



Utility investigation of automated techniques in hematopoietic progenitor cell count and viability assessment in the Good Manufacturing Practice (GMP) setting

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ABSTRACT

Aim: To compare our parameters as regards: i) cell count via two different automated cell count techniques, and ii) viability via automated trypan blue exclusion and 7-aminoactinomycin D (7-AAD) staining.

Method: We used the trypan blue exclusion technique and an automated cell counter and for viability testing, and the trypan blue exclusion technique and the 7-AAD evaluation by flow cytometry. The trypan blue exclusion and the radio frequency techniques were used for automated cell counting. Flow cytometric analysis was performed by evaluating the yielded cellular products for 7-AAD uptake during the cell count of CD34+ cells.

Results: The mean values for cell count were estimated as $3.44 \pm 1.22 \times 10^6/\text{ml}$ (range, 2.48-5.71 $\times 10^6/\text{ml}$) and $4.14 \pm 1.94 \times 10^6/\text{ml}$ (range, 1.77-7.43 $\times 10^6/\text{ml}$) for the trypan blue exclusion and radio frequency techniques, respectively. Additionally, the mean values for viability analyses via the automated trypan blue exclusion and 7-AAD were $93.38 \pm 6.09\%$ (range, 79.00-98.00%) and $99.49 \pm 0.60\%$ (range, 98.40-100.00%), respectively.

Conclusions: Our study has responded to two fundamental questions: whether the results of both of the automated techniques for cell count correspond with each other, and whether the results of the automated viability assessment conform those of the 7-AAD technique during the manufacturing processes of cellular therapy products intended for clinical use. Even though we have the opportunity to use the hemocytometer in our laboratory setting, the automated trypan blue exclusion technique gives cell count results in concordance within the range of the expectations of our Quality Management System (QMS).

Keywords: 7-AAD, automated cell count, cellular therapy, quality, safety, viability.

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Introduction

Promising to be first-line therapy for many kinds of different diseases in the future, cellular therapies are gradually expanding as a treatment option in many clinics. Cellular therapies can be bluntly defined as *in vitro*-manipulated human cells which require certain safety and quality parameters as prerequisites – two of which are cell count and viability– at the time of release from the laboratory to the clinic. Cell counting techniques can either be performed manually (e.g. hemocytometer) or by the use of automatic devices which are operated by certain principles such as the automated trypan blue exclusion [1,2] and radio frequency [3,4]. Viability assessment is also possible via the trypan blue exclusion technique [5]. Also some manual methods– i.e. acridine orange, eosin staining can be used for viability detection [6]. Alternatively, the viability dye 7-aminoactinomycin D (7-AAD) can also be used to determine the number of viable CD34+ cells [7,8]. In this manner, automated techniques present themselves to be utile in performing both cell count and viability estimation, and when necessary being able to give both results realtime.

Previously, researchers made various comparisons of manual and automated cell count and viability techniques in different cell types [2,5,6,9-11]. However, none of these studies show comparison between automatic and/or manual cell count and viability techniques by means of hematopoietic progenitor cells. In this study, we aimed to compare our parameters as regards: cell count via two different automated cell count techniques, and ii) viability via automated trypan blue exclusion and 7-AAD staining. As noticed, studies practicing the comparison of different automated cell counting methods on human stem cells have not yet been performed.

Under the scope of the current Good Manufacturing Practices (cGMP) activities at Ankara University Stem Cell Institute Tissue and Cell Manufacturing Center, we obtained purified CD34+ hematopoietic stem cell products intended for use in patients mainly suffering from severe combined immune deficiency (SCID). We had previously reported our local experience with the CliniMACS (magnetic-activated cell separation system) in hematologic malignancies and immune failure disease. There, we evaluated our CliniMACS CD34+ cell enrichment process by revealing absolute cell count and viability besides other parameters for the end products [12]. This study has two objectives: one is to determine whether the two automated techniques' results for cell count match each other, and whether the automated cell counter results for viability match those of the 7-AAD technique during the manufacturing processes of cellular therapy products intended for clinical use. This is the first time that hematopoietic progenitor cell count and viability testing are compared between different automated techniques in order to suggest automated cell counters as simple-use devices with the ability to produce reliable and timely results.

