MSCGlo™ SC-IPS / MSCGlo™ RS

Mesenchymal Cell Identity, Purity and Strength (Potency) Assay for Regenerative Medicine

Technical Manual

(Version 8-19)

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

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

Preferred Cell Systems™

KM029.001

TABLE OF CONTENTS

1. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. *MSCGlo™ SC-IPS is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)*
2. *MSCGlo™ SC-IPS 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. Safety data sheets (SDS) are included in each literature packet.*

2. Introduction

The field of regenerative medicine has paid little attention to the potency of cells being manufactured for this purpose. Mesenchymal cells represent an population of cells that are present in the bone marrow, cord blood, adipose tissue and other sources that are often used in the regenerative medicine field.

Mesenchymal cells are represented by several different names: Mesenchymal stem cells (MSC), also called Mesenchymal Stromal Cells (MSC) or Mesenchymal Progenitor Cells (MPS). The cells are characterized by their ability to adhere to a growth surface and to produce colonies in the colony-forming unit - fibroblast (CFU-F) assay. The clonogenicity and, therefore, the question of whether mesenchymal cells are actually stem cells, is debatable, since the CFU-F assay detects the appearance of proliferating fibroblastoid-like cells that may be a progenitor of mesenchymal cells. However, mesenchymal cells are often detected by their phenotypic profile. The cells are usually CD73, CD90 and CD105 positive as well as CD29, CD44 and CD166 positive, but are negative for CD45 and CD34. Mesenchymal cells are, in part, responsible for producing the hematopoietic stroma facilitating hematopoiesis. In the presence of specific growth factors and/or cytokines, mesenchymal cells are responsible for chondrogenesis, adipogenesis and osteogenesis, but can also produce several other cell types.

Mesenchymal cells are difficult to obtain as a native population due to their low frequency. As a result, mesenchymal cells are usually first grown in culture and then expanded by passaging the cells in tissue culture flasks or cell reactors. Mesenchymal cells also have a finite life span, with proliferation potential and ability declining with time in culture. This means that with increased passaging of the cells, the quality and potency will decline. When mesenchymal cells are prepared for regenerative medicine purposes, they are often passages just a few times prior to cryopreservation or use to try and maintain the highest quality (proliferation ability) and greatest potency (proliferation potential). Unless a standardized and validated assay is used, it is impossible to compare batches or lots of mesenchymal cells even when the cells are prepared using the same procedure. Furthermore, when cells are cryopreserved, both their quality and potency will suffer. The strength or potency of a mesenchymal cell preparation can be defined as "the quantitative measure of biological activity based on the attribute of the product, which is linked to the relevant biological properties". The attributes measured in this case are proliferation ability or quality and proliferation potential or potency. When these two parameters are measure, the biological identity and purity can also be determined.

To measure these parameters, Preferred Cell Systems™ developed two assays, namely MSCGlo™ RS to establish mesenchymal cell reference standards (RS) and MSCGlo™ SC-IPS to compare samples of mesenchymal cells with the established RS. The comparison can only be performed if the assays being used have been properly standardized and validated. To do this, the most sensitive and reliable non-radiation signal detection system has been used.

Like all mammalian cells, the viability and functional ability to proliferate, is based on the availability of chemical energy in the form of intracellular adenosine triphosphate (iATP). The amount of iATP produced by the 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. Reagents are included in the assay kits to calibrate the luminescence plate reader and standardize the assay. This allows measurement assurance parameters to be compared (s**ee Section 14**) prior to measuring the samples. In this way, the user can ensure that the results obtained will be trustworthy and manufacturing consistency has been attained.

If required, MSCGlo™ SC-IPS can also be multiplexed with phenotypic analysis by flow cytometry or even genetic analysis of the cells to provide additional, non-functional, parameters.

3. Use and Availability

MSCGlo™ RS is used to establish batched or lots of in-house reference standards (RS) from different sources of MSCs. The MSCGlo™ RS assay kit contains a vial of cryopreserved cells that can be used to compare and establish an in-house RS.

MSCGlo™ SC-IPS is used to compare a sample of MSCs with the in-house RS established using the MSCGlo™ RS assay kit.

Both assays determined the identity and purity and measure the strength of human MSCs.

MSCGlo™ RS and MSCGlo™ SC-IPS are available with the following choice of growth medium:

- MSCGro™ complete, low serum medium
- MSCGro™ complete, serum-free and xeno-free medium
- MSCGro™ complete humanized medium
- CRUXRUFA™ Human Platelet Lysate (HPL), fibrinogen-depleted and GMP grade.

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

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

MSCGlo™ RS Assay Kit Availability

Catalog No.	Cryopreserved Cells Included	Medium Formulation	No. of Plates
KLMC-CRRS-1CB	Cord Blood MSC	CRUXRUFA™ HPL	1
KLMC-LSRS-1CB	Cord Blood MSC	MSCGro™ Low Serum	1
KLMC-SFRS-1CB	Cord Blood MSC	MSCGro™ Serum-Free	1
KLMC-HMRS-1CB	Cord Blood MSC	MSCGro™ Humanized	1
KLMC-CRRS-1BM	Bone Marrow MSC	CRUXRUFA™ HPL	1
KLMC-LSRS-1BM	Bone Marrow MSC	MSCGro™ Low Serum	1
KLMC-SFRS-1BM	Bone Marrow MSC	MSCGro™ Serum-Free	1
KLMC-HMRS-1BM	Bone Marrow MSC	MSCGro™ Humanized	1

MSCGlo™ RS is only required once to begin establishing an in-house RS. Other batches or lots of in-house RS would be compared and established using MSCGlo™ SC-IPS.

To establish in-house MSC reference standards from other tissue sources, use MSCGlo™ SC-IPS.

