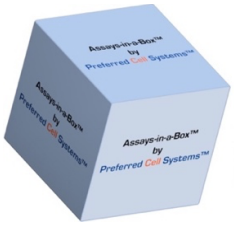

Assays-in-a-Box™
by
Preferred Cell Systems™

MSCGlo™

A Bioluminescence Proliferation Assay for Mesenchymal Cells (MSC)

Technical Manual

(Version 06-24)

This manual should be read in its entirety prior to using this product

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

No part of this instruction manual may be copied, duplicated or used without the express consent of Preferred Cell Systems™

KM022.002

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1. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. *MSCGlo™ is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)*
2. *MSCGlo™ is for research use only and has not been approved for clinical diagnostic use.*
3. *Reagents and supplies in this kit are STERILE. Perform all procedures under sterile conditions, except where indicated.*
4. *This kit should not be used beyond the expiration date on the kit label.*
5. *Do not mix or substitute reagents or other kit contents from other kit lots or sources.*
6. *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.*
7. *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.*

2. Introduction

MSCGlo™ is a proliferation assay platform that detects and quantitatively measures mesenchymal stem cells (MSC).

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 tissue sources. The cells can also be produced from induced pluripotential stem (iPS) cells. 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, MSC are responsible for chondrogenesis, adipogenesis and osteogenesis, but can also produce several other cell types.

Mesenchymal stem cells are often detected by their phenotypic (or lack of) profile. The MSC populations are usually CD73, CD90 and CD105 positive as well as CD29, CD44 and CD166 positive, but are negative for CD45 and CD34. Besides the aforementioned membrane characteristics, MSC are also characterized by their ability to adhere to a growth surface and their ability to produce colonies in the colony-forming unit - fibroblast (CFU-F) assay. Since MSCs are a proliferating cell population, the CFU-F assay has the distinct disadvantage of not only being subjective and lacking quantitative evaluation, but cultures have to be enumerated when the colonies are discrete and not growing together.

Mesenchymal stem cells are rarely used as a native population. Instead, they are often passaged and expanded in tissue culture flasks or reactors. The cells also have a definite life span, with proliferation potential usually decreasing with time in culture. Often, the cell doubling time, rather than passage number is used as a measure of proliferation ability or potential.

All mammalian cells require chemical energy in the form of intracellular adenosine triphosphate (iATP), which is also a biochemical indicator of viability, functionality and cell proliferation. The amount of iATP produced by a cell correlates directly with its functional status. The most sensitive non-radioactive readout to measure cell proliferation is iATP using a luciferin/luciferase bioluminescence signal detection system. This concept is used in the MSCGlo™ assay.

MSCGlo™ contains everything necessary to culture and measure MSC proliferation. An optional ATP Bioluminescence Standardization and Calibration Kit (Catalog Number: K-ATPSC-1) is available to convert and standardized ATP concentrations (µM) from Relative Luminescence Units (RLU) produced by the luminescence plate reader. Although this is not required, it is highly recommended, since it provides the measurement assurance that the assay is working correctly and that experimental results can be directly compared. MSCGlo™ can also be multiplexed with phenotypic analysis by flow cytometry or even genetic analysis of the cells.

3. Use and Availability

MSCGlo™ is used to quantitatively measure MSC proliferation under different research conditions and from different sources and species. As a standardized assay, MSCGlo™ can be used to enhance cell doubling time or replace it.

MSCGlo™ can be used for MSCs from the following tissues:

- Bone marrow
- Umbilical cord blood
- Adipose tissue
- Wharton's jelly
- iPS or even ES cells
- Any other sources

MSCGlo™ can be used with the following tested species:

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

MSCGlo™ is a 1 x 96-well plate assay kit, but is also available in larger kit sizes upon request.

MSCGlo™ Complete is a "turnkey" assay kit that include a vial of cryopreserved MSCs derived from either of the following sources:

- Human bone marrow
- Human umbilical cord blood
- Mouse bone marrow

MSCGro™ Media

- Low serum, complete
- Serum-free, xeno-free, complete

4. The Concept of ATP Bioluminescence Assays

MSCGlo™ is a ATP bioluminescence assay. The fundamental concept underlying the assay is the measurement of the cell's chemical energy in the form of intracellular adenosine triphosphate (iATP). If a cell is producing iATP, it is demonstrating cellular and mitochondrial integrity and is therefore viable. When hematopoietic cells are stimulated to proliferate, in cultured with growth factors and/or cytokines, the iATP concentration increases several fold. The iATP concentration produced is directly dependent on:

