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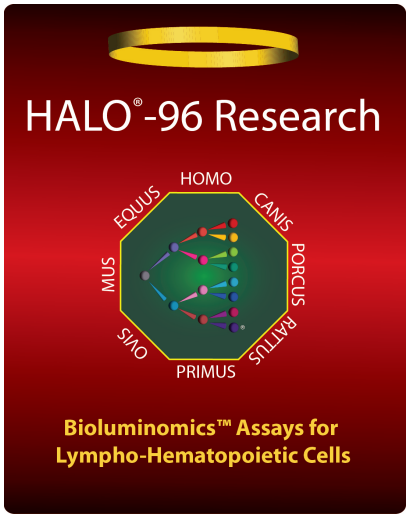
NOT FOR CLINICAL DIAGNOSTIC USE



HALO®-96 Research

Instruction Manual

(Version 4-2012)

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

Please read this manual in its entirety before using the assay kit

HemoGenix®, Inc

TABLE OF CONTENTS

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Changing the Paradigm
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13. REFERENCES

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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 Research has been developed for human and animal stem cell and basic research applications as well as veterinary applications.

Stem and progenitor cell proliferation is measured by virtue of the fact that when lympho-hematopoietic cells are stimulated with growth factors and/or cytokines, the intracellular ATP (iATP) concentration varies proportionately. Indeed, the iATP concentration and therefore the proliferation status correlates directly not only with the concentration of the growth factors and/or cytokines used to stimulate the cells, but also with the number of cells plated.

HALO® is one of several assay platforms (the others being LUMENESC™ for mesenchymal stem cells, ImmunoGlo™ for immune cells, STEMGlo™ for other primary stem cells and stem cell lines, XVPrime-Glo™ for *ex vivo* primary explanted cells and CLGlo™ for transformed cell lines and tumor cells) developed by HemoGenix® that incorporate bioluminomics™ technology. Bioluminomics™ is the instrument-based, non-subjective measurement of iATP cell concentrations using a luciferin/luciferase bioluminescence signal detection system, the most sensitive and accurate, non-radioactive readout available. However, bioluminomics™ is much more than simply measuring iATP concentrations. Bioluminomics™ also incorporates the calibration, standardization and, if necessary, the validation of the assay being used. Bioluminomics™ ensures that the results obtained are reliable and reproducible, not only within one laboratory, but between laboratories.

All HALO® assays also incorporate Suspension Expansion Culture (SEC) Technology. SEC technology means that lympho-hematopoietic stem and progenitor cells are not cultured under clonal conditions in methylcellulose. SEC technology allows fast and easy use, greater sensitivity and accuracy and significantly shorter culture times. (For methylcellulose colony-forming cell (CFC) assays, HemoGenix® has developed CAMEO™-4, a miniaturized CFC assay and CAMEO™-96, a bioluminomics™, 96-well plate assay system that allows both proliferation and differentiation to be detected using the same assay and also standardizes the CFC assay).

HALO®-96 Research is the most advanced research assay for lympho-hematopoietic stem and progenitor cells available. Yet it is fast to learn and easier to use than any CFC assay. By incorporating standards and controls, the user is assured of reliable results with the ability to compare those results over time.

2. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. **Please this manual in its entirety prior to using the HALO®-96 Research Assay Kit. To technical questions after reading the manual, please contact HemoGenix® prior to use.**
2. **HALO®-96 Research is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)**
3. **HALO®-96 Research is for research use only and has not been approved for clinical diagnostic use.**
4. **Reagents and supplies in this kit are STERILE. Perform all procedures under sterile conditions, except where indicated.**
5. **This kit should not be used beyond the expiration date on the kit label.**
6. **Do not mix or substitute reagents or other kit contents from other kit lots or sources.**
7. **Always use professionally calibrated and, preferably, electronic pipettes for all dispensing procedures. Small discrepancies in pipetting can lead to large pipetting errors. Although electronic pipettes self-calibrate themselves, they still need to be professionally calibrated on a regular basis.**
8. **Good laboratory practices and universal protective precautions should be undertaken at all times when handling the kit components as well as human cells and tissues. Material safety data sheets (MSDS) are included in each literature packet.**
9. **Please ware a laboratory coat, safety glasses and laboratory gloves for all procedures.**

iv. Contamination: Scan plate(s) under an inverted microscope to ensure no contamination occurs. If contamination occurs in all cultures, one or more of the reagents is contaminated. Replace the reagents. 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.

iii. 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.