MSCGlo™ SC-IPS Assay Kit Availability

Catalog No.	MSC Source	Serum Formulation	No. of Plates
KLMC--CRP-1	Any	CRUXRUFA™ HPL	1
KLMC-LSP-1	Any	MSCGro™ Low Serum	1
KLMC-SFP-1	Any	MSCGro™ Serum-Free	1
KLMC-HMP-1	Any	MSCGro™ Humanized	1

MSCGlo™ RS and MSCGlo™ SC-IPS are available in larger kit sizes upon request.

4. Establishing a Reference Standard (RS) for MSCs using MSCGlo™ RS

To measure potency, a reference standard is required. This is because the measure of potency is the potency ratio. To estimate the potency ratio, the sample must be compared with that of a standard. For traditional drugs, establishing RSs for a compound is relatively easy since large quantities of the drug or compound are usually available. This is not the case for cells. The establishment of a primary cell RS is not a standard procedure and there is no consensus on how this should be performed for cellular therapeutic products. The following is a suggestion for establishing internal primary, secondary and even tertiary reference standards for MSCs. One of the advantages of using a calibrated, standardized and validated assay such as MSCGlo™ RS or MSCGlo™ SC-IPS is that results can be directly compared over time. This means that one RS

can be compared to another, both for intra- and inter-laboratory comparisons.

MSCGlo™ RS: The Strength or Potency Reference Standard Assay Kit
MSCGlo™ RS is an assay that helps establish an in-house RS. MSCGlo™ RS includes a cryopreserved RS of cord blood- or bone marrow-derived MSC. To establish a RS in-house, a batch of umbilical cord blood or bone marrow should be red cell depleted and the mononuclear cell (MNC) fraction prepared by density gradient fractionation (DGF). An MSC culture is then established from the fractionated cord blood or bone marrow source. It may take more than two weeks to begin observing the production of MSCs. This can be monitored using either MSCGlo™ or MSCGlo QC assays. Once MSC growth has been established, the cells should be expanded 2-3 times prior to cryopreserving the cells in aliquots of 1-2 million and storing the cryovials in liquid nitrogen. Using an aliquot of the newly prepared, in-house cryopreserved RS cells, the potency is determined against the cryopreserved RS included with the MSCGlo™ RS assay kit. This is performed using the general protocol described in **Section 9.** This procedure establishes the Primary Reference Standard (1° RS).

Once a 1° RS has been established, a second batch of cells can be processed in the same way to produce a Secondary Reference Standard (2° RS). This 2° RS is tested against the 1° RS. The secondary RS should demonstrate similar or better potency parameters than the primary RS. The same procedure and testing is performed for a Tertiary Reference Standard (3° RS).

The last RS to be established and stored is the RS used to test against the unknown samples.

At regular intervals or when the number of aliquots remaining of the RS is low, a new RS should be established and compared to a previously established RS batch. In this way, several RS batches can be maintained at the same time.

The same procedure and testing is used for establishing reference standards of purified stem cells.

Please note MSCGlo™ RS is usually used only once. When one or more in-house reference standards have been established, all further reference standards can be tested using MSCGlo™ SC-IPS, unless a new tissue RS is required.

When is the Reference Standard Used?
A reference standard is used every time a MSC product is to be released for use. Approximately 1-2 weeks prior to use, the potency of a sample of the product should be tested. As described later in this manual (**Section 14**), there are specific parameters that are required for release of the product. These include a potency ratio of greater than 1 (>1) and an iATP concentration of greater than 0.04μM (> 0.04μM). Other parameters might also be included in the release criteria.

When is Potency Measured?
Depending on the source of the cells, potency should be measured just prior to use in a patient. For cryopreserved samples, the potency and quality should be determined shortly after cryopreservation and again prior to use. Potency can not be measured after the product has been used in the patient since potency is a predictor of cell growth and dose.

5. The Concept of ATP Bioluminescence Assays

MSCGlo™ RS and MSCGHlo™ SC-IPS are ATP bioluminescence assays. 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.

Mesenchymal cells are allowed to adhered to the growth surface for 6-24h in either HPL or MSCGro™ medium. The HPL or medium is then replace and the cells further incubated for a reasonable amount of time in order to measure MSC proliferation. 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 assay can be calibrated and standardize, and controls and standards are included for this purpose. 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 14**. 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 14**, 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

6. QuickGuide to MSCGlo™ SC-IPS (Figure 1)

QuickGuide to MSCGlo™ SC-IPS/RS

Identity, Purity, Strength (Potency)

CELL SAMPLE

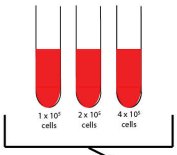
STEP 2
For sample or new in-house RS, prepare 3 cell dilutions in CRUXRUFA™ Human Platelet Lysate or MSCGro™ Medium.

COATING PLATE

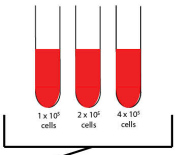
Step 1
It is recommended to coat the wells of the sterile 96-well plate with rat collagen

REFERENCE STANDARD (RS)

STEP 3
Thaw vial of cryopreserved reference standard MSCs from same source, passage number, specific doubling time or other parameter and prepare 3 dilutions using the same growth medium for the sample.

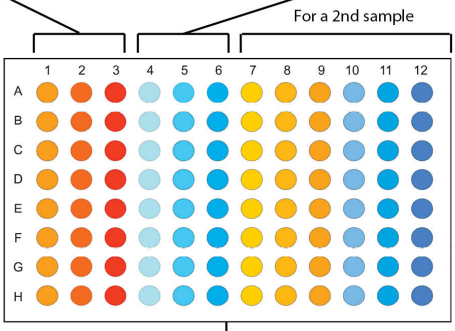


STEP 4
Dispense 0.1mL from each cell dose into 8 replicate wells of a 96-well plate provide. Coat plate prior to use. 3 columns for sample and 3 the reference standard.



TIPS

- The reference standard must be of the same source and purity as the sample.
- An in-house RS is established using MSCGlo™ RS using exactly the same method and comparing against a known MSC preparation.
- Always perform 8 replicates/cell dose.
- The potency can only be measured using a cell dose response and against a reference standard.
- 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.
- Use sterile, adhesive foil (included) to maintain unused well sterility.



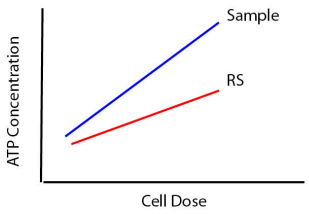
STEP 5
Incubate for 24h to allow cells to adhere.

STEP 6
Replace growth medium and incubate for 2-3 days

STEP 7
Calibrate and standardize assay

STEP 8
Measure bioluminescence

STEP 9
Compare slopes.
Sample slope/RS slope = Potency Ratio



7. Kit Contents and Storage Conditions

MSCGlo™ SC-IPS and MESCGlo™ RS 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	Vial of cryopreserved cord blood or bone marrow MSC. Included in MSCGlo™ RS only.	Liquid nitrogen
2	CRUXRUFA™ Human Platelet Lysate or MSCGlo™ medium of choice	-20°C until used
3	Medium (IMDM) for dilution of the ATP standard only.	-20°C until used
4	ATP standard.	-20°C until used
5	ATP extra high, high and low controls.	-20°C until used
6	ATP Enumeration Reagent (ATP-ER)*	-20°C in the dark until used
7	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
8	Sterile, individually wrapped, adherent, white, clear-bottom, 96-well plate for cell culture	Can be kept with other kit components
9	Non-sterile, adherent, white, clear-bottom, 96-well plate(s) for ATP standard curve determination.	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 2-8°C for 48h once thawed and is stable for 20 weeks when stored at -20°C. This reagent is light sensitive. Keep in the dark.

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

8. 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. CRUXRUFA™ Human Platelet Lysate (available from Preferred Cell Systems™) or MSCGro™ Growth Medium (available from Preferred Cell Systems™).
2. Type I bovine or rat tail collagen at 50µg/mL in 0.02M acetic acid.
3. Phosphate buffered saline (PBS).
4. Density-gradient centrifugation medium (to prepare a mononuclear cell fraction).
5. 7-AAD, propidium iodide or trypan blue for viability assay.
6. LIVEGlo™ metabolic viability assay (Preferred Cell Systems™).

9. The MSCGlo™ RS and SC-IPS Protocol

PLEASE READ THE FOLLOWING PROTOCOL CAREFULLY
SEE SECTION 10 BEFORE PERFORMING THE PROTOCOL

Performing MSCGlo™ RS or MSCGlo™ SC-IPS is a 4-step process.

Step 1 - Coating the 96-well plate.
Step 2 – Cell preparation.
Step 3 – MSCGlo™ RS or MSCGlo™ SC-IPS cell culture.
Step 4 – Luminescence measurement. An ATP dose response is performed prior to sample luminescence measurements with conversion of RLUs to µM ATP.

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

STEP 1 - Coating 96-Well Plates

If using serum-free or low serum medium, it is recommended to coat the sterile, white, clear-bottom, 96-well plate with bovine or rat tail collagen prior to performing the assay. Dispense 65µL of a sterile 50µM type 1 collagen in 0.02M acetic acid solution into the required number of wells of the sterile, white 96-well plate supplied with the assay kit. Coat the wells, in the dark, at 37ºC for 1hr or overnight at room temperature. After coating, remove the collagen solution and what each well 2 times with sterile PBS. Leave the last PBS wash in the plate until it is used.

STEP 2 – Cell Preparation

Cell preparation will depend on the 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.

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.

Thawing of Cells Included with MSCGlo™ RS

1. Prepare 20mL of thaw medium (IMDM + 10% fetal bovine serum, FBS) warmed to 37°C in a 50mL conical tube.
2. Prior to thawing the cells, thaw a vial of DNase (100µL at 4,000U/mL).
3. Add 100µL of thawed DNase to the 20mL of thaw medium in the 50mL conical tube and mix by inversion.
4. Carefully remove the vial of cells from liquid nitrogen and partially unscrew the cap.
5. Thaw the cells in a 37°C water bath by gently swirling the vial for about 1 min or until a small ball of ice remains in the vial.
6. Remove the vial and spray with 70% alcohol.
7. Carefully remove the lid of the vial and transfer all of the contents down the side of the 50mL tube containing the thaw medium. Mix gently.
8. Remove about 1mL from the 50mL tube and use it to rinse the vial, returning the cell suspension back to the 50mL tube.
9. Close the 50mL and mix gently by inversion.
10. Centrifuge the cells in the 50mL tube at 300 x g for 10min at room temperature.
11. After centrifugation, aspirate and discard the supernatant.
12. Add 1mL of IMDM to the tube and resuspend the cells.
13. Count the cells, preferably using an electronic cell counter, and perform a viability assessment. (See Step 1, Section C.).

Cell Viability, Cell Counting and Cell Culture Suspension Preparation

1. For dye exclusion viability methods, use trypan blue and a hemocytometer or automated method, flow cytometry using 7-AAD or another vital stain.
   Note *that dye exclusion viability methods detect membrane integrity. They do not detect cellular and mitochondrial integrity and therefore metabolic viability.*
   A viability of 85% or greater should be obtained when using dye exclusion viability methods only. It is recommended not to use cell suspensions with a viability of less than 85% since these cells will not be able to sustain proliferation ability. Use LIVEGlo™ (Preferred Cell Systems™) as a metabolic viability assay.
2. 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.
3. Adjust the cell concentration using CRUXRUFA™ HPL or MSCGro™ medium.

Flow Cytometry

Prior to 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

To establish an in-house RS or to measure the potency of a MSC cell population, it is necessary to perform a cell dose response. For 96-well plates, the maximum cell dose/well should not be greater than 2,000 cells/well or 20,000 cells/mL. Perform a serial dilution to obtain working cell concentrations of 10,000 cells/mL and 5,000 cells/mL equivalent to 1,000 cells/well and 500 cells/well when 0.1mL from each cell dilution is dispensed. The dilutions should be performed using either CRUXRUFA™ HPL or MSCGro™ that was included with the assay kit.

STEP 3. MSCGlo™ RS or MSCGlo™ SC-IPS Cell Culture

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

1. If required, coat and prepare the sterile 96-well plate. These plates are provided with a high affinity growth surface for adherent cells.
2. Prepare the RS MSC cells and the cell dose response at the working concentrations of 5,000, 10,000 and 20,000 cells/mL in the HPL or MSCGro™ provided with the assay kit. It is recommended to prepare a total volume of 1mL for each cell dilution. This will be sufficient to setup 8 replicates of 0.1mL for each cell dose.
3. Prepare the sample MSC cells and the cell dose response at the working concentrations of 5,000, 10,000 and 20,000

cells/mL in the HPL or MSCGro™ provided with the assay kit. It is recommended to prepare a total volume of 1mL for each cell dilution. This will be sufficient to setup 8 replicates of 0.1mL for each cell dose.

4. Using a calibrated pipette, preferably an electronic pipette, dispense the cell suspensions into the sterile, 96-well plate as follows:
   (i) Starting with the sample at the working concentration of 5,000 cells/mL, dispense 0.1mL into each of the 8 replicate wells of the first column (A1-H1).
   (ii) Dispense 0.1mL from the 10,000 cells/mL dilution into each of the 8 replicate wells A2 - H2.
   (iii) Dispense 0.1mL from the 20,000 cells/mL dilution into each of the 8 replicate wells A3 - H3.
   (iv) Now starting with the RS, dispense 0.1mL from the 5,000 cells/mL dilution into each of the 8 replicate wells A4 -H4.
   (v) Dispense 0.1mL from the 10,000 cells/mL dilution into each of the 8 replicate wells A5 - H5.
   (vi) Dispense 0.1mL from the 10,000 cells/mL dilution into each of the 8 replicate wells A6 - H6.
   By dispensing 0.1mL, each of the cell dilutions has been reduced 10 fold, thereby providing the final cell concentrations of 500, 1,000 and 2,000 cells/well for the sample and RS.
5. Replace the lid of the 96-well plate.
6. Place the 96-well plate in a humidity chamber (see **Section 10 (v)** 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 at least 48hr incubation, monitor cell growth under an inverted microscope. The cells should be approaching 70%-80% confluency. If this is not the case, incubate the cells for another 24hr.
9. Once the cells are 70%-80% confluent, ATP bioluminescence can be measured. Go to Step 4.

STEP 4 – 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 13 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 11 for recommendations and tips prior to starting this part of the procedure. In particular, please refer to Section 11 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 37°C incubator.***
- ***If the assay is to be calibrated and standardized, remove the ATP standard, controls and reagents from the freezer and thaw to room temperature or in cold running water prior to analysis.***
- ***ATP standard curves performed on previous days or for previous experiments or studies must not be used since the ATP-ER intensity changes with time and lot number.***
- ***Use the unwrapped, non-sterile, 96-well plate provided with the kit to perform the ATP standard dose response curve.***

A. Calibrating and Standardizing the Assay

To perform a potency assay, it is necessary to calibrate the luminescence plate reader and standardize the assay prior to measuring any samples. This will allow a comparison with the expected measurement assurance values (**see Section 14**) that should be obtained prior to measuring samples. Use the non-sterile, 96-well white plate provided with the assay kit for this purpose.

Both MSCGlo™ RS and MSCGlo™ SC-IPS include the following reagents to calibrate and standardize the ATP bioluminescence step.

- IMDM medium: Used only for ATP standard serial dilution.
- ATP Standard at 10µM. Serially diluted to produce the ATP standard curve.
- Low ATP Calibration Control. Used for normal and extra high cell proliferation.
- High ATP Calibration Control. Used for normal cell proliferation.

- Extra High ATP Calibration Control. Used for extra high cell proliferation.

B. Deciding Which Calibration Controls to Use and ATP Standard Curve Range

PROTOCOL 1: It is possible that even after 3 days of incubation, the cells have not reach optimum growth. If this has occurred, use the low and high calibration controls and perform an ATP standard curve from 0.01µM to 1µM. **See Page 21**.

PROTOCOL 2: For MSCs that have reach 70-% to 80% confluency with 2-3 days, use the low and extra high calibration controls and perform an ATP standard curve from 0.03µM to 3µM. **See Page 22**.

IMPORTANT. It is important that the sample and RS ATP values are within the limits of the ATP standard curve, otherwise the interpolation of Relative Luminescence Unit (RLU) values from the luminescence plate reader into ATP concentrations will not be accurate. If Protocol 2 has been used and values are not as high as 0.03µM ATP, perform Protocol 1. In some cases, MSC cell proliferation could be greater than 3µM ATP. If ATP values are greater than 3µM , it is recommended to dilute the sample with additional medium so that the values are within the ATP standard curve range. This may require removing an aliquot from the replicate wells, transferring the aliquot to a new wells and diluting each aliquot with additional medium. The replicate wells would then be reread.

C. Sample Measurement

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

1. Remove the sample plate from the incubator and let it attain room temperature under the hood.
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, 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 11**, Adhesive Plate Covering Film).
3. Using a multichannel pipette (8 channel pipette), add 0.1mL of ATP-ER to each well of the first column (A1-H1). Mix the contents as described in Section 11. The color of the medium should change from red to light orange.
4. Repeat this procedure for each column 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 or transfer the plate, without the lid, to the luminescence plate reader and close the draw of the instrument. During this time, the cells will be lysed and the luminescence signal stabilized.
6. Unused ATP-ER may be returned to the bottle and refrozen. See **Section 7** for ATP reagent storage conditions and stability.

D. Using a plate luminometer with automatic dispenser

Do not use the automatic dispenser to dispense ATP-ER, since the contents of the well will not be mixed sufficiently.

10. Recommendations and Tips Prior To Using MSCGlo™ RS and MSCGlo™ SC-IPS.

(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™ RS and MSCGlo™ SC-IPS kits. 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.

11. 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™ RS and MSCGlo™ SC-IPS include solid white, clear bottom 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˚C.
- 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˚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 2-8°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)).

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

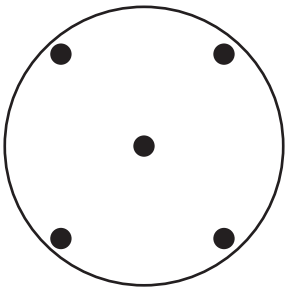
To help keep the plate(s) sterile, adhesive, air permeable, sterile films are provided so that the part of the plate that is not being used can be covered and kept sterile until required. If using the adhesive film provided, the plate cover should be removed in a laminar air-flow hood and replaced with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

Mixing the Contents of 96-well Plate

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



12. Luminescence Plate Reader Setup and Conversion of RLU Values to ATP Values Using the ATP Standard Curve

It is very important that the luminescence or multimode plate reader is setup correctly, otherwise false results could occur. Preferred Cell Systems™ has provided a separate document to help the investigator setup their instrument and perform the calculations in order to convert Relative Luminescence Units (RLU) into ATP concentrations using the ATP standard curve. It is strongly recommended that the investigator consult this document prior to performing any ATP bioluminescence assay. This document can be downloaded with this manual.

13. How to Analyze the Results

MSCGlo™ RS and MSCGlo™ SC-IPS provide an instrument-based, non-subjective, quantitative readout of MSC total proliferation ability and potential.

Many regenerative medicine procedures use both fresh and cryopreserved cells and MSCGlo™ RS and MSCGlo™ SC-IPS can be used on fresh or cryopreserved cells. If testing fresh cells, it should be emphasized that results produced will not reflect the actual viability or proliferation ability and potential after thawing frozen cells; proliferation ability will be 2-3 fold lower for a cryopreserved sample than a fresh sample.

The strength or potency of a sample cannot be performed using a non-validated assay. Assay validation can occur when controls and standards are used. It is, therefore, required that the user perform the calibration and ATP standardization procedure described in this manual and available as an Instructional Video on the Preferred Cell Systems™ website under Resources. The ATP controls calibrate the luminescence plate reader. The ATP standard curve allows non-standardized RLU values to be converted into standardized ATP concentrations (µM). The results should be compared with those provided in **Section 14** below. Providing the results are within the ranges specified in **Section 14**, it is then possible to continue with sample processing and measurement. If results do not conform to those in **Section 14**, repeat the calibration and standardization process and go to **Section 15**, Troubleshooting. If the problem persists, contact Preferred Cell Systems™ for help.

These measurement assurance parameters also indicate whether the sample being tested provides acceptable viability and proliferation activity, or whether it should be rejected for use. Please be aware that acceptance criteria for the sample may not necessary apply to the unit of cells from which the sample was obtained. It should also be emphasized that potency can NOT be determined with a single value; a dose response is required.

Analyzing the Results

To determine if an in-house RS can be considered similar to or better than the frozen cells provided with the MSCGlo™ RS kit or to determine if a sample of cells can be used for clinical purposes, it is necessary to (1), convert the RLU values obtained from the plate reader into standardized ATP concentrations and (2) plot the data.

To convert RLU values into standardized ATP concentrations see **Section 12** of this manual.

Once all RLU values have been converted to standardized ATP concentrations (µM), the results are plotted as follows:

1. If the instrument software allows graphing, plot the raw data with cell dose on the X-axis and mean ATP concentration (µM)/well on the Y-axis. If the instrument software does not allow graphing, use a third-party software such as Excel, GraphPad Prism or SigmaPlot to plot the raw data.
2. The data are plotted for both the sample and the RS.
3. A linear regression curve fit for each cell dose response is performed and the slope of the dose response curve calculated.
4. Calculate the potency ratio for the RS and sample by dividing the slope of the sample cell dose response by that for the slope of the RS.
5. The potency ratio is the measure of potency.

Interpreting the Results

MSCGlo™ RS and MSCGlo™ SC-IPS are used to measure two basic parameters of cell proliferation:

- Proliferation ability, is the amount of cell proliferation, in standardized ATP units (µM), at a specific cell dose. Proliferation ability is equivalent to cell quality.
- Proliferation potential is the capacity of cells to proliferate and is determined by the slope of the cell dose response curve.

The combination of these two parameters provides all the information needed to interpret the results. By performing a cell dose response for the sample and the RS, both proliferation ability and potential are determined simultaneously. In addition, when comparing the sample with the RS several other parameters are defined:

- By comparing the slope of different MSC sample cell dose response curves, it will become apparent that the steeper the slope, the more primitive the cells and the greater their proliferation potential. This property can be used to identify and distinguish primitive from mature MSC populations.
- The purity of the sample being tested can be compared with other samples that are less pure. Purer samples will also have a steeper dose response curve than less pure samples.
- Finally, the slope of the MSC cell dose response curve is also a direct measure of its proliferation potential. The steeper the slope, the more primitive the cell population, the greater its potency. Thus, proliferation potential not only defines cell identity, but also strength or potency.

With this information, it is now possible for the user to interpret the data provided by MSCGlo™ RS and MSCGlo™ SC-IPS. For example:

1. The ATP concentration produced at a specific cell dose for the sample, e.g. at 1,000 or 2,000 cells/well, should be at least 2-3 x greater than the lowest ATP concentration indicating unsustainable stem cell proliferation. This value is ~0.04µM ATP (**Section 14**). Therefore, the ATP concentration produced by the sample at a specific dose is defined as the proliferation ability or cell quality. Thus, the cell quality of the sample must be equal to or greater than the required value for the cells to be considered "high quality".
2. The potency ratio of the reference standard is always consider to be 1 (one).
3. If the potency ratio for the sample measured is greater than 1, the sample exhibits a potency greater than the RS. This means that fewer sample cells are required to produce the same response as the RS. If cell "quality" and potency are greater than that for the RS, the MSC unit of cells might be considered for use, providing other factors (e.g. cell number, viability, phenotypic markers etc) are also consistent.
4. If the MSC sample potency ratio is less than 1, the sample exhibits a potency ratio less than the RS. This means that more cells will be required to produce a similar response to that of the RS. If MSC "quality" is also below the minimum level, the unit of MSC cells being considered should probably not be used, even if other parameters, (e.g. cell number, viability, phenotypic markers) might indicate otherwise. Do not forget that the ATP concentration being measured is also a metabolic viability measurement. If the ATP concentration is indicating that the cells might not or will not sustain proliferation they will be non-viable and other parameters will not override this indication.

These are the best and the worst scenarios. Many scenarios will lie in between. Accumulation of historical data will provide more specific information.

14. MSCGlo™ RS and MSCGlo™ SC-IPS Assay Measurement Assurance and Validation Parameters

If MSCGlo™ RS and MSCGlo™ SC-IPS have been calibrated and standardized, ATP bioluminescence technology allows the User's results to be compared to the measurement assurance parameters shown in the Table below. For each control, ATP standard dose and the log-log linear regression curve fit parameters provided, the User's results must lie within the ranges provided. If this is the case, then the following are applicable:

1. The User has performed and passed the integrated proficiency test.
2. The instrument and assay readout reagents are working correctly.
3. The User can continue to process and measure samples.
4. The User can trust results of the assay.

IMPORTANT. If the User's results DO NOT comply with those in the table, DO NOT measure the samples. Perform a repeat of the controls and ATP standard curve. If the results still do not comply with those in the Table, contact Preferred Cell Systems for help.

ATP Controls and Standard Curve Measurement Assurance Parameters

Expected Parameter	Observed Value	Mean ± 15%(*)	Min / Max	%CV (where applicable)
0.01µM ATP	0.0099µM ATP	0.00972 - 0.0114	0.009 - 0.01	2.34%
0.03µM ATP	0.029µM ATP	0.285 - 0.0336	0.028 - 0.03	1.67%
0.05µM ATP	0.0497µM ATP	0.0486 - 0.0571	0.048 - 0.051	1.57%
0.01µM ATP	0.1026µM ATP	0.1003 - 0.118	0.099 - 0.107	1.96%
0.3µM ATP	0.317µM ATP	0.310 - 0.364	0.302 - 0.325	1.51%
0.5µM ATP	0.5023µM ATP	0.491 - 0.578	0.491 - 0.515	1.19%
1.0µM ATP	1.048µM ATP	1.024 - 1.205	0.977 - 1.117	3.7%
3.0µM ATP	2.722µM ATP	2.661 - 3.130	2.633 - 2.934	2.09%
Intercept	6.533	6.386 - 7.513	5.86 - 6.7	1.84%
Slope	0.9656	0.944 - 1.110	0.947 - 0.988	1.21%
r^2 goodness of fit)	0.9993	-	0.998 - 1	0.05%
R (correlation coefficient)	1	-	0.999 - 1	0.02%
Low control, (0.05µM ATP	0.0487µM ATP	0.0476 - 0.0560	0.042 - 0.063	6.79%
High control 0.7µM ATP	0.725	0.710 - 0.836	0.655 - 0.904	5.35%
Extra high control (1.75µM ATP)	1.756	1.717 - 2.019	1.61 - 2.198	5.24%
The above values represent results from 71 control and ATP standard curve studies performed from January 2016 to June 2018				

(*) 15% represents the acceptable range of values for FDA Bioanalytical Method Validation Guidelines

Samples Values:

- Lowest ATP value indicating unsustainable cell proliferation for many cell types: ~0.04µM. This should be determined for a specific cell type.
- ATP value below which cells are not metabolically viable: ~0.01µM.
- All samples values must lie on the ATP standard curve for accurate RLU to ATP conversion. If ATP values are greater

than 3µM, the replicate samples should be diluted with medium provided in the kit and re-measured. Take the dilution value into account when estimating the true ATP concentration. Alternatively, repeat the culture and ATP measurement using fewer cells.

Assay Validation Parameters
MSCGlo™ RS and MSCGlo™ SC-IPS exhibit the following validation parameters:

- Assay ATP linearity => 4 logs
- Assay ATP sensitivity: ~ 0.001µM
- Assay cell sensitivity: 20-25 cells/well (depending on cell type and purity)
- 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).

15. Troubleshooting

If Calibration and Standardization Results Do Not Conform to Measurement Assurance Parameters (Section 14)
If the investigator has elected to calibrate and standardize the assay using the ATP controls and standard supplied with the kit, the results should be within the ranges provided in **Section 14**. If the values obtained conform to the measurement assurance parameters, the investigator can continue the assay and process and measure the samples with the assurance that the results can be trusted.

If any of the values obtained during calibration and standardization do not conform or are not within the ranges provided in **Section 14,** the user should repeat the calibration and standardization. Often discrepancies occur due to pipetting and/or dilution errors. Accurate and careful dilution of the ATP stock solution is important. It is also possible that if pipettes have not been professionally calibrated, errors can occur. These will also be picked up during this phase of the assay. Finally, if the ATP-ER has not be handled or stored correctly, it will decay, leading to erroneous results. Please contact Preferred Cell Systems™ to obtain new ATP-ER.

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

- Accurate reagent dispensing and mixing are of prime importance. Since the volumes dispensed are small it is imperative to use instruments that have been properly calibrate to avoid pipetting error.
- Insufficient mixing of components prior to 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 10** (v) for instructions on how to build a humidity chamber.

16. References

1. **Reems J-A, Hall KM, Gebru LH, Taber G, Rich IN.** Development of a novel assay to evaluate the functional potential of umbilical cord blood progenitors. Transfusion (2008) 48:620-628.
2. Rich IN. Potency, Proliferation and Engraftment Potential of Stem Cell Therapeutics: The Relationship between Potency and Clinical Outcome for Hematopoietic Stem Cell Products. J Cell Sci Therapy. (2013).
3. **Hall KM, Harper H, Rich IN**. Hematopoietic stem cell potency for cellular therapeutic transplantation. In: Hematopoietic Stem Cells, Ed.: RP Camacho. ISBN 978-953-307-746-8 (2011).
4. **Patterson J, Moore CH, Palser E, Hearn JC, Dumitru D, Harper HA, Rich IN**. Detecting primitive hematopoietic stem cells in total nucleated and mononuclear cell fractions from umbilical cord blood segments and units. J Translat Med (2015) 13:94.
5. Rich IN. Improving quality and potency testing for umbilical cord blood: A New Perspective. Stem Cells Translational Medicine. 4:967-973 (2015).
6. Rich IN. Short Primer in Stem Cell Biology. In: Stem Cell Protocols. Methods in Molecular Biology, 1235, Ed. Rich IN. Published by Human Press (2015).
7. Rich IN. Measurement of Hematopoietic Stem Cell proliferation, Self-Renewal and Expansion Potential. In: Stem Cell

Protocols. Methods in Molecular Biology, 1235, Ed. Rich IN. Published by Human Press (2015).
8. Harper H and Rich IN. Measuring the Potency of a Stem Cell Therapeutic. In: Stem Cell Protocols. Methods in Molecular Biology, 1235, Ed. Rich IN. Published by Human Press (2015).
9. Harper H and Rich IN. Bioluminescence Potency Measurement of Cellular Therapy Products. In: Cellular Therapy: Principles, Methods, and Regulations, 2nd Edition (2016), Eds. Areman EM and Loper K. Published by AABB.

Ordering Information
Toll free: 1-888-436-6869
Tel: (719) 264-6251
Fax: (719) 264-6253
Email: info@preferred-cell-systems.com
Order online at preferred-cell-systems.com

Technical Support
Tel: (719) 264-6251
Email: info@preferred-cell-systems.com

Preferred Cell Systems™
1485 Garden of the Gods Road
Suite 152
Colorado Springs, CO 80907
U.S.A.
Website: www.preferred-cell-systems.com

Preferred Cell Systems™

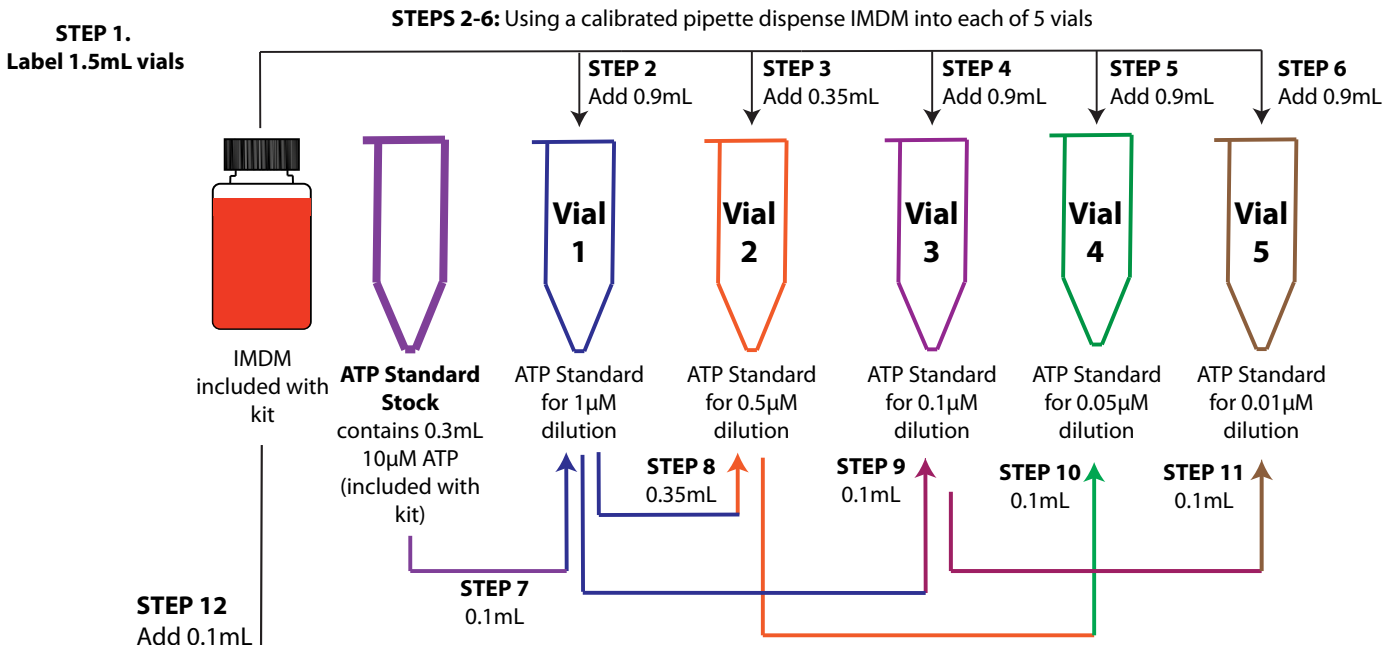
Calibration and Standardization Protocol of an ATP Bioluminescence Assay