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

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

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

The bioluminescence emitted is detected and measured in a luminescence plate reader as relative luminescence units (RLU). The ATP Bioluminescence Standardization and Calibration Kit (Catalog Number: K-ATPSC-1) can be used to calibrated and standardize the MSCGlo™ Assay. Performing an ATP standard curve and controls has the following advantages:

1. The controls calibrate the instrument and also ensure that the reagents are working correctly.

2. The ATP standard curve also ensures 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.
5. The results obtained from controls and standard curve should be compared with those provided in Section 12. These are the measurement assurance parameters that allow the investigator to ensure that the assay is working correctly prior to measuring samples. When the values from the controls and ATP standard curve are within the ranges provided in Section 12, the investigator can consider the results trustworthy.

The ATP standard curve and controls need only be measured once on the day samples are to be processed. Do not use previous results from an ATP standard curve and controls performed on a different day. This will cause erroneous results.

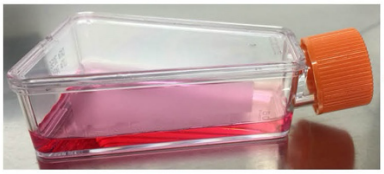
The ATP standard curve is used to convert sample RLU results into ATP concentrations by interpolation. This procedure can often be performed automatically by the instrument software. If the software does not allow this, it will be necessary to use third-party software to perform this operation.

NOTES

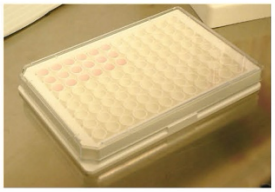
5. QuickGuide to MSCGlo™ (Figure 1)

QuickGuide to MSCGlo™

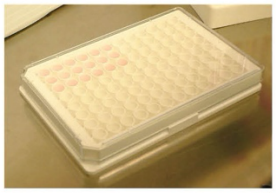
STEP 1
Expand and/or passage MSCs on a coated surface in flask or 96-well plate (provided) in MSCGro medium for a specified time at 37°C in 5% CO_2 and 5% O_2 (preferred)



STEP 2
Remove cells from flask using trypsin/EDTA, count, adjust cell concentration and dispense into white 96-well plate provided. Include replicate wells with culture medium only.



If cells are cultured in the 96-well plate provided, cell proliferation/ viability can be measured in the same plate



TIPS

- MSCs from any source can be used in the assay.
- If possible, use self-calibrating electronic pipettes.
- Always dispense cell suspensions and reagents into the bottom of the well, never on the side.
- Always incubate plates in a humidified chamber to prevent drying out.
- Always use gloves when measuring ATP.
- Consider performing the calibration and standardization procedure since this has several advantages.
- Use sterile, adhesive foil (included) to maintain unused well sterility.

STEP 3
Culture cells for 2-3 days at 37ºC, 5% CO_2 and 5% O_2 (preferred)

STEP 4
Prior to measuring sample proliferation, it is recommended to calibrate the instrument and standardize the assay to provide measurement assurance

Setup extra wells from the same sample so that the assay can be multiplexed with flow cytometry to analyze the phenotype of the MSCs

STEP 5
Add 0.1mL of ATP-ER to each sample well, mix and measure bioluminescence after 10 min incubation in the dark

6. Kit Contents and Storage Conditions

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

Item	Component	Storage
1	MSCGro™ Medium of choice	-20°C until used
2	ATP Enumeration Reagent (ATP-ER)*	-20°C in the dark until used
3	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
4	Sterile, individually wrapped, 96-well plate for cell culture	Can be kept with other kit components
	Technical manual downloaded from www.preferred-cell-systems.com	Can be kept with other kit components

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

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

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

This kit contains a reagent for measuring luminescence (ATP-ER) that decays with time. Preferred Cell Systems™ recommends that this kit be used before the expiry date of this reagent. Preferred Cell Systems™ does not take responsibility for the quality of reagents beyond their expiry date. If the kit cannot be used prior to the expiry date of this reagent, fresh reagent can be obtained from Preferred Cell Systems™.

