PROLIFERATION ASSAYS PROVIDE NON-SUBJECTIVE and STANDARDIZED ALTERNATIVES for ASSESSING the POTENCY of STEM CELL PRODUCTS for STORAGE and TRANSPLANTATION

I. N. Rich and Karen M[1]. Hall,

[1]HemoGenix, Inc. Colorado Springs, Colorado

THE PROBLEM:
How Should Stem Cell Potency be Measured?

Since 1971, when Pike and Robinson first modified the the Colony Forming Cell (CFC) Assay to detect human cells, this assay has been used to measure stem cell product potency for stem cell transplantation and eventually for cell storage. But the CFC assay was never designed and developed as a potency assay, but rather an assay for investigational use. In accordance with the Code of Federal Regulations, article 21 CFR 610.10 states that "tests for potency shall consist of either an in vitro or in vivo test, or both, which have been specifically designed for each product so as to indicate its potency". Now with improved technology, the CFC assay is not the assay of choice for four reasons:

1. Subjectivity: A potency assay should be based on an independent, instrument-based reading and not manual enumeration of colonies.

2. Calibration and Standardization: A potency assay should be calibrated against an external standard, thereby allowing the assay to be intra- and interlaboratory validated.

3. Assay time: According to the FDA, "product potency for living cell products may be compromised by extensive assay times". The 14 day culture compromises results.

4. Function: The CFC assay is a differentiation assay. Differentiation is the process whereby an undifferentiated cells, i.e. a stem cell (by definition), acquires the features of a specialized cell. Proliferation is defined as the expansion of cells by the continuous division into two identical daughter cells. Proliferation occurs prior to differentiation. Without proliferation, differentiation would not occur. And differentiation is a default program requiring prior proliferation. The CFC assay therefore measures differentiation potential. As such, not only was the CFC assay not designed to measure potency, but it provides indirect information. The CFC assay is the incorrect assay to use for stem cell potency measurements.

THE SOLUTION TO MEASURING STEM CELL POTENCY

These, and many other drawbacks of the CFC assay were taken into account in the development of the *HALO™* Stem and Progenitor Cell - Quality Control (SPC-QC) Platform. The original HALO™ (Hematopoietic Assays via Luminescence Output) was a 96-well plate, 7 day, methylcellulose clonogenic assay, which instead of counting colonies, directly measured cell proliferation of individual cell populations by virtue of the fact that, when stem and progenitor cells are stimulated with growth factors, an increase in intracellular ATP (iATP) concentration occurs that directly correlates with proliferation. By releasing iATP from the cells, it acts as a limiting substrate for a luciferin / luciferase reaction to produce bioluminescence in the form of light detected in a plate luminometer. HALO™ is calibrated and therefore standardized using an ATP standard dose response curve prior to measuring the samples. This allows intra- and inter-laboratory comparison of results. It also allows various procedures which rely sample potency, e.g. sample preparation, cryopreservation and thawing to name but a few, to be compared and standardized between laboratories.

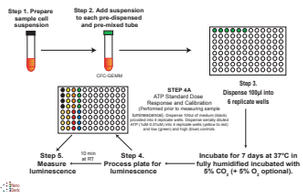
Although a clonogenic assay has traditionally be considered the only way to detect lympho-hematopoietic stem and progenitor cells, we now demonstrate that stem and progenitor cell potency can be performed in the absence of methylcellulose. This assay, now called HALO™-96 SEC (Suspension Expansion Culture) is more rapid and sensitive, easier to use and can be interchanged with either HALO™-96 MeC (Methylcellulose) or the CFC Assay as demonstrated by the direct correlations obtained between these assays.

In addition, we now demonstrate for the first time, that even the 14 days CFC assay can be standardized providing the CFC assay and HALO™-96 MeC are performed under exactly the same conditions.

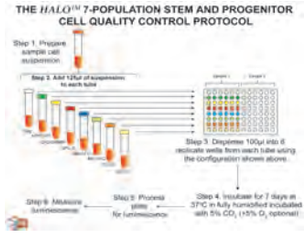
The results from these new assays is that colony counts can now be expressed as standardized ATP equivalent concentrations.

