Ordering Information
Tel: (719) 264-6250
Fax: (719) 264-6253
Email: info@hemogenix.com
Order online at www.hemogenix.com

Technical Support
Tel: (719) 264-6250
Email: info@hemogenix.com

HemoGenix®, Inc
1485 Garden of the Gods Road
Suite 152
Colorado Springs, CO 80907
U.S.A.
Tel: (719) 264-6250
Fax: (719) 264-6253
Web: www.hemogenix.com

FOR *IN VITRO* RESEARCH USE ONLY (RUO)
NOT FOR CLINICAL DIAGNOSTIC USE

HALO®-Tox HT

for *In Vitro* Predictive Hemotoxicity Testing.

96- and 384-Well Plate, 4, 5 and 7 Population

"Global" Hemotoxicity Platform

Instruction Manual

(Version 3-2013)

For *In Vitro* Research Use Only. Not for clinical diagnostic use.

HemoGenix®, Inc

TABLE OF CONTENTS

HemoGenix®
Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

12. REFERENCES

1. **Bradley TR, Metcalf D**: The growth of mouse bone marrow cells in vitro. Aust J Exp Biol Med (1966), 44:287.
2. **Pluznik DH, Sachs L**: The induction of clones of normal mast cells by a substance from conditioned medium. Exp Cell Res (1966), 43:553.
3. **Stephenson JR, Axelrad AA, McLeod DL, Shreeve MM**: Induction of colonies of hemoglobin-synthesizing cells by erythropoietin in vitro. Proc Natl Acad Sci USA (1971), 44:517.
4. **Axelrad AA, McLeod DL, Shreeve MM, Heath DS**: Properties of cells that produce erythrocytic colonies in vitro. In Hemopoiesis in Culture. Edited by Robinson W. Washington D.C.: U.S. Gorvernment Printing Office; (1973) pp. 226.
5. **Iscove NN, Sieber F, Winterhalter KH**: Erythroid colony formation in cultures of mouse and human bone marrow; analysis of the requirement for erythropoietin by gel filtration and affinity chromatography on agarose-concanavalin A. J Cell Physiol (1974), 83:309-320.
6. **Iscove NN, Sieber F**: Erythroid progenitors in mouse bone marrow detected by macroscopic colony formation in culture . Exp Hemat (1975), 3:32-43.
7. **Rich IN & Kubanek B**: The effect of reduced oxygen tension on colony formation of erythropoietic cells in vitro. Brit J Haematol (1982), 52:579-588.
8. **Rich IN**: A role for the macrophage in normal hemopoiesis: II. Effect of varying oxygen tensions on the release of hemopoietic growth factors from bone marrow-derived macrophages in vitro. Exptl. Hemat. (1986), 8:746-751.
9. **Crouch SPM, Kozlowski R, Slater KJ, Fletcher J**: The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicty. J Immunol Meth (2000), 160:81-88.
10. **Musk P, Rich IN**: A new high throughput stem cell and multilineage progenitor cell assay for hemotoxicity testing. The Toxicologist (2001), 66.
11. **Rich IN, Musk P**: HALO - A multifunctional colony-forming-based assay platform for drug development and basic and clinical research. Blood (2002), 100:618a
12. **Rich IN**: In vitro hemotoxicity testing in drug development. A review of past, present and future applications. Current Opinion in Drug Discovery and Development. (2003) 6:100-109.
13. **Rich IN and Hall KM**: Validation and development of a predictive paradigm for hemotoxicology using a multifunctional bioluminescence colony-forming proliferation assay. Tox Sci (2005) 87:427-441.
14. **Rich IN:** High-throughput *in vitro* hemotoxicity testing and *in vitro* cross-platform comparative toxicity. Expert Opin. Drug Metab. Toxicol. (2007) 3:295-307.
15. **Reems J-A, Hall KM, Gebru LH, Taber G, Rich IN.** Development of a novel assay to evaluate the functional potential of umbilical cord blood progenitors. Transfusion (2008) 48:620-628.
16. **Hall KM, Harper H, Rich IN**. Hematopoietic stem cell potency for cellular therapeutic transplantation. In: Hematopoietic Stem Cells, Ed.: RP Camacho. ISBN 978-953-307-746-8. (In preparation).

