC recovery (%)			PLT removal (%)			PBMC purity* (%)		
		2ml	3ml	1ml	2ml	3ml	1ml	2ml	3ml	1ml
ST	E1	55.8	79.7	85.9	81.1	76.0	72.8	95.1	98.6	97.4
	E2	55.5	79.7	87.9	83.5	70.6	67.4	98.0	98.2	96.4
	Avg.	55.7	79.7	86.9	82.3	73.3	70.1	96.6	98.4	96.9
MB	E1	40.3	52.8	51.8	96.7	97.2	97.1	97.2	97.5	98.3
	E2	37.5	55.2	34.2	96.8	96.3	96.7	93.1	94.7	94.0
	Avg.	38.9	54.0	43.0	96.7	96.8	96.9	95.2	96.1	96.2

E1 – examiner no.1; E2 – examiner no.2; Avg. – average value; *PBMC purity accounts only for granulocyte/erythrocyte contamination (PLTs are treated separately)

cell population composition, both examiners had similar results, independent of protocol and blood sample volume. The average PBMC population composition throughout Phase-1 is 86.4% lymphocytes and 10.2% monocytes (data not shown).

For Phase-2, all numerical values are presented in Table III. As expected, PBMC recovery significantly increases with sample volume ($p=0.002$ for 2ml vs. 1ml; $p=0.04$ for 3ml vs. 2ml). Average cell recovery values reported in million cells per ml of blood are as follows: 1.00 for 1ml, 1.57 for 2ml and 1.81 for 3ml. It should be stressed that PBMC recovery, PLT removal and purity values are very similar between HY and MB protocols (see Tables II and III). PLT removal was significantly greater for the 1ml lot than for its 2ml ($p=0.006$) and 3ml ($p=0.04$) counterparts. As shown in Figure 2c, in this study, cells considered viable are FITC Annexin V and PI negative; cells in early apoptosis are FITC Annexin V positive and PI negative; and cells in late apoptosis or already dead are both FITC Annexin V and PI positive. Lymphocyte viability was generally high and

also significantly increases with sample volume ($p=0.001$ for 2ml vs. 1ml; $p=0.02$ for 3ml vs. 2ml). On the contrary, we recorded low viabilities for monocytes, while no influence of sample volume was observed ($p>0.05$). Although increasing with sample volume, the viability of non-lymphocyte-non-monocyte events was also low with only the 3ml lot showing significantly higher viability than the 1ml and 2ml lots ($p=0.04$ and $p=0.008$, respectively).

Discussion

Density gradient centrifugation – advantages and limitations

There are several ways of isolating PBMCs, each technique having its own advantages and limitations [17,18]. The density gradient separation principle was first described by Böyum A. [30, 31] and quickly became the most popular PBMC preparation method. A major advantage of this method for lymphocyte immunophenotyping is the removal of most erythrocytes, granulocytes and nonvi-

Table III. Average numerical values from Phase-2

	1ml	2ml	3ml	
PBMC recovery (%)	37.2	57.3	63.7	
PLT removal (%)	98.1	95.5	93.9	
PBMC purity (%)	97.0	95.4	92.6	
Lymphocytes	86.2	90.2	91.7	
Viability (%)	Monocytes	16.1	17.0	17.5
Other*	25.7	26.2	33.1	

*Other – all non-lymphocyte-non-monocyte events recorded by the main gate

able cells from the sample [17]. Therefore, initial purification of cell populations speeds up the acquisition process while saving expensive antibodies by reducing non-specific binding. Density centrifugation is the most typical density gradient method to isolate PBMCs. Despite the advent of other enhanced, easier-to-use techniques, density centrifugation reportedly remains the most employed density-based PBMC isolation method. Besides yielding good results, this method is also less expensive [19] and less complicated than many of the other methods, allowing for relatively rapid processing with minimal laboratory equipment requirements. The main limitation of our study was the lack of population-specific staining markers such as CD3, CD4, CD8 for lymphocytes and CD14 for monocytes. Although morphology-based gating (FSC/SSC) allows for good population identification, it gives no information about lymphocyte sub-populations or any of the non-debris gated events that were too spread out to be accurately identified.

There are two critical steps in PBMC separation by density centrifugation. The first one is layering blood over the gradient medium which is a delicate process where patience and self control prevent catastrophic mixing of the two layers. At the end of this step, one should clearly see a distinct separation between the clear bottom layer and the upper blood layer. Inadequate layering during this step may result in partial or complete loss of the target cells. The second critical step is extracting the buffy coat which is

found at the medium-plasma interface. Contamination is inevitable but should be kept minimal as the osmotic stress exerted by the gradient medium may result in decreased viability, while the upper plasma layer is a rich source of undesired platelets.