THE ASSAYS

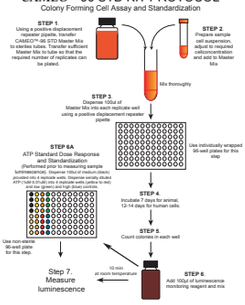
HALO™-96 MeC SPC-QC PROTOCOL for a SINGLE STEM CELL POPULATION
using 3rd Generation Luminescence Reagent



THE *HALO™* 7-POPULATION STEM AND PROGENITOR CELL QUALITY CONTROL PROTOCOL

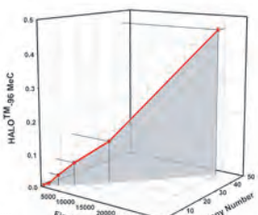


***CAMEO™*-96 STD KIT PROTOCOL**
Colony Forming Cell Assay and Standardization



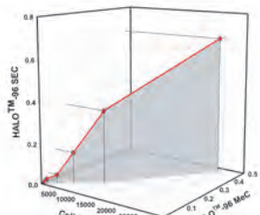
CORRELATION of the COLONY FORMING CELL ASSAY with HALO™-96 MeC and HALO™-96 SEC as a FUNCTION of CELL DOSE: An interchangeable assay system

Correlation of Colony Number with HALO™-96 MeC as a Function of Cell Dose



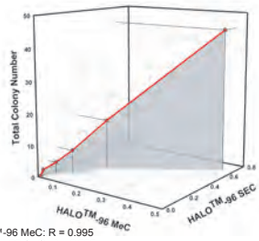
Correlations:
Cells & CFC: R = 0.99
Cells & HALO™-96 MeC: R = 0.993
CFC & HALO™-96 MeC: R = 0.997

Corellation of HALO™-96 MeC with HALO™-96 SEC as a Function of Cell Dose



Correlations:
Cells & HALO™-96 MeC: R = 0.993
Cells & HALO™-96 SEC: R = 0.987
HALO™-96 MeC & HALO™-96 SEC: R = 0.964

Correlation of Colony Number with HALO™-96 MeC and HALO™-96 SEC as a Function of Cell Dose

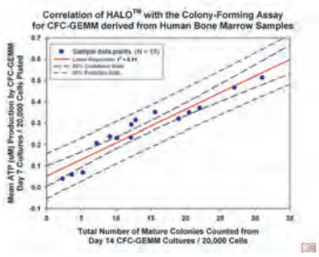


Correlations:
CFC & HALO™-96 MeC: R = 0.995
CFC & HALO™-96 SEC: R = 0.986
HALO™-96 MeC & HALO™-96 SEC: R = 0.964

These results validate HALO™-96 MeC and HALO™-96 SEC against the Colony Forming Cell Assay in one direction. Since all three assays were performed under the same conditions, colony counts counted on day 14 can be expressed as ATP equivalent concentrations on day 7 for HALO™-96 MeC and on day 5 from HALO™-96 SeC. All three assays are interchangeable and therefore the type of assay used will depend on the application.

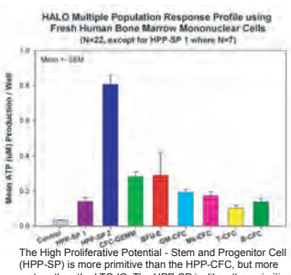
RANGE of PROLIFERATION of HUMAN BONE MARROW LYMPHO-HEMATOPOIETIC STEM and PROGENITOR CELLS

Correlation of HALO™ with the Colony-Forming Assay for CFC-GEMM derived from Human Bone Marrow Samples



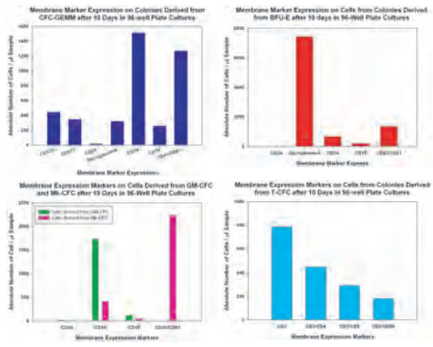
A similar correlation between ATP production at 7 days incubation, as a measure of proliferation potential of pCFC-GEMM, and total number of colonies counted, derived from CFC-GEMM, after 14 days of incubation was also found for umbilical cord blood samples. These results, as well as those shown above, indicate that HALO™ is an alternative, and in most cases can replace, the colony-forming cell assay.

