HemoGenix®

Changing the Paradigm

Validating a Cell Viability Measurement

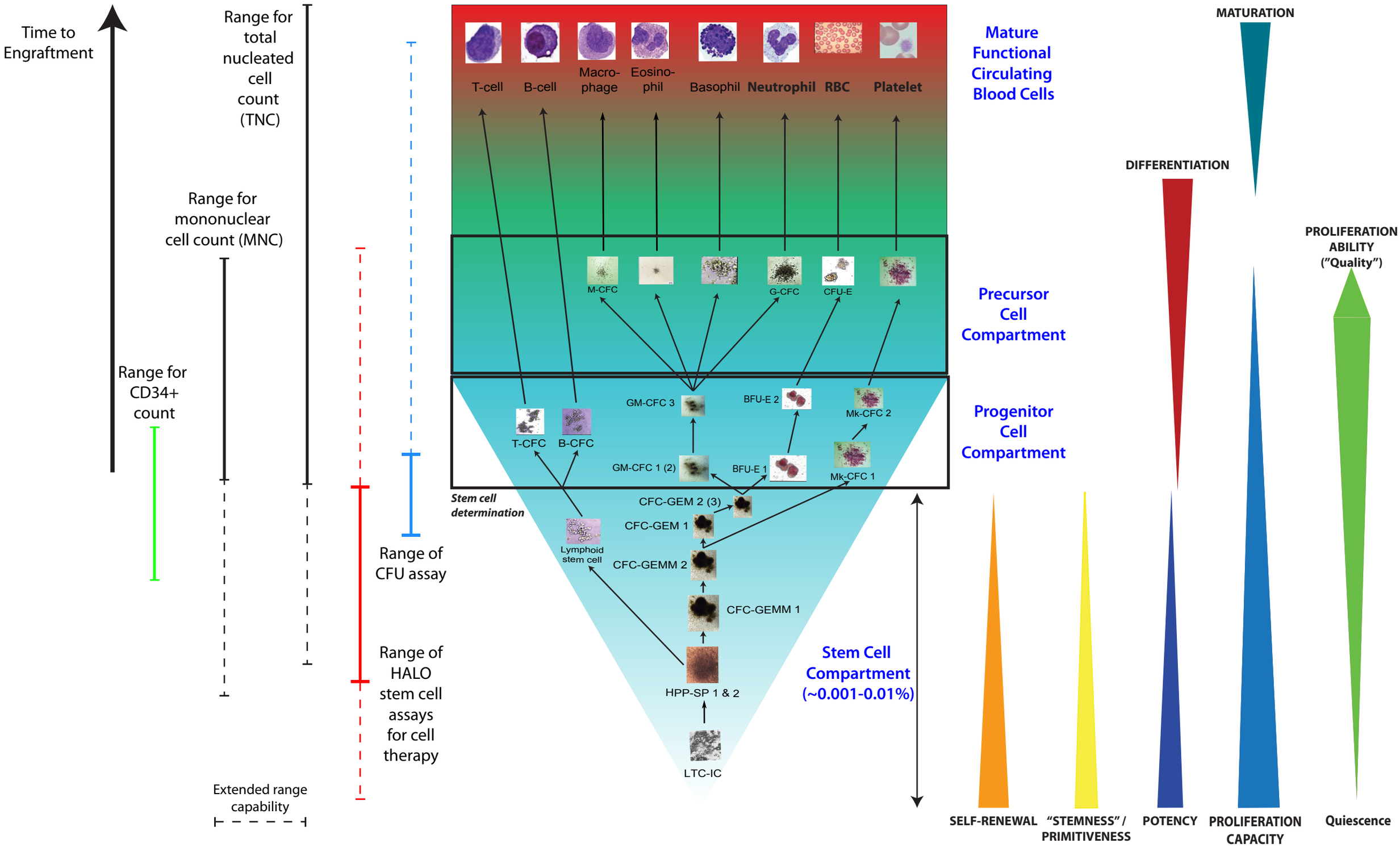
NIST Workshop: "Strategies to Achieve Measurement Assurance for Cell Therapy Products" May 11-12, 2015

Ivan N. Rich, PhD
Founder, Chairman & CEO
1485 Garden of the Gods Road
Colorado Springs, CO
www.hemogenix.com
ivannr@hemogenix.com

Developing a Stem Cell Therapeutic Assay

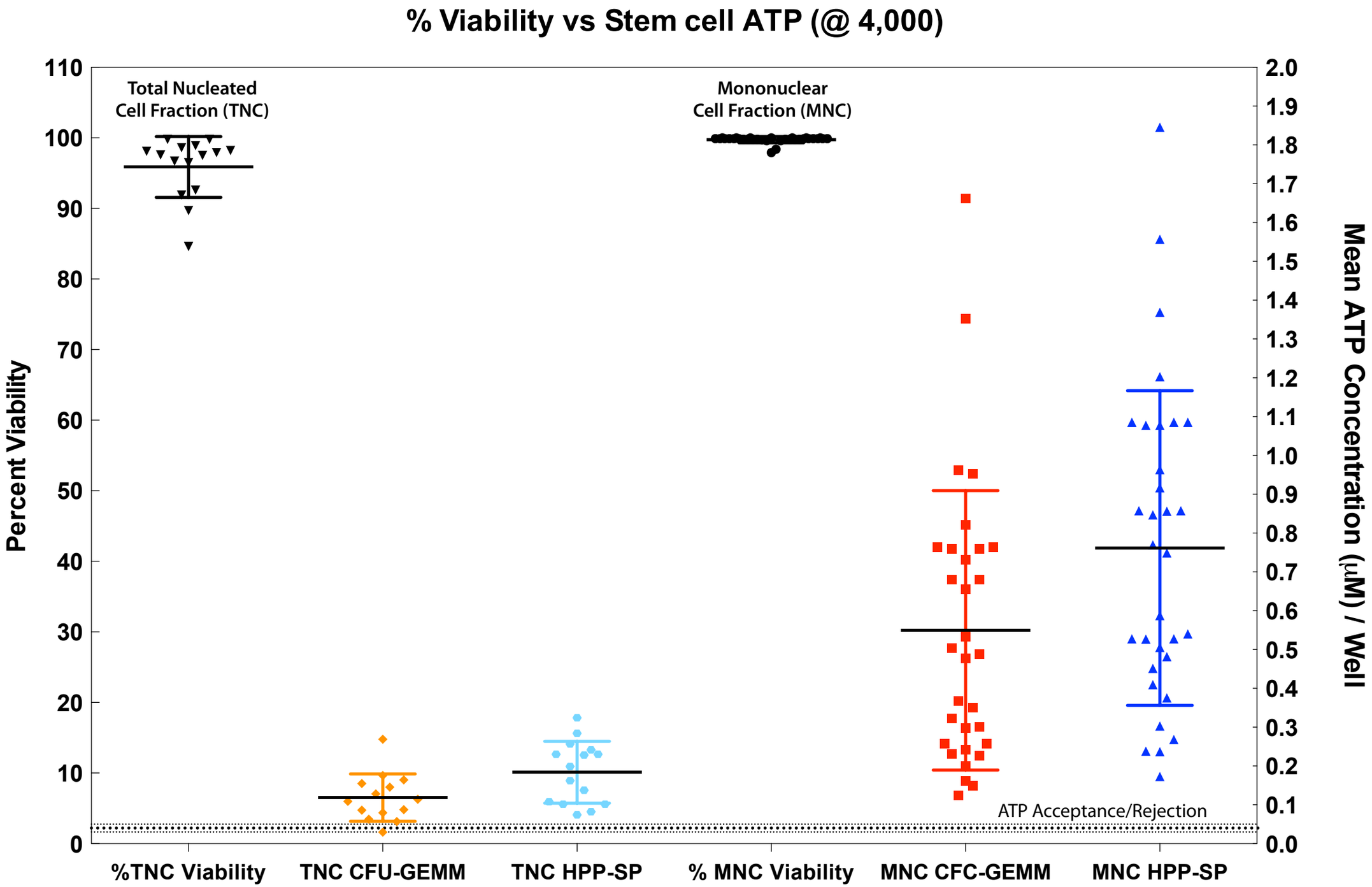
- Target cells? Primary stem cells, e.g. cord blood
- Goal of the assay? Quality and potency
- Biological process to be measured? Stem cell proliferation ability and potential
- Endpoint to be measured? Intracellular ATP concentration
- Readout of the endpoint? Bioluminescence

