Assay Validation:

How a CFU Replacement Assay is Validated for Cord Blood, Mobilized Peripheral Blood and Bone Marrow

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1. What is Assay Validation?

Assay validation is defined as establishing documented evidence which provides a high degree of assurance that a specific process will consistantly produce a product meeting its predetermined specifications and quality attributes. Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications.

The goal of assay validation is to confirm the operating characteristics of the procedure for its intended use.

Cross-Validation is a comparison of validation parameters when two or more methods are used to generate data within the same study or across different studies. Cross-validation would be a situation where an original validated method serves as a reference and the revised method the comparator. The comparisons should be done both ways.

An assay is validated when it demonstrates:
Accuracy: the proportion of correct outcomes.
Sensivity: the proportion of correctly identified positive samples.
Selectivity: the proportion of correctly identified negative samples.
Precision (Reliability and Reproducibility): intra- and inter-laboratory variability.
Robustness: the ability of the assay to withstand changes and transferability.

References:
FDA Guidance for Industry. Bioanalytical Method Validation. 2001.
ICH Topic Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products.

2. The Principle of Bioluminomics™ Assays for Cellular Therapy

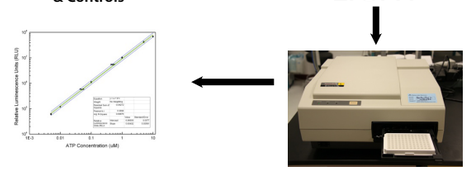
Chemical Energy can be used as a Biochemical Marker for Multiple Readouts

Induced Changes in iATP correlate directly with:

Proliferation — Cytotoxicity — Cell Number — Apoptosis

Released iATP + Luciferin + Luciferase = Bioluminescence

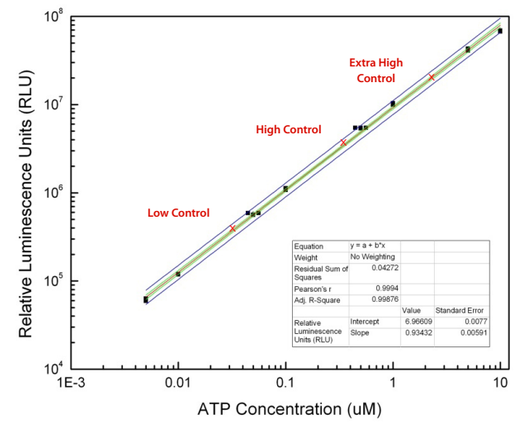
LIGHT



ATP Standard Curve & Controls

Calibrated, Standardized & Validated Assays for Specific Cell Therapy Applications

3. The ATP Standard Curve and Controls



- The ATP standard curve allows Relative Luminescence Units (RLU) to be converted to standardized ATP concentrations (µM).
- The controls calibrate the instrument.
- ATP correlation coefficient (R) => 0.997.
- ATP Standard Curve Slope = 0.937 (range: 0.8-1.1).
- Assay ATP sensitivity: =< 0.001µM.
- Low ATP Control range: 0.043 - 0.058µM.
- High ATP Control range: 0.595 - 0.805µM.
- Extra High ATP Control range: 1.488 - 2.013µM.
- Lowest ATP value indicating unsustainable cell proliferation: = <0.04µM.
- ATP value below which cells are not metabolically viable: =<0.01µM.

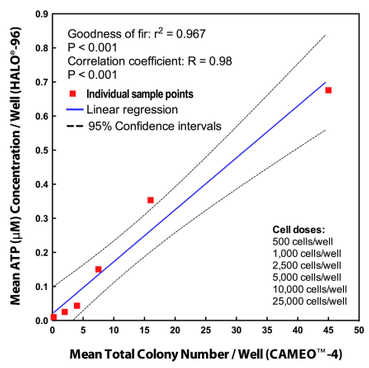
4. ATP Bioluminomics™ CFU Replacement Assays that have been Validated

The colony-forming unit (CFU) assay (manual or image analysis colony counting) cannot be calibrated or standardized due to the lack of standards and controls and can therefore not be validated. In contrast, ATP bioluminomics™ assays for hematopoietic stem and progenitor cells, which have been developed from the CFU assay, are not only fully validated, but some have FDA Master File status and can be referenced in a BLA or IND. ATP assays for hematopoietic stem and/or progenitor cells that have been validated include:

- HALO®-96 PCA^{EQ}: A progenitor cell equivalent assay to CFU.
- STEMPredict™: A 3 day stem cell assay for cord blood and mobilized peripheral blood to predict stem cell functionality and viability.
- HALO®-96 SPC-QC: An assay to ensure stem cell quality during processing and cryopreservation. (Assay submitted for FDA Master File status).
- HALO®-96 PQR: A reference standard-based potency assay to predict engraftment potential of cord blood, mobilized peripheral blood and bone marrow prior to transplantation. (Assay has FDA Master File status).
- HALO®-96 PMT: An assay to predict time to engraftment and detect lympho-hematopoietic reconstitution.
- LUMENESC™-96 QC: Similar to HALO-96 SPC-QC, but for mesenchymal stem cells.
- LUMENESC™-96 PQR: Similar to HALO-96 PQR, but for mesenchymal stem cells.

5. Cross-Validation and Accuracy

ATP Assays Correlate Directly with CFU, Demonstrating Cross-Validation



This relationship shows that the total colony number from the CFU assay can be interpolated into ATP concentrations. This is the only relationship that allows the CFU assay to be standardized

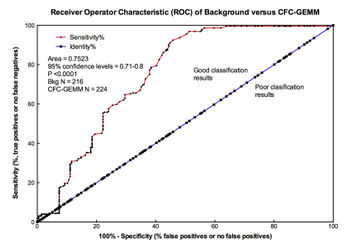
Accuracy

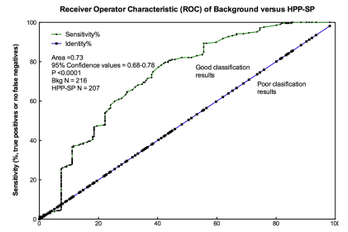
ATP Standard Curve Concentrations					
Expected ATP Concentrations	0.01µM	0.05µM	0.1µM	0.5µM	1µM
Mean (µM)	0.00963	0.05218	0.10206	0.49838	0.98645
St. Dev. (µM)	0.00057	0.00343	0.00434	0.02318	0.04955
%CV	**5.95**	**6.58**	**4.25**	**4.65**	**5.02**
N =	76	76	76	76	76

Percent CVs must be =<15%, with a Lower Level of Quantitation (LLOQ) =<20%

6. Sensitivity and Selectivity

Receiver Operator Characteristic (ROC) curve fitting is used to measure sensitivity and selectivity. A total of 216 background values were compared with 224 hematopoietic CFC-GEMM stem cell and 207 lympho-hematopoietic HPP-SP primitive stem cell ATP values. In essence the test looks for false positives. The Area Under the Curve (AUC, red line) is calculated and must be between 0.5 and 1 and will be to the left of the diagonal. If the AUC is below 0.5, the curve will be to the right of the diagonal. This means that the assay cannot distinguish either false positives or false negatives. In other words, the assay would be useless. The AUC value for both curves is > 0.73, indicating that the assay exhibits both sensitivity and selectivity for different stem cell populations.





7. Precision (Reliability & Reproducibility) and Robustness

Precision is defined as the closeness of the individual measures of an analyte or cell sample when the procedure is applied repeatedly to multiple aliquots. Precision is usually subdivided into:
Within-run, intra-batch precision, which measures the precision during a single run, and
Between-run, inter-batch precision, which measures the precision with time and could also involve several users performing the same test, equipment, reagents and laboratories.

Within-Run, Intra-Batch Precision

5 separate samples

ATP Concentrations (µM)							
		Hematopoietic Stem Cells			Primitive Lympho-Hematopoietic Stem Cells		
	Background	CFC-GEMM 2,500 cells/well	CFC-GEMM 5,000 cells/well	CFC-GEMM 7,500 cells/well	HPP-SP 2,500 cells/well	HPP-SP 5,000 cells/well	HPP-SP 7,500 cells/well
Mean (µM)	0.006	0.027	0.118	0.202	0.067	0.187	0.32
St. Dev. (µM)	0.001	0.005	0.019	0.018	0.01	0.024	0.041
%CV	**13.4**	**17.4**	**15.8**	**9**	**15.3**	**12.7**	**12.7**

Between-Run, Inter-Batch Precision

9 batches performed on 9 separate days

Background Controls				
Cells / Well	2,500	5,000	7,500	10,000
No. of Cultures	184	192	88	88
Mean %CV	**5.0**	**4.3**	**3.7**	**3.4**

