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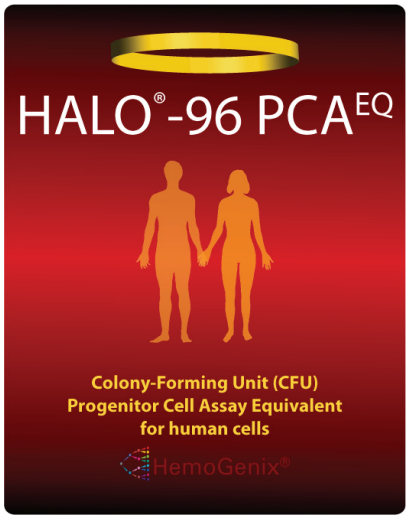

HALO®-96 PCA EQ
Progenitor Cell Assay Equivalent

for hematopoietic stem cell processing laboratories

Instruction Manual

(Version 1-2014)

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Not for clinical diagnostic use.

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1. INTRODUCTION

HemoGenix® divides its cell-based assays into proliferation and differentiation assays. HALO® is a proliferation assay platform for stem and progenitor cells of the blood-forming (lympho-hematopoietic) system. HALO® assays have been developed for specific applications. HALO®-96 PCA^{EQ} has been developed for stem cell processing laboratories to compare the "classic" colony-forming unit (CFU) assay with a more advanced equivalent assay system.

The CFU assay, first published in 1966 for mouse cells and later modified to detect the growth of human hematopoietic cells is an established assay in stem cell processing laboratories. Based on the ability of cells to form colonies in a semi-solid, viscous medium (usually methylcellulose), the CFU assay detects the ability of cells to differentiate. This is because the cells producing the colonies and scored by manual counting with the aid of a dissection or inverted microscope, have to differentiate and mature in order to identify the colonies as belonging to one or other hematopoietic lineage. Although colony growth requires proliferation, the CFU assay does not measure cell proliferation. The subjective nature of scoring colonies together with the lack of standards and controls has meant that the CFU assay cannot be validated as required by regulatory agencies.

HALO®-96 PCA^{EQ}, (Progenitor Cell Assay, Equivalent) was designed to replace the present CFU assays used in stem cell processing laboratories with an advanced non-subjective cell culture and readout system that uses exactly the same growth factor cocktails as CFU reagents for use with human cells. HALO®-96 PCA^{EQ} incorporates two proprietary technologies, Bioluminomics™ and Suspension Expansion Culture™ (SEM™).

Bioluminomics™ is the instrument-based measurement of intracellular ATP (iATP) concentrations using a luciferin/luciferase bioluminescence signal detection system. When hematopoietic stem and progenitor cells are stimulated with growth factors and/or cytokines, the iATP concentration changes in direct proportion to the cell population being measured, the types and concentrations of growth factors and/or cytokines and the cell dose. Detection iATP concentrations is the most sensitive and accurate *in vitro* readout available. Bioluminomics™ also incorporates the calibration, standardization and, if necessary, the ability to validate the assay. Bioluminomics™ ensures that the results obtained are reliable and reproducible, not only within one laboratory, but between laboratories.

Suspension Expansion Culture™ (SEC™) is a methylcellulose-free culture system that is fast and easy to use, exhibits greater sensitivity and accuracy and significantly shorter culture times than any "classic" colony-forming unit (CFU) assay.

By implementing HALO®-96 PCA^{EQ} in the stem cell processing laboratory, the user is assured of trustworthy and reliable results with the ability to compare those results over time with any CFU assay. However, HALO®-96 PCA^{EQ} is also meant as a stepping stone to even more advanced hematopoietic cellular therapeutic assays, HALO®-96 SPC-QC and HALO®-96 PQR.

2. INTENDED USE

HALO®-96 PCA^{EQ} is intended to replace the CFU assay used in cell processing laboratories that manufacture and produce hematopoietic stem cell products for transplantation purposes. HALO®-96 PCA^{EQ} can be used to determine the proliferation ability of the cell populations derived from the following tissues:

- Bone marrow
- Peripheral blood (normal or mobilized)
- Umbilical cord blood.

Table 1 shows the different HALO®-96 PCA^{EQ} assays available that are equivalent to the "classic" CFU assays produced by both HemoGenix® (CAMEO™-4) and Stem Cell Technologies (MethoCult®).

TABLE 1. HALO®-96 PCA^{EQ} CFU-Equivalent Assays

HALO®-96 PCA^{EQ}	Equivalent MethoCult® Product	Growth Factor/ Cytokine Cocktail	Cell Populations Stimulated
K2-PCA1-1 K2-PCA1-2 K2-PCA1-4	H4434 "Classic"	EPO, GM-CSF, IL-3, SCF	BFU-E, CFU-E, GM-CFC, G-CFC, M-CFC
K2-PCA2-1 K2-PCA2-2 K2-PCA2-4	H4034 "Optimum"	EPO, GM-CSF, G-CSF. IL-3, SCF	BFU-E, CFU-E, GM-CFC, G-CFC, M-CFC
K2-PCA3-1 K2-PCA3-2 K2-PCA3-4	H4534 "Classic"	GM-CSF, IL-3, SCF	GM-CFC, G-CFC, M-CFC
K2-PCA4-1 K2-PCA4-2 K2-PCA4-4	H4035 "Optimum"	GM-CSF, G-CSF, IL-3, SCF	GM-CFC, G-CFC, M-CFC
K2-PCA5-1 K2-PCA5-2 K2-PCA5-4	H4435 "Enriched"	EPO, GM-CSF, G-CSF, IL-3, IL-6, SCF + TPO(#)	CFC-GEMM, BFU-E, GM-CFC, G-CFC, M-CFC, Mk-CFC

(#) Please not that thrombopoietin (TPO) is not included in the H4435 "Enriched" MethoCult® product, but is included in the HALO®-96 PCA^{EQ} for the PCA5 products. This means the H4435 stimulates a more mature stem cell population than the HALO® product and will not stimulate cells of the megakaryopoietic lineage.

IMPORTANT:
HALO®-96 PCA^{EQ} is for research use only and has not been approved for clinical diagnostic use.

- *Low humidity*. Plates dry out (see below) and cell growth declines.
- *Contamination*: Cells cultured in 96-well plates cannot be view under a microscope. If contamination occurs it will usually be seen by the difference in color of the cultures. Contaminated cultures will usually be bright yellow in color and probably cloudy in appearance. Cell cultures that demonstrate high proliferation will usually appear orange to light orange, but will not be cloudy. If only "spot" contamination occurs, this is usually due to pipette or repeater tips coming in contact with materials other than the reagents. Contamination will usually lead to outlier RLU values.

Luminescence Reagent Mixing.
The luminescence reagent has to be added and thoroughly mixed with the culture components. The ATP-ER lyses the cells and releases intracellular ATP. If mixing is not adequate, only a proportion of the cells will be lysed and the RLU values will be low. Conversely, too much mixing can lead to ATP degradation and low luminescence readings.

Culture Plates Drying Out

- Due to the relatively small culture volume (0.1ml), drying out of the culture wells, particularly around the outside of the plate, can be a problem. These are called "edge effects". An incubator with insufficient humidity will cause this problem. To ensure that this does not occur, the incubator water reservoir should be full and the humidity in the chamber checked using a hygrometer.
- If drying out continues, use of a humidity chamber is recommended. A humidity chamber can be assembled using containers just large enough to hold several plates and deep enough to place both the plate(s) and a layer of 2-5ml serologic pipettes with the cotton plugs removed (cut to fit the container). Sterile water is placed in the container to just below the height of the pipettes. Do not let the plate sit in water. Place the plate on the pipettes and cover loosely with either aluminum foil or with a fitted lid that has holes in it to facilitate exchange of gases in and out of the container. Call HemoGenix® for more information on constructing a humidity chamber.

