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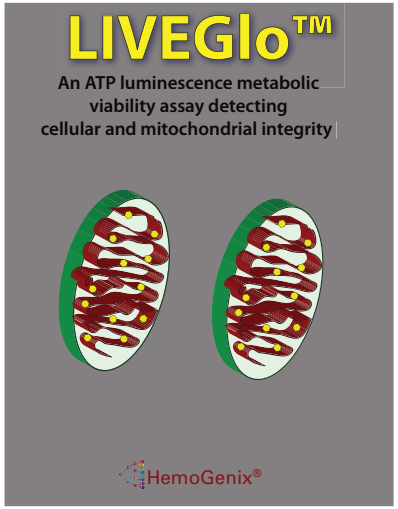

LIVEGlo™

An ATP Bioluminescence Metabolic Viability Assay

Assay Manual

(Version 2-2014)

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Not for clinical diagnostic use.

HemoGenix®, Inc

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1. 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. In other words, dye exclusion viability does not detect cellular and membrane integrity, which is a more significant indication of cell viability. 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 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 cell suspensions from a variety of organs and tissues to measure metabolic viability. If cells are alive, they will be producing chemical energy in the form of adenosine triphosphate or ATP, which is 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. To measure metabolic viability, the cells in questions are added to wells of a 96-well plate and a single reagent is added that lyses the cells and releases the intracellular ATP (iATP). This then reacts with a luciferin/luciferase reagent to produce bioluminescence in the form of light, which is measured in a luminescence plate reader. The amount of light produced is directly related to the viability of the cells being tested.

Metabolic viability does not produce a percentage 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.

2. INTENDED USE

LIVEGlo™ is intended to be used as a metabolic viability assay for virtually all types of mammalian cells.

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.

IMPORTANT:
LIVEGlo™ is for research use only and has not been approved for clinical diagnostic use.

3. The CONCEPT and PRINCIPLE of BIOLUMINOMICS™ ASSAYS

LIVEGlo™ is a metabolic ATP viability assay. The assay incorporates Bioluminomics technology to standardize the assay. The fundamental concept underlying bioluminomics is the measurement of the cell's chemical energy in the form of intracellular ATP (iATP). If a cell is producing iATP, it is demonstrating cellular and mitochondrial integrity and is therefore viable. The iATP concentration is directly dependent on:

- The basal level of metabolism by the cells.
- The plated cell concentration.

The viability of any tissue or organ made into a cell suspension prior to *in vitro* culture can be measured using LIVEGlo™. This represents the basal level of metabolism by the cells. The level of metabolism and therefore the concentration of iATP varies from one cell type to another. The cell suspension is dispensed into replicate wells of a 96-well plate (provided) and a single reagent is added to each well containing cells 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:

$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{LIGHT}$$

The bioluminescence emitted is detected and measured in a plate luminometer as relative luminescence units (RLU). To standardize the assay, an ATP standard curve is performed. **Performing the ATP standard curve allows the results from the instrument to be converted to iATP concentrations (µM)** The ATP standard curve should be performed prior to processing the samples.

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11. Troubleshooting

High Coefficients of Variation (%CV)

Coefficients of variation (%CV) should be =< 15%. The percent coefficient of variation is calculated as standard deviation/mean x 100. Outliers can be obtained resulting in large variations between replicates. 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.
- 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.
- Perform a minimum of 4 replicates per point.

ATP Standard Curve

Performing an ATP standard curve prior to sample measurement can help detect problems prior to sample measurement. If the slope of the standard curve is out of range or 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 cells. 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.

10. LIVEGlo™ Assay Characteristics and Validation Parameters

LiveGlo™ bioluminomics technology allows the assay to be standardized.

Assay Characteristics

When performing the ATP standard curve, the following parameters should be obtained:

- Log-log linear regression goodness of fit (r^2) = > 0.995
- Log-log linear regression correlation coefficient (r) = > 0.997
- Log-log linear regression slope = 0.937 ± 15% (slope range: 0.796 - 1.07)

For many cell types, the following acceptance/rejection values have been found:

- Lowest ATP value indicating unsustainable cell proliferation: ~0.04µM at 5,000 cells/well.
- ATP value below which cells are not metabolically viable: ~0.01µM at 5,000 cells/well.

These arbitrary acceptance/rejection values should be ascertained for each cell type used.

