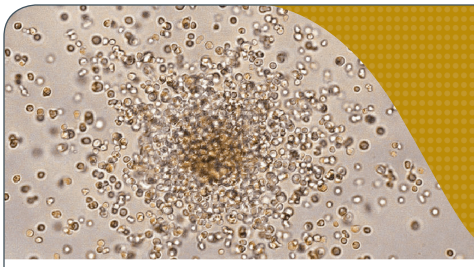

WHITE PAPER

# "Potency" Assays for Measuring the Engraftment Potential of Hematopoietic Stem and Progenitor Cells

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## Introduction

The transplantation of hematopoietic stem cells (HSCs) obtained from bone marrow (BM), mobilized peripheral blood (MPB) and umbilical cord blood (CB) has become an established therapy to regenerate the immuno-hematopoietic system in cancer patients following ablative chemo/radiotherapy and in patients with non-malignant hematological disorders. An important question in this field is how to measure the number of hematopoietic stem and progenitor cells (HSPCs) in a cellular product prior to its infusion. This information can be used to assist in graft selection and, ideally, to prospectively predict the likelihood of successful engraftment. While numerous "potency" assays have been adopted for this purpose, investigators disagree about the value of the different methods used, and there remains a critical need for assay standardization. These assays are particularly important for CB transplantation because most allogeneic CB units currently stored in public banks contain low numbers of viable HSPCs and are thus unsuitable for transplantation, particularly of adults.[1,2] Data from the National Marrow Donor Program (NMDP) presented at the 2012 Cord Blood Symposium indicated that ~80% of currently stored CB units fall into this category. The need to improve the quality of the CB inventory should drive adoption of the most biologically informative and accurate potency assays but this will need to be balanced against the cost and practicality of performing each type of assay in a routine lab setting. Cord blood banks are facing increased global competition and cost pressures due to constrained funding. Clearly, continuing to expend increasingly limited resources to bank unsuitable CB units is not sustainable. The choice of assay can be used by CB banks to champion their products to make them more attractive for transplant physicians and parents. This in turn should increase profitability and improve the quality of the worldwide CB repository. This article reviews the assays that are most commonly used to measure the "potency" of hematopoietic cell grafts, with specific consideration given to what these assays do and do not measure, and summarizes key studies that demonstrate a positive correlation between these assay end-points and the rate and success of hematopoietic engraftment.

## Hematopoietic Stem Cell Transplantation

All mature blood cells are produced by a small population of multi-potential hematopoietic stem cells that reside primarily in the BM in adults. In the late 1950's, it was first shown that HSCs could be harvested from the BM of a donor, transplanted into a patient whose own hematopoietic system had been ablated by chemotherapy or radiation, and that the transplanted cells could regenerate the blood and immune systems.[3] Stem cell transplantation is now a widely used therapy for a variety of malignant and non-malignant hematological disorders. HSCs can also be collected from "mobilized" peripheral blood following treatment with agents that stimulate the migration of stem/progenitor cells from the BM into the circulation (e.g. granulocyte colony-stimulating factor, or the small molecule drug Mozobil® [Plerixafor]). Umbilical cord blood has become recognized as a rich source of HSCs and is now often cryopreserved for future use by parents who elect to bank their child's cells for autologous transplantation, or who donate the cells to a public bank for allogeneic transplantation.

Engraftment following HSPC transplantation is typically considered to be successful when the numbers of neutrophils and platelets in the circulation have recovered to a sufficient level that the patient no longer requires treatment with supportive drugs (e.g. human growth factors, antibiotics) or blood products (e.g. red cells, platelets). Typically, this is defined as >500 and >50,000 neutrophils and platelets per µL of blood, respectively. Successful treatment for the underlying hematological disorder (usually cancer) is defined by the duration of disease-free and overall survival.



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Hematopoietic cell grafts are typically manipulated in different ways in the laboratory before transplantation. Common procedures for CB-derived grafts involve automated processing to reduce the overall volume to a size that can be more readily stored and infused. Removal of red blood cells is also advantageous as many are lysed during cryopreservation. Infusion of this erythrocyte lysate can increase the likelihood of renal and/or cardiotoxicity in the patient. T lymphocytes can be depleted from allogeneic grafts to reduce the possibility of graft-versus-host disease. Alternatively, T cells can also mediate beneficial graft-versus-leukemia effects that improve survival. Processed hematopoietic cells are often cryopreserved for future use. This procedure and the subsequent thawing and washing steps often result in reduced recovery of viable $CD34^+$ cells and functional progenitors. In order to monitor the effects of these different manipulations on HSPCs, it is critical that appropriate assays be used to assess the functional potential of the final cell product for transplantation.

