A Cord Blood Potency Assay that Accurately Predicts Engraftment Potential

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INTRODUCTION

The Stem Cell Therapeutic and Research Act, 2005, 2010.
"To provide for the collection and maintenance of cord blood stem cells for the treatment of patients and research ..... to authorize the C.W. Bill Young Cell Transplantation Program". Throughout this statute, the statement "high quality cord blood units" refers to the stem cells that have to be of "high quality" in the units as required in the first sentance of the statute.

The Goal
The goal is to transplant the patient with sufficient stem cells of high enough potency and "quality" that allows engraftment and reconstitution.

FDA Guidance for Industry on Potency Testing for Cellular and Gene Therapy Products (2011)
"All potency assays used for release testing of licensed biological drug products must comply with applicable biologics and cGMP regulations including":

- ♦ Indication of biological activity specific to the product.
- ♦ Results that allow for release of the product.
- ♦ Provide quantitative data.
- ♦ Meet pre-defined acceptance/rejection criteria.
- ♦ Include reference materials, standards and controls.
- ♦ Demonstrate validation.
- ♦ Measure the identity and activity of the "active" ingredients or components.

Definition of Potency for Hematopoietic Stem Cell Therapy Products
Stem cell potency is the quantitative and validated measurement of biological activity of the "active" stem cell components that are responsible for and result in an engraftment response.

Requirements for a Stem Cell Potency Assay

1. A quantitative and validated readout system that measures biological function.
2. The ability to identify and specifically measure the "active" stem cell components in a dose-dependent manner.
3. A reference standard of the same source so that the response of the sample can be compared and the potency ratio evaluated.

The First Requirement for a Stem Cell Potency Assay

A quantitative and validated readout system that measures biological activity

To meet the specific requirements for a potency assay it is necessary to develop an assay specific for the product that can be validated according to FDA guidelines. The readout of the assay must be able to quantitatively measure a specific biological activity that is a property or characteristic of the cells for which potency is to be measured. Since the goal of the assay is to measure rare populations of stem cells that are responsible for the engraftment process, the assay readout must be highly sensitive and accurate. This sensitivity and accuracy can only be achieved using a bioluminescence readout.

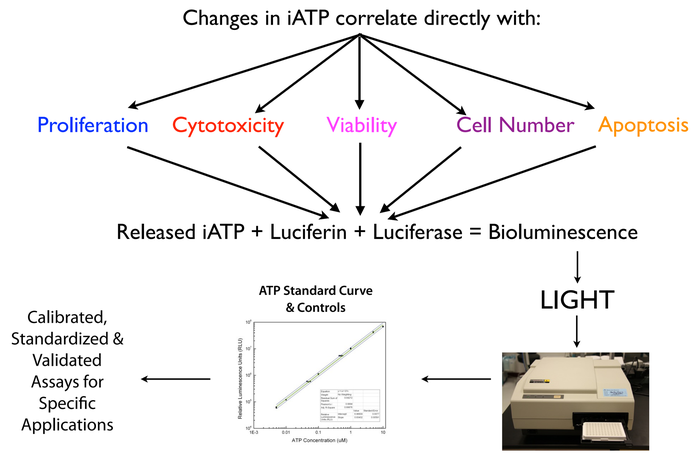
Adenosine triphosphate (ATP) is produced in the mitochondria (the cell's chemical energy plant). The concentration of ATP correlates directly with the state of proliferation or inhibition (cytotoxicity), viability, cell number and apoptosis. The concentration of ATP can be quantitately measured using a luminescence plate reader.

Prior to processing and measuring the samples, the assay is calibrated and standardized:

- ♦ The controls included with the assay calibrate the instrument.
- ♦ The ATP standard curve standardizes the assay and allows the output of the instrument in Relative Luminescence Units (RLU) to be interpolated into standardized ATP concentrations.
- ♦ The process ensures that all the reagents are working correctly prior to measuring the samples.
- ♦ It provides and immediate proficiency test by comparing results with expected results and ranges.
- ♦ It allows results to be directly compared over time without normalization.