NOTE: Prior to proceeding with a sample, the slope of the log-log linear regression should be in the proper range.

If these parameters are not attainable, please refer to Troubleshooting (Section 12).

Assay Validation Parameters

- Assay ATP linearity => 5 logs
- Assay cell linearity: 1,000 - >25,000 cells/well
- Assay ATP sensitivity: ~ 0.001µM
- Assay cell sensitivity: 20-25 cells/well (depending on purity and growth factor cocktail)
- Accuracy (% correct outcomes): ~95%
- Sensitivity and specificity detected by Receiver Operator Characteristics (ROC) curve fit and detected as area under the curve (AUC): 0.73 - 0.752 (lowest possible value, 0.5; highest possible value, 1).
- Precision (Reliability and Reproducibility) =< 15%. At lower limit of quantification (LLOQ): 20%
- Robustness (intra- and inter-laboratory): ~95%.

Assay validation parameters will vary depending upon the species, cell source and cell type.

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4. OVERVIEW OF THE LIVEGlo™ PROTOCOL

Using LIVEGlo™ is a 2-step procedure.

Step 1 – Cell Preparation
Cells are not provided with LIVEGlo™ assay kits. Cells should be prepared using a user-defined, pre-validated protocol. LIVEGlo™ is performed after a nucleated cell count has been established and before cells are cultured. LiveGlo™ can be performed in parallel with a dye exclusion viability test.

No cell incubation is necessary since the metabolic viability, that is, the cellular and mitochondrial integrity, of the original cell suspension is to be measured in order to determine if the cells are alive and therefore capable of cell culture.

Step 2 – Bioluminescence measurement
To measure bioluminescence, the iATP is released from the cells. This is accomplished by the addition of 0.1ml of an ATP Enumeration Reagent (ATP-ER) using a multichannel pipette. The ATP-ER also contains luciferin and luciferase, the reagents used to produce bioluminescence. After mixing and a 10 min incubation period, the bioluminescence is measured in a plate luminometer. Prior to sample luminescence measurements, an ATP standard curve is performed.

5. KIT CONTENTS

LIVEGlo™ kits contain the following components:

1. Medium (IMDM) for ATP standard dilution and background.
2. ATP standard.
3. ATP Enumeration Reagent (ATP-ER)*
4. Adhesive Plate Covering—a sterile foil to protect and keep unused wells sterile.
5. Non-sterile 96-well plates, used for both the ATP standard curve and samples.
6. Assay manual.

Exact volumes/amounts 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 HemoGenix®.

This kit contains a reagent for measuring luminescence (ATP-ER) that decays with time.

HemoGenix® suggests that this kit be used before the expiry date of this reagent. HemoGenix® 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 reagents can be purchased from HemoGenix®. Please contact HemoGenix®.

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.

6. EQUIPMENT AND SUPPLIES REQUIRED, BUT NOT PROVIDED

Equipment and Supplies

- Plate luminometer (e.g. Molecular Devices, SpectraMax L; Berthold, CentroLia)
- Capped, plastic tubes (5ml)
- Single channel pipettes, preferably electronic (e.g. ViaFow or Rainin EDP pipettes for variable volumes up to 1ml.
- 8-channel pipette, preferably electronic (e.g. ViaFlow or Rainin EDP pipettes volumes up to 0.1ml).
- Reservoir for 8-channel pipette.
- Pipette tips.
- Vortex mixer.
- 1.5ml plastic vials (5 for each ATP dose response).
- Hemocytometer or electronic cell counter to determine cell concentration.

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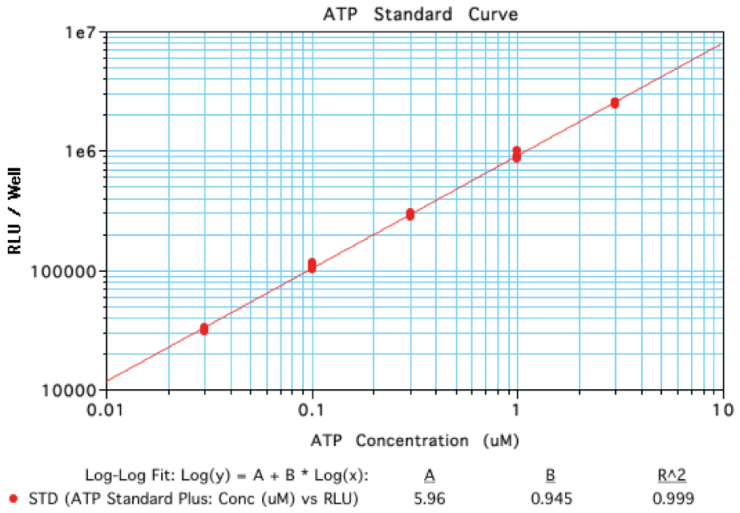
calculations and graphs:

- 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 HemoGenix®.