HemoGenix®
Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

1. INTRODUCTION

Hemo- or hematotoxicity is toxicity to the hematopoietic or blood-forming system. Traditional hemotoxicity testing is performed during pre-clinical animal studies when changes to circulating blood elements and pathological changes in blood-producing tissues and organs (bone marrow, spleen and (fetal) liver) can occur as a result of drug administration. The changes seen in the circulation and tissues actually reflect changes that have occurred in more primitive cell populations, especially the stem and progenitor cells that give rise to the mature functional circulating cells. The response of stem and progenitor cells can only be detected using *in vitro* assays. Any response by the stem cells to drugs or other agents are amplified throughout the system. Moreover, with more than 60 years of accrued knowledge of the hematopoietic system and how it reacts to different agents and perturbations, it is not surprising that, in contrast to traditional hemotoxicity testing, *in vitro* stem and progenitor cell testing is highly predictive.

In 2002, HemoGenix® launched the first instrument-based, high throughput, *in vitro* hemotoxicity assay developed specifically for the biopharmaceutical industry. This assay was called HALO®. Since that time, HALO® has developed into a highly refined *in vitro* toxicity platform that can be performed in either 96- and 384-well plate formats. HALO® was the first of several *in vitro* toxicity assay platforms to incorporate bioluminomics™ technology. Bioluminomics™ is the instrument-based, non-subjective measurement of intracellular ATP (iATP) concentrations using a luciferin/luciferase bioluminescence signal detection system. It is the most sensitive and accurate, non-radioactive readout available. Bioluminomics™ incorporates the calibration, standardization and, if necessary, the validation of the assay.

In contrast to traditional *in vitro* assays for hematopoietic cells (the colony-forming cell or CFC assay), HALO® assays incorporate Suspension Expansion Culture™ (SEC) Technology. SEC™ technology is a methylcellulose-free culture system that is fast and easy to use, exhibits greater sensitivity and accuracy and requires significantly shorter culture times than any "classic" CFC assay.

HALO®-Tox HT for 4, 5 or 7 cell populations is a "global" predictive hemotoxicity platform (PHP) that detects and measures the response of one or two stem cell populations and three hematopoietic cell lineages (4 and 5 population assay) and two additional lymphopoietic cell lineages (7 population assay). In this way, the investigator can obtain a "global" view of potential toxicity either to the hematopoietic system alone (4 and 5 population assay) or the lympho-hematopoietic system (7 population assay).

2. INTENDED USE

HALO®-Tox HT is primarily intended for use by the biopharmaceutical industry, environmental institutions and academic toxicology and pharmacology departments. HALO®-Tox HT has been designed to detect, measure and predict toxicity to the stem and progenitor cells of blood-forming organs and tissues from the following species:

- Human
- Non-human primate
- Horse
- Pig
- Sheep
- Dog
- Rat
- Mouse

HALO®-Tox HT can be used with bone marrow, peripheral blood (normal or mobilized) and umbilical cord blood from human donors and large animals. For smaller animals (dog, rat and mouse), hematopoietic organs such as the spleen and liver may also be tested using the assay.

In addition, HALO®-Tox HT may also be used to detect the response of compounds on purified stem cell populations such as CD34 and CD133 cells.

The cell populations detected using HALO®-Tox HT 5- and 7 Population Assay Kits are shown in Table 1.

TABLE 1

Description	4 and 5 Population Kit	7 Population Kit
Primitive lympho-hematopoietic stem cell	HPP-SP (not included in 4 population kit	HPP-SP
Mature hematopoietic stem cell	CFC-GEMM	CFC-GEMM
Erythropoietic progenitor	BFU-E	BFU-E
Granulocyte-macrophage progenitor	GM-CFC	GM-CFC
Megakaryopoietic progenitor	Mk-CFC	Mk-CFC
T-cell progenitor	Not included	T-CFC
B-cell progenitor	No included	B-CFC
Control (no growth factors)	Included	Included

IMPORTANT:
HALO®Tox HT is for research use only.
It has not been approved for clinical use.

- *Contamination*: Cells cultured in 96-well plates cannot be viewed 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 or 0.025ml), 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. Please refer to Section 8 (vii) for instructions on how to build 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. Since the volumes dispensed are small it is imperative to use instruments that have been properly calibrate to avoid pipetting error.
- 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.
- *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.
- *Low humidity*. Plates dry out (see below) and cell growth declines.

HemoGenix® Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

3. The CONCEPT and PRINCIPLE of BIOLUMINOMICS™ ASSAYS

HALO®-Tox HT 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 types and 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 Mixes 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:

$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{LIGHT}$$

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®-Tox HT. 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.
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®-Tox HT PROCEDURE

PLEASE NOTE: This manual is for both 96- and 384-well plates. Please read the manual carefully to ensure that the correct volumes and concentrations are used for the respective plate size.

There are 4 steps to use HALO®-Tox HT.

Step 1 – Cell Preparation
Cells are not provided with HALO®Tox HT assay kits. Mononuclear cell (MNC) preparations should be used as target cells. Cells are prepared either by using 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 assay and nucleated cell count should be performed on all samples.

