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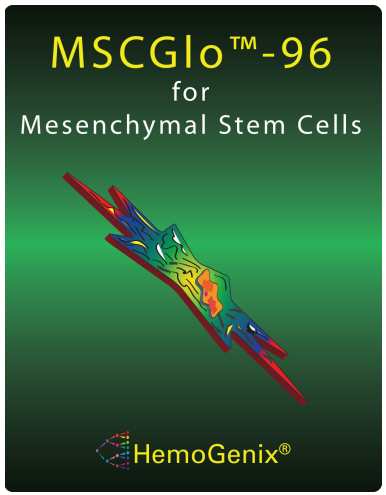

MSCGlo™-96 & MSCGlo™-96 Complete
for Mesenchymal Stem Cells

Assay Manual

(Version 4-2014)

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

Please read this manual in its entirety prior to using the assay kit

HemoGenix®, Inc

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1. INTRODUCTION

Mesenchymal stem cells (MSC), also called mesenchymal stromal cells (MSC) or mesenchymal progenitor cells (MPS) are fibroblastoid-like proliferating cells found in bone marrow, umbilical cord blood and several other sources. The cells can also be produced from induced pluripotential stem (iPS) cells and iPS cells can be produce MSCs. Mesenchymal stem/stromal cells are, in part, responsible for producing the hematopoietic stroma facilitating hematopoiesis. In the presence of specific growth factors and/or cytokines, they are responsible for chondrogenesis, adipogenesis and osteogenesis, but can also produce several other cell types.

Mesenchymal stem/stromal cells are difficult to detect as primary, explanted cells from normal sources. They are usually grown on adherent surfaces from a mixed population of cells. They are identified by the presence of membrane expression markers detected by flow cytometry. The MSC population is normally positive for CD73, CD90 and CD105 as well as CD29, CD44 and CD166, but negative for CD45 and CD34. The only functional assay for MSC has been the colony-forming unit - fibroblast (CFU-F) assay first developed by Friedenstein and colleagues in 1974. This assay requires that the MSC source cells or expanded MSC be cultured on adherent growth surfaces at 2-3 cell concentrations during which time, fibroblast-like colonies are produced. Since the CFU-F is a proliferating cell population, the time at which the colonies are counted has to be chosen so that the colonies do not grow together and form a confluent layer of cells over the culture plate. Not only is this CFU-F assay subjective and inaccurate, but also lacks standards and controls. As a result, the CFU-F assay cannot be calibrated or standardized and therefore cannot be validated.

MSCGlo™-96 and MSCGlo™-96 Complete directly measure the proliferation, viability and cellular functionality of MSCs. The assay can also be used to measure the number of MSCs after culture. The MSCGlo™ Platform is one of several assay platforms from HemoGenix® that incorporate bioluminomics™ technology. Bioluminomics™ is the instrument-based, quantitative measurement of iATP concentrations using a luciferin/luciferase bioluminescence signal detection system, the most sensitive and accurate, non-radioactive readout available. Bioluminomics™ technology encompasses the ability to calibrate, standardize and, if required, fully validate the assay. This technology ensures that the results obtained are trustworthy, reliable and reproducible, not only within one laboratory, but between laboratories.

All MSCGlo™ assay kits include high performance MSCGro™ Medium. The medium obtained with the kit and/or separately to grow and expand MSC is available containing either low-serum, serum-free or humanized formulations. MSCGlo™-96 Complete is a "turnkey" assay system that includes cryopreserved cells, medium and bioluminomics™ readout.

MSCGlo™-96 and MSCGlo™-96 Complete allows the user to either culture MSCs directly in the 96-well plates provided or to use the assay to monitor MSCs proliferation, expansion and even differentiation.

2. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. **Please read this manual in its entirety prior to using the MSCGlo™-96 Assay Kit. For technical questions after reading the manual, please contact HemoGenix® prior to use.**
2. **MSCGlo™-96 and MSCGlo™-96 Complete are not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)**
3. **MSCGlo™-96 and MSCGlo™-96 Complete are for research use only and neither have 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 wear a laboratory coat, safety glasses and laboratory gloves for all procedures.**

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

Luminescence Reagent Mixing.