## What Assays Can Be Used to Quantitate Hematopoietic Stem and Progenitor Cells?

Hematopoietic cell grafts can be analyzed using several different assays to determine their content of HSPCs (Table 1). This information is useful for the purposes of quality control in CB banks and transplant labs where it is critical to determine the impact of various manipulations and cryopreservation on progenitor cell viability and number, and to assist in predicting engraftment potential. Such tests are often referred to as "potency" assays. This term is imprecise, however, and a better approach is to use terminology that describes the property being measured in operational terms. Stem/progenitor cell assays can be broadly categorized into two types: phenotypic and functional assays. Phenotypic assays measure a physical property of the cells, most commonly the expression of a cell surface or intracellular protein that is

**TABLE 1.** "Potency" assays commonly used to evaluate hematopoietic cells for transplantation.

| ASSAY END-POINT | TYPE (METHOD) | TIME TO RESULT | ADVANTAGES | DISADVANTAGES | WHAT ASSAY MEASURES | WHAT ASSAY DOES NOT MEASURE |
|---|---|---|---|---|---|---|
| TNC | Cell count (hemacytometer or automated cell counter) | ~10 mins | • Simple<br>• Fast<br>• Inexpensive | • Low biological relevance | Viable nucleated cells | Stem/progenitor cell number or function |
| $CD34^+$ cells | Phenotypic (flow cytometry) | ~3 hrs | • Fast<br>• Standardized kits | • Misses apoptotic cells<br>• Phenotypes can change<br>• High instrument cost<br>• High variability | Cell surface marker expressed on most HSPCs | Stem/progenitor cell function |
| CFU | Functional (in vitro culture) | 7-14 days | • Biological read-out<br>• Standardized reagents | • Long assay duration<br>• Variability of manual colony counting | Viable and functional progenitor cells | Long-term repopulating HSCs |

**TNC:** total nucleated cells; **CFU:** colony-forming unit; **CD34:** cluster of differentiation antigen 34

present at higher levels on more primitive than differentiated cells. Functional assays measure a biological property of the cells, such as the ability to proliferate and differentiate into mature blood cells in vitro or in vivo. A key advantage of functional assays for evaluating hematopoietic cells intended for transplantation is that these latter properties are directly relevant to engraftment.

The simplest method used routinely for assessing hematopoietic cell grafts is to count the total number of viable nucleated cells (TNC). This is typically done by staining with vital dyes (e.g. trypan blue) that are passively absorbed across the cell membrane of dead or damaged cells but are not quickly absorbed by healthy living cells. The TNC content (or overall size) of a graft predictably correlates with successful engraftment following transplantation. This finding naturally drives physicians to select larger grafts for transplantation. TNC counts alone are of limited value, however, because the stem and progenitor cells that mediate engraftment represent only a very small fraction of non-purified BM or blood cells. As the frequency (percent) of stem and progenitor cells in a cell sample varies, for example between individual donors or between fresh vs. thawed cryopreserved cells, the reliability of the TNC count alone as an accurate measurement of HSPC numbers diminishes significantly. The largest graft may not always be the one containing the highest number of viable HSPCs.

### i) Phenotyping Assays:

The discovery in the mid-1980's that most HSPCs express the cell surface protein CD34 facilitated a more direct measurement of primitive cells that mediate engraftment.[4] CD34-positive cells typically represent approximately ~1-4% of BM, MPB or CB cells and can be readily enumerated by flow cytometry following staining with a CD34-specific monoclonal antibody. The advantage of the CD34 assay is that it can be completed in a few hours so the number of $CD34^+$ cells can be used prospectively to predict the engraftment potential of a stem cell product. The International Society for Cellular Therapy (ISCT; formerly the International Society for Hematotherapy and Graft Engineering [ISHAGE]) has established a protocol for staining cells with anti-CD34 antibodies and standardized kits are available commercially. The gating criteria are complex, however, and there remains widespread variation in how the assay is deployed. This has resulted in a considerable variation in the accuracy of $CD34^+$ cell enumeration that is probably not widely appreciated.[5]