Step 2 - Compound Dose Response and Controls
The compound dose response and appropriate controls are prepared.

Step 3 – Cell Culture
HALO®Tox HT for 4 or 5 populations contains 4 or 5 separate HALO® Master Mixes plus a control Master Mix and 6 solid, white-walled plates. HALO®Tox HT for 7 populations contains 7 separate HALO® Master Mixes plus a control and 8 solid, white-walled plates. Sufficient HALO® Master Mix for each cell population is provided for each plate. Tubes are prepared containing the HALO® Master Mix for each cell population. Mononuclear target cells are then added to prepare the HALO® Culture Master Mix. In addition, a tube for each of 4 separate controls is also prepared. These controls include the no growth factor control, vehicle-only control, growth factor-only control and growth factor+vehicle control. For 96-well plates, 11µl of each test compound dose is dispensed into replicate wells, followed by 0.1ml of the HALO® Culture Master Mix. For 384-well plates 25µl of the HALO® Culture Master Mix is dispensed into replicate wells followed by 2.8µl of the test compound dilution. For 384-well plates, all dispensing should be performed using a liquid handler, which allows automated and reliable dispensing of small well volumes . The plates are cultured for the prescribed period of time in a fully humidified incubator at 37°C with 5% CO_2 and preferably 5% O_2.

Step 4 – Bioluminescence measurement
To measure bioluminescence, the iATP is released from the cells. This is accomplished by the addition of ATP Enumeration Reagent (ATP-ER) as follows:

1. For 96-well plates, add 0.1ml of ATP-ER manually using a multichannel pipette or automatically using a liquid handler.
2. For 384-well plates dispense 0.25µl for to each well using a liquid handler.

The ATP-ER also contains luciferin and luciferase, the reagents used to produce bioluminescence. After a short incubation period the bioluminescence is measured in a plate luminometer. Prior to sample luminescence measurements, the assay is calibrated and standardized by performing an ATP standard curve with controls (provided with the kit).

HemoGenix® Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

11. HALO®Tox HT 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)
- Extra 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 unsustainable stem cell proliferation: ~0.04µM
- ATP value below which cells are not metabolically viable: ~0.01µM.

If these parameters are not attainable, 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 purity 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).

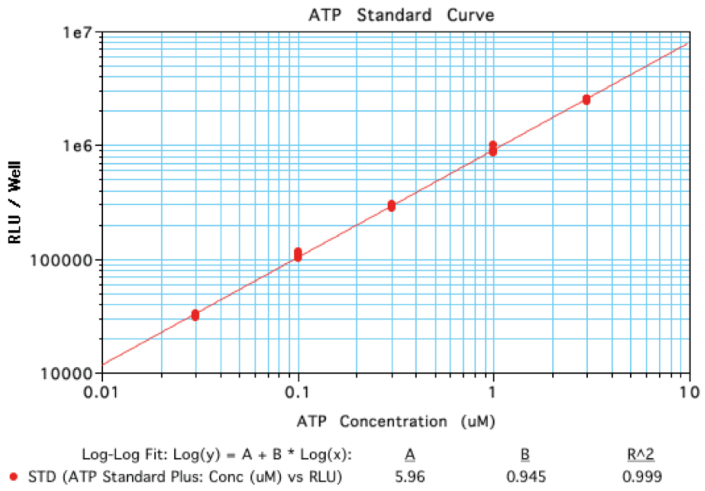
Assay validation parameters will vary depending upon the species, cell source and cell population detected.

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 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



HemoGenix®
Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

5. KIT CONTENTS

HALO®Tox HT kits contain the following components:

1. A HALO® Master Mix for each cell population to be detected.
2. A control HALO® Master Mix (no growth factors).
3. Medium (IMDM) to prepare the ATP standard curve.
4. ATP standard.
5. Controls; low, high and extra high.
6. ATP Enumeration Reagent (ATP-ER)*
7. Sterile, 96- or 384-well plates for cell culture.
8. Non-sterile 96- or 384-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 2-8ºC for 48h once thawed and is stable for 20 weeks when stored at -20ºC. 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. 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 reagents 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.; Promega GloMax®-96, Molecular Devices, SpectraMaxL; TECAN, GENios)
3. Sterile, capped, plastic tubes (5ml, 10ml, 50ml)
4. Single channel pipettes, preferably electronic (e.g. ViaFow 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. hemocytometer or flow cytometer for determining viability.
13. Liquid handler for dispening reagents into 384-well plates.
14. Adhesive Plate Covering: sterile foils to protect and keep unused wells sterile (optional).

