

LIVEGlo™

An ATP Bioluminescence Metabolic Viability Assay

Technical Manual

(Version)

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

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

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

Preferred Cell Systems™

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1. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. *LIVEGlo™ is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)*
2. *LIVEGlo™ 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

A cell viability assay determines whether cells are alive or dead. There are two types of viability assay. The first is dye exclusion viability and the second is metabolic viability. Dye exclusion viability measures the percentage of live or dead cells by using a dye such as trypan blue, propidium iodide, 7-aminoactinomycin D (7-AAD) or acridine orange. Many of these dyes pass through the membrane and bind to DNA. The ability of a dye to pass through the membrane indicates the cell's membrane integrity. If the dye can pass through the membrane and enter the cell and bind to components within the cells, the presence of the dye is considered an indication that the cells are dead. However, dye exclusion viability does not take the metabolic or cellular activity into account and therefore does not detect cellular and membrane integrity.

As an example of this, cells can demonstrate a high proportion of "live" cells by dye exclusion viability, but the cells could be metabolically "dead" by not producing chemical energy, in the form of adenosine triphosphate or ATP, to support all the required cellular and nuclear activities. This false positive result can have important consequences, especially in the cellular therapy and regenerative medicine fields.

LIVEGlo™ is a fast and simple assay that can be used on fresh primary cell suspensions from a variety of organs and tissues or from thawed, previously cryopreserved cells, to measure metabolic viability. If cells are alive, they will be producing chemical energy in the form of ATP, produced in the mitochondria.

A viability assay is usually performed prior to cell culture or other cell treatment. Some cells, such as primary stem cells, and especially quiescent stem cells, have very low levels of metabolism. Other cell types, for example hepatocytes or cells of the kidney will exhibit high basal levels of intracellular ATP.

The LIVEGlo™ Metabolic Viability Assay does not produce the customary percentage value of dead/live cells. Instead, it produces a concentration of iATP in micromolar (μM) amounts. Therefore, it is important to ascertain the acceptance/rejection limits for a specific cell type as this can vary between different types of cells and different species.

3. Use and Availability

LIVEGlo™ is intended to be used as a metabolic viability assay for the following mammalian cell types:

- Fresh, primary, explanted cells.
- Thawed, previously cryopreserved cells.
- Transformed cell lines, tumor cell lines.

LiveGlo™ can be used in combination with any dye exclusion viability assay. However, LIVEGlo™ provides a quantitative evaluation of the concentration of intracellular ATP produced by the cells. For each cell type used, it is recommended to ascertain the acceptance/rejection ATP concentration that will indicate sustained metabolism or proliferation.

LIVEGlo™ is available as a 1 plate assay kit with the catalog number: PS-96-1.

4. The Concept of ATP Bioluminescence Assays

LIVEGlo™ is a ATP bioluminescence metabolic viability 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 produces iATP, it is demonstrating cellular and mitochondrial integrity and is therefore viable.

Cells are prepared as a single cell suspension and plated into the 96-well plate provided with the assay kit. The ATP reagent is added and the plate 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:



The bioluminescence emitted is detected and measured in a luminescence plate reader as relative luminescence units (RLU). The assay instrument can be calibrated and the assay standardized using controls and standards that are included with the assay kit.

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.
6. Obtaining similar measurement assurance parameters with those in this manual indicates that the user has performed the assay correctly and has demonstrated proficiency, i.e. this is a proficiency test.

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.

5. QuickGuide to LIVEGlo™ (Figure 1)

STEP 1
Prepare single
cell suspension
of sample.

STEP 2
Measure cell
concentration

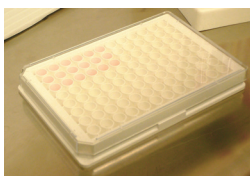


STEP 3
Adjust cell
concentration
to working cell
concentration. If
necessary, setup a cell
dose response.

STEP 4
Gently mix cells by vortexing

STEP 5
If instrument calibration
and assay standardization
is to be performed, do this
prior to Step 6 (see separate
QuickGuide)

STEP 6
Dispense 0.1 mL of the sample working cell concentration into
replicate wells of the non-sterile 96-well plate provided. The
final cell concentration will be 10x lower than the working
concentration



STEP 7
Add 0.1 mL of ATP-ER to each well, mix and measure
bioluminescence after 10 min incubation in the dark

TIPS

- If possible, use self-calibrating electronic pipettes.
- Always use gloves when measuring ATP.
- Performing calibration and standardization provides measurement assurance for trustworthy results.
- Always ensure well contents are mixed properly (see manual).
- Use adhesive foil (included) to maintain unused well sterility.