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

Culture Plates Drying Out

- Due to the relatively small culture volume (0.1ml), drying out of the culture wells, particularly around the outside of the plate, can be a problem. These are called "edge effects". An incubator with insufficient humidity will cause this problem. To ensure that this does not occur, the incubator water reservoir should be full and the humidity in the chamber checked using a hygrometer.
- If drying out continues, use of a humidity chamber is recommended. Please refer to Section 8 (vi) for instructions on how to build a humidity chamber.

12. Troubleshooting

High Coefficients of Variation (%CV)

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

- Accurate reagent dispensing and mixing are of prime importance. Since the volumes dispensed are small it is imperative to use instruments that have been properly calibrate to avoid pipetting error.
- Insufficient mixing of components prior to plating the cell culture master mix and insufficient mixing during the addition of luminescence reagents to cultures in the 96-well plate can also lead to high C--Vs.
- 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. Fractionations of the cells on a density gradient can dramatically improve the cell viability.
- *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 plates without a transparent growth surface cannot be viewed under a microscope. If contamination occurs it will usually

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3. INTENDED USE

MSCGlo™-96 is intended for use only with mesenchymal stem/stromal cells (MSCs) derived from, but not limited to:

- Bone marrow
- Umbilical cord blood
- Wharton's jelly
- iPS cells

and obtained from the following species:

- Human
- Non-human primate
- Rat
- Mouse

Primary applications using MSCGlo™-96 include:

- MSC production from different sources
- MSC proliferation under different conditions
- MSC proliferation potential (primitiveness) to distinguish different MSC populations
- Monitor growth and expansion of MSC
- *In vitro* to *in vivo* studies during regenerative research applications
- Differentiation of MSC from proliferating to non-proliferating cells
- Multiplexing to combine MSC proliferation with phenotypic or gene expression analysis as well as other MSC functions.

For MSC quality control and potency in cellular therapeutic or regenerative medicine applications, it is recommended to use MSCGlo™-96 HuQC or MSCGlo™-96 PQR, respectively. For detecting and measuring MSC toxicity, MSCGlo™-Tox HT, in 96- or 384-well plate formats, can be used for medium to high throughput screening.

MSCGlo™-96 Complete is similar to MSCGlo™-96, but includes a vial of cryopreserved cord blood-derived mesenchymal stem/stromal cells. MSCGlo™-96 Complete is intended to be used for similar applications to MSCGlo™-96, but would be cord blood MSC-specific.

4. The CONCEPT and PRINCIPLE of BIOLUMINOMICS™ ASSAYS

MSCGlo™-96 and MSCGlo™-96 Complete are bioluminomics™ assays. 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 increases several fold. The iATP concentration produced is directly dependent on:

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

The target cells are incubated with growth medium 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 MSCGlo™-96 and MSCGlo™-96 Complete. 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 standardizes the assay.
2. The controls calibrate the assay and ensure that the reagents are working correctly.
3. The ATP standard curve allows the luminometer output in Relative Luminescence Units (RLU) to be converted to standardized ATP concentrations (µM).
4. Performing the ATP standard curve allows results to be compared over time.

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

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

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11. MSCGlo™-96 and MSCGlo™-96 Complete ASSAY PARAMETERS

MSCGlo™-96 bioluminomics™ technology allows the assay to be calibrated and standardized. Inclusion of an ATP standard and controls are requirements for assay validation.

Assay Characteristics

When performing the ATP standard curve using IMDM to dilute the ATP standard provided, the following parameters should be obtained:

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

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

For samples:

- Lowest ATP value indicating unsustainable stem cell proliferation: ~0.04µM
- ATP value below which cells are not usually metabolically viable: ~0.01µM.

If these parameters are not attainable, please refer to Troubleshooting (Section 12).