Reagents

1. Sterile Phosphate Buffered Saline (PBS)
2. Iscove's Modified Dulbecco's Medium (IMDM)
3. DNase (Sigma-Aldrich, Catalog No. D4513-1VL)
4. Density-gradient medium (e.g. NycoPrep 1.077, Axis-Shield).
5. ACK Lysis buffer (Cat. No. K-Lysis-100, HemoGenix®, Inc)
6. 7-AAD, propidium iodide, trypan blue or other dye exclusion viability assay.
7. Vehicle to dissolve the test compound(s).

- 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)

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 calculated 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 trend ATP values 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

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 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 calculated 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 Lmax 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)

HemoGenix® Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

7. The HALO®Tox HT PROTOCOL

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

Performing HALO®Tox HT is a 4-step process.

Step 1 – Cell preparation.
Step 2 – Compound dose response preparation.
Step 3 – HALO®Tox HT cell culture master mix preparation, plating and cell culture.
Step 4 – Luminescence measurement. An ATP dose response is performed prior to sample luminescence measurements with conversion of RLUs to µM ATP.

Steps 1, 2 and 3 must be performed in a laminar flow biohazard hood

STEP 1 – Cell Preparation

HALO®-Tox HT can be used with cells derived from different tissues and organs. However, it is important that depending on the cell source, the red blood cell content is below 10%. This is because red blood cells contain high levels of ATP. Depletion of red blood cells is essential since they can interfere with the assay when present at high concentrations and cause false positive results.

With the exception of murine bone marrow, mobilized peripheral blood from human donors and purified stem cell populations, virtually all other hematopoietic organs and tissues from all species should be fractionated using a density gradient centrifugation to prepare a mononuclear cell (MNC) preparation. This procedure removes red blood cells and other contaminating cells (e.g. granulocytes, platelets) from the cell suspension and also improves cell viability.

A. Preparation of a Mononuclear Cell (MNC) Fraction using Density Gradient Centrifugation from Fresh, Primary Tissues or Solid Organs.
If using solid organs, e.g. spleen liver (adult or fetal) it is necessary to first prepare a single cell suspension by disaggregation. The investigator should use a pre-tested and pre-validated procedure to obtain the cells. After preparing the cell suspension, it is recommended to prepare a mononuclear cell suspension as described below.

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 temperature 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 greater than 3ml, 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 1ml 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 temperature.
9. Aspirate the supernatant after centrifugation taking care not to aspirate 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 1ml 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 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

HemoGenix® Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

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.

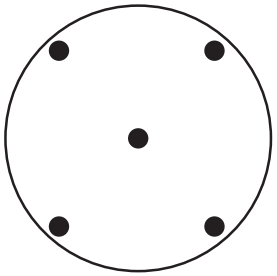
a. First set the integration time to 2 seconds.
b. Next, set the "gain". This must be determined empirically and is best per formed when the ATP standard curve is measured. The gain should be adjusted so that the percent coefficients of variation (%CV) for the mean of the replicates are the lowest value. 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 programmed 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

Figure 1. Positions of pipette tip for mixing the well contents in 96-well plates only. For 384-well plates, this multipoint mixing is not necessary.



HemoGenix®
Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

granulocyte and platelet contamination of the MNCs.

- In some cases, a trade-off 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.

B. Samples with High Red Blood Cell Concentrations

Tissues, such as normal peripheral blood and umbilical cord blood must be red blood cell depleted prior to use in the toxicity assay. For non-cryopreserved cell samples with high red blood cell concentrations, a cold red blood cell lysis should be performed. For cryopreserved samples containing high concentrations of red blood cells, a density gradient centrifugation procedure, rather than cell lysis, is recommended after thawing.

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 suspension. 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. 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%.

C. Mononuclear Cell Suspensions from Cryopreserved Samples

Instead of fresh samples, cryopreserved cells may also be used. This may be the case for bone marrow, mobilized peripheral blood and umbilical cord blood. If cryopreserved bone marrow is used, this will probably already be a MNC preparation and no further cell separation procedures will be necessary.

Similarly, if a human mobilized peripheral blood preparation is used, these cells will usually be produced using an apheresis procedure which separates the MNCs from the other cell components. These preparations contain low red blood cell concentrations and no further separation procedure will normally be necessary.

For normal peripheral blood and umbilical cord blood samples high concentrations of red blood cells are usually present even in cryopreserved samples. The red blood cells and other contaminating cells must be removed prior to using the cells in the toxicity assay. The density gradient centrifugation procedure described above is recommended.

Procedure for 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 and progenitor 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 used 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 DNase to the total volume in the vial to achieve a concentration of 6µg/ml 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 6µg/ml 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. If necessary, add 6µg/ml DNase.