(iv) Culture plates dry out:
a. 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.
b. 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

(i) 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:

a. 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 long periods of time have elapsed between calibrations, can lead to high CVs.
b. 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 to dispense reagents. Use calibrated or self-calibrating electronic pipettes or dispensers to add and mix the luminescence reagents.
c. 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.
d. Perform 4-8 replicates per point.

(ii) 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.
a. Reagent decay: The ATP-ER decays with time, even when frozen. This can lead to low luminescence. 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.
b. Inadequate cell growth: Cells did not exhibit high enough viability. Measure cell viability prior to adding the cells to the master mix. A cell viability lower than 85% should not be used.
c. Reagent deterioration: Reagents arrived thawed, at room temperature or greater or were not stored correctly as indicated in Section 5 of this manual.
d. Inadequate incubator conditions: Maintaining a correct humidified gaseous atmosphere in the incubator is essential.
i. Carbon dioxide concentration is inadequate. Ensure that the carbon dioxide concentration in the incubator is correct using a Fyrite gas analyzer.
ii. Use low oxygen tension. Using an oxygen concentration of 5% can increase plating efficiency. Check that the incubator oxygen concentration is correct using a Fyrite gas analyzer.
iii. Low humidity. Plates dry out (see below) and cell growth declines.

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3. INTENDED USE

HALO®-96 Research is used to detect and measure the proliferation ability and/or potential of lympho-hematopoietic stem and progenitor cells for virtually any research application. Traditionally, the colony-forming cell (CFC) assay has been used for many cell-based assays involving blood-forming stem and progenitor cells. It should be emphasized that although the growth of colonies in the CFC assay requires proliferation, the CFC assay does not measure proliferation. Instead, it detects the ability of stem and progenitor cells to differentiate and produce mature colonies of cells that allow the colonies to be identified as being derived from a specific cell type. As such, the CFC assay is a differentiation assay and cannot be used to measure cell proliferation because the latter requires a different readout.

Separate HALO®-96 Research assay kits are used to detect specific stem and progenitor cell populations.

HALO®-96 Research can be used for stem and progenitor cell proliferation research applications from multiple tissues, including:
- Bone marrow
- Peripheral blood
- Umbilical cord blood
- Spleen
- Fetal liver
- Embryonic tissue (e.g. yolk sac)

HALO®-96 Research Kits are available for cells from the following species:
- Human
- Non-human primate
- Horse
- Pig
- Sheep
- Dog
- Rat
- Mouse

Please note that HALO®-96 Research should not be used for human clinical applications or for toxicity and safety applications. HemoGenix® has developed specialized assays for these application areas.

Suspension Expansion Culture (SEC) versus Methylcellulose

All HALO® assays incorporate Suspension Expansion Culture (SEC) technology. The equivalent methylcellulose assay is CAMEO™-96. Suspension expansion culture technology has several advantages over using methylcellulose. These include:
- Ease and accuracy of dispensing reagents.
- Cell-cell interaction.
- Greater assay sensitivity.
- Shorter cell incubation times.
- Coefficients of variation ≤15%.

It is recommended to use CAMEO™-4 to:

- To culture hematopoietic cells under clonal conditions.
- Detect differentiation ability or potential.

It is recommended to use CAMEO™-96 to:

- To culture hematopoietic cells under clonal conditions.
- To measure proliferation of cells in colonies.
- Detect and measure both proliferation and differentiation using the same assay system.
- To standardize the "classic" CFC assay.

Replacing the CFC Assay with HALO®

The same culture reagents are used for CFC assays (CAMEO™-4 and CAMEO™-96) and HALO®-96 Research. Providing the same culture reagents and conditions are used to compare CAMEO™ and HALO®, there is a direct correlation between the two assay platforms. This allows HALO® to be used as an alternative or replacement for the CFC assay.

6. Calculated mean ATP values
7. Standard deviation of calculated ATP values
8. % CV of calculated ATP values.

d. 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.