Indeed, the coefficient of variation of the CD34 assay is not significantly lower than that of the colony-forming unit (CFU) assay when colonies are counted manually.[6] Numerous clinical studies have demonstrated a positive correlation between the number of $CD34^+$ cells in different hematopoietic cell grafts and the speed of hematological recovery; for an example see.[7] However, the CD34 assay also has several disadvantages. Foremost, it is a phenotyping assay that measures a physical property of HSPCs as a surrogate assessment of their biological function. The physical properties of primitive hematopoietic cells, like CD34 expression, can change when cells are subjected to different manipulations, for example when they are maintained in culture. Second, in thawed cryopreserved cells, measuring the total number of viable $CD34^+$ cells alone will typically over-estimate the number of functional stem and progenitor cells. This is because the CD34 phenotyping assay used routinely in clinical laboratories does not identify cells that have been damaged by this process and that have begun to undergo apoptosis.[8] Such cells will die within days after thawing and will not contribute to engraftment following transplantation. Viable and functional stem and progenitor cells typically comprise only a minority (~10-20%) of $CD34^+$ cells, depending on the tissue.

## When is a Phenotyping Assay Not Enough?

Arguably the most important advantage of phenotyping assays is their speed. The percentage of 'positive' cells can typically be quantitated within hours by flow cytometry. A rapid answer affords the opportunity to gain insight into the anticipated rate of engraftment and, in the case of allogeneic CB transplantation, can help guide selection of the most appropriate cryopreserved unit(s) for infusion. The advantage of assay speed diminishes, however, when the surrogate end-point being measured (e.g. the percent of $CD34^+$ cells) changes in a different way than the biological function of the cell that the phenotypic assay is meant to detect. A good example of the divergence of phenotypic and functional assay results is found in a comparison of fresh and cryopreserved cells. In fresh CB, for example, progenitor cells identified by their functional ability to produce colonies in semi-solid cultures, called colony-forming cells (CFCs) or colony-forming units (CFUs), represent a low but relatively predictable proportion of the total $CD34^+$ population (~10-20%).

**TABLE 2.** Key studies demonstrating a positive correlation between graft CFU content and hematopoietic engraftment following HSPC transplantation

| STUDY | NO. PATIENTS | CANCER | HSC SOURCE | CELLS TESTED IN CFU ASSAY | CORRELATIONS IN UNIVARIATE ANALYSIS BETWEEN THE INDICATED ASSAY AND CLINICAL PARAMETER | |
|---|---|---|---|---|---|---|
| Hogge et al.[9] | 65 | Lymphoid cancers, solid tumors | MPB | Post-thaw | $CD34^+$ vs. PLT:<br>CFU vs. PLT: | $p = 0.0001$<br>$p < 0.0001$ |
| Migliaccio et al.[10] | 204 | Leukemias, MDS, genetic diseases | Unrelated CB | Pre-cryo | TNC vs. NEU:<br>TNC vs. PLT:<br>CFU vs. NEU:<br>CFU vs. PLT: | $p < 0.0001$<br>$p = 0.007$<br>$p < 0.0001$<br>$p = 0.0001$ |
| Iori et al.[11] | 20 (long-term follow-up) | Leukemias | Unrelated CB | Post-thaw | TNC vs. OS, LFS, EFS:<br>$CD34^+$ vs. OS, LFS, EFS:<br>CFU vs. OS:<br>CFU vs. DFS:<br>CFU vs. EFS: | NS<br>NS<br>$p = 0.001$<br>$p = 0.002$<br>$p = 0.002$ |
| Yoo et al.[12] | 35 | Leukemias, BM failure, solid tumors | Unrelated CB | Post-thaw | TNC vs. NEU:<br>TNC vs. PLT:<br>$CD34^+$ vs. NEU:<br>$CD34^+$ vs. PLT:<br>CFU vs. NEU:<br>CFU vs. PLT: | $p = 0.04$<br>NS<br>$p = 0.004$<br>NS<br>$p = 0.004$<br>$p = 0.02$ |
| Kozlowska-Skrzypczak et al.[13] | 52 | AML | Autologous BM | Post-thaw | CFU vs. NEU:<br>$CD34^+$ vs. NEU:<br>TNC vs. NEU: | $p = 0.056$<br>NS<br>NS |
| Prasad et al.[14] | 159 | Inherited metabolic disorders | Unrelated CB | Post-thaw | TNC vs. NEU:<br>TNC vs. PLT:<br>TNC vs. OS:<br>$CD34^+$ vs. NEU:<br>$CD34^+$ vs. PLT:<br>$CD34^+$ vs. OS:<br>CFU vs. NEU:<br>CFU vs. PLT:<br>CFU vs. OS: | $p < 0.01$<br>$p = 0.02$<br>NS<br>$p < 0.01$<br>$p = 0.02$<br>NS<br>$p < 0.0001$<br>$p < 0.0001$<br>$p = 0.01$ |
| Page et al.[2] | 435 | Cancer, BM failure, Inherited metabolic disorders | Unrelated CB | Post-thaw | TNC vs. NEU:<br>TNC vs. PLT:<br>$CD34^+$ vs. NEU:<br>$CD34^+$ vs. PLT:<br>CFU vs. NEU:<br>CFU vs. PLT: | HR = 2.1<br>HR = 2.4<br>HR = 2.3<br>HR = 2.6<br>HR = 3.6<br>HR = 3.2 |

