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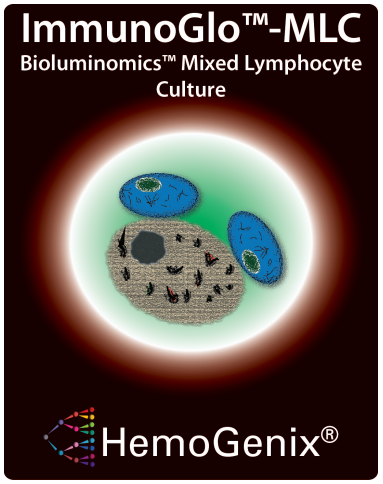
NOT FOR CLINICAL DIAGNOSTIC USE



ImmunoGlo™-MLC

An ATP Bioluminescence Mixed Lymphocyte Culture Assay Kit

Instruction Manual

(Version 7.2015)

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

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

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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.

A specific type of immune reaction occurs when peripheral blood lymphocytes from different donors are mixed together in different proportions and cultured together for several days. The histocompatibility complex present on cells detects whether the donors are compatible. If the cells from each donor are compatible, little or no reaction will occur. If the cells are not compatible, stimulation will occur resulting in a dramatic increase in cell proliferation. This type of reaction is called a mixed lymphocyte reaction (MLR) and is performed in a mixed lymphocyte culture (MLC). There are two types of MLC. In a one-way (1-way) MLC, the lymphocytes of one individual are inactivated by first treating the cells with mitomycin-C or radiation to inhibit proliferation. If the donors are incompatible, the cells from the untreated donor react to the foreign histocompatibility antigens resulting in cell proliferation. In a two-way (2-way) MLC, the cells from both donors are left untreated and can stimulate each other to proliferate. Under these conditions, however, the direction of the stimulation will not be obvious. A MLR is usually used to examine T-lymphocyte help cells (T_H cells, CD4) or to generate cytotoxic T-lymphocytes (CTLs). The MLR is also used to test the compatibility of donor and patient for cell transplantation purposes.

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 might be detected using a colorimetric (absorbance) or fluorescence readout. 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. 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.

2. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. **ImmunoGlo™-MLC is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA).**
2. **ImmunoGlo™-MLC is for research use only (RUO) 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.**

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3. USE

ImmunoGlo™-MLC is a research tool to measure a 1- or 2-way stimulation of lymphocytes due to histocompatibility differences.

ImmunoGlo™-MLC is used with peripheral blood lymphocytes or purified immune cell populations from human and other animal species.

ImmunoGlo™-MLC Assays Available

Catalog Nos.	No. of Samples* for 1-Way MLC	No. of Samples* for 2-Way MLC	No. of Plates/Kit
KM1-MLC-1	3	5	1
KM1-MLC-2	6	10	2
KM1-MLC-4	12	20	4

* Based on performing 6 replicates/sample and incorporating 4 controls for a 1-way and 2 controls for a 2-way MLC.

4. The CONCEPT and PRINCIPLE of BIOLUMINOMICS™ ASSAYS

ImmunoGlo™-MLC 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 usually incubated for 5 days. 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™-MLC 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.

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

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5. OVERVIEW OF THE ImmunoGlo™-MLC PROCEDURE

There are 3 - 4 steps to use ImmunoGlo™-MLC.

Step 1 – Cell Preparation

Cells are not provided with ImmunoGlo™-MLC assay kits. Cells should be prepared using a user-defined, pre-validated protocol to obtain a single cell suspension of mononuclear cells or 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 - Mitomycin-C Treatment for a 1-Way MLC

If a 2-way MLC is to be performed, this step is omitted. For a 1-way MLC, the stimulator cells are first treated with a final concentration of 25µg/ml mitomycin-C to inhibit cell proliferation. The treated cells should be washed to remove mitomycin-C prior to use. Depending on the number of controls and samples to be tested, sufficient mitomycin-C - treated cells should be prepared so that multiple replicate wells can be seeded prior to the addition of the responder cells. It is suggested to perform a cell dose response to ascertain the optimal stimulator cell concentration.

Step 3 – Cell Culture

Normally a cell dose response or cell titration curve for both stimulator and responder cells in a 1-way MLR or both cell types for a 2-way MLR is usually performed for a mixed lymphocyte culture. Cell concentrations should be calculated so that two times (2 x) the final required cell concentration is in a total volume of 0.05ml/well. For controls, this volume is then diluted with 0.05ml of medium to the final required concentration. When cells are mixed together for a 1- or 2-way MLC, 0.05ml from each donor is added to the same well. The final cell concentrations of each will then be similar to the individual controls The cells are cultured in a 37°C, fully humidified incubator containing an atmosphere of 5% CO_2. Culturing cells under low oxygen tension (5% O_2) is advantageous. For a MLC, cells are usually cultured for 5 days, although this may vary depending on the cell source.

