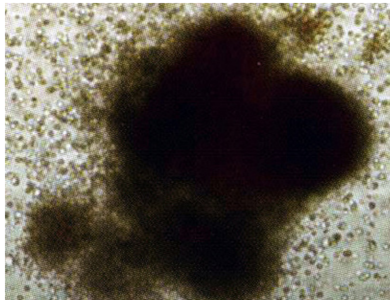
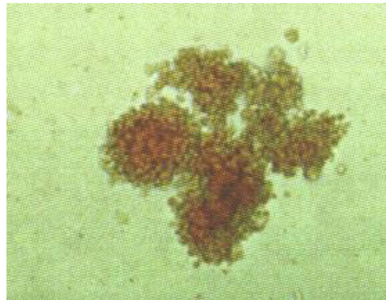
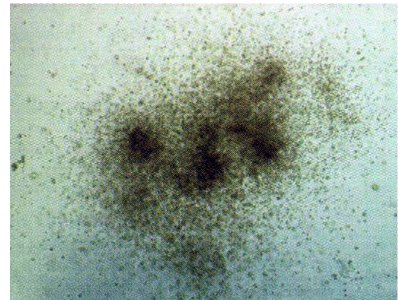
Application Note

50 Years of Colony-Forming Assays for Hematopoietic Stem and Progenitor Cells: *A Comparison and the Future*

This Application Note is accompanied by a video that can be accessed either on the HemoGenix® website or directly on the HemoGenix® YouTube Channel

December 2016

50 Years of Colony-Forming Cell Assays

2016 marks the 50th anniversary of the first published articles on the *in vitro* clonal culture of hematopoietic cells by Bradley and Metcalf[1] in Melbourne, Australia and Pluznik and Sachs[2] in Rehovot, Israel.

Using basic biological and physiological concepts, these scientists hypothesized that if hematopoietic cells could grow as colonies in the spleen of lethally irradiated mice transplanted with normal bone marrow cells from an untreated donor, hematopoietic cells could also grow as colonies in the culture dish, provided they were given the right conditions. The concept that proliferating hematopoietic cells could grow as colonies both *in vivo* and *in vitro* laid the fundamental groundwork upon which our understanding, use and applications of the hematopoietic system are based.

In vivo, this concept produced the field of stem cell biology. *In vitro*, this concept furthered our knowledge of the organization, hierarchy and regulation of many stem cell systems.

The clonal growth of cells is based on the simple concept that the number of clones correlates with the number of cells used. When the straight line graph is extrapolated, it passes through the origin or zero, indicating that each clone was derived from a single cell.

The *in vitro* methodology introduced in 1966 showed that mouse bone marrow cells grown in agar could be stimulated with a conditioned medium to produce colonies of granulocytes and macrophages over a 7 day culture period. The conditioned medium, we now know, contained granulocyte-macrophage colony-stimulating factor (GM-CSF). The colonies were originally designated as CFU-C or colony-forming units - culture. We now know that the colonies were not derived from a "unit" or group of cells, but rather a single cell called the colony-forming cell or CFC.

During culture, the CFC undergoes considerable proliferation and the cells growing outwards from the center differentiate and mature into a colony consisting of all developmental stages of the lineage with functionally mature cells on the outside of the colony. It is these mature cells that identifies the colony as being derived from a specific cell type .

Colonies are only produced by cells that have the capacity for proliferation. Since the CFC that produced the colony is so primitive that it cannot be morphologically identified, the colony-forming assay is used to demonstrate not only the presence, but also the functional ability of the CFC to proliferate and produce differentiated cells that mature into functional end cells. Even though cell proliferation is an absolute requirement to produce a colony, the CFC assay does not, and cannot, quantitatively measure cell proliferation. The CFC assay is a differentiation and maturation assay.

Exactly the same concepts and principles apply to cells that are grown in a plasma clot or in viscous methylcellulose, a method that was developed by Norman Iscove and his colleagues to grow erythropoietic cells in culture in the early 1970s[3]. It is the methylcellulose CFC assay technique that is predominantly still used today. However, despite advances in many other areas of hematopoietic research, scientists using the CFC assay have, for one reason or another, either failed to keep up, are oblivious to, or have denied the presence of new technology that could help them better understand their own field.

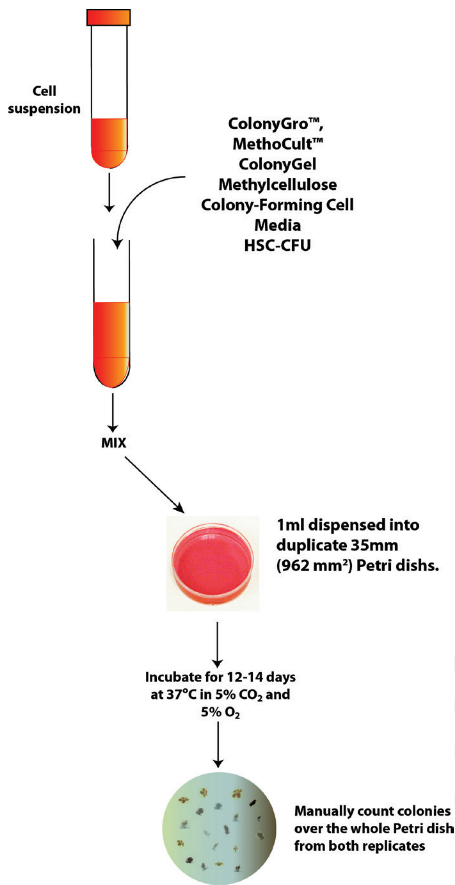
Surprisingly, few have realized that 2016 marks the 50th anniversary of the CFC assay. As a result, HemoGenix® considered it would be a salute and appreciation to the fathers of this technology to provide an Application Note describing the assay and the advances made over 50 years. It is hoped that this Application Note will be an enlightening guide for those who wish to further the science of the hematopoietic system.

The Methylcellulose Colony-Forming Cell Assays

Methylcellulose is a powder available at different viscosities that is water-soluble and has numerous uses. Prior to companies like HemoGenix® and Stem Cell Technologies commercially producing methylcellulose reagents for tissue culture, investigators had to produce their own stock solutions and mix the methylcellulose with the other components required to allow the hematopoietic cells to grow under sterile conditions. These components usually include a source of protein, usually fetal bovine serum (FBS), the concentration of which can be as high as 30%, iron-saturated transferrin, a reducing agent such as β-mercaptoethanol or α-thioglycerol, culture medium that is usually Iscove's Modified Dulbecco's Medium (IMDM) and growth factors and/or cytokines, depending on the cell population being stimulated. Serum-free cultures, meaning no FBS, do include other sources of protein such as bovine serum albumin (BSA) and other components that are usually proprietary.

Several companies produce methylcellulose reagents containing different cocktails of growth factors. Traditionally, a total volume of 3mL is usually dispensed into a tube followed by 0.3mL of the target cell suspension. The contents are mixed thoroughly and 1mL is dispensed into duplicate 35mm Petri dished as shown in Figure 1. Dispensing is traditionally performed using a syringe and needle, which is one of the reasons why the method is characterized by high coefficients of variation (%CVs). This can be significantly reduced using more accurate and calibrated positive displacement pipettes.

Figure 1: The Traditional CFC Method

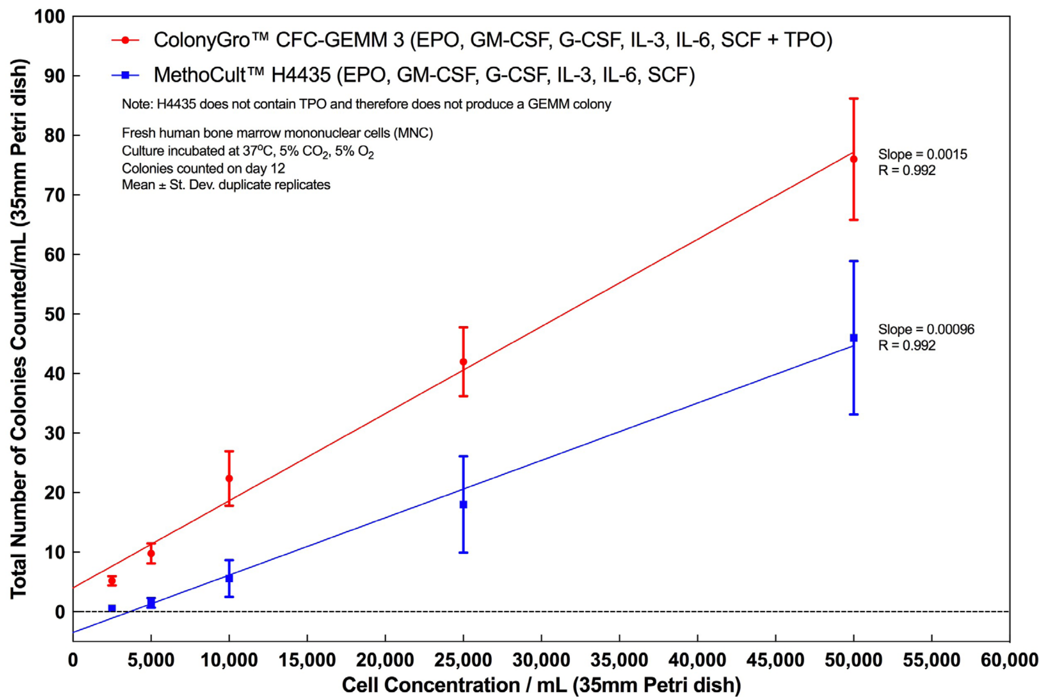


The duplicate methylcellulose cultures are usually placed in a larger container with a dish of sterile water to maintain humidity and prevent the cultures from drying out. Even though incubators are humidified, the humidity can vary depending on where the cultures are placed in the incubator and how full the incubator is with other cultures. Although most providers of methylcellulose reagents suggest culturing the cells at 37ºC in an atmosphere containing 5% CO_2, there has been mounting scientific evidence to show that using physiological levels of oxygen (5% O_2) combined with reducing agents increases plating efficiency by reducing oxygen toxicity.

Most methylcellulose reagent providers will also suggest counting colonies after 7 days for animal cells and 14 days for human cells. The incubation time depends on the formulation of the methylcellulose reagent and the growth surface area. ColonyGro™ produced by HemoGenix® is a methylcellulose reagent that allows colonies to be counted between 10 and 12 days of culture.

Counting colonies is the most difficult part of the CFC procedure that not only takes the most time to learn and perform, but also significantly increases the statistical unreliability of the assay. To reduce this problem, colony atlases have been produced and companies have developed automated colony counters that will scan each plate taking images, combine those images and, using algorithms,

Figure 2



automatically count and differentiate the colonies. For detecting primitive stem cell populations and even lineage-specific cell populations, false results can occur, which in turn, can lead to a false interpretation of the data. This will be discussed in more detail later.