D. Hematopoietic Subpopulations and Purified Cell Populations

Providing sufficient cells are available, subpopulations of stem and lineage-specific cells can be isolated and purified for use in the HALO®Tox HT assay. HemoGenix® recommends using magnetic cell isolation procedures (e.g. Miltenyi Biotech), since these allow standardized and rapid isolation of stem cell populations with substantial purity, viability and yield. Alternatively, purified cell populations can be obtained from vendors. Please see table of recommended cell concentrations to determine the optimal, final cell concentration to use in HALO®Tox HT.

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

1. Dye exclusion viability can be performed using, for example, trypan blue, and a hemocytometer or automated method. Flow cytometry using 7-AAD or another fluorescence vital stain can also be used.
   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

standard dose responses.

- The amount of ATP-ER added to each well is 0.10ml for 96-well plates and 0.025ml for 384-well plates. Therefore:
  Total amount of ATP-ER (µl) required = 0.1ml or 0.025ml x (number of wells used + 32 (background, ATP dose response wells and ATP controls)).

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 samples are processed 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 can be used. If using the adhesive film, 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- or 384-Well Plates

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®Tox HT 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 enumeration reagent is supplied to perform 1 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 Enumeration 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 one or two ATP

HemoGenix® Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

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 in a 96-well plate, and 400 x the final concentration per well in a 384-well plate.* If cells have been treated prior to cell culture, higher or lower cell concentrations may be required.
4. Prepare the total volume of working cell suspension required using IMDM or PBS. **NOTE** that the working cell concentration should be 100 x the final cell concentration/well for a 96-well plate and 400 x the final cell concentration/well for a 384-well plate. See Table 2 below.

TABLE 2
Recommended Cell Types and Concentrations for Use with HALO®Tox HT

Species	Cell Type	Cell Preparation	Cell State	Working Cell Concentration Required (96-wells/ 384-wells)	Final Cell Dose / Well
Human	Bone marrow	MNC	Fresh/ Frozen	0.5-0.75 x 10^6/ 2.0-3.0 x 10^6	5,000-7,500
	Peripheral blood	MNC	Fresh/ Frozen	0.5-0.75 x 10^6/ 2.0-3.0 x 10^6	5,000-7,500
	Mobilized peripheral blood	MNC	Fresh/ Frozen	0.5-0.75 x 10^6/ 2.0-3.0 x 10^6	5,000-7,500
	Umbilical cord blood	MNC	Fresh/ Frozen	0.5-0.75 x 10^6/ 2.0-3.0 x 10^6	5,000-7,500
	Umbilical cord blood	MNC	Frozen	0.5-0.75 x 10^6/ 2.0-3.0 x 10^6	5,000-7,500
	Bone marrow	$CD34^+$	Fresh	0.1-1 x 10^5/ 0.4-4 x 10^5	100-1,000
	Mobilized peripheral blood	$CD34^+$	Fresh/ Frozen	0.1-5 x 10^5/ 0.4-2.0 x 10^5	100-5,000
	Umbilical cord blood	$CD34^+$	Fresh/ Frozen	0.1-5 x 10^5/ 0.4-2.0 x 10^5	100-5,000
NHP, Horse, Pig, Sheep, Dog, Rat,	Bone marrow	MNC	Fresh/ Frozen	0.5-0.75 x 10^6/ 2.0-3.0 x 10^6	5,000-7,500
NHP, Horse, Pig, Sheep, Dog, Rat, Mouse	Peripheral blood	MNC	Fresh/ Frozen	0.5-0.75 x 10^6/ 2.0-3.0 x 10^6	5,000-7,500
Horse	Umbilical cord blood	MNC	Fresh/ Frozen	0.5-0.75 x 10^6/ 2.0-3.0 x 10^6	5,000-7,500

Species	Cell Type	Cell Preparation	Cell State	Working Cell Concentration Required (96-wells/ 384-wells)	Final Cell Dose / Well
Mouse	Bone marrow	Whole	Fresh	0.5-0.75 x 10^6/ 2.0-3.0 x 10^6	5,000-7,500
Rat, Mouse	Spleen	MNC	Fresh	0.5-1.0 x 10^6/ 2.0-4.0 x 10^6	5,000-10,000
Rat, Mouse	Fetal liver	MNC/Whole	Fresh	0.5-0.75 x 10^6/ 2.0-3.0 x 10^6	5,000-7,500
Mouse	Embryo, blood islands	Whole	Fresh	0.25-0.75 x 10^6/ 1.0-3.0 x 10^6	2,500-7,500

STEP 2. Controls and Test Compound Dose Response

A. Controls

Depending on whether the test compound is dissolved in aqueous medium or a solvent, up to 4 controls should be performed for each solvent used. The recommended controls are:

Control 1. Control contains cells, but no growth factors. (Control Master Mix provided).