12. Troubleshooting

High Coefficients of Variation (%CV)
Coefficients of variation (%CV) should be =< 15%. The percent coefficient of variation is calculated as standard deviation/mean x 100. High %CVs are usually an indication of incorrect dilutions or pipetting error. Although outliers can be obtained, these being observed for the more primitive stem cells than for the more mature proliferating cells, large variations between replicates should not be obtained. Please consider the following:

- Accurate reagent dispensing and mixing are of prime importance. Small volumes are dispensed and the use of instruments that have not been calibrated correctly or have not be calibrated for a long period of time, can lead to high CVs.
- Insufficient mixing of components prior to plating the culture master mix and insufficient mixing during the addition of luminescence reagents to cultures in the 96-well plate can also lead to high CVs. Use repeater pipettes. Never use syringes with needles to dispense reagents. Use calibrated or self-calibrating electronic pipettes or dispensers to add and mix the luminescence reagents.
- If the luminometer requires determining the "gain" empirically, it is possible that this parameter has not been optimally set and will result in an incorrect signal to noise ratio. Once the optimal "gain" has been set for the instrument, it should not be changed.
- Perform a minimum of 6 replicates per point.

Low RLU Values
Performing an ATP dose response prior to sample measurement can help detect problems prior to sample measurement. If low RLU values occur, this could be due to the following reasons.

- *Reagent decay:* The ATP-ER decays with time, even when frozen. This can lead to low bioluminescence. Once thawed the reagent can be refrozen up to 11 cycles without significant loss of sensitivity. Do not use the reagent after expiry date has elapsed. As a rule of thumb, the RLU value for the lowest ATP standard should be 10 times greater than that of the background value.
- *Inadequate cell growth:* Cells did not exhibit sufficiently high viability. Measure cell viability prior to adding the cells to the master mix. A cell viability lower than 85% should not be used. Viabilities lower than 85% can be an indication that the sample was not processed in a time-sensitive manner or that the processing procedures were not standardized and controlled.
- *Reagent deterioration:* Reagents arrived thawed, at room temperature or greater or were not stored correctly as indicated in Section 5 of this manual.
- *Inadequate incubator conditions:* Maintaining a correct humidified gaseous atmosphere in the incubator is essential (See Culture Plate Drying Out).
- *Carbon dioxide concentration is inadequate.* Ensure that the carbon dioxide concentration in the incubator is correct using a Fyrite gas analyzer.
- *Use low oxygen tension*. Using an oxygen concentration of 5% reduces oxygen toxicity due to free radical production and increases plating efficiency. Check that the incubator oxygen concentration is correct using a Fyrite gas analyzer.

3. The CONCEPT and PRINCIPLE of BIOLUMINOMICS™ ASSAYS

HALO®-96 PCA^{EQ} is a bioluminomics™ assay. The fundamental concept underlying bioluminomics™ is the measurement of the cell's chemical energy in the form of intracellular ATP (iATP). If a cell is producing iATP, it is demonstrating cellular and mitochondrial integrity and is therefore viable. When cells are stimulated to proliferate, which is the case when hematopoietic cells are cultured in the presence of growth factor and/or cytokines, the iATP concentration increases several fold. The iATP concentration produced is directly dependent on:

- The proliferation potential (or primitiveness) of the cell population being detected.
- The concentration of the growth factor(s) and/or cytokine(s) used to stimulate the cells.
- The plated cell concentration.

Lympho-hematopoietic cells are incubated in the HALO® Master Mix(s) provided with this kit for a specific period of time. When the culture period has elapsed, a single ATP Enumeration Reagent is dispensed into each culture well and the contents mixed. The plate is incubated at room temperature in the dark for 10 minutes. During this time, the cells are lysed and the released iATP acts as a limiting substrate of a luciferin/luciferase reaction to produce bioluminescence in the form of light according to the following equation:

$$\text{ATP} + \text{Luciferin} + \text{O2} \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{LIGHT}$$

The bioluminescence emitted is detected and measured in a plate luminometer as relative luminescence units (RLU). To calibrate and standardize the assay, an ATP standard and high and low controls are provided. **Performing the ATP standard curve and controls is the most important part of HALO®-96 PCA^{EQ}. Failing to perform the ATP standard curve and controls can invalidate the results.** The ATP standard curve and controls must be performed prior to processing the samples for the following reasons:

1. Performing an ATP standard curve calibrates and standardizes the assay.
2. The controls ensure that the reagents are working correctly and indicate pipetting error.
3. The ATP standard curve allows the luminometer output in Relative Luminescence Units (RLU) to be converted to standardized ATP concentrations (µM).
4. Performing the ATP standard curve allows results to be compared over time.

The ATP standard curve and controls are measured once, on the day samples are to be processed. DO NOT use results from an ATP standard curve or controls performed on one day for samples processed on another day.

The ATP standard curve is used to convert sample RLU results into ATP concentrations by interpolation. This procedure can often be performed automatically by the luminometer software. If the software does not allow this, it will be necessary to use third-party software to perform this operation.

4. OVERVIEW OF THE HALO® PROCEDURE

There are 3 steps to use HALO®.

Step 1 – Cell Preparation
Cells are not provided with HALO®-96 PCA^{EQ} assay kits. Cells are prepared either with a user-defined, pre-validated protocol to obtain a single cell suspension or procedures that are suggested in this manual. A dye exclusion viability and/or metabolic viability and nucleated cell count should be performed on all samples.

Step 2 – Cell Culture
Each HALO®-96 PCA^{EQ} assay contains a Master Mix to stimulate the cell populations shown in Table 2. However, the assay does not differentiate individual cell populations. It measure the proliferation of all cells in all cell populations. (To measure proliferation of individual cell populations, separate assays must be used). In addition, medium is also provided for the dilution of the ATP standard. Sufficient HALO® Master Mix volumes are provided to culture 16 samples in a single 96-well plate. For each sample tested, 0.72ml of the HALO® Master Mix is dispensed into a sterile plastic tube followed by 0.08ml of the cell suspension adjusted to the correct working concentrating. The contents of the tube(s) are mixed and 0.1ml dispensed into 6 replicate wells in rows across the plate. The plates are incubated for 5, 6 or 7 days. The incubation time is usually dependent on preference and work schedule. (Please note that cultures incubated for longer incubation period will also exhibit increased coefficients of variation).

Step 3 – Bioluminescence measurement
To measure bioluminescence, the iATP is released from the cells. This is accomplished by the addition of 0.1ml of the ATP Enumeration Reagent (ATP-ER), which also contains luciferin and luciferase, the reagents used to produce bioluminescence. The ATP-ER is added using a multi-channel pipette. After a short incubation period the bioluminescence is measured in a plate luminometer. Prior to sample luminescence measurements, the instrument is calibrated and the assay standardized by performing an ATP standard curve with controls (provided with the kit).

PLEASE NOTE: Although HALO®-96 PCA^{EQ} is performed in a 96-well plate, it is not similar and should not be performed in the same manner as any enzyme-linked immunsorbant assay (ELISA).

