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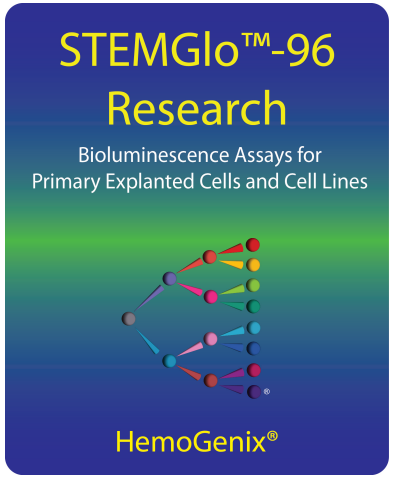

STEMGlo™-96 Research

A Proliferation and Viability Assay for Primary Explanted Stem and Progenitor Cells, Stem Cell Lines and Transformed Cell Lines, including Cancer Cell Lines

Assay Manual

(Version 3-2015)

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

HemoGenix®, Inc

TABLE OF CONTENTS

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13. REFERENCES

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1. Limitations of the Assay and Precautions

1. **STEMGlo™-96 Research is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA).**
2. **STEMGlo™-96 Research is for research use only (RUO) and have not been approved for clinical diagnostic use.**
3. **This kit should not be used beyond the expiration date on the kit label.**
4. **Do not mix or substitute reagents or other kit contents from other kit lots or sources.**
5. **Always use professionally calibrated and, preferably, electronic pipettes for all dispensing procedures. Small discrepancies is pipetting can lead to large pipetting errors. Although electronic pipettes self-calibrate themselves, they still need to be professionally calibrated on a regular basis.**
6. **Good laboratory practices and universal protective precautions should be undertaken at all times when handling the kit components and human cells and tissues. Material safety data sheets (MSDS) are included in each literature packet.**

2. INTRODUCTION

STEMGlo™-96 Research Research is a proliferation, viability and functionality assay for primary explanted cells, including primary stem and progenitor cells from a variety of organs and tissues as well as stem cell lines (e.g. embryonic stem (ES) cells and induce pluripotential stem (iPS) cells), transformed cell lines and cancer cells. STEMGlo™-96 Research Research is available for adherent or non-adherent cell populations and has been designed to multiplex with other assay readouts, thereby providing the assay with exceptional flexibility.

STEMGlo™-96 Research Research include a base medium that is used to dilute the standards included with the kit. The assay kit provides the user with the flexibility to use their own growth medium and stimulation factors, that is, growth factors and/or cytokines.Cells are first cultured using the investigator's own protocol. After incubation, proliferation is then determined by releasing the intracellular ATP (iATP) and measuring its concentration using a luciferin/luciferase bioluminescence signal detection system. Since iATP is a biochemical marker that increases proportionately in response to (a) the cell population being measured, (b) the type and concentration of the growth factor/cytokines used to stimulate the cells and (c) the cell dose, iATP is an ideal and highly sensitive marker to measure metabolic viability, proliferation, cytotoxicity or cell number.

An important advantage of using STEMGlo™-96 Research Research over other assays is that it is calibrated and standardized prior to measuring samples. This provides an internal proficiency test that ensures the assay is working correctly before results from valuable samples are measured. Standardization of the assay also allows results to be compared directly over time without normalizing the results. When cells from different lots or batches are cultured under similar conditions, STEMGlo™-96 Research Research provides an important measure of "quality" for the lots produced.

IMPORTANT:
STEMGlo™-96 Research Research is for research use only and has not been approved for clinical diagnostic use.

color and probably cloudy in appearance. Cell cultures that demonstrate high proliferation will usually appear orange to light orange, but will not be cloudy. If only "spot" contamination occurs, this is usually due to pipette or repeater tips coming in contact with materials other than the reagents. Contamination will usually lead to outlier RLU values.

Luminescence Reagent Mixing.

