A NOVEL STEM CELL POTENCY and RELEASE ASSAY for UMBILICAL CORD BLOOD.

Correlation of Stem Cell Proliferation with Engraftment

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ABSTRACT

Acceptance or rejection of an umbilical cord blood (UCB) unit is determined by total nucleated cell count (TNC). Yet stem cell potency will decide whether a UCB unit will engraft and repopulate the patient after transplantation. A cell potency and release assay is required by standards organizations for UCB. Neither TNC nor viability and CD34 number provide stem cell potency assessment. Although the colony-forming cell (CFC) assay is required, it is used only as a "growth or no-growth assay" and not as a measure of cell potency because, due to lack of standardization and validation, cell potency cannot be measured. In a recent article in Transfusion (2008, 28:620-628), Reems et al describe a novel instrument-based, ATP bioluminescence proliferation assay used for UCB and compared at two geographical locations. Results indicated a correlation for the assay between the locations with a correlation coefficient of R=0.94 (p<0.001; r2=0.89). In addition there was a correlation between the ATP and CFC assays (R=0.79). The ATP assay has now been further developed into a cell potency and release assay using a UCB reference standard. The reference standard and UCB sample(s) are measured at 3 cell doses. The linear regression of the sample must be parallel to that of the reference standard. The horizontal displacement provides an estimate of the cell potency compared to the reference standard. A displacement to the left indicates greater potency, while a displacement to the right indicates decrease cell potency. In addition, background controls are performed for both reference standard and sample. The release criteria for a sample can be arbitrarily set from the standard deviation of the background control. This is the first stem cell potency and release assay that is instrument-based, non-subjective and fully standardized against an external standard, allowing intra- and inter-laboratory comparisons and therefore validation.

REGULATORY ISSUES

International Standard for Cell Processing

According to FACT-JACIE International Standards, 3rd Edition:

Section D6.13 states, "Laboratory processes shall include the establishment of appropriate and validated assays and test procedures of the evaluation of cell therapy products".

According to NetCord-FACT Standards for Cord Blood, 3rd Edition:

Section D14.1.1.1 states, "The use of established and validated appropriate assays, standards, and test procedures for the evaluation of the CD unit".

Section D14.1.1.2 states, "Adequate provisions for monitoring the reliability, accurcy, precision and performance of CBB Processing Facility test procedures and instruments".

According to AABB Standards for Cellular Therapy Product Service:

Section 5.14C states, "Potency assay appropriate for the cellular therapy, if applicable".

Docket No. 2007D-0025 from the FDA / CBER.
Guidance for Industry: Class II Special Controls Guidance Document: Cord Blood Processing System Storage Container. For immediate implementation.

- ..."Total colony-forming unit granulocyte macrophage (CFC-GEM)
- Total burst-forming units - erythroid (BFU-E), if applicable
- Total colony-forming unit - granulocyte erythrocyte monocyte macrophage (CFC-GEMM), if applicable..."

What is an "Appropriate" and "Validated" Assay for Cord Blood?

Is the CFC Assay an "appropriate" and "validated" assay for cell potency? NO.

The CFC assay is an indicator of differentiation potential, but does NOT measure proliferation potential.

To ensure that a stem cell transplantation product actually contains stem cells which exhibit the ability to proliferate and therefore exhibit a measure of poetency, the stem cells themselves, not their lineage-specific decendents (CFC-GM, BFU-E etc) have to be determined.

The potency of a stem cell product is not an "all-or-nothing" / "growth - no growth" subjective determination, but a quantitative measurement of the cell's "stemness" and therefore its proliferation potential compared to a reference standard.