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11. HALO®-96 PCA^{EQ} Characteristics and Validation Parameters

HALO® bioluminomics™ technology allows the assay to be calibrated and standardized. Inclusion of an ATP standard and controls are requirements for assay validation. The HALO® Platform has been verified against the CFU assay and has been validated not only by HemoGenix®, but also by universities and biopharmaceutical companies that have implemented the assay.

Assay Characteristics

When performing the ATP standard curve, the following parameters should be obtained:

- Log-log linear regression goodness of fit (r^2) = > 0.995
- Log-log linear regression correlation coefficient (r) = > 0.997
- Log-log linear regression slope = 0.937 ± 15% (slope range: 0.796 - 1.07)

- Low ATP control = 0.05µM ± 15% (allowable range: 0.043µM - 0.058µM)
- High ATP control for ATP standard curves up to 1µM = 0.7µM ± 15% (allowable range: 0.595µM - 0.805µM)
- High ATP control for ATP standard curves up to 3µM = 1.75µM ± 15% (allowable range: 1.488µM - 2.013µM).

For samples:
- Lowest ATP value indicating non-sustainable cell proliferation: ~0.04µM
- ATP value below which cells are not metabolically viable: ~0.01µM.

If these parameters are not attainable, it is usually an indication that one or more of the following needs to be addressed:
1. Pipettes used are not properly calibrated
2. Pipetting error
3. Incorrect serial dilutions
4. Cultures drying out.

Please refer to Troubleshooting (Section 12).

Assay Validation Parameters

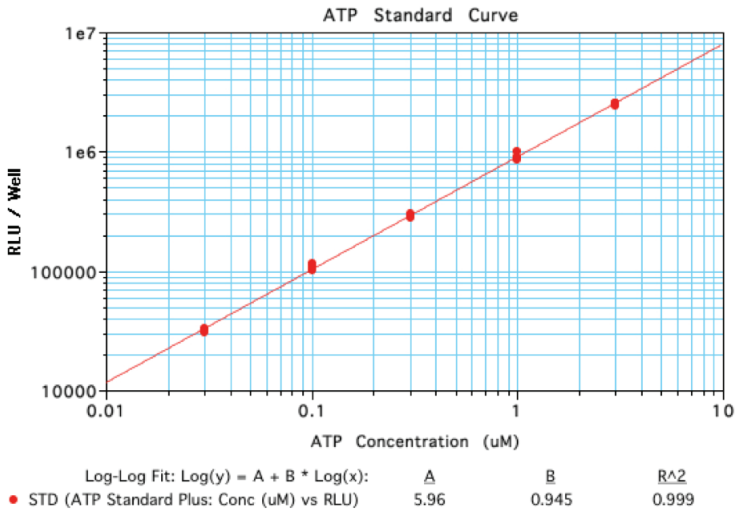
- Assay ATP linearity => 5 logs
- Assay cell linearity: 1,000 - >25,000 cells/well
- Assay ATP sensitivity: ~ 0.001µM
- Assay cell sensitivity: 20-25 cells/well (depending on pruity and growth factor cocktail)
- Accuracy (% correct outcomes): ~95%
- Sensitivity and specificity detected by Receiver Operator Characteristics (ROC) curve fit and detected as area under the curve (AUC): 0.73 - 0.752 (lowest possible value, 0.5; highest possible value, 1).
- Precision (Reliability and Reproducibility) =< 15%. At lower limit of quantification (LLOQ): 20%
- Robustness (intra- and inter-laboratory): ~95%.
- High throughput capability (Z-Factor): >0.76 (lowest possible value, 0.5; highest possible value, 1).

calculations and graphs:

- GraphPad Prism version 5.0d
- TableCurve 2D from Systat Software, Inc.
- OriginLab version 8.1or higher from Origin Software.

For technical assistance using these third-party software packages, please contact HemoGenix®.

Figure 2. Typical ATP Standard Dose Response Curve Using SoftMax Pro Software



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5. KIT CONTENTS

HALO®-96 PCA^{EQ} kits contains the following components:

1. HALO®-96 PCA^{EQ} Master Mix.
2. Medium (IMDM) to dilute the ATP standard.
3. ATP standard.
4. ATP high and low controls.
5. ATP Enumeration Reagent (ATP-ER)*
6. Adhesive Plate Covering—a sterile foil to protect and keep unused wells sterile.
7. Sterile, 96-well plates for stem cell culture.
8. Non-sterile 96-well plates for ATP standard curve determination.
9. Instruction manual.

Exact volumes of the kit reagents and supplies are provided on a separate sheet included with this assay kit.

*The ATP-ER should not be thawed until needed and can be refrozen 11 times without significant loss of sensitivity. It can be kept at 2-8°C for 48h once thawed and is stable for 20 weeks when stored at -20°C. This reagent is light sensitive. Keep in the dark. The ATP-ER must not be used past the expiration date.

IMPORTANT

All kit components are quality controlled and optimized so that they work together. Please do not replace kit components with those of a different product. This will invalidate the warranty provided by HemoGenix®.

This kit contains a reagent for measuring luminescence (ATP-ER) that decays with time. HemoGenix® suggests that this kit be used before the expiry date of this reagent. HemoGenix® does not take responsibility for the quality of reagents beyond their expiry date. If the kit cannot be used prior to the expiry date of this reagent, fresh reagent can be purchased from HemoGenix®. Please contact HemoGenix®.

Good laboratory practices and universal protective precautions should be undertaken at all times when handling the kit components as well as cells and tissues. Material safety data sheets (MSDS) are included in each literature packet.

6. EQUIPMENT, SUPPLIES AND REAGENTS REQUIRED, BUT NOT PROVIDED

Equipment and Supplies

1. Laminar Flow Biohood
2. Plate luminometer (e.g. Molecular Devices, SpectraMaxL; Berthold, CentroLIA)
3. Sterile, capped, plastic tubes (5ml, 10ml, 50ml)
4. Single channel pipettes, preferably electronic (e.g. ViaFlow or Rainin EDP pipettes for variable volumes between 1µl and 1000µl).
5. 8 or 12-channel pipette, preferably electronic (e.g. ViaFlow or Rainin EDP pipettes for fixed or variable volumes between 10µl and 100µl).
6. Reservoir for 8- or 12 channel pipette
7. Sterile pipette tips.
8. Vortex mixer.
9. Tissue culture incubator, humidified at 37°C with 5% CO_2 (minimum requirement) and 5% O_2 (preferable).
10. 1.5ml plastic vials (5 for each ATP dose response).
11. Hemocytometer or electronic cell counter to determine cell concentration.
12. Flow cytometer or hemocytometer for determining viability.

Reagents

1. Sterile Phosphate Buffered Saline (PBS)
2. Iscove's Modified Dulbecco's Medium (IMDM)
3. Density-gradient medium (e.g. NycoPrep 1.077, Axis-Shield).
4. ACK Lysis buffer (Cat. No. K-Lysis-100, HemoGenix®, Inc)
5. 7-AAD, propidium iodide, trypan blue or other dye exclusion viability assay.

c. The third set of measurements to be performed will be those of the samples. Setup the software to perform the following calculations:

1. Group or sample designation
2. Sample number
3. Well number
4. RLU/well
5. ATP values/well (calculated from the ATP standard dose response curve)
6. Calculated mean ATP values
7. Standard deviation of calculated ATP values
8. % CV of calculated ATP values.