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

Culture Plates Drying Out

- Due to the relatively small culture volume (0.1ml), drying out of the culture wells, particularly around the outside of the plate, can be a problem. These are called "edge effects". An incubator with insufficient humidity will cause this problem. To ensure that this does not occur, the incubator water reservoir should be full and the humidity in the chamber checked using a hygrometer.
- If drying out continues, use of a humidity chamber is recommended. 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. Outliers can be obtained resulting in large variations between replicates. 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 calibrated 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. 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 standard curve 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.
- *Contamination*: Cells cultured in 96-well plates cannot be view under a microscope. If contamination occurs it will usually be seen by the difference in color of the cultures. Contaminated cultures will usually be bright yellow in

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3. The CONCEPT and PRINCIPLE of BIOLUMINOMICS™ ASSAYS

STEMGlo™-96 Research 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, the iATP concentration can increase several fold. The opposite is true if proliferation is inhibited by a drug or other agent. The iATP concentration produced is directly dependent on:

- The proliferation potential (or primitiveness) of the stem 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.

Stem cells are incubated in the desired medium either with or without growth factors/cytokines and the cells incubated 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 STEMGlo™-96 Research. 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 instrument software does not allow this, it will be necessary to use third-party software to perform this operation.

4. OVERVIEW OF THE STEMGlo™-96 Research PROTOCOL

Using STEMGlo™-96 Research is a 3-step procedure.

Step 1 – Cell Preparation
Cells are not provided with STEMGlo™-96 Research assay kits. Cells should be prepared using a user-defined, pre-validated protocol. A dye exclusion viability and/or metabolic viability and nucleated cell count should be performed on all samples.

Step 2 – Cell Culture
STEMGlo™-96 Research does not include growth medium since this will vary depending on the stem cells used. Similarly, growth factors and/or cytokines to stimulate the stem cells are not included and have to be provided by the user. A base medium is provided for the dilution of the ATP standard. It should not be used for cell culture. Since this is an assay kit for research purposes, the user can perform as many replicate wells/sample as needed. For statistical purposes, HemoGenix® recommends 6 replicates/sample. A Master Mix should be prepared in a tube containing cells adjusted to a working cell concentration that will provide the correct final cell concentration/0.1ml, medium and growth factors/cytokines. The contents of the tube(s) are mixed on a vortex mixer and 0.1ml dispensed into wells of the 96-well plate using a calibrated pipette. The plates are transferred to a 37°C, fully humidified incubator gassed with 5% CO_2. It is recommended to incubate stem cells in an atmosphere containing low oxygen tension (e.g. 5% O_2) since this reduced oxygen toxicity and increases plating efficiency.

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) using a multichannel pipette. The ATP-ER also contains luciferin and luciferase, the reagents used to produce bioluminescence. After mixing and a 10 min 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. STEMGlo™-96 Research Characteristics and Validation Parameters

STEMGlo™ bioluminomics™ technology allows the assay to be calibrated and standardized. Inclusion of an ATP standard and controls are requirements for assay validation. The STEMGlo™ readout has been validated.

Assay Characteristics

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

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

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

For samples:
- Lowest ATP value indicating 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).

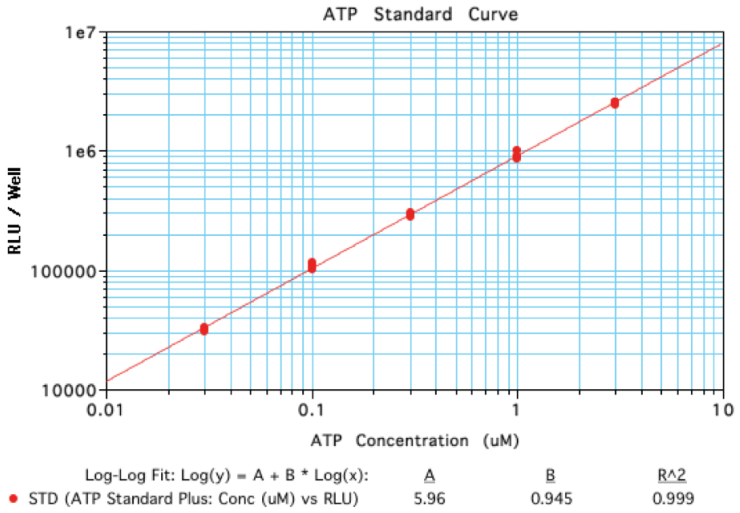
Assay validation parameters will vary depending upon the species, cell source and cell population detected.