Materials and Methods

As defined in the study of Kilic et al. [12], the apheresis products were transferred from Ankara University School of Medicine İbn-i Sina Hospital Therapeutic Apheresis Unit Center to our Tissue and Cell Manufacturing Center, within a sterile container, with the facility to transport at a stable temperature recorded by a data logger [12]. The records containing the results of complete blood count (CBC), CD34+ cell enumeration and viability obtained by flow cytometric evaluation were accompanied with each sample. The study was

approved by the Turkish Ministry of Health Turkish Medicines and Medical Devices Agency, with the approval number 2014/2 for manufacturing of human medical products. The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. Written informed consent was collected from each subject.

Samples were used to perform 8 separate CliniMACS CD34+ enrichment process cycles. The enrichment process was carried out as described in the CliniMACS® User Manual (CliniMACS User Manual), and was followed in accordance with the study of Leong et al. [9]. The CD34+ cell selection technique is used to deplete T cells from collected human-based cell products before allogeneic HSC transplantation. The CD34+ cells can be separated by various devices, one of which is the CliniMACS (Miltenyi, Biotec, GmbH, Bergish, Gladbach, Germany) [9,12-14]. The CliniMACS CD34+ cell enrichment process is performed within the Quality Management System of our Tissue and Cell Manufacturing Center, as described by Kilic et al. [12]. Cell count and viability are the most critical release criteria besides other tests, such as sterility and endotoxin test, for each CD34+-enriched end product. In the 8 cell count processes included in this study, we used the trypan blue exclusion technique and an automated cell counter for cell count, and for viability testing, the trypan blue exclusion technique and the 7-AAD evaluation by flow cytometry [12].

Cell count

Automated cell count via trypan blue exclusion

In the beginning, the trypan blue exclusion test was performed by the use of the TC20™ Automated Cell Counter [1]. This device provides cell counts within the range of 5×10^4

to 1×10^7 cells/ml [2]. Twenty microliters of the 0.04% trypan blue staining solution and 20 μ L of each sample were mixed within the test tube. Ten microliters taken from this mixture was pipetted and placed on the counting chamber. If the cell number exceeded 1×10^7 cells/ml, the samples were diluted with saline solution at the ratio of 1:9, and the count was repeated thereafter [6].

Automated cell count via radio frequency

The radio frequency principle was used as the second automated cell count technique (Sysmex Europe GmbH. Sysmex XN-3000). Devices operating under such principle are only certified for testing blood samples, hence do not guarantee use of other bodily fluids. The Sysmex XN-3000 is a fully automated complete blood count (CBC) hematology analyzer including 6-part differential count. This analyzer differentiates white blood cells (WBC) and tests 28 standard diagnostic CBC parameters. The XN-3000 processes 200 samples/hour and includes the SP-10 slidemaker/stainer for reflexive slide preparation [15]. Cells in an aliquot (1 cc) of each of the end products were automatically counted with the Sysmex XN-3000 Automated Cell Counter.

Viability

Automated cell counter via trypan blue exclusion

Viability was assessed via an automated cell counter, using the trypan blue exclusion technique [1]. The automated device was used after preparation of samples as explained in section “2.1.1 Automated Cell Count via Trypan Blue Exclusion”. In this technique, the automated cell counter detects the dead cells, which are instantly stained with the trypan blue, within the total cell population. The viability of

the cells is displayed on the screen in terms of percentage of viability.

The 7-aminoactinomycin dye (7-AAD) method

Flow cytometric analysis was performed at Ankara University Hematology Laboratory as explained in the studies of Varan et al. [6] and Kilic et al. [12]. Briefly, the yielded cellular products were evaluated for 7-AAD uptake during the flow cytometric count of CD34+ cells. At the end of the CD34+ enrichment process, the end products were evaluated for cells expressing CD34 and also for CD45, CD3 CD56, CD19, and CD14 to further characterize the cell content of the product. Cell viability was checked for each sample using the viability dye 7-AAD, and all counts were reported in terms of viable cells. The Kaluza software ver2.1 (Beckman Coulter Miami, USA) was used to analyze the collected data using the Navios 3L10C device (Beckman Coulter Miami, USA). CD34+ cell counts were calculated according to the single platform ISHAGE protocol [16]. The statistical data of the charts were retrieved from the statistical results of the report and the ratio of dead cells (cells stained with 7-AAD) was determined. The percentage of living cells was determined by subtracting the percentage of dead cells from 100.