Most, if not all, the calculations and results can be obtained automatically directly from the luminometer without any further manipulation. By automatically converting the RLU values into ATP concentrations (µM) directly from the ATP standard curve, results from the samples can be graphically displayed via the software.

Most software packages can export the results to MS Excel either directly or via text files.

(iii) Instrument Setup for Luminometers without Software Analysis Capabilities

Many plate luminometers do not come with analysis software. Instead, the data is either automatically exported or has to be manually exported to a Microsoft Excel file for calculation and analysis. Excel has functions to perform the necessary calculations for interpolating RLU values into ATP concentrations using the ATP standard curve. The basic Excel procedure is as follows:

1. Column 1: Make a column for the actual ATP concentrations used for the ATP standard curve.
2. Column 2: Copy the RLU values for the standard curve.
3. Column 3. Transform the RLU values into log RLU values using the LOG function.
4. Column 4. Transform the ATP values in column 1 into log ATP values.
5. Column 5. Using the Excel TREND function, perform a Trend analysis for the log RLU values in Column 3.
6. Column 6. Transform the log values back into actual values using the Excel ANTI-LOG function.
7. Column 7. Perform a TREND function for the log ATP values.
8. Column 8. Transform the log tend ATP back into actual ATP values using the Excel ANTILOG function.
9. Column 9. Copy the sample RLU values.
10. Column 10. Transform the sample RLU values into LOG RLU values.
11. Column 11. Using the Excel TREND function, perform a trend analysis for the sample.
12. Column 12. Convert the calculated sample values back into ATP concentrations.

(iv) Using Third-Party Software

Instead of using Microsoft Excel, third party software can also be used. In this case, the raw data in the Excel file must be copied and pasted or copied into the clipboard and imported into the software program. It is important that the third-party software can either perform a log-log linear regression analysis on the raw data or can transform the data into log values. The following software has been tested to perform the necessary

manufacturer to determine whether the software can provide the information below and whether it can perform the necessary calculations so that the procedure can be automated.

a. The first measurement to be performed will be to detect the background (Bkg) luminescence in wells A1 – D1. Setup the software to produce the following results:
   1. Well numbers
   2. RLU/well
   3. Mean RLU
   4. RLU Standard Deviation (St. Dev)
   5. RLU Percent Coefficient of Variation (%CV)

b. The second set of measurements to be performed will be the ATP standard curve. Setup the software to give the following information:
   1. Group or sample designation
   2. ATP standard dose response values (these are the actual values of the ATP concentrations used for the dose response)
   3. Well numbers
   4. RLU/well
   5. Mean RLU (optional)
   6. Standard deviation of Mean RLU (optional)
   7. %CV of Mean RLU (optional)
   8. Predicted ATP concentration/well calculated by interpolating the RLU values from the ATP standard curve into ATP concentrations actually obtained. This should be performed automatically by the luminometer software. This is actually a back calculation of the ATP doses used to generate curve. The calculated ATP concentrations should correspond to the expected ATP values.
   9. Mean predicted ATP
   10. Standard deviation of mean predicted ATP
   11. %CV of mean predicted ATP.

The software should be capable of performing a log-log linear regression curve fit according to the equation:

$$\log Y = A + B * \log X$$

where A is the Y-intercept and B is the slope of the dose response curve. Do not use the equation Y = A + B*X as this will normally produce negative values for the lowest ATP dose. In addition, converting the X- and Y-axes to log is not equivalent to the curve fit shown above.

Figure 2 shows a typical ATP standard dose response using SoftMax Pro software that controls a Molecular Devices SpectraMax L luminometer. The curve fit is for a 5-point ATP dose response ranging from 0.03µM to 3µM. If the log-log linear regression curve fit is performed as stated above, then the curve fit parameters should fall within the following guidelines obtained for 93 individual ATP dose response curves:

- Goodness of fit (r^2) = 0.999 ± 0.001 (%CV = 0.071)
- Y-Intercept (A) = 6.71 ± 0.63 (%CV = 9.37)
- Slope (B) = 0.969 ± 0.18 (%CV = 1.9). This is slightly different to the value given in Section 11.

(Values are the Mean ± 1 Standard Deviation)

7. The HALO®-96 PCA^{EQ} PROTOCOL

PLEASE READ THE FOLLOWING PROTOCOL CAREFULLY.
SEE SECTION 8 BEFORE PERFORMING AN ASSAY

Performing HALO®-96 PCA^{EQ} is a 3 step process.

Step 1 – Cell preparation.
Step 2 – HALO®-96 PCA^{EQ} cell culture master mix preparation, plating and incubation in the 96-well plate.
Step 3 – Luminescence measurement. An ATP dose response is performed prior to sample luminescence measurements with conversion of RLUs to µM ATP.

Step 1 and Step 2 must be performed in a laminar flow biohazard hood

STEP 1 – Cell Preparation

HALO® PCA^{EQ} assay kits have been designed to be used with peripheral blood (normal or mobilized), bone marrow or umbilical cord blood. Depletion of erythrocytes is essential since they can interfere with the assay when present at high concentrations (hematocrits > 10%) and cause false positive results.

A. Umbilical cord blood

A mononuclear cell (MNC) suspension is required for best results. It is recommended to deplete the cord blood of red blood cells using a current Hetastarch® protocol, cell lysis or density gradient fractionation per the manufacture's protocol. The red blood cell content of the test sample should be 10% or less so that it does not interfere with the assay. If the red blood cell content is reduced as suggested, the final concentration of red blood cells in culture will be 0.1% or less. The small number of enucleated and nucleated erythrocytes present should not interfere with the assay. However, if depletion is not complete or it appears that erythrocytes are present in a high concentration, it is recommended that a density gradient centrifugation or cold lysis is performed. Please see below for details.

B. Human peripheral blood (normal or mobilized) or bone marrow cells

If normal peripheral blood or bone marrow aspirates are used, it is essential that the red blood cell concentration be reduced. Mobilized peripheral blood samples are usually produced during apheresis of the patient. They normally contain a very low level of red blood cells and are essentially a MNC fraction. However, if the red blood cell content is high, the sample must be further processed.

C. Thawing of Cells and Procedures to Reduce Red Blood Cell Content and Produce a Mononuclear Cell Suspension

(i) Thawing of Cells

If cells are cryopreserved as a total nucleated cell product, they will contain red blood cells, granulocytes and other cell populations that do not contribute to cell engraftment and result in a dilution of the stem cell content. When the cells are

thawed, granulocytes and other cell components will rupture and release DNA. Large amounts of released DNA will clump together encasing valuable stem cells. If the cell preparation originally cryopreserved was a MNC or similar fraction, the chances of clumping will be low. However to reduce or alleviate the possibility of clumping during cell thawing, it is recommended that DNase be added to the cell suspension. The following procedure is for small aliquots of cells only (1-1.5ml).

1. Thawing of the vial contents is initiated in a 37°C water bath, by swirling the vial for approx. 1 min.
2. When a small ball of ice still remains in the vial (1-2 min), remove the vial from the water bath, sterilized the outside of the vial by spraying with 70% ethanol and carefully unscrew the vial lid.
3. It is possible that clumping can occur at this stage, in which case, add approx. 2% DNase to the total volume in the vial before proceeding to the next step.
4. Using a 1ml pipette, gently mix the contents of the vial and transfer to a 50ml tube containing 20ml of thaw medium. Up to 3 vials of the same cells can be added to this 20ml of thaw medium. However, clumping can also occur at this stage. In this case, DNase at a final concentration of 2% should be added before proceeding to the next step.
5. Gently mix the cells by swirling the contents of the tube. Do not use repeat pipetting to mix the cells. This could cause further rupture of cells and the release of DNA resulting in increased clumping.
6. Centrifuge the cells at 300 x g for 10 min at room temperature and discard the supernatant after centrifugation.
7. Resuspend the cells in 1ml of culture medium containing 2% DNase.