6. Kit Contents and Storage Conditions

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

TABLE 1

Item	Component	Storage
1	Medium (IMDM) for dilution of the ATP standard.	-20°C until used
2	ATP standard.	-20°C until used
3	ATP extra high, high and low controls.	-20°C until used
4	ATP Enumeration Reagent (ATP-ER)*	-20°C in the dark until used
5	Adhesive Plate Covering to protect unused wells.	Can be kept with other kit components
6	Non-sterile 96-well plate(s) for ATP standard curve determination.	Can be kept with other kit components
7	Non-sterile 96-well plate(s) for sample viability measurement.	

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

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

IMPORTANT

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

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

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

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

Although it is not necessary to perform the assay under sterile conditions, it is good practice to do so.

Equipment and Supplies

1. Laminar Flow Biohood
2. Plate luminometer (e.g. Berthold LB962 CentroLIA/pc; Molecular Devices, SpectraMaxL)
3. Plastic tubes (5ml)
4. Single channel pipettes, preferably electronic (e.g. Rainin EDP pipettes for variable volumes between 1µl and 100µl).
5. 8-channel pipette, preferably electronic (e.g. Rainin EDP pipettes for fixed or variable volumes between 10µl and 100µl).
6. Reservoir for 8-channel pipette
7. Pipette tips for different pipettes.
8. Vortex mixer.
9. 1.5ml plastic vials (5 for each ATP dose response).
10. Hemocytometer, electronic cell counter or flow cytometer to determine cell concentration and/or dye exclusion viability.

8. The LIVEGlo™ Protocol

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

Performing LIVEGlo™ is a 2-step process.

Step 1 - Cell preparation.

Step 2 - Cell viability, cell counting and sample preparation for LIVEGlo™ measurement

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

Prepare or thaw cells using a validated cell preparation protocol. If cells are to be processed prior to cell culture, it is good practice to measure metabolic cell viability before and after the procedure to ensure that cell death has not occurred.

STEP 2 - Cell Viability, Cell Counting and Sample Preparation for LIVEGlo™

Dye exclusion viability can also be determined using a hemocytometer, automated method or flow cytometer. However, please be aware that percentage values obtained with dye exclusion methods may not conform to the quantitative measurement obtained using LIVEGlo™.

Determine the cell count using a validated protocol. Do not base the cell count on dye exclusion viability. Adjust the working cell concentration so that it is 10 times the final cell concentration/mL in the well. A total of 0.1 mL of the working cell concentration will be added to each well, thereby reducing the cell concentration 10 fold.

For example, if the final cell concentration in the well is to be 10,000 cells, the working cell concentration will be 100,000 cells/mL.

For a specific cell type and condition, it may be necessary to perform a cell dose response in order to determine the optimal cell concentration to measure metabolic viability. This can be done over a dose range from 1,000 cells up to 50,000 cells. Once an optimal cell dose is determined for the type of cells, maintain this cell dose for all future measurements so that they can be compared over time to obtain historical data.

It is recommended to initially perform a minimum of 6 replicates/cell dose so that statistical variation can be determined and pipetting errors can be minimized. Once this has been established, it is possible to reduce the number of replicates/samples, if required.

When dispensing cells or the ATP reagent into wells of the 96-well plate provided, ensure that the pipette tip is directed into the center of the well. Never dispense cells or reagents onto the walls of the well, since this can cause erroneous results.

Cell samples are added to each replicate well in a 0.1 μL volume. Depending on the number of replicate wells being prepare for each sample, these can be configured either in rows or in columns to maximize the number of samples/plate. (See Section 9).

STEP 3 – BIOLUMINESCENCE MEASUREMENT

Please note the following important points:

- **FOR ALL OF THE FOLLOWING STEPS, WEAR LABORATORY GLOVES.** ATP is present on the skin and can cause erroneous results
- **PLEASE REFER TO SECTION 11 ON HOW TO SETUP THE PLATE LUMINOMETER.** The instrument should be setup and prepared for use prior to any of the following steps being performed.
- Please refer to Section 10 for recommendations and tips prior to starting this part of the procedure. In particular, please refer to Section 10 for important information on mixing components.
- Remove the ATP Enumeration Reagent (ATP-ER) from the freezer and thaw at room temperature or in cold running water prior to analysis. Do not thaw the ATP-ER in a water bath or 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 one of the non-sterile, 96-well plates 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 12**) that should be obtained prior to measuring samples. Use one of the non-sterile, 96-well white plate provided with the assay kit for this purpose.