Assay Validation Parameters

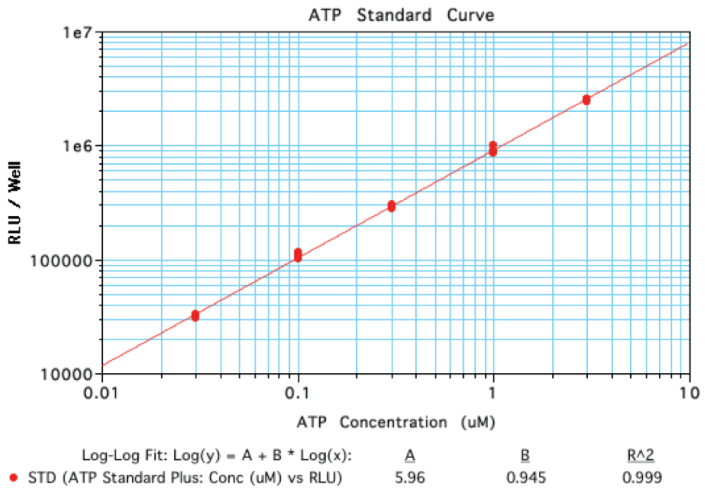
- Assay ATP linearity => 4 logs
- Assay cell linearity: 1,000 - >25,000 cells/well
- Assay ATP sensitivity: ~ 0.001µM
- Assay cell sensitivity: 20-25 cells/well (depending on purity and growth factor cocktail)
- Accuracy (% correct outcomes): ~95%
- Sensitivity and specificity detected by Receiver Operator 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 are for human cells and may be different for different species and cell sources as well as the passage number.

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

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

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



5. OVERVIEW OF THE MSCGlo™-96 and MSCGlo™-96 COMPLETE PROCEDURE

There are 3 steps to using MSCGlo™-96 and MSCGlo™-96 Complete.

Step 1 – Cell Preparation
Cryopreserved MSC are only provided with the MSCGlo™-96 Complete assay kit. Cells are not provided with MSCGlo™-96 assay kits. Source cells for MSC should be prepared according the investigator's own protocol. A dye exclusion viability and/or metabolic viability and nucleated cell count should be performed on all samples.

Step 2 – Cell Culture
MSCGlo™-96 can be used in either of two ways. Cells can be produced and expanded in tissue culture vessels and after each passage, the MSC measured in a small, but representative aliquot of the cells. Alternatively, the cells can be cultured directly in the sterile 96-well plates provided with the assay kit and their proliferation measured directly in the well without removing the cells. Regardless of whether MSC proliferation is measured after passage and expansion or directly in the 96-well plate, the MSCs should be cultured using MSCGro™ medium. This is provided with the kit for growing MSCs in the 96-well plate(s). For passaging and expansion purposes, it is recommended to purchase MSCGro™ medium in larger quantities. MSCGro™ media is available as a low serum or with humanized serum or as a serum-free formulation.

MSCGlo™-96 Complete includes a vial of cryopreserved cord blood-derived MSCs. The cells should be thawed and expanded by passaging at least twice prior to using the cells and the assay kit. Once expanded, MSCs can be used in the same manner as MSCs for the MSCGlo™-96 assay kit. The MSCGlo™-96 Complete kit includes MSCGro™ medium of choice; low serum, serum-free or humanized. Sufficient medium is included to initially expand the cells included with the kit. It is recommended to purchase extra medium for continued use of the cells.

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 a short incubation period (10min) 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 diluted in IMDM provided and controls (all provided with the kit).

6. MSCGlo™-96 and MSCGlo™-96 COMPLETE KIT CONTENTS and STORAGE CONDITIONS

MSCGlo™-96 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. MSCGlo™-96 Complete contains cryopreserved MSC shipped on dry ice. The following components are included:

Item	Component	Storage
1	MSCGro™ Medium (low serum, serum-free or humanized).	Store at 4°C in a refrigerator
1A	1 vial of frozen, cord blood-derived MSCs. Included in the MSCGlo™-96 Complete Assay Kit only.	Remove from shipping container and immediately transfer to liquid nitrogen for continued storage. DO NOT store cells at -80°C
2	Iscove's Modified Dulbecco's Medium (IMDM)	-20°C until used
3	ATP standard.	-20°C until used
4	ATP extra high, high and low controls.	-20°C until used
5	ATP Enumeration Reagent (ATP-ER)*	-20°C in the dark until used
6	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Store at room temperature
7	Sterile, 96-well plates for stem cell culture	Store at room temperature
8	Non-sterile 96-well plates for ATP standard curve determination.	Store at room temperature
9	Instruction manual.	

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

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

IMPORTANT
All kit components are quality controlled and optimized so that they work together. 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®.

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 calculations and graphs:

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) = 0.945 ± 0.18 (%CV = 1.9). This is slightly different to the value given in Section 11.