(ii) Reduction or Removal of Red Blood Cells

The red blood cell content must be reduced to 10% or less for assay cell suspensions used in HALO®-96 PCA^{EQ}. This is because red blood cells contain high levels of ATP that will cause false positive results. It is therefore necessary to deplete the sample of red blood cells. If the sample to be assayed is fresh, the red blood cells can be subjected to a cold lysis procedure. Lysis should not be used to remove red blood cells from a cryopreserved umbilical cord blood sample. Density gradient centrifugation is recommended for cryopreserved umbilical cord blood samples.

Red Blood Cell Cold Lysis

To perform a cold lysis, ACK Lysis Buffer (Cat. No. K-Lysis-100, HemoGenix®) is recommended. The procedure is performed as follows:

1. Refrigerate the ACK Lysis Buffer.
2. Prepare an ice water bath in a 200ml beaker.
3. Transfer the sample to a centrifuge tube.
4. Add 10 parts of the cold ACK Lysis Buffer to 1 part cell supension. For 1ml of cell suspension, add 10ml ACK Lysis Buffer.
5. Place the tube in the ice water bath and set a lab timer for 5 min.
6. At 2.5 min, invert the tube once and replace in the ice water bath.
7. At 5 min, remove the tube. There should be a cherry red clear solution. If lysis has not taken place, a cloudy cell suspension will still be observed. Replace the tube in the ice water bath for another 2.5 min. Continue this for a maximum of 10 min.
8. Centrifuge the tube at 400 x g for 5 min and discard the supernatant after centrifugation.
9. Resuspend the cells in IMDM to the original cell volume.

10. LUMINOMETER SETUP AND CONVERSION OF RLU VALUES TO ATP VALUES USING THE ATP STANDARD DOSE RESPONSE CURVE

The readout from all plate luminometers is Relative Luminescence Units (RLU). The term "relative" is used because luminometers from different manufacturers produce different RLU ranges. The RLU range may be from 0-100 for one instrument and 1-1,000,000 for another. A RLU value is a non-standardized unit of measurement. It is therefore difficult to compare results from one experiment to another. The term "bioluminomics™" includes not only the procedure for measuring cell proliferation or inhibition by virtue of the iATP concentration, but also the procedures for standardizing the assays. This is done by interpolating the RLU values into ATP concentrations using the ATP standard dose response curve and a log-log linear regression least squares analysis.

(i) Luminometer Setup

Multiparameter instruments, i.e. those that can detect absorbance, fluorescence and luminescence, often need to be manually set for both the integration time and the "gain". Dedicated instruments, i.e. those that only detect luminescence, usually only have to be set for the "integration time". It is therefore necessary to first know whether the instrument is a multiparameter or multipurpose instrument and whether "integration time" and "gain" need to be set. The instrument instruction manual will provide this information. If the "gain" has to be set, the instruction manual will explain how the correct "gain" is established. Once the "integration time" and "gain" are set, they should not be changed.

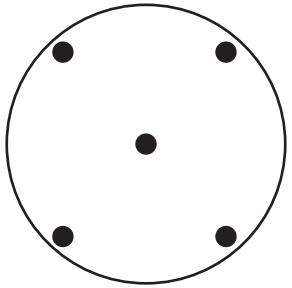
a. First set the integration time to 2 seconds.
b. Next, set the "gain". This must be determined empirically and is best performed when the ATP standard curve is measured. The gain should be adjusted so that the percent coefficients of variation (%CV) for the mean of the replicates are the lowest value. These values should be about 5% or less.
c. The measurement temperature of the instrument should be set to between 22°C and 24°C or turned off.
d. Most luminometers are equipped with a plate shaking protocol. It is not necessary to use the plate shaker mode.
e. Do not use injectors if the instrument is so equipped.

(ii) Instrument Setup for Luminometers with Software Analysis Capabilities

The luminometer is usually controlled by software installed on a computer using a serial or USB interface cable. The software for some luminometers comes with extensive analysis capabilities. This allows all the calculations to be programed and performed by the luminometer software. If the software does not include analysis capabilities, the results are usually exported directly to a Microsoft Excel file for calculation and analysis.

Before using any luminometer, ensure that you are familiar with the software that controls the instrument. For luminometer software that has analysis capabilities, setting up the software properly prior to any measurements can save considerable time and produce an optimized report. It may be necessary to contact the instrument

Figure 1. Positions of pipette tip for mixing the well contents



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10. Perform a cell and differential count and viability assay.
11. The white blood cell recovery should be at least 95% and the viability must be greater than 85%.

Density Gradient Centrifugation

Red blood cells and other contaminating cells can be removed by density gradient centrifugation. It should be emphasized that when the red blood cell content is high in some post-thaw umbilical cord blood samples, even density gradient centrifugation may not provide optimal depletion. In most cases, however, density gradient centrifugation will produce a MNC fraction that will be far superior to any total nucleated cell (TNC) fraction for analyzing stem cell quality.

HemoGenix® does not recommend using a Ficoll density gradient centrifugation procedure. This is because all Ficoll-derived reagents are toxic to cells. It is recommended to use NycoPrep 1.077, which is non-toxic and can also be added to cultures without any deleterious effects. It is essential that the density gradient centrifugation reagent is brought to room temeperature prior to use. The procedure for density gradient centrifugation using NycoPrep is as follows:

1. Dilute the sample to be fractionated with an equal volume of Dulbecco's phosphate buffered saline (dPBS) and mix gently by inversion.
2. For samples 3ml or less use a 15ml conical plastic tube for separation. For samples greater than 3ml, use a 50ml conical, plastic tube for separation.
3. For samples of 3ml diluted to 6ml with dPBS, dispense 5ml of the density gradient reagent into the tube. For samples 3ml or greater, dispense 15ml of the density gradient reagent into a 50ml tube.
4. Using a sterile, serological pipette, dispense the diluted sample gently on top of the density gradient reagent by holding the tube at approx. 45° and using a Pipette Aid on slow delivery. The cleaner the interface between the density gradient reagent and cell suspension, the better the separation will be. Do not allow the cells to mix with the reagent.
5. Centrifuge for 10 min at 1,000 x g or 20 min at 600 x g at room temperature with NO brake.
6. After centrifugation, remove the tube(s) gently and carefully aspirate the top layer above the MNC interface leaving approx. ¼" above the interface. Discard the supernatant.
7. Harvest the MNCs from the interface and transfer the cells to another sterile tube. It is best to harvest the cells using a manual 1 ml pipette. Do not remove cells below the interface.
8. Add approx. 10-20 ml dPBS, mix gently and centrifuge the cells for 10 min at 300 x g at room temeperature.
9. Aspirate the supernatant after centrifugation taking care not to apirate the cell pellet.
10. Add 1-2ml of IMDM and resuspend the cells, breaking up any clumps using a 1ml manual pipette.
11. Perform a nucleated cell count and viability. The cell viability must be greater than 85%. Using cells with a viability lower than 85% will produce results with low proliferation ability.

Fractionating the MNCs by density gradient centrifugation will usually dramatically improve viability.

