

**METHODOLOGICAL ARTICLE**

**COLONY-FORMING UNIT ASSAY AS A POTENCY TEST FOR  
HEMATOPOIETIC STEM/PROGENITOR CELL PRODUCTS**

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**Abstract:** Colony-forming unit (CFU) assay is a short-term culture assay used for the detection of functionally active hematopoietic progenitor cells (HPCs) in the *in vitro* setting. This potency assay enables the identification of colony-producing HPCs in any type of hematopoietic stem cell/progenitor product (HSC/P) including bone marrow (BM), cord blood (CB) and mobilized peripheral blood (MPB). It has been shown that the frequency of HPCs in BM, MPB and CB cellular products directly correlates with the engraftment of both neutrophils and platelets as well as with the overall survival of recipients following hematopoietic stem cell transplantation which makes the CFU assay a good quality parameter for the prediction of engraftment success. The aim of this article is to provide an overview of different approaches to setting up the CFU assay with an emphasis on different sample preparation techniques, cell plating density and colony identification and enumeration used by different laboratories.

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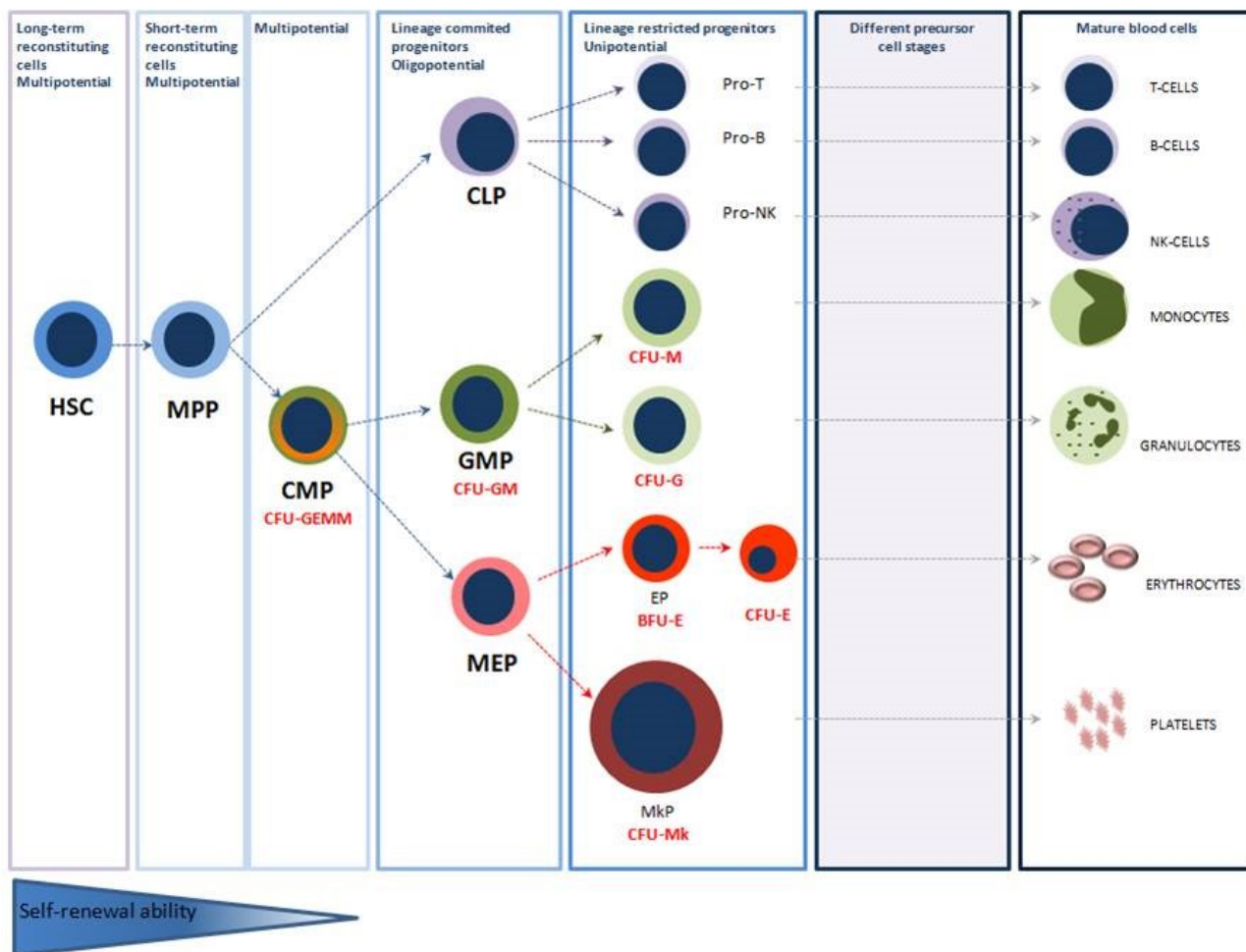
**Key words:** CFU assay, hematopoietic progenitor cell, potency test, bone marrow, mobilized peripheral blood, cord blood, CFU-GM, BFU-E, CFU-GEMM