Step 4 – 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).

PLEASE NOTE: Although ImmunoGlo™-MLC is performed in a 96-well plate, it is not an ELISA and should not be considered as an ELISA.

6. KIT CONTENTS and STORAGE

ImmunoGlo™-MLC 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	Mitomycin-C (solid substance). Add 1mL of PBS to each vial just prior to use.	-20°C until used. 1-2 weeks dissolved. Keep at 2-8°C in the dark.
2	Medium (IMDM) for dilution of the ATP standard.	-20°C until used
3	ATP standard.	-20°C until used
4	ATP extra high, high and low controls.	-20°C until used
5	ATP Enumeration Reagent (ATP-ER)*	-20°C in the dark until used
6	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
7	Sterile, 96-well plates for cell culture	Can be kept with other kit components
8	Non-sterile 96-well plates for ATP standard curve determination.	Can be kept with other kit components
9	Instruction 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.

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12. ImmunoGlo™-MLC Assay Characteristics and Validation Parameters

ImmunoGl™-MLC 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

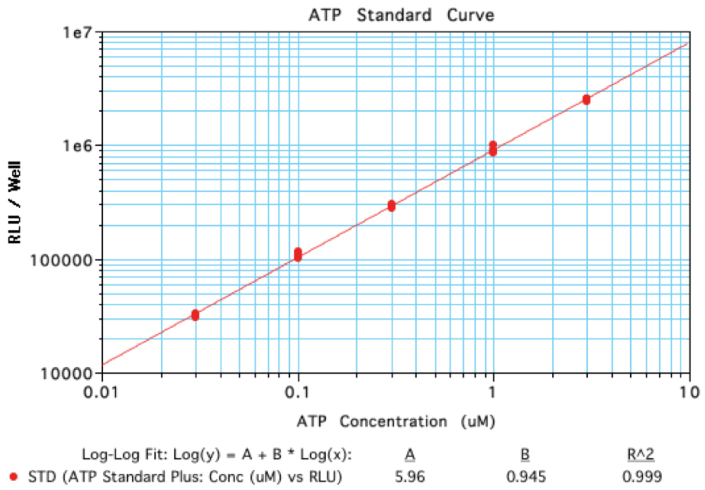
- Assay ATP linearity => 5 logs
- Assay ATP sensitivity: ~ 0.001µM
- Assay cell sensitivity: 20-25 cells/well (depending on cell type and purity)
- Accuracy (% correct outcomes): ~95%
- Sensitivity and specificity detected by Receiver Operator Characteristics (ROC) curve fit and detected as area under 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 5. Typical ATP Standard Dose Response Curve Using SoftMax Pro Software



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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)
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™-MLC PROTOCOL

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

Performing ImmunoGlo™-MLC is a 3 to 4 - step process.

Step 1 – Cell preparation.
Step 2 -- Treatment of stimulator cells with mitomycin-C for a 1-way MLR.
Step 3 – Cell culture and incubation in the 96-well plate.
Step 4 – Luminescence measurement. An ATP dose response is performed prior to sample luminescence measurements with conversion of RLUs to µM ATP.

Steps 1 to 3 must be performed in a laminar flow biohazard hood

STEP 1 – Cell Preparation

A MLC assay is usually performed using human peripheral blood lymphocytes, but cells from other species can also be used. It is best, however, to start with a mononuclear cell (MNC) fraction that has been significantly depleted of red blood cells (RBCs). Depletion of erythrocytes is essential since they can interfere with the assay when present at high concentrations (hematocrits > 10%) and cause false positive results.

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 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%.

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 5 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)

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(ii) Density Gradient Centrifugation

Red blood cells 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, which 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 dramatically improve viability.

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. Prepare a working cell suspension concentration for each of the two different samples. If a cell dose response is required, prepare sufficient volumes to allow for serial dilution. ***Note*** *that the working cell concentration per ml will be 20 x of the final cell concentration per well.* If stimulator cells have been treated with mitomycin-C for a 1-way MLR prior to cell culture, higher or lower cell concentrations may be required.
4. The total volume of cell suspension in each well should not be greater than 0.1ml. This is the recommended volume since an additional 0.1ml of the ATP-ER will be added to each well in order to measure proliferation. Since the contents of each well will be mixed, sufficient space is necessary in each well to accommodate for these later procedures.

STEP 2. Treatment of Cells with Mitomycin-C

IMPORTANT: Mitomycin-C is a toxic compound. Handle with care. Use laboratory gloves and universal protective clothing.

NOTE: Mitomycin-C decomposes rapidly. Please follow storage conditions on Page 6.

This step is only required for a 1-way MLC. If a 2-way MLC is required, omit this step and continue to Step 3.