The Principle of ATP Bioluminescence Proliferation Assays

Using the Cell's Chemical Energy (Adenosine Triphosphate, ATP) as a Biochemical Marker for Multiple Readouts



ATP Bioluminescence Assay Characteristics

The following assay characteristics must be obtained prior to sample processing and measurement:

- ♦ Standard curve correlation coefficient (R) => 0.997
- ♦ Standard curve slope = 0.937 (range: 0.8 - 0.11)
- ♦ Low control = 0.05µM (Range: 0.043µM - 0.58µM)
- ♦ High control = 0.7µM (Range: 0.595µM - 0.805µM)
- ♦ Acceptance/Rejection Cutoff = 0.04µM (Range: 0.034µM - 0.046µM)
- ♦ Non-viability cutoff =< 0.01µM

Assay Validation Parameters

The following parameters must be determined for any potency assay:

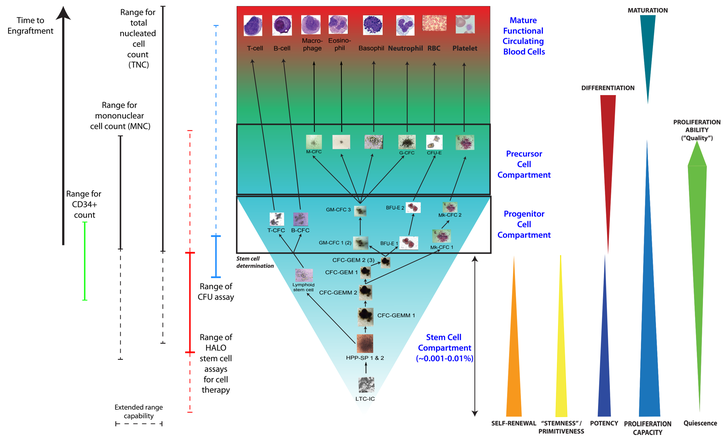
- ♦ Assay linearity: > 4 logs
- ♦ Assay ATP sensitivity: ≤ 0.001µM
- ♦ Assay cell sensitivity: 20-25 cells/well
- ♦ Accuracy (% correct outcomes): 90-95%
- ♦ Sensitivity and Specificity (Receiver Operator Characteristics): 0.73 - 0.752 (range: 0.5 - 1)
- ♦ Precision (Reliability and Reproducibility): =< 15% (LLOQ: =< 20%)
- ♦ Robustness (Intra- and inter-lab): => 95%.
- ♦ High throughput capability (Z-Factor): > 0.76 (range: 0.5-1)

Assay validation performed in accordance with FDA Bioanalytical Method Validation Guidelines (2002)

The Second Requirement for a Stem Cell Potency Assay

To identify and specifically measure the "active" stem cell components in a dose-dependent manner

If the purpose of the product is a stem cell therapy, then the "active" components of the potency assay are the stem cells. No other cells are relevant. It is therefore necessary to identify and measure the stem cells, since only these are responsible for engraftment. The diagram below shows tests presently used for cord blood testing and the properties of cord blood stem cells that need to be measured for a cord blood stem cell potency assay.



Stem Cell Proliferation is the Property Used to Measure Stem Cell Potency and "Quality"

- ♦ **Proliferation potential:** The inherent capacity of cells to proliferate. Proliferation potential is used to measure stem cell self-renewal, primitiveness and potency because all of these parameters correlate with each other.
- ♦ **Proliferation ability:** The status of cell proliferation at a specific point in time and concentration. This is used to measure stem cell "quality".