LIVEGlo™ includes the following to calibrate and standardize the ATP bioluminescence part of the assay to measure quantitative metabolic cell viability.

- 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

Please see the graphical protocols at the end of this manual that show the steps for calibrating and standardizing LIVEGlo™.

Instructional videos for calibrating and standardizing and ATP bioluminescence assay are also available on the Preferred Cell Systems website at: <http://preferred-cell-systems.com/videos.php>.

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 14.** Thawed, previously cryopreserved cells would fall into this group.

PROTOCOL 2: For fresh, primary cells or cells with expected high intrinsic metabolism, e.g. hepatocytes, kidney cells, the low and extra high calibration controls can be used and an ATP standard curve from 0.03µM to 3µM. **See Page 15.**

PROTOCOL 3. All 3 ATP controls and an ATP standard curve from 0.01µM to 3µM can also be used.

It is important that the sample ATP values lie 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.

C. Sample Measurement

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

1. 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).
2. Using, preferably, a multichannel pipette, add 0.1 mL of ATP-ER to all replicate wells. Mix the contents as described in Section 10 or as seen in the instructional video.
3. Repeat this procedure for each column or row. Always change the pipette tips between each ATP-ER addition.
4. When ATP-ER has been added to all wells, replace the plastic cover and incubate for 10 min at room temperature in the dark to lyse the cells and stabilize the luminescence signal. Alternatively, transfer the plate to the luminescence plate reader, close the draw of the reader and incubate in the dark for 10 minutes
5. Unused ATP-ER may be returned to the bottle and refrozen. See section 10 for ATP reagent storage conditions and stability.

D. Using a plate luminometer with automatic dispenser

The user may have a plate luminometer that allows reagents to be dispensed automatically directly into the well.

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

E. Using a liquid handler

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

9. Recommendations and Tips Prior To Using LIVEGlo™.**(i) Number of Replicates Performed**

A minimum of 4 replicates/sample can be used, although 6 replicates will provide better statistics. Please remember that using fewer replicates may save components in the short term, but may also cause inconclusive results. If outliers are encountered, which may have to be removed from the analysis, the consequence could be that extra experiments would be required resulting in extra time and costs.

(ii) Plate Configuration

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

(iii) 96-Well Plates Provided

The reagents have been optimized to work with the 96-well plate(s) provided in the LIVEGlo™ kit. Please do not replace the plates included with the kit with those of another manufacturer. 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.

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® 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 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 -20°C for 20 weeks.

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 standard curve and ATP controls)).

ATP Standard Curve

Depending on the size of the kit purchased, non-sterile, 96-well plates have been included to perform an ATP standard curve prior to processing the sample cultures. Performing an ATP standard curve and controls on each day samples are processed is an essential part of the assay because it has 4 functions:

- It tests whether the instrument is working properly and calibrates it.
- It ensures that the reagents are working correctly.
- It calibrates and standardizes the assay and allows the assay system to be validated, if required.
- It allows the output of the plate luminometer, in relative luminescence units (RLU), to be converted to ATP concentrations, thereby standardizing the procedure so that intra- and inter-laboratory experiments can be compared.

Adhesive Plate Covering Film

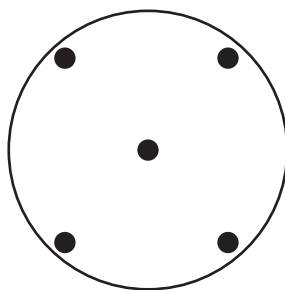
To help keep the plate(s) sterile, adhesive, air permeable, sterile films are provided so that the part of the plate that is not being used can be covered and kept sterile until required. If using the adhesive film provided, the plate cover should be removed in a laminar air-flow hood and replaced with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

Mixing the Contents of 96-well Plate

Mixing the contents of the wells after adding ATP-ER is one of the most important procedures of the assay. It is recommended that the addition of ATP-ER is performed using a multi-channel pipette to achieve consistency and reduce variability. Addition of the reagent and mixing should be performed in the following manner:

1. Take up the required amount of reagent and add it to the well without inserting the tip into the well contents.
2. Starting from the center of the well, aspirate and dispense the contents twice without removing the pipette tip from the contents of the well.
3. Move the pipette tip to one corner of the well and aspirate and dispense the contents twice without removing the tip from the contents of the well.
4. Repeat this operation as shown in Figure 4 for each corner of the well.
5. Try not to cause excessive bubbles in the culture and DO NOT over mix since this can result in drastically reduced luminescence values.
6. This procedure effectively and optimally mixes the contents well.