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 Molecular Devices SoftMax Pro Software



5. KIT CONTENTS

STEMGlo™-96 Research kits contain the following components:

1. Medium (IMDM) for ATP standard dilution and background.
2. ATP standard.
3. ATP controls (low, high and extra high).
4. ATP Enumeration Reagent (ATP-ER)*
5. Adhesive Plate Covering—a sterile foil to protect and keep unused wells sterile.
6. Sterile, 96-well plates for cell culture.
7. Non-sterile 96-well plates for ATP standard curve determination.
8. Assay manual.

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

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

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

This kit contains a reagent for measuring luminescence (ATP-ER) that decays with time. HemoGenix® suggests that this kit be used before the expiry date of this reagent. HemoGenix® does not take responsibility for the quality of reagents beyond their expiry date. If the kit cannot be used prior to the expiry date of this reagent, fresh 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.; Berthold SpectraLIA, Molecular Devices, SpectraMaxL; TECAN, GENios)
3. Sterile, capped, plastic tubes (5ml, 10ml, 50ml)
4. Positive displacement (preferably electronic) syringe pipette (e.g. Eppendorf Repeater® Stream)
5. Single channel pipettes, preferably electronic (e.g. ViaFow or Rainin EDP pipettes for variable volumes between 1µl and 1000µl).
6. 8 or 12-channel pipette, preferably electronic (e.g. ViaFlow or Rainin EDP pipettes for fixed or variable volumes between 10µl and 100µl).
7. Reservoir for 8- or 12 channel pipette
8. Sterile pipette tips.
9. Vortex mixer.
10. Tissue culture incubator, humidified at 37°C with 5% CO_2 (minimum requirement) and 5% O_2 (preferable).
11. 1.5ml plastic vials (5 for each ATP dose response).
12. Hemocytometer or electronic cell counter to determine cell concentration.
13. Flow cytometer or hemocytometer for determining viability.

Reagents

1. Sterile Phosphate Buffered Saline (PBS)
2. Iscove's Modified Dulbecco's Medium (IMDM) or other culture growth medium.
3. DNase (Sigma-Aldrich, Catalog No. D4513-1VL)
4. Density-gradient medium (e.g. NycoPrep 1.077, Axis-Shield).
5. 7-AAD, propidium iodide, trypan blue, acridine orange or other dye exclusion viability assay.

Kits

LIVEGlo™ (HemoGenix®, Inc), for metabolic viability.

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 imported into the software program. It is important that the third-party software can either perform a log-log linear regression analysis on the raw data or can transform the data into log values. The following software has been tested to perform the necessary

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

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

b. The second set of measurements to be performed will be the ATP standard curve. Setup the software to give the following information:
   1. Group or sample designation
   2. ATP standard dose response values (these are the 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)
- Slope (B) = 0.969 ± 0.18 (%CV = 1.9). This is slightly different to the value given in Section 11.

(Values are the Mean ± 1 Standard Deviation)

7. The STEMGlo™-96 Research PROTOCOL

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

Performing STEMGlo™-96 Research is a 3 step process.

Step 1 – Cell preparation.
Step 2 – STEMGlo™-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

Perform all cell preparation procedures under sterile conditions in a biosafety cabinet.

Always ware protective clothing, including gloves.

Primary stem cells are present in many organs and tissues. A user-defined and pre-validated stem cell preparation protocol should be used for the specific stem cells to be analyzed. Similarly, stem cell lines should also be prepared using protocols that have been previously used and tested.

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 cytometer 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. The cell suspension is adjusted to a specific working concentration/ml that is usually 10 - 100 x the final concentration per well. It may be necessary to perform a cell dose response to optimized the cell dose.
4. Prepare the total volume of cell suspension required culture medium that has been used previously to growth the stem cells . The volume of the adjusted cell suspension required will be 10% of the total volume of the STEMGlo™-96 Research Culture Master Mix prepared.

Important. When preparing the cell suspension, it is essential that the suspension contains no red blood cells as these will interfere with the assay.

STEP 2. STEMGlo™-96 Research 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.

Always ware protective clothing, including gloves.

It is always good practice to include positive and/or negative controls in any study.

Always use calibrated and preferably electronic pipettes for accurate dispensing of reagents.