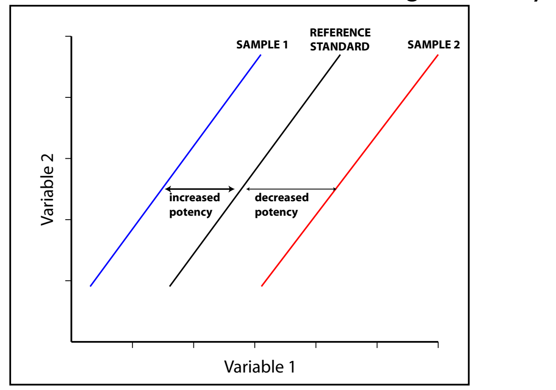
WHY IS A CELL POTENCY ASSAY SO IMPORTANT?

- To control for batch manufacturing consistancy.
- Product stability.
- Product bridging studies.
- Product performance prediction / assurance
- Evaluate / correlate clinical dose response
- Avoid product failure / toxicity due to improper potency.

HOW IS CELL POTENCY MEASURED?

POTENCY:

A Specific Measure of Standardized Biological Activity



21 CFR 600.3 and 21 CFR 610.10

The HALO®-96 PQR Cell Potency and Release Assay

THE HALO® PRINCIPAL

As cells proliferate, the concentration of the biochemical marker, intracellular ATP (iATP) increases proportionately. When iATP is released after incubation it acts as a limiting substrate for the following reaction:

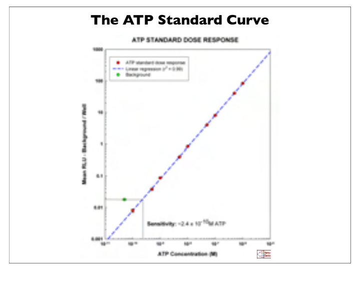
$$iATP + luciferin + O_2 \xrightarrow{luciferase,\ Mg^{2+}} Oxyluciferin + AMP + PP_i + CO_2 + \text{Bioluminescence (Light)}$$

Bioluminescence is measured in a plate luminometer as Relative Luminescence Units (RLU) which are standardized against an external ATP standard.

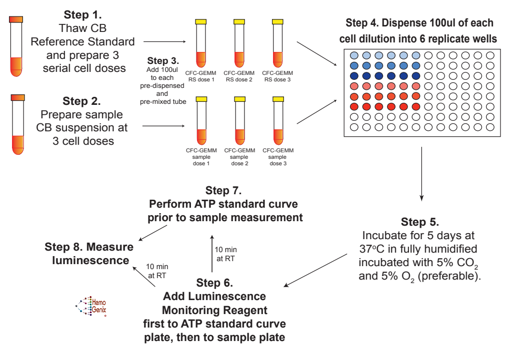
The ATP standard curve:

1. Calibrates the instrument.
2. Ensures the reagents work correctly.
3. Controls for pipetting error and bad technique.
4. Standardizes the assay.
5. Helps validate the assay

The ATP Standard Curve



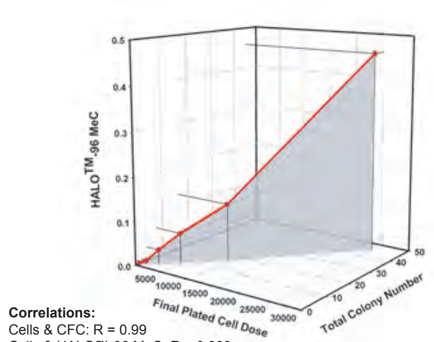
THE HALO®-96 PQR STEM CELL (CFC-GEMM) POTENCY ASSAY


Step 1. Thaw CB Reference Standard and prepare 3 serial cell doses
Step 2. Prepare sample CB suspension at 3 cell doses
Step 3.
Step 4. Dispense 100ul of each cell dilution into 6 replicate wells
Step 5. Incubate for 5 days at 37°C in fully humidified incubated with 5% CO_2 and 5% O_2 (preferable).
Step 6. Add Luminescence Monitoring Reagent first to ATP standard curve plate, then to sample plate
Step 7. Perform ATP standard curve prior to sample measurement
Step 8. Measure luminescence