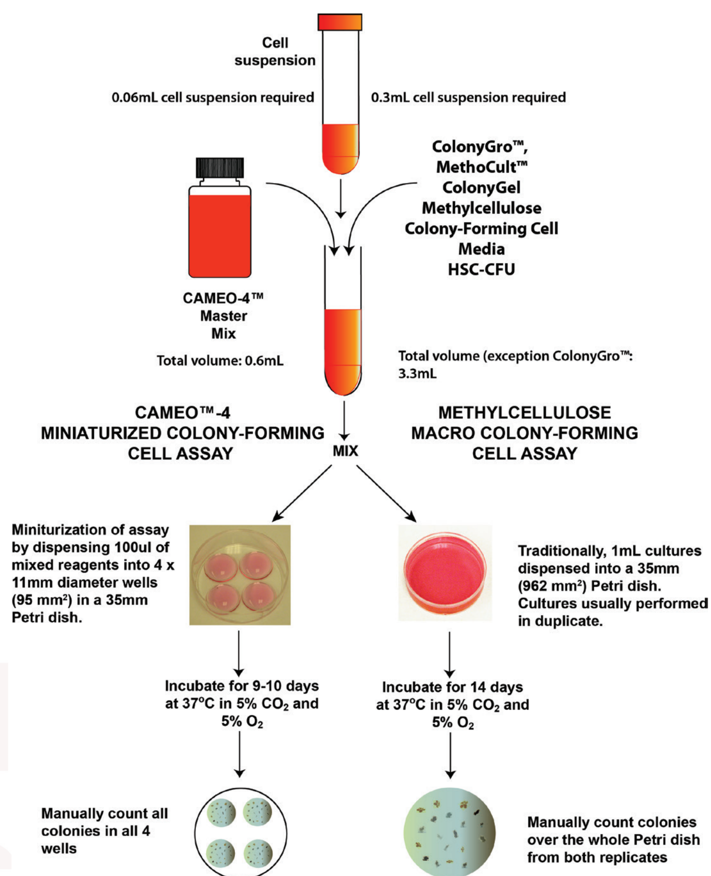
When detecting the presence of stem cells, the growth factor cocktail is all important. Figure 2 shows the difference between two methylcellulose reagents that have the same combination of growth factors and cytokines, with one exception. ColonyGro™ includes thrombopoietin or TPO[4], which together with the other factors stimulates a stem cell population designated as CFC GEMM 3. (See Figure 9 and Table 4 for a more detailed explanation of the different stem cell populations detected). The equivalent reagent, without TPO, is produced by Stem Cell Technologies as MethoCult™ H4435[5]. Since TPO is not present, the stem cell population detected and the colony produced cannot be designated as CFC-GEMM meaning that the colony contains granulocytes, erythroid cells, macrophages and megakaryocytes. The stem cell colony produced by MethoCult™ H4435 is therefore more mature than the CFC-GEMM 3. It is important to emphasize that many aspects can influence the interpretation and conclusion of the results. Assuming results that are based on a false designation can have serious scientific repercussions, especially if the results are used for clinical purposes.

One aspect of the traditional CFC assay that few take into account is the large overage volume needed to setup just duplicate cultures each of 1mL. Whether a 100mL bottle, sufficient for 30-33 duplicate cultures, or individual tubes each containing 3mL of the reagent are purchased, this not only wastes money, but also valuable cell suspension. HemoGenix® addressed this problem by reformulating ColonyGro™ so that the total volume required for each sample is just 2.5mL, thereby allowing 44 samples to be obtained from a 100mL bottle of methylcellulose reagent. HemoGenix® also addressed this problem in a different manner.

The 2nd Generation of CFC Assays: CAMEO™-4 - A Miniaturized Methylcellulose Colony-Forming Cell Assay.

Often, the number of cells or other components are limiting factors for an assay. For example, segments of cord blood samples may only be 0.1-0.2mL in total volume. A traditional CFC assay cannot be performed when the sample volume is so small. Similarly, for other applications, a small sample volume might result in fewer replicates being prepared that would call the statistical relevance (see below) of the results into question. These problems were addressed in the early 1980s by miniaturizing the CFC assay as shown in Figure 3. HemoGenix® calls this assay CAMEO™-4[6].

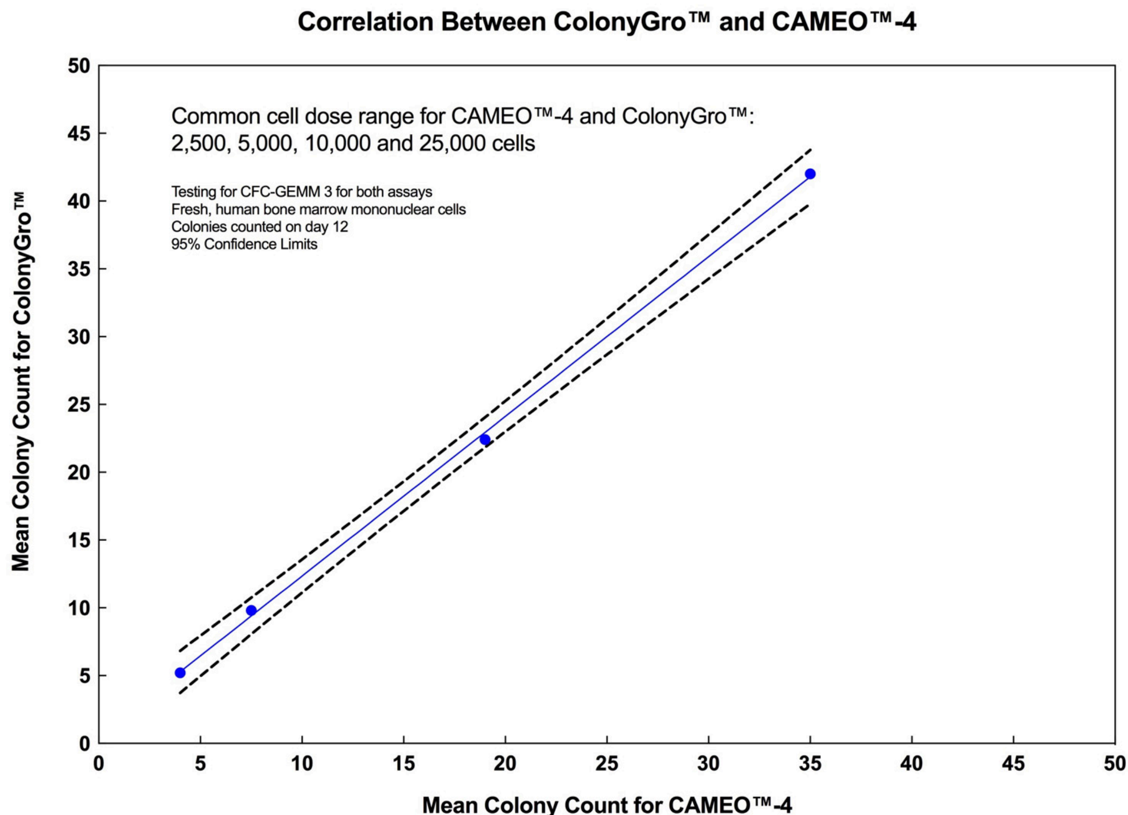
Figure 3. A Miniaturized CFC Assay (CAMEO™-4)



CAMEO™-4 demonstrates a number of important advantages over the traditional, macro CFC assay.

1. The total volume is just 0.6mL; 0.54mL of the CAMEO™-4 Master Mix is dispensed into a 5mL sterile tube followed by only 0.06mL of the adjusted cell suspension.
2. After mixing, 0.1mL is dispensed into each of 4 replicate wells in a 35mm culture dish.
3. Cultures are incubated for 9-10 days for human cells and just 5-7 days for animal cells. The decreased incubation time is due to the smaller growth surface, which in turn, induces improved cell interaction and growth.
4. The very low height of the well walls means that expensive dishes are not required to eliminate the meniscus because colonies can be easily view and differentiated up to the walls of each well.
5. Sufficient reagent is included for 50 samples.
6. Culture dishes are also included with the kit.
7. CAMEO™-4 assay kits are the same price as ColonyGro™.
8. As shown in Figure 4, there is a direct correlation between colony counts using the traditional macro CFC assay and CAMEO™-4, indicating that the latter can replace the former assay.

Figure 4. The Correlation Between Colony Counts Obtained Using ColonyGro™ and CAMEO™-4



The Difference Between "Standardized Reagents" and a Standardized Assay

Suppliers of methylcellulose reagents will often designate their products as "standardized media" or "standardized reagents"[7]. This misleads the investigator into thinking that they are buying a "standardized assay".

The terms simply mean that the components are manufactured under standardized or rather quality-controlled conditions, usually GMP or Good Manufacturing Procedures or Practices. The terms do nor mean that the assay is standardized.

A standardized assay[8] is one that includes the necessary external standards and controls. If an assay does not include these components, and this encompasses instruments that count colonies in methylcellulose, it is not a standardized assay and does not allow intra- and inter-laboratory comparisons or validation. The traditional version of the CFC assay as well as CAMEO™-4, are not standardized assays because they do not have the reagents that are required for assay calibration and standardization[9].

Proliferation and Differentiation Cannot Be Measured Using the Same Endpoint

It is obvious that to produce a colony of cells, the cell giving rise to the colony must exercise its ability to proliferate. Cells that do not proliferate cannot produce a colony. It is also obvious that to identify the colony, the cells must also differentiate and mature. It follows that prior to the cells differentiating and maturing, the cells must proliferate. In other words, proliferation occurs prior to differentiation and maturation. Under normal conditions, differentiation is a default program that occurs at a specific point in time during the cell's development, but can also overlap with proliferation. However, proliferation and differentiation are two different biological processes that cannot be determined using the same endpoint.

It is often thought that the CFC assay is a proliferation assay. From the previous discussion, it follows that this is a misleading and false assumption. Cells growing as colonies in methylcellulose need to proliferate, but the CFC assay is not a proliferation assay. It is a differentiation assay, because the colonies obtained are identified by the ability of the cells to differentiate and mature. This is why colonies are counted after 12-14 days of culture in the traditional CFC assay.

The 3rd Generation of CFC Assays: CAMEO™-96

Although cell proliferation is not directly measured using the CFC assay, the incorporation of two endpoint measurements plus the ability to standardize the CFC assay, provides the third iteration of the colony-forming assay, namely CAMEO™-96[10].

It would appear that during the lat ten years of the 50 year history of the CFC assay, many have tried to raise the status of the assay to a level it cannot accommodate: the use of "standardized media or reagents" to imply a standardized assay; designating the assay as a proliferation assay when cell proliferation cannot be measured; even calling the CFU assay a "potency assay", when the assay methodology has changed little in 50 years[11,12]. These and other statements have misled investigators into a pervasive and false state of understanding that has, unfortunately, found its way into peer-reviewed articles.

In fact, the traditional, methylcellulose CFC assay has neither the accuracy nor the sensitivity to "drill down" into the cellular details and subtleties of the hematopoietic system. To do that, far superior tools are needed.

In 2002, HemoGenix® developed the first duel endpoint, methylcellulose CFC assay. Performed in a 96-well plate and incorporating the most sensitive, non-radioactive, endpoint available to measure cell proliferation, CAMEO™-96 was also the first major advancement[13] of the CFC assay since it was first reported in 1966.
Based on similar volumes and formulations as CAMEO™-4, target cells are suspended in the methylcellulose reagent containing the growth factor cocktail to stimulate the cells of interest. Using a positive displacement pipette, the CFC master mix is dispensed into replicate wells of a 96-well plate provided with the assay kit. Human cells are incubated for just 7-9 days, while animal cells are incubated for only 4-5 days. After culture, the colonies are counted, just as they would be for a CFC assay. However, this is where CAMEO™-96 diverges from other CFC assays.