Density Gradient Centrifugation Troubleshooting

- Specimens that are not fresh or greater than 24 hours old, may not separate cleanly. These specimens may need to have the procedure performed twice.
- Fibrin strands present after separation can be attached to the mononuclear cell layer. Care should be taken in this situation.
- Aspirate the supernatant using a serological pipette and save to a sterile tube. Do not discard the supernatant until a clean transfer has been made. Try to avoid the fibrin strands.
- If fibrin strands get caught up with the cells, transfer entire supernatant to a sterile tube and centrifuge for 10 minutes @ 300 x g. The resulting cells will have some residual platelets; however, most of the platelets will be removed with the supernatant after centrifugation.
- Bone marrow often has fat globules and bone spicules; these can get caught in the cell interface. The fat will rise to the top during the second spin and the spicules can be "picked out" using a manual 1000ul pipette.
- If the cell separation is not clean and distinct, transfer the supernatant to a sterile tube using a serological pipette and reserve until the cell count is performed. If the cell count is low, centrifuge the supernatant to recover additional cells.
- Specimens that have a high red cell concentrations can be separated again or treated with ACK lysis buffer.
- Removing excess density gradient reagent below the interface can result in granulocyte and platelet contamination of the the MNCs.
- In some cases, a tradeoff of higher cell recovery with increased platelet contamination may be necessary. In these cases, cells can be centrifuged for 10 minutes at 400 x g instead of 300 x g, with no detrimental effect on the cells.

E. Cell Viability, Cell Counting and Cell Culture Suspension Preparation

1. For dye exclusion viability methods, use trypan blue and a hemocytometer or automated method, flow cytometry using 7-AAD or another vital stain.
   Note *that dye exclusion viability methods detect membrane integrity. They do not detect cellular and mitochondrial integrity and therefore metabolic viability.*
   A viability of 85% or greater should be obtained when using dye exclusion viability methods only. It is recommended not to use cell suspensions with a viability of less than 85% since these cells will not be able to sustain proliferation ability. Use ProliferSTEM™ (HemoGenix®, Inc) as a metabolic viability assay.
2. Determine the cell concentration using either a hemocytometer or electronic cell/particle counter.
3. Adjust the cell suspension concentration to that recommended in Table 1.
   Note *that the working cell concentration per ml is 100 x the final cell concentration per well.* If cells have been treated prior to cell culture, higher cell concentrations may be required.
4. Prepare the total volume of cell suspension required using IMDM or PBS. The volume of the adjusted cell suspension required will be 10% of the total volume of HALO® Culture Master Mix prepared.

dose responses.

- The amount of ATP-ER added to each well is 0.10ml. Therefore:
  Total amount of ATP-ER (µl) required = 100 x (number of wells used + 24 (background and ATP dose response wells)).

ATP Standard Curve

Depending on the size of the kit purchased, non-sterile, 96-well plates have been included to perform an ATP standard curve prior to processing the sample cultures. Performing an ATP standard curve and controls on each day is an essential part of the assay because it has 4 functions:

- It tests whether the instrument is working properly and calibrates it.
- It ensures that the reagents are working correctly.
- It calibrates and standardizes the assay and allows the assay system to be validated, if required.
- It allows the output of the plate luminometer, in relative luminescence units (RLU), to be converted to ATP concentrations, thereby standardizing the procedure so that intra- and inter-laboratory experiments can be compared.

Adhesive Plate Covering Film

To help keep the plate(s) sterile, adhesive, air permeable, sterile films are provided so that the part of the plate that is not being used can be covered and kept sterile until required. If using the adhesive film provided, the plate cover should be removed in a laminar air-flow hood and replaced with the film to ensure sterility.

Mixing the Contents of 96-well Plate

Besides mixing the contents of the tubes after cells have been added prior to plating the HALO® Culture Master Mix, mixing the contents of the wells after adding ATP-ER is probably the most important procedure. It is recommended that the addition of ATP-ER is performed using a multi-channel pipette to achieve consistency and reduce variability. Addition of the reagent and mixing should be performed in the following manner:

1. Take up the required amount of reagent and add it to the well without inserting the tip into the well contents.
2. Starting from the center of the well, aspirate and dispense the contents twice without removing the pipette tip from the contents of the well.
3. Move the pipette tip to one corner of the well and aspirate and dispense the contents twice without removing the tip from the contents of the well.
4. Repeat this operation as shown in Figure 1 for each corner of the well.
5. Try not to cause excessive bubbles in the culture and DO NOT over mix since this can result in drastically reduced luminescence values.
6. This procedure effectively and optimally mixes the contents well.

9. RECOMMENDATIONS AND TIPS PRIOR TO MEASURING BIOLUMINESCENCE

Always wear laboratory (e.g. latex) gloves during this operation to avoid ATP contamination from skin.

DO NOT wipe the pipette tip with tissue etc as this will wick the reagent from the tip and cause an erroneous ATP standard curve and false sample results.

Always change pipette tips after use.

Each day bioluminescence is measured, a standard curve MUST be performed. The ATP-ER decays with time. A new ATP standard curve must be performed to ensure accurate conversion of the RLU values to ATP concentrations so that results can be compared.

HALO®-96 PCA^{EQ} includes solid white plates for both cell culture and the ATP standard curve and controls. Do not use different plates for the assay. Doing so will result in inaccurate results and invalidation of the assay kit warranty. Extra plates can be purchased from HemoGenix®.

Bioluminescence Assay Kit Components

- Prior to measuring bioluminescence, remove the ATP standard, 1 set of ATP controls and the ATP Enumeration Reagent (ATP-ER) from the freezer and thaw at room temperature or at 22 - 23˚C.
- Enough ATP standard and monitoring reagent is supplied to perform 4 standard curves and controls for each sterile plate provided.
- If thawing more than one bottle of ATP-ER for analysis, mix the contents of the bottles together before dispensing into reagent reservoir.
- ATP-ER can be refrozen up to 11 cycles without significant loss of sensitivity. Thawed ATP-ER can be kept at 2-8˚C, in the dark, for 48h or is stable at -20ºC for 20 weeks.

Reconstitution of Lyophilized Monitoring Reagent (if included)

- Thaw the ATP Enumeration Reagent Buffer at room temperature, in cold running water, or at 2-8ºC overnight.
- Do not use any form of heat to thaw this reagent.
- Allow the lyophilized ATP-ER substrate (brown glass bottle) to come to room temperature.
- Remove the closures from both bottles.
- Carefully pour the entire contents of the buffer bottle into the lyophilized ATP-ER substrate bottle. Swirl gently or invert slowly to mix. Do not shake.
- Allow the ATP-ER mix to reconstitute for 10 minutes at room temperature.
- Reconstituted ATP-ER is stable for 8 hours at room temperature, 48 hours at 2-8ºC, or 20 weeks at -20ºC.
- ATP-ER can be refrozen up to 11 cycles without significant loss of sensitivity.

Volumes of Luminescence Kit Components Required

- Each vial of ATP standard contains enough volume to perform four (4) standard

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TABLE 1
HALO®-96 PCA^{EQ} Recommended Human Tissues, Cell States and Cell Concentrations

Cell Type	Cell Preparation	Cell State	Working Cell Concentration Required (100 x Final Cells/Well)	Final Cell Dose / Well
Bone marrow	MNC	Fresh/Frozen	0.5-0.75 x 10^6	5,000-7,500
Peripheral blood	MNC	Fresh/Frozen	0.5-0.75 x 10^6	5,000-7,500
Mobilized peripheral blood	MNC	Fresh/Frozen	0.5-0.75 x 10^6	5,000-7,500
Umbilical cord blood	MNC	Fresh/Frozen	0.5-0.75 x 10^6	5,000-7,500

STEP 2. HALO®-96 PCA^{EQ} Cell Culture

Please refer to Section 8 for recommendations and tips prior to beginning this stage of the procedure.