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



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 at <http://preferred-cell-systems.com/LIVEGlo.php>.

12. LIVEGlo™ Assay Measurement Assurance and Validation Parameters

If LIVEGlo™ has been calibrated and standardized, ATP bioluminescence technology allows the User's results to be 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 \pm 15% ^(*)	Min / Max	%CV (where applicable)
0.01 μ M ATP	0.0099 μ M ATP	0.00972 - 0.0114	0.009 - 0.01	2.34%
0.03 μ M ATP	0.029 μ M ATP	0.285 - 0.0336	0.028 - 0.03	1.67%
0.05 μ M ATP	0.0497 μ M ATP	0.0486 - 0.0571	0.048 - 0.051	1.57%
0.01 μ M ATP	0.1026 μ M ATP	0.1003 - 0.118	0.099 - 0.107	1.96%
0.3 μ M ATP	0.317 μ M ATP	0.310 - 0.364	0.302 - 0.325	1.51%
0.5 μ M ATP	0.5023 μ M ATP	0.491 - 0.578	0.491 - 0.515	1.19%
1.0 μ M ATP	1.048 μ M ATP	1.024 - 1.205	0.977 - 1.117	3.7%

Expected Parameter	Observed Value	Mean \pm 15% ^(*)	Min / Max	%CV (where applicable)
3.0 μ M ATP	2.722 μ M 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, (0.05 μ M ATP)	0.0487 μ M ATP	0.0476 - 0.0560	0.042 - 0.063	6.79%
High control 0.7 μ M ATP	0.725	0.710 - 0.836	0.655 - 0.904	5.35%
Extra high control (1.75 μ M 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: ~0.04 μ M
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: ~0.01 μ M.
- All samples values must lie on the ATP standard curve for accurate RLU to ATP conversion. If ATP values are greater than 3 μ M, 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

LIVEGlo™ exhibits the following validation parameters:

- Assay ATP linearity => 4 logs
- Assay ATP sensitivity: ~ 0.001 μ M
- 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).

13. Troubleshooting

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

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 12. 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 been 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 $\leq 20\%$. The percent coefficient of variation is calculated as standard deviation/mean $\times 100$. High %CVs are usually an indication of incorrect dilutions or pipetting error. Although outliers might be obtained between similar samples, large variations between replicates should not be obtained. Please consider the following:

- Accurate reagent dispensing and mixing are of prime importance. Since the volumes dispensed are small it is imperative to use instruments that have been properly calibrated to avoid pipetting error.
- Insufficient mixing of components prior to cell plating and insufficient mixing during the addition of luminescence reagents to cultures in the 96-well plate can also lead to high CVs. Use repeater pipettes. Use calibrated or self-calibrating electronic pipettes or dispensers to add and mix the luminescence reagents.
- If the luminometer requires determining the “gain” empirically, it is possible that this parameter has not been optimally set and will result in an incorrect signal to noise ratio. Once the optimal “gain” has been set for the instrument, it should not be changed.

Low RLU Values

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

- *Reagent decay:* The ATP-ER decays with time, even when frozen. This can lead to low bioluminescence. Once thawed the reagent can be refrozen up to 11 cycles without significant loss of sensitivity. Do not use the reagent after expiry date has elapsed. As a rule of thumb, the RLU value for the lowest ATP standard should be 10 times greater than that of the background value.
- *Reagent deterioration:* Reagents arrived thawed, at room temperature or greater or were not stored correctly.

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. Please follow the mixing instructions shown in Section 10 on the instruction video for ATP bioluminescence calibration and standardization.

Plates Drying Out

- The cell volume in each well is 0.1 μL . For LIVEGlo™ it is usual for the cell suspension to be prepared and a sample added to the plate and measured almost directly for metabolic viability. If cells are left in the plate for long periods prior to the addition of ATP-ER and measuring bioluminescence, it is possible that the cell volume will decrease due to drying out of the wells.