Cell proliferation occurring within all of the colonies is then quantitatively measured using a concept shown in Figure 9 and will be discussed in detail later. Suffice is to say that colony cell proliferation is determined by the measurement of intracellular adenosine triphosphate or iATP, whose concentration correlates directly with the state of cell proliferation.

Prior to measuring iATP, the instrument is calibrated by external ATP high and low controls. The assay is also standardized by performing an ATP standard curve. The instrument used is a luminescence plate reader. The output of the instrument is in relative luminescence units or RLU. By performing the ATP standard curve, the RLU values can be directly converted into standardized ATP concentrations.

As seen in Figure 5, when the number of colonies counted/ well is plotted against the mean ATP concentration per well, the results correlate directly with each other. This means that

Figure 5

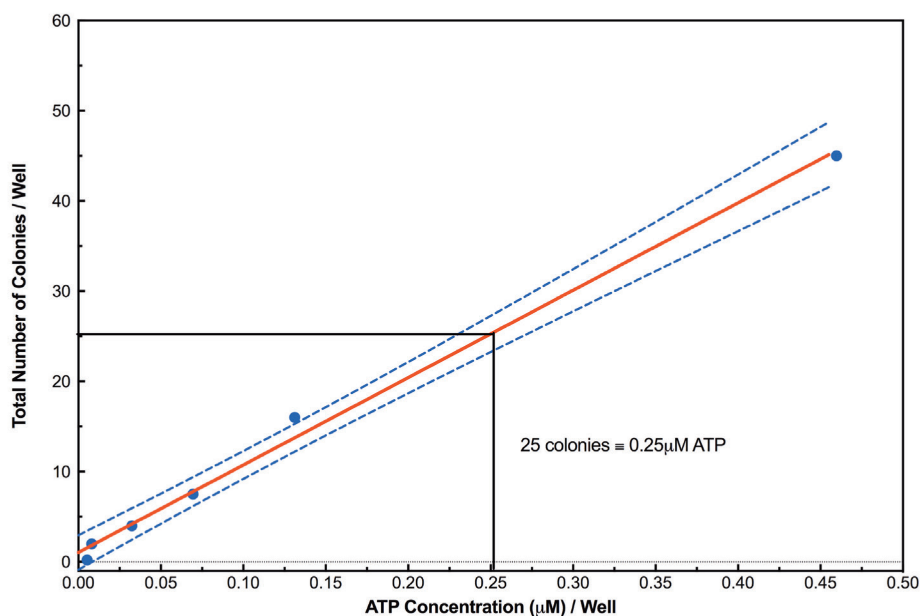
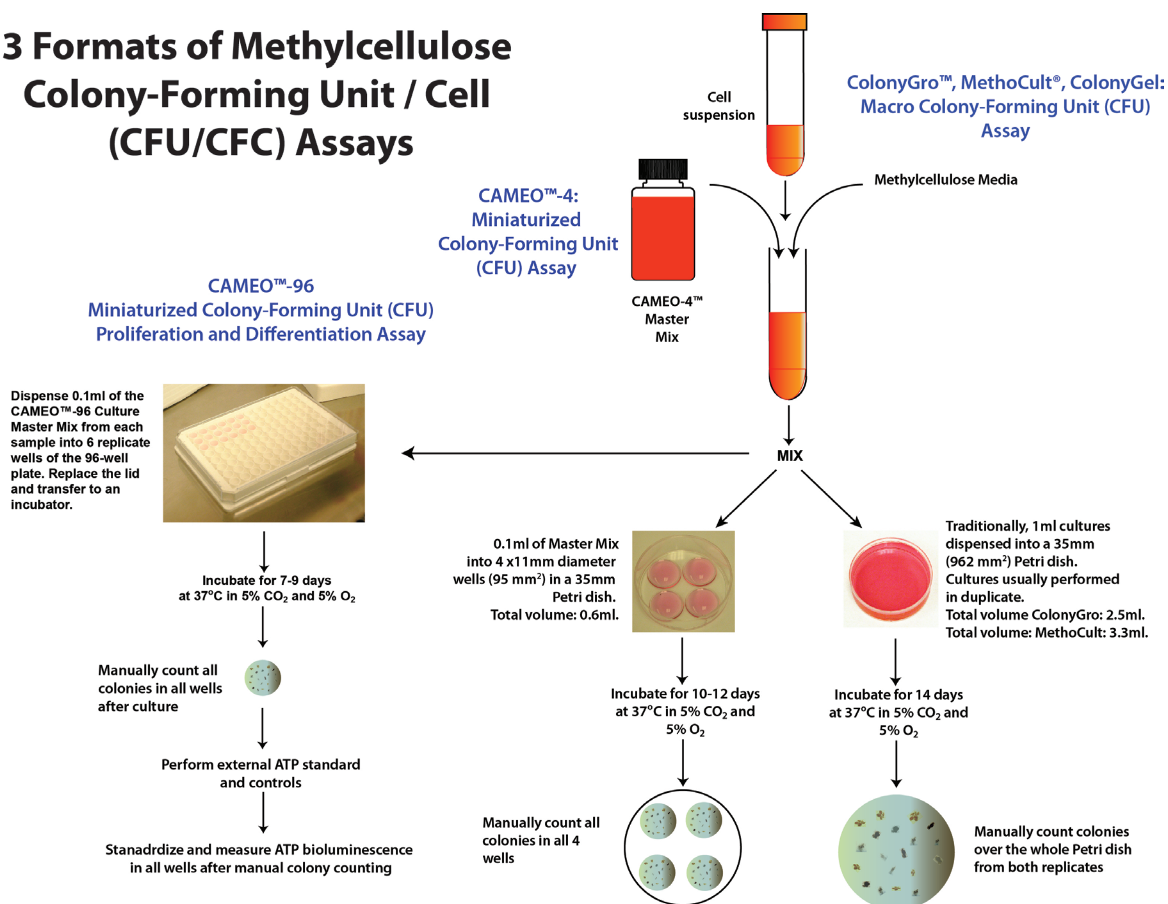


Figure 6. A Comparison of CFU Assay Methodologies



the number of colonies counted can be directly converted into standardized ATP concentrations. In other words, since the ATP endpoint is a standardized measurement, it can be used to back-standardize the CFC assay[14,15].

Since CFC growth requires both proliferation and differentiation, and the latter can only occur if cell proliferation has also taken place, it follows that a correlation between the two processes must occur. It is this basic principle that allows the CFC assay to be standardized in µM of ATP rather than colony counts.

Investigators, now have 3 options to perform a methylcellulose CFC assay. The 3 options available are shown in Figure 6 and a direct comparison is given in Table 1. There are a number points to emphasize. First, notice how the amount of information obtained increases from a traditional CFC assay to CAMEO™-96. Second, notice how the number of samples assayed and the flexibility also increases in the same direction. Third, the incubation time decreases from 14 days for the traditional assay, down to between 7 and 9 days for CAMEO™-96. This is directly related to the growth surface area; the smaller the growth surface area, the increased chance of cell interaction and the greater the sensitivity of the assay. However, this decrease in culture time down to just 7 days is based on a new concept that had previously never been possible.

CAMEO™-96: The Concept of Predicting Colony Formation Without Counting Colonies

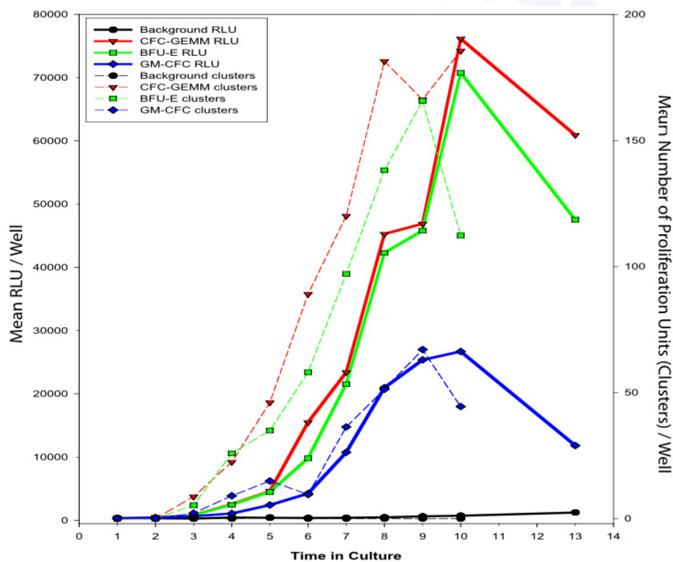
CAMEO™-96 was originally developed as a high-throughput hemotoxicity assay funded by the National Cancer Institute. Development was initiated in January 2002 by rebuilding every aspect of the traditional CFC assay into one that was optimized for a 96-well plate format. CAMEO™-96 was launched as a contract service at the Society of Toxicology meeting in March 2002.

During the three months of intensive development, one observation predominated, namely that once cells started to proliferate to produce colonies in methylcellulose, the colonies only grew larger; no new colonies were formed at later time points. During the first 7 days, only undifferentiated cell aggregates were observed. These small aggregates represented centers or clusters of proliferation that would eventually build the colony into one that could be later

TABLE 1. A Comparison of Commercially Available CFC Products

	MethoCult™/ ColonyGel / HSC-CFU / Methylcellulose Media	ColonyGro™	CAMEO™-4	CAMEO™-96
Application:	For virtually any hematopoietic cell application that involves cell differentiation			
Use:	Traditional CFU assays	Cost-effective alternatives to competitor reagents		Standardized CFC assay
Special Use:	No products for special applications	Specific reagents and assays for cellular therapy (cord blood banks and cell processing laboratories)		Proliferation and differentiation assay
Available for:	Limited number of cell populations and flexibility	Up to 8 different stem cell populations Up to 9 different progenitor cell populations 3 different precursor cell populations (not available for CAMEO™-96)		
Species:	Limited number of species	Human, non-human primate, hose, pig, sheep, dog, rat and mouse		
Number of Samples:	Max. 33 samples at 2 replicates/sample	44 samples at 2 replicates/ sample	50 samples at 4 replicates/ sample	Completely flexible
What's included:	100mL bottle of reagent	100mL bottle of ColonyGro™ reagent	CAMEO™-4 reagent + 50 culture plates	CAMEO™-96 reagent, 96-well plates, standards, calibrator controls and ATP Enumeration Reagent
Total culture volume:	3.3mL, including 0.3mL cell suspension	2.5mL, including 0.25mL cell suspension	0.6mL, including 0.06mL cell suspension	Varies depending on number of replicates performed
Culture incubation time:	14 days	10-12 days	9-10 days	7-10 days
Instrument required:	Inverted microscope. Automated colony counter requiring special supplies	Inverted microscope	Inverted microscope	Inverted microscope and luminescence plate reader (capable of multiple applications)

Figure 7. Time Course of the Development of Proliferation Units or Cell Clusters and the Amount of Proliferation During Colony Development in Methylcellulose for Human Bone Marrow Cells



Separate 96-well plates were prepared for each day of the study. Each cell population was setup in columns of 8 replicates. On each day, the number of proliferation units or cell clusters were manually counted followed by the processing and measurement of bioluminescence to determine the amount of cell proliferation.

