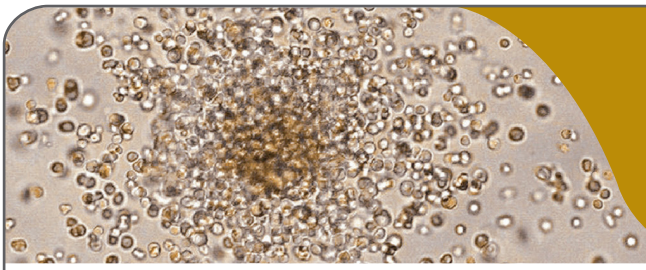

TECHNICAL BULLETIN
The CFC Assay For Cord Blood

Introduction

Umbilical cord blood (CB) is becoming increasingly important as a source of hematopoietic stem and progenitor cells for clinical transplantation for a variety of malignant, hematologic, immunodeficiency and genetic disorders (for recent reviews see 1, 2). Hematopoietic engraftment after cord blood transplantation (CBT) is relatively slow as compared to bone marrow and mobilized peripheral blood cell transplantation.[3] This problem has been attributed to a lack of potency of CB units, which is determined by the relatively small number of stem and progenitor cells in CB and the potentially negative effects of CB processing, cryopreservation and thawing procedures on cell yield and viability.

To improve the overall success of CBT, adequate assays are needed to select the most suitable CB products and monitor the effectiveness of procedures for CB banking and graft selection. The most commonly used parameters include volume and numbers of total, nucleated, mononuclear, $CD34^+$ and colony-forming cells that are measured on pre-cryopreservation and post-thaw samples of candidate CB units. Only the colony-forming cell (CFC) assay (also know as colony-forming unit (CFU) assay) can provide information about the frequencies, viability and hematopoietic capacity of progenitor cells in the unit.

The Colony-Forming Cell Assay

The CFC assay is the standard in vitro functional assay for measuring hematopoietic progenitors in bone marrow, blood and other hematopoietic tissues. In its basic form the assay involves plating a single cell suspension in a semi-solid medium that contains an optimal combination of nutrients, cytokines and other supplements which supports the proliferation and differentiation of hematopoietic progenitors, and a gelling agent (usually methylcellulose) which increase the viscosity of the medium.

At a sufficiently low plating density, the daughter cells of each original progenitor cell remain together in the high-viscosity medium allowing the identification and enumeration of distinct colonies after a suitable culture period, usually 14 days.

The size and composition of each colony is representative of the developmental potential of the original progenitor cell. Together these properties facilitate measurement of the frequencies and growth properties of erythroid progenitors (CFU-E and BFU-E), granulocyte/macrophage progenitors (CFU-GM) and multipotential granulocyte, erythroid, macrophage and megakaryocyte progenitors (CFU-GEMM).

Colony identification and enumeration are usually done by eye, using an inverted microscope, but can also be done with an automated colony imaging and enumeration platform (STEMvision™, see back page).

TOLL-FREE T. 1 800 667 0322 • T. +1 604 877 0713 • TOLL-FREE F. 1 800 567 2899 • F. +1 604 877 0704
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Why Use The CFC Assay As A Potency Assay?

Several studies have demonstrated that the CFC content of CB, particularly in post-thaw cells, is the single parameter that can most precisely predict time to neutrophil and platelet engraftment, and overall survival after unrelated CBT.

Migliaccio et al. (2000) reviewed engraftment results in 204 patients with leukemias, other cancers, myelodysplastic disease or genetic disorders who received unrelated CB grafts in which the CFC content had been measured before cryopreservation.[4] They found that the total CFC 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. (2004) examined engraftment and survival of 42 leukemia patients after unrelated CBT.[5] They found that the three main pre-transplant parameters tested, nucleated and $CD34^+$ cell counts, and CFC numbers measured on post-thaw cell samples, were positively correlated with each other, but did not affect hematopoietic recovery significantly. However, the CFC dose (specifically the CFU-GM dose) 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. (2007) examined pre-transplant and post-CBT results for 53 young patients (<18 years) with leukemias, marrow failure or solid tumors.[6] In this study, post-thaw CFU-GM, TNC and $CD34^+$ cell numbers correlated with the speed of neutrophil engraftment, but only the CFU-GM counts correlated significantly with platelet engraftment, and also predicted engraftment of the predominant unit in double CB transplants. The CFU-GM and $CD34^+$ cell numbers 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 (on average 1 x 10^5 cells/kg), whereas TNC and $CD3^+$ cell numbers did not show differences between both groups.

Prasad et al. (2008) examined the impact of pre-freeze and post-thaw characteristic in CB engraftment and survival of 159 young patients (median age: 1.5 years) with inherited metabolic disorders.[7] The number of total CFCs post-thaw 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 (Page et al., 2011) recently confirmed and extended these findings in a larger study (435 patients, median age 5.3 years).[8] The CFC 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 CFC recovery in the post-thaw sample was relatively low (~20%) as compared to the pre-cryopreservation CB, indicating that many hematopoietic progenitor cells in the CB products did not survive banking, thawing and washing using standard clinical procedures. The actual CFC number available for infusion may thus be much lower than the number that was present before cryopreservation. This indicates that measuring the viability and hematopoietic potential of post-thaw cells is necessary to select the most suitable grafts for transplantation, while functional assays on pre-freeze CB cells are important to select the most suitable grafts for banking. Comparison between pre-cryopreservation and post-thaw CFC numbers is important for quality control of CB collection, processing, banking, thawing and washing procedures.

Studies in several laboratories have shown that CFC numbers in attached cryopreserved samples are representative of the CFC content of the main CB unit.[9-11] Thus, it is feasible to obtain information on the post-thaw CFC content prior to thawing of the main unit and to include CFC assay results in the selection criteria for the most suitable CB units for transplantation.

Standardization Of The CFC Assay

The CFC asay is a specialized culture assay that requires a high level of expertise and dedication by the people that set up and score the assays to maintain high consistency and reproducibility. Reliable CFC assay results are routinely achieved in many individual laboratories, as evidenced by the strong correlations between CFC assay results and clinical outcomes in single-center studies. However, CFC assay results in different laboratories can be variable and results between different laboratories may be difficult to compare due to differences in cell processing procedures, culture media, scoring criteria and skill levels of the people performing the assays. The relatively long assay duration of standard CFC assays, i.e. 14 days, is a concern in situations when information on the hematopoietic potential is needed quickly, for example, for CB banking and for graft selection prior to transplantation. There are several ways that these problems can be addressed.

1. Standardized Culture Media: MethoCult™

The culture medium is a key element in the standardization of the CFC assay within and between laboratories as differences in medium composition and quality have large effects on hematopoietic progenitor cell proliferation and differentiation, and on the number, types and sizes of colonies that are produced.

MethoCult™ media contain carefully screened and optimized components. Each batch is subjected to rigorous performance testing prior to release, ensuring high lot-to-lot consistency. MethoCult™ media are available in formulations that contain all cytokines that are necessary for optimal outgrowth of erythroid, myeloid and multilineage colonies in the same assay. Formulations without erythropoietin are available for applications in which only the enumeration of myeloid progenitors (CFU-GM) is required.

2. Shorter Assay Duration: MethoCult™ Express

MethoCult™ Express is a culture medium that has been specifically developed for accelerated progenitor cell proliferation and colony formation, permitting total CFC scoring after only 7 days of culture, but without distinction of colony types. Total CFC numbers after 7 days of culture in MethoCult™ Express are very close to total CFC numbers after 14 days in MethoCult™ media used in standard CFC assays (e.g. MethoCult™ H4034 Optimum), demonstrating that the 7-day assay in this medium can replace 14-day assays for detecting and enumerating total CFCs.

3. Training and Proficiency Testing

STEMCELL Technologies Inc. regularly organizes hands-on courses and provides training tools (colony atlases and online tutorials) to assist laboratory staff in maintaining and improving their skills. In collaboration with the National Marrow Donor Program (NMDP), a proficiency testing (PT) program has been established to monitor the reproducibility of the CFC assay and other parameters, specifically TNC and $CD34^+$ counts, between participating laboratories.[12] In the five programs performed between 2006 and 2010 the interlaboratory variability of TNC counts ranged between 6 and 17%. The variability of CFC and $CD34^+$ counts was much higher, with CV of 32.8% and 27.8%, respectively, for the PT program run in 2010, indicating that neither the CFC assay nor the $CD34^+$ assay are sufficiently standardized between laboratories.

Results of a single interlaboratory comparison of the 7-day CFC assay in MethoCult™ Express medium suggested that differences in colony counting reproducibility between different people are a major source of CFC assay variability and that training can improve assay reproducibility.[13] In this study the variability between colony counts by inexperienced people was ~20%. The variability between colony counts obtained by people with at least half a year of experience with the 7-day CFC assay was much lower, i.e. 5-7%. A similar difference between experienced and inexperienced individuals has been observed in the consecutive proficiency testing programs for standard 14-day CFC assays on frozen CB samples. These results suggest that with training, inter-laboratory variability associated with the the CFC assay may be substantially reduced.

4. Automated Colony Enumeration

Automated colony scoring may help to standardize CFC assays within and between laboratories by providing uniform and consistent colony scoring criteria. STEMvision™, developed by STEMCELL Technologies, is the first instrument and computer platform for imaging and automated scoring of hematopoietic colonies in CFC assays using MethoCult™ media. STEMvision™ can be used to score total CFCs in 7-day CFC assays using MethoCult™ Express, as well as total CFCs, erythroid and myeloid colonies using MethoCult™ media for 14-day CFC assays. It also provides a permanent digital image of each CFC assay, which can be used for record keeping, data review and re-analysis at later times if required.

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