Control 2. Vehicle-only control contains cells, but no growth factors. (Control Master Mix provided).

Control 3. Growth factor control contains cells with growth factors for cell populations being tested. (Master Mix bottles).

Control 4. Growth factor + vehicle control contains cells with growth factors for population being tested and the vehicle. (Master Mix bottle).

The vehicle control should be prepared at the same concentration of vehicle used in the highest working test compound dose.

B. Test Compound Dose Response

The following points should be considered when preparing the test compound dose response.

1. It is recommended to perform a minimum of 6 compound doses to obtain a full dose response curve and estimation of IC or EC values.
2. It is also recommended to perform 8 replicate wells/dose configured in columns across the plate. If possible, include at least the growth factor+vehicle control on each plate. Otherwise, controls can use the extra 8th plate provided with the kit.
3. Alternative plate configurations can be performed depending on the number of compound doses, compounds and replicates. However, for statistical purposes, 8 replicates are recommended, although 6

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 supermarket 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

Human and non-human primate cells should be incubated for 5 days. All other animal cells should be incubated for 4 days. It is possible to extend the culture incubation time by 1-2 days to increase sensitivity. However, with increase in incubation time, there will also be an increase in coefficients of variation. The validation parameters provided in Section 11 are for optimal days of incubation. To enable comparison of results, it is essential that once an incubation time period has been elected, the same culture period should be maintained thereafter.

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

(i) IMPORTANT INFORMATION REGARDING 7 POPULATION KITS

Assay kits to detect 7 populations include two lymphopoietic cell populations, T-CFC and B-CFC. These populations are stimulated with IL-2 and IL-7 respectively. Under normal conditions, T- and B-cells are not meant to be stimulated. Only when co-stimulators (e.g. CD28) or mitogens are present will the T-CFC and B-CFC be induced to proliferate to a significant level. As a result, stimulating T-CFC with IL-2 and B-CFC with IL-7 will only produce a minimum stimulation above background levels. This response is normal. If the effect of a cytotoxic agent on these populations is to be analyzed, the amount of inhibition may not be significantly different from the background control. Consider co-stimulation or mitogenic stimulation in the absence and presence of the test compound in order to obtain a satisfactory and more meaningful result.

(ii) Cell Suspension

a. The preferred cell suspension is a mononuclear cell suspension (MNC) .
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 red blood cell concentration 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.

(iii) 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 after mixing and frozen until the expiration date.
c. HALO® Master Mix is stable at 2-8°C for 1 month after thawing.

(iv) 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 for greater accuracy. Mix frequently during dispensing. For 384-well plates, a liquid handler is recommended.

(v) Number of Replicates Performed

A minimum of 6 replicates/test compound dose should be used. However, 8 replicates/test compound dose is recommended. 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.

(vi) Culture Plates Provided

The reagents have been optimized to work with the culture plates provided in the HALO® kit. Please do not replace the plates included with the kit with those

replicates can also be used. These considerations are for both 96- and 384-well plates.

4. If possible, it is best to configure the plate so that at least Controls 3 and 4 are on the same plate as the test compound.
5. If the test compound can be dissolved in water or aqueous medium, the vehicle controls (Controls 2 and 4) are not absolutely necessary.
6. If the test compound has to be dissolved in a solvent, such as dimethylsulphoxide (DMSO) or in the presence of fetal bovine serum (FBS), all controls should be included.
7. For 96-well plates, the test compound or vehicle is dispensed directly into each well before to the addition of the HALO® Culture Master Mix containing the cell suspension.
8. For 384-well plates, the test compound is dispensed after the addition of the HALO® Culture Master Mix.
9. The original test compound stock concentration is defined as the concentration of the test compound after it is dissolved in water, aqueous medium, solvent or FBS.
10. The working concentration is defined as the test compound dose prepared prior to dispensing into a well, and should be 10 x the final concentration in culture.
11. The final concentration is defined as the test compound dose that is present in the culture well.
12. The volume of test compound dispensed into each well of a 96-well plate should be 11µl.
13. For 384-well plates, 2.8µl is dispensed using a liquid handler directly into the HALO®-Culture Master Mix.
14. If using a solvent to dissolve the test compound, the final concentration of the solvent in the culture well should not exceed 0.1%.
15. The first or highest working concentration of a test compound dissolved in a solvent should be diluted 1:100 from the original test compound stock concentration so that when 11µl of the test compound working concentration is added to the well, the final concentration of the solvent is reduced 1:1000 or 0.1% in the final culture. Example: Test compound stock concentration dissolved in DMSO = 10mM. First working concentration diluted to 0.1mM of compound. DMSO diluted in this stage is 1:100. Final concentration of compound when 11µl is added to the well followed by 0.1ml of Culture Master Mix = 10µM. Final dilution of DMSO in culture is 1:1000 or 0.1%.
16. If the test compound is dissolved in a solvent, all further dilutions must be either in water, PBS, aqueous medium or FBS. The best diluent should be determined empirically prior to preparing the full dose response. If precipitation of the test compound occurs at the first dilution, a different diluent has to be used. If FBS has to be used in the diluent, try using a 10% FBS concentration in medium to determine if the compound, when diluted, will produce a clear solution. If