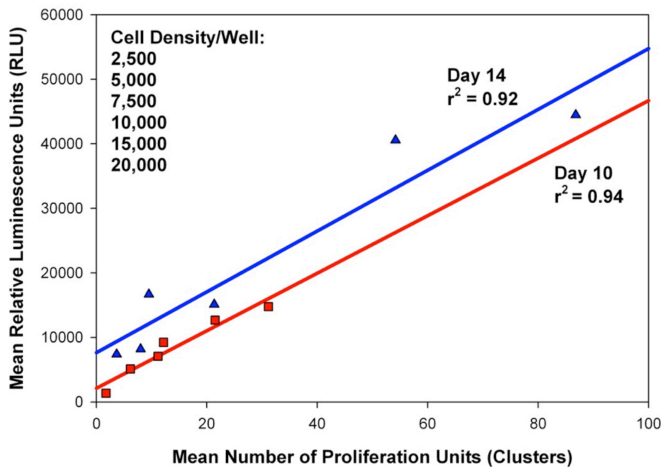
identified. These centers of proliferation were called "proliferation units"; they represent the dark centers of cell expansion observed in virtually every methylcellulose colony. They are the reason why the majority of colonies have an irregular shape

Figure 7 shows the correlation between the amount of proliferation measured using bioluminescence (as RLUs) and the number of cell aggregates or cell clusters manually counted on every day from 1 to 13 of culture for three hematopoietic cell populations, CFC-GEMM, BFU-E and GM-CFC. The time course of cluster formation directly parallels the amount of proliferation measured in the culture wells. The exponential increase in proliferation begins at about day 4 (see also HALO®-Real Time) and continues until day 10, after which proliferation decreases. Differentiation begins after day 7. In other words, up to day 7, undifferentiated cell clusters can be counted, but measurement of their cell proliferation gives exactly the same result.

When the bioluminescence endpoint in RLUs was measured on day 7 of culture for hematopoietic stem cells (CFC-GEMM) for human bone marrow and plotted against the number of proliferation units manually counted and separated on days 10 and 14 as a function of cell concentration, a direct correlation between the parameters as a function of cell dose was obtained (Figure 8).

This correlation states that measuring proliferation of cells

Figure 8. Measuring Cell Proliferation on Day 7 of Methylcellulose Culture Predicts Colony Formation on Days 10 and 14.



growing in methylcellulose predicts colony formation on days 10 and 14. Therefore, counting colonies on day 10, 14 or any other time, can be replaced by measuring cell proliferation on day 7.

The fundamental and important relationships shown in Figures 7 and 8 were the basis of what is now the HALO® Platform. In fact, these relationships were so enticing and important that Stem Cell Technologies copied them to develop a 7-day, 35mm Petri dish methylcellulose assay, which they call MethoCult™ Express.

Is There a Need for Clonal Assays?

It is now possible to determine both proliferation and differentiation of hematopoietic cells in a single assay using two endpoints that are specific for the biological processes in question. CAMEO™-96 opens up a new realm of basic, stem cell and applied applications for hematopoietic research, providing the clonality of the cells is also required. As stated previously, only cells that proliferate have the ability to form colonies, both *in vivo* and *in vitro*.

The use of clonal hematopoietic assays has helped in understanding many important aspects of the hematopoietic system. However, the use of clonal assays, and the CFC assay in particular is, in part, based on the fact that these assays were the only assays available for 40 years. During the last 10 years and now in 2016, new technology calls into question the use of the colony-forming assays.

1. If colony formation relies only on cell proliferation, which cannot be measured using a clonal assay without incorporating a second endpoint, why use a colony-forming assay?
2. Since differentiation is a default program requiring prior proliferation, stimulating cells with a specific cocktail of growth factors will predict the lineage-specific cells being produced so that a colony-forming assay is unnecessary.
3. Figure 5 demonstrates that equivalent results can be obtained using an instrument-based cell proliferation assay compared to a manual enumeration colony-forming cell assay. Why would it be necessary to spend so much time learning and counting colonies, when the same data can be obtained in just minutes using a different endpoint?
4. If the assay cannot be standardized so that results can be compared between samples and over time, why use such an assay?
5. Indeed, why invest in expensive equipment that can only be used for one assay and requires additional special supplies, when a plate reader for less than half the cost can be purchased and used for multiple applications and automating the process?
6. Some vendors have found a way of misleading their customers into thinking that if they perform a colony counting proficiency test, they are actually standardizing their assay against others who are performing the same assay. If the assay cannot be standardized in the first place, this type of proficiency testing wastes time and money. There is no useful information that is obtained from such an exercise, other than demonstrating the large variability of results between laboratories.

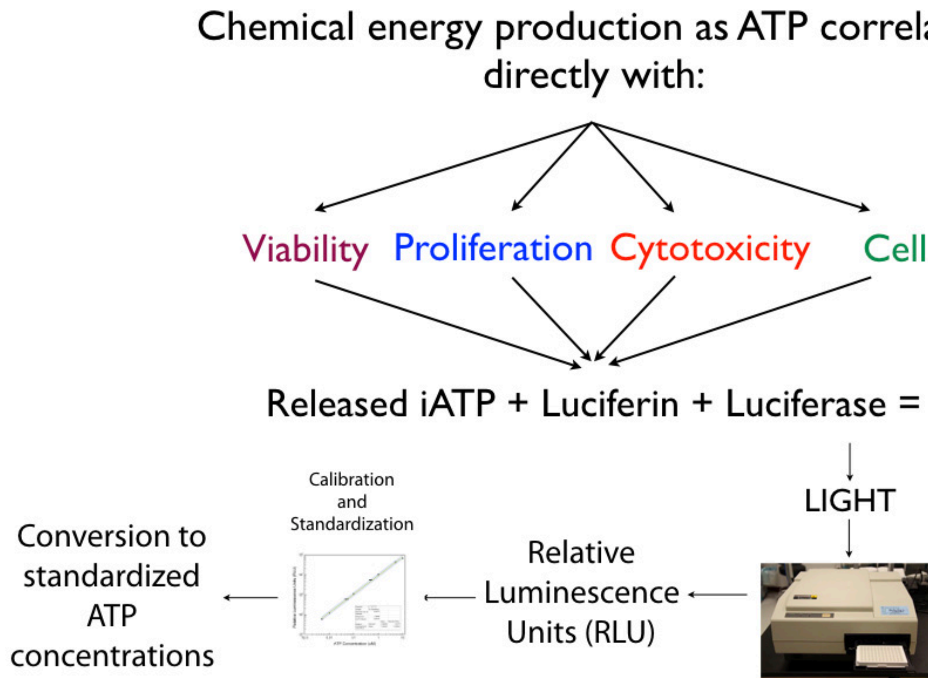
Hematopoietic Assays Without Methylcellulose and Colony Counting

Researchers site two main reasons for continuing to use the CFC assay. First, it is an established (grandfathered) assay that everyone in the field uses. Second, the principle investigator or head of the research group used it and therefore people in the laboratory also use it. These are not reasons that would instill much confidence in researchers entering the field. Indeed, researchers should be acutely aware of the limitations of any assay they are using not just the CFC assay. Many articles have been published on the limitations of the CFC assay[9]. Yet the field either fails to rectify, or denies, the situation.

With more precise, sensitive and reliable technology that is faster, easier to learn and more cost-effective, there are few reasons for continuing to use the CFC assay.

- Due to the viscosity of methylcellulose and the difficulty in dispensing the reagent accurately, whether from a stock solution or diluted with culture components, high coefficients of variation (CV) make for difficult statistical evaluation of results.

Figure 9. The Concept and Principle of Measuring Intracellular ATP Using Bioluminescence



- Similarly, high CVs within sample replicates and lack of data correlation between laboratories caused by high pipetting error and colony counting does not allow standardization of the assay or studies to be repeated.
- The lack of any external standards and controls means that the assay can be neither standardized nor validated. This is especially important for clinical applications.
- The inability to quantitatively measure the basic process of cell proliferation (except with CAMEO™-96) severely inhibits the use of the assay.
- The inability to combine other assay readouts on the same sample limits the amount of information acquired by the assay.
- The use of liquid handlers or robots to perform routine and accurate dispensing operations and screening are impossible to perform, again limiting the use of the CFC assay in many applications.

A clear understanding of these deficiencies prompted the consideration of a world without methylcellulose assays.

The First Innovation that Led to a World Without Colony Counting

The first innovation that led to the removal of colony counting was the incorporation of the ATP bioluminescence endpoint used for the first time in the CAMEO™-96 assay[14].

Why ATP bioluminescence? It is the most sensitive and accurate signal detection endpoint system available. It is just as sensitive, if not more sensitive than radioactive endpoints and outshines both fluorescence and absorbance in many aspects. It has been used by biopharmaceutical companies for many years. The ability to standardize and validate the readout is unquestionable. With few exceptions, measurement of intracellular ATP can be reliably and reproducibly used for measuring metabolic viability, cell proliferation and cytotoxicity, cell concentrations and even apoptosis. The basic methodology is shown in Figure 9.

When cells proliferate or are inhibited from proliferation, the intracellular ATP concentration varies proportionately. The amount of ATP is dependent upon mitochondrial and cellular integrity and activity and therefore provides a measurement of metabolic viability. After culture, a single reagent is added that lyses the cells within 10 minutes and releases the intracellular ATP into the supernatant where it reacts with luciferin and luciferase to produce a "glow" of light that is measured in a luminescence plate reader. The output of the reader is in relative luminescence units or RLU. This is a non-standardized measurement that depends on the instrument characteristics. Using calibration controls and an external ATP standard, a standard curve can be performed that allows the RLU values to be interpolated and converted into standardized ATP concentrations. This quantitative output allows the assay to be validated[16].

It was this technology that allowed the CFC assay to be

standardized and cell proliferation within the colonies to be measured using CAMEO™-96.

The Second Innovation that Led to a World Without Methylcellulose

Even when intracellular ATP is measured, it follows that many of the problems associated with methylcellulose, mentioned above, are still present. The need to remove methylcellulose from the equation was met with the second innovation, namely the development of Suspension Expansion Culture™ (SEC™) Technology, the importance of which cannot be underestimated.

Suspension Expansion Culture™ Technology has the following advantages over methylcellulose.

- Normal pipettes can be used. No need for inaccurate syringes and needles).
- Greater dispensing accuracy reduces pipetting error.
- Significant increase in assay sensitivity.
- Allows the use of different formulated media.
- Allows cell-to-cell interactions to occur.
- Shorter proliferation onset lag time (within 24 hours).
- Shorter culture time: 3-7 days, instead of 10-14 days.
- It allows for the expansion of different cell types.
- Easy to remove cells for multiplexing with other assay endpoints.
- Ability to use 96- or 384-well plates.
- Allows high-throughput capability and the use of liquid handlers (robots) for routine dispensing operations and screening that also reduces dispensing errors.
- Allows for non-subjective, instrument-based measurement and analysis that can be fully automated.

Hematopoietic Assays via Luminescence Output (HALO®)

The combination of both innovations led HemoGenix® to develop Hematopoietic Assays via Luminescence Output, otherwise known as the HALO® Platform[17].

HALO® is a non-clonal, methylcellulose-free, proliferation assay for lympho-hematopoietic stem and progenitor cells. In addition to the advantages provided by SEC™ technology, HALO® is capable of detecting and measuring the response of up to 9 different stem cell populations (Figure 9) and up to 11 different progenitor cell populations from 8 species, simultaneously (Table 4). HALO® is also the only hematopoietic assay platform that can be performed in 384-well plates.

