ATP Bioluminescence Standardization & Calibration Kit

(Catalog Number: K-ATPSC-1)

Technical Manual

(Version 1-22)

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™

KM034.001

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1. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. *The ATP Bioluminescence Standardization & Calibration Kit is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)*
2. *The ATP Bioluminescence Standardization & Calibration Kit is for research use only and has not been approved for clinical diagnostic use.*
3. *This kit should not be used beyond the expiration date on the kit label.*
4. *Do not mix or substitute reagents or other kit contents from other kit lots or sources.*
5. *Always use professionally calibrated and, preferably, electronic pipettes for all dispensing procedures. Small discrepancies 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.*
6. *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

Adenosine triphosphate (ATP) bioluminescence assays are considered to be the sensitive non-radioactive assays available. They are fast and easy to setup and provide extremely accurate and rapid results using a luminescence plate reader. Many ATP bioluminescence research assays available from Preferred Cell Systems™ do not include the ability to calibrate the plate reader and standardize the assay. This reduces the cost of the assay to the research, but also has the disadvantage of not providing standardized results.

The readout of the luminescence plate reader is in Relative Luminescence Units or RLU. It is "relative" because different instruments have different sensitivities and therefore the output of the instrument may be from 0 1,000 or 0 - several million RLUs. Although all Preferred Cell Systems™ "Glo" assays are highly reproducibile, using RLUs does not provide the ability to directly compare either intra- or inter-laboratory results. This is because the ATP Enumeration Reagent (ATP-ER) decays with time, so that slightly different results will occur for exactly the same experiment over time.

For all assays (including all "Glo" assays) from Preferred Cell Systems™, it is recommended to include a background control (i.e. cells cultured in medium alone), so that true differences between the background and stimulated/proliferating cells can be assessed.

However, this is not an alternative for assay standardization. For this reason, Preferred Cell Systems™ provides an optional add-on ATP Bioluminescence Standardization & Calibration Kit that can be purchased separated.

Calibration and assay standardization are an integral part of the assay validation procedure, which in turn, is part of trusting the results obtained with an assay. There are several inportant reasons for assay standardization. These include:

1. Calibrating the instrument and also ensure that the reagents are working correctly.
2. The standard curve also ensures that the reagents are working correctly.
3. The standard curve allows the luminescence output in Relative Luminescence Units (RLU) to be converted to standardized ATP concentrations (µM).
4. Allows proficiency testing without perfoming additional assays.
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.
6. Performing the ATP standard curve allows results to be compared over time.

3. Use and Availability

The ATP Bioluminescenc Standardization & Calibration Kit is meant to be used as an add-on to any "Glo" assays available from Preferred Cell Systems™

The assay kit includes everything required to perform 3 instrument calibrations and 3 ATP standard curves. The assay kit also includes a non-sterile, 96-well plate.

4. The Concept of ATP Bioluminescence Assay Standardization

The fundamental concept underlying any cellular assay that measures the cell's chemical energy is the detection of the concentration of intracellular adenosine triphosphate (iATP). If a cell is producing iATP, it is demonstrating cellular and mitochondrial integrity and is therefore viable. When cells are stimulated to proliferate, the iATP concentration increases several fold. The iATP concentration produced is directly dependent on:

- The proliferation potential (or primitiveness) of the cell population being detected.
- The concentration of the cell stimulants.

- The plated cell concentration.

After cells are cultured, a single-step addition of an ATP Enumeration Reagent (ATP-ER) 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 present assay kit, allows the non-standardized RLU values to be converted into standardized ATP concentrations in micro molar (µM) values. This allows results to be directly compared over time regardless of the possible decay of the ATP-ER reagent over time.

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 convertion of RLU results into ATP concentrations is performed by interpolation from the ATP standard curve. 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.

5. Assay Standardization Videos

To see and understand the stanadrdization procedure, we highly recommend viewing Part 1 and Part 2 of the videos entitled "How to Calibrate and Standardize Any ATP Bioluminescence Assay" by navigating to this web address.

Alternatively, you can view these videos our the Preferred Cell Systems YouTube Channel:

Part 1: How to Calibrate and Standardize Any ATP Bioluminescence Assay
Part 2: How to Calibrate and Standardize Any ATP Bioluminescence Assay

6. Kit Contents and Storage Conditions

The ATP Bioluminescence Standardization & Calibration Kit contains 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
3	Medium (IMDM) for dilution of the ATP standard.	-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
9	Non-sterile 96-well plate(s) for ATP standard curve determination.	Can be kept with other kit components
	Technical manual. Downloaded from the Preferred Cell Systems™.	