(Values are the Mean ± 1 Standard Deviation)

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

Equipment and Supplies

1. Laminar Flow Biohood.
2. Plate luminometer (e.g. Molecular Devices, SpectraMaxL; Berthold, CentroLia).
3. Gloves to handle cells stored in dry ice and liquid nitrogen (for MSCGlo™-96 Complete kit only).
4. Water bath at 37°C for thawing frozen cells (MSCGlo™-96 Complete Kit only).
5. Sterile, capped, plastic tubes (5ml, 10ml, 50ml).
6. Sterile tissue culture flasks (for MSCGlo™-96 Complete).
7. Single channel pipettes, preferably electronic (e.g. ViaFow or Rainin EDP pipettes for variable volumes between 1µl and 1000µl).
8. 8 or 12-channel pipette, preferably electronic (e.g. ViaFlow or Rainin EDP pipettes for fixed or variable volumes between 10µl and 100µl).
9. Reservoir for 8- or 12 channel pipette .
10. Sterile pipette tips.
11. Vortex mixer.
12. Humidity chamber.
13. Tissue culture incubator, humidified at 37°C with 5% CO_2 (minimum requirement) and 5% O_2 (preferable).
14. 1.5ml plastic vials (5 for each ATP dose response).
15. Hemocytometer or electronic cell counter to determine cell concentration.
16. Flow cytometer or hemocytometer for determining viability and characterization of MSCs.

Reagents

1. Sterile Phosphate Buffered Saline (PBS).
2. DNase (Sigma-Aldrich, Catalog No. D4513-1VL) for thawing cryopreserved samples.
3. Density-gradient medium (e.g. NycoPrep 1.077, Axis-Shield).
4. Trypsin/EDTA or Accutase (Innovative Cell Technologies, San Diego, CA) to remove cells from the growth surface.
5. 7-AAD, propidium iodide, trypan blue or other dye exclusion viability assay.
6. Additional MSCGro™ medium (HemoGenix®, Inc).

8. The MSCGlo-96™ and MSCGlo™-96 Complete PROTOCOL

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

Using MSCGlo-96™ or MSCGlo™-96 Complete is a 3-step process.

Step 1 – Cell preparation.
Step 2 – Cell culture, plating and incubation.
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

Preparation of the cells depends on the species and source of cells.

MSC Derived from Fresh Tissue
When MSC are to be obtained from fresh, primary tissues, e.g. bone marrow, umbilical cord blood etc., it is recommended to start with a mononuclear cell (MNC) population that has been prepared by density gradient centrifugation to remove red blood cells, granulocytes and platelets. For density gradient centrifugation, it is recommended to use NycoPrep 1.077 since this is non-toxic to cells. For MSCs derived from species other than human cells, the density and osmolarity of the medium may have to be changed.

MSCGlo™-96 Complete and Cryopreserved MSC
MSCGlo™-96 Complete assay kits include cryopreserved, umbilical cord blood MSC. A separate protocol for thawing and expanding the cells is provided with this kit.

Cells that have been passaged and expanded followed by cryopreservation and storage in liquid nitrogen should be thawed using DNase to reduce the possibility of clumping. Clumping occurs when large amounts of DNA are released from thawed cells that rupture during the process. DNase should be included with the thawing medium at a final concentration of 6µg/ml.

Cell Viability
Cell viability can be determined either manually using a dye exclusion stain and a hemocytometer or can be determined by flow cytometry using 7-AAD. The viability should be 85% or greater for optimal results. Viabilities lower than 85% will produce sub-optimal results or unsustainable MSC proliferation.

Flow Cytometry
Prior to and after MSC culture (regardless of the method used for MSC culture, see below), it is recommended to perform and ascertain the proportions of membrane expression markers that are used to define MSCs as well as markers for the presence of non-MSC, contaminating cells.

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11. LUMINOMETER SETUP AND CONVERSION OF RLU VALUES TO ATP VALUES USING THE ATP STANDARD 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 luminescence 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 curve and a log-log linear regression least squares analysis.