Studies use different statistical methods to determine correlations and significance. The individual papers should be consulted for details. In most cases, p values are shown. In one study the hazard ratios (HR) are shown only for the patients transplanted with the highest of several cell doses tested, but this cell dose is equivalent for all clinical parameters. In studies that determined correlations for both pre-cryopreservation (pre-cryo) and post-thaw cells, only the latter are shown for brevity.

**BM:** bone marrow; **CB:** cord blood; **TNC:** total nucleated cells; **CFU:** colony-forming units; **MDS:** myelodysplastic syndrome; **PLT:** platelet engraftment to 50,000/µL; **NEU:** neutrophil engraftment to 500/µL; **OS:** overall survival; **LFS:** leukemia-free survival; **EFS:** event-free survival. **NS:** not significant

Cell viability in both the $CD34^+$ and CFU population is high (>95%) and $CD34^+$ cells can thus be used as a relatively reliable surrogate measurement of CFU numbers. The picture changes, however, after cryopreservation. In thawed CB cells, for example, the proportion of viable $CD34^+$ cells usually remains relatively high, at least when measured using conventional methods that do not identify cells that have begun to undergo apoptosis. Compared to their numbers in the pre-cryopreservation sample, the recovery of $CD34^+$ cells after thawing thus often approaches >80%. Conversely, a functional measurement of CB CFU content demonstrates that only ~20% are recovered after thawing.[2] In this case, the use of a phenotyping assay will over-estimate the actual number of viable progenitor cells. As hematopoietic grafts from different donors will be impacted in different ways by manipulations such as freezing and thawing, the use of a phenotyping assay has the potential to adversely affect decisions about which graft might contain the highest number of stem/progenitor cells and compromise clinical outcome.

### ii) Functional Assays:

Human HSCs can only be definitively identified using a functional assay that directly measures properties that are relevant to engraftment, such as proliferation and differentiation. In a research lab setting, human HSCs are assayed by transplantation into genetically immune-compromised (e.g. *NOD-SCID*) mice. HSCs are measured retrospectively when human blood cells of multiple lineages are detected in the recipient mice at least 5 weeks after transplantation. Unfortunately, this type of xenotransplantation assay is expensive and impractical for routine use in a clinical laboratory. In this setting, an in vitro assay that measures the number of functional progenitor cells able to produce colonies of hematopoietic cells in methylcellulose-based culture medium supplemented with stimulatory growth factors offers the best alternative. The CFU assay has several important advantages over the phenotyping assays described above. First, it measures a functional property directly relevant to engraftment; i.e. the ability of a progenitor cell to divide and produce daughter blood cells of different lineages. Second, only viable cells that have not begun to undergo apoptosis are detected in the CFU assay. This ensures that the number of progenitor cells is not over-estimated as can happen with some phenotyping assays. Third, the CFU assay directly measures the number of clonogenic cells. In other words, unlike in the CD34 assay, in which only a minority and unknown (unless explicitly measured using a functional assay) proportion of the measured $CD34^+$ cells actually possess the ability to proliferate and differentiate, in the CFU assay one colony equates to one functional progenitor cell.