Statistics analysis

Statistical analysis was performed by using the SPSS 22 version package program. Correlation between cell count and viability results,

obtained from different methods, were tested using the 2-tailed Pearson correlation analysis ($p=0.05$).

Results

An example of three consecutive cell count and viability results obtained by our automated cell counter is presented in Figure 1.

The distribution of the cells was checked in two-dimensional dot plot graph (SSC vs 7-AAD) and upon gating of 7-AAD unstained viable cells this gate was applied to a CD45-SSC dot plot graph (Figure 2).

Cell count and viability results for the CD34+ end products are summarized in Table 1.

Mean values for cell count were estimated as $3.44 \pm 1.22 \times 10^6/\text{ml}$ (range, 2.48 - $5.71 \times 10^6/\text{ml}$) and $4.14 \pm 1.94 \times 10^6/\text{ml}$ (range, 1.77 - $7.43 \times 10^6/\text{ml}$) for the trypan blue exclusion and radio frequency tests, respectively. Additionally, viability mean values for the automated trypan blue exclusion and 7-AAD were $93.38 \pm 6.09\%$ (range, 79.00 - 98.00%) and $99.49 \pm 0.60\%$ (range, 98.40 - 100.00%), respectively.

Discussion

Automated techniques facilitate the work load of researchers by requiring less time for analysis and no need for complementary devices, and leave negligible effort. There is debate about the efficiency between the current automated cell count techniques. This issue has

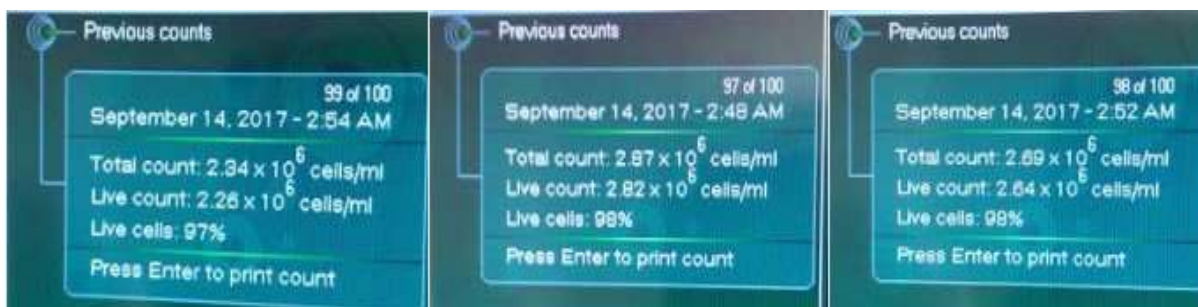


Figure 1. An example of three consecutive cell count and viability results obtained by the use of the automated cell counter.