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

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

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

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

Please see the corresponding "Glo" Assay Kit requirements for each tissue type being measured.

8. The ATP Bioluminescence Standardization & Calibration Procedure

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 10 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 9 for recommendations and tips prior to starting this part of the procedure. In particular, please refer to Section 9 for important information on mixing components.***
- ***Remove the ATP Enumeration Reagent (ATP-ER) from the freezer and thaw at room temperature or in cold running water prior to analysis. Do not thaw the ATP-ER in a water bath or 37ºC incubator.***
- ***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 non-sterile, 96-well plate provided with the kit to perform the ATP standard dose response curve.***

A. Calibrating and Standardizing the Assay
It is highly recommended to calibrate the luminescence plate reader and standardize the assay prior to measuring samples. This will allow a comparison with the expected measurement assurance values (**see Section 11**) that should be obtained prior to measuring samples. Use the non-sterile, 96-well white plate provided with the assay kit for this purpose.

The ATP Bioluminescence Standardization & Calibration Kit includes the following to calibrate and standardize the ATP bioluminescence part of the assay to measure cell proliferation occurring in the colonies.

- 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: If it is expected that the cells have a low proliferation ability, use the low and high calibration controls and perform an ATP standard curve from 0.01µM to 1µM. **See Page 6**. Human bone marrow and cord blood and all animal bone marrow, with the exception of mouse and sometime rat, will fall into this group.

PROTOCOL 2: For human mobilized peripheral blood, mouse bone marrow and purified cell populations, use the low and extra high calibration controls and perform an ATP standard curve from 0.03µM to 3µM. **See Page 7**.

PROTOCOL 3: It is also possible to perform a 6-point standard curve from 0.01µM to 3µM. However, this will reduce the number of standardizations possible to two (2).

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. If Protocol 2 has been used and values are not as high as 0.03µM ATP, perform Protocol 1. In some cases, cell proliferation could be greater than 3µM ATP. If ATP values from the samples 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. Using a plate luminometer with automatic dispenser
The user may have a plate luminometer that allows reagents to be dispensed automatically directly into the well. Preferred Cell Systems™ does <u>not</u> recommend using the automatic dispensers, since the contents of the well are not mixed sufficiently using this method.

D. Using a liquid handler
The ATP bioluminescence standardization and calibration procedure can be performed using a liquid handler (robot). If you intend to perform any part of the assay procedure using a liquid handler, please contact Preferred Cell Systems™ for information on setting up the instrument. Extra ATP-ER is required when using a liquid handler.

9. Recommendations and Tips Prior To 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.***
- ***The kit includes a solid white plate for 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 3 standard curves and controls/assay kit.
- 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

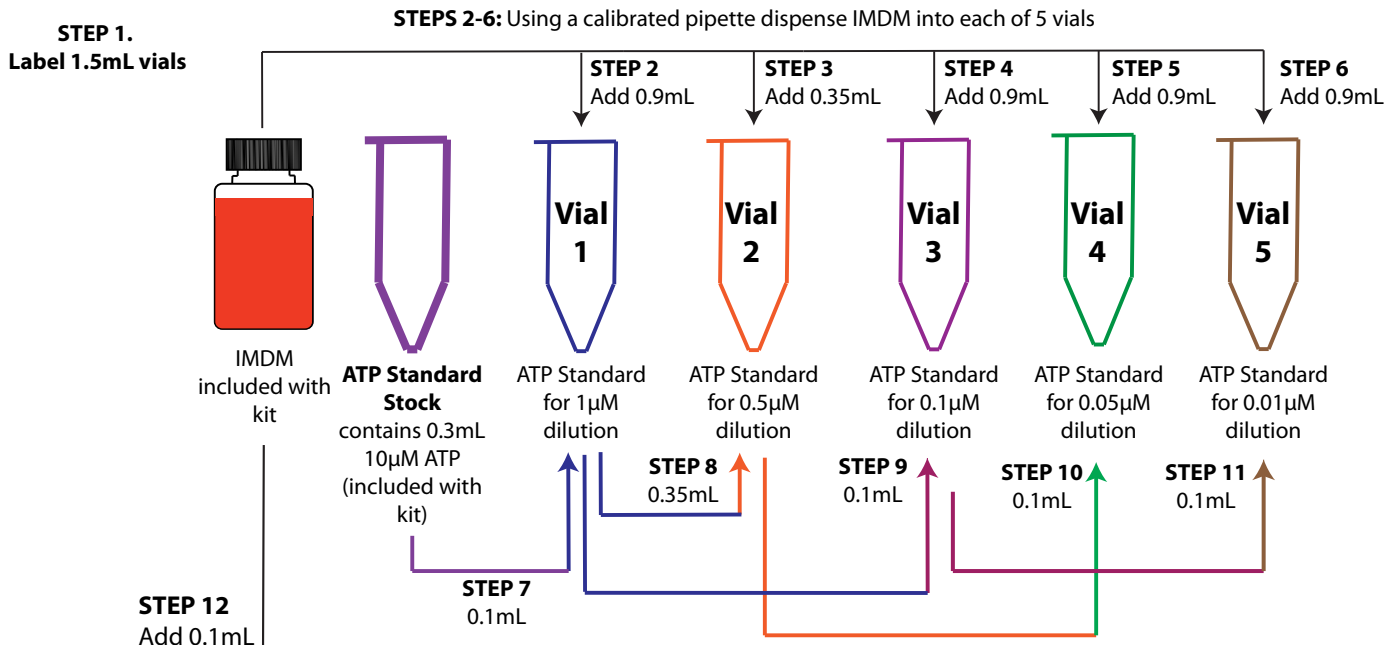
Calibration and Standardization Protocol of an ATP Bioluminescence Assay