The ability to directly and accurately measure two separate, but related, parameters of cell proliferation, has allowed the development of assays for a multitude of different applications that has hitherto been impossible. Thus, instead of trying to adapt a CFC reagent to fit a specific application, HALO® allows the application to dictate the assay itself. This means, for example, that the properties of stem cells can be directly applied to applications that require the measurement of stem cell self-renewal, expansion, cytotoxicity, quality, and potency.

Although cell proliferation is more important than differentiation, since without proliferation there would be no differentiation, the majority of investigators believe that counting and identifying (differentiating) colonies is the only way to study hematopoiesis. As a result, they fail to comprehend a very basic aspect of the CFC assay.

If Colonies are not Formed and Cannot be Counted and Differentiated, How is it Possible to Know Which Cells are Being Produced?

The answer to this question was given by the evidence in Figures 7 and 8 for CAMEO™-96. However, a second answer to this question is the same as asking, what cell types would be expected and produced if a colony-forming cell was stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF), but no colonies were produced or could be counted? The answer is simple; granulocytes and macrophages could be expected, and would be produced. If this is known, why is a CFC assay needed?

The growth of granulocyte and/or macrophage colonies in the original agar culture performed by Bradley and Metcalf and Pluznik and Sachs more than 50 years ago was due to the addition of conditioned medium to the cultures, which we now know contained colony stimulating factor or CSF. In fact, in the same year, 1966, John Paul and Robin Cole used erythropoietin (EPO) to stimulate the production of erythropoietic cells in non-clonal, mouse fetal liver cultures and measured the response using the uptake of radioactive iron into red blood cells[18]. With the development of recombinant technology, EPO, was the first growth factor to be manufactured in recombinant form for clinical use to alleviate the symptoms of anemia in patients with chronic renal failure. No one asked whether EPO would not stimulate red blood cell production in this clinical situation; it was already known. Recombinant EPO was followed by recombinant GM-CSF and G-CSF and eventually all of the other growth factors and cytokines that are used today for research and clinical purposes.

Lineage-specific factors such as EPO, the CSFs and thrombopoietin (TPO), specifically stimulate and/or maintain cells of the erythropoietic, myelomonocytic and

TABLE 2
The Proportion of Different Cell Types Determined by Flow Cytometry After Stimulating the Equivalent Colony-Forming Cell with the Growth Factor Cocktail Shown

Marker	SC-GEMM [CFC-GEMM] (EPO, GM-CSF, IL-3, IL-6, SCF, Flt3-L, TPO)	E-Progenitor, [BFU-E] (EPO, IL-3, SCF)	GM-Progenitor, [GM-CFC] (GM-CSF, IL-3, SCF)	Macrophage Precursor [M-CFC] (M-CSF)	Mk-Progenitor, [Mk-CFC] (TPO. IL-3, SCF)	T-Cell Progenitor [T-CFC] (IL-2)	B-Cell Progenitor [B-CFC] (IL-7)
CD34	0.05%	0.06%	0.08%	-	-	-	-
CD117	2.55%	-	-	-	-	-	-
CD133	3.24%	-	-	-	-	-	-
Glycophorin-A	25%	54%	-	-	-	-	-
CD14	9.3%	3.9%	16.8%	17.8%	9.7%	-	-
CD15	1.6%	1.1%	1.1%	-	1.1%	-	-
CD41/CD61	9.2%	8.5%	-	-	48.1%	-	-
CD3	-	-	-	-	-	31.4%	-
CD3/CD4	-	-	-	-	-	17.9%	-
CD3/CD8	-	-	-	-	-	11.6%	-
CD3/CD56	-	-	-	-	-	7.0%	-
CD19	-	-	-	-	-	-	21.9%

Abbreviations in round brackets () indicate the growth factor cocktails used to stimulate the cell population.
EPO = Erythropoietin. GM-CSF = Granulocyte-macrophage colony-stimulating factor. IL-3 = Interleukin-3. IL-6 = Interleukin-6. SCF = Stem cell factor. Flt3-L = Flt3-Ligand. TPO = Thrombopoietin.
Abbreviations in square brackets [] indicate the equivalent colony-forming cell detected in the methylcellulose assay.

megakaryopoietic lineages, respectively. Some, however, and TPO is an example, together with other factors such as interleuin-3 (IL-3), interleukin-6 (IL-6), stem cell factor (SCF), Flt3-ligand, may result in a potentiation as well as a stimulation of cells. These and many other factors have a pliotropic effect on cells; that is, they can effect more than one function and/or cell type.

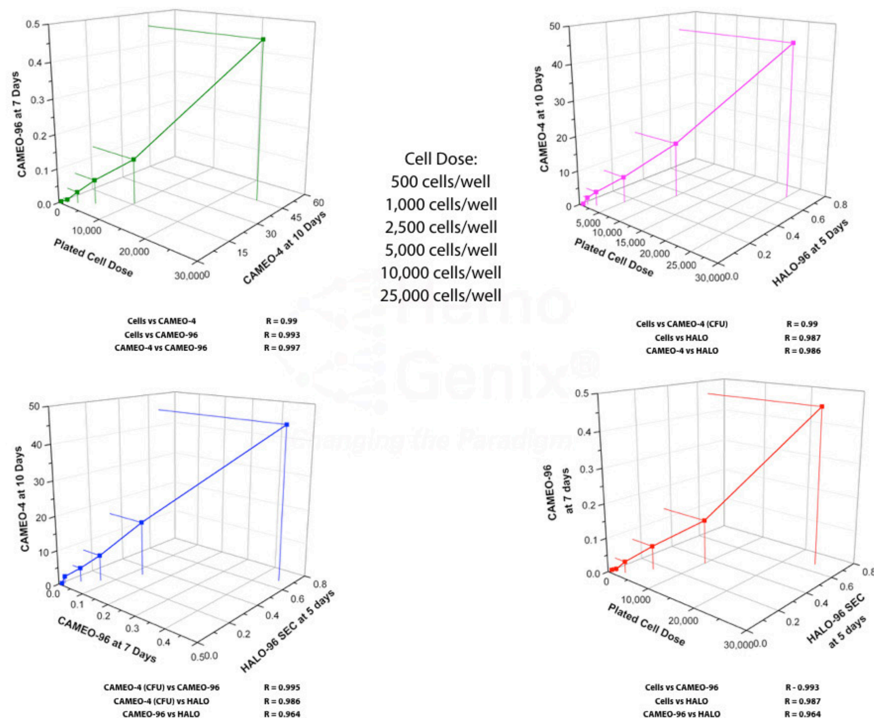
The stimulation of stem cells of the lympho-hematopoietic system usually requires a combination or cocktail of growth factors and cytokines to detect their presence. As the cells mature and enter a specific cell lineage, their requirement for multiple factors decreases until it is only necessary to stimulate and/or maintain cells of a lineage using a single factor.

Since a considerable amount of study has gone into examining the effect of different growth factors, the response by different cell types is relatively well known. This is taken into account when HALO® is used. As in the clinical situation, it is known that if cells are stimulated with EPO alone, only those cells in the erythropoietic progenitor and precursor compartments will be stimulated. If IL-3 and SCF is added to EPO, this will result in an influx of cells from the stem cell compartment, which, in the presence of EPO, enter the erythropoietic lineage to increase the number of erythropoietic progenitor cells that can be measured. Using HALO®, it is therefore possible to compare the number of BFU-E responding to EPO alone in the BFU-E compartment, and the BFU-E produced by an influx of cells from the stem cell compartment. These are subtle, but important differences in understanding the regulation of different compartments and the system as a whole.

To demonstrate that the cell types produced in a HALO® culture are those that would be expected, the multiplexing capability of HALO® is used. This involves culturing the cells using different cocktails of growth factors. After a specific time period, the cells can be removed and labeled with fluorescent-conjugated antibodies that identify the different cell types in the culture. An example of this analysis is shown for a HALO® 7-population assay in Table 2.

The results indicate that depending on the growth factor cocktail used to stimulate the equivalent CFC population in question, the cell types found in HALO® cultures are those that would be expected. Therefore the population SC-GEMM (stem cell-GEMM), which is equivalent to the CFC-GEMM or colony-forming cell - granulocyte, erythroid, macrophage, megakaryocyte, produced in methylcellulose, gives rise to all of these cell types, together with very low proportions of other stem cells. This is precisely what would be expected and it is why the population is called CFC-GEMM. Similarly, if BFU-E are stimulated with EPO, IL-3 and SCF, it would be expected that the majority of cells would be glycophorin-A^+ erythroid cells, but with the presence of IL-3 and SCF, some stem cells might be present as well as others stimulated by IL-3. Since the genetic sequence of EPO is partially similar to that of TPO[19], it might not be surprising that megakaryocytes can also be detected. In short, the use of different growth factor cocktails to induce proliferation of primitive cells will predict the type of cells produced without the need for counting colonies. In fact,

Figure 10. The Correlation Between CAMEO™-4 and CAMEO™-96 Methylcellulose CFC Differentiation Assays With the HALO® Non-Methylcellulose, Non-Colony-Forming, Proliferation Assay



as demonstrated in Table 2, cells (and not necessarily colony-forming cells) may be produced that might otherwise not be detected by the CFC assay.

It should be emphasized that like the CFC assay, HALO® detects cell populations that cannot be morphologically identified, because they are so few in number. There should be no misunderstanding that both assays use exactly the same growth factors and cytokines and therefore stimulate the same cell populations and types. However, unlike the CFC assay, no colonies are formed in HALO®, because cell stimulation and proliferation occurs in suspension culture. This means that it is inaccurate to designate cell populations detected and measured using HALO® as CFC; they are equivalent to CFC, but are not colony-forming cells. Therefore, the non-colony-forming equivalent designation will be used for all non-methylcellulose, SEC™ assays (see Figure 11 and Table 4).

Comparison of HALO® with the Methylcellulose CFC Assays

The correlation of results of two or more assays with different endpoints or readouts is a process called assay verification; one assay is verified against another to demonstrate equivalent results. Such an assay verification is shown in Figure 10 in which CAMEO™-4, CAMEO™-96 and HALO® are compared with each other as a function of cell dose and culture time.

That each assay format correlates with each other even though the endpoint is determined at different time points, indicates that one assay can predict the other, but more importantly, that one assay can replace the other[20].

Figure 4 demonstrated a correlation between the traditional ColonyGro™ methylcellulose CFC assay and the miniaturized CAMEO™-4 CFC assay. Figure 10 shows this same relationship between CAMEO™-4, CAMEO™-96 and HALO®. This is to be expected since all use the same hematopoietic target cells and all use the relationship between cell differentiation which is dependent upon cell proliferation.

The scientific evidence speaks for itself. The only reason to use a clonal, methylcellulose CFC assay is to study hematopoietic cell differentiation and maturation, even though flow cytometric phenotypic analysis might provide a more informative readout (Table 2). Since the majority of stem cell characteristics, e.g. self-renewal, primitiveness, engraftment etc are dependent upon the proliferation (not differentiation) ability and potential of the stem cells, it follows that a proliferation assay and not a differentiation assay is going to provide the information needed to study these and related properties. Similarly, since the main property of hematopoietic progenitor cells is to amplify and provide sufficient functionally mature end cells, it also follows that measuring progenitor cell proliferation is more important than differentiation, since the latter is, under normal circumstances, a default program of the former. It is therefore easy to see why many view HALO® as a threat to CFC use.