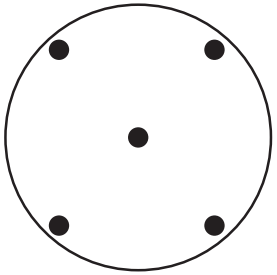
(i) Luminometer Setup
Multiparameter instruments, i.e. those that can detect absorbance, fluorescence and luminescence, often need to be manually set for both the integration time and the "gain". Dedicated instruments, i.e. those that only detect luminescence, usually only have to be set for the "integration 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 22ºC and 24ºC 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 manufacturer to determine whether the software can provide the information below

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



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Cell Concentrations
The assay shows linearity prior to the cells becoming confluent. However, for normal MSC proliferation analysis, a final concentration of 1,500 - 2,000 cells/well is sufficient. The working cell suspension concentration (cells/ml) must be 10 x the concentration of the final cell concentration/well. The cell suspension (cells/ml) is prepared in MSCGro™ medium provided. Each well receives 0.1ml of the cell suspension.

STEP 2. Cell Culture

Please refer to Section 9 for recommendations and tips prior to beginning this stage of the procedure.

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

MSC Expansion prior to ATP Measurement.
Mesenchymal stem cells are often passaged and expanded in flask cultures or other vessels as well as 3D-culture systems. The initial tissue source is usually started in small flask cultures. The cells should be expanded in MSCGro™ medium at a concentration between 5,000 and 10,000 cells/cm^2. Each time the cells are passaged for expansion, an aliquot of the cells should be analyzed by measuring intracellular ATP. MSCs are adherent cells and require an established trypsin/EDTA or Accutase® protocol to release the cells from the growth surface of the flask every time the cells are passaged.

Once the cells have been removed, perform a cell count. To measure MSC proliferation, only a single cell dose is usually required. A final concentration of 5,000 cells/well is usually sufficient. Dispense 0.1ml of each cell concentration into 6 replicate wells of the sterile 96-well plate(s) provided. The intracellular ATP concentration, as a measure of proliferation, can be detected immediately. Alternatively, the cells can be incubated for 24h and then measured.

Special Instructions for MSCGlo™-96 Complete
When passaging and expanding cells provided with MSCGlo™-96 Complete, it is possible to perform a minimum 3-point cell dose response after each passage. The 3 point cell dose response can also be performed so that the final cell concentration/well lies between 1,000 and 5,000 cells/well. For example, 1,250 cells/well. 2,500 cells/well and 5,000 cells/well. The ATP results should indicate an approximate doubling of ATP concentration with cell dose. Calculate the slope of linear regression cell dose response. The steeper the slope, the greater the proliferation potential of the MSCs. If the slope of the dose response curve starts to decrease with time, the MSCs are beginning to loose their proliferation potential and therefore their effectiveness.

MSC Culture in 96-Well Plates
Mesenchymal stem/stromal cells can also be cultured directly in the sterile, 96-well plates provided with the assay kit. It is recommended to culture cells at between 1,500 - 2,000 cells/well. Alternatively, a time and/or cell concentration growth curve for MSC can also be performed. The time growth curve will provide information on the MSC growth kinetics, which can then be compared with different MSC batches to help determine optimal procedures.

Regardless of the method used to grow MSCs, stringent control of culture time periods

and cell concentrations prior to measuring bioluminescence is important to be able to compare results.

To culture MSCs in the sterile, 96-well plates provided, the following procedure is recommended:

1. Prepare cells according to the user-defined procedures (STEP 1).
2. Remove the MSCGro™ medium from the kit and warm to 37°C in an incubator or water bath.
3. Determine the cell concentration of the cell suspension.
4. Prepare a working cell dilution that is 10 fold greater in concentration than the required final dilution in the well. For example, if the final dilution is to be 2,000 cells/well, prepare a dilution that is 20,000 cells/ml. Prepare a min. 1ml of this working concentration in MSCGro™ medium.
5. Using a calibrated pipette, preferably an electronic pipette, dispense 0.1ml into each of 6 wells of the sterile 96-well plate. This reduces the cell concentration 10 fold so that the final cell concentration in each replicate well will be the desired final cell dose. When dispensing 6 wells, dispense in rows, i.e. A1-A6, B1-B6 etc. This will allow 16 samples to be tested on a single plate.
   NOTE: It is not necessary to use the whole plate at the same time. See Step 3, Sample Measurement.
6. Place the 96-well plate in a humidity chamber (see Section 9 (iv) and transfer the humidity chamber to a humidified incubator.
7. Incubate the cells at 37°C in a fully humidified atmosphere containing 5% CO_2 and, if possible, 5% O_2. The plating efficiency of MSC is increased under low oxygen tension compared to atmospheric oxygen tension (approx. 21% O_2).
8. After 24hr, the MSC will have attached to the growth surface of the plate. Gently swirl the plate to suspend the non-adherent cells in the media. Using a manual pipette or vacuum apparatus, remove 50-75% of the medium from each well being careful not to touch the bottom of the well.
9. Dispense another 0.1ml of pre-warmed (37°C) fresh MSCGro™ medium to each well and return plate to the incubator.
10. Monitor the MSC growth using an inverted microscope.
11. When the cells have grown to approx 70%-80% confluency, the proliferation status can be measured.
12. If performing a growth curve, 6 replicate wells should be prepared for every day of the study that will be measured.