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



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 ANTI-LOG 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

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7. The LIVEGlo™ PROTOCOL

PLEASE READ THE FOLLOWING PROTOCOL CAREFULLY.

Performing LIVEGlo™ is a 2 step process.

Step 1 – Cell preparation.
Step 2 – Luminescence measurement. An ATP standard curve is performed prior to sample luminescence measurements with conversion of RLUs to µM ATP.

STEP 1 – Cell Preparation and Addition of Cells to the Sample Plate

Perform all cell preparation procedures under sterile conditions in a biosafety cabinet. However, LIVEGlo™ does not require aseptic technique.

Always ware protective clothing, including gloves.

Primary stem cells are present in many organs and tissues. A user-defined and pre-validated cell preparation protocol should be used for the cells to be analyzed.

Prior to using LIVEGlo™, a cell count should be performed. If required, a dye exclusion viability test can also be carried out at this time. The following general protocol can be used for preparing cells for LIVEGlo™.

1. Using aseptic technique, either transfer a small aliquot of the cells to another tube from which a working cell concentration can be prepared or remove a calculated volume of cells from the original cell suspension under sterile conditions so that a working cell concentration can be produced. A maximum volume need not be greater than 1ml.
2. To prepare a final cell concentration of 5,000 cells/well for example, a working cell concentration of 50,000 cell/ml is prepared.
3. Accurately dispense 0.1ml into each replicate well of a non-sterile 96-well plate provided. It is recommended to perform a minimum of 4 replicates wells for each sample. Dispensing 0.1ml into each well from a 50,000 cell/ml working concentration reduces the cell concentration 10 fold to produce a final cell concentration of 5,000 cells/well.

Important. When preparing the cell suspension, it is essential that the suspension contains no red blood cells as these will interfere with the assay.

STEP 2 – BIOLUMINESCENCE MEASUREMENT

FOR ALL OF THE FOLLOWING STEPS, WEAR LABORATORY GLOVES
ATP is present on the skin and can cause erroneous results

IMPORTANT: PLEASE REFER TO SECTION 9 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.

IMPORTANT. Please refer to Section 8 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.

PLEASE NOTE: ATP standard curves performed on previous days or for previous experiments or studies should not be used since the ATP-ER intensity changes with time and lot number.

Remove the ATP standard and reagents from the freezer and thaw to room temperature prior to analysis.

Use a separate, non-sterile, 96-well plate provided with the kit to perform the ATP standard curve.

A. Performing the ATP Standard Curve

1. Prepare and label 5 vials (e.g. 1.5ml volume) for the ATP dose response consisting of the following ATP concentrations:
   Vial #1: 1µM
   Vial #2: 0.5µM
   Vial #3: 0.1µM
   Vial #4: 0.05µM
   Vial #5: 0.01µM
2. Using the medium provided with the kit, dispense:
   0.90ml of the medium to vial #1.
   0.35ml of the medium to vial #2
   0.90ml of the medium to vial #3
   0.90ml of the medium to vial #4
   0.90ml of the medium to vial #5
3. Remove 0.1ml of the supplied stock ATP solution (at 10µM) and transfer it to vial #1. Mix by vortexing. This ATP concentration is 1µM.
4. Dispense 0.35ml from vial #1 to vial #2. Mix. This concentration is 0.5µM.
5. Dispense 0.10ml from vial #2 to vial #4. Mix. This concentration is 0.05µM.
6. Dispense 0.10ml from vial #1 to vial #3. Mix. This concentration is 0.1µM.
7. Dispense 0.10ml from vial #3 to vial #5. Mix. This concentration is 0.01µM.
8. Dispense 0.10ml of the supplied medium alone into wells A1, B1, C1 and D1. NOTE: This is to measure background luminescence.
9. Dispense 0.10ml from ATP dilution in vial #5 to wells E1, F1, G1, and H1.
10. Dispense 0.10ml from ATP dilution in vial #4 to wells A2, B2, C2, and D2.
11. Dispense 0.10ml from ATP dilution in vial #3 to wells E2, F2, G2, and H2.
12. Dispense 0.10ml from ATP dilution in vial #2 to wells A3, B3, C3, and D3.
13. Dispense 0.1ml from ATP dilution in vial #1 to wells E3, F3, G3 and H3.