Our Goal is to Measure the "Quality" and Potency of Primitive Stem Cells that are used for Transplantation



Stem Cell "Quality"

Dye Exclusion Viability does not Correlate with Metabolic Viability for Umbilical Cord Blood

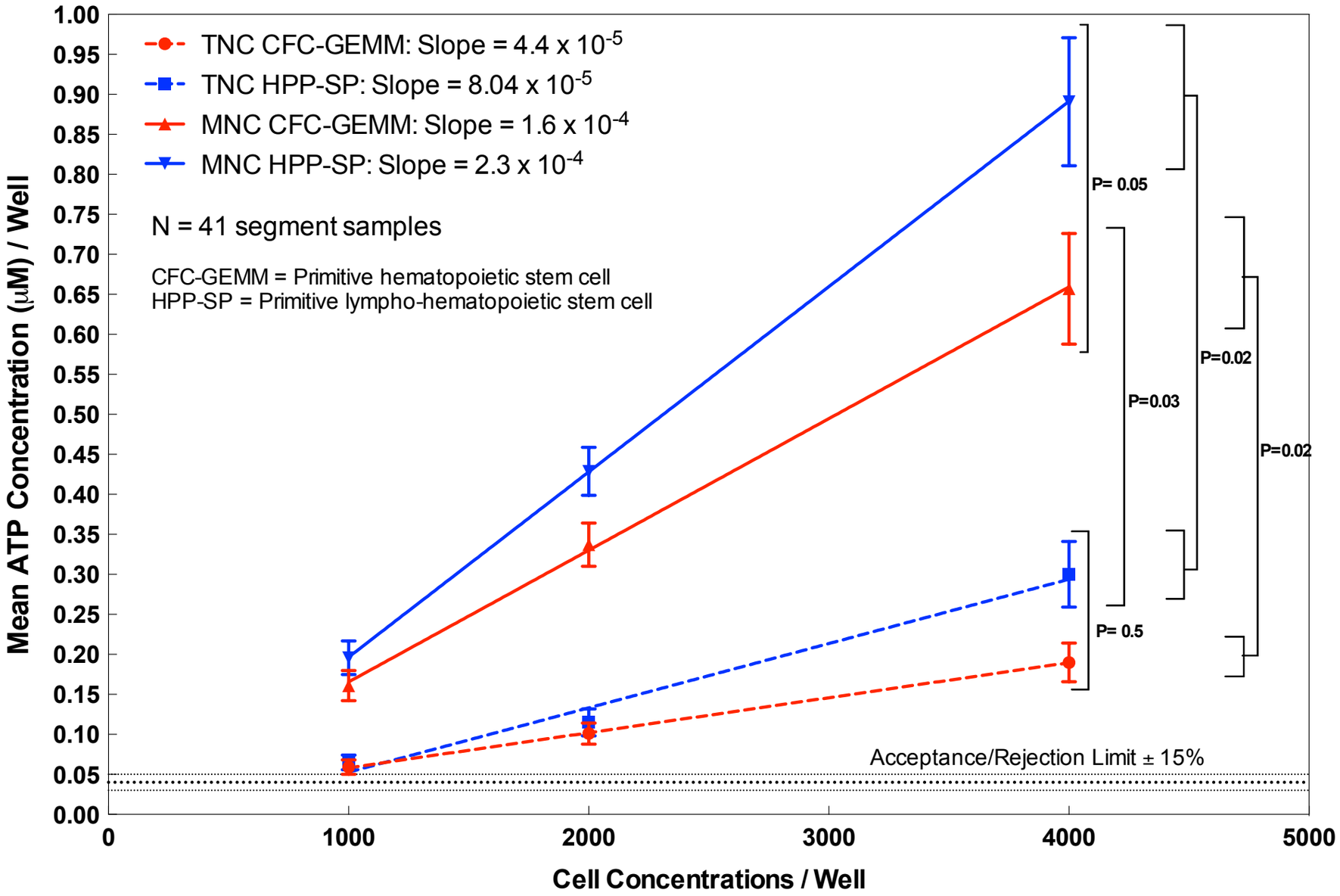


Adapted from: Patterson et al. J. Translation Medicine (2015) 13:94

Stem Cell Potential:

Cell Viability Depends on Cell Purity

Umbilical cord blood is usually processed to a Total Nucleated Cell (TNC) fraction that contains cell impurities, which dilute, mask and result in a severe underestimation of the true viability and functionality of the cord blood unit