PBMC recovery, purity, viability and platelet contamination

This study showed that ST protocol is superior to MB in terms of PBMC recovery (Figure 3a vs. 3b), while MB excels in platelet removal, with significantly lower platelet contamination (Figure 3c). For MB, there are no differences between blood sample lots regarding platelet contamination while for ST, the 1ml lot was significantly less contaminated than its 2ml and 3ml counterparts. Regarding cell recovery, our results are consistent with previous studies reporting an average cell recovery by Ficoll isolation between 0.6 and 3 million cells per ml of blood [19-22]. However, we consider that when assessing performance, "cells per ml of blood" is an inappropriate manner of reporting cell recovery since it provides no information about initial cell count and cell population composition. Both protocols showed high and comparable PBMC purities, with sporadic contamination due to residual erythrocytes and granulocytes. HY protocol consisted of a complete ST protocol to which we added the last two washing steps from MB. All performance indicators were similar between MB and HY protocols (compare Tables II and III, see Figure 3d,e). Therefore, the lower cell recovery and PLT contamination observed in MB/HY can only be explained by the two additional washing steps that were performed for these protocols but not for ST. Monocytes are well known for their fragility in such experiments, while lymphocytes are typically more resistant, hence the generally high-lymphocyte/low-monocyte viabilities seen in our hybrid protocol (Figure 3f). We would like to emphasize the ascending trend seen in Phase-2, where lymphocyte viability

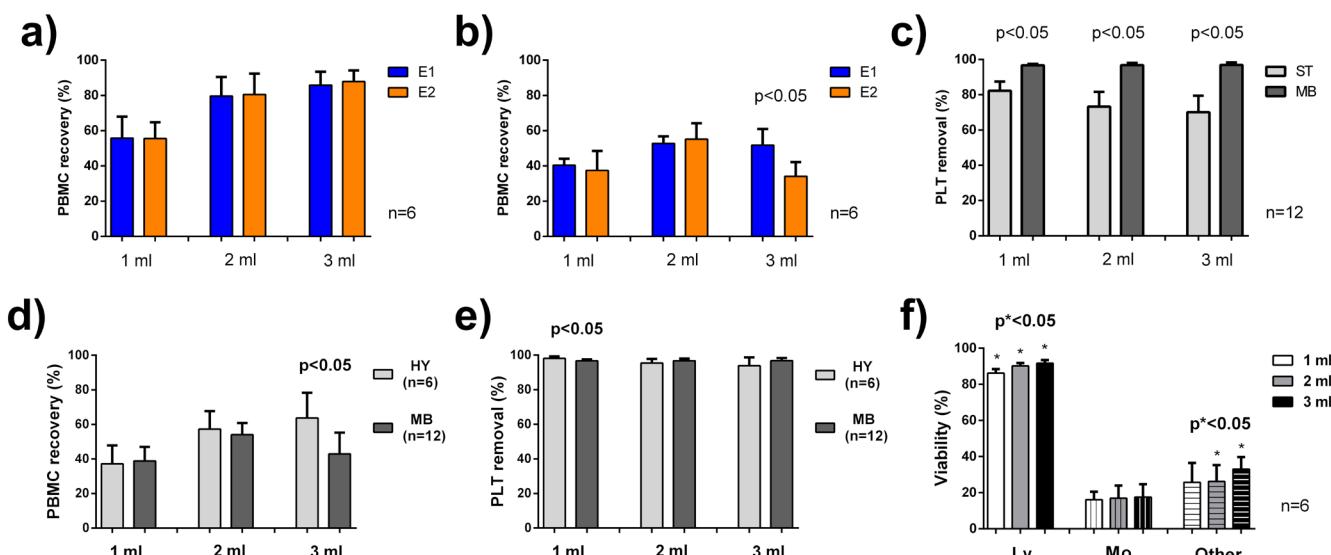


Fig. 3. Column bar graph representing average variation of recovery rates with sample volume for a) ST; b) MB; d) HY. Average platelet removal is compared between MB and c) ST and e) HY. Sub-figure e) shows average viability for cell populations as

ity significantly increases with blood sample volume. HY protocol's resemblance to MB in both design and outcome supports the idea that, if tested for viability, MB would yield similar results. Due to its single washing step, ST protocol may have higher levels of residual Histopaque-1077 which can cause cellular toxicity. On the other hand, ST may yield a higher viability since this protocol is notably faster and requires less centrifugation and cell manipulation than HY/MB. It's also worth mentioning that in our experiment, cells were resuspended and washed in PBS at RT while complex downstream applications require that washing steps be done with culture media. In this case, an increase in viability should be expected.

Blood sample volumes and variability

An important aspect of our study was the intra- and interindividual variability of the two examiners. Each of them processed a total of 36 samples that is 18 samples per protocol. For each protocol, the 18 samples were divided in 3 lots of 6, namely 1ml, 2ml and 3ml. Regardless of which protocol and/or examiner was considered, there were no relevant differences between cell recovery rates within any of the lots, meaning that both examiners showed insignificant intraindividual variability. Regarding the interindividual variability, it has been previously reported that differences up to 60% in cell recovery were recorded between a well-trained but inexperienced technician and a technician experienced in cell isolations [19]. In our experiment, the examiners were similarly trained/experienced and processed the samples at the same time in identical conditions. When the corresponding lots were compared between the two, we found no considerable differences in cell recovery, except for the 3ml lot from MB protocol (Figure 3a,b). The most likely cause of this disparity is that MB-3ml was the only lot where samples were processed in (2/3 empty) 50ml centrifuge tubes, thus making the buffy coat removal an even more challenging task. Nonetheless, we conclude that in our experiment, the degree of interindividual variability is negligible.

