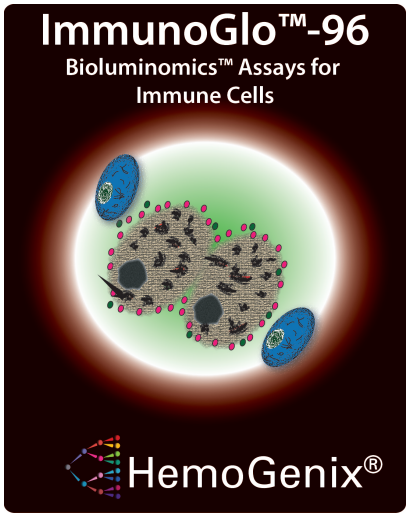
Ordering Information
Tel: (719) 264-6250
Fax: (719) 264-6253
Email: info@hemogenix.com
Order online at www.hemogenix.com

Technical Support
Tel: (719) 264-6250
Email: info@hemogenix.com

HemoGenix®, Inc
1485 Garden of the Gods Road
Suite 152
Colorado Springs, CO 80907
U.S.A.
Tel: (719) 264-6250
Fax: (719) 264-6253
Web: www.hemogenix.com

FOR *IN VITRO* RESEARCH USE ONLY (RUO)
NOT FOR CLINICAL DIAGNOSTIC USE



ImmunoGlo™-96

Lymphocyte Proliferation Assay

Assay Manual

(Version 12.12)
This manual should be read in its entirety prior to using this product

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

HemoGenix®, Inc

TABLE OF CONTENTS

HemoGenix®
Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

14. REFERENCES

1. **Rich IN & Kubanek B**: The effect of reduced oxygen tension on colony formation of erythropoietic cells in vitro. Brit J Haematol (1982), 52:579-588.
2. **Rich IN**: A role for the macrophage in normal hemopoiesis: II. Effect of varying oxygen tensions on the release of hemopoietic growth factors from bone marrow-derived macrophages in vitro. Exptl. Hemat. (1986), 8:746-751.
3. **Crouch SPM, Kozlowski R, Slater KJ, Fletcher J**: The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicty. J Immunol Meth (2000), 160:81-88.
4. **Musk P, Rich IN**: A new high throughput stem cell and multilineage progenitor cell assay for hemotoxicity testing. The Toxicologist (2001), 66.
5. **Rich IN, Musk P**: HALO - A multifunctional colony-forming-based assay platform for drug development and basic and clinical research. Blood (2002), 100:618a
6. **Rich IN**: In vitro hemotoxicity testing in drug development. A review of past, present and future applications. Current Opinion in Drug Discovery and Development. (2003) 6:100-109.
7. **Rich IN and Hall KM**: Validation and development of a predictive paradigm for hemotoxicology using a multifunctional bioluminescence colony-forming proliferation assay. Tox Sci (2005) 87:427-441.
8. **Rich IN:** High-throughput *in vitro* hemotoxicity testing and *in vitro* cross-platform comparative toxicity. Expert Opin. Drug Metab. Toxicol. (2007) 3:295-307.
9. **Reems J-A, Hall KM, Gebru LH, Taber G, Rich IN.** Development of a novel assay to evaluate the functional potential of umbilical cord blood progenitors. Transfusion (2008) 48:620-628.
10. **Hall KM, Harper H, Rich IN**. Hematopoietic stem cell potency for cellular therapeutic transplantation. In: Hematopoietic Stem Cells, Ed.: RP Camacho. ISBN 978-953-307-746-8. (In preparation).

HemoGenix®
Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

1. INTRODUCTION

Under normal, steady-state conditions, immune cells demonstrate little or no proliferation. When stimulated, however, different types of immune cells can exhibit different degrees of proliferation activity. The proliferation activity will be dependent upon the type of inducer, concentration and any co-stimulation that might be present.

ImmunoGlo™-96 is an assay designed to measure lymphocyte proliferation by immune cells obtained from various tissues and organs derived from different species.

Immune or lymphocyte proliferation has traditionally been measured using a radioactive marker, usually tritiated thymidine (^{3}H-Tdr), or more recently a non-radioactive marker that incorporates into the cell's DNA, such as bromodeoxyuridine (BrdU) which can be detected using a colorimetric (absorbance) or fluorescence readout. Absorbance or fluorescence readouts are not as sensitive as radioactive readouts. The radioactive marker has usually been the method of choice because of the high sensitivity. However, use of any radioactive compound is a hazardous operation that also involves regulated waste removal.