## The Number of CFUs in a Graft Positively Correlates With Hematopoietic Engraftment

Several studies have demonstrated that the number of CFUs infused per kg of recipient body weight is the single parameter that best correlates with the number of days to neutrophil and platelet engraftment, and overall patient survival after HSC transplantation. This positive correlation between the CFU content of a graft and the rate and success of hematopoietic reconstitution has been observed with BM, MPB and CB transplantation. The correlation is particularly strong when the CFU assay is performed on the cells actually infused into the patient, i.e. in the case of cryopreserved cells, on the cells recovered after thawing and washing. Several such studies are summarized below and in Table 2. It is noteworthy that not all studies that have been conducted to study this question have demonstrated a positive correlation between the number of CFUs in a graft and the rate of hematopoietic reconstitution. The underlying reasons for the poor predictive utility of graft CFU content in these latter studies are difficult to determine, but are very likely related to differences in cell processing (freezing, thawing), methods and materials used to enumerate CFUs, and clinical study design.

Hogge et al. compared the rate of platelet engraftment in 65 patients who were transplanted with autologous leukapheresis cells following mobilization with cyclophosphamide and different hematopoietic growth factors.[9] Despite significant inter-individual variation in the efficacy of mobilization of different progenitor cell types, the number of CFUs in the MPB graft was strongly correlated with the rate of platelet recovery. The number of $CD34^+$ cells also correlated well with engraftment, although slightly less than did CFU content. Migliaccio et al. reviewed engraftment results in 204 patients with leukemias, other cancers, myelodysplastic disease or genetic disorders who received unrelated CB grafts in which the CFU content was measured before cryopreservation.[10] They found that the total CFU dose in the CB grafts was more closely correlated with neutrophil and platelet engraftment and post-transplantation events than the TNC dose. Iori et al. examined engraftment and survival of 42 leukemia

patients after unrelated CB transplantation.[11] They found that the numbers of TNCs, CD34$^+$ cells and CFUs measured on post-thaw cell samples were positively correlated with each other, but did not significantly affect hematopoietic recovery. In 20 patients subjected to long-term follow-up, however, the CFU dose (specifically the dose of granulocyte/macrophage progenitors; CFU-GM) was the most important factor that affected overall and leukemia-free survival, and the only factor that significantly affected event-free survival. Yoo et al. studied 35 young patients (<18 years) with leukemias, marrow failure or solid tumors who were transplanted with a single CB unit.[12] In this study, post-thaw CFU-GM, TNC and CD34$^+$ cell numbers correlated with the speed of neutrophil engraftment. Only the CFU-GM counts correlated significantly with platelet engraftment in these single CB unit recipients, and also predicted engraftment of the predominant unit in 18 patients transplanted with two CB units. The numbers of CFU-GM and CD34$^+$ cells infused were higher in patients who had successful engraftment (on average 2 x $10^5$ CFU-GM and CD34$^+$ cells per kg body weight) than in patients who did not show donor-cell engraftment (each on average 1 x $10^5$ cells/kg). The number of TNCs infused were not different between the two groups. Few studies have examined the relationship between bone marrow CFU content and the speed of hematopoietic recovery because cell numbers are usually not limiting in BM transplantation. One recent study of 52 patients with acute myeloid leukemia (AML) found that the CFU-GM dose in transplanted autologous BM was the only factor close to significance in univariate analysis of variables that correlate with neutrophil recovery.[13] Prasad et al. examined the impact of pre-cryopreservation and post-thaw graft characteristics on CB engraftment and survival of 159 young patients (median age 1.5 years) with inherited metabolic disorders.[14] The total number of post-thaw CFUs correlated best with neutrophil and platelet engraftment, and overall survival. Other parameters, specifically pre- and post-thaw TNCs, and post-thaw CD34$^+$ numbers, were less predictive. The same group recently confirmed and extended these findings in a larger study (435 patients, median age 5.3 years).[2] The CFU dose in pre-cryopreservation and post-thaw CB units best predicted neutrophil and platelet engraftment, whereas post-thaw TNC and CD34$^+$ cell dose were less predictive. In addition they reported that the CFU recovery in the post-thaw sample was only ~20% as compared to the pre-cryopreservation CB. This indicates that many hematopoietic progenitor cells in CB products do not survive banking, thawing and washing using standard clinical procedures. The actual number of CFUs available for infusion is thus often much lower than the number that was present before cryopreservation. This argues strongly that investigators must measure the viability and CFU content of post-thaw cells to select the most suitable grafts for transplantation. CFU assays of pre-freeze CB cells are important to select the most suitable units for banking. Comparison between pre-cryopreservation and post-thaw CFU numbers is important for quality control of CB processing, banking, thawing and washing procedures. Several studies have shown that the number of CFUs present in cryopreserved segments attached to the main unit accurately reflect its CFU content.[15, 16] Thus, it is feasible to obtain information on the post-thaw CFU content prior to thawing of the main unit and to include CFU assay results in the selection criteria for the most suitable CB units for transplantation. A more rapid CFU assay that can be completed in only 7 days has been developed specifically for this purpose (see www.stemcell.com/express and below).