HALO®-96 PQR is a SPECIAL 5 DAY HUMAN HALO®-96 SEC (Suspension Expansion Culture) for CFC-GEMM STEM CELLS

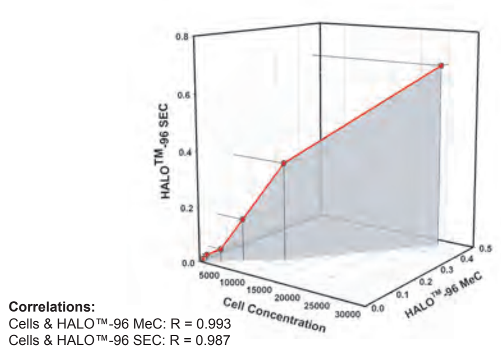
The following 3-D dose response curves show a direct correlation between the traditional CFC assay, HALO®-96 MeC (methyl cellulose) and HALO®-96 SEC (Suspension Expansion Culture)

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



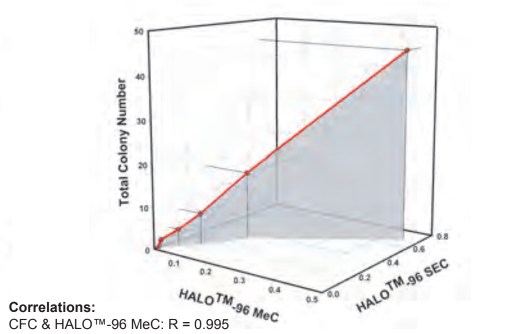
Correlations:
Cells & CFC: R = 0.99
Cells & HALO™-96 MeC: R = 0.993
CFC & HALO™-96 MeC: R = 0.997

Correlation 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



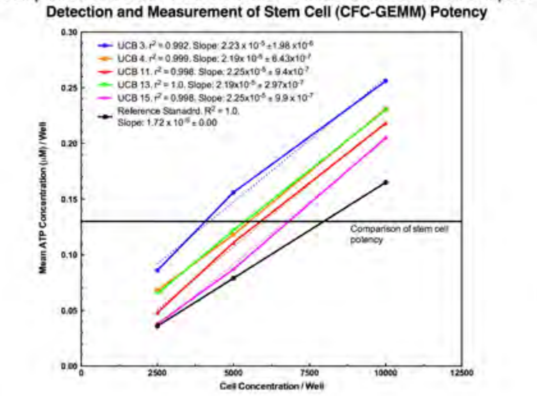
Correlations:
CFC & HALO™-96 MeC: R = 0.995
CFC & HALO™-96 SEC: R = 0.986
HALO™-96 MeC & HALO™-96 SEC: R = 0.964

These results validate HALO®-96 MeC and HALO®-96 SEC against the CFC Assay. 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 and HALO®-96 PQR.

Any HALO®-96 format is an alternative to, and can completely replace, the colony-forming cell (CFC) assay.

HALO®-96 PQR can MEASURE STEM CELL (CFC-GEMM) POTENCY from UMBILICAL CORD BLOOD SAMPLES

Comparison of a Cord Blood Reference Standard with Cord Blood Samples: Detection and Measurement of Stem Cell (CFC-GEMM) Potency