Perform all cell culture under sterile conditions in a biosafety cabinet.

The HALO®-96 PCA^{EQ} kit contains a pre-mixed master mix. Only the cells need to be added. This allows the assay to be performed rapidly and with a minimum of manipulation.

1. Transfer the frozen pre-mixed bottle of Master Mix to a 37ºC incubator or water bath or allow the bottles to thaw at room temperature.
2. When thawed, mix the contents of the bottle thoroughly using inversion or swirling.
3. Prepare the cell suspension as required.
4. Table 3 shows the recommended cell concentrations required for different tissues. Calculate the adjusted cell concentration for each sample accordingly. NOTE: the concentration of the cell suspension in cells/ml is 100 x the concentration for cells/well. For example: 5,000 cells/well requires a working cell suspension concentration of 500,000 cells/ml.
5. Prepare and label one 5ml tube for each sample.
6. Using calibrated and preferably electronic pipettes, accurately dispense 0.72ml of the HALO®-96 PCA^{EQ} Master Mix into each sterile 5ml tube.
7. Using calibrated and preferably electronic pipettes, dispense 0.08ml of the adjusted cell suspension into each tube. Use the same volume and cell concentration for each stem cell population to be measured.
8. Mix the contents of each tube thoroughly using a vortex mixer. This is now the HALO® Culture Master Mix and the total volume will be 0.8ml.
9. Remove the sterile, 96-well plate from plastic covering under the hood.
10. Using calibrated and preferably electronic pipettes, dispense 0.1ml of the Culture Master Mix into each of 6 replicate wells according to the configuration given in Section 9.
11. Place the lid on the 96-well plate and transfer the culture plate to a humidity

chamber to ensure high humidity during incubation (See section 7(vii)).

12. Place the humidity chamber into a fully humidified incubator set at 37˚C and gassed with 5% CO_2 and, if available, 5% O_2.
13. The cells can be incubated for 5, 6 or 7 days depending on the users schedule. IMPORTANT. Once an incubation period has been elected, it should be used continually and should not be changed. If the incubation period is changed, it will not be possible to compare results between different samples setup on different days. It is therefore important to maintain the elected incubation period.

STEP 3 – LUMINESCENCE MEASUREMENT

FOR ALL OF THE FOLLOWING STEPS, WEAR LABORATORY GLOVES
ATP is present on the skin and can cause erroneous results

IMPORTANT: PLEASE REFER TO SECTION 10 ON HOW TO SETUP THE PLATE LUMINOMETER. The instrument should be setup and prepared for use prior to any of the following steps being performed.

IMPORTANT. Please refer to Section 9 for recommendations and tips prior to starting this part of the procedure. In particular, please refer to Section 9 for important information on mixing components.

PLEASE NOTE: ATP standard curves performed on previous days or for previous experiments or studies should not be used since the ATP-ER intensity changes with time and lot number.

Remove the ATP standard, controls and reagents from the freezer and thaw to room temperature prior to analysis.

Use the unwrapped, non-sterile, 96-well plate provided with the kit to perform the ATP standard dose response curve.

A. ATP Standard Dose Response Preparation for Use with Mobilized Peripheral Blood or Purified Stem Cell Populations.
Mobilized peripheral blood samples usually exhibit a high iATP concentration. For this reason, it is necessary to modify the ATP standard curve range so that sample ATP values fall within this range. If mobilized peripheral blood samples or purified stem cell populations are found outside of this range from 0.03µM to 3µM, the the ATP standard curve will have to be repeated using the dose range from 0.01µM to 1µM (see below). If samples are found to exhibit higher iATP concentrations than 3µM, the sample will have to be diluted so that the iATP falls within the ATP standard curve dose range. The dilution would then be taken into account when calculating the ATP value of the sample.

1. Label 5 vials (e.g. 1.5ml volume) for the ATP dose response consisting of the following ATP concentrations:
   Label the ATP Standard supplied as Vial #1 (3µM)
   Vial #2: 1µM
   Vial #3: 0.3µM

of another manufacturer. Cell growth and bioluminescence output can be seriously affected and the assay kit warranty will be void. Additional plates can be purchased from HemoGenix® if required.

(vii) Humidity Chamber
A humidity chamber is recommended due to the small sample volume. Even fully humidified incubators do not keep the humidity level high enough to keep the sample from evaporating. This usually results in so-called "edge effects". A humidity chamber can be assembled using plastic lunch boxes or other plasticware available from a supermaket or discount stores. Holes must be made in the lid to enable adequate gas exchange. Disposable serological pipettes are cut to an appropriate length to fill the bottom of the container. Distilled/deionized water is poured into the container to just below the level of the pipettes. This allows for adequate water to keep the humidity high without the plates sitting in water. Please contact HemoGenix® for further information about assembling and using humidity chambers.

(viii) Incubation Times
Cultures can be incubated for 5, 6 or 7 days. The iATP values will increase from 5 to 7 days since the cells are in the exponential growth phase of proliferation. With increase in incubation time, there will also be an increase in coefficients of variation. The validation parameters provided in Section 11 are for 5 days of incubation. In addition, to enable comparison of results, it is essential that once an incubation time period has been elected (5, 6 or 7 days), the same culture period should be maintained thereafter.

8. RECOMMENDATIONS AND TIPS PRIOR TO USING THE HALO® KIT CELL CULTURE MIXES.

(i) Cell Suspension

a. The preferred cell suspension is a mononuclear cell suspension (MNC) of bone marrow, peripheral blood or umbilical cord blood.
b. Extraneous ATP, red blood cells (which have high concentrations of ATP) and hemoglobin interfere with the ATP analysis. The cell suspension must have a hematocrit of 10% or less.
c. If cells have been treated prior to cell culture, higher cell concentrations than those shown in Table 1 may be required.

(ii) Thawing of HALO® Master Mix

a. Prior to using the HALO® Master Mix, remove the bottle from the kit box and thaw either at 37ºC or at room temperature.
b. The HALO® Master Mix may be aliquotted into 5ml tubes and frozen until the expiration date.
c. HALO® Master Mix is stable at 2-8°C for 1 month after thawing.

(iii) Dispensing the Culture Master Mix

Once the cell suspension has been added to produce the Culture Master Mix, we strongly recommend using electronic pipettes to dispense the Culture Master Mix into individual wells. Mix frequently during dispensing.

(iv) Number of Replicates Performed

HALO®-96 PCA^{EQ} has been designed for samples to be tested using 6 replicates/sample. Please remember that using fewer replicates may save components in the short term, but may also cause inconclusive results. If outliers are encountered, which may have to be removed from the analysis, the consequence could be that extra experiments would be required resulting in extra time and costs. For quality control purposes, extra samples may not be available to perform an additional experiment. For these reasons, performing 6 replicates/sample should be maintained.