Table 3. Comparison Between Proliferation Assays

Assay Type	Measures Cell Viability	Cell Proliferation Readout
Isotope labeling	No	^{3}H-Tdr, ^{51}Cr
Cell counts using dyes (neutral red, methylene blue)	Yes, by dye exclusion	Manual/Automated
MTT, MTS, XTT, BudR, Edu	Only those that rely on metabolic activity	Absorbance
Calcein, BudR, Live Protease, WST-1, CSFE, CellQuant	Only those that rely on metabolic activity	Fluorescence
Intracellular ATP	Yes	Bioluminescence
Reduction potential	Yes	Bioluminescence

It is ironic that those who complain about the drawbacks of the CFC assay and advocate new technologies, are the same who have shown no incentive or innovation to improve the assay or change to new technology that is already available. For whatever reason, they would prefere to keep the CFC assay[21]. However, the world does not stop for the CFC assay!

The Importance of Measuring Stem and Progenitor Cell Proliferation

Cell differentiation is a very difficult parameter to measure. In fact, there are very few quantitative differentiation assays, since biochemical markers for different stages of differentiation are often not available.

In contrast, nearly all proliferation assays are quantitative and the majority are based on a specific biochemical marker that correlates with the proliferation process. Table 3 lists some of the proliferation markers available.

Many of the proliferation assays are also cell viability assays, the most accurate of which are those assays that detect changes in metabolic state. As seen in Figure 9, intracellular ATP is a metabolic marker for viability, proliferation and cytotoxicity. Several absorbance and fluorescence readouts can be used for the same purpose (see Table 4).

There are two parameters of proliferation that most of these readouts can measure. The first is proliferation ability, which is defined as the amount of cell proliferation at a specific cell dose and point in time. The second is proliferation potential. This is a predictive measurement of how much proliferation a cell or cell population can undertake. As shown in Figure 11, there is a relationship between stem cell proliferation ability and stem cell proliferation potential; that is, as stem cells enter proliferation from their quiescent state and begin maturing from more primitive stem cells with the highest potency and greatest capability for self-renewal, their proliferation potential decreases. This is clearly seen in practice. Figure 12, shows

Figure 11. The Hematopoietic Stem Cell Compartment and the Relationship Between Self-Renewal, Primitiveness, Potency, Proliferation Potential and Proliferation Ability

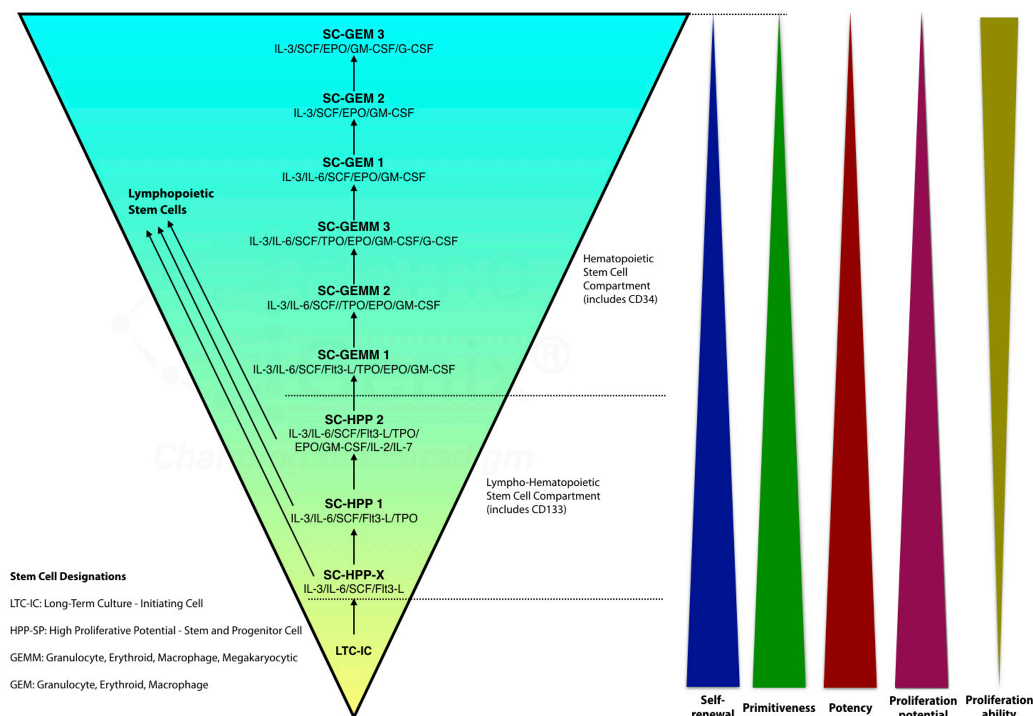


Figure 12. The Relationship Between Proliferation Potential, Proliferation Ability and Stem Cell Primitiveness Detected using HALO®

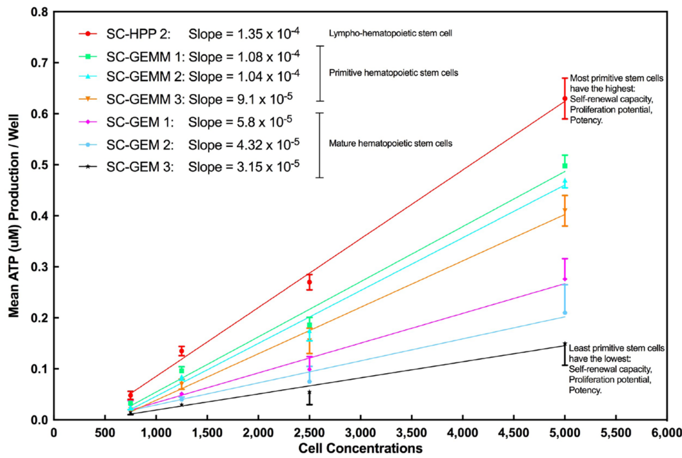
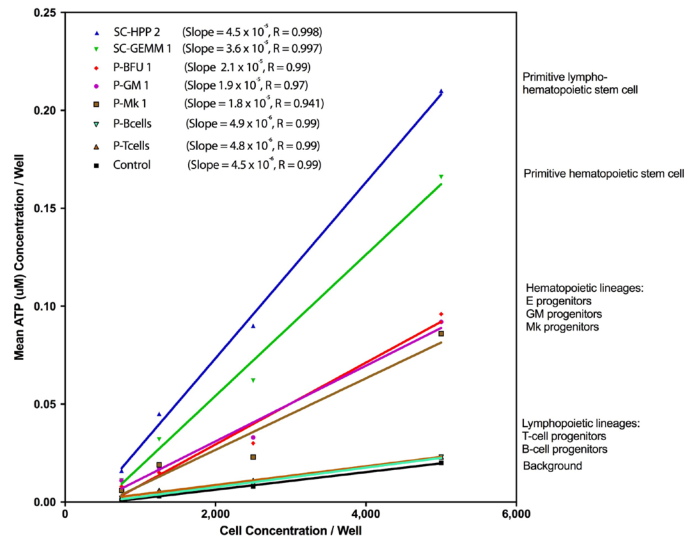


Figure 13. Changing Proliferation Potential Between Stem and Progenitor Cells Detected using HALO®



how proliferation potential decreases as stem cells become more mature and closer to the point of being determined into a specific cell lineage[22]. Proliferation potential is measured by performing a minimum 3-point cell dose response curve. To detect different hematopoietic stem cell populations, human bone marrow was cultured for 5 days with cocktails of growth factors specified in Figure 9. Using the standardized readout from HALO®, proliferation at each cell dose was determined by ATP bioluminescence and linear regression of the cell dose responses performed. The slope of each linear regression is shown for each stem cell population measured. As stem cells become more mature (less primitive), their proliferation potential, i.e. the slope of the linear regression, decreases.

This concept has been shown to be the basis of measuring stem cell potency prior to umbilical cord blood, bone marrow or mobilized peripheral blood stem cell transplantation[16,23,24].

However, this concept is not just applicable to stem cells. Figure 13 demonstrates that proliferating lympho-hematopoietic cells can be divided into three groups, namely the stem cells with the greatest proliferation potential, hematopoietic progenitor cells and lymphopoietic cells, which have the lowest proliferation potential depending on how they are stimulated.

It is obvious that this type of analysis has far-reaching implications, not only for hematopoietic research, and especially in cell expansion technology for cellular therapy, but for any biological system in which changes in the properties of proliferating cells needs to be determined. In addition, even cells that do not undergo proliferation, but have high levels of metabolic activity, such as brain, liver, heart or kidney cells for example, will demonstrate a similar characteristic.

Measuring Hematopoietic Cell Proliferation in Real Time

A new assay that takes hematopoietic cell proliferation to a different level was introduced by HemoGenix® in 2016. Although a bioluminescence assay, HALO®-Real Time[25] is not based on intracellular ATP concentrations. Instead, it is based on mitochondrial reduction potential. In addition, HALO™-Real Time is a non-lytic or non-destructive assay. However, like all HALO® assays, cell populations are detected and stimulated in the same manner and everything is included in the assay kit. Hematopoietic cell cultures, for any cell population available (Table 4), are setup in the plates provided. The HALO®-Real Time reagent can be added directly to the wells at the beginning of cell culture or at any time during the cell culture phase. The plate is transferred to the incubator and at any time can be removed, the luminescence measured and the plate replaced back into the incubator.

Figure 14.
HALO®-Real Time to Measure Stem Cell Growth Kinetics

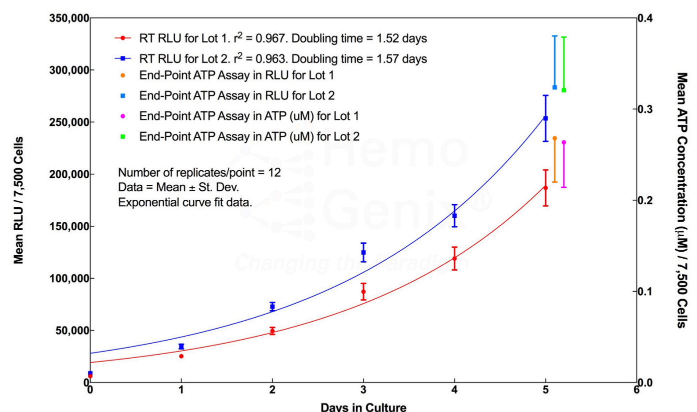


Figure 14 shows an example of the growth kinetic curves for primitive human hematopoietic bone marrow stem cells (SC-GEMM 1, see Figure 9 and Table 4) from two separate donors. In this particular case, the time course was taken out to 5 days, after which culture wells were processed to measure the amount of proliferation using intracellular ATP as an end point. HALO®-Real Time cannot be standardized in the same manner as the ATP HALO® assay, but the instrument can be calibrated prior to use. Nevertheless, by 5 days, high levels of intracellular ATP were measured corresponding to the level of proliferation by the stem cells. Using HALO®-Real Time, the exponential increase of stem cells is clearly discernible and corroborates why HALO® for human cells can be completed in just 5 days as opposed to longer incubation times for the methylcellulose CFC assay. Fitting an exponential curve fit to the data indicates that the doubling time is approximately 1.5 days. In addition, at 3 days of incubation, the RLU values are significantly greater than at the initiation of culture or at 1 and 2 days of incubation. This too, corroborates the basis for the 3-day stem cell viability and functionality assay specifically developed to triage high from low quality umbilical cord blood units for cord blood banking using STEMpredict[26].