Another important aspect is the effect of blood sample volume on separation performance. We have chosen to investigate blood volumes of 1ml, 2ml and 3ml as such small volumes are likely obtainable in most clinical contexts. Moreover, assuming the donor's CBC is within normal range, even a 50% recovery rate should deliver roughly between 0.5 and 2.0 million PBMCs/ml of blood, which is enough for immunophenotyping purposes and other preliminary analyses and processing. In our study, regardless of the employed protocol, both examiners recorded the same ascending trend where cell recovery rates increased with blood sample volume (Figure 3a,b). This trend could also be observed in Phase-2 with the hybrid protocol for both cell recovery and viability (Figure 3d,f), but the extent and limitations of this effect remain to be further investigated.

Practical aspects

The low interindividual variability recorded in our experiment allowed us to cumulate the results from both examiners and perform a global and statistically more powerful analysis that further consolidated our conclusions. However, a series of aspects needs to be taken in consideration. Given that MB is considerably more expensive and time consuming than ST protocol, routinely employing MB for PBMC isolation is questionable unless low platelet contamination is required. In our study, the two examiners were similarly experienced/trained, hence the minimal intra- and interindividual variability. However, this is not always the case, especially with large studies employing both experienced and newly recruited personnel. Therefore, we stress that variability should not be overlooked. It was clearly shown that blood sample volume influences separation performance. Although some volumes proved superior to others, it is sometimes physically impossible or ethically questionable to collect the desired amount of blood. Also, any study involving PBMC isolation as a first step should take in consideration whether granulocytes and PLTs can at any point interfere and influence the results. Consequently, the most appropriate protocol should be chosen. Another question is when to stop washing the cells. It is obvious that additional wash cycles considerably reduce PLT contamination, but the absolute PBMC count also decreases with each cycle. Therefore, a convenient compromise should be reached between cell recovery and platelet contamination. Last but not least, obtaining viable cells is the main objective of most experiments involving PBMCs. During separation, PBMCs are exposed to chemical, mechanical and osmotic stress, hence the importance of minimal cell manipulation techniques and appropriate buffers and culture media. If satisfactory, the shortest and less stressful protocol should be employed as each minute of in vitro processing takes its toll on viability. We have already described the two critical steps of PBMC isolation by density gradient centrifugation and we suggest that individuals using this technique should be at least averagely trained and experienced in order to achieve satisfactory results.

A set of general indications was formulated to serve as a guideline for contextual adjustment of density gradient centrifugation protocols for PBMC isolation:

1. If kept within normal range, CF, CT and Blood: PBS: gradient medium ratio can be varied
2. MB protocol should not be employed on a routine basis unless otherwise specified
3. For maximum PBMC recovery, ST/ST-like protocol is recommended; if possible, use blood samples of 3ml in order to obtain a total amount of 1.8-12.0 million PBMCs (computed for the worst/best-case scenarios of a physiological CBC)
4. For minimal PLT contamination, a multi-wash-ST/HY protocol is recommended
5. For maximum viability, use samples of 3ml

Following this study, ST and HY protocols were successfully tested and implemented as SOPs in the CCAMF Cellular Immunology Laboratory. However, for future studies, these protocols may be applied for both healthy and pathological subjects. Selection of 'healthy' control subjects will require preliminary basic screening and subsequent exclusion of inflammatory status and/or abnormal CBCs. Depending on clinical context and each study's design, aims and particularities, pathological subjects may be either excluded or required to donate an increased amount of blood in order to obtain the desired number of target cells (e.g. leukopenia, abnormal leukocyte subpopulations distribution). However, further investigations are required in order to properly establish the applicability of ST/HY protocols for this particular cases.

Conclusions

Overall, this study provided us with a valuable insight on how density centrifugation separation protocols could be adjusted in order to meet the performance criteria required by various downstream applications. We confirm that both ST and MB are reliable protocols, yielding satisfactory cell recovery rates and purities. ST is considerably faster and cheaper than MB and also yields more cells while MB stands out for its exceptionally low PLT contamination. Multiple washing steps decrease both cell recovery and PLT contamination. The hybrid protocol is as good and solid as MB but less expensive and shows acceptable lymphocyte viability despite its many steps and long duration. Regarding sample volumes, we conclude that, up to at least 3ml, the larger the blood sample, the higher the cell recovery rates will be. Intra- and interindividual variability should not be an issue if the examiners are equally trained and experienced. Another conclusion is that differences in Blood: PBS: Ficoll ratio, CF and CT between ST and MB/HY may have little to no effect on cell recovery and erythrocyte/granulocyte contamination.

Conflict of interest

None to declare.

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