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

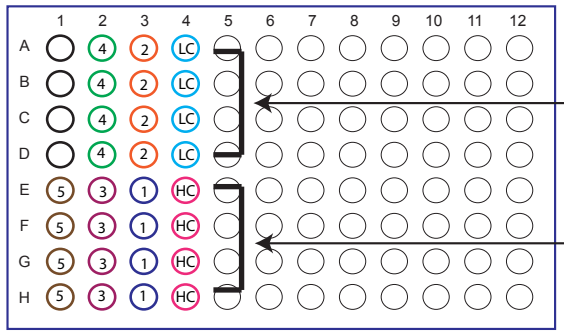
Reagents & Materials Needed
1. 1.5mL vials or similar (not included)
2. IMDM (included)
3. ATP Standard (included)
4. ATP Controls (included)
5. Non-sterile, 96-well plate (included)

TIPS

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



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

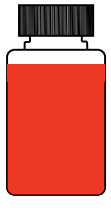
Calibration and Standardization Protocol of an ATP Bioluminescence Assay

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



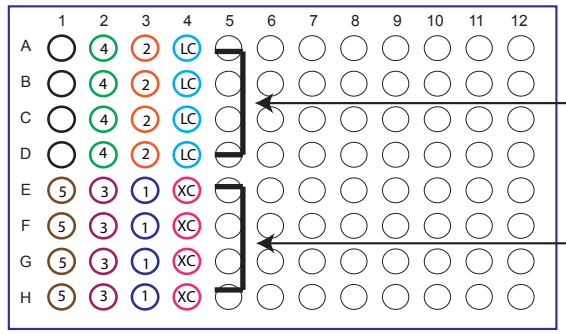
IMDM included with kit

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

the dark, for 48h or is stable at -20°C for 20 weeks.

Volumes of Luminescence Kit Components Required

- Each vial of ATP standard contains enough volume to perform 3 ATP standard dose responses.
- The amount of ATP-ER added to each well is 0.10mL.

ATP Standard Curve

The ATP standard and controls (calibrators) are used for the following purposes:

- 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.
- It tests for proficiency of the user to perform the assay correctly without dispensing errors.

Adhesive Plate Covering Film

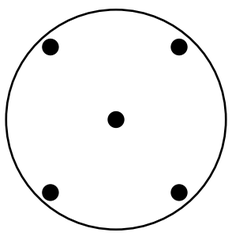
To help keep the plate(s) sterile, adhesive, air permeable, sterile films are provided so that the part of the plate that is not being used can be covered and kept sterile until required. If using the adhesive film provided, the plate cover should be removed in a laminar air-flow hood and replaced with 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 1 for each corner of the well.
5. Try not to cause excessive bubbles in the culture and DO NOT over mix since this can result in drastically reduced luminescence values.
6. This procedure effectively and optimally mixes the contents well.