Properties and Characteristics When Considering a Cell Proliferation Assay

Every assay has its advantages and disadvantages. No single assay is going to be ideal. However, when deciding on an assay, the researcher should start by asking the question; what is the goal that has to be investigated? This will inevitably define the type of assay that has to be used to achieve that goal. If the assay for a particular application is not commercially available, then it will have to be developed, but this, in turn, may prove to be a difficult undertaking. Deciding which type of cell proliferation assay to use is based on a number of factors, which should be taken into account prior to actually purchasing and using the assay.

1. **Radioactive or non-radioactive**. A non-radioactive assay such as ATP bioluminescence is as sensitive, if not more sensitive than a radioactive assay, without the hazardous waste and documentation that accompanies the use of a radioactive marker.
2. **Destructive or non-destructive assay**. Several proliferation assays are lytic or destructive; the cells have to be lysed in order to release the substrate that is used for the reaction. For example, intracellular ATP, MTT, MTS, XTT are all examples of destructive assays. This, however, does not prohibit them from being multiplexed with other endpoint assays. In most cases, additional replicates, from the same sample, can be setup to determine other endpoints.
3. **Assay interference**. Culture supplements or other compounds can interfere with the assay result. For example, large numbers of red blood cells can cause a false positive result if using an ATP bioluminescence readout (see also Target Cell Purity below). The opposite is true when too much hemoglobin is present if blood cells are not washed after lysis to remove red blood cells. Some compounds interfere with metabolism and therefore an assay based on metabolic activity could give false results. Performing an assay with insufficiently pure cell populations can introduce large numbers of cell impurities that can drastically affect the results. This also applies to the CFC assay. Knowing the limitations of the assay can save a lot of time, effort and cost.
4. **Assay multiplexing**. Assay multiplexing is when the same sample is used to measure different assay readouts or endpoints. It allows a large amount of information to be obtained from the same sample, rather than setting up multiple assays on different samples, producing results that often cannot be compared with each other. HALO®, HALO®-Real Time, HemoFLUOR™ [27]and HemoLIGHT™[28], all of which are non-clonal assays using SEC™ technology, were designed for multiplexing. Non-destructive assays such as HALO™-Real Time and HemoFLUOR™ can be used very efficiently for multiplexing purposes. Nevertheless, if, for example, cell proliferation needs to be coordinated with measuring cell differentiation, then all of these assays can be multiplexed with flow cytometric phenotypic analysis or gene expression analysis.
5. **Assay standardization and validation**. Most investigators purchase a reagent or an assay kit from a supplier and hope that it works immediately. One problem often encountered and not discovered until it is too late, is how to compare the results over time or from one assay to another. This is why controls and standards are necessary and how assay standardization comes into play. The other question is, can the results can be trusted? This is especially important if results are going to be used for clinical purposes. Results can only be trusted if the assay is validated, and to validate an assay, controls and standards are needed, otherwise it would not be known whether the instrument, assay or the experimental protocol is to blame for incorrect or lack of results.
6. **Target cell purity**. The goal of the study will define, to a certain extent, the preparation and purity of the primary cells used for the assay, regardless of whether proliferation or differentiation is to be measured. In general, the more pure a cell population, the greater the response and visa versa. If a primitive stem cell population, whose normal concentration is less than 0.1%, is to be measured, it would be like searching for a needle in a haystack if the starting cell preparation contained high concentrations of other cell impurities, such that the stem cells would be diluted and their activity severely underestimated. If this appears a ridiculous scenario, it is not. This scenario occurs every time a CFC assay is used to test an umbilical cord blood

sample prior to cryopreservation[16]. However, sometimes a highly purified cell population might be "overkill" because the cost of purchasing or purifying a cell population may far outweigh the quality of the results.

7. **Cell interaction**. In the past, cell interactions have been considered rather unimportant in the CFC assay. In fact, one of the reasons for using the CFC assay was to reduce the possibility of cell interactions. This may be the reason for the low plating efficiency and sensitivity exhibited by the CFC assay. However, cell interactions are an absolute requirement for the regulated cell proliferation in hematopoietic tissues. Incorporation of SEC™ Technology in HALO®, HALO®-Real Time, HemoFLUOR™ and HemoLIGHT™ assays allows cell interactions to occur that are necessary, not only to detect the presence of different cell populations, but to provide the assay itself with the needed sensitivity to measure cell proliferation at very low levels.
8. **Assay sensitivity and other parameters**. Assay sensitivity, accuracy, reliability, reproducibility (which are incidentally all part of the validation process), are very important parameters to take into account. Hematopoietic stem cells represent a minute population consisting of cells with different degrees of primitiveness. In flow cytometry, stable and intensely fluorescent markers are ususually used to identify primitive, rare cell populations. The same principle should be used for cell populations assays. The most sensitive and accurate assay available should be chosen for primitive, rare cell populations. In general, cell proliferation assay sensitivity is as follows:
   ATP bioluminescence => isotope labeling > fluorescence > absorbance > cell counting. Fluorescence is between 10-100 times more sensitive than absorbance and bioluminescence is 10-100 times more sensitive than fluorescence.
9. **Outliers and statistics**. When using assays that measure primary and primitive cells that are few in number, outliers often occur. Assays, even proliferation assays, are often performed with the minimum of replicates to save money and/or time. When deciding on an assay, it is worth considering the statistical power of the results. If just 2 replicates are performed and the percent coefficient of variation (%CV) is greater than about 20%, it is not possible to remove the outlier that is causing the high %CV; there will be no statistics from one replicate sample. Using 3-4 replicate samples, it might be possible to remove one outlier. The higher the number of replicate samples, the better the statistics. This is particularly important when using, for example, the subjective CFC assay with only 2 replicate samples for clinical applications. Remember, it costs more to repeat an assay (and often this may be impossible) than it does to increase the number of replicates.
10. **Proliferation or differentiation**. Once again, what is the goal of the study? The goal of the study will determine the assay to be used, not visa versa. If the endpoint of the assay is to determine whether cells can proliferate or the amount of proliferation, a differentiation endpoint is not the assay of choice. If, on the other hand, the starting population is already well differentiated, then a proliferation assay may not be of much use because of the limited proliferation ability and potential of the cells. Understanding the properties of the cells being studied and what the assay results can deliver are key to reducing costly failures and false interpretations and conclusions. Since proliferation can predict differentiation, it is often unnecessary to perform an assay to detect non-quantitative, differentiation when a quantitative proliferation assay can provide superior results.

50 Years of Colony-Forming Assays and its Future

The CFC assay should be celebrated for providing the methodology that has played such a crucial role into the workings of the hematopoietic system by deciphering the organization and hierarchy, the role played by different growth factors and cytokines and its regulation under different conditions. Its importance cannot be underestimated. Indeed, without the CFC assay, HemoGenix® would not have been able to develop different formats and more advanced assays, and that is all thanks to Ray Bradley, Don Metcalf, Dov Pluznik and Leo Sachs.

However, new technologies, at both the molecular and cellular levels, have left the traditional CFC assay in the dust. This is clearly shown in Table 4, where a comparison is provided of the traditional CFC assay from five different suppliers, including HemoGenix®, with other commercially available methylcellulose and non-methylcellulose assays[29]. With the exception of HemoGenix®, all other suppliers only offer a 35mm Petri dish traditional CFC assay to their customers and a very limited number of cell populations to detect. After 50 years of using virtually the same assay, it might be expected that suppliers would have updated their products to keep pace with technology. After all, everyone expects their computer operating system and smartphones to be updated to remove "bugs" so that they work better without crashing. This is not the mantra of methylcellulose CFC providers. They know that over the years, these "bugs" have been accepted, despite researchers complaining about the lack of standardization, high variability, colony counting etc. As a result, they have not deemed it necessary to improve the CFC assay or even offer alternatives. On the one hand, it is up to the researcher to understand why, what and how they need to measure a particular endpoint and the limits of the technology they are using, and demand improved products from their

suppliers. On the other hand, it is up to the supplier to provide better products that have fewer limitations.

Compared to other suppliers of assays for hematopoietic cells, HemoGenix® has lead the way for more than 14 years. It has done this since the introduction of CAMEO™-96 and the HALO® Platform. In fact, HALO® is the most trusted *in vitro* hemotoxicity testing platform for half of the top 50 biopharmaceutical companies. At HemoGenix®, it is not sufficient to merely say that our assays are standardized; the scientific proof is provided.

Subjectivity is no longer an option. Yet to combat subjectivity, expensive image analysis instruments and special accessories have been introduced that are supposed to provide "standardized" colony counting. When standardization is based on a comparison with non-standardized manual colony counting, rather than the use of non-subjective, external standards and controls, this is, in effect, pulling the wool over customer's eyes. It is making the instrument sound "grander" than it actually is.

That syringes and needles are still recommended and sold to dispense methylcellulose reagents demonstrates the inability to change to more accurate dispensing technology that can reduce pipetting errors. A bottle of methylcellulose media costs several hunderd dollars, yet such antiquated dispensing methods shows the lack of incentive and innovation for moving the field forward. This is precisely why the development of non-methylcellulose assays was pursued.

Colony counting and identification has been replaced by higher quality, more cost-effective, quantitative, instrument-based assays performed in a matter of days that can predict results normally obtained in 2 weeks. Moreover, such instrument-based assays are easy to learn by multiple users in a single day, compared with the time-consuming and expensive training needed to count and differentiate colonies.

The inclusion of assay standardization for instrument-based assays, and often discussed, but which never materializes for the CFC assay, is a reality allowing both intra- and inter-laboratory comparison of results and further standardization of procedures and processes, especially in the clinical therapy arena. With standardization, also comes the ability to instantly perform a proficiency test, not only for the user's benefit, but also for the assay itself, so that time-consuming and expensive proficiency tests, that merely provide an indication of how one laboratory compares to another, can be discarded.

It is unfortunate that over the last ten years, misinformation and the misconception that the basic CFC assay is a "one for all, all for one" assay has been propagated. In fact, there is no area of hematopoietic research where this is so deep-rooted than in cellular therapy. The inability to accept new scientific data and put these results into practice, and the false desire to continue using the CFC assay at whatever cost, has, in all likelihood, set hematopoietic stem cell transplantation back decades. Indeed, the damage caused to patients is probably indeterminable. The ingrained and misconstrued notion that it is an absolute necessity to use the differentiation process and count colonies, rather than measure the more important and earlier process of cell proliferation, to establish whether cord blood, bone marrow or mobilized peripheral blood cells will grow, has been perpetuated to such an extent, that it is now considered a scientific fact. During the "discovery phase" of hematopoietic research, when the CFC assay was the only *in vitro* research tool available, considerable care was taken to ensure that reported and published facts were based on sound, repeatable, high quality science. If there is one thing half a century of the CFC assay has taught us, it is that, regrettably, "sound, repeatable, high quality science" is probably a thing of the past, at least in the cellular therapy arena.

It is time to realize that the traditional CFC assay has run its course and better technology and better results await. As stated previously, the world does not stop for the CFC assay! Assays should not stand still; they are always a work in progress to achieve better more reliable and reproducible results. It is in everyone's interest to use the best tools to achieve better scientific results. Only the implementation of non-subjective, instrument-based assays can help keep the legacy of the CFC assay alive for the next 50 years, as one of the most important methodologies developed for hematopoietic research.

HemoGenix®
Changing the Paradigm

Feedback, Comments and Questions

Send all feedback, comments and questions to info@hemogenix.com.