**INTRODUCTION**

During adulthood, the majority of multipotent hematopoietic stem cells (HSCs) reside in bone marrow (BM). There they sustain the balance between the processes of self-renewal and differentiation, thus at the same time providing the pool of cells with self-renewal ability and the progeny of cells that proceed further into differentiation. Differentiation of HSCs to mature blood cells is a multiphase process that includes several intermediate stages. Multipotential progenitors give rise to committed progenitors of the two main branches of the hematopoietic lineage; common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) (Figure 1). Downstream differentiation of CMPs and CLPs results in lineage-restricted progenitors that subsequently differentiate to completely functional mature blood cells.<sup>1</sup>

Although the best demonstration of the existence and function of HSCs *in vivo* is the hematopoietic recovery of a myeloablated recipient after HSC transplantation (HSCT), several *in vitro* assays have been developed in order to provide a comprehensive insight into the biological properties, activity and quantity of HSCs.

The most primitive HSCs can be identified in long-term culture (LTC) assays that are performed by culturing hematopoietic cells on an adherent monolayer of stromal cells during several weeks. In this model, stromal cells derived from human BM promote survival, self-renewal, proliferation and differentiation of so-called long-term culture-initiating cells (LTC-ICs). Following 5-8 weeks of cell culture, newly produced non-adherent cells can be collected and cultured in a short-term culture assay in order to determine the clonogenic potential of initial LTC-ICs.<sup>2</sup> Another variant of the LTC assay is the cobblestone area-forming cell (CAFC) assay in which phase dark areas of proliferating cells („cobblestone areas“) are visually detected beneath the stromal layer after several weeks of culture. The CAFC numbers have been shown to directly correlate to the



**Figure 1. Schematic representation of hematopoietic development.**

Legend: HSC - hematopoietic stem cell; MPP - multipotential progenitor; CMP - common myeloid progenitor; CLP - common lymphoid progenitor; GMP - granulocyte/macrophage progenitor; MEP - megakaryocyte/erythroid progenitor; EP - erythroid progenitor; Mkp - megakaryocyte progenitor. Depending on the formulation of the culture medium, colony-forming unit (CFU) assay can identify multipotential progenitor (CFU-GEMM, CFU-granulocyte, erythroid, monocyte/macrophage, megakaryocyte) and lineage-directed progenitors (BFU-E - burst-forming unit erythroid; CFU-E, CFU-erythroid; CFU-M, CFU-monocyte/macrophage; CFU-G, CFU-granulocyte; CFU-Mk, CFU-megakaryocyte).

BM repopulating ability in the murine model. However, in the case of human samples the results of CAFC assays showed variable results depending on the type of sample and the feeder layer used.<sup>3</sup>

Although LTC assays can provide important information about HSC biological properties, their use is mainly limited to scientific research. On the other hand, the short-term culture assay, popularly called the colony-forming unit (CFU) assay, is frequently used as a potency test in the case of HSCT. The CFU assay was developed with the aim of detecting hematopoietic progenitor cells (HPCs) that originate from HSCs.<sup>4</sup> It enables the identification of multipotential CFU-granulocyte, erythroid, monocyte/macrophage, megakaryocyte (CFU-GEMM) progenitors that have multi-lineage differentiation potential and limited self-renewal ability (Figure 1). Furthermore, the CFU assay enables the detection of more mature lineage-restricted progenitors including CFU-granulocyte/macrophage (CFU-GM, CFU-M and CFU-G), burst-forming unit

erythroid (BFU-E) and CFU-erythroid (CFU-E) that lack self-renewal ability and can differentiate into cells of one or two hematopoietic lineages (Figure 1).<sup>5</sup>