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



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

The readout from all luminescence plate readers or luminometers is Relative Luminescence Units (RLU). The term "relative" is used because luminometers from different manufacturers produce different RLU ranges. The RLU range may be from 0-100 for one instrument and 1-1,000,000 for another. A RLU value is a non-standardized unit of measurement. However, to compare results it is necessary to standardize the assay.

(i) Luminescence Plate Reader Setup

Multimode instruments, i.e. those that can detect absorbance, fluorescence and luminescence, often need to be manually set for both the integration time and the "gain". Dedicated instruments, i.e. those that only detect luminescence, usually only have to be set for the "integration time". It is therefore necessary to first know whether the instrument is a multimode or multipurpose instrument and whether "integration time" and "gain" need to be set. The instrument instruction manual will provide this information. If the "gain" has to be set, the instruction manual will explain how the correct "gain" is established. Once the "integration time" and "gain" are set, they should not be changed.

a. First set the integration time to 2 seconds.
b. Next, set the "gain". This must be determined empirically and is best performed when the ATP standard curve is measured. The gain should be adjusted so that the percent coefficients of variation (%CV) for the mean of the replicates are the lowest value. These values should be about 5% or less.
c. The measurement temperature of the instrument should be set to between 22°C and 24°C or turned off.
d. Do not use plate shaking or the injectors if the instrument has this capability.

(ii) Instrument Setup for Luminometers with Software Analysis Capabilities

The luminometer is usually controlled by software installed on a computer using a serial or USB interface cable. The software for some luminometers comes with extensive analysis capabilities. This allows all the calculations to be programmed and performed by the luminometer software. If the software does not include analysis capabilities, the results are usually exported directly to a Microsoft Excel file for calculation and analysis.

Before using any luminometer, ensure that you are familiar with the software that controls the instrument. For luminometer software that has analysis capabilities, setting up the software properly prior to any measurements can save considerable time and produce an optimized report. It may be necessary to contact the instrument manufacturer to determine whether the software can provide the information below and whether it can perform the necessary calculations so that the procedure can be automated.

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

b. The second set of measurements to be performed will be the ATP standard curve. Setup the software to give the following information:
   1. Group or sample designation
   2. ATP standard dose response values (these are the calculated values of the ATP concentrations used for the dose response)
   3. Well numbers
   4. RLU/well
   5. Mean RLU (optional)
   6. Standard deviation of Mean RLU (optional)
   7. %CV of Mean RLU (optional)
   8. Predicted ATP concentration/well calculated by interpolating the RLU values from the ATP standard curve into ATP concentrations actually obtained. This should be performed automatically by the luminometer software. This is actually a back calculation of the ATP doses used to generate curve. The calculated ATP

concentrations should correspond to the expected ATP values.
9. Mean predicted ATP
10. Standard deviation of mean predicted ATP
11. %CV of mean predicted ATP.

The software should be capable of performing a log-log linear regression curve fit according to the equation:

$$\log Y = A + B * \log X$$

where A is the Y-intercept and B is the slope of the dose response curve. Do not use the equation Y = A + B*X as this will normally produce negative values for the lowest ATP dose. In addition, converting the X- and Y-axes to log is not equivalent to the curve fit shown above.

Figure 2 shows a typical ATP standard dose response using SoftMax Pro software that controls a Molecular Devices Lmax luminometer. The curve fit is for a 5-point ATP dose response ranging from 0.03µM to 3µM. If the log-log linear regression curve fit is performed as stated above, then the curve fit parameters should fall within the following guidelines obtained for 93 individual ATP dose response curves:

- Goodness of fit (r^2) = 0.999 ± 0.001 (%CV = 0.071)
- Y-Intercept (A) = 6.71 ± 0.63 (%CV = 9.37)
- Slope (B) = 0.969 ± 0.18 (%CV = 1.9). This is slightly different to the value given in Section 11. (Values are the Mean ± 1 Standard Deviation)

c. The third set of measurements to be performed will be those of the samples. Setup the software to perform the following calculations:
1. Group or sample designation
2. Sample number
3. Well number
4. RLU/well
5. ATP values/well (calculated from the ATP standard dose response curve)
6. Calculated mean ATP values
7. Standard deviation of calculated ATP values
8. % CV of calculated ATP values.

Most, if not all, the calculations and results can be obtained automatically directly from the luminometer without any further manipulation. By automatically converting the RLU values into ATP concentrations (µM) directly from the ATP standard curve, results from the samples can be graphically displayed via the software.