Please Note: Although MSCGro™ medium is supplied with the MSCGlo™-96 and MSCGlo™-96 Complete assay kits, other medium can be used. It is, however, recommended to compare results using MSCGro™ and any other medium that is being tested. In addition, the above procedure can be modified to accommodate the investigator's own protocols. Always compare other protocols with that described above.

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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)).

96-Well Plates for 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 master mix tubes after cells have been added prior to plating, 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.

10. 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.

MSCGlo-96™ includes clear-bottom white plates for both cell culture and the ATP standard curve and controls. Do not use different plates for the assay. Doing so will result in inaccurate results and invalidation of the assay kit warranty. Extra plates can be purchased from HemoGenix®.

Bioluminescence Assay Kit Components

- Prior to measuring bioluminescence, remove the ATP standard (low or high), 1 set of ATP controls (low, high or extra high) 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 2 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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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 11 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 10 for recommendations and tips prior to starting this part of the procedure. In particular, please refer to Section 10 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.

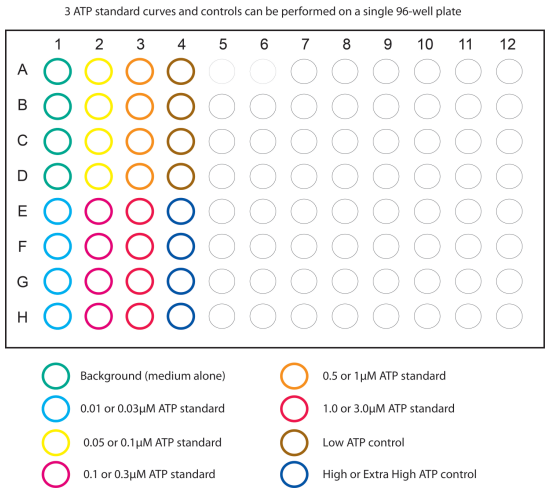
Use the unwrapped, non-sterile, 96-well plate(s) provided with the kit to perform the ATP standard curve and measure the controls.

To measure the ATP concentration accurately, it is necessary for the sample ATP concentration to lie within the range of the ATP standard curve. MSCGlo™-96 and MSCGlo™-96 Complete assay kits include sufficient ATP standard so that a high or low ATP standard curve can be performed depending on the proliferation capability of the cells being tested. When MSCs are grown in MSCGro™ medium, they will usually exhibit high proliferation rates. It can be expected that with time and repeated passaging of MSCs, the proliferation and, therefore the generation time, will decrease. If the cells are in an early passage, the cells should be expected to demonstrate high proliferation and contain high intracellular ATP concentrations. If this is the case, perform an ATP standard curve from 0.03µM to 3µM. If cells are expected to have low proliferation, then an ATP standard curve from 0.01µM to 1µM should be performed.

IMPORTANT: ***For the background and ATP standard curve dilutions, it is important to use the IMDM provided with the assay kit.***

NOTE: To perform an ATP standard curve, background and controls, 4 columns of the 96-well plate will be used. The remaining wells will be used to perform the ATP standard curve, background and controls on other days samples are analyzed. **The configuration of the non-sterile, 96-well plate to perform the ATP standard curve and controls is shown in Figure. 1.**

Figure 1. Plate Configuration for the ATP Standard Curve and Controls



A. High ATP Standard Curve Preparation for Use with MSCs Exhibiting High Proliferation or High Intracellular ATP (iATP) Concentrations.

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 to vial #2
   0.90ml IMDM to vial #3
   0.90ml IMDM to vial #4
   0.90ml IMDM to vial #5
3. Dispense 0.7ml of the IMDM 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 IMDM 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.