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

7. Equipment, Supplies and Reagents Required, but not Provided

Equipment and Supplies

1. Laminar Flow Biohood
2. Plate luminometer (e.g. Berthold LB962 CentroLIA/pc; Molecular Devices, SpectraMaxL)
3. Sterile plastic tubes (5ml, 10ml, 50ml)
4. Single channel pipettes, preferably electronic (e.g. Rainin EDP pipettes for variable volumes between 1µl and 1000µl).
5. 8 or 12-channel pipette, preferably electronic (e.g. Rainin EDP pipettes for fixed or variable volumes between 10µl and 100µl).
6. Reservoir for 8- or 12 channel pipette

7. Sterile pipette tips.
8. Vortex mixer.
9. Tissue culture incubator, humidified at 37°C with 5% CO_2 (minimum requirement) and 5% O_2 (preferable).
10. 1.5ml plastic vials (5 for each ATP dose response).
11. Hemocytometer or electronic cell counter to determine cell concentration.
12. Flow cytometer or hemocytometer for determining viability.

Reagents

1. ATP Bioluminescence Standardization and Calibration Kit (Catalog No. K-ATPSC-1 from Preferred Cell Systems™).
2. MSCGro™ Growth Medium (available in 100mL or 500mL bottles from Preferred Cell Systems™).
3. Density-gradient centrifugation medium (to prepare a mononuclear cell fraction).
4. 7-AAD, propidium iodide or trypan blue for viability assay.
5. LIVEGlo™ metabolic viability assay (Preferred Cell Systems™).

8. The MSCGlo™ Protocol

PLEASE READ THE FOLLOWING PROTOCOL CAREFULLY
SEE SECTION 9 BEFORE PERFORMING THE PROTOCOL

Performing MSCGlo™ is a 3-step process.

Step 1 – Cell preparation.
Step 2 – MSCGlo™ cell culture master mix preparation, plating and incubation in the 96-well plate.
Step 3 – Luminescence measurement.

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

STEP 1 – Cell Preparation
Cell preparation will depend on the species and source of cells.

MSC Derived from Fresh Tissue
When MSCs are prepared from fresh, primary tissues, 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 MSCs derived from species other than human cells, the density and osmolarity of the medium may have to be changed.

MSCGlo™ Complete with Cryopreserved Cells
MSCGlo™ Complete assay kits include a vial of cryopreserved MSC. The characteristics of the cells are provided on a separate document included with the assay kit. A separate protocol for thawing and expanding the cells is also provided with the MSCGlo™ Complete Assay 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 Counting and Cell Culture Suspension Preparation

1. For dye exclusion viability methods, use trypan blue and a hemocytometer or automated method, flow cytometry using 7-AAD or another vital stain.
   Note that dye exclusion viability methods detect membrane integrity. They do not detect cellular and mitochondrial integrity and therefore metabolic viability.
2. 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™ (Preferred Cell Systems™) as a metabolic viability assay.
3. Determine the cell concentration using either a hemocytometer or electronic cell/particle counter. **NOTE**: Do not base the working concentration on the number of viable cells as this will give erroneous results.
4. Adjust the cell concentration using MSCGro™ medium.

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.

Cell Concentrations

For normal MSC proliferation analysis, a final concentration of 1,500 - 2,000 cells/well is usually 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) should be prepared in MSCGro™ that was included with the assay kit . Each well receives 0.1ml of the cell suspension.

STEP 2. MSCGlo™ Cell Culture Preparation

- ***Perform all procedures under a laminar flow, bio-hazzard hood.***
- ***Wear protective clothing, including gloves for all operations.***

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.
- MSCs should be expanded in MSCGro™ medium of choice, at a concentration between 5,000 and 10,000 cells/cm^2.
- The initial MSC concentration should be documented if the cell doubling time is to be estimated.
- Each time the cells are passaged for expansion, an aliquot of the cells should be counted and analyzed by measuring intracellular ATP (see below, MSC Culture in 96-well plates).

- 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. This will allow an estimation of the cell doubling time. Alternatively, MSCGlo™ Real Time may be used to fit an exponential curve to the data to estimate the cell doubling time.
- 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 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 lose 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.

STEP 3 – BIOLUMINESCENCE MEASUREMENT

Please note the following important points:

- ***FOR ALL OF THE FOLLOWING STEPS, WEAR LABORATORY GLOVES. ATP is present on the skin and can cause erroneous results***
- ***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.***
- ***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.***
- ***Remove the ATP Enumeration Reagent (ATP-ER) from the freezer and thaw at room temperature or in cold running water prior to analysis. Do not thaw the ATP-ER in a water bath or 37oC incubator.***

A. Sample Measurement

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

1. If possible, place the sample plate(s) in a humidified incubator set at 22-23℃ 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 cover foil from the kit box, remove the backing 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 0.1mL of ATP-ER to each well of the first column (A1-H1) or row (A1-12). Mix the contents as described in Section 10.
4. Repeat this procedure for each column or row using new tips.
5. When ATP-ER has been added to all wells, replace the plastic cover and incubate for 10 min at room temperature in the dark to lyse the cells and stabilize the luminescence signal. Incubate the plate in the reader for the last 2 min to stabilize the plate.
6. Unused ATP-ER may be returned to the bottle and refrozen. See section 10 for ATP reagent storage conditions and stability.