The Purity of the Cord Blood Sample Affects both Stem Cell Proliferation Ability ("Quality") and Proliferation Potential (Potency)

Cord blood units are usually red blood cell reduced, producing a Total Nucleated Cell (TNC) fraction that contains varying concentrations of red blood cells, granulocytes and platelets. The presence of these cells in a cord blood unit dilute and mask the rare stem cells so that potency cannot be measured. Removal or reduction of these unwanted cells produces a mononuclear cell (MNC) fraction in which both stem cell proliferation potential (potency) and ability ("quality") can be reliably and accurately measured.

The Effect of Removing Unwanted Cells on Stem Cell "Quality":
From TNC Fraction to MNC Fraction

Potency regulations require that ALL "active" ingredients be measured. Since cell systems are not static and are "continuums", it is not only impossible to measure ALL stem cell components, but would be time and cost prohibitive.

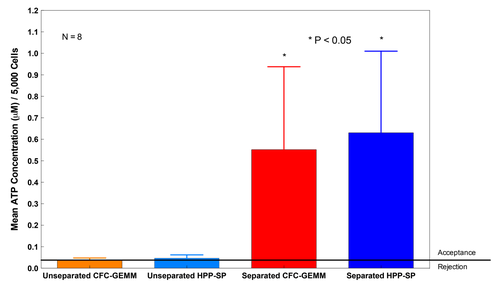
A minimum of two stem cell populations have been chosen to measure potency with an accuracy of > 90%:

- ♦ A primitive lympho-hematopoietic stem cell population called HPP-SP or High Proliferative Potential Stem and Progenitor Cell.
- ♦ A primitive hematopoietic stem cell equivalent to the CFC-GEMM

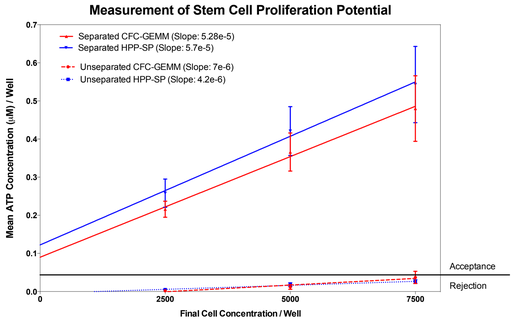
To measure stem cell proliferation potential, a minimum 3-point cell dose response is required.

Proliferation potential is then measured by the slope of the cell dose response linear regression.

- ♦ The steeper the slope,
- ♦ The greater the proliferation potential
- ♦ The more primitive the stem cell population
- ♦ The greater the potency.



Measurement of Stem Cell Proliferation Potential



NOTE: Significant differences in stem cell potency and "quality" can occur between cord blood segments of the same unit (data not shown).

The Third Requirement for a Stem Cell Potency Assay

A reference standard of the same source so that the response of the sample can be compared and the potency ratio evaluated

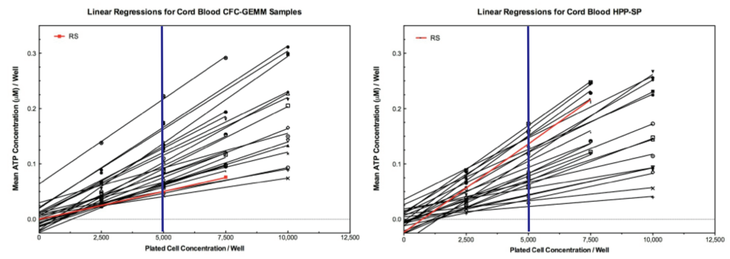
- ♦ There are no international or "global" reference standards for cellular therapeutic products, because the quantities of the product are too low.
- ♦ Instead, an in-house reference standard is established. There are 2 alternative procedures:
  1. If samples are available from a patient that demonstrated both short- and long-term engraftment and reconstitution, this sample can be used to established the primary reference standard.
  2. A sample demonstrating high stem cell potency and quality *in vitro* can be used to establish the primary reference standard.
- ♦ To establish a reference standard, it is imperative that a standardized and validated assay (e.g. HALO®) is used, otherwise intra- and inter-laboratory reference standards cannot be compared.

For more information, please visit the HemoGenix® YouTube® Channel, HemoGenix® website (www.hemogenix.com) to see how in-house hematopoietic stem cell reference standards are established.

Combining the 3 Requirements into a Unified Standardized and Validated Assay for Hematopoietic Stem Cell Potency Assay for Umbilical Cord Blood that is compliant with statutes, regulations and standards

Measuring Stem Cell Potency and Quality in a Single Assay

28 cryopreserved cord blood samples were prepared as MNC fractions and the proliferation potential of 2 stem cell populations (HPP-SP and CFC-GEMM) were measured and compared against a cord blood reference standard (shown in red)

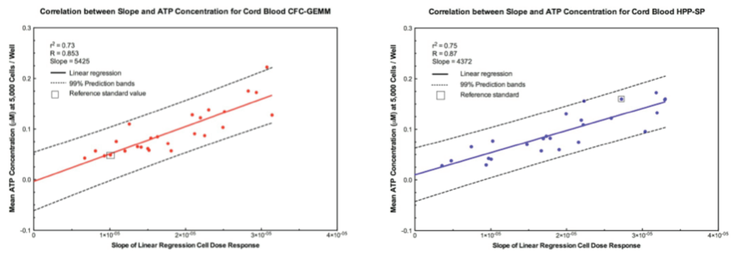


The slope of each cell dose response linear regression gives the proliferation potential for each stem cell population. Then:

Potency Ratio = Slope of the Sample / Slope of the Reference Standard

Stem cell "quality" is provided by the proliferation ability at a specific cell dose (shown by the blue vertical line).

The Relationship between Stem Cell Potency Ratio and "Quality"

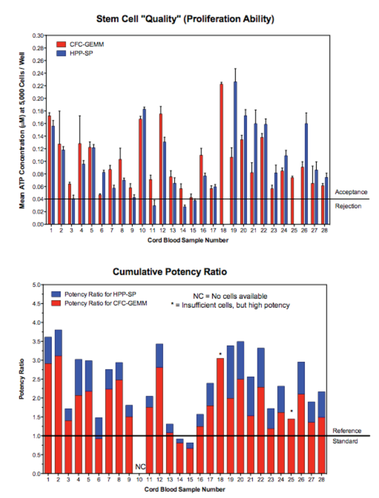


There is a direct and highly significant correlation between the stem cell potency ratio and and "quality", with prediction and confidence limits within the 99% range.

This means that both the stem cell potency ratio and "quality" must be taken into account to release the sample for transplantation.

Release Criteria for a Umbilical Cord Blood Stem Cell Product

Release Criteria



A cord blood unit can be released for use if:

1. Stem cell "quality" (proliferation ability) for both stem cell populations is > 0.04µM ATP, or the pre-determined acceptance/rejection criteria.

2. The cumulative potency ratio for both stem cell populations is => 1 (the potency of a reference standard).

No engraftment would have been predicted for samples 14 and 15. However, all 28 units demonstrated short-term engraftment.

Under these conditions, the accuracy of HALO®-96 PQR to predict engraftment potential is >90%.

NOTE: Engraftment potential is NOT the same as Time to Engraftment, which is downstream of the engraftment process and does NOT correlate with either any stem cell parameter, e.g. potency or "quality".

CONCLUSIONS

1. **The stem cells are the "active" components required for engraftment.**
2. **Present cord blood tests and assays do not measure the stem cells required by U.S. statutes.**
3. **To measure potency a standardized and validated assay is an absolute requirement.**
4. **To measure both potency and "quality", a MNC fraction (not a TNC fraction) must be prepared.**
5. **Measuring 2 stem cell populations using HALO®-96 PQR can measure both potency and "quality" in a single assay with an accuracy of >90%.**
6. **HALO®-96 PQR should be used by cord blood banks prior to shipment of the cord blood unit to the transplant center.**

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