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

(iii) Alternative Methods for Converting RLU Values into ATP Concentrations

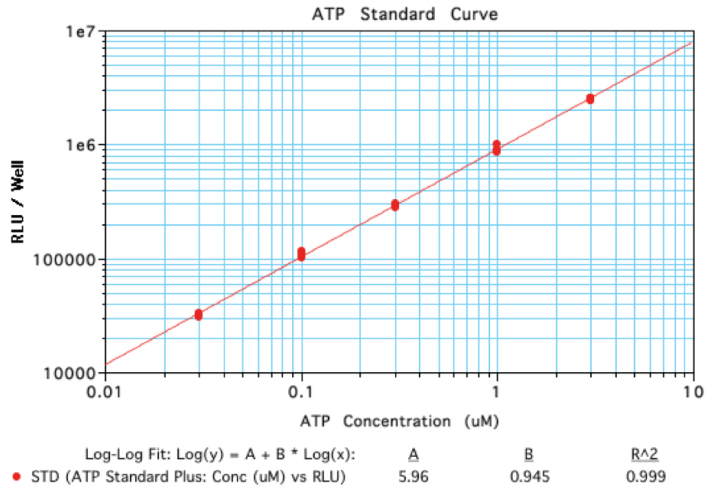
a. Manual method

Manually convert all values to log and plot the values on a log scale. Interpolate the results from the graph and convert back using antilog values.

b. Third-party software curve fitting

Several software programs are available to perform the required curve fit and interpolate the ATP concentrations from the RLU values. The TableCurve 2D software program from Systat Software, Inc. can be used for this purpose, but other software packages are also available, e.g. Origin software from OriginLab, Inc. In some cases, the equation and parameters have to be manually programed into the software. TableCurve 2D software has the ability to perform the curve fit and perform the interpolation producing similar values to that of SoftMax Pro from Molecular Devices.

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



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
6. Standard deviation of Mean RLU
7. %CV of Mean RLU
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)
- Slope (B) = 0.969 ± 0.18 (%CV = 1.9)

(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)

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4. The CONCEPT and PRINCIPLE of BIOLUMINOMICS™ ASSAYS

HALO® 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 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 by 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 the HALO® assay.** 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 the instrument.
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 and controls must be performed on the day samples are to be processed.

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.

5. OVERVIEW OF THE HALO® PROCEDURE

There are 3 steps to use HALO®.

Step 1 – Cell Preparation
Cells are not provided with HALO®-96 Research assay kits. Cells are prepared using a user-defined, pre-validated protocol to obtain a single cell suspension. A viability and cell count are performed.

Step 2 – Cell Culture
Two reagent mixes are provided. A base master mix containing growth media and a support media; and a growth factor mix. In addition, medium is also provided for the dilution of the ATP standard. Depending on the number of samples and replicates to be tested, tubes are prepared and each of the Mixes is added to the tubes in ratios defined in the detailed protocol in Section 7 of this manual. The target cells are added and the tubes mixed thoroughly. This HALO® Culture Master Mix is then dispensed at 100µl/well to the 96-well plate(s) provided with the kit. The plates are incubated for the prescribed time period.

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 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).

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11. 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 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 standardizing the assays. This is done by interpolating the RLU into ATP concentrations using the ATP standard dose response curve and a 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" need 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 1 second.
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 the injectors if the instrument is so equipped.

(ii) Analysis Setup
The luminometer is usually controlled by software installed on a computer using a serial or USB interface cable. Before using the luminometer, ensure that you are familiar with the software that controls the instrument. Although all of the necessary calculations can be performed manually, 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

a. It tests whether the instrument is working properly and calibrates it.
b. It ensures that the reagents are working correctly.
c. It standardizes the assay and allows the assay system to be validated, if required.
d. 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.

(vii) Sterile Adhesive Plate Covering Film

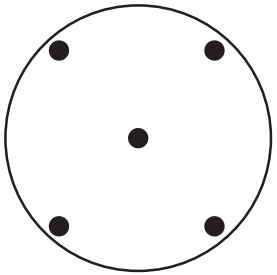
To help keep the plate(s) sterile, adhesive, air permeable, sterile films are provided so that the part of the plate that is not being used can be covered and kept sterile until required. If using the adhesive film provided, the plate cover should be removed in a laminar air-flow hood and replaced with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

(viii) 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:

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

Figure 2. Positions of pipette tip for mixing the well contents



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6. KIT CONTENTS and STORAGE CONDITIONS