Resources

1. Bradley TR, Metcalf D. (1966) The growth of mouse bone marrow cells in vitro. Aust J Exp Biol med Sci 44:287-299.
2. Pluznik DH, Sachs L. (1966) The induction of clones of normal mast cells by a substance from conditioned medium. Exp Cell Res 43: 553-563.
3. Iscove NN, Sieber F, Winterhalter KM. (1974) Erythroid colony formation in cultures of mouse and human bone marrow: analysis of the requirement for erythropoietin by gel filtration and affinity chromatography on agarose-concanavalin A. J Cell Physiol 83: 309-320.
4. Access ColonyGro™
5. Access MethoCult™ H4435
6. Access CAMEO™-4
7. Access Standardized Media (1)
8. Access Assay Calibration, Standardization, Proficienty Testing and Validation
9. Pamphilon S, Selogie E, McKenna D et al. (2013) Current

practices and prospects for standardization of the hematopoietic colony-forming unit assay: a report by the cellular therapy team of the Biomedical Excellence for Safer Transfusion (BEST) Collaborative. Cytotherapy 15: 255-262.
10. Access CAMEO™-96
11. The CFC assay as a potency assay
12. Reems JA, Hall KM, Gebru LH, Rich IN (2006) Development of a potency assay for umbilical cord blood. Blood 108:5225
13. Access Proliferation and Differentiation
14. Rich IN (2003) In vitro hemotoxicity testing in drug development. A review of past, present and future applications. Current Opinion in Drug Discovery and Development. 6:100-109.
15. Rich IN, Hall KM (2005) Validation and development of a predictive paradigm for hemotoxicology using a multifunctional bioluminescence colony-forming proliferation assay. Tox Sci 87:427-441.
16. Patterson J, Moore CH, Palser E et al. (2015) Detecting primitive hematopoietic stem cells in total nucleated and mononucleated cell fractions from umbilical cord blood segments and units. J. Translational Medicine 13:94. http://www.translational-medicine.com/content/13/1/94.
17. Access HALO®
18. Cole RJ, Paul J (1966) The effects of erythropoietin on haem sythesis in mouse yolf sac and cultured fetal liver cells. J Embryol Exp Morph 15: 245-260.
19. Foster DC, Sprecher CA, Grant FJ et al. (1994) Human thrombopoietin: Gene structure, cDNA sequence, expression, and chromosomal localization. Proc Natl Acad Sci USA 91: 13023-13027.
20. Rich IN (2007) High-throughput in vitro hemotoxicity testing and in vitro cross-platform comparative toxicity. Expert Opin Drug Metab Toxicol. 3:295-307.
21. Spellman S, Hurley CK, Brady C et al. (2011) Guidelines for the development and validation of new potency assays for the evaluation of umbilical cord blood. Cytotherapy 13: 848-855.
22. Rich IN (2015) Measurement of hematopoietic stem cell proliferation, self-renewal, and expansion potential. In: Stem Cell Protocols, Methods in Molecular Biology, Editor IN Rich. Vol. 1235: 7-17.
23. Hall KM, Harper H, Rich IN. (2012) Hematopoietic Stem Cell Potency for Cellular Therapeutic Transplantation. Advances in Hematopoietic Stem Cell Research, Rosana Pelayo (Ed.), ISBN: 978-953-307-930-1, InTech.
24. Rich IN (2015) Improving quality and potency testing for umbilical cord blood: A new perspective. Stem Cell Translational Medicine 4: 967-973. http://stemcellstm.alphamedpress.org/content/4/9/967.short?rss=1.
25. Access HALO®-Real Time
26. Access STEMpredict
27. Access HemoFLUOR™
28. Access HemoLIGHT™
29. Access Availability of Assays for Hematopoietic Cells from HemoGenix® and Other Suppliers

About HemoGenix®

HemoGenix® is a privately owed company specializing in developing, manufacturing and producing advanced assays for testing cells of the hematopoietic and immune systems, mesenchymal system, hepatocyte, neural and renal systems, ES, iPS, transformed cell lines and cancer cells. The company also performs *in vitro* toxicity screening and testing contract services for the biopharmaceutical industry as well as basic research studies. HemoGenix is located in Colorado Springs, Colorado, U.S.A.

This Application Note is a forward-looking document and contains material that is proprietary to HemoGenix®, Inc. No part of this document may be copied or used without the written consent of HemoGenix®. It represents the present state of the art and future technology in the field of hematopoietic research, cellular therapy and toxicology. The views expressed in this Application Note are those of HemoGenix®, Inc.

Table 4. Availability of Assays for Different Hematopoietic Cell Populations from HemoGenix and Other Suppliers

Cell Type	Cell Population CFC / Non-CFC Equivalent Designation	Growth Factor Cocktail used for Stimulation	Methylcellulose CFC Reagents and Kits			Non-Methylcellulose, Proliferation Assays
			Traditional 35mm Plate Assay Format	4-Well, 35mm Plate Format	96-Well Plate Format	96- and/or 384-Well Plate Format
For any cell population		No cocktail included. User adds their own.	ColonyGro, MethoCult, ColonyGel, MeC Media, HSC-CFU	CAMEO-4	CAMEO-96	HALO, HALO-RT, HemoFLUOR, HemoLIGHT
STEM CELL POPULATIONS	SC-HPP-X	3, 6, SCF, Flt3-L	NA	NA	NA	HALO, HemoFLUOR, HemoLIGHT
	SC-HPP-XT	3, 6, SCF, Flt3-L, 2, CD3, CD28	NA	NA	NA	HALO, HemoFLUOR, HemoLIGHT
	CFC-HPP-SP 1 (SC-HPP 1)	3, 6, SCF, Flt3-L, TPO	ColonyGro	CAMEO-4	CAMEO-96	HALO, HALO-RT, HemoFLUOR, HemoLIGHT
	SC-HPP-1T	2, 3, 6, SCF, Flt3-L, TPO, CD3, CD28	NA	NA	NA	HALO, HemoFLUOR, HemoLIGHT
	CFC-HPP-SP 2 (SC-HPP 2)	EPO, GM, 2, 3, 6, 7, SCF, Flt3-L, TPO	ColonyGro	CAMEO-4	CAMEO-96	HALO, HALO-RT, HemoFLUOR, HemoLIGHT
	CFC-GMM 1 (SC-GEMM 1)	EPO, GM, 3, 6, SCF, Flt3-L, TPO	ColonyGro	CAMEO-4	CAMEO-96	HALO, HALO-RT, HemoFLUOR, HemoLIGHT
	CFC-GEMM 2 (SC-GEMM 2)	EPO, GM, 3, 6, SCF, TPO	ColonyGro	CAMEO-4	CAMEO-96	HALO, HALO-RT, HemoFLUOR, HemoLIGHT
	CFC-GEMM 3 (SC-GEMM 3)	EPO, GM, G, 3, 6, SCF + TPO	ColonyGro, MethoCult, ColonyGel, MeC Media, HSC-CFU (only ColonyGro contains TPO)	CAMEO-4	NA	HALO
	CFC-GEM 1 (SC-GEM 1)	EPO, GM, 3, 6, SCF	ColonyGro	CAMEO-4	CAMEO-96	HALO, HALO-RT, HemoFLUOR, HemoLIGHT
	CFC-GEM 2 (SC-GEM 2)	EPO, GM, 3, SCF	ColonyGro, MethoCult, ColonyGel, MeC Media, HSC-CFU	CAMEO-4	CAMEO-96	HALO, HALO-RT, HemoFLUOR, HemoLIGHT
	CFC-GEM 3 (SC-GEM 3)	EPO, GM, G, 3, SCF	ColonyGro, MethoCult, HSC-CFU	CAMEO-4	NA	HALO
PROGENITOR CELL POPULATIONS	BFU-E 1 (P-BFU 1)	EPO, 3, SCF	ColonyGro	CAMEO-4	CAMEO-96	HALO, HALO-RT, HemoFLUOR, HemoLIGHT
	BFU-E 2 (P-BFU 2)	EPO alone (high dose)	ColonyGro, MethoCult	CAMEO-4	CAMEO-96	HALO, HALO-RT, HemoFLUOR, HemoLIGHT
	GM-CFC 1 (P-GM 1)	GM, 3, SCF	ColonyGro, MethoCult, ColonyGel, MeC Media	CAMEO-4	CAMEO-96	HALO, HALO-RT, HemoFLUOR, HemoLIGHT
	GM-CFC 2 (P-GM 2)	GM, G, 3, SCF	ColonyGro, MethoCult	CAMEO-4	NA	HALO
	GM-CFC 3 (P-GM 3)	GM alone	ColonyGro	CAMEO-4	CAMEO-96	HALO, HALO-RT, HemoFLUOR, HemoLIGHT
	Mk-CFC 1 (P-Mk 1)	TPO, 3, SCF	ColonyGro	CAMEO-4	CAMEO-96	HALO, HALO-RT, HemoFLUOR, HemoLIGHT
	Mk-CFC 2 (P-Mk 2)	TPO alone	ColonyGro	CAMEO-4	NA	NA
	T-CFC (P-Tcell)	2 alone	ColonyGro	CAMEO-4	CAMEO-96	HALO, HALO-RT, HemoFLUOR, HemoLIGHT
	B-CFC (P-Bcell)	7 alone	ColonyGro	CAMEO-4	CAMEO-96	HALO, HALO-RT, HemoFLUOR, HemoLIGHT
PRECURSOR CELL POPULATIONS	CFU-E	EPO alone (low dose)	ColonyGro, MethoCult	CAMEO-4	NA	NA
	G-CFC	G alone	ColonyGro	CAMEO-4	NA	NA
	M-CFC	M alone	ColonyGro	CAMEO-4	NA	NA

Suppliers:
HemoGenix: ColonyGro, CAME4, CAMEO-96, HALO, HALO-Real Time, HemoFLUOR, HemoLIGHT
Stem Cell Technologies: MethoCult
ReachBio: ColonyGel
R & D Systems: Methylcellulose Media
Miltenyi Biotech: HSC-CFU

Abbreviations:
SC = Stem cell. P = Progenitor cell.
HPP-SP = High proliferative potential-stem and progenitor
GEMM = Granulocyte, erythroid, macrophage, megakaryocyte
GEM = Granulocyte, erythroid, macrophage
BFU-E = Burst-forming unit erythroid
CFU-E = Colony-forming unit - erythroid
CFC = Colony-forming cell
Mk = Megakaryocyte
T = T-cell
B = B-cell
G = Granulocyte
M = Macrophage
MeC = Methylcellulose
EPO = Erythropoietin
GM = Granulocyte-macrophage colony-stimulating factor (GM-CSF)
G = Granulocyte colony-stimulating factor (G-CSF)
M = Macrophage colony-stimulating factor (M-CSF)
2 = interleukin2 (IL-2)
3 = Interleukin-3 (IL-3)
6 = Interleukin-6
7 = Interleukin-7 (IL-7)
SCF = Stem cell factor
TPO = Thrombopoietin
SF = Serum-free
NA = Not available

Availability is shown for human cells.
ColonyGro, CAMEO-4, CAMEO-96, HALO, HALO-Real Time, HemoFLUOR and HemoLIGHT are available for up to 8 different species:
- Human
- Non-human primate
- Horse
- Pig
- Sheep
- Dog
- Rat
- Mouse

Serum-free assays are available for human, primate and mouse assays for:
- HALO
- HALO-Real Time
- HemoFLUOR
- HemoLIGHT

All assays for all species are otherwise available with a low serum formulation