HemoGenix® has been developing highly sensitive, non-radioactive assay kits to detect different cell types for specific applications since 2003. The most sensitive non-radioactive readout to measure cell proliferation is the detection of intracellular adenosine triphosphate (iATP) using a luciferin/luciferase bioluminescence signal detection system. When cells proliferate or are inhibited from proliferation, the iATP concentration varies proportionately. This concept is used in both the ImmunoGlo™-96 and ImmunoGlo™-MLC assays from HemoGenix®. ImmunoGlo™-96 is used for the direct induction/stimulation of immune cells, for example using mitogens or co-stimulators. ImmunoGlo™-MLC is used to measure a 1- or 2-way mixed lymphocyte reaction.

Both assay kit types incorporate the principles of Bioluminomics™ that includes calibration, standardization and, if necessary, the validation of the assay being used. Unlike other assay detection systems, Bioluminomics™ ensures that the results obtained are reliable and reproducible, not only within one laboratory, but between laboratories. Bioluminomics™ also allows results to be compared over time, thereby providing a measure of quality control. ImmunoGlo™-96 is a rapid, easy to use assay that can replace all other methods for measuring lymphocyte proliferation.

2. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. **ImmunoGlo™-96 is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)**
2. **ImmunoGlo™-96 is for research use only and has not been approved for clinical diagnostic use.**
3. **Reagents and supplies in this kit are STERILE. Perform all procedures under sterile conditions, except where indicated.**
4. **This kit should not be used beyond the expiration date on the kit label.**
5. **Do not mix or substitute reagents or other kit contents from other kit lots or sources.**
6. **Always use professionally calibrated and, preferably, electronic pipettes for all dispensing procedures. Small discrepancies in pipetting can lead to large pipetting errors. Although electronic pipettes self-calibrate themselves, they still need to be professionally calibrated on a regular basis.**
7. **Good laboratory practices and universal protective precautions should be undertaken at all times when handling the kit components as well as human cells and tissues. Material safety data sheets (MSDS) are included in each literature packet.**

HemoGenix® Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

microscope. If contamination occurs it will usually be seen by the difference in color of the cultures, if the medium contains an indicator, e.g. phenol red. Contaminated cultures will usually be bright yellow in color and probably cloudy in appearance. Cell cultures that demonstrate high proliferation will usually appear orange to light orange, but will not be cloudy. If only "spot" contamination occurs, this is usually due to pipette or repeater tips coming in contact with materials other than the reagents. Contamination will usually lead to outlier RLU values.

Luminescence Reagent Mixing.
The luminescence reagent has to be added and thoroughly mixed with the culture components. The ATP-ER lyses the cells and releases intracellular ATP. If mixing is not adequate, only a proportion of the cells will be lysed and the RLU values will be low. Conversely, too much mixing can lead to ATP degradation and low luminescence readings.

Culture Plates Drying Out

- Due to the relatively small culture volume (0.1ml), drying out of the culture wells, particularly around the outside of the plate can be a problem. These are called "edge effects". An incubator with insufficient humidity will cause this problem. To ensure that this does not occur, the incubator water reservoir should be full and the humidity in the chamber checked using a hygrometer.
- If drying out continues, use of a humidity chamber is recommended. Please refer to Section 9 (v) for instructions on how to build a humidity chamber.

13. 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. High %CVs are usually an indication of incorrect dilutions or pipetting error. Although outliers can be obtained, these being observed for the more primitive stem cells than for the more mature proliferating cells, large variations between replicates should not be obtained. Please consider the following:

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

Low RLU Values

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

- *Reagent decay:* The ATP-ER decays with time, even when frozen. This can lead to low bioluminescence. Once thawed the reagent can be refrozen up to 11 cycles without significant loss of sensitivity. Do not use the reagent after expiry date has elapsed. As a rule of thumb, the RLU value for the lowest ATP standard should be 10 times greater than that of the background value.
- *Inadequate cell growth:* Cells did not exhibit sufficiently high viability. Measure cell viability prior to using cells. A cell viability lower than 85% should not be used. Viabilities lower than 85% can be an indication that the sample was not processed in a time-sensitive manner or that the processing procedures were not standardized and controlled.
- *Reagent deterioration:* Reagents arrived thawed, at room temperature or greater or were not stored correctly.
- *Inadequate incubator conditions:* Maintaining a correct humidified gaseous atmosphere in the incubator is essential (See Culture Plate Drying Out).
- *Carbon dioxide concentration is inadequate*. Ensure that the carbon dioxide concentration in the incubator is correct using a Fyrite gas analyzer.
- *Use low oxygen tension*. Using an oxygen concentration of 5% reduces oxygen toxicity due to free radical production and increases plating efficiency. Check that the incubator oxygen concentration is correct using a Fyrite gas analyzer.
- *Low humidity*. Plates dry out (see below) and cell growth declines.
- *Contamination*: Cells cultured in 96-well plates cannot be view under a