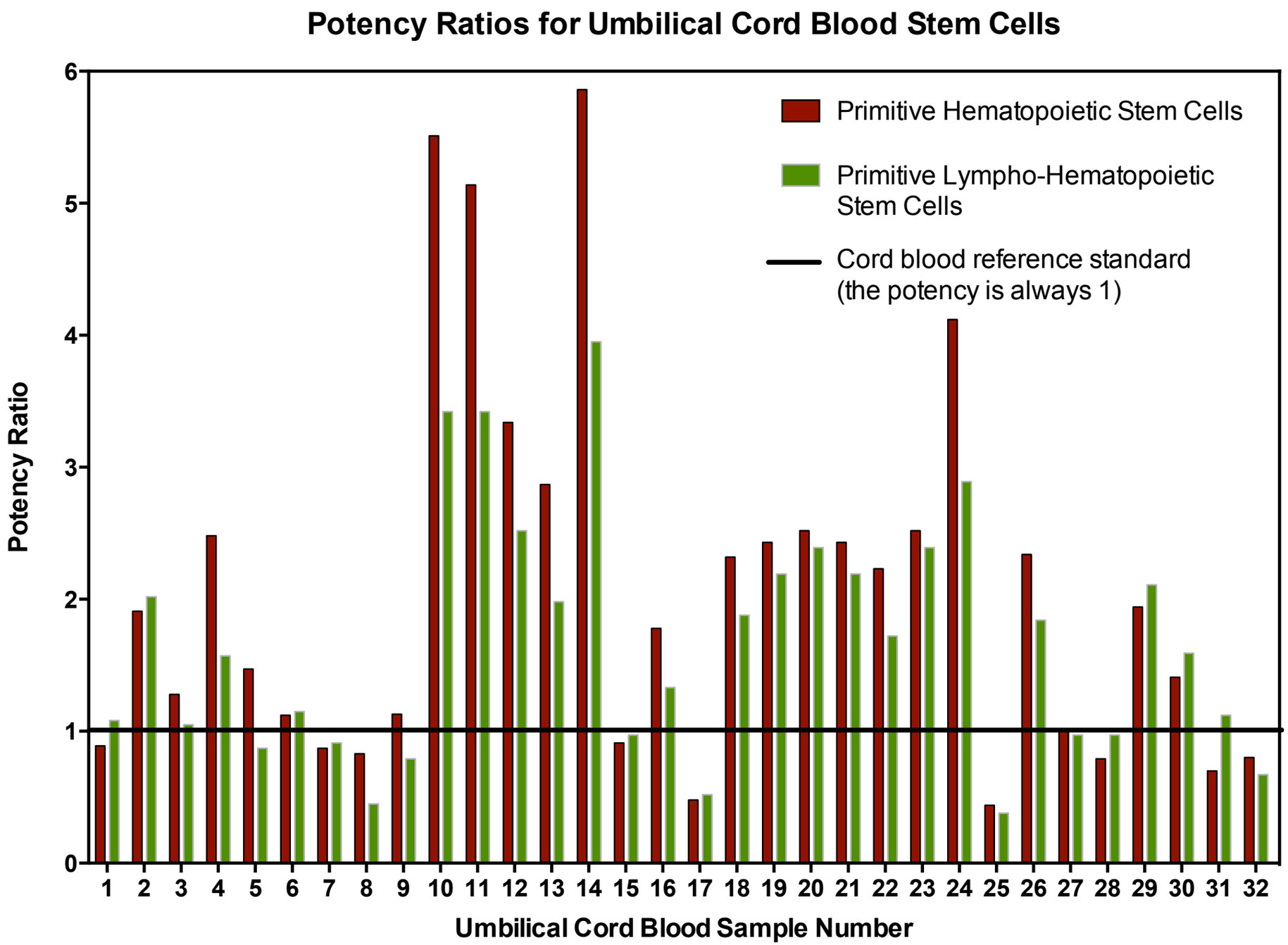
Slope of the cell dose response = Proliferation potential
The steeper the slope, the greater the proliferation potential, the more primitive the cells being measured

Adapted from: Patterson et al. J. Translation Medicine (2015) 13:94

A Potency Assay is a Very Special Viability Assay

A potency assay must:

- Measure the "active" components
- Quantitatively measure biological activity
- Predict the intended response
- Release the product for use
- Include a reference standard, to determine potency ratio
- Include standards and controls for validation



Dose response slope = Proliferation potential = Primitiveness = Potency = Engraftment potential
Potency Ratio = Slope of the stem cell dose response / Slope of the dose response for the Reference Standard
Stem cells with potency lower than the reference standard should not be used for transplantation

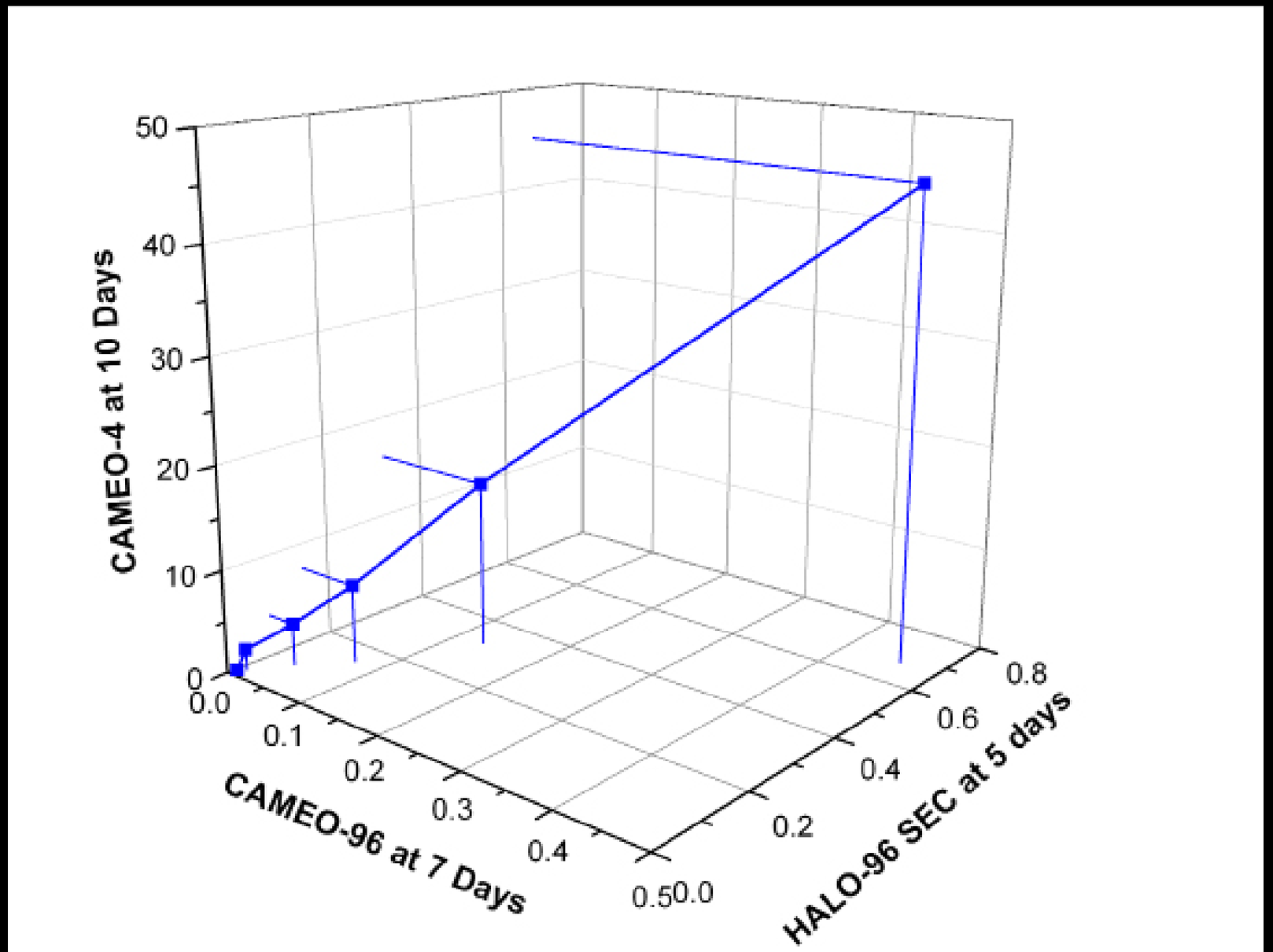
Can You Trust These Results?