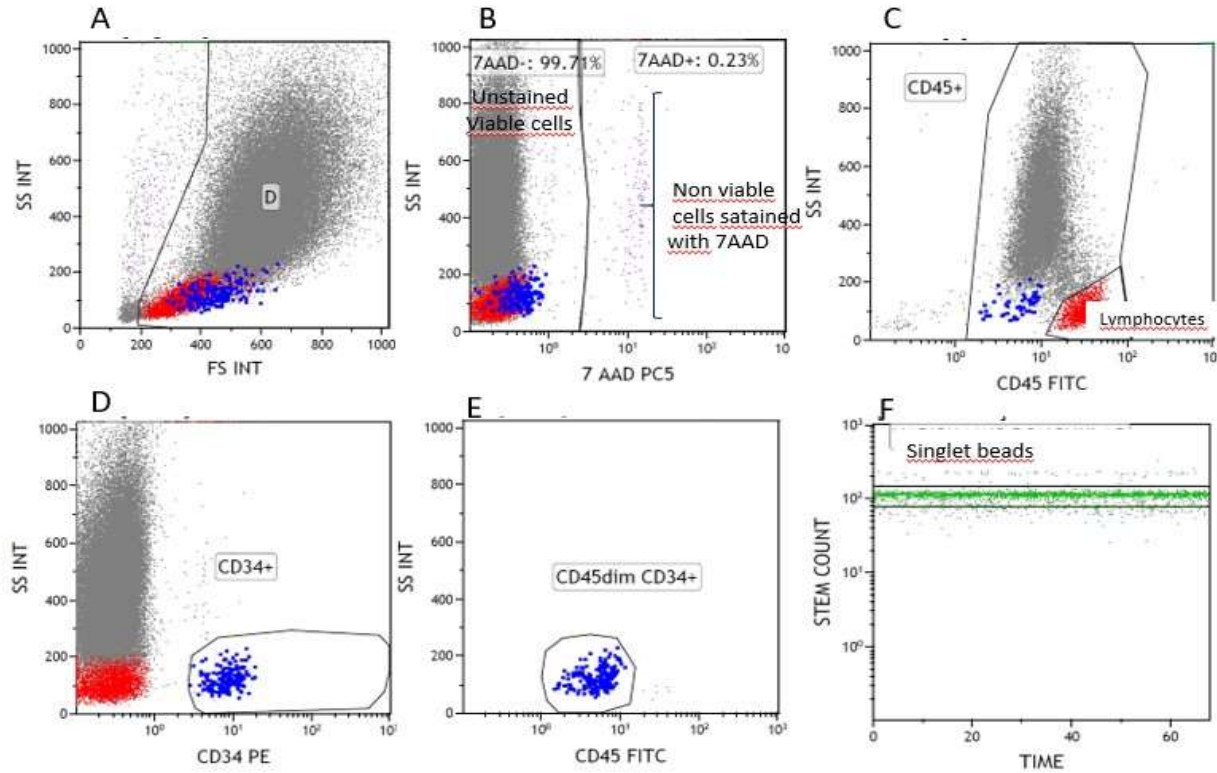


Figure 2. Before starting the enrichment of the hematopoietic stem cells; CD34⁺ cell count was performed using the ISHAGE protocol: A: The artifacts were eliminated from the leukocytes, B: The viable cells that were unstained were selected, C: CD45⁺ cells were selected from viable cells, D: CD34⁺ cells among viable CD45⁺ cells were marked, E: CD45^{dim+} cells were selected from CD45⁺ and CD34⁺ viable cells and upon checking for particles smaller than lymphocytes (unseen) the actual viable CD34⁺ cell numbers were detected. As seen in F, non-adhered beads, pipetted into the same tube, were selected and used for the absolute count of CD34⁺ cells using the single platform analysis.

Table 1. Cell count and viability results by means of the two automated cell count techniques, and by means of an automated cell counter and flow cytometric evaluation by 7-AAD.

	Cell Count		Viability	
	Automated trypan blue exclusion (x10 ⁶ /ml)	Automated radio frequency (x10 ⁶ /ml)	Automated trypan blue exclusion (%)	7-AAD (%)
1	2.80	3.46	95.00	99.00
2	2.63	3.00	98.00	99.50
3	2.60	3.50	92.00	99.90
4	4.87	4.97	94.00	100.00
5	3.68	7.43	96.00	99.10
6	2.71	1.77	79.00	98.40
7	2.48	2.68	96.00	100.00
8	5.71	6.34	97.00	100.00

been addressed in a number of studies. The use of such techniques force the researchers to analyze the reliability of the automated systems. A number of previous studies have compared the various cell count and viability assessment methods [5,6,9-11,17].