HemoGenix® Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

3. INTENDED USE

ImmunoGlo™-96 is intended as a research tool to measure lymphocyte proliferation of immune cells from various tissues and organs derived from different species. Specific uses if ImmunoGlo™-96 include, but are not limited to:

- Testing unprimed T-cells in the presence of antibodies, enterotoxins, mitogens etc.
- Cellular immune response studies.
- Testing DLI (Donor Lymphocyte Infusion) samples for stimulation/induction ability prior to use.
- Effect of accessory (non T-cells) on T cell induction.
- Effect of co-stimulators on T-cell induction.
- Effect of epitope sequences and novel peptides or proteins.
- Test the response of primed T-cells *in vitro*.
- Single-cell, T-cell cloning studies.

It is usual however, to study peripheral blood lymphocytes or purified immune cell populations from human and other animal species.

ImmunoGlo™-MLC Assays Available

Catalog Nos.	Additions	No. of Plates/Kit
KM1-LPA-1	No growth factors, mitogens or co-stimulators included	1
KM1-LPA-2	No growth factors, mitogens or co-stimulators included	2
KM1-LPA-4	No growth factors, mitogens or co-stimulators included	4

IMPORTANT:
ImmunoLIGHT™-96 is for research use only and has not been approved for clinical diagnostic use.

4. The CONCEPT and PRINCIPLE of BIOLUMINOMICS™ ASSAYS

ImmunoGlo™-96 is a bioluminomics™ 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. 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 type and concentration of the stimulator cells.
- The plated cell concentration.

Cells are cultured for defined period of time. When the culture period has elapsed, a single 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 for 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 calibrate and standardize the assay, an ATP standard and high and low controls are provided. **Performing the ATP standard curve and controls is a very important part of the ImmunoGlo™-96 assay. Failing to perform the ATP standard curve and controls can invalidate the results.** The ATP standard curve and controls must be performed prior to processing the samples for the following reasons:

1. Performing an ATP standard curve calibrates and standardizes the assay.
2. The controls ensure 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.

The ATP standard curve and controls are measured once, on the day samples are to be processed. DO NOT use results from an ATP standard curve or controls performed on one day for samples processed on another day.

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 luminometer software. If the software does not allow this, it will be necessary to use third-party software to perform this operation.

HemoGenix®
Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

12. ImmunoGlo™-96 Assay Readout Characteristics and Validation Parameters

ImmunoGlo™96 bioluminomics™ technology allows the assay to be calibrated and standardized. Inclusion of an ATP standard and controls are requirements for assay validation.

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)

- Low ATP control = 0.05µM ± 15% (allowable range: 0.043µM - 0.058µM)
- High ATP control for ATP standard curves up to 1µM = 0.7µM ± 15% (allowable range: 0.595µM - 0.805µM)
- High ATP control for ATP standard curves up to 3µM = 1.75µM ± 15% (allowable range: 1.488µM - 2.013µM).

For samples:

- Lowest ATP value indicating unsustainable cell proliferation: ~0.04µM
- ATP value below which cells are not metabolically viable: ~0.01µM.

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

Assay Validation Parameters

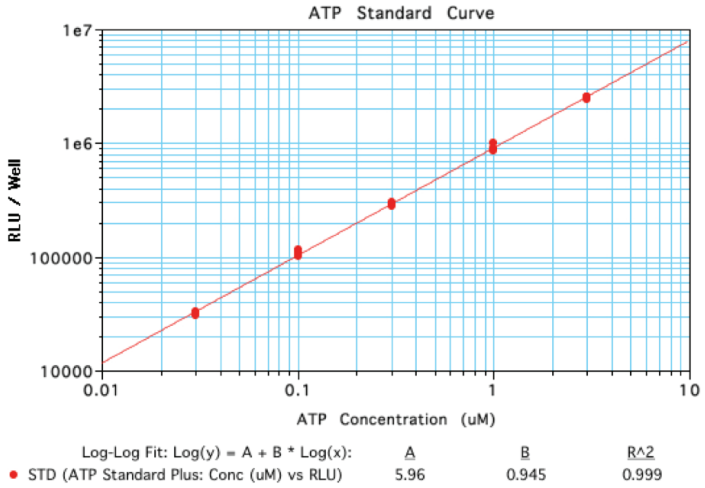
- Assay ATP linearity => 5 logs
- Assay ATP sensitivity: ~ 0.001µM
- Assay cell sensitivity: 20-25 cells/well (depending on cell type and purity)
- Accuracy (% correct outcomes): ~95%
- Sensitivity and specificity detected by Receiver Operator Characteristics (ROC) curve fit and detected as area under the curve (AUC): 0.73 - 0.752 (lowest possible value, 0.5; highest possible value, 1).
- Precision (Reliability and Reproducibility) =< 15%. At lower limit of quantification (LLOQ): 20%
- Robustness (intra- and inter-laboratory): ~95%.
- High throughput capability (Z-Factor): >0.76 (lowest possible value, 0.5; highest possible value, 1).