1. Prepare the growth factor and/or cytokine mix necessary to stimulate the stem cells being analyzed. It is suggested to prepared this master mix as a 100 x or 1,000 x solution.
2. Prepare the cell suspension as required and adjust the cell concentration so that it is 10 - 100 x the final cell dose required. For example: if 5,000 cells/well is required as the final cell concentration, a working cell suspension concentration of 50,000 cells/ml or 500,000 cell/ml will be needed. To determine the optimum cell dose, it is recommended to perform a cell dose response and use a cell dose that is on the linear part of the cell dose response.
3. Prepare and label one or more 5ml sterile, plastic tubes for each sample to be tested.
4. For 6 replicate wells/sample, it is suggested to make up a total volume of 1ml Culture Master Mix containing medium, growth factors/cytokines and cells. For 4 replicate wells/sample a total volume of 0.6ml Culture Master Mix will be sufficient. If the growth factor/cytokine Master Mix is prepared as a 100 x fold solution and the cells are also at a 100 x concentration higher than the final concentration required, then 0.1ml of the growth factor/cytokine mix and 0.1ml of the cell suspension will be added to 0.8ml of growth medium. When 0.1ml of the Culture Master Mix is dispensed into each well, the final required concentrations will be attained.
5. Mix the contents of each tube containing all components thoroughly using a vortex mixer. This is now the STEMGlo™-96 Research Culture Master Mix.
6. Remove the sterile, 96-well plate from the plastic covering under the hood.
7. Using calibrated and preferably electronic pipette, take up as much of the Culture Master Mix as possible without including bubbles.
8. Prior to dispensing into the first well, discard the first amount back into the tube. This ensures that the tip of the syringe is completely full and that the correct amount (0.1ml) will be dispensed into the well.
9. Carefully dispense 0.1ml of the Culture Master Mix into the bottom of each well according. If 4 replicate wells are used/sample, these can be dispensed either in columns or in rows. If 6 replicate wells/sample are prepared, they should be dispensed in rows across the plate.
10. After dispensing all of the sample wells, replace the lid on the 96-well plate and transfer the culture plate to a humidity chamber to ensure high humidity during incubation (See section 8). **NOTE**: It is not necessary to use the whole

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

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

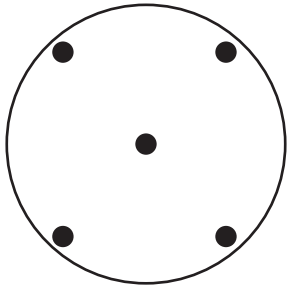

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plate at once. Sterile adhesive foils are included so that unused wells can remain sterile for later use (Section 9).

11. 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.
12. The cell incubation time will depend on the species and cell population being determined. It may be necessary to perform a time course to determine the optimum culture period. IMPORTANT. Once an incubation period has been elected, it should be used continually and should not be changed. If the incubation period is changed, it will not be possible to compare results between different samples setup on different days. It is therefore important to maintain the elected incubation period.

STEP 3 – LUMINESCENCE MEASUREMENT

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

IMPORTANT: PLEASE REFER TO SECTION 9 ON HOW TO SETUP THE PLATE LUMINOMETER. The instrument should be setup and prepared for use prior to any of the following steps being performed.

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

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

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

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

A. ATP Standard Curve for Use with Samples that are Expected to Exhibit High Proliferation.
It is important that the sample ATP values measured are within the range of the ATP dose response curve. This is, in part, determined by the number of cells plated, since the ATP concentration correlates directly with the cell dose. For samples expected to produce high ATP concentrations (high cell proliferation), it is recommended to perform an ATP standard curve from 0.03µM to 3µM. 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. For samples with low proliferation, use the ATP dose range from 0.01µM to 1µM. The ATP standard curve as follows.

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
   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 high control into wells E4, F4, G4, H4.
17. Add the required amount of ATP-ER to a non-sterile reagent reservoir.

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. Using a multichannel pipette, add 0.10ml to each well of the first column (A1-H1).
19. Mix the contents as directed in Section 9 by repeated pipetting and discard the tips.
20. Change tips for each new addition of ATP-ER.

B. ATP Standard Curve for Samples that Exhibit Low Proliferation

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

standard dose responses.

- The amount of ATP-ER added to each well is 0.10ml. Therefore:
  Total amount of ATP-ER (µl) required = 0.1ml x (number of wells used + 24 (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 are provided so that the part of the plate that is not being used can be covered and kept sterile until required. If using the adhesive film provided, the plate cover should be removed in a laminar air-flow hood and replaced with the film to ensure sterility.

