

# Automation of Colony-Forming Cell Assays for Measuring Hematopoietic Progenitors in Cord Blood

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## Introduction

The colony forming cell (CFC) assay is the most widely accepted method for quantifying the frequency of functional hematopoietic progenitors in cell products such as cord blood (CB), mobilized peripheral blood, and bone marrow. While providing a powerful predictive measure of hematopoietic recovery and survival following clinical transplantation, the usefulness of the CFC assay for routine evaluation of graft quality and potency could be improved by a shorter assay duration and higher reproducibility of assay results, both within and between centers. To achieve these improvements, a semisolid medium (MethoCult® Express) was formulated to enable the quantification of total colonies after only 7 days of culture, but without distinction of colony types. In addition, automation of the assay was achieved with an image-based analysis system specifically designed for colony enumeration. The automated system was used in combination with cultureware designed to improve image quality and colony distribution by eliminating the meniscus at the periphery of the culture dish. Automated colony counts were closely correlated with manual counts of the same assay cultures, but considerably less variable than standard manual colony counts.

## Method

CFC assays were conducted by inoculating mononuclear cells, derived from human umbilical cord blood by centrifugation over a Ficoll™ gradient, into semisolid media. Experiments were also done with CB cells from which red blood cells had been removed using ACK-lysis buffer. The cells were inoculated into both MethoCult® Express and MethoCult® H4034 (STEMCELL Technologies Inc.) and incubated for 7 and 14 days, respectively. Both media were inoculated with different cell numbers to result in a colony density in the range of 20 to 100 colonies per 35mm dish (approximately 1-4 x 10<sup>4</sup> cells/mL of MethoCult®). Following incubation, the total colony numbers were determined by manual observation using an inverted light microscope and by imaging and automated analysis using the STEMvision™ imaging instrument (STEMCELL Technologies Inc.). In addition, for a subset of the samples analyzed, microscope counts as well as manual counts of colonies from the acquired images were conducted independently by four different assay operators. Multiple automated counts of CFC assay cultures were obtained by repeated imaging and analysis of the same assay dishes.

Variability of the assay outcome was assessed by determining the coefficient of variation (CV) between the multiple manual and automated counts and the correlation between the two different media and manual and automated counts was assessed by regression analysis.

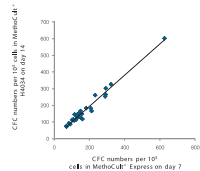
## Results

The total colony counts after 7 days in MethoCult® Express were closely correlated with total colonies counted in MethoCult® H4034 after 14 days ( $r = 0.97$ ,  $p < 0.01$ ,  $n=32$ , see **Figure 1**), demonstrating that the shorter assay accurately estimates progenitor numbers in CB samples. In addition, the MethoCult® Express assay exhibited greater reproducibility for counting total CFC numbers than the 14 day CFC assay, with a significantly smaller CV between results obtained by multiple assay operators ( $6 \pm 3\%$  vs.  $11 \pm 5\%$  for the 7 day assays in MethoCult® Express vs. 14 day assays in H4034).

The automated colony counts of CFC assays conducted in MethoCult® Express were highly correlated to manual microscope counts obtained from a large number of assays conducted by a single operator ( $r^2 = 0.96$ ,  $p < 0.01$ ,  $n=75$ ; see **Figure 3**). When a number of CFC assay dishes were counted manually by four different operators by either using a microscope or by counting colonies on the acquired images, the average manual colony counts correlated well with the results from the automated analysis ( $r^2 = 0.95$  vs.  $0.98$  for microscope and image counts respectively,  $n=16$ , see **Figure 3**). Likewise, automated total colony counts of 14 day CFC assays conducted in MethoCult® H4034 were highly correlated with the average of manual counts conducted by four trained operators ( $r^2 = 0.95$ ,  $p < 0.01$ ,  $n=21$ , see **Figure 7**). However, the variability in the manual counts was considerably greater than for the automated system (CV of 13% vs. 4% and 12% vs. 5%, for assays conducted in MethoCult® Express and MethoCult® H4034, respectively). The reduced variability of the automated assay is clearly demonstrated by the narrowing of the confidence intervals around the mean colony counts, as compared to the manual assay (**Figures 4 and 8** for 7 and 14 day assays, respectively).

The use of specially-designed cultureware to prevent formation of a meniscus and the resulting dark peripheral boundary during microscopic observation and imaging resulted in greater ease of detection of colonies near the dish edge and improved distribution of colonies throughout the dish (**Figure 5**).

**FIGURE 1: Correlation Between Day 7 CFC Frequencies with MethoCult® Express and Day 14 CFC Frequencies with a Standard Medium for 14 Day CFC Assays, MethoCult® H4034**



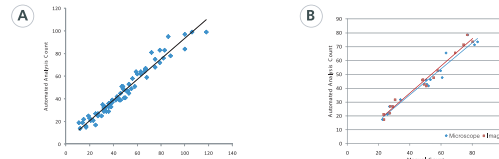
7 day CFC numbers in MethoCult® Express are significantly correlated with 14 day CFC numbers in standard MethoCult® H4034 ( $R^2=0.97$ , Slope=0.94,  $P < 0.01$ ,  $n=32$ ).

## 7 Day Assay

**FIGURE 2: Composite STEMvision™ Image**

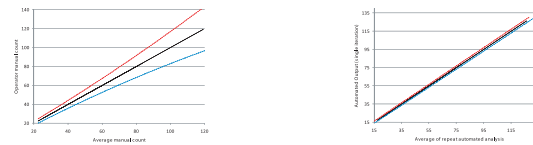


**FIGURE 3: Correlation Between Manual Counts of 7 Day CFC Assay and STEMvision™ Automated Counts**



Analysis of 7 day CFC assay cultures conducted in MethoCult® Express demonstrates the high degree of correlation between manual microscope counting by a single operator and automated analysis (A). Both methods are significantly correlated ( $R^2=0.96$ , Slope=1.05,  $P < 0.01$ ,  $n=75$ ). Similar results are obtained when the average of manual counts by multiple operators is taken to minimize the effects of operator bias (B). Manual counts were conducted by inspection of assay cultures using a microscope or by counting of colonies from the acquired images. Results of automated analysis are closely correlated to both manual microscope ( $r^2=0.95$ , slope = 0.93) and manual image counts ( $r^2=0.98$ , slope = 0.98).

**FIGURE 4: Variability of STEMvision™ Automated Colony Analysis vs. Manual Counting of 7 Day CFC Assay**



Manual microscope analysis of 7 day CFC assay cultures conducted in MethoCult® Express. The 95% confidence intervals are shown for any given individual operator manual count. The confidence interval diverges with the total colony number counted per plate.

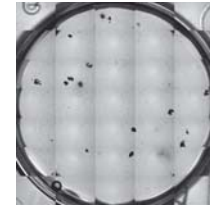
Automated analysis of 7 day CFC assay cultures conducted in MethoCult® Express. The 95% confidence intervals are shown for any given iteration of the automated analysis. The confidence interval is uniform throughout the typical range of plating densities for CFC assays and reduced significantly compared to the manual count.

**FIGURE 5: STEMvision™ Fully Automated CFC Assay Reader (A), Colony Image in Regular Dish (B) vs. Meniscus-Free SmartDish (C)**

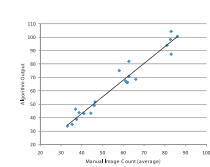


## 14 Day Assay

**FIGURE 6: Composite STEMvision™ Image**



**FIGURE 7: Correlation Between STEMvision™ and Average of Multiple Operator Manual Counts of 14 Day CFC Assay**



Analysis of 14 day CFC assay cultures conducted in MethoCult® H4034. The correlation shown is between automated analysis results and average manual counts of images by four operators (total colonies). Results of automated analysis are closely correlated to the manual image counts ( $r^2=0.95$ , slope = 1.2).

**FIGURE 8: Variability of STEMvision™ Automated Colony Analysis vs. Manual Counting of 14 Day CFC Assay**



Manual microscope analysis of 14 day CFC assay cultures conducted in MethoCult® H4034. The 95% confidence intervals are shown for any given individual operator's manual count. A minimum confidence interval is seen at a plating density near 40 colonies per 35mm dish, with the intervals diverging above and below this point.

Automated analysis of 14 day CFC assay cultures conducted in MethoCult® H4034. The 95% confidence intervals are shown for any given iteration of the automated analysis. The confidence interval is uniform throughout the typical range of plating densities for CFC assays and reduced significantly compared to the manual count.

## Conclusions

- MethoCult® Express provides a short and highly reproducible CFC assay for counting total colonies derived from hematopoietic progenitors in cord blood.
- Automated quantification of total colonies for both the 7 day and 14 day CFC assays using the STEMvision™ instrument is equivalent to manual analysis methods and is a reliable substitute for microscopic counting.
- Colony counting with the automated system reduces counting variability and provides a new method for standardizing the CFC assay within and between CB banks and cell therapy labs.