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 3. Typical ATP Standard Dose Response Curve Using SoftMax Pro Software



HemoGenix®
Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

5. OVERVIEW OF THE ImmunoGlo™-96 PROCEDURE

There are 3 step to use ImmunoGlo™-96.

Step 1 – Cell Preparation
Cells are not provided with ImmunoGlo™-96 assay kits. Cells should be prepared using a user-defined, pre-validated protocol to obtain a single cell suspension of mononuclear cells or purified lymphocytes. If using peripheral blood cells, the lymphocytes are contained in the mononuclear fraction that can be obtained by density gradient centrifugation. A dye exclusion viability and/or metabolic viability and nucleated cell count should be performed on all samples.

Step 2 – Cell Culture
The investigator determines how the cells should be cultured. Considerable pipetting error with high coefficients of variation (%CV) can result if individual culture components are added separately to the wells. It is recommended that all of the components required to grow and culture the cells be prepared in a Culture Master Mix prior to adding the cells and dispensing the complete Cell Culture Master Mix into the wells of the plate(s) provided. Sufficient volumes should be prepared for both the Culture Master Mix and cell suspension to accommodate all replicates wells and samples.

Step 3 – Bioluminescence measurement
To measure bioluminescence, the iATP is released from the cells. This is accomplished by the addition of 0.1ml of the ATP Enumeration Reagent (ATP-ER) using a multichannel pipette. The ATP-ER also contains luciferin and luciferase, the reagents used to produce bioluminescence. After a short incubation period the bioluminescence is measured in a plate luminometer. Prior to sample luminescence measurements, the instrument is calibrated and the assay standardized by performing an ATP standard curve with controls (provided with the kit).

6. KIT CONTENTS and STORAGE

ImmunoGlo™-96 kits contain reagents that have been frozen and stored at -80°C prior to shipment. The kit is shipped either with dry ice or blue ice. The following components are included:

Item	Component	Storage
1	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: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
6	Sterile, 96-well plates for cell culture	Can be kept with other kit components
7	Non-sterile 96-well plates for ATP standard curve determination.	Can be kept with other kit components
8	Assay manual.	Can be kept with other kit components

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

*The ATP-ER should not be thawed until needed and can be refrozen 11 times without significant loss of sensitivity. It can be kept at 2-8ºC for 48h once thawed and is stable for 20 weeks when stored at -20ºC. 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.

HemoGenix® Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

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

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 3 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)

7. EQUIPMENT, SUPPLIES AND REAGENTS REQUIRED, BUT NOT PROVIDED

Equipment and Supplies

1. Laminar Flow Biohood
2. Plate luminometer (e.g.; Promega GloMax®-96, Molecular Devices, SpectraMaxL; TECAN, GENios)
3. Sterile, capped, plastic tubes (5ml, 10ml, 50ml)
4. Single channel pipettes, preferably electronic (e.g. ViaFow or Rainin EDP pipettes for variable volumes between 1µl and 1000µl).
5. 8 or 12-channel pipette, preferably electronic (e.g. ViaFlow or Rainin EDP pipettes for fixed or variable volumes between 10µl and 100µl).
6. Reservoir for 8- or 12 channel pipette
7. Sterile pipette tips.
8. Vortex mixer.
9. Tissue culture incubator, humidified at 37°C with 5% CO_2 (minimum requirement) and 5% O_2 (preferable).
10. 1.5ml plastic vials (5 for each ATP dose response).
11. Hemocytometer or electronic cell counter to determine cell concentration.
12. Flow cytometer or hemocytometer for determining viability. A flow cytometer might also be required to determine the proportion of immune cell types in a cell suspension.

Reagents

1. Sterile Phosphate Buffered Saline (PBS)
2. Iscove's Modified Dulbecco's Medium (IMDM) or other growth medium.
3. DNase (Sigma-Aldrich, Catalog No. D4513-1VL)
4. Density-gradient medium (e.g. NycoPrep 1.077, Axis-Shield).
5. ACK Lysis buffer (Cat. No. K-Lysis-100, HemoGenix®, Inc)
6. 7-AAD, propidium iodide, trypan blue or other dye exclusion viability assay.

8. The ImmunoGlo™-96 PROTOCOL

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

Performing ImmunoGlo™-96 is a 3-step process.