Several studies have focused on cell count and viability of human cells. Leong et al. [9] used the flow cytometry technique for counting CD34+-selected hematopoietic stem cells and a manual trypan blue exclusion technique via the Neubauer chamber for viability assessment. We analyzed the same kind of cells and used the 7-AAD for viability assessment. However, while they preferred the manual technique for trypan blue exclusion, we used an automated cell counter. In the study of Leong et al. [9], the CD34+-selected products showed a median viability of 98%. (range 92 - 99%). We found only one published study which specifically addresses comparison of various techniques for cell count. Nevertheless, this study was not performed on human stem cells [11]. Until today, only two reports have studied the techniques on human stem cells on a viability perspective [6,17].

With the trypan blue exclusion method, Humpe et al. [17] detected the mean viability as 95.8% (range, 72.6%-98.7%) for 8 patients, with slight similarity to our automated viability results, 93.38±6.09% (range, 79.00-98.00%). Like us, Varan et al. [6] studied the comparison of viability results belonging to hematopoietic progenitor cells in 20 samples by trypan blue uptake and measurement of 7-AAD staining by flow cytometry. However, no remarks were made on cell count [6]. The median viability obtained by the 7-AAD was 78±16%, much lower from our 7-AAD results, of 99.49±0.60% (range, 98.40-100.00%). The Intraclass Correlation Coefficient (ICC) between the trypan blue and 7-AAD methods was found as

0.47 ($p > 0.05$) in the study Varan et al. [6], and no statistically significant concordance was detected. In our study, no significant correlation was detected between viability results obtained by the two different techniques, the automated cell counter and the 7-AAD. Reich-Slotky et al. [10] determined the number of CD34+ cells by flow cytometry and the viability by trypan blue uptake and by the measurement of 7-AAD staining using flow cytometry. The average viability was 98.8% with trypan blue exclusion, and 97.0% with 7-AAD [10]. Our study suggested mean values of viability obtained by the automated cell counter as 93.38±6.09% (range, 79.00-98.00%) and of 7-AAD as 99.49±0.60% (range, 98.40-100.00%). Such values were respectively lower and higher than that of Reich-Slotky et al. [10]. These findings are controversial and thus make it questionable as to which method would be more reliable.

Previously, there have been different results comparable with ours obtained from mammalian cell types of non-human origin. Similar with our study, Camacho-Fernández et al. [11] performed cell counting by comparing manual and automated techniques in isolated eggplant microspore cultures. This study is significant as it has compared several techniques for cell count. In our study, when the two different automated cell counters were compared by means of cell count results, a significant correlation value of 0.72 was observed, in concordance with that of Camacho-Fernández et al. [11]. On the other hand, Kwizera et al. [5] counted *Cryptococcus* yeast cells in cerebrospinal fluid culture by trypan blue staining and rapidly quantified viable cells with an automated cell counter. The study of Kwizera et al. [5] is the first article practicing the comparison of different automated cell counting methods on human

stem cells and mainly focusing on the validation of the repeatability of results.

Although human donor-based, cellular therapies are classified as human medicinal products, they differ from the conventional medicines in the sense that each batch release is equivalent to one donor per manufacture. In this manner, it is not so easy to reach high sample sizes as it would be for serial manufacturing. Additionally, one end user, a patient, has to be matched with the appropriate donor in order to start manufacturing a cellular therapy product. This does not always happen in a serial manner, which also contributes to the bottleneck of reaching high sample sizes. In the setting of our study, the current sample size for hematopoietic stem cell manufacturing is 8. We plan further studies to enhance this sample size and continue our studies in depth.

Conclusion

In conclusion, our study has responded to two questions at the same time: whether the results of both automated techniques for cell count correspond with each other, and whether the results of the automated viability assessment conform those of the 7-AAD technique when manufacturing cellular therapy products intended for clinical use. Even though we have the opportunity to use the hemocytometer in our laboratory setting, the automated cell counter that make use of trypan blue exclusion gives the cell count results in concordance within the range of the expectations of the Quality Management System (QMS) of our Tissue and Cell Manufacturing Center. However, for viability results, the 7-AAD technique, which is already validated at our premises, might be more accurate when products are intended for clinical use.

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Ethical statement: The study was approved by the Turkish Ministry of Health Turkish Medicines and Medical Devices Agency, with the approval number 2014/2 for manufacturing of human medical products.

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