precipitation still occurs, increase the concentration of the FBS in steps of 10%. Use the same diluent for all remaining serial dilutions.

17. It is possible that the test compound can only be dissolved at low pH. If this is the case, dilution to the first working concentration should include achieving a normal pH. The medium used in the HALO® Culture Master Mix contains HEPES buffer and therefore can accommodate a change in pH that will not harm the target cells.
18. Prepare enough vehicle control for the number of wells to be used.

STEP 3. HALO®Tox HT 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®Tox HT kit contains sufficient plates and Master Mixes so that each cell population tested is performed on a separate plate. The extra plate included is for controls.

1. Transfer the frozen bottles 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 each bottle of HALO® Master Mix thoroughly using inversion or swirling.
3. Prepare the cell suspension and adjust to the working cell concentration.
4. Prepare the test compound dose response.
5. Calculate the total volume of each HALO® Master Mix and volume of cells to be added to each Master Mix required for each cell population tested as follows:

   Step 1 - Sample Master Mix required:
   No. replicates/dose * No. doses * Vol. of well * 1.2 (overage) = Vol. of Culture Master Mix (sample).

   Step 2 - Growth Factor Control Mix required:
   No. replicates Control 3 * Vol. of well * 1.2 (overage) = Vol. of Culture Master Mix (Growth Factor Control).

   Step 3 - Growth Factor + Vehicle Control Mix required:
   No. replicates Control 4 * Vol. of well * 1.2 (overage) = Vol. of Culture Master Mix (Growth Factor + Vehicle).

   Total Growth Factor Master Mix Required =(Step 1 + Step 2 + Step 3) * 0.9 (90% of final culture master mix).

   Total Cell volume = (Step 1 + Step 2 + Step 3) * 0.1 (10% of final culture master mix).

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.

Extra ATP standards and controls can be obtained from HemoGenix®.

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. Otherwise, allow the plate to come to room temperature for 30 min.
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 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 samples. (See Section 9, Adhesive Plate Covering Film).
3. Using a multichannel pipette (8- or 12-channel depending on the plate configuration), add 0.10ml (96-well plate) or 0.025ml (384-well plate) of ATP-ER to each well of the first column (A1-H1 or A1-P1) or row (A1-12 or A1 to A24). Mix the contents as described in Section 9.
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.

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.

Using a liquid handler

HALO®Tox HT using both 96- and 384-well plates 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.

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 (96-well plate) or 0.025ml (384-well plate) from ATP dilution in vial #5 to wells E1, F1, G1, and H1.
10. Dispense 0.10ml (96-well plate) or 0.025ml (384-well plate) from ATP dilution in vial #4 to wells A2, B2, C2, and D2.
11. Dispense 0.10ml (96-well plate) or 0.025ml (384-well plate) from ATP dilution in vial #3 to wells E2, F2, G2, and H2.
12. Dispense 0.10ml (96-well plate) or 0.025ml (384-well plate) from ATP dilution in vial #2 to wells A3, B3, C3, and D3.
13. Dispense 0.10ml (96-well plate) or 0.025ml (384-well plate) 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 (96-well plate) or 0.025ml (384-well plate) from the ATP low control to wells A4, B4, C4, D4.
16. Dispense 0.1ml (96-well plate) or 0.025ml (384-well plate) 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 (96-well plate) or 0.025ml (384-well plate) 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.
21. Repeat additions of 0.1ml (96-well plate) or 0.025ml (384-well plate) to all four columns, followed by mixing.