(v) Plate Configuration

Performing 6 replicates/well means that the samples are plated across the plate, for example from A1 to A6, A7 to A12 or B1 to B6. In this manner 16 samples can be tested on a single plate If samples are tested across the plate from, for example A1 to A6 and then from A7 to A12, use a 12-channel pipette to dispense and mix the ATP-ER. If it is preferred to dispense the samples from A1 to A6 followed by B1 to B6 etc., then it is suggested to use an 8-channel pipette. If fewer than 8 samples have been plated, only attached sufficient pipette tips for the number of samples to be processed. Thus, if samples correspond to wells A1 to A6, B1 to B6 and C1 to C6, then attached only 3 pipette tips to the 8-channel pipette.

(vi) 96-Well Plates Provided

The reagents have been optimized to work with the 96-well plate(s) provided in the HALO® kit. Please do not replace the plates included with the kit with those

Vial #4: 0.1µM
Vial #5: 0.03µM

2. Now dispense:
   0.40ml IMDM medium to vial #2
   0.90ml IMDM medium to vial #3
   0.90ml IMDM medium to vial #4
   0.90ml IMDM medium to vial #5
3. Dispense 0.7ml of the IMDM medium into Vial #1.
4. Dispense 0.20ml from vial #1 to vial #2. Mix. This concentration is 1µM.
5. Dispense 0.10ml from vial #2 to vial #4. Mix. This concentration is 0.1µM.
6. Dispense 0.10ml from vial #1 to vial #3. Mix. This concentration is 0.3µM.
7. Dispense 0.10ml from vial #3 to vial #5. Mix. This concentration is 0.03µM.
8. Dispense 0.10ml of the supplied medium alone into wells A1, B1, C1 and D1 on the non-sterile luminescence plate(s) provided. NOTE: This is to measure background luminescence. *PLEASE NOTE that it is important to use the same medium to measure background luminescence as used for the ATP standard curve and cell cultures. Using different media will cause discrepancies in the results.*
9. Dispense 0.10ml from ATP dilution in vial #5 to wells E1, F1, G1, and H1.
10. Dispense 0.10ml from ATP dilution in vial #4 to wells A2, B2, C2, and D2.
11. Dispense 0.10ml from ATP dilution in vial #3 to wells E2, F2, G2, and H2.
12. Dispense 0.10ml from ATP dilution in vial #2 to wells A3, B3, C3, and D3.
13. Dispense 0.10ml from ATP dilution in vial #1 to wells E3, F3, G3 and H3.
14. Mix the vials containing the low and high controls provided.
15. Dispense 0.1ml from the ATP low control to wells A4, B4, C4, D4.
16. Dispense 0.1ml from the ATP high control into wells E4, F4, G4, H4.
17. Add the required amount of ATP-ER to a non-sterile reagent reservoir.
18. Using a multichannel pipette, add 0.10ml to each well of the first column (A1-H1).
19. Mix the contents as directed in Section 9 by repeated pipetting and discard the tips.
20. Change tips for each new addition of ATP-ER.

B. ATP Standard Dose Response Preparation for Umbilical Cord Blood, Bone Marrow or Normal Peripheral Blood

1. Prepare and label 5 vials (e.g. 1.5ml volume) for the ATP dose response consisting of the following ATP concentrations:
   Vial #1: 1µM
   Vial #2: 0.5µM
   Vial #3: 0.1µM
   Vial #4: 0.05µM
   Vial #5: 0.01µM
2. Now dispense:
   0.90ml of the medium to vial #1.
   0.35ml of the medium to vial #2
   0.90ml of the medium to vial #3
   0.90ml of the medium to vial #4
   0.90ml of the medium to vial #5
3. Remove 0.1ml of the supplied stock ATP solution (at 10µM) and transfer it to vial #1. Mix by vortexing. This ATP concentration is 1µM.

4. Dispense 0.35ml from vial #1 to vial #2. Mix. This concentration is 0.5µM.
5. Dispense 0.10ml from vial #2 to vial #4. Mix. This concentration is 0.05µM.
6. Dispense 0.10ml from vial #1 to vial #3. Mix. This concentration is 0.1µM.
7. Dispense 0.10ml from vial #3 to vial #5. Mix. This concentration is 0.01µM.
8. Dispense 0.10ml of the supplied medium alone into wells A1, B1, C1 and D1 on the non-sterile luminescence plate(s) provided. NOTE: This is to measure background luminescence.
9. Dispense 0.10ml from ATP dilution in vial #5 to wells E1, F1, G1, and H1.
10. Dispense 0.10ml from ATP dilution in vial #4 to wells A2, B2, C2, and D2.
11. Dispense 0.10ml from ATP dilution in vial #3 to wells E2, F2, G2, and H2.
12. Dispense 0.10ml from ATP dilution in vial #2 to wells A3, B3, C3, and D3.
13. Dispense 0.10ml from ATP dilution in vial #1 to wells E3, F3, G3 and H3.
14. Add the required amount of ATP-ER to a non-sterile reagent reservoir.
15. Using a multichannel pipette, add 0.10ml to each well of the first column (A1-H1).
16. Mix the contents as directed in Section 9 by repeated pipetting and discard the tips.
17. Change tips for each new addition of ATP-ER.

IMPORTANT: Mixing the contents of each well properly is a very important procedure. PLEASE REFER TO SECTION 9 ON HOW TO MIX THE WELL CONTENTS CORRECTLY. THIS PROCEDURE IS ALWAYS USED WHEN MIXING REAGENTS FOR BIOLUMINESCENCE MEASUREMENT IS REQUIRED.

18. Add 0.10ml of ATP-ER to each well of the other columns, mixing the contents as described in Section 9.
19. Repeat the procedure for each new column.
20. Place the ATP plate in the luminometer and incubate 2 min before initiating measurement.

NOTE: The non-sterile 96-well plate(s) is/are used for all ATP standard curves and controls. Continue using empty wells on the plate for additional ATP standard curves.

C. Sample Measurement

The addition of ATP-ER is performed in the same manner as that for the ATP Standard Curve.

1. If possible, place the sample plate(s) in a humidified incubator set at 22-23˚C gassed with 5% CO_2 for 30min to equilibrate or allow to come to room temperature.
2. If only part of the plate has been used, transfer the plate to a bio-safety hood and remove the lid under sterile conditions. Take a sterile adhesive plate coverfoil from the kit box and layer it over the top of the plate. Using a sharp knife or scalpel, cut away the foil that covers the wells to be processed. The unused, empty wells will now remain sterile for the next experiment. (See Section 9, Adhesive Plate Covering Film).
3. Using a multichannel pipette (8- or 12-channels depending on the plate configuration), add 0.10ml of ATP-ER to each well of the first column (A1-H1) or row (A1-12). Mix the contents as described in Section 9.

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4. Repeat this procedure for each column or row using new tips.
5. When ATP-ER has been added to all wells, replace the plastic cover and incubate for 10 min at room temperature in the dark to lyse the cells and stabilize the luminescence signal. Incubate the plate in the reader for the last 2 min to stabilize the plate.
6. Unused ATP-ER may be returned to the bottle and refrozen. See section 9 for ATP reagent storage conditions and stability.

D. Using a plate luminometer with automatic dispenser

The user may have a plate luminometer that allows reagents to be dispensed automatically directly into the well. HemoGenix® does not recommend using the automatic dispensers, since the contents of the well are not mixed sufficiently using this method.

E. Using a liquid handler

HALO®-96 PCA^{EQ} can be performed in high throughput mode. If you intend to perform any part of the HALO® procedure using a liquid handler, please contact HemoGenix® for information on setting up the instrument. Extra ATP-ER is required when using a liquid handler.