Most software packages can export the results to MS Excel either directly or via text files.

(iii) Instrument Setup for Luminometers without Software Analysis Capabilities

Many plate luminometers do not come with analysis software. Instead, the data is either automatically exported or has to be manually exported to a Microsoft Excel file for calculation and analysis. Excel has functions to perform the necessary calculations for interpolating RLU values into ATP concentrations using the ATP standard curve. The basic Excel procedure is as follows:

1. Column 1: Make a column for the calculated ATP concentrations used for the ATP standard curve.
2. Column 2: Copy the RLU values for the standard curve.
3. Column 3. Transform the RLU values into log RLU values using the LOG function.
4. Column 4. Transform the ATP values in column 1 into log ATP values.
5. Column 5. Using the Excel TREND function, perform a Trend analysis for the log RLU values in Column 3.
6. Column 6. Transform the log values back into actual values using the Excel ANTILOG function.
7. Column 7. Perform a TREND function for the log ATP values.
8. Column 8. Transform the log trend ATP values back into actual ATP values using the Excel ANTILOG function.
9. Column 9. Copy the sample RLU values.
10. Column 10. Transform the sample RLU values into LOG RLU values.
11. Column 11. Using the Excel TREND function, perform a trend analysis for the sample.
12. Column 12. Convert the calculated sample values back into ATP concentrations.

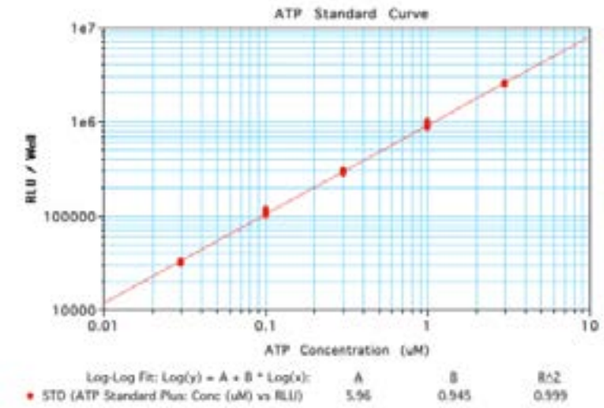
(iv) Using Third-Party Software

Instead of using Microsoft Excel, third party software can also be used. In this case, the raw data in the Excel file must be copied and pasted or copied into the clipboard and imported into the software program. It is important that the third-party software can either perform a log-log linear regression analysis on the raw data or can transform the data into log values. The following software has been tested to perform the necessary calculations and graphs:

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

For technical assistance using these third-party software packages, please contact Preferred Cell Systems™.

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



11. ATP Bioluminescence Standardization & Calibration Measurement Assurance and Validation Parameters

If any Preferred Cell Systems™ "Glo" assay has been calibrated and standardized with the present kit, 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 4, 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 hematopoietic cells: ~0.04µM
  Please note that human B-cells, especially cryopreserved cells, may exhibit very low ATP values. It is important to compare the stimulated B-cells with their background (no growth factors added) to determine B-cell activity.
- 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 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

By using the ATP Bioluminescence Standardization & Calibration Kit, it is possible to expect 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).

These parameters have been obtained from numerous experiments performed at Preferred Cell Systems™.

12. Troubleshooting

If Calibration and Standardization Results Do Not Conform to Measurement Assurance Parameters (Section 12)

If the investigator has elected to calibrate and standardize an ATP bioluminescence assay for the tissue concerned, results should be within the ranges provided in Section 11. 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 11, 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.

High Coefficients of Variation (%CV)

Coefficients of variation (%CV) for the scalibration and tandardization procedure 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. 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 Bioluminescence Values

Performing the calibration and standardization procedure prior to sample measurement can help detect problems prior to sample measurement. If low RLU values occur, this could be due to several 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. **NOTE:** Even though the cell viability might be 85% or higher, this does not necessarily mean that the cells will proliferate and grow. This is because a dye exclusion viability measurement does not predict metabolic viability, e.g. intracellular ATP product. It is possible to have a high dye exclusion viability, but the metabolic viability, indicating the ability to proliferate, might be very low or zero, indicating at the cells will either not proliferate or are dead, respectively.
- *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.

Ordering Information
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The ATP Bioluminescence Standardization & Calibration Kit was designed and developed by Preferred Cell Systems™, Inc.

Preferred Cell Systems™