Step 1 – Cell preparation.
Step 2 – Cell culture and incubation in the 96-well plate.
Step 3 – Luminescence measurement. An ATP dose response is performed prior to sample luminescence measurements with conversion of RLUs to µM ATP.

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

STEP 1 – Cell Preparation

Cells should be prepared according to the investigator's own protocol. Different organs and tissues usually require a specific protocol for preparing a single cell suspension. Regardless of the protocol, it will be necessary to measure the cell viability and cell number prior to culturing the cells. It is not recommended to plate a cell concentration based on viability. This will result in a greater cell concentration (consisting of many dead cells) being plated.

It should be emphasized that the type of viability method used can influence the result of the assay. There are essential two viability methods:

- Dye exclusion viability
- Cellular and metabolic integrity viability.

Dye exclusion viability uses dyes that can enter the cell and usually bind with DNA. The dyes enter the cell due to a leaky cell membrane or loss in membrane integrity due to apoptosis and cell death. Using dyes such as typan blue, propridium iodide, acridine orange and 7-aminoactinomycin D (7-AAD) are membrane integrity assays and do not detect loss of viability due to cellular and mitochondrial integrity.

ImmunoGlo™-96 is essentially a cellular and metabolic integrity viability assay because it relies of the cell performing a biochemical reaction based on ATP production. However, when cells are stimulated or induced into proliferation, ImmunoGlo™-96 can be used to detect and measure these responses.

Often peripheral blood lymphocytes are used as the target cells. To use peripheral blood lymphocytes or any other tissue that may contain high concentrations of red blood cells (RBCs), it is necessary to remove the RBCs prior to assay. The RBC concentration should be reduced to below 10%. Otherwise they will interfere with the ATP bioluminescence readout.

A. Reduction or Removal of Red Blood Cells
Reduction or removal of RBCs is usually performed by lysis or by density gradient centrifugation. If the latter, this will also produce a MNC fraction. A protocol is provided

HemoGenix® Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

11. 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.

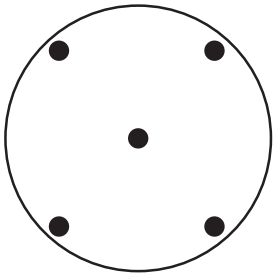
(i) Luminometer Setup
Multiparameter instruments, i.e. those that can detect absorbance, fluorescence and luminescence, often need to be manually set for both the integration time and the "gain". Dedicated instruments, i.e. those that only detect luminescence, usually only have to be set for the "integration 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 per formed 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

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



HemoGenix® Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

below for both procedures.

(i) Cold Lysis

To perform a cold lysis, ACK Lysis Buffer (Cat. No. K-Lysis-100, HemoGenix®) is recommended. The procedure is performed as follows:

1. Refrigerate the ACK Lysis Buffer.
2. Prepare an ice water bath in a 200ml beaker.
3. Transfer the sample to a centrifuge tube.
4. Add 10 parts of the cold ACK Lysis Buffer to 1 part cell suspension. For 1ml of cell suspension, add 10ml ACK Lysis Buffer.
5. Place the tube in the ice water bath and set a lab timer for 5 min.
6. At 2.5 min, invert the tube once and replace in the ice water bath.
7. At 5 min, remove the tube. There should be a cherry red clear solution. If lysis has not taken place, a cloudy cell suspension will still be observed. Replace the tube in the ice water bath for another 2.5 min. Continue this for a maximum of 10 min.
8. Centrifuge the tube at 400 x g for 5 min and discard the supernatant after centrifugation.
9. Resuspend the cells in IMDM or other medium to the original cell volume.
10. Perform a cell and differential count and viability assay.
11. The white blood cell recovery should be at least 95% and the viability must be greater than 85%.

(ii) Density Gradient Centrifugation

Red blood cells, dead and other contaminating cells can be removed by density gradient centrifugation. This procedure will also produce a MNC fraction.

HemoGenix® does not recommend using a Ficoll density gradient centrifugation procedure. This is because all Ficoll-derived reagents are toxic to cells. It is recommended to use NycoPrep 1.077 especially for human cells, since it is non-toxic and can also be added to cultures without any deleterious effects. It is essential that the density gradient centrifugation medium is brought to room temperature prior to use. The procedure for density gradient centrifugation using NycoPrep is as follows:

1. Dilute the sample to be fractionated with an equal volume of Dulbecco's phosphate buffered saline (dPBS) and mix gently by inversion.
2. For samples 3ml or less use a 15ml conical plastic tube for separation. For samples greater than 3ml, use a 50ml conical, plastic tube for separation.
3. For samples of 3ml (or less) diluted to 6ml with dPBS, dispense 5ml of the density gradient reagent into the tube. For samples greater than 3ml, dispense 15ml of the density gradient reagent into a 50ml tube.
4. Using a sterile, serological pipette, dispense the diluted sample gently on top of the density gradient reagent by holding the tube at approx. 45° and using a Pipette Aid on slow delivery. The cleaner the interface between the density gradient reagent and cell suspension, the better the separation will be. Do not allow the cells to mix with the reagent.
5. Centrifuge for 10 min at 1,000 x g or 20 min at 600 x g at room temperature with NO brake.
6. After centrifugation, remove the tube(s) gently and carefully aspirate the top layer above the MNC interface leaving approx. ¼" above the interface. Discard the supernatant.
7. Harvest the MNCs from the interface and transfer the cells to another sterile

tube. It is best to harvest the cells using a manual 1ml pipette. Do not remove cells below the interface.

8. Add approx. 10-20 ml dPBS, mix gently and centrifuge the cells for 10 min at 300 x g at room temperature.
9. Aspirate the supernatant after centrifugation taking care not to aspirate the cell pellet.
10. Add 1-2ml of IMDM and resuspend the cells, breaking up any clumps using a 1ml manual pipette.
11. Perform a nucleated cell count and viability (see below). Using cells with a viability lower than 85% will usually produce results with low proliferation ability.

Fractionating the MNCs by density gradient centrifugation will usually also dramatically improve viability in addition to removing RBCs, dead and other cells.

B. Cell Viability, Cell Counting and Cell Culture Suspension Preparation

1. For dye exclusion viability methods, use trypan blue and a hemocytometer or automated method such as flow cytometer using 7-AAD or another vital stain. ***Note*** *that dye exclusion viability methods detect membrane integrity. They do not detect cellular and mitochondrial integrity and therefore metabolic viability.* A viability of 85% or greater should be obtained when using dye exclusion viability methods only. It is recommended not to use cell suspensions with a viability of less than 85% since these cells will not be able to sustain proliferation ability. It is recommended to use LIVEGlo™ (HemoGenix®, Inc) as a metabolic viability assay.
2. Determine the cell concentration using either a hemocytometer or electronic cell/particle counter.
3. Adjust the cell suspension concentration to the desired working cell concentration. This will usually be 10-100 fold greater than the final cell concentration/well. For cell culture, the optimal cell concentration/well should be determined using a cell dose response.

STEP 2. ImmunoGlo™-96 Cell Culture

Please refer to Section 9 for recommendations and tips prior to beginning this stage of the procedure.

Perform all cell cultures under sterile conditions in a biosafety cabinet.

Use calibrated pipettes and sterile tips throughout.

NOTE: The number of replicate wells prepare is arbitrary. However, a minimum of 4 replicate wells/sample is recommended for statsistical purposes.

Cell culture should be performed according to the investigator's own protocols and reagents. The following is a general procedure for culturing cells in the 96-well plates provided.

Volumes of Luminescence Kit Components Required

- Each vial of ATP standard contains enough volume to perform one or two ATP standard dose responses.
- The amount of ATP-ER added to each well is 0.10ml. Therefore:
  Total amount of ATP-ER (µl) required = 0.1ml x (number of wells used + 24 (background, ATP dose response wells and ATP controls)).

ATP Standard Curve

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

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

Adhesive Plate Covering Film

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

Mixing the Contents of 96-well Plate

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

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

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.

ImmunoGlo™-96 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 HemoGenix®.

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.
- Enough ATP standard and monitoring reagent is supplied to perform 2 standard curves and controls for each sterile plate provided. Additional ATP standards and controls can be obtained 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.

1. Prepare a master mix of culture components without the cells. Ensure that the concentrations of all components are increased such that when dispensed into the wells of a 96-well plate, the correct concentrations and doses are attained. Depending on the number of replicate wells to be assayed, prepare the master mix with approx. 10-20% increase in volume.
2. Prepare the cell suspension as required and adjust the cell concentration to a working cell concentration.
3. Prepare and label one 5ml tube for each sample tested at a single cell dose to be assayed.
4. Dispense the master mix of culture components into the tube followed by the appropriate cell suspension volume. For example, if 6-8 replicates are to be assayed, dispense 0.9ml of the culture component master mix and 0.1ml of the cell suspension.
5. Mix the contents of each tube thoroughly using a vortex mixer.
6. Remove the sterile, clear 96-well plate from plastic covering under the hood.
7. Dispense 0.1ml of the Cell Culture Master Mix into each of the replicate wells.
8. Place the lid on the 96-well plate and transfer the culture plate to a humidity chamber to ensure high humidity during incubation (See section 9).
9. Place the humidity chamber into a fully humidified incubator set at 37˚C and gassed with 5% CO_2 and, if available, 5% O_2. Culturing cells under low oxygen tension is usually advantageous because it reduces the production of dangerous free radicals and improves plating efficiency.
10. Incubate the cells for the required period of time.