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 0.1ml (96-well plate) or 0.025ml (384-well plate) of ATP-ER to each well of

6. **Example.**
   No. of replicates for test compound = 8
   No. of test compound doses = 6
   Volume/well = 0.1ml
   No. of replicates for Control 3 = 8
   No. of replicates for Control 4 = 8
   Therefore:
   Volume of Culture Master Mix for Test Compound = 8 * 6 * 0.1 * 1.2 = 5.76ml
   Volume of Culture Master Mix for Control 3 = 8 * 0.1 x 1.2 =0.96
   Volume of Culture Master Mix for Control 4 = 8 * 0.1 x 1.2 = 0.96
   Total volume of Growth Factor Master Mix for each cell population = (5.76 + 0.96 + 0.96) * 0.9 = 6.91ml.
   Total volume of cells to be added = (5.76 + 0.96 + 0.96) * 0.1 = 0.768ml.
   In this example, 6.91ml of HALO® Growth Factor Master Mix for each cell population tested would be dispensed into each tube followed by 0.768ml of cells at the adjusted working cell concentration.
7. Mix the contents of each HALO® Master Mix tube thoroughly using a vortex mixer. This is now the HALO® Culture Master Mix.
8. Remove the sterile plates from their plastic covering under the hood. Use 1 plate for each cell population.
9. If only part of the plate is to be used, the remaining unused wells can be covered with a sterile, adhesive coverfoil. Alternatively, the coverfoil can be applied prior to processing the plate. See Section 9.
10. For 96-well plates: Using a calibrated and preferably electronic pipette, dispense 0.011ml (11µl) of each working test compound dose into the bottom and center of each replicate well. Do not dispense down the side of the well. Also transfer the same amount of vehicle into replicate wells for Controls 2 and 4.
11. For 96-well plates: Using a calibrated and preferably electronic pipette, transfer 0.1ml from each HALO® Culture Master Mix into replicate wells. Dispense into the bottom of the well so that the HALO® Culture Master Mix mixes with the test compound. No additional mixing is necessary.
12. For 384-well plates: Using a liquid handler, transfer 25µl from the HALO® Culture Master Mix into each replicate well.
13. For 384-well plates: Using a liquid handler, dispense and mix 2.8µl of each working test compound dilution into the center of each replicate well.
14. Place the lid on the plate and transfer the culture plates to a humidity chamber to ensure high humidity during incubation (See section 8 (vii)).
15. 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.
16. Incubate cells from human and non-human primate for 5 days. Cells from all other species are incubated for 4 days.

STEP 4 – 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. If the assay kit contains lyophilized ATP-ER, please refer to Section 9 for instructions on reconstituting the reagent.

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.

To measure the ATP concentration accurately, it is necessary for the sample ATP concentration to lie within the range of the ATP standard curve. HALO®-Tox HT assay kits include sufficient ATP standard so that a high or low ATP standard curve can be performed depending on the proliferation capability of the cells being tested. If the cells are expected to demonstrate high proliferation ability or contain high intracellular ATP concentrations, then an ATP standard curve from 0.03µM to 3µM should be used. If cells are expected to have low proliferation ability, then an ATP standard curve from 0.01µM to 1µM should be employed. If a sample is found to exhibit an iATP concentration greater 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.

NOTE: For the background and ATP standard curve, it is important to use the same base medium (matrix) as that used to culture the cells.

NOTE: To perform an ATP standard curve, background and controls, 4 columns of the 96-well plate will be used. The remaining wells will be used to perform the ATP standard curve, background and controls on other days samples are analyzed.

A. Extra High ATP Standard Curve Preparation (0.03µM - 3.0µM.

1. Label 5 vials (e.g. 1.5ml volume) for the ATP dose response consisting of the following ATP concentrations:

Label the ATP Standard (containing 0.3ml of 10µM ATP) supplied as Vial #1 (3µM).
Vial #2: 1µM
Vial #3: 0.3µM
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. Mix. This concentration is 3µM.
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 (96-well plate) or 0.025ml (384-well plate) from ATP dilution in vial #5 to wells E1, F1, G1, and H1.
10. Dispense 0.10ml (96-well plate) or 0.025ml (384-well plate) from ATP dilution in vial #4 to wells A2, B2, C2, and D2.
11. Dispense 0.10ml (96-well plate) or 0.025ml (384-well plate) from ATP dilution in vial #3 to wells E2, F2, G2, and H2.
12. Dispense 0.10ml (96-well plate) or 0.025ml (384-well plate) from ATP dilution in vial #2 to wells A3, B3, C3, and D3.
13. Dispense 0.10ml (96-well plate) or 0.025ml (384-well plate) 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 (96-well plate) or 0.025ml (384-well plate) from the ATP low control to wells A4, B4, C4, D4.
16. Dispense 0.1ml (96-well plate) or 0.025ml (384-well plate) from the ATP extra 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.
21. Repeat additions of 0.1ml (96-well plate) or 0.025ml (384-well plate) to all four columns, followed by mixing.

B. Low ATP Standard Curve Preparation (0.01µM - 1µM)

1. Prepare and label 5 vials (e.g. 1.5ml volume) for the ATP dose response consisting of the following ATP concentrations: