HALO® PMT "Global"

Short- and Long-Term Lympho-Hematopoietic Patient Monitoring after Stem Cell Transplantation

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™

KM021.001

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1. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. *HALO® PMT "Global" is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)*
2. *HALO® PMT "Global" 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

When a patient has been transplanted with hematopoietic stem cells from umbilical cord blood, bone marrow or mobilized peripheral blood, it is necessary to determine whether the transplanted cells have reconstituted into the patient. There are two types of reconstitution. The first is short-term reconstitution that can be measured, in part, by Time to Engraftment (TE) using HALO® PMT "Global" Assay Kits, which can predict erythroid, neutrophil and/or platelet engraftment. However, HALO® PMT "Global" does not measure stem cell reconstitution. This is performed using HALO® PMT (Patient Monitoring after Transplantation) "Global".

The second type of lympho-hematopoietic reconstitution is the long-term repopulation, not only of hematopoietic stem and progenitor cells, but also lympho-hematopoietic stem cells and the lymphopoietic system. Long-term lympho-hematopoietic reconstitution can take many months. It can also be determined using HALO® PMT "Global".

HALO® PMT "Global" has been designed to measure 4-, 5- or 7-populations of stem and progenitor cells individually, to obtain a "global" picture of the state of the lympho-hematopoietic system.

Medical directors and stem cell processing laboratory personnel might be familiar with the ability to differentiate different types of colonies in a single methylcellulose colony-forming unit (CFU) assay. Unfortunately, this aspect of analyzing stem or progenitor cell differentiation provides only the tip of the iceberg with respect to stem and progenitor cell functionality. To understand whether the stem cell compartment as well as different lineages are being repopulated, the CFU assay cannot be used.

To determine if balanced short- and long-term reconstitution is or has taken place in the patient after stem cell transplantation, it is necessary to determine multiple cell population individually. Each population measured using HALO® PMT"Global" is stimulated with a specific growth factor cocktail. If cells are stimulated, the intracellular ATP (iATP) concentration of these cells increases proportionately. Indeed, the iATP concentration and, therefore the proliferation status, correlates directly with the number of cells plated. An increase in iATP is a direct indication that cells are proliferating and that differentiated and functionally mature cells can be expected in the circulation. This can be determined from from a bone marrow biopsy or from peripheral blood.

HALO® PMT "Global" incorporates a fully standardized and validated ATP bioluminescence readout to measure progenitor cell proliferation ability. HALO® PMT "Global" is, therefore, an instrument-based, non-subjective measurement of iATP using a luciferin/luciferase reaction. It is the most efficient, sensitive, accurate and reliable readout available for hematopoietic cells and is robust so that results, not only within a single laboratory, but between different laboratories can be compared.

Like all HALO® assays, HALO® PMT "Global" also incorporates Suspension Expansion Culture™ (SEC) Technology. SEC™ technology is a methylcellulose-free culture system that is fast and easy to use, exhibits greater sensitivity, accuracy and precision than any CFU assay and requires significantly shorter culture times (only 5 days) than the CFU assay. The investigator can be assured that when using HALO® PMT, they will obtain reliable and trustworthy results that can be compared over time. This is of importance when monitoring a patient after transplantation.

3. Use and Availability

HALO® PMT "Global" is intended for stem cell processing laboratories or other clinical research laboratories that monitor engraftment and reconstitution of patients after hematopoietic stem cell transplantation. HALO®-96 PMT is intended to be used with:

- Peripheral blood from patients.
- Bone marrow biopsies

HALO® PMT are "Global" assays to monitor patient reconstitution after umbilical cord blood, bone marrow or mobilized peripheral blood transplantation. They are available to detect and measure 4-, 5- or 7-populations individually

TABLE 1

	HALO® PMT "Global" Assay Kits		
Description	**4-Population Kit**	**5-Population Kit**	**7-Population Kit**
Primitive lympho-hematopoietic stem cell	Not included	SC-HPP 2	SC-HPP 2
Mature hematopoietic stem cell	SC-GEMM 1	SC-GEMM 1	SC-GEMM 1
Erythropoietic progenitor	P-BFU 1	P-BFU 1	P-BFU 1
Granulocyte-macrophage progenitor	P-GM 1	P-GM 1	P-GM 1
Megakaryopoietic progenitor	P-Mk 1	P-Mk 1	P-Mk 1
T-cell progenitor	Not included	Not included	P-Tcell 3
B-cell progenitor	Not included	No included	P-Bcell 2
Control (no growth factors)	Included	Included	Included

(*) To distinguish between colony-forming unit (CFU) cells and cells that are grown in suspension expansion cultures™ (HALO®), stem cell populations are designated with the prefix "SC", while progenitor cells are designated with the prefix, "P".

Suspension Expansion Culture™ (SEC™) Technology

HALO® PMT "Global" incorporate Suspension Expansion Culture (SEC) Technology. No methylcellulose is used. This has the following advantages over traditional CFU assay methodology:

- All reagents can be dispensed using normal pipettes.
- Allows cell-cell interaction.
- Produces greater assay sensitivity.
- Rapid results in just 5 days.
- Coefficients of variation ≤15%.

HALO® PMT "Global" can be obtained low serum or serum-free, SEC™ Master Mix formulations.

HALO® PMT "Global" Assay Availability

Catalog No.	Number of Populations	Serum Formulation	No. of Samples/ Plate	No. of Plates
K2-4PMT-5	4 + background	Low serum	8	5
K2-5PMT-6	5 + background	Low serum	8	6
K2-7-PMT-8	7 + background	Low serum	8	8
K2SF-4PMT-5	4 + background	Serum-free	8	5
K2SF-5PMT-6	5 + background	Serum-free	8	6
K2SF-7PMT-8	7 + background	Serum-free	8	8

4. The Concept of ATP Bioluminescence Assays

HALO® PMT "Global" is an 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.

Stem and progenitor cells are incubated in the respective HALO® PMT "Global" Master Mixes provided with this kit for 5

days. The incubation time for stem cells can be extended from 5 to 7 days to increase assay sensitivity. 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 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.

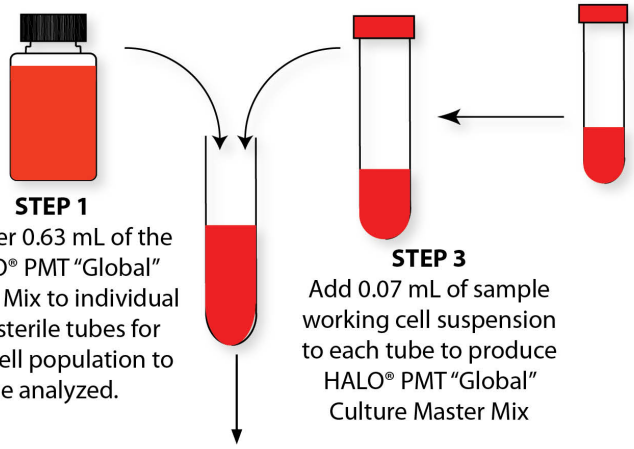
The ATP standard curve and controls need only be measured once on the day samples are to be processed. Do not use previous results from an ATP standard curve and controls performed on a different day. This will cause erroneous results.

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 HALO® PMT "Global" (Figure 1)

QuickGuide to HALO® PMT "Global"



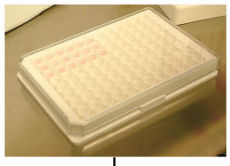
STEP 1
Transfer 0.63 mL of the HALO® PMT "Global" Master Mix to individual 5mL sterile tubes for each cell population to be analyzed.

STEP 2
Prepare cell suspension to a mononuclear cell (MNC) fraction. Perform cell count and viability and adjust to working cell concentration

STEP 3
Add 0.07 mL of sample working cell suspension to each tube to produce HALO® PMT "Global" Culture Master Mix

STEP 6
Gently mix by vortexing

STEP 5
Using an electronic pipette, dispense 0.1mL of Culture Master Mix into each of 6 replicate wells. Use 1 96-well plates for each cell population and background



STEP 6
Incubate for 5-7 days at 37°C in 5% CO_2 and 5% O_2 (preferred)

STEP 7
Calibrate and standardize proliferation assay prior to sample measurement (see separate QuickGuide)

STEP 8
Add 0.1mL of ATP-ER to each well, mix and measure bioluminescence after 10 min incubation in the dark

TIPS

- HALO™ PMT "Global" includes separate 96-well plates for each cell population to be analyzed. Do not mix populations on the same plate. An addition plate is included for a background control.
- Try to use at least a mononuclear cell (MNC) or purified fraction.
- If possible, use self-calibrating, electronic pipettes.
- Always dispense the Master Mix into the bottom of the well, never on the side.
- Incubate plates in a humidified chamber to prevent drying out.
- Culture human cells for 5-7 days.
- Always use gloves when measuring ATP.
- Performing the calibration and standardization procedure ensures reliability and reproducibility.
- Use sterile, adhesive foil (included) to maintain unused well sterility.

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6. Kit Contents and Storage Conditions

HALO® PMT "Global" 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
1a	HALO® PMT "Gobal" 4-Population Assay Kit: 4 Master Mixes to detect SC-GEMM 1, P-BFU 1, P-GM 1 and P-Mk 1 + a Background Master Mix (no growth factors).	-20°C until used
1b	HALO® PMT "Global" 5-Population Assay Kit: 5 Master Mixes to detect SC-HPP 2, SC-GEMM 1, P-BFU 1, P-GM 1 and P-Mk 1 + a Background Master Mix (no growth factors).	-20°C until used
1b	HALO® PMT "Global" 7-Population Assay Kit: 7 Master Mixes to detect SC-HPP 2, SC-GEMM 1, P-BFU 1, P-GM 1 and P-Mk 1, P-Tcell 3 and P-Bcell 2 + a Background Master Mix (no growth factors).	-20°C until used
2	Medium (IMDM) for dilution of the ATP standard.	-20°C until used
3	ATP standard.	-20°C until used
4	ATP extra high, high and low controls.	-20°C until used
5	ATP Enumeration Reagent (ATP-ER)*	-20°C in the dark until used
6	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
7	Sterile, solid white 96-well plates for cell culture	Can be kept with other kit components
8	Non-sterile, solid white 96-well plates for ATP standard curve determination.	Can be kept with other kit components
	Technical manual can be downloaded from the HALO® PMT page on the Preferred Cell Systems™ webiste	

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

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

IMPORTANT
All kit components are quality controlled and optimized so that they work together. Please do not replace kit components with those of a different product. This will invalidate the warranty provided by 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. HemoGro™ Hematopoietic Growth Medium for cell suspensions and dilutions (Preferred Cell Systems™).
2. Iscove's Modified Dulbecco's Medium (IMDM).
3. Density-gradient medium (e.g. LymphoPrep).
4. 7-AAD, propidium iodide or trypan blue for viability assay.
5. LIVEGlo™ metabolic viability assay (Preferred Cell Systems™)
6. CD19 magnetic microbeads (Miltenyi Biotech) for separation of P-Bcell 2 responsive progenitor cells from the MNC fraction. Necessary to determine this population.
7. CD3 magnetic microbeads (Miltenyi Biotech) for separation of P-Tcell 3 responsive progenitor cells from the MNC fraction (can be used for greater accuracy).

8. The HALO® PMT "Global" Protocol

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

Performing HALO® PMT "Global" is a 3-step process.

Step 1 – Cell preparation.
Step 2 – HALO® PMT "Global" cell culture procedure, plating and incubation in the 96-well plates.
Step 3 – Luminescence measurement. An ATP dose response is performed prior to sample luminescence measurements with conversion of RLUs to µM ATP.

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

STEP 1 – Cell Preparation

HALO® PMT "Global" can be performed using tissues with the following purity:

1. A total nucleated cell (TNC) fraction can be used from the original sample, but it is not recommended as it will severely dilute and underestimate the concentration of stem and progenitor cells. It is highly recommended to use a mononuclear cell fraction or higher for these assays.
2. For peripheral blood or bone marrow samples, a mononuclear cell (MNC) fraction is the cell preparation of choice. This fraction can be prepared by density gradient centrifugation using, for example, LymphoPrep.
3. Further purification of B-lymphopoietic progenitor cells is required and, additional purification of T-lymphopoietic progenitor cells might improve accuracy of the HALO® PMT "Global" 7-Population Assay (See Step 2 and Section 9).

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 TNC count or the number of viable cells as this will give erroneous results.
3. Adjust the cell suspension concentration to that recommended in Table 1.

Note *the working cell concentration per ml is 100 x the final cell concentration per well.* If cells have been treated prior to cell culture, higher cell concentrations may be required.

4. Prepare the total volume of cell suspension required using HemoGro™. The volume of the adjusted cell suspension required will be 10% of the total volume of HALO® PMT "Global" Culture Master Mix prepared.

TABLE 1
Recommended Cell Doses for Cell Types, Cell Preparations and Cell States for HALO® PMT "Global"

Cell Type	Cell Preparation	Cell State	Working Cell Concentration Required (100 x Final Cells/Well)	Final Cell Dose / Well
Bone marrow	MNC	Fresh/Frozen	0.5-0.75 x 10^6	5,000-7,500
Peripheral blood	MNC	Fresh/Frozen	0.5-0.75 x 10^6	5,000-7,500

STEP 2. HALO® PMT "Global" Cell Culture Procedure

- ***HALO® PMT "Global" Master Mixes are complete and ready-.to-use.***
- ***Perform all procedures under a laminar flow, bio-hazzard hood.***
- ***Wear protective clothing, including gloves for all operations.***

IMPORTANT: ***If using HALO® PMT "Global" 7-Population Assays, this includes Master Mixes for T- and B-progenitor cells (P-Tcell 3 and P-Tcell2). Preferred Cell Systems™ highly recommends that to detect the P-Bcell 2 population accurately, the MNC fraction should be further purified into CD19$^+$ cells using Miltenyi magnetic microbeads. If required, the CD3$^+$, P-Tcell 3 population can also be purified using Miltenyi magnetic microbeads, although the MNC fraction usually suffices.***

1. Remove the HALO® PMT "Global" Master Mixes for all cell populations to be measured, including the background, that came with the assay kit from the freezer and thaw either at room temperature or in a beaker of cold water. Do not thaw in a 37°C water bather or incubator.
2. Label sufficient 5mL tubes for the number of samples to be tested. Each cell population HALO® PMT "Global" will have its own tube.
3. Using a calibrated pipette, preferably an electronic pipette with repeat function, dispense a volume of the HALO® PMT "Global" Master Mix minus 10%, that will allow 0.1mL to be dispensed into the required number of replicate wells. See Table 2. Performing a minimum of 6 replicate wells provides the necessary statistical relevance.
4. In addition to setting up culture tubes for each cell population, remember to include an extra tube for the background.

TABLE 2

Number of Replicate Wells Required	Volume of HALO® PMT "Global" Master Mix	Volume of Cells (10% of final volume)	Total Volume
6	0.63mL	0.07mL	0.7mL

5. Adjust the cell concentration to the working cell concentration shown in Table 1. The working cell concentration should be 100 fold higher than the final cell concentration in the well. Thus, if the final cell concentration is to be 5,000 cells/well, the working cell concentration should be 100 times 5,000 or 500,000 (5 x 10^5) cells/mL.
6. Dispense the required volume of cells into each tube containing the HALO® PMT "Global" Master Mix.
7. Mix the contents gently on a vortex mixer. Do not cause bubbles.
8. Remove all sterile, individually wrapped, 96-well plates from the assay kit box. Use one plate for each cell population plus the background.

9. For each cell population, use a calibrated pipette, preferably an electronic pipette with repeat function, to dispense 0.1mL into each of 8 replicate wells of the 96-well plate.
   TIP: For 8 replicates, each sample is dispensed in columns.
10. After replacing the lid, transfer the 96-well plate to a humidified container (see Section 9).
11. Transfer the humidified container to the incubator. The incubator should be fully humidified at 37°C and gassed with 5% CO_2 and, if possible, 5% O_2. This reduced oxygen tension improves plating efficiency by reducing oxygen toxicity cause by the producing of free radicals.
12. Incubate the cells for 5 days.

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 DOWNLOAD THE DOCUMENT 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 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. Instrument Calibration and Assay Standardization

An Instructional Tutorial on "How to Calibrate and Standardize Any ATP Bioluminescence Assay" is available on the Preferred Cell Systems website under the Resources tab.

It is highly recommended 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 13**) that should be obtained prior to measuring samples. Use the non-sterile, 96-well white plate provided with the assay kit for this purpose.

HALO® PMT "Global" Assay Kit includes the following to calibrate and standardize the ATP bioluminescence part of the assay to measure cell proliferation.

- 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: In the majority of cases, values for all stem and progenitor cells are going to be within the range provided by Protocol 1. Use the low and high calibration controls and perform an ATP standard curve from 0.01µM to 1µM. **See Page 19**. ATP concentrations should be within this ATP standard curve range. If they are found to be higher, perform Protocol 2.

PROTOCOL 2: In some cases, proliferation of one or more cell populations might exceed the ATP standard curve for Protocol 1. If this occurs, repeat the ATP standard curve from 0.03µM to 3µM and include the extra high control. **See Page 20**. If calibration and assay standardization has been performed, it is extremely important that the ATP standard curve slope, and ATP controls lie within the ranges specified in **Section 13**.

It is important that the sample 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. For short-term engraftment, it will be very rare that the cell proliferation will be greater than 3µM ATP. However, if ATP values from a sample is 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.

IMPORTANT. ***Only when the ATP standard curve and controls are within the measurement assurance parameters depicted in Section 13, should the samples be measured. Obtaining these values within the ranges shown in Section 13, indicate that the assay is working correctly and the results obtained can be trusted. It is for this reason why performing the ATP standard curve and control is high recommended.***

C. 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˚C gassed with 5% CO_2 for 30min. Otherwise, allow the plate to come to room temperature for 30 min.
2. If only part of the plate has been used, transfer the plate to a bio-safety hood and remove the lid under sterile conditions. Take a sterile adhesive plate coverfoil from the kit box, 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 an 8-channel pipette, add 0.1mL of ATP-ER to each wells in the first column (A1-H1). Mix the contents as described in Section 10.
4. Repeat this procedure for each column using new tips.
5. When ATP-ER has been added to all wells, transfer the plate to the luminometer and close the lif or draw. Incubate the plate in the dark for 10 min at room temperature. During this period the cells are lysed and the luminescence signal stabilized.
6. Without taking the plate out of the reader, measure the samples.
7. Unused ATP-ER may be returned to the bottle and refrozen. See section 10 for ATP reagent storage conditions and stability.

D. Luminescence Plate Readers with automatic dispensers

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

9. Recommendations and Tips Prior To Using HALO® PMT "Global" .

(i) Cell Suspension

a. The preferred cell suspension is a mononuclear cell suspension (MNC) or higher purity. **PLEASE NOTE:** Although it is normal for cell processing laboratories to prepare a red blood cell-/plasma-depleted total nucleated cell (TNC) fraction using instrumentation, using this fraction will severely underestimate the results.
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

For clinical purposes, a minimum of 6 replicates/sample is recommended. 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
When performing 6 replicates, cultures are plated across the plate in rows.

(iv) 96-Well Plates Provided
The reagents have been optimized to work with the 96-well plates provided in the HALO® PMT "Global" 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 plastic ware 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) Detecting Lymphopoietic Progenitor Cell Populations
HALO® PMT "Global" 7-Population Assay Kits contain Master Mixes to detect both T- and B-lymphopoietic progenitor cell populations (P-Tcell 3 and P-Bcell 2). To detect the B-lymphopoietic cell population, it is necessary to further purify $CD19^+$ cells from the MNC fraction. This will reduce the possibility detecting proliferation by an impure population. Further purification of T-lymphopoietic cells is not necessary, although a more accurate response is obtained with purified $CD3^+$ cells.

(vii) Incubation Times
The recommended incubation time is 5 days. This can be increased for the stem cell populations to 7 days to improve assay sensitivity. There is a 2-3 fold increase in ATP concentration from day 5 to day 7 of culture. However, this increase can be at the expense of higher variability (increased coefficients of variation, CVs) between wells.

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.***
- ***HALO® PMT "Global" 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˚C.
- Sufficient ATP standard, controls and ATP-ER are supplied to usually 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 2-8ºC overnight.
- Do not use any form of heat to thaw this reagent.
- Allow the lyophilized ATP-ER substrate (brown glass bottle) to come to room temperature.
- Remove the closures from both bottles.
- Carefully pour the entire contents of the buffer bottle into the lyophilized ATP-ER substrate bottle. Swirl gently or invert slowly to mix. Do not shake.
- Allow the ATP-ER mix to reconstitute for 10 minutes at room temperature.
- Reconstituted ATP-ER is stable for 8 hours at room temperature, 48 hours at 2-8ºC, or 20 weeks at -20ºC.
- ATP-ER can be refrozen up to 11 cycles without significant loss of sensitivity.

Volumes of Luminescence Kit Components Required

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

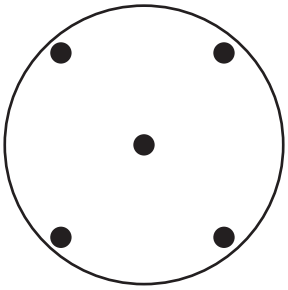
Please refer to the Instructional Tutorial on "How to Calibrate and Standardize Any ATP Bioluminescence Assay" is available on the Preferred Cell Systems website under the Resources tab.

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 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 from the Preferred Cell Systems™ website.**

12. Results

Reconstitution after stem cell transplantation occurs in two steps. The first is short-term reconstitution during which erythroid, neutrophil and platelet engraftment takes place. Mature stem cells are usually responsible for this process. Long-term reconstitution requires the establishment of primitive lympho-hematopoietic stem cells that are responsible for both lymphopoietic and hematopoietic repopulation. The latter can require many weeks, if not months, to occur. HALO® PMT "Global" has the potential to predict both short-term hematopoietic, and long-term lympho-hematopoietic reconstitution.

In order to monitor and assess patient reconstitution at various time after stem cell transplantation, it is necessary to calibrate and standardize the assay HALO® PMT "Global" prior to measuring any samples. This will allow results from different time points to be directly compared with each other.

If reconstitution occurs, gradual increases in all cell populations over time will be apparent and should eventually reach a plateau, whereby a steady-state situation with respect to stem and progenitor cell numbers has been attained.