STEP 3 – Measurement of Lymphocyte Proliferation using ATP Bioluminescence

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 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.

IMPORTANT. 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.

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, controls and reagents from the freezer and thaw to room temperature prior to analysis.

Use the unwrapped, non-sterile, 96-well plate provided with the kit to perform the ATP standard dose response curve.

ImmunoGlo™-96 includes ATP standards for an "extra high" ATP standard curve (from 0.01µM to 3µM ATP. It is important for the sample ATP values to be within the limits of the ATP standard curve, otherwise the interpolation of Relative Luminescence Unit (RLU) values from the plate luminometer into ATP concentrations will not be accurate. Lymphocyte proliferation could be greater than 3µM ATP. If ATP values from the samples are greater than this ATP concentration, 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 well and diluting each aliquot with additional medium. The replicate wells would then be reread. If low proliferation is expected, use the "normal" ATP standard curve and controls.

A. "Extra High" ATP Standard Curve Preparation.
If the expected lymphocyte proliferation is unknown or could be high, it is recommended to perform the "Extra High" ATP standard curve as follows using a similar plate configuration as shown in Fig. 1:

1. Label 5 vials (e.g. 1.5ml volume) for the ATP dose response consisting of the following ATP concentrations:
   Label the ATP Standard (containing 0.3ml) supplied as Vial #1 (3µM)
   Vial #2: 1µM
   Vial #3: 0.3µM
   Vial #4: 0.1µM
   Vial #5: 0.03µM
2. Now dispense:
   0.40ml IMDM medium to vial #2
   0.90ml IMDM medium to vial #3
   0.90ml IMDM medium to vial #4
   0.90ml IMDM medium to vial #5
3. Dispense 0.7ml of the IMDM medium into Vial #1. Mix.
4. Dispense 0.20ml from vial #1 to vial #2. Mix. This concentration is 1µM.
5. Dispense 0.10ml from vial #2 to vial #4. Mix. This concentration is 0.1µM.
6. Dispense 0.10ml from vial #1 to vial #3. Mix. This concentration is 0.3µM.
7. Dispense 0.10ml from vial #3 to vial #5. Mix. This concentration is 0.03µM.
8. Dispense 0.10ml of the supplied medium alone into wells A1, B1, C1 and D1 on the non-sterile luminescence plate(s) provided. NOTE: This is to measure background luminescence. *PLEASE NOTE that it is important to use the same medium to measure background luminescence as used for the ATP standard curve and cell cultures. Using different media will cause discrepancies in the results.*
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 0.10ml from ATP dilution in vial #1 to wells E3, F3, G3 and H3.
14. Mix the vials containing the low and high controls provided.
15. Dispense 0.1ml from the ATP low control to wells A4, B4, C4, D4.
16. Dispense 0.1ml from the ATP extra high control into wells E4, F4, G4, H4.
17. Add the required amount of ATP-ER to a non-sterile reagent reservoir.
18. Using a multichannel pipette, add 0.10ml to each well of the first column (A1-

HemoGenix®
Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

sensitivity might improve with longer incubation times, but usually at the expense of higher variability between wells. Once an optimal incubation time has been found, the same time period should be maintained for all future experiments so that results can be directly compared.

9. RECOMMENDATIONS AND TIPS PRIOR TO USING THE ImmunoGlo™-96 KIT CELL CULTURE MIXES.

(i) Cell Suspension

a. The preferred cell suspension is a mononuclear cell suspension (MNC).
b. Extraneous ATP, red blood cells (which have high concentrations of ATP) and hemoglobin interfere with the ATP analysis. The cell suspension must have a hematocrit of 10% or less.
c. If cells have been treated prior to cell culture, higher cell concentrations than those shown in Table 1 may be required.

(ii) Number of Replicates Performed

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

(iii) Plate Configuration

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

(iv) 96-Well Plates Provided

The reagents have been optimized to work with the 96-well plate(s) provided in the ImmunoGlo™-96 kit. Please do not replace the plates included with the kit with those of another manufacturer. Cell growth and bioluminescence output can be seriously affected and the assay kit warranty will be void. Additional plates can be purchased from HemoGenix® if required.

(v) Humidity Chamber

A humidity chamber is recommended due to the small sample volume. Even fully humidified incubators do not keep the humidity level high enough to keep the sample from evaporating. This usually results in so-called "edge effects". This phenomenon is observed when ATP values in the outside wells are lower than those in the inside wells. A humidity chamber can be assembled using plastic lunch boxes or other plasticware available from a supermarket or discount stores. Holes must be made in the lid to enable adequate gas exchange. Disposable serological pipettes are cut to an appropriate length to fill the bottom of the container. Distilled/deionized water is poured into the container to just below the level of the pipettes. This allows for adequate water to keep the humidity high without the plates sitting in water. Please contact HemoGenix® for further information about assembling and using humidity chambers.

(vi) Incubation Times

The incubation time may vary depending on cell type and species. Assay

H1).
19. Mix the contents as directed in Section 9 by repeated pipetting and discard the tips.
20. Change tips for each new addition of ATP-ER.

B. "Normal" ATP Standard Curve Preparation

If lymphocyte proliferation is not expected to be high, then a "normal" ATP standard curve can be performed as follows using the plate configuration shown in Fig. 1:

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. Now 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 on the non-sterile luminescence plate(s) provided. 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 0.10ml from ATP dilution in vial #1 to wells E3, F3, G3 and H3.
14. Mix the vials containing the low and high controls provided.
15. Dispense 0.1ml from the ATP low control to wells A4, B4, C4, D4.
16. Dispense 0.1ml from the ATP extra high control into wells E4, F4, G4, H4.
17. Add the required amount of ATP-ER to a non-sterile reagent reservoir.
18. Using a multichannel pipette, add 0.10ml to each well of the first column (A1-H1).
19. Mix the contents as directed in Section 9 by repeated pipetting and discard the tips.
20. 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 9 ON HOW TO MIX THE WELL CONTENTS CORRECTLY. THIS PROCEDURE IS ALWAYS USED WHEN MIXING REAGENTS FOR BIOLUMINESCENCE MEASUREMENT IS REQUIRED.

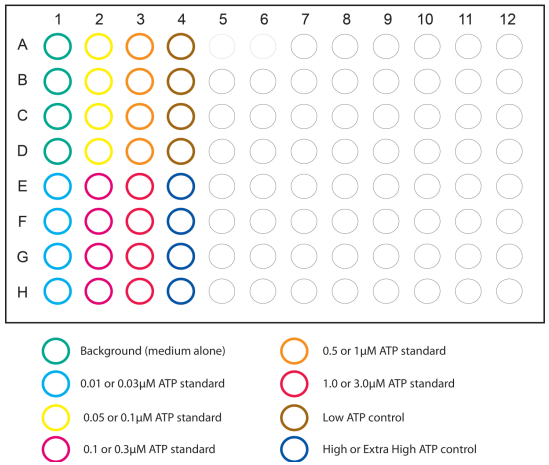
18. Add 0.10ml of ATP-ER to each well of the other columns, mixing the contents as described in Section 9.
19. Repeat the procedure for each new column.
20. Place the ATP plate in the luminometer and incubate 2 min before initiating measurement.

NOTE: The non-sterile 96-well plate(s) is/are used for all ATP standard curves and controls. Continue using empty wells on the plate for additional ATP standard curves.

FIGURE 1

96-Well Plate Configuration for ATP Standard Curve and Controls

3 ATP standard curves and controls can be performed on a single 96-well plate



C. Sample Measurement

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

1. If possible, place the sample plate(s) in a humidified incubator set at 22-23˚C gassed with 5% CO_2 for 30min. Otherwise, allow the plate to come to room temperature for 30 min.
2. If only part of the plate has been used, transfer the plate to a bio-safety hood and remove the lid under sterile conditions. Take a sterile adhesive plate coverfoil from the kit box and layer it over the top of the plate. Using a sharp knife or scalpel, cut away the foil that covers the wells to be processed. The unused, empty wells will now remain sterile for the next samples. (See Section 10, Adhesive Plate Covering Film).
3. Using a multichannel pipette (8- or 12-channel depending on the plate configuration), 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 10.

HemoGenix®
Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

4. Repeat this procedure for each column or row using new tips.
5. When ATP-ER has been added to all wells, replace the plastic cover and incubate for 10 min at room temperature in the dark to lyse the cells and stabilize the luminescence signal. Incubate the plate in the reader for the last 2 min to stabilize the plate.
6. 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. HemoGenix® does not recommend using the automatic dispensers, since the contents of the well are not mixed sufficiently using this method.

E. Using a liquid handler

ImmunoGlo™-96 can be performed in high throughput mode. If you intend to perform any part of the ImmunoGlo™-96 procedure using a liquid handler, please contact HemoGenix® for information on setting up the instrument. Extra ATP-ER is required when using a liquid handler.