Mixing the Contents of 96-well Plate

Besides mixing the contents of the tubes prior to plating the STEMGlo™-96 Research 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.

STEMGlo™-96 Research includes solid, white plates that have been optimized for use. Do not use different plates for the assay. Doing so will result in inaccurate results and invalidation of the assay kit warranty. Extra plates can be purchased from HemoGenix®.

Bioluminescence Assay Kit Components

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

Reconstitution of Lyophilized Monitoring Reagent (if included)

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

Volumes of Luminescence Kit Components Required

- Each vial of ATP standard contains enough volume to perform one or two ATP

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2. Now dispense:
   0.90ml of the medium to vial #1.
   0.35ml of the medium to vial #2
   0.90ml of the medium to vial #3
   0.90ml of the medium to vial #4
   0.90ml of the medium to vial #5
3. Remove 0.1ml of the supplied stock ATP solution (at 10µM) and transfer it to vial #1. Mix by vortexing. This ATP concentration is 1µM.
4. Dispense 0.35ml from vial #1 to vial #2. Mix. This concentration is 0.5µM.
5. Dispense 0.10ml from vial #2 to vial #4. Mix. This concentration is 0.05µM.
6. Dispense 0.10ml from vial #1 to vial #3. Mix. This concentration is 0.1µM.
7. Dispense 0.10ml from vial #3 to vial #5. Mix. This concentration is 0.01µM.
8. Dispense 0.10ml of the supplied medium alone into wells A1, B1, C1 and D1 on the non-sterile luminescence plate(s) provided. NOTE: This is to measure background luminescence.
9. Dispense 0.10ml from ATP dilution in vial #5 to wells E1, F1, G1, and H1.
10. Dispense 0.10ml from ATP dilution in vial #4 to wells A2, B2, C2, and D2.
11. Dispense 0.10ml from ATP dilution in vial #3 to wells E2, F2, G2, and H2.
12. Dispense 0.10ml from ATP dilution in vial #2 to wells A3, B3, C3, and D3.
13. Dispense 0.10ml from ATP dilution in vial #1 to wells E3, F3, G3 and H3.
14. Add the required amount of ATP-ER to a non-sterile reagent reservoir.
15. Using a multichannel pipette, add 0.10ml to each well of the first column (A1-H1).
16. Mix the contents as directed in Section 9 by repeated pipetting and discard the tips.
17. Change tips for each new addition of ATP-ER.

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

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

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

C. Sample Measurement

IMPORTANT. It is very important that the ATP-ER is mixed properly with the well contents using the procedure described in Section 9. If the cells are not mixed correctly with the ATP-ER, the cells may not be lysed and lower ATP concentrations will be measured.

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 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 samples. (See Section 9, Adhesive Plate Covering Film).
3. Using a calibrated multichannel pipette (8- or 12-channel 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 **always using new pipette 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.
6. Incubate the plate in the reader for the last 2 min in the dark to stabilize the plate.
7. Unused ATP-ER may be returned to the bottle and refrozen. See section 9 for ATP reagent storage conditions and stability.

D. Using a plate luminometer with automatic dispenser

The user may have a plate luminometer that allows reagents to be dispensed automatically directly into the well. Please do not use the automatic dispensers, since the contents of the well will not mixed sufficiently using this method.

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8. RECOMMENDATIONS AND TIPS PRIOR TO USING STEMGlo™-96 Research ASSAY KIT.

(i) Cell Suspension

A pre-defined and validated protocol for prepared the cell suspension or purifying the stem cells for culture should already be in place prior to performing STEMGlo™-96 Research.

(ii) Number of Replicates Performed

STEMGlo™-96 Research can be used to measure as many replicates as necessary. However, it is recommended to use 6 replicates. Please remember that using a lower number of 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.

(iii) 96-Well Plates Provided

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

(iv) 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.

(v) Incubation Times

The culture time will depend on the cells, growth factor/cytokine concentration, cell dose and species. The 96-well plate provided are usually solid, white plates for bioluminescence. To determine an optimum incubation time period, select a cell dose (e.g. 5,000 cells/well) and perform a time course experiment (e.g. 2-7 days). The plates have a solid growth surface to reduce light scatter from one well to another. 96-well plates with a transparent growth surface are also available.