Evaluation of Stem Cell Potency from a Reference Standard

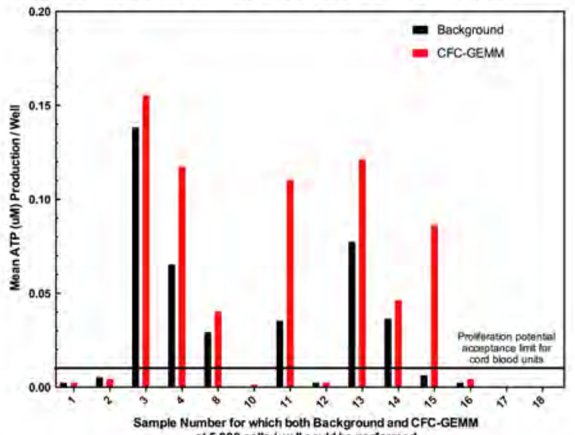
	Stem Cell Potency compared to Reference Standard / Equivalent Cell Concentration / Well					
ATP Concentration (µM)	Cord Blood Reference Standard	UCB Sample 3	UCB Sample 4	UCB Sample 11	UCB Sample 13	UCB Sample 15
0.1	6126	2.13 / 2871	1.51 / 4049	1.31 / 4691	1.51 / 4055	1.14 / 5388
0.11	6728	2.02 / 3320	1.49 / 4506	1.31 / 5136	1.49 / 4511	1.15 / 5833
0.12	7330	1.94 / 3769	1.47 / 4964	1.31 / 5581	1.47 / 4967	1.16 / 6278
0.13	7932	1.88 / 4217	1.46 / 5421	1.31 / 6026	1.46 / 5423	1.18 / 6723
0.14	8534	1.82 / 4666	1.45 / 5879	1.31 / 6470	1.45 / 5878	1.19 / 7169
0.15	9136	1.78 / 5115	1.44 / 6336	1.32 / 6915	1.44 / 6334	1.12 / 7614

Linear regression analysis must demonstrate that the slope of the reference standard and the cord blood samples are parallel within 90-95% confidence limits. In the present case, the pooled slope was 2.14×10^{-5}. The probability that all linear regressions were within 95% of this slope was P = 0.048. If the slopes are similar, as shown in the diagram above, then the horizontal displacement from the reference standard at one or more ATP values can be calculated and the corresponding cell concentration estimated (see table). Displacement to the left, as found for all samples shown, indicates a greater potency than the reference standard.

HALO®-96 PQR is used to DEFINE ACCEPTANCE LIMITS for RELEASE CRITERIA of UMBILICAL CORD BLOOD UNITS for STEM CELL TRANSPLANTATION PURPOSES.

The acceptance limit is arbitrary unit set by individual laboratories. In this example, the acceptance limit is arbitrarily set at an ATP concentration of 0.01µM. Above this ATP concentration level, the CFC-GEMM were signficantly stimulated and, as shown in the diagram and table below, the response of all of these samples were correlated with engraftment. Below this limit, there was little, if any, proliferation of the background or CFC-GEMM. However, three cord blood samples did exhibit engraftment, despite the fact that the ATP concentrations of the CFC-GEMM and background were virtually zero (see table below).

Defining Acceptance Limits for Release Criteria of Cord Blood Units for Stem Cell Transplantation

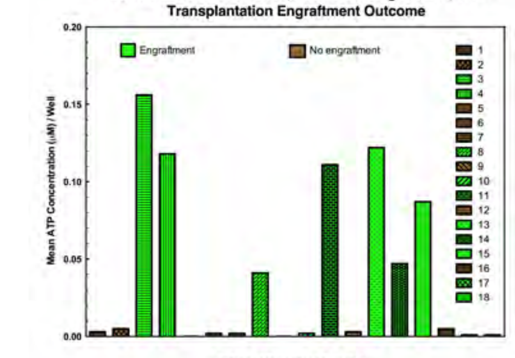


Sample Number for which both Background and CFC-GEMM at 5,000 cells / well could be performed

HALO®-96 PQR PREDICTS ENGRAFTMENT OUTCOME for UMBILICAL CORD BLOOD

All of the samples used in this study were cryopreserved cord blood pellets some of which were frozen for more than 10 years. After performing HALO®-96 PQR on 18 "blind" cord blood samples, the results of CFC-GEMM proliferation were correlated with the engraftment of the same samples in patients. Of the 18 samples tested, 7 exhibiting CFC-GEMM proliferation above the 0.01µM ATP arbitrary acceptance limit predicted engraftment and were found to have engrafted in the patient. Three samples that were not predicted to have engrafted, apparently did engraft. All other samples that were below the acceptance limit, did not engraft.