Ordering Information

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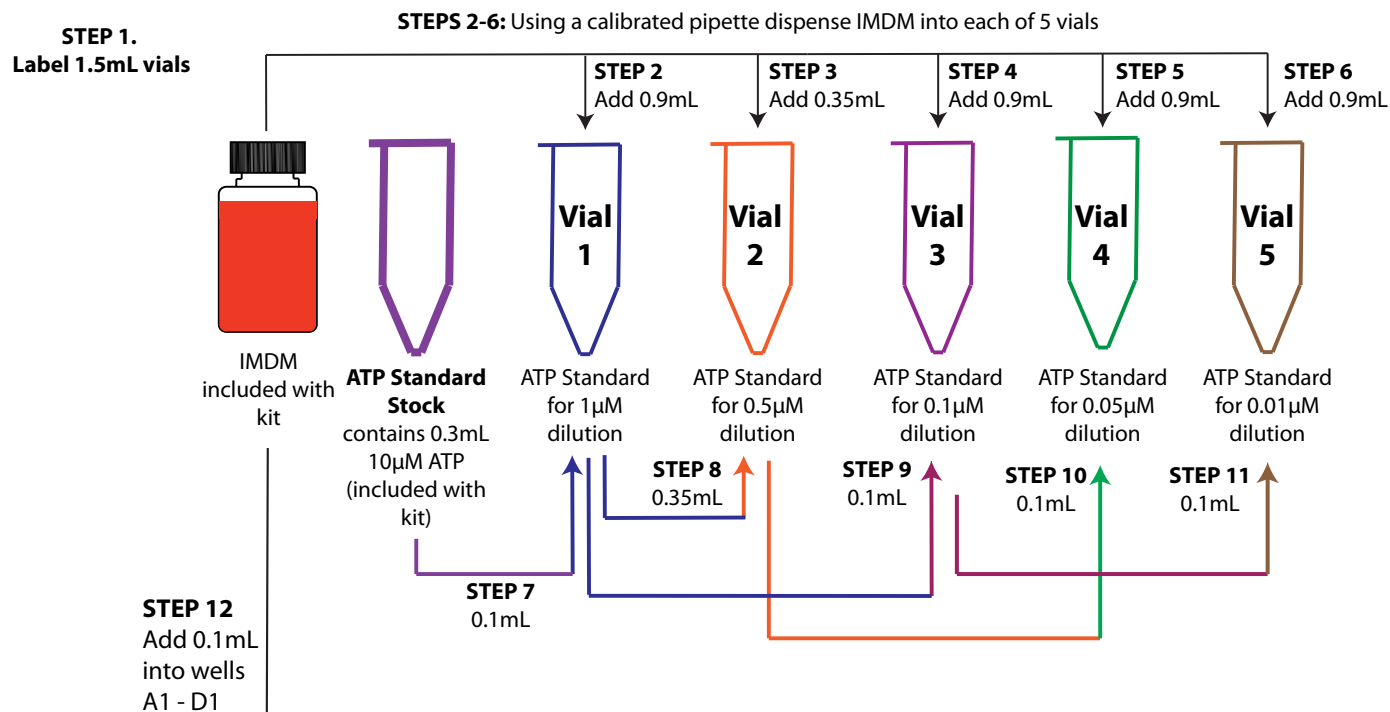
LIVEGlo™ is a trademark of Preferred Cell Systems™

LIVEGlo™ incorporates patents: 7,354,729, 7,354,730, 7,666,615, 7,709,258, 7,883,861, 7,700,354.

LIVEGlo™ was designed and developed by Preferred Cell Systems™, Inc

Calibration and Standardization Protocol of an ATP Bioluminescence Assay

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



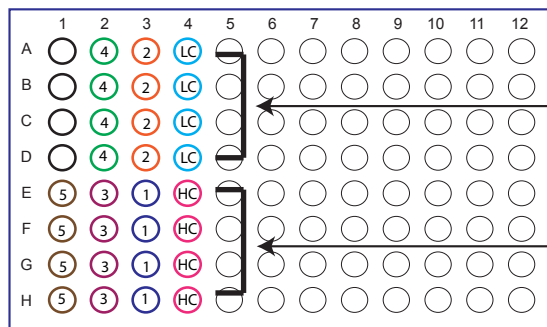
Reagents & Materials Needed

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



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

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 20: Add ATP-ER to reservoir 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 0.03 μ M - 3 μ M For Samples with Known or Expected High Cell Proliferation