B. 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. Preferred Cell Systems™ does <u>not</u> recommend using the automatic dispensers, since the contents of the well are not mixed sufficiently using this method.

C. Using a liquid handler

MSCGlo™ can be performed in high throughput mode. If you intend to perform any part of the MSCGlo™ procedure using a liquid handler, please contact Preferred Cell Systems™ for information on setting up the instrument. Extra ATP-ER is required when using a liquid handler.

9. Recommendations and Tips Prior To Using MSCGlo™.

(i) Cell Suspension

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

(ii) Number of Replicates Performed

A minimum of 4 replicates/sample can also be used, although 6 replicates will provide better statistics. 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.

(iii) Plate Configuration

Using 4 replicates/sample can be performed either in rows across the plate or in columns. If 6 replicate wells/sample are used, these should be plated in rows across the plate. If 8 replicates/sample are used, the sample should be plated n columns across the plate.

(iv) 96-Well Plates Provided

The reagents have been optimized to work with the 96-well plate(s) provided in the MSCGlo™ 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 Preferred Cell Systems™ if required.

(v) 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”. This phenomenon is observed when ATP values in the outside wells are lower than those in the inside wells. 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 Preferred Cell Systems™ for further information about assembling and using humidity chambers.

(vi) Incubation Times

The incubation time may vary depending on cell type and species. Assay sensitivity might improve with longer incubation times, but usually at the expense of higher variability between wells. Once an optimal incubation time has been found, the same time period should be maintained for all future experiments so that results can be directly compared.

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 each 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™ includes solid white plates for both cell culture and the ATP standard curve and controls. Do not use different plates for the assay. Doing so will result in inaccurate results and invalidation of the assay kit warranty. Extra plates can be purchased from Preferred Cell Systems™.***

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℃.
- Sufficient ATP standard, controls and ATP-ER are supplied to perform 2 standard curves and controls/assay kit. Additional ATP standards and controls can be obtained from Preferred Cell Systems™.
- 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℃, in the dark, for 48h or is stable at -20°C 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 2-8°C 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 2-8°C, or 20 weeks at -20°C.
- 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 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)).

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.

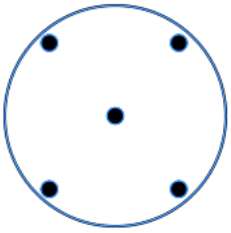
Mixing the Contents of 96-Well Plate

Mixing the contents of the wells after adding ATP-ER is one of the most important procedures of the assay. 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 4 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.

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



11. Luminescence Plate Reader Setup

Multimode 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 multimode 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" (if required). 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. Do not use plate shaking or the injectors if the instrument has this capability.
e. The output of the luminescence plate reader is in Relative Luminescence Units (RLU). To convert RLU values into iATP concentrations (µM), it is necessary to calibrate the instrument with controls and perform an ATP standard curve. If this is required, it will be necessary to purchase the ATP Bioluminescence Standardization and Calibration Kit from Preferred Cell Systems™ (Cat. No. K-ATPSC-1).

13. Troubleshooting

High Coefficients of Variation (%CV)

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

- Accurate reagent dispensing and mixing are of prime importance. Since the volumes dispensed are small it is imperative to use instruments that have been properly calibrated to avoid pipetting error.
- Insufficient mixing of components prior to cell plating 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.

Low RLU Values

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

- *Reagent decay:* The ATP-ER decays with time, even when frozen. This can lead to low bioluminescence. Once thawed the reagent can be refrozen up to 11 cycles without significant loss of sensitivity. Do not use the reagent after expiry date has elapsed. As a rule of thumb, the RLU value for the lowest ATP standard should be 10 times greater than that of the background value.
- *Inadequate cell growth:* Cells did not exhibit sufficiently high viability. Measure cell viability prior to using cells. 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, if the medium contains an indicator, e.g. phenol red. 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 9 (v) for instructions on how to build a humidity chamber.

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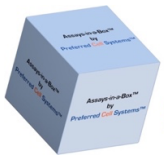

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