Correlation of Proliferation, Detected using HALO®, with Transplantation Engraftment Outcome



Individual Cord Blood Samples

CORRELATION OF HALO® WITH TRANSPLANTATION ENGRAFTMENT OUTCOME

Ability to Detect and Measure Stem Cell Proliferation as a Function of Cryopreservation Time

Sample No.	Frozen Date (Mo/Day/Year)	Engraftment	TNC after thawing	Percent Viability (7-AAD)	Background @ 5,000/well	CFC-GEMM @ 5,000 cells/well
1	11/2/99	No	1.58E+05	97.6	0.003	0.003
2	1/10/99	No	2.62E+05	99.8	0.006	0.005
3	10/10/00	Yes	3.31E+06	99.8	0.139	0.156
4	10/12/00	Yes	2.07E+06	99.8	0.066	0.118
5	5/5/98	No	5.83E+04	81.7		0
6	6/10/98	No	1.12E+05	99.4		0.002
7	10/20/98	No	2.00E+04	99.5		0.002
8	6/20/00	Yes	3.85E+06	99.6	0.03	0.041
9	9/10/98	No	1.01E+05	91.4		0
10	8/30/99	Yes*	1.54E+06	94.9	0.01	0.002
11	7/20/01	Yes	2.60E+06	99.4	0.036	0.111
12	11/10/99	No	1.00E+05	97.2	0.003	0.003
13	2/15/01	Yes	1.07E+06	99.9	0.078	0.122
14	10/17/01	Yes	4.65E+06	99.9	0.037	0.047
15	10/4/01	Yes	5.00E+06	98.4	0.007	0.087
16	9/5/97	No	2.89E+06	97.9	0.003	0.005
17	6/13/98	Yes*	5.35E+05	96	0	0.001
18	9/10/98	Yes*	4.19E+05	83.5	0.001	0.001

* = questionable outcome

CONCLUSIONS

HALO®-96 PQR is the only *in vitro* assay available that can:

1. Reliably measure and evaluate cord blood stem cell potency against a reference standard.
2. Define acceptance limits for release criteria of cord blood units for stem cell transplantation.
3. Predict cord blood transplantation engraftment outcome.

Despite the mandated use of "acceptable" and "validated" assays to detect and measure cell potency and release criteria for umbilical cord blood by Standards Organizations and Regulatory Agencies, stem cell transplantation centers and cord blood banks continue to use outdated, subjective, non-standardized and non-validated assays. The CFC assay was designed as a research tool and for the reasons given above, should be considered totally unacceptable for use in patient-related clinical applications.

The HALO®-96 Stem and Progenitor Cell - Quality Control (SPC-QC) and HALO®-96 PQR (Potency, Quality, Release) Platforms have been specifically designed for stem cell transplantation and cord blood bank processing laboratories. The HALO® Platform has been validated many times not only against the CFC assay, but also against the Registry of Cytotoxicity Prediction Model as a validated cytotoxicity assay and by large biopharmaceutical companies that now consider HALO® the "GOLD STANDARD" in hemotoxicity testing.

Further studies are ongoing both with frozen and fresh cord blood samples. It is expected that furture results will consolidate and cement those reported here, thereby providing users with an easy to use, validated and predictive assay for routine use and increased patient safety.

REFERENCES

"Developemnt of a Novel Assay to Evaluate the Functional Potential of Umbilical Cord Blood Progenitors". Reems, Hall, Luladay, Taber, Rich. Transfusion, 48:620-628 (2008).

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"Validation and Development of a Predictive Paradigm for Hemotoxicity Using a Multifunctional Bioluminescence Colony-Forming Proliferation Assay". Rich IN, Hall KM. Tox Sci 87: 427-441 (2005).

ACKNOWLEDGEMENT

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