HALO®-96 Research assay kits contain reagents that have been frozen and stored at -80°C prior to shipment. The kit is shipped either with dry ice or blue ice. The following components are included:

Item	Component	Storage
1	HALO® Base Master Mix - contains support media.	-20°C until used
2	HALO® Growth Factor Mix - contains the growth factor cocktail formulated to support the specific cell population of interest.	-20°C until used
3	Medium (IMDM) for dilution of the ATP standard.	-20°C until used
4	ATP standard.	-20°C until used
5	ATP extra high, high and low controls.	-20°C until used
6	ATP Enumeration Reagent (ATP-ER)*	-20°C in the dark until used
7	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
8	Sterile, 96-well plates for stem cell culture	Can be kept with other kit components
9	Non-sterile 96-well plates for ATP standard curve determination.	Can be kept with other kit components
10	Instruction manual.	Can be kept with other kit components

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 cycles 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.

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 obtained 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.

7. EQUIPMENT, SUPPLIES AND REAGENTS REQUIRED BUT NOT PROVIDED

Equipment and Supplies

1. Laminar Flow Biohood
2. Plate luminometer (e.g.; Molecular Devices, SpectraMaxL; TECAN, GENios)
3. Sterile plastic tubes (5ml, 10ml, 50ml)
4. Single channel pipettes, preferably electronic (e.g. Rainin EDP pipettes for variable volumes between 1µl and 1000µl).
5. 8 or 12-channel pipette, preferably electronic (e.g. 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. 7-AAD, propidium iodide or trypan blue for viability assay.

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10. RECOMMENDATIONS AND TIPS PRIOR TO MEASURING BIOLUMINESCENCE

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

(ii) 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.

(iii) Bioluminescence Assay Kit Components

a. 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.
b. Enough ATP standard and monitoring reagent is supplied to perform 2 standard curves and controls for each sterile plate provided.
c. Unused ATP standard can be refrozen once.
d. If thawing more than one bottle of ATP-ER for analysis, mix the contents of the bottles together before dispensing into reagent reservoir.
e. 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.

(iv) Reconstitution of Lyophilized Monitoring Reagent (if included)

a. Thaw the ATP Enumeration Reagent Buffer at room temperature, in cold running water, or at 2-8ºC overnight.
b. Do not use any form of heat to thaw reagent.
c. Allow the lyophilized ATP-ER substrate (brown glass bottle) to come to room temperature.
d. Remove the closures from both bottles.
e. 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.
f. Allow the ATP-ER mix to reconstitute for 10 minutes at room temperature.
g. Reconstituted ATP-ER is stable for 8 hours at room temperature, 48 hours at 2-8ºC, or 20 weeks at -20ºC.
h. ATP-ER can be refrozen up to 11 cycles without significant loss of sensitivity.

(v) Volumes of Luminescence Kit Components Required

a. Each vial of ATP standard contains enough volume to perform two (2) standard dose responses.
b. 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)).

(vi) 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 to be processed is an essential part of the assay because it has 3 functions:

b. If performing column replicates, addition of the ATP-ER will be performed using an 8-channel pipette from left to right across the plate. If performing row replicates, a 12-channel pipette could be used to dispense the reagents from the top to the bottom of the plate.

(vii) 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 of other manufacturer. Cell growth and bioluminescence output can be seriously affected and the assay kit warranty will be void. HemoGenix® can supply additional plates if required.

(viii) Growth Factor-Free Assay Kits

If you have purchased a kit without growth factors, please refer to the special instructions at the end of Step 2 of the HALO® Protocol.

8. The HALO®-96 RESEARCH PROTOCOL

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

Performing HALO®-96 Research is a 3-step process.

Step 1 – Cell preparation.
Step 2 – HALO®-96 Research 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

A. Human, Non-Human Primate, Horse, Pig, Sheep or Canine Cells

1. The HALO®-96 assay requires that target cells be separated from red blood cells. Neutrophils and platelets should also be removed. A mononuclear cell (MNC) suspension is the cell suspension of choice. HemoGenix® recommends separating the MNCs using NycoPrep 1.077 (Axis-Shield) density gradient centrifugation media. This separation procedure should be used for all bone marrow and peripheral blood samples from the aforementioned species. Ficoll-Paque can also be used, although this is toxic to cells. Follow the manufacturer's protocol to prepare the MNCs. A hematocrit of 10% or less is required to avoid interference with the ATP analysis.
2. Resuspend the cells in IMDM or PBS.