## Standardization of the CFU Assay

Since its development in the mid 1960's, simultaneously by Pluznick and Sachs,[17] and Bradley and Metcalf,[18] the CFU assay has been the "gold standard" in vitro assay for the identification and quantitation of hematopoietic progenitor cells. Originally, cells were grown in semi-solid agar cultures stimulated with medium that had been "conditioned" by different cell types, most commonly unfractionated leukocytes. Over the past ~25 years, these crude preparations have been replaced with high quality defined culture media and recombinant human growth factors that have enabled the assay to be standardized for reproducible and consistent measurements of CFU numbers. STEMCELL Technologies Inc. has pioneered this work and produces numerous products for laboratories that perform human CFU assays. An enriched methylcellulose-based medium (MethoCult™ Express) has been developed to shorten the duration of the CFU assay from 14 to 7 days. This quicker assay is ideal for CB banking where it can be used to determine the frequency of viable post-thaw CFUs in attached frozen segments prior to thawing the main cryopreserved unit and in sufficient time to guide selection of the most suitable unit(s) for transplantation. Another recent innovation is the development of STEMvision™, an instrument for automated counting of 7-day CB CFU assays that reduces the inter-individual and inter-laboratory variability associated with manual CFU assay counting from ~34%[6] to only ~5% (see www.stemcell.com/stemvision7day). Image analysis software for BM and MPB cells, conventional 14-day CFU assays, and for full differential scoring of CFU sub-types will be available in 2012. While various options for the final read-out of the CFU assay are available, e.g., counting on day 7 or day 14, counting total CFUs, CFU-GM only, or all CFU sub-types, the field will benefit from adoption of a standard CFU assay end-point, particularly as globalization of allogeneic CB banking and dissemination of CB units to different countries for transplantation will become routine.

## Summary and Future Directions

Cord blood banks and transplant laboratories employ several assays to measure the number of HSPCs in hematopoietic cell products. Such assays are used for quality control to measure the impact of processing on cell viability, to identify CB units that meet minimal thresholds to justify long-term banking, and ultimately to guide selection of allogeneic grafts for transplantation. Phenotyping assays such as the CD34 assay are advantageous because they are quick, but do not provide any information about the functional potential of the $CD34^+$ cells and can over-estimate HSPC numbers, particularly in previously frozen cells. The CFU assay is currently the only assay that can measure the absolute number of viable and functional progenitor cells. Clinical studies have shown that the total number of CFUs in a CB unit is the single parameter that best correlates with the time to neutrophil and platelet engraftment, and overall survival following unrelated allogeneic CB transplantation. Several regulatory bodies (FDA[19], AABB[20], NetCord-FACT[21]) recommend or require measurement of CFU content before CB units can be listed in national registries or released for transplantation. The field is moving toward increased regulation. A Biological License from the US FDA is now required by laboratories engaged in the manufacture of minimally manipulated unrelated allogeneic CB cells for hematopoietic reconstitution to demonstrate that the cell product meets prescribed requirements of safety, purity and potency. Although the technical procedures for performing and reading the CFU assay have been standardized, these regulatory requirements underscore the need for standardizing the way in which CFU assay results are reported. The current use of disparate CFU measurements in different countries and regions makes it more complicated for transplant physicians to compare and utilize CB units from different banks and reduces the utility of the global CB stem cell repository. Thus while the CFU assay protocol has been standardized, the challenge for the future will be to standardize its implementation in the clinic.

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