Assay Verification:

Comparison of a new assay against an established assay

Verification of ATP Bioluminescence Assay Against Two Methylcellulose Colony-Forming Unit (CFU) Assays

From: Rich IN. Expert Opinion Drug Metab Toxicol (2007) 3:295-307



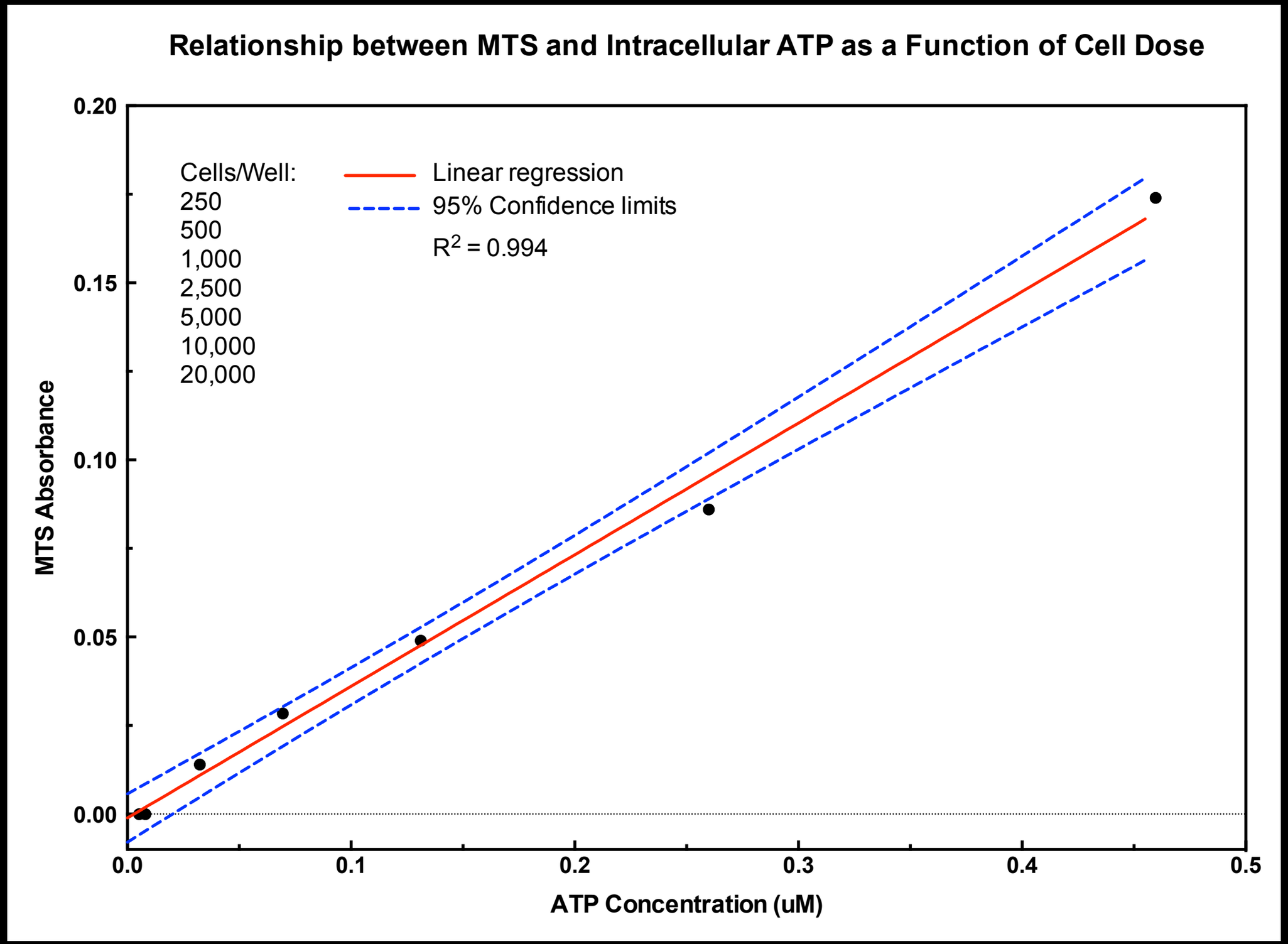
Cell Concentrations:
250
500
1,000
2,500
5,000
10,000
20,000

CAMEO™-4: A miniaturized colony-forming unit (CFU) assay with a manual, subjective readout.

CAMEO™-96: A fully standardized, methylcellulose CFU assay capable of determining both hematopoietic cell proliferation and differentiation using two different readouts in the same assay.