PROTOCOL 1: ATP Standard Curve from 0.01µM to 1µM
For Samples with Known or Expected Normal Cell Proliferation

Reagents & Materials Needed
1. 1.5mL vials or similar (not included)
2. IMDM (included)
3. ATP Standard (included)
4. ATP Controls (included)
5. Non-sterile, 96-well plate (included)

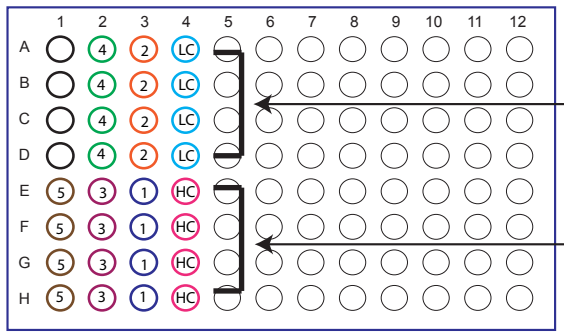
TIPS

> Use calibrated pipettes throughout.
> Vortex thoroughly between each dilution.
> Change tips between each dilution.
> Follow color coding.



STEP 12
Add 0.1mL into wells A1 - D1

Change pipette tips for each well



Follow Color Coding
STEP 13: Add 0.1ml from **Vial 5** into wells E1-H1
STEP 14: Add 0.1mL from **Vial 4** into wells A2-D2
STEP 15: Add 0.1mL from **Vial 3** into wells E2-H2
STEP 16: Add 0.1mL from **Vial 2** into wells A3-D3
STEP 17: Add 0.1mL from **Vial 1** into wells E3-H3

STEP 18: LOW CONTROL (LC, included with kit)
Vortex and lightly centrifuge to remove liquid from cap
Add 0.1mL from low control to wells A4-D4

STEP 19: HIGH CONTROL (HC, included with kit)
Vortex and lightly centrifuge to remove liquid from cap
Add 0.1mL from high control to wells E4-H4

STEP 20: Add ATP-ER to reserviour and using a multichannel pipette, dispense 0.1mL into each replicate well
STEP 21: Mix replicate wells as described for Figure 2 in this manual. Change tips for each new addition of ATP-ER
STEP 22: Transfer 96-well plate to luminescence plate reader
STEP 23: Incubate in the dark for 2 minutes and measure luminescence

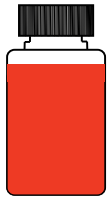
Calibration and Standardization Protocol of an ATP Bioluminescence Assay

PROTOCOL 2: ATP Standard Curve from 0.03µM - 3µM
For Samples with Known or Expected High Cell Proliferation

STEP 1.
Label 1.5mL vials

STEPS 2-6: Using a calibrated pipette dispense IMDM into each of 5 vials

STEP 6 Add 0.7mL
STEP 2 Add 0.4mL
STEP 3 Add 0.9mL
STEP 4 Add 0.9mL
STEP 5 Add 0.9mL



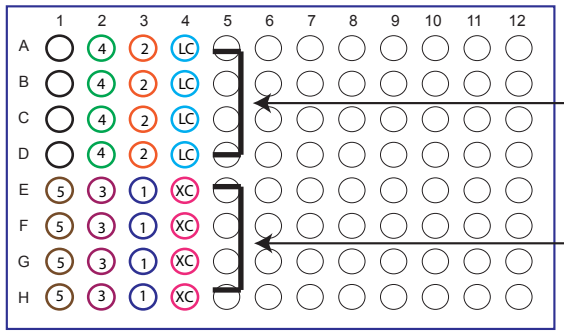
IMDM included with kit

Vial 1 — ATP Standard contains 0.3mL 10µM ATP (included with kit)
Vial 2 — ATP Standard for 1µM dilution
Vial 3 — ATP Standard for 0.3µM dilution
Vial 4 — ATP Standard for 0.1µM dilution
Vial 5 — ATP Standard for 0.03µM dilution

STEP 7 0.2mL
STEP 8 0.1mL
STEP 9 0.1mL
STEP 10 0.1mL

STEP 11
Add 0.1mL into wells A1 - D1

Change pipette tips for each well



Follow Color Coding
STEP 12: Add 0.1ml from **Vial 5** into wells E1-H1
STEP 13: Add 0.1mL from **Vial 4** into wells A2-D2
STEP 14: Add 0.1mL from **Vial 3** into wells E2-H2
STEP 15: Add 0.1mL from **Vial 2** into wells A3-D3
STEP 16: Add 0.1mL from **Vial 1** into wells E3-H3

LC

STEP 17: LOW CONTROL (LC, included with kit)
Vortex and lightly centrifuge to remove liquid from cap
Add 0.1mL from low control to wells A4-D4

XC

STEP 18: EXTRA HIGH CONTROL (XC, included with kit)
Vortex and lightly centrifuge to remove liquid from cap
Add 0.1mL from extra high control to wells E4-H4

Reagents & Materials Needed
1. 1.5mL vials or similar (not included)
2. IMDM (included)
3. ATP Standard (included)
4. ATP Controls (included)
5. Non-sterile, 96-well plate (included)

TIPS

> Use calibrated pipettes throughout.
> Vortex thoroughly between each dilution.
> Change tips between each dilution.
> Follow color coding.
> A 0.01µM ATP Standard can be made from the Vial 4 and added to the plate.

STEP 19: Add ATP-ER to reserviour and using a multichannel pipette, dispense 0.1mL into each replicate well
STEP 20: Mix replicate wells as described for Figure 2 in this manual. Change tips for each new addition of ATP-ER
STEP 21: Transfer 96-well plate to luminescence plate reader
STEP 22: Incubate in the dark for 2 minutes and measure luminescence