Since its introduction into transplantation medicine, the CFU assay has become the benchmark *in vitro* assay that can be used for the detection of biologically active HPCs in any type of hematopoietic stem cell/progenitor product (HSC/P) including BM, cord blood (CB) and mobilized peripheral blood (MPB). The quality of a HSC/P product is usually assessed at several different processing and storage points in order to monitor the effect of manipulations such as volume reduction, red blood cell (RBC) removal, cryopreservation and thawing on viability and functional capacity of the cells.<sup>5-8</sup> In this regard, one of the most important quality assessment parameters is CD34-positive cell count.<sup>6</sup> The CD34 marker, the so-called “stem cell marker”, is expressed by a very small proportion of HSCs. The majority of CD34-positive cells in HSC/P products are HPCs, and not all of them participate in the engraftment

process following transplantation. In this regard, CFU assay can provide information about the quantity of functionally active CD34-positive HPCs that are capable of producing hematopoietic colonies. In this way, CFU assay can be used to predict engraftment success together with other product quality parameters. For this reason, many transplant centres include the CFU assay in the quality control of HSC/P products despite the fact that its results are a release criterion only in the case of CB products.<sup>6</sup>

The aim of this article is to provide an overview of the CFU assay as a tool for defining HPC content in human cell products with an emphasis on different culture techniques and colony enumeration approaches used by different laboratories.

## SETTING UP THE CFU ASSAY

The CFU assay is usually performed by culturing a sample of HSC/P product in a semi-solid matrix supplemented with a combination of recombinant human cytokines. Different types of complete media formulated for optimal growth and differentiation of hematopoietic cells are now commercially available. The matrix of choice in most media is methylcellulose since it provides optimal viscosity necessary to support the formation of colonies derived from individual progenitor cells. Furthermore, methylcellulose is chemically inert and stable in the environment with a variable pH.<sup>8</sup> Nevertheless, agar- and collagen-based media are also available and can be used for the detection of pure megakaryocyte progenitors since their colonies normally do not grow in methylcellulose.<sup>9</sup> Proliferation and differentiation of progenitor cells during culture are stimulated by the presence of recombinant cytokines in the culture medium. Such a cocktail of cytokines usually includes, but is not limited to, stem cell factor (SCF), granulocyte macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), interleukin 3 (IL-3) and erythropoietin (EPO). The choice of cytokine formulation of the medium depends on the purpose of the CFU assay.<sup>10</sup>

### **RBC depletion**

Samples collected from BM and CB products with high hematocrit levels usually have to be processed prior to setting up the CFU assay. Large number of RBCs in short-term cell culture can interfere with colony growth and reduce the accuracy of the CFU assay. Therefore, in such cases RBC depletion needs to be performed.

In the case of BM samples, RBCs can be lysed with ammonium chloride or, alternatively, isolation of BM mononuclear cells by density gradient separation can be performed. The latter method provides cell suspension enriched with hematopoietic progenitors deprived of interfering RBCs, while cell suspension obtained by

RBC lysis provides all leukocyte populations. Difference in the content of cell populations resulting from different methods of cell suspension preparation should be considered when adjusting cell seeding density.

The gravity sedimentation using RBC aggregating agents is a standard method of CB processing by use of an automated system. However, depending on the processing method used, hematocrit of post-processed CB units might remain high. In such cases, further depletion of RBCs in CB samples is required before setting up the CFU assay. There are two common methods of eliminating RBCs from CB samples. One strategy includes the use of a gravity sedimentation medium that enables the isolation of nucleated cells from CB samples by promoting the formation of RBC aggregates that sediment much faster than nucleated cells. The other strategy includes magnetic depletion of RBCs using immunomagnetic particles that bind to Glycophorin AB+ cells. This method is less laborious and time consuming and it has shown good results in both fresh and frozen CB samples.

In the case of MPB samples, the RBC depletion step is not needed as the hematocrit level in this type of products is very low.

The choice of the RBC depletion method used for preparing the cell suspension for setting up the CFU assay greatly depends on the expectations from the assay results. If the expected information is just the qualitative result (growth/no growth of CFU colonies), any of the described methods of sample preparation will be appropriate. However, if the expected information is the quantitative result that could be correlated with absolute CD34-positive cell number, then a more volumetric approach to setting up the CFU assay should be considered.