HALO Multiple Population Response Profile using Fresh Human Bone Marrow Mononuclear Cells (N=22, except for HPP-SP 1 where N=7)



The High Proliferative Potential - Stem and Progenitor Cell (HPP-SP) is more primitive than the HPP-CFC, but more mature than the LTC-IC. The HPP-SP is, like other primitive stem cells, quiescent. To induce the cell into cell cycle, a "priming" cytokine cocktail is used over a 7 day incubation period. The proliferation potential shown as HPP-SP 1. When the HPP-SP is "fully stimulated", however, the proliferation potential shown as HPP-SP 2 is obtained, indicating its proliferation capacity. Combined as a "priming" and "full stimulation" 7+7 day, re-plating and secondary culture procedure, produces the HALO™ Expansion Potential Assay.

PHENOTYPIC ANALYSIS of CELLS DERIVED from 10 DAY 96-WELL PLATE CULTURES

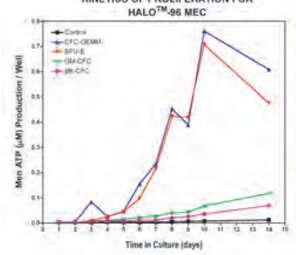


These results demonstrate that the cell types expected to be produced by stimulation with different growth factor / cytokine cocktails are actually produced. The number or proportion might be different depending on conditions, but the same cell types are produced.

STANDARDIZING the COLONY-FORMING CELL ASSAY against HALO™-96 MeC to PRODUCE a DUEL PROLIFERATION / DIFFERENTIATION POTENCY ASSAY at 14 DAYS: CAMEO™-96 STD

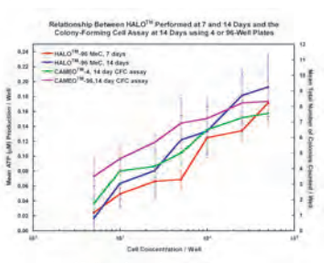
The only procedure to standardize the traditional colony-forming cell assay is against another assay that is validated and can be calibrated and standardized. Since HALO™-96 MeC has be validated against the colony-forming cell assay and is a calibrated and standardized assay, the reverse process can standardize the colony-forming cell assay, providing both assays are performed under exactly the same conditions.

KINETICS OF PROLIFERATION FOR HALO™-96 MEC



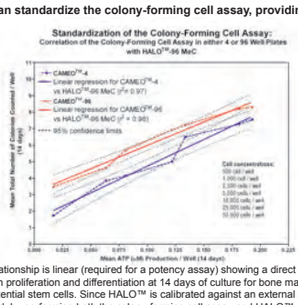
The time course for proliferation of hematopoietic cell populations may decrease after 10 days, but this "residual" proliferation can be used to measure both proliferation AND differentiation at 14 days.

Relationship Between HALO™ Performed at 7 and 14 Days and the Colony-Forming Cell Assay at 14 Days using 4 or 96-Well Plates



Normally, HALO™-96 MeC is performed at 7 days. However, when the proliferation assay is performed at 14 days, a relationship with the colony-forming cell differentiation assay is produced.

Standardization of the Colony-Forming Cell Assay:
Correlation of the Colony-Forming Cell Assay in either 4 or 96 Well Plates with HALO™-96 MeC



This relationship is linear (required for a potency assay) showing a direct correlation between proliferation and differentiation at 14 days of culture for bone marrow multipotential stem cells. Since HALO™ is calibrated against an external ATP standard, by performing both the colony-forming cell assay and HALO™ under exactly the same conditions, standardization of the colony-forming cell assay can be achieved. Colony number can be expressed as ATP concentrations.
This assay is now called CAMEO™-96 STD.