B. Rat or Murine Bone Marrow

1. Remove organs (femora and tibia (optional)) under aseptic conditions.
2. Remove as much muscle from the bones as possible.
3. Using a sterile blade, first cut off the proximal (hip joint) end below the ball joint at right angles to the longitudinal length of the bone. Then cut off the distal end (above the patella or knee).
4. Transfer sufficient sterile medium to a tube so that it will cover the whole bone, approximately 1-2ml. (Some of the medium provided with the kit can be used for this purpose).
5. Half fill a syringe (1-3ml) with sterile medium and, using a needle gauge that will enter the bone cavity without cracking the bone, insert the needle into the proximal end and immerse the whole bone in the medium contained in the tube.
6. Flush out the marrow through the bone cavity and withdraw part of the cell suspension through the bone and into the syringe.
7. Flush the cell suspension through the bone and repeat steps 6 and 7 two to three times. When finished, the bone should appear translucent, indicating that most of the cells have been flushed out of the cavity.
8. Remove the empty bone and replace it with the next bone until the marrow from all bones has been flushed out of the cavities.

9. Let the cell suspension settle for 1-2 minutes to allow large debris to fall to the bottom of the tube.
10. Using a small gauge (22-25) needle and syringe, slowly withdraw the cell suspension leaving the large debris in the tube and transfer it to a new tube, noting the volume.
11. If necessary, add medium to achieve the required volume.

To further purify a cell suspension, it is recommended to underlay 1-2ml of NycoPrep 1.077 (Axis-Shield) under the cell suspension in a sterile, 15ml conical tube with screw cap and centrifuge the cells according to the manufacturer's protocol. This is recommended if using the rat cell suspensions. See Table 1 for recommended cell concentrations.

C. Isolation of Hematopoietic Subpopulations

Providing sufficient cells are available, subpopulations of stem and progenitor cells can be isolated and purified for use in the HALO® assay. We recommend using magnetic cell isolation procedures (e.g. Miltenyi Biotech), since these allow rapid isolation of different cell populations with substantial purity, viability and yield. Please see Table 1 of recommended cell concentrations to determine the optimal, final cell concentration to use in the HALO®-96 Research procedure.

D. 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 LIVEGlo™ (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 *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.

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9. 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. Cell suspensions from other tissue may also be used.
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. Cell lysis is not recommended, however, if cell lysis is used, a cold lyse procedure should be performed and the cells washed prior to introducing into the culture mix.
c. If cells have been treated prior to cell culture, higher cell concentrations may be required.

(ii) HALO® Base Master Mix

The Base Master Mix has a proprietary mix of medium and other components that increase the plating efficiency of the cells. Do not use this in place of medium. The amount of Base Master Mix provided is for the number of 96-well plates determined by the kit. However, if dispensed carefully, there is sufficient Base Master Mix so that several smaller experiments can be performed using the plate provided.

(iii) Dispensing Individual Component Mixes and Reagents

We recommend using electronic pipettes to dispense all mixes and reagents.

(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. Mix frequently during dispensing.

(v) Number of Replicates Performed

The number of replicates is determined by the user. HemoGenix® recommend at least 6 replicates per culture point. Please remember that low numbers of replicate wells may save components in the short term, but may also cause inconclusive results and statistics, especially 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) Plate Configuration

a. The configuration of the 96-well plate for an experiment is arbitrary. However, the number of replicates and the way in which the reagents are added prior to luminescence measurement, will determine the plate configuration. For example, if performing 4 or 8 replicates per culture point, HemoGenix® recommends configuring the plate in columns; that is, A1 to D1 and E1 to H1 etc. for quadruplicate cultures and A1 to H1, A2 to H2 etc. for 8-replicate cultures. If performing 6 or 12 replicates, HemoGenix® recommend configuring the plate in rows, that is, A1-A6 and A7 to A12 for 6 replicates and A1-A12, B1 to B13 for 12 replicates.

C. 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.

D. Using a liquid handler

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.