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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 10.

(Values are the Mean ± 1 Standard Deviation)

9. LUMINOMETER SETUP AND CONVERSION OF RLU VALUES TO ATP VALUES USING THE ATP STANDARD DOSE RESPONSE CURVE

The readout from all plate 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. It is therefore difficult to compare results from one experiment to another. The term "bioluminomics" includes not only the procedure for measuring cell proliferation or inhibition by virtue of the iATP concentration, but also the procedures for standardizing the assays. This is done by interpolating the RLU values into ATP concentrations using the ATP standard dose response curve and a log-log linear regression least squares analysis.

(i) Luminometer Setup

Multiparameter 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 multiparameter 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. Most luminometers are equipped with a plate shaking protocol. It is not necessary to use the plate shaker mode.
e. Do not use injectors if the instrument is so equipped.

(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

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14. Add the required amount of ATP-ER to a non-sterile reagent reservoir.
15. Using a multichannel pipette, add 0.10ml to each well of the first column (A1-H1).
16. Mix the contents as directed in Section 8 by repeated pipetting and discard the tips.
17. Change tips for each new addition of ATP-ER.

IMPORTANT: Mixing the contents of each well properly is a very important procedure. PLEASE REFER TO SECTION 8 ON HOW TO MIX THE WELL CONTENTS CORRECTLY. THIS PROCEDURE IS ALWAYS USED WHEN MIXING REAGENTS FOR BIOLUMINESCENCE MEASUREMENT IS REQUIRED.

18. Add 0.10ml of ATP-ER to each well of the other columns, mixing the contents as described in Section 8.
19. Repeat the procedure for each new column.
20. Place the ATP plate in the luminometer and incubate 2 min before initiating measurement.

B. Sample Measurement

IMPORTANT. It is very important that the ATP-ER is mixed properly with the well contents using the procedure described in Section 8. If the cells are not mixed correctly with the ATP-ER, the cells may not be lysed and lower ATP concentrations will be measured.

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

1. If not already at room temperature, allow the sample plate to come to room temperature for 30 min.
2. If only part of the plate has been used, take an adhesive plate coverfoil from the kit box 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. In this way, other wells will remain clean for future samples. (See Section 9, Adhesive Plate Covering Film).
3. Using a calibrated multichannel pipette (8-channel), add 0.1ml of ATP-ER to each well of the first column (A1-H1) or row (A1-12). Mix the contents as described in Section 8.
4. Repeat this procedure for each column or row **always using new pipette tips**.
5. When ATP-ER has been added to all wells, replace the plate lid and incubate for 10 min at room temperature in the dark to lyse the cells and stabilize the luminescence signal.
6. Unused ATP-ER may be returned to the bottle and refrozen. See section 9 for ATP reagent storage conditions and stability.

8. 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 use.

Each day bioluminescence is measured, an ATP standard curve should 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.

LIVEGlo™ includes solid, white plates that have been optimized for use. 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 HemoGenix®.

Bioluminescence Assay Kit Components

- Prior to measuring bioluminescence, remove a vial containing the ATP standard and the ATP Enumeration Reagent (ATP-ER) from the freezer and thaw at room temperature or at 22 - 23˚C.
- Enough ATP standard and monitoring reagent is supplied to perform 4 standard curves for each plate of samples. Additional ATP standard can be purchased from HemoGenix®.
- 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.

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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 samples. Performing an ATP standard curve on each day samples are required is an essential part of the assay because it has 4 functions:

- It tests whether the instrument is working properly.
- It ensures that the reagents are working correctly.
- It standardizes the assay.
- It allows the output of the plate luminometer, in relative luminescence units (RLU), to be converted to ATP concentrations.

Adhesive Plate Covering Film

To help keep unused wells clean, adhesive films are provided.

Mixing the Contents of a 96-well Plate

Besides mixing the cell suspensions, mixing the contents of the wells after adding ATP-ER is probably the most important procedure. 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