CAMEO™-4 vs CAMEO™-96: R = 0.997
CAMEO™-4 vs HALO®: R = 0.986
CAMEO™-96 vs HALO®: R = 0.964

Verification of Two Metabolic Viability Assays



Assay Validation:

Measurement Assurance

The Principle of Bioluminomics™ Assays

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

The intracellular concentration of ATP correlates directly with:

Viability

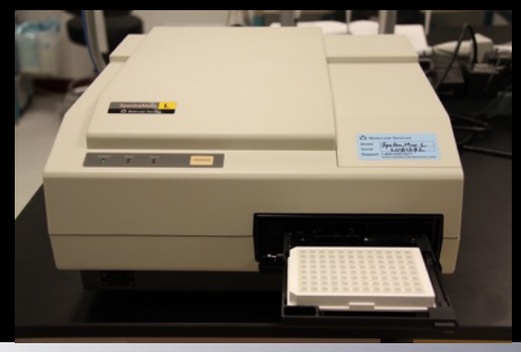
Proliferation

Cytotoxicity

Cell Number

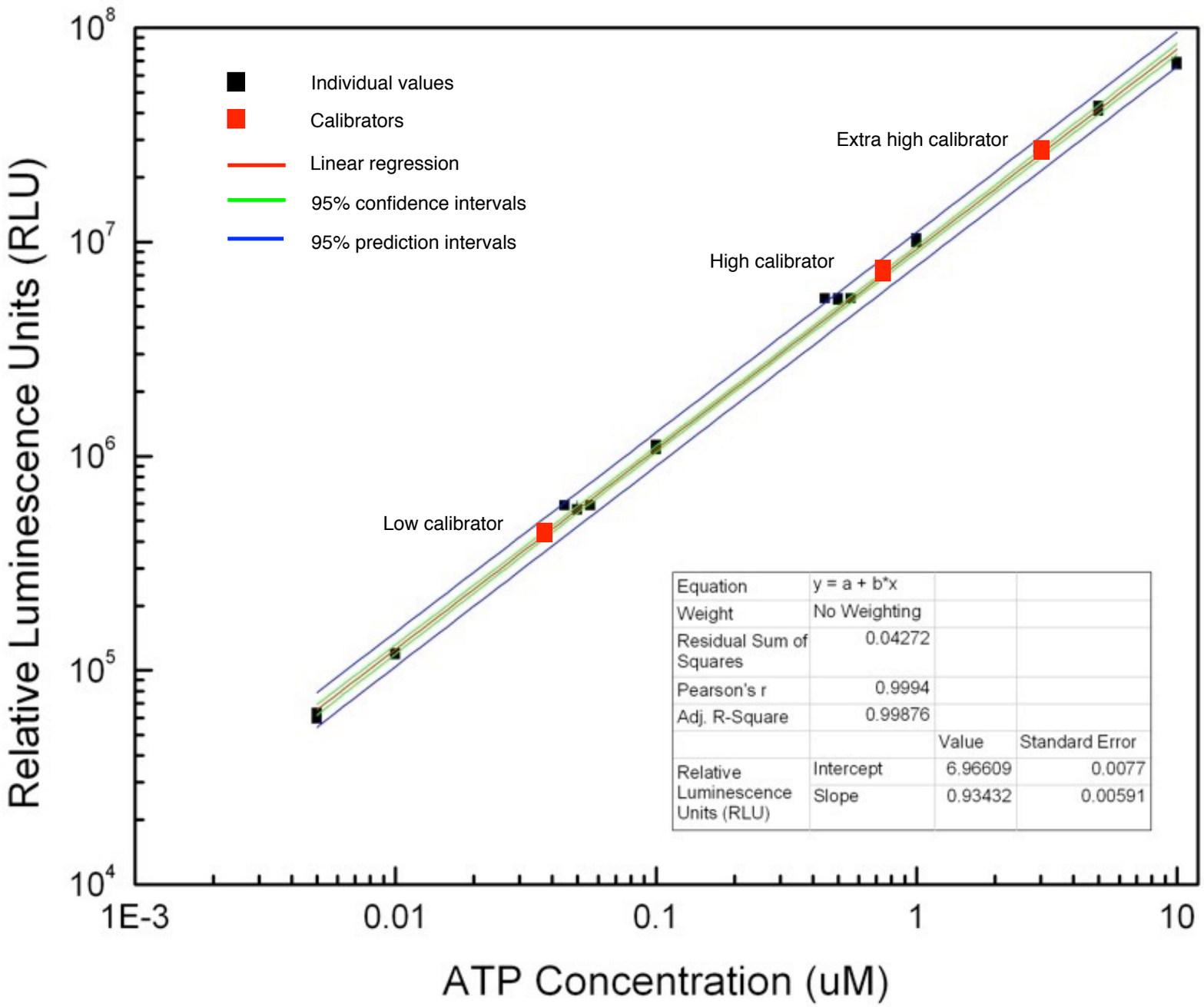
Released iATP + Luciferin + Luciferase = Bioluminescence

LIGHT



Relative Luminescence Units (RLU)

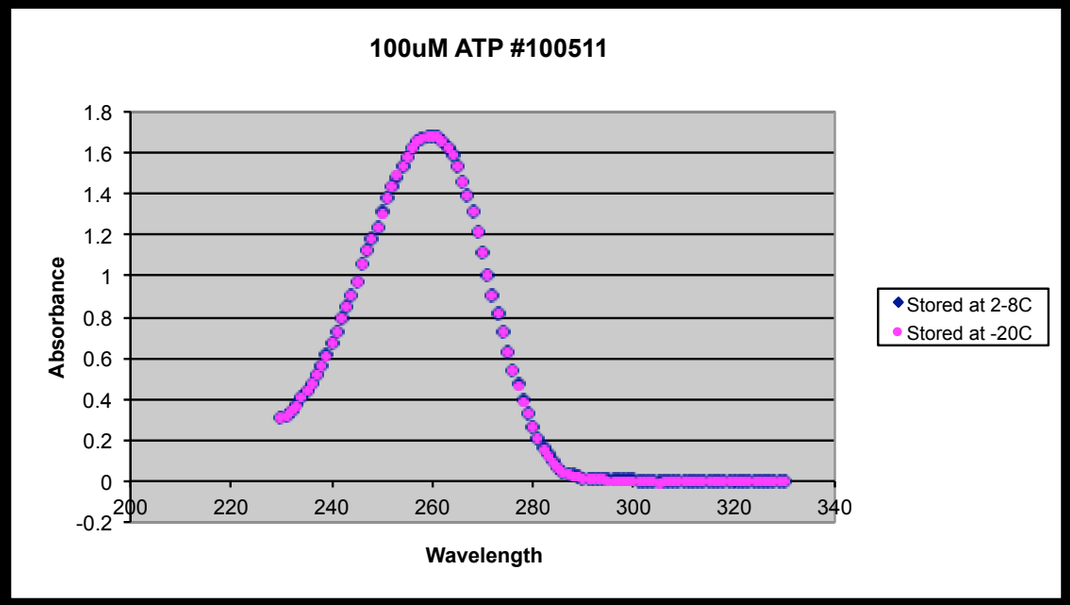
The ATP Standard Curve



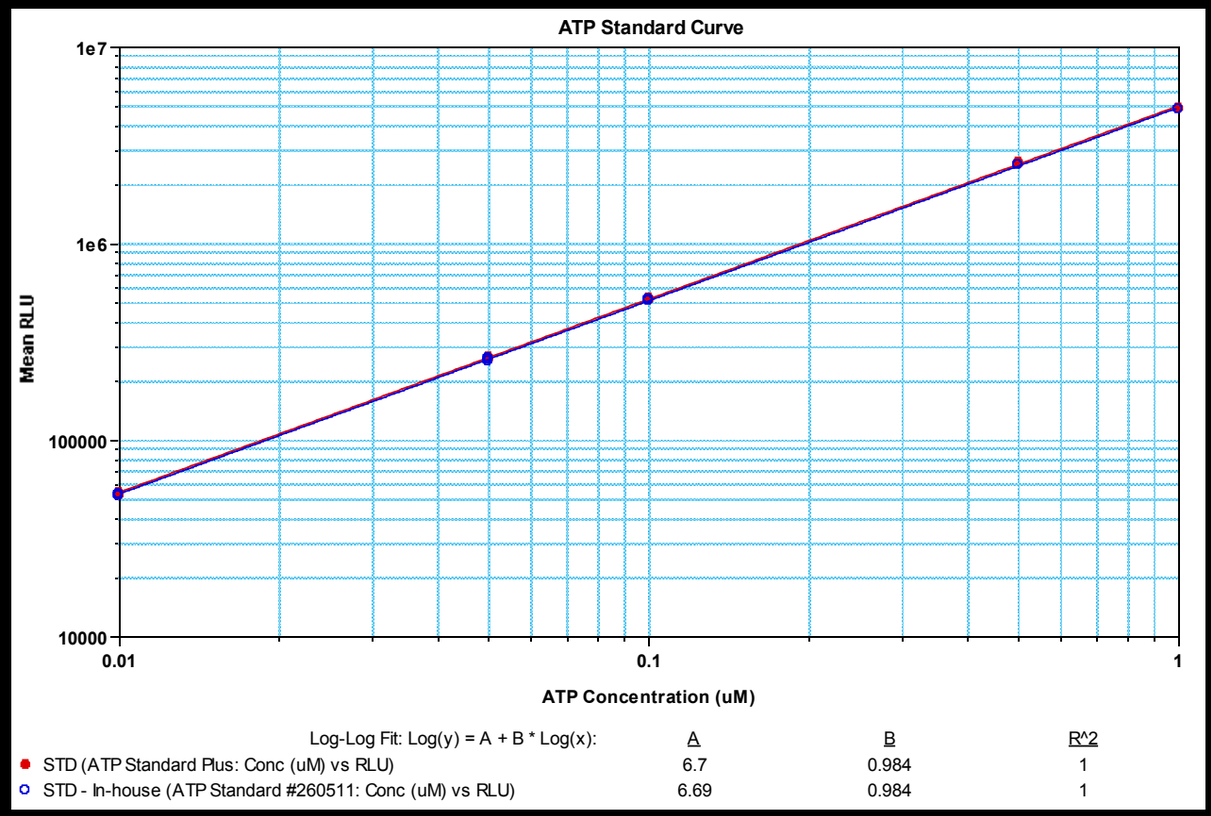
Equation	y = a + b*x		
Weight	No Weighting		
Residual Sum of Squares	0.04272		
Pearson's r	0.9994		
Adj. R-Square	0.99876		
		Value	Standard Error
Relative Luminescence Units (RLU)	Intercept	6.96609	0.0077
	Slope	0.93432	0.00591

Establishment of an ATP Standard

Absorbance Profile of ATP



Comparison of an ATP Standard with an ATP In-House Preparation



Stock 100µM ATP Diluted to 10µM ATP

Sample No.	Stock [ATP] (UV-Vis)	Abs (@lambda max)	Final ATP Conc (UV-Vis)
1	97.66	0.154	10.00
2	96.95	0.152	9.87
3	95.0	0.155	10.06
4	91.49	0.153	9.94
5	97.66	0.156	10.13
6	96.95	0.158	10.26

Lambda max at 259-260nm.
According to Beer's Law:
ATP Concentration (µM) = Absorbance at lambda max / Extinction coefficient of ATP (0.0154)

Accuracy of the ATP Standard Curve

Do the measured values agree with the expected values?

	ATP Standard Curve Concentrations				
Expected ATP Concentrations	0.01µM	0.05µM	0.1µM	0.5µM	1µM
Mean (µM)	**0.00965**	**0.05186**	**0.10210**	**0.49873**	**0.98799**
St. Dev (µM)	0.00057	0.00321	0.00443	0.02338	0.05048
%CV	5.93	6.21	4.35	4.69	5.11
N=	72	72	72	72	72

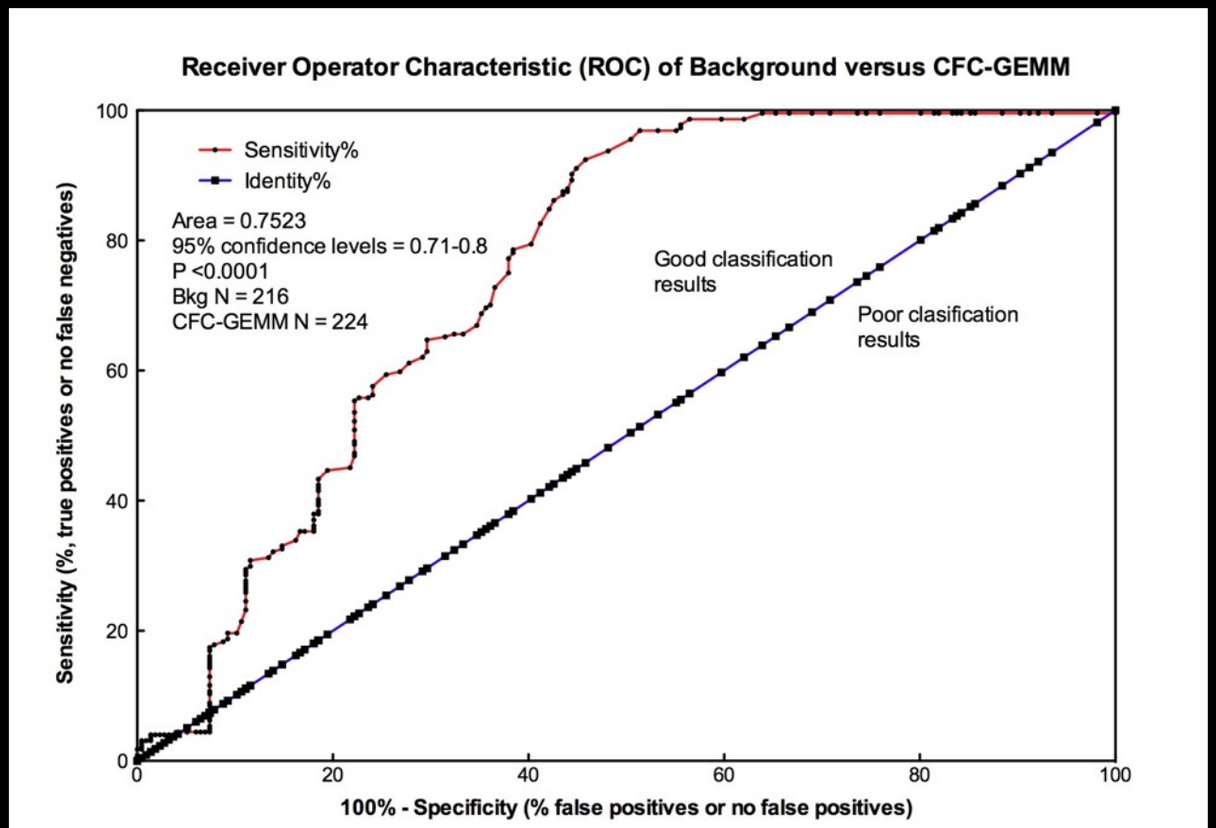
Accuracy of the ATP Standard Curve

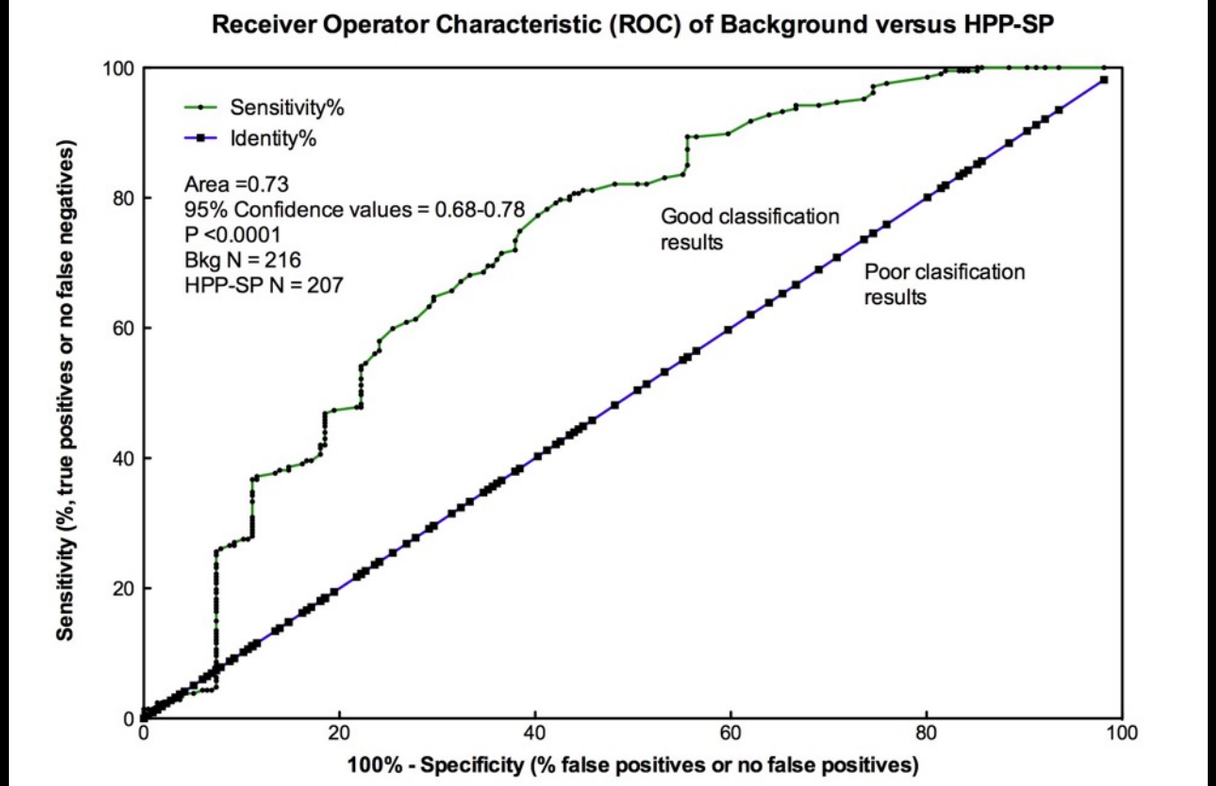
Dose Response Number	Intercept	Slope	Correlation Coefficient (R)
1	1.68	0.96	1
2	2.21	0.97	0.999
3	2.06	0.985	1
4	2.14	0.976	1
5	6.1	0.944	0.998
6	6.03	0.979	1
7	6.22	0.964	0.999
8	6.09	0.986	1
9	6.13	0.978	1
10	6.08	0.962	0.999
11	6.1	0.976	1
12	6.11	0.979	1
13	5.85	0.982	1
14	5.81	0.987	1
15	5.85	0.965	1
16	6.07	0.979	1
Mean	**5.033**	**0.973**	**1**
St. Dev.	**1.802**	**0.012**	**0.0006**
%CV	**35.804**	**1.233**	**0.06**

Sample Accuracy, Sensitivity and Specificity Receiver Operator Characteristics

(ROC Curves)

- A plot of "true positive rate" or sensitivity against the "false positive rate" or specificity.
- A tradeoff between sensitivity and specificity (an increase in sensitivity = a decrease in specificity).
- The close the curve follows the left axis and the top boarder, the more accurate the test.
- A 45-degree diagonal indicates that the assay is "worthless".
- Area under the curve (AUC) is a measure of accuracy. Must be between 0.5 and 1, 0.5 being a "worthless" assay and 1 being an ideal assay.





PRECISION: Reliability and Reproducibility

Within-Run, Intra-Batch Precision for Cord Blood MNC

			Hematopoietic Stem Cells			Lympho-Hematopoietic Stem Cells		
Sample No.		Background, 5, 000 cells	2,500 cells	5,000 cells	7,500 cells	2,500 cells	5,000 cells	7,500 cells
1	Mean(µM)	0.006	0.027	0.118	0.202	0.019	0.153	0.169
	%CV	**13.4**	**17.4**	**15.8**	**9**	**27**	**11.8**	**13.1**
2	Mean(µM)	0.007	0.034	0.079	0.126	0.067	0.187	0.32
	%CV	**7.1**	**19.6**	**10.3**	**16.8**	**15.3**	**12.7**	**12.7**
3	Mean(µM)	0.036	0.139	0.394	0.532	0.154	0.443	0.635
	%CV	**12.9**	**19.8**	**12.4**	**7.7**	**16.2**	**13**	**8.1**
4	Mean(µM)	0.009	0.014	0.042	0.078	0.029	0.088	0.111
	%CV	**11.5**	**29.2**	**9.9**	**13.4**	**16.1**	**13.1**	**14.5**
5	Mean(µM)	0.02	0.131	0.271	0.448	0.117	0.36	0.571
	%CV	**7.3**	**15.5**	**20**	**11.8**	**19.9**	**12.3**	**13.5**