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TABLE 1
Recommended Cell Doses for Different Species, Cell Types, Cell Preparations and Cell States for HALO®-96 Research

Species	Cell Type	Cell Preparation	Cell State	Working Cell Concentration Required (100 x Final Cells/Well)	Final Cell Dose / Well
Human	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
	Umbilical cord blood	MNC	Frozen	0.5-0.75 x 10^6	5,000-7,500
	Bone marrow	$CD34^+$	Fresh	0.1-1 x 10^5	100-1,000
	Mobilized peripheral blood*	$CD34^+$	Fresh/ Frozen	0.1-5 x 10^5	100-5,000
	Umbilical cord blood	$CD34^+$	Fresh/ Frozen	0.1-5 x 10^5	100-5,000
Non-human primate	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
Horse, Pig, Sheep	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
Dog	Bone marrow	MNC	Fresh/ Frozen	0.5-0.75 x 10^6	5,000-7,500
Rat	Bone Marrow	MNC	Fresh	0.5-0.75 x 10^6	5,000-7,500
	Peripheral blood	MNC	Fresh	0.5-0.75 x 10^6	5,000-7,500
Mouse	Bone Marrow	MNC	Fresh	0.5-0.75 x 10^6	5,000-7,500
	Spleen	MNC	Fresh	0.5-1 x 10^6	5,000-10,000
	Fetal liver	MNC	Fresh	0.5-0.75 x 10^6	5,000-7,500

STEP 2. HALO®-96 Research Cell Culture Master Mix Preparation and Cell Culture

All of the culture components required, with the exception of the cells, are included. HALO®-96 Research kits have been designed so that only 2 components need to be mixed together. The components are mixed together in fixed ratios or proportions to achieve the correct concentrations. Each kit contains a:

- Base Master Mix and
- Growth factor mix or Medium.

These two kit components are first mixed together before the target cell suspension (from Step 1) is added.

A. The HALO® Method

1. Determine the number of sample categories required for testing and the number of replicates for each sample. Categories can be defined as cell concentrations, populations or different target cells used.
2. Multiply the number of sample categories by the number of replicates to obtain the number of wells to be prepared. (Although the number of replicates wells is user dependent, statistics and possible outliers should be taken into account when deciding how many replicates to perform).
   Total number of wells = Number of sample categories x Number of replicates.
3. Multiply the number of wells to be used by 0.1ml (volume of each well) plus an extra 10%. This will give the total volume of Culture Master Mix required.
   Total volume (ml) to be prepared = (Number of wells x 0.1ml) + 10%.
4. Label the tubes required for the number of samples and replicates.
   Tip: Total volumes of 3,500µl (3.5ml) or less should be prepared in sterile 5ml plastic tubes with caps. Volumes greater than 3,500µl (3.5ml), but less than 9,500µl (9.5ml) should use 10-15ml sterile plastic tubes. Volumes greater than 9,500µl (9.5ml) should use 50ml sterile plastic tubes. If using sufficient reagents to prepare 1 batch for all 96-wells, use a 15ml tube.
5. For each sample tube, mix the following components in the ratios shown in Table 2:

TABLE 2

Component	Number of Parts
HALO® Base Master Mix	8
HALO® Growth Factor Mix or Medium Mix	1
Target Cells	1

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

B. 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 cover foil 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.
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.

0.90ml IMDM medium to vial #5

3. Dispense 0.7ml of the IMDM medium into Vial #1. Mix.
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 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.

B. Low ATP Standard Curve Preparation for Use with Low Proliferation Cell Populations

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

For example, if a total volume of 1.0 ml is required (sufficient for 6-8 replicate wells), the following volumes would be necessary:

TABLE 3

Component	Number of Parts	Volumes (ml)
HALO® Base Master Mix	8	0.8
HALO Growth Factor Mix or Medium Mix	1	0.1
Target Cells	1	0.1
	Total volume:	**1.0**

IMPORTANT: Although the total volume is 1.0ml, 10 x 0.1ml wells cannot be plated. In general, about 10% extra volume has to be prepared in order to achieve the desired number of wells. Thus, from a 1.0ml volume, 9 wells of 0.1ml each can be dispensed.

NOTE: Before adding the target cells, ensure that a single cell suspension has been prepared.