It is important to realize that HALO® PMT "Global" is NOT a potency assay and does not provided quantitative data that would indicate that the cell source was of high quality or high potency. Although clinical outcome is often considered to indicate potency, this parameter is used as a predictor of stem cell capacity and capability used for transplantation and not of the cell populations produced by the transplanted stem cells.

13. HALO® PMT "Global" Assay Measurement Assurance and Validation Parameters

HALO® PMT "Global" must be calibrated and standardized, to allow results of reconstitution to be compared over time.

This is achieved by performing a calibration and assay standardization prior to measuring the samples. The results of the calibration and standardization are compared to the measurement assurance parameters shown in Table 4. 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 4, contact Preferred Cell Systems for help.

Table 4: 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 hematopoietic cells: ~0.04µM. The proliferation ability (growth) of the cells in this situation is extremely questionable.
- ATP concentration below which cells are not metabolically viable, i.e. the cells are dead: ~0.01µM. The cells should not be used in this situation.
- 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 IMDM 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
HALO® PMT "Global" exhibits 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 comparison): ~95%.
- High throughput capability (Z-Factor): >0.76 (lowest possible value, 0.5; highest possible value, 1).

14. Troubleshooting

If Calibration and Standardization Results Do Not Conform to Measurement Assurance Parameters (Section 13)
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 13. 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 12, 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. **PLEASE NOTE:** Dye exclusion viability can produce a false positive results. Even if the cell viability by dye exclusion methods is greater than 85%, this does not mean that the metabolic viability allows the cells to proliferate and grow. It is possible to have high dye exclusion viability values, but the proliferation ability of the cells will be low or non-existent, i.e. the cells might be dead.
- *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.

Other Hematopoietic Cellular Therapy Assay Products from Preferred Cell Systems™

HALO® PCA is a 96-well ATP bioluminescence progenitor cell assay to replace the CFU assay for cell processing laboratories.

HemoFLUOR™ PCA is a 96-well, fluorescence readout version of HALO® PCA .

HemoLIGHT™ PCA is a 96-well, absorbance readout version of HALO® PCA .

STEMpredict™ is a 3-day, fully standardized, ATP bioluminescence, stem cell quality assay designed primarily for cord blood banks to triage high from low quality cord blood units prior to cryopreservation. STEMpredict™ is the only assay for hematopoietic cellular therapy products designed for both high-throughput 96- and 384-well plate formats.

HALO® RS is a 7 day, 96-well, standardized ATP bioluminescence assay to establish cord blood, bone marrow or peripheral blood reference standards for HALO® SC-IPS assays.

HALO® SC-IPS is a 7-day, standardized, 96-well, ATP bioluminescence assay to measure the identity, purity and strength (potency) of 2 primitive stem cell populations in cord blood, bone marrow or peripheral blood samples, prior to use in patients.

HALO® TE, HemoFLUOR™ TE and **HemoLIGHT™ TE** are Time to Engraftment assays using luminescence, fluorescence or absorbance, respectively

HemoFLUOR™ PMT "Global" and **HemoLIGHT™ PMT** "Global" are 4-, 5- or 7-population assays to monitor patient reconstitution after transplantation.

Preferred Cell Systems™

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

Technical Support
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Website: www.preferred-cell-systems.com

Preferred Cell Systems™

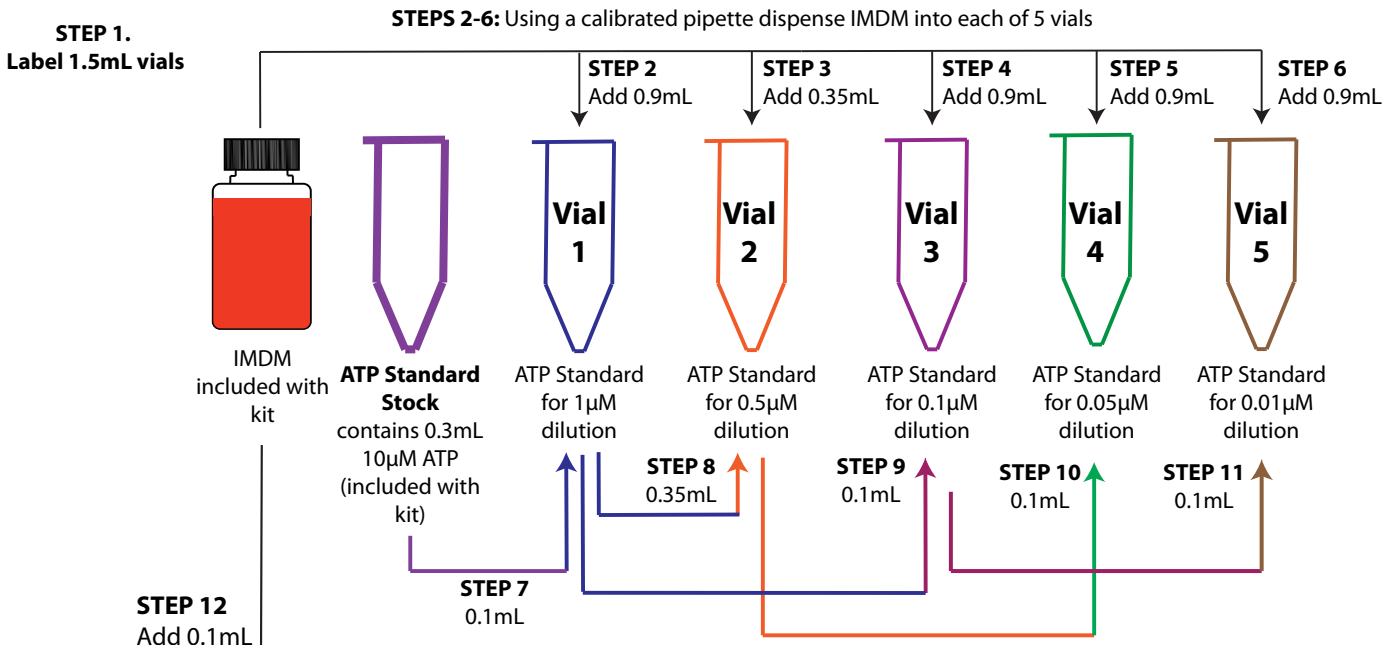
Calibration and Standardization Protocol of an ATP Bioluminescence Assay

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)

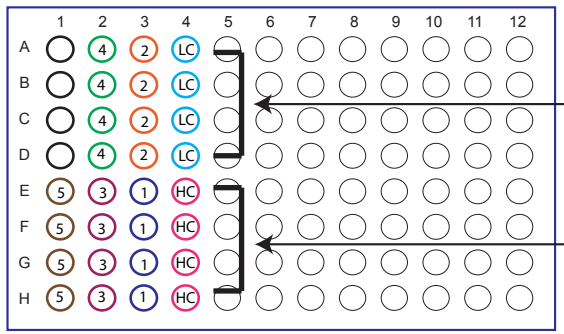
TIPS

> 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

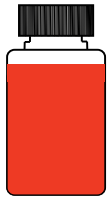
Calibration and Standardization Protocol of an ATP Bioluminescence Assay

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



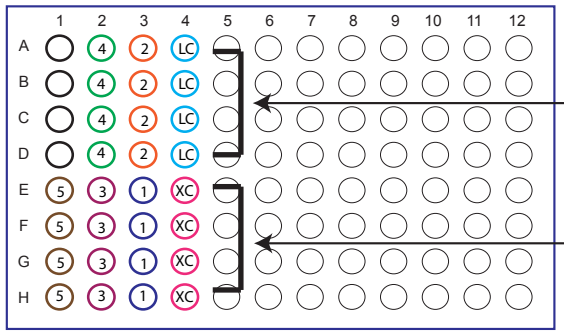
IMDM included with kit

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