### **Plating cell density**

Plating cell density should be adjusted according to the cell source (Table 1). It is considered that cultures with 20-80 colonies per dish ensure statistically accurate enumeration. Underplating of cells can result in a small number of colonies which can lead to large variations between replicates. On the other hand, overplating of cells can result in overgrowth of colonies which can cause difficulties in identifying individual colonies and/or inhibit colony growth due to excessive consumption of nutrients in the medium. Therefore, in order to set up the optimal CFU assay strategy in any laboratory, validation studies focusing on the methods of cell suspension preparation and cell plating density should be performed.

If the aim of the CFU assay is only to demonstrate the presence of biologically active HPCs in a certain HSC/P product, then the initial plating cell density should be adjusted to those expectations. Following the initial RBC depletion step where needed, plating cell density should be adjusted according to the cell source as shown

**Table 1. Recommended plating concentrations for different cell samples**

HSC/P product	TNC/ml of complete media
BM - RBC depleted	$5 \times 10^4$ ( $2 \times 10^4 - 1 \times 10^5$ )
BM - mononuclear cells	$2 \times 10^4$ ( $1 \times 10^4 - 5 \times 10^4$ )
CB - RBC depleted	$2 \times 10^4$ ( $1 \times 10^4 - 4 \times 10^4$ )
MPB - mononuclear cells	$2 \times 10^4$ ( $1 \times 10^4 - 5 \times 10^4$ )
CD34+ selected cells (BM, CB, MPB)	500 ( $500 - 2 \times 10^3$ )

Modified according to Helgason et al. (5) and Technical Manual: Human Colony-Forming Cell (CFC) Assays Using MethoCult, Version 4.0.0 (STEMCELL Technologies)

Legend: HSC/P - hematopoietic stem cell/progenitor; TNC - total nucleated cells; BM - bone marrow; RBC - red blood cells; CB - cord blood; MPB - mobilized peripheral blood

in Table 1. In this case, the adjustment of cell concentration can include several dilutions in order to achieve the final concentration of cells in the culturing semi-solid medium.

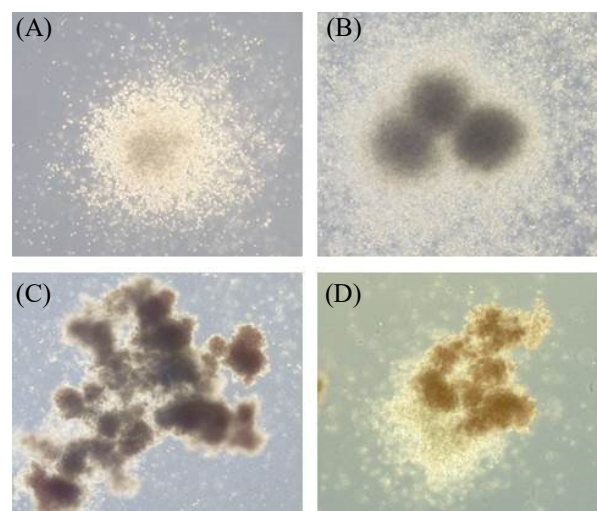
On the other hand, if the aim of the CFU assay is to provide the number of CFU colonies per volume of HSC/P product and correlate it with the absolute number of CD34-positive cells per volume, then multi-dilution approach is not appropriate because with every further dilution the number of cells deviates more from initial cell concentration in the product. Although one dilution step is almost always necessary, multiple dilutions should be avoided. In the case of BM and CB samples with high hematocrit levels, the RBC depletion step is inevitable. In this situation the immunomagnetic RBC depletion approach should be considered since it includes only one dilution step and provides better approximation of CFU-producing HPCs in the product. In the case of MPB samples, the concentration of cells in the product is usually very high, and the volume of cells that should be transferred directly to cultivating media using semi-automatic pipette is too low to measure. Therefore, in these samples, one dilution step is also needed in order to obtain the cell concentration in the volume unit that can be collected with a semi-automatic pipette.

### Enumeration of colonies

The majority of hematopoietic colonies growing in culture achieve their maximum number and size after approximately 14 days, when they can be identified based on their morphology. If the information about the total colony number needs to be obtained within a shorter time frame, the CFU assay can be performed using a specially formulated medium that enables the enumeration of colonies after 7 days of culture. In this type of CFU assay, the identification of different colony types is not possible since colonies are much smaller and contain less differentiated cells.<sup>8</sup> Therefore, this type of

assay can only provide the total number of colonies but cannot give information about progenitor lineages.

In standard 14-day CFU assays, normal CFU-GM colonies consist of at least 40 granulocyte and macrophage cells that commonly spread out from a distinct dense colony centre (Figure 2A). In some cases, one CFU-GM colony can consist of multiple clusters that are grouped together (Figure 2B). BFU-E and CFU-E colonies are easily recognizable due to their red or brownish color that is derived from hemoglobin.<sup>8</sup> BFU-E colonies arise from more immature progenitors and although they can appear as only one compact cluster, they usually form several clusters or bursts (Figure 2C). On the other hand, CFU-E colonies are derived from more mature erythroid progenitors and are typically found in BM samples. They contain up to 200 erythroblasts in one or two clusters.<sup>8</sup> Colonies derived from multipotential CFU-GEMM progenitors are usually more abundant in CB samples. A typical CFU-GEMM colony is often large and contains both cells of erythroid and non-erythroid lineages including granulocytes, macrophages and megakaryocytes (Figure 2D).



**Figure 2. Hematopoietic colony identification under inverted microscope (10x objective). (A) Colony derived from CFU-GM with one dense centre. (B) Multi-cluster colony derived from CFU-GM. (C) Multi-cluster colony derived from BFU-E. (D) Colony derived from multipotential CFU-GEMM progenitor.**

Enumeration of colonies is usually performed by trained individuals using an inverted microscope. Nevertheless, manual scoring of colonies is inherently subjective, which implies that inter-individual variations associated with colony counting can never be completely eliminated.<sup>10</sup> Recently, automated imaging instrument equipped with sophisticated image acquisition and analysis software that identifies, classifies and counts hematopoietic colonies has been developed (Figure 3). This automated system has been designed with the aim to standardize the enumeration of colonies, enhance



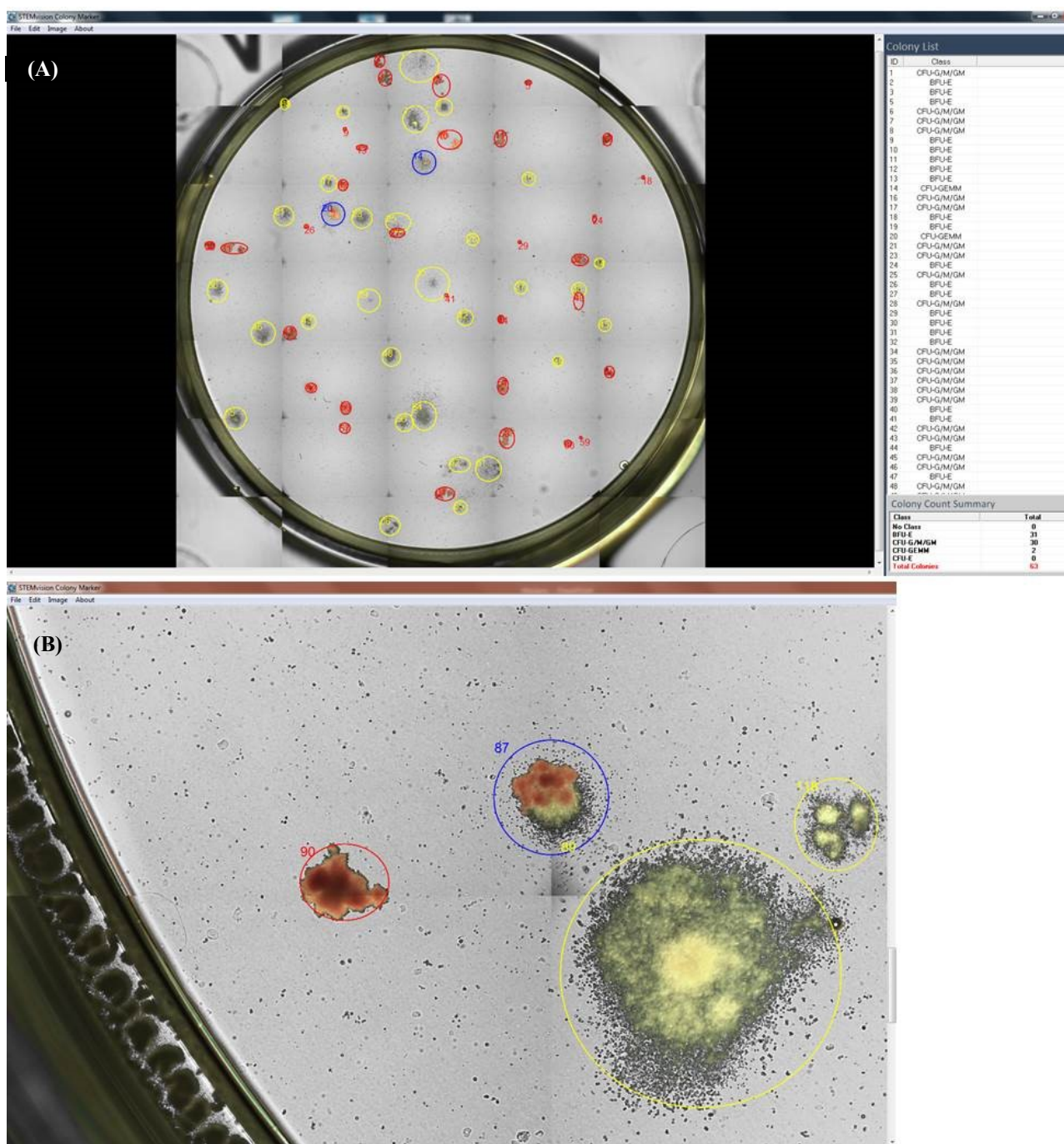


Figure 3. Image and analysis of a CFU assay using automated CFU assay reader STEMvision™ (StemCell Technologies). (A) Display of colony analysis results in STEMvision™ Colony Marker Application. (B) Enlarged view of different types of hematopoietic colonies.

reproducibility of the CFU assay and improve inter-laboratory comparability of the results.

## CONCLUSION

Although the amount of CD34-positive cells in transplanted HSC/P products usually correlates with the engraftment in the patients, in some cases the potency of the transplant is lower than expected, which might

consequently result in graft failure.<sup>11</sup> Therefore, if the quality assessment of the HSC/P product is based primarily on the flow cytometric analysis of CD34-positive cells, the information about the real potency of the graft is lacking.<sup>12</sup> This is the rationale for use of functional assays like the CFU assay which can give a much better correlation with potency. It has been shown that the frequency of HPCs in BM, MPB and CB cellular products directly correlates with engraftment of both neutrophils and platelets as well as with the overall survival of recipients following HSCT.<sup>13-17</sup>

However, performing this assay can be very challenging from the technical point of view for several reasons. For each tissue source, thorough validation procedures need to be performed to ensure that the assay results will not over- or underestimate the potency of the tested product. The number of the produced colonies depends on the plating cell density and on the CD34-positive cell content in the HSC/P product. The ideal situation would be to adjust the plating density according to the CD34-positive cell count. However, due to technical or organizational difficulties this information is not always available at the time of the CFU assay set-up, and in such cases the plating density has to be adjusted according to the total nucleated cell (TNC) count instead.

Another issue is that cell plating, enumeration and identification of colonies is usually performed manually, which also contributes to subjectivity and, consequently, to a high level of variation in inter-laboratory results. In this regard, the proficiency testing program for the CFU assay has been developed. This program is aimed at critical points in setting up the CFU assay and enables inter-laboratory comparison of the obtained results. In addition to that, an automated imaging system has been designed with the aim of increasing the objectivity of the enumeration and identification of colonies. Although this is a step forward in the standardization of CFU assay results, there is only one type of automated CFU assay reader commercially available at the moment (STEMvision™, StemCell Technologies).

In conclusion, the CFU assay is a laborious and time consuming assay but it provides valuable and relevant information about biological properties of the HSC/P product which could be correlated with engraftment success. The CFU assay can indicate the real potency of the graft and should be included in the quality assessment of all HSC/P products. Therefore, in order to ensure that the engraftment process does not fail because of low HSC/P potency, the decision about the release of the HSC/P product for clinical transplantation should be based not only on the CD34-positive cell content but on the graft potency as well.

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