6. Mix the contents of the tube vigorously, either by vortexing or by shaking to obtain a homogenous culture master mix.
7. Dispense 0.10ml of the culture master mix into each of the replicate wells.
8. Mix the culture master mix well between every few replicates.
9. Transfer the culture plate to a 37˚C, fully humidified incubator with an atmosphere of 5% CO_2. If possible, use an incubator gassed with nitrogen in order to reduce the atmospheric oxygen concentration (21%) to 5% O_2 since this helps increase the plating efficiency.
10. Incubate the cells for the time periods shown in the Table 4 below:

TABLE 4

Species	Cell Type	Incubation Period (days)
Human	Bone marrow, normal and mobilized peripheral blood, umbilical cord blood	5
Non-human primate	Bone marrow, peripheral blood	5
Horse, Pig, Sheep	Bone marrow, peripheral blood	4
Dog, Rat	Bone marrow	4
Mouse	All	4

PLEASE NOTE that incubation times can be extended. Human and non-human primate cell incubation can be extended to 6 or 7 days, while all other animal cells can be extended to 5 days. Extending the incubation time will result in high ATP values and therefore greater sensitivity, but also slightly greater coefficients of variation (%CV).

B. Special instructions for using the growth factor-free HALO® kit.

The growth factor-free HALO® kits was designed so that users can add their own

growth factors. The growth factor-free HALO®-96 Research kit does not come with a growth factor mix. Instead, the user adds their growth factor mix and cell suspension to the HALO® Base Master Mix.

1. User's Growth Factor Mix
Add your growth factor mix at a concentration 10x the desired concentration in culture. A 1:10 dilution is made when adding growth factor mix at 10% of the culture master mix volume.

2. Dose Response Studies

a. Follow the steps 1-4 for determining the number of tubes, replicates and wells. For example, if you wish to perform a 6- or 9-point dose response for a specific growth factor, 6 or 9 tubes will be required for each growth factor dose and might also include a control tube to which no growth factor is added
b. For each of the growth factor doses used, determine the total volume required and prepare separate labeled tubes for each dose.
c. For each growth factor dilution, add the required amount from the stock growth factor concentration to tubes and make up to the required volume with medium supplied.
d. Each growth factor dose is now the growth factor mix. Follow steps 5-10.

Example: An erythropoietin (EPO) dose response is required from 0.1U/ml to 10U/ml using doses 0.1U/ml, 0.25U/ml, 0.5U/ml, 1.0 U/ml, 2.5U/ml, 5U/ml and 10U/ml One ml of each EPO dose has to be prepared. A stock concentration of 1,000U/ml is available for dilution. First prepare a 1ml dilution containing 100U/ml by adding 0.10ml of the 1,000U/ml EPO stock and 0.90ml of the medium provided. For each further dilution required, use the medium supplied with the kit keeping in mind the Growth Factor mix must be 10x the desired concentration in culture.

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.

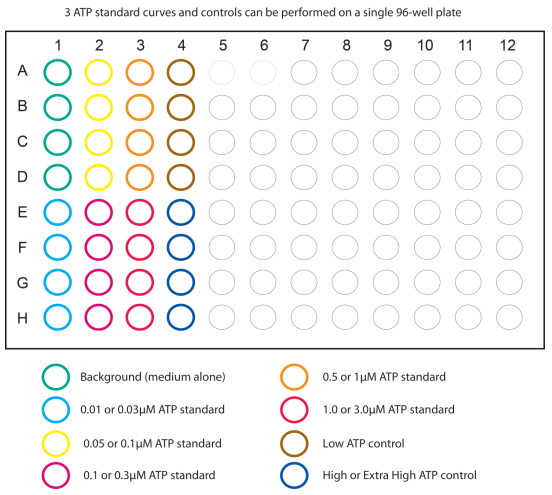
IMPORTANT. Please refer to Section 9 for recommendations and tips prior to starting this part of the procedure. In particular, please refer to Section 9 for important information on mixing components.

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.

Figure 1: ATP standard curve and control plate configuration using non-sterile, 96-well plates.



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.

A. High ATP Standard Curve Preparation for Use with High Proliferation 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 the ATP concentrations for mobilized peripheral blood samples or purified stem cell populations are found outside of the range from 0.03µM to 3µM, the ATP standard curve will have to be repeated using the dose range from 0.01µM to 1µM (see below). 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.

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) 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