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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.

(vii) Incubation Times

The incubation time is dependent upon a number of factors. It is important to regularly measure cell proliferation when the cells are growing exponentially. With increase in incubation time, there may also be an increase in coefficients of variation. Once an incubation time has been determined, this incubation time should be maintained so that results can be directly compared over time.

9. RECOMMENDATIONS AND TIPS PRIOR TO USING THE MSCGlo™-96 KIT.

(i) Cell Suspension

a. For MSC preparation, a nucleated cell suspension is recommended.
b. Extraneous ATP, red blood cells (which have high concentrations of ATP) and hemoglobin interfere with the ATP analysis. Wear laboratory gloves at all times and ensure that the cell suspension does not contain high concentrations of red blood cells or hemoglobin.
c. If cells have been treated prior to cell culture, higher cell concentrations may be required.

(ii) Dispensing the Cell Culture Suspensions

It is strongly recommend using electronic pipettes to perform all dispense operations and to dispense into individual wells. This provides greater accuracy. Mix frequently during dispensing. Mesenchymal stem/stromal cells are large and can sink rapidly.

(iii) Number of Replicates Performed

It is recommended to use 6 replicates/sample. Please remember that using fewer replicates may save components in the short term, but may also cause inconclusive results. If outliers are encountered, which may have to be removed from the analysis, the consequence could be that extra experiments would be required resulting in extra time and costs.

(iv) Plate Configuration

Performing 6 replicates/well means that the sample replicates are plated across the plate, for example from A1 to A6, A7 to A12 or B1 to B6. In this manner 16 samples can be tested on a single plate. If samples are tested across the plate from, for example A1 to A6 and then from A7 to A12, use a 12-channel pipette to dispense and mix the ATP-ER. If it is preferred to dispense the samples from A1 to A6 followed by B1 to B6 etc., then it is suggested to use an 8-channel pipette. Only attached sufficient pipette tips for the number of samples to be processed.
For 8 replicates/sample, configure the plate to use columns, e.g. A1-H1, A2-H2 etc. and an 8-channel pipette.

(v) Multiwell Plates Provided

The reagents have been optimized to work with the plate(s) provided in the MSCGlo™-96 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.

(vi) Humidity Chamber

A humidity chamber is recommended due to the small sample volumes used (0.1ml). 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

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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 10 by repeated pipetting and discard the tips.
20. 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 10 ON HOW TO MIX THE WELL CONTENTS CORRECTLY. THIS PROCEDURE IS ALWAYS USED WHEN MIXING REAGENTS FOR BIOLUMINESCENCE MEASUREMENT IS REQUIRED.

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. Low ATP Standard Curve Preparation for MSCs Exhibiting 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
2. Now dispense:
   0.90ml of IMDM to vial #1.
   0.35ml of IMDM to vial #2
   0.90ml of IMDM to vial #3
   0.90ml of IMDM to vial #4
   0.90ml of IMDM 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 IMDM 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. 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.

IMPORTANT: Mixing the contents of each well properly is a very important procedure. PLEASE REFER TO SECTION 10 ON HOW TO MIX THE WELL CONTENTS CORRECTLY. THIS PROCEDURE IS ALWAYS USED WHEN MIXING REAGENTS FOR BIOLUMINESCENCE MEASUREMENT IS REQUIRED.

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.

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

C. Sample Measurement

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

1. If possible, place the sample plate(s) in a humidified incubator set at 22-23˚C gassed with 5% CO_2 for 30min. 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 10, Adhesive Plate Covering Film).
3. Using a 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 using new tips.
5. When ATP-ER has been added to all wells, replace the plastic cover and incubate for a total of 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. Remember to remove the lid prior to putting the plate in the luminometer.
6. Unused ATP-ER may be returned to the bottle and refrozen. See section 9 for ATP reagent storage conditions and stability.

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D. Using a plate luminometer with automatic dispenser

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

E. Using a liquid handler

MSCGlo™-96 can be performed in high throughput mode. If you intend to perform any part of the MSCGlo™-96 procedure using a liquid handler, it will be necessary to obtain additional ATP-ER since larger volumes of ATP-ER are required when using a liquid handler. Please contact HemoGenix® for information on setting up the instrument.