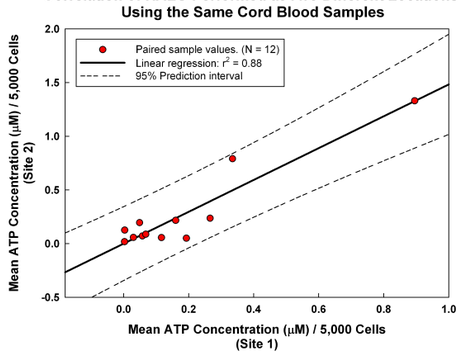
CFC-GEMM				
Cells / Well	2,500	5,000	7,500	10,000
No. of Cultures	184	192	88	88
Mean %CV	**16.6%**	**10.4%**	**9.4%**	**8.0%**

HPP-SP				
Cells / Well	2,500	5,000	7,500	10,000
No. of Cultures	184	192	88	88
Mean %CV	**12.0%**	**9.7%**	**6.7%**	**5.7%**

Percent CVs must be =<15%, with a Lower Level of Quantitation =<20%

Robustness

Correlation of HALO Performed at Two Different Locations Using the Same Cord Blood Samples



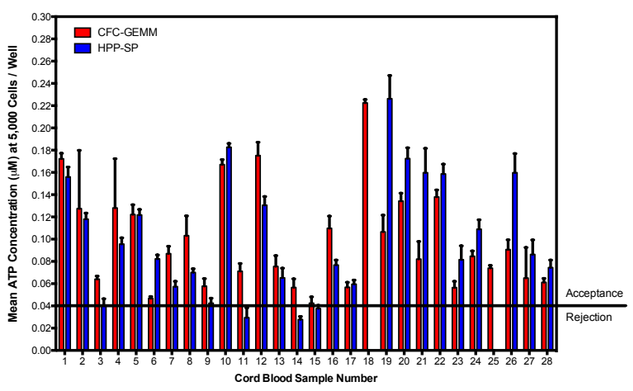
The assay can be easily transferred from one laboratory to another and is therefore robust.

8. How to Measure Cord Blood Potency using a Validated Bioluminomics Assay

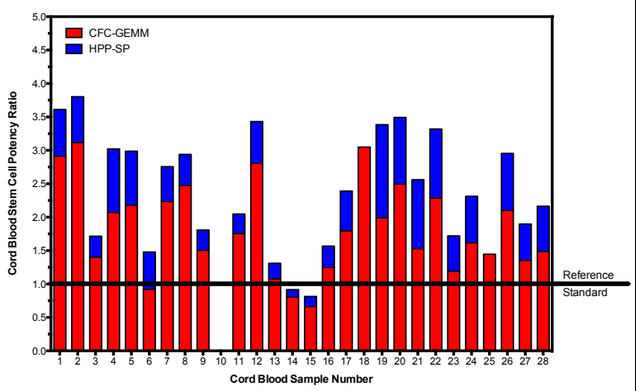
Potency is defined as a quantitative measure of the biological activity of the active contituents of a product. According to FDA and EMA guidelines on potency for cellular therapeutic products, a potency assay must (A) quantitatively measure biological activity, (B) include reference materials, standards and controls, (C) be validated, (D) measure the identity and stregth of the active ingredients, (E) provide results for release of the product, and (F) meet pre-defined acceptance/rejection criteria. Unless the product is "pure", total nucleated cell count, viability and CD34$^+$ and/or ALDH$^+$ cells do not comply with potency assay regulations. Although the CFU assay detects biological activity, it too, cannot comply with potency assay regulations. Furthermore, even a combination of all tests and assays will not comply with potency assay regulations.

HALO®-96 PQR meets all regulatory requirements in a single potency, quality and release assay. The results shown below for 28 cord blood samples produced an accuracy to predict engraftment of over 80%. The first step is to perform a 3-point cell dose response for at least 2 of the active constituents, i.e. stem cell populations, and compare the slope of the cell dose response linear regression with that of a cord blood reference standard to calculate the potency ratio. The quality, or stem cell proliferation ability, is provided by the ATP concentration at a specific cell dose. Since quality and potency are directly correlated with each other, both parameters must be used to provide the acceptance and release criteria.

Cord Blood Stem Cell "Quality" as Part of the Release Criteria



Cord Blood Cumulative Stem Cell Potency as Part of the Release Criteria



ATP concentrations less than 0.04µM do not usually support sustained proliferation (see Panel 3). Therefore, this ATP value corresponds to the acceptance/rejection cutoff. The potency ratio of the reference standard is always 1. A cumulative potency ratio less than 1 AND an ATP value below the cutoff will indicate that the cells have low or no engraftment potential and should not be released for use. NOTE that engraftment potential and therefore potency is not the same as time to engraftment and do not correlate with each other.

REFERENCE: Karen M. Hall, Holli Harper and Ivan N. Rich (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.