FDA Guidelines for Industry: Bioanalytical Method Validation. LLOQ =< 20% CV; otherwise =< 15% CV

PRECISION: Reliability and Reproducibility

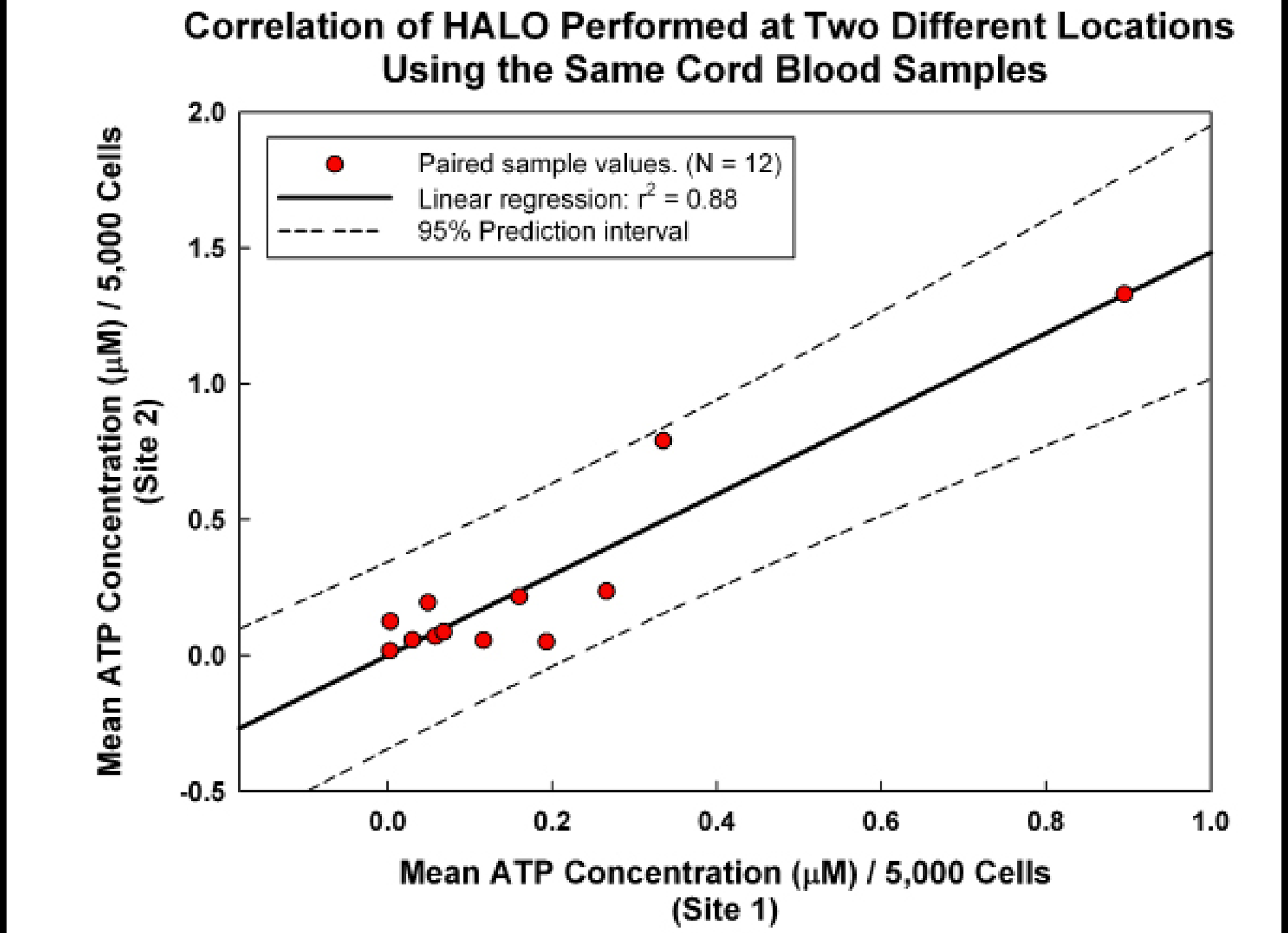
Between-Run, Inter-Batch Precision for Cord Blood MNC

16 cord blood batches (different units) measured over a 30 day period

	Hematopoietic Cord Blood Stem Cells			Lympho-Hematopoietic Stem Cells		
	2,500 Cells	5,000 Cells	7,500 Cells	2,500 Cells	5,000 Cells	7,500 Cells
Mean (µM)	0.024	0.049	0.078	0.055	0.123	0.205
St. Dev. (µM)	0.0023	0.0053	0.0076	0.0062	0.016	0.0276
%CV	**9.75**	**10.74**	**9.77**	**11.21**	**12.71**	**13.48**

FDA Guidelines for Industry: Bioanalytical Method Validation. LLOQ =< 20% CV; otherwise =< 15% CV

Inter-Laboratory Robustness



From: Reems et al. Transfusion (2008) 48: 620-628

Why are Standards, Calibrators and Controls so Important?

- The output of a luminometer or fluorimeter is in non-standardized relative units, e.g. RLU or RFU
- Calibrators ensure that the instrument is working correctly.
- Standards and controls ensure that the reagents are working correctly with low dispensing error.
- The standard curve standardizes the assay to allow the conversion of non-standard units into standardized concentrations.
- This, in turn, allows direct comparison of results over time.
- Incorporates an internal proficiency test for both the user and the assay.
- Allows assay validation: Measuring accuracy, sensitivity and specificity, precision and robustness.

Assay Characteristics and Validation Parameters

- Linear regression correlation coefficient (R) = >0.997
- Linear regression slope = 0.937 (slope range allowed: 0.796-1.07)
- Low calibrator = 0.05uM ATP (range allowed: 0.043-0.058uM ATP)
- High calibrator = 0.7uM ATP (range allowed: 0.595-0.805uM ATP)
- Extra high calibrator = 1.75uM (range allowed: 1.488-2.013uM)
- Lowest ATP value indicating unsustainable proliferation = 0.04uM ATP +- 15%
- ATP value below which cells are metabolically dead = 0.01uM ATP
- Assay linearity = >4 logs
- Assay cell linearity: 1,000 - > 25,000 cells
- Assay sensitivity = 0.001uM ATP
- Assay cell sensitivity: 20-25 cells

- Accuracy (% correct outcomes): ~95%
- Sensitivity and specificity: Receiver Operator Characteristics (ROC) AUC: 0.73-0.752
- Precision: = <15% (LLOQ): 20%
- Robustness (intra- and inter-laboratory): ~95%
- High throughput capability (Z-factor): >0.76

Thank You

The Siamese Twins in the Garden of the Gods Park with Pike's Peak in the background