For a 1-way MLC, one cell sample acts as the stimulator, while the other acts as the responder. Although it is usual to treat the stimulator cells (whichever donor is designated the stimulator) with an inhibitor of proliferation prior to use, it is recommended to also treat an aliquot of the responder cells as a control. This is shown in Fig. 1. The cells can be subjected to irradiation or treated with mitomycin-C prior to use to inhibit proliferation. The following is a suggested protocol for mitomycin-C - treated cells.

1. Prepare cell suspensions so that after treatment and 2-3 cell washing steps, sufficient cells will be available for controls and MLC.
2. Transfer an aliquot of the cells to a sterile tube.
3. Mitomycin-C is dissolved in physiological saline or PBS and prepare a 10 fold working dilution so that when added to the target cell suspension the final mitomycin-C concentration will be 25µg/well. Add 1.0mL of sterile PBS or saline to each vial containing 250µg of mitomycin-C, producing a solution of 250µg/mL. When added at 10% of the well volume, the final concentration is 25µg/well.
4. Add mitomycin-C working concentration to the cells.
5. Incubate for 30 minutes at 37°C in a fully humidified atmosphere containing 5% CO_2.
6. After the incubation time has elapsed, remove the tube from the incubator

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.

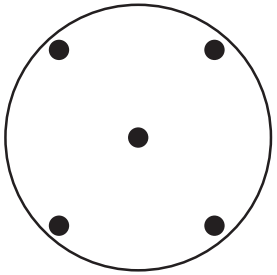
a. First set the integration time to 2 seconds.
b. Next, set the "gain". This must be determined empirically and is best per formed when the ATP standard curve is measured. The gain should be adjusted so that the percent coefficients of variation (%CV) for the mean of the replicates are the lowest value. 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 4. Positions of pipette tip for mixing the well contents



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and add 5-10ml of sterile physiological saline or PBS to dilute the agent and centrifuge the cells at 200 x g for 10 minutes at room temperature.

7. Remove and discard the supernatant.
8. Resuspend the cells in another 5-10 mls of saline or PBS and centrifuge again under the same conditions as in Step 6.
9. If necessary, Steps 7 and 8 can be repeated for a third cell wash. Note that with each centrifugation step, approx. 10% of the cells will be lost.
10. After the last wash, resuspend the cells in a volume of culture medium.
11. Perform a nucleated cell count and, if required, a viability determination.
12. Adjust the cell concentrations to the required working concentration that is 20 times (20 x) the final concentration/well.

STEP 3. ImmunoGlo™-MLC - Cell Culture Protocol

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

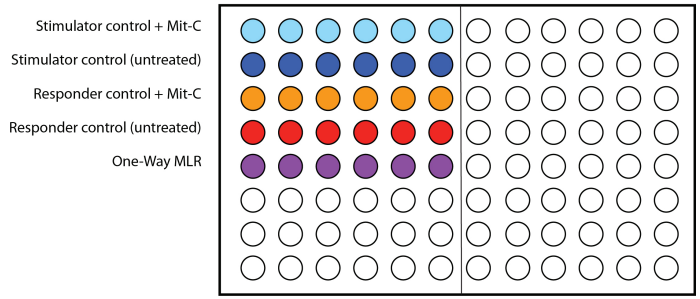
Perform all cell culture under sterile conditions in a biosafety cabinet.

Use calibrated pipettes and sterile tips throughout.

A. One-Way MLR

Figure 1 shows the suggested 96-well plate configuration for a 1-way MLR and includes four controls. It may be necessary to perform a cell titration curve to obtain optimal cell concentrations.

FIGURE 1
ImmunoGlo™-MLC Plate Configuration* for a One-Way MLR



* Suggested number of replicate wells = 6

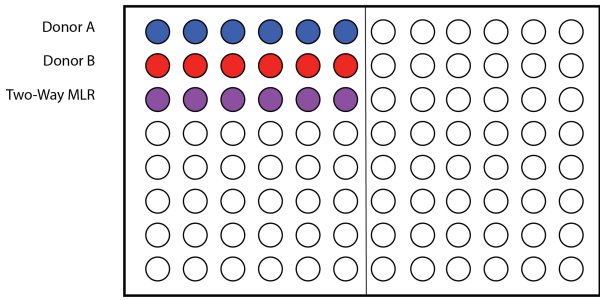
1. Prepare the stimulator and responder cells ± Mit-C at the required working concentration, equivalent to twenty times (20 x) the final concentration/well. For example, if the final concentration is to be 1 x 10^5 cells/well, then the stimulator + Mit-C and responder cells will both have to be 2 x 10^6 cells/ml.
2. Dispense 0.05ml of culture medium into all control wells. See Fig. 1.

3. Dispense 0.05ml of the stimulator control cells treated with mitomycin-C (Mit-C) into each replicate well. Figure 1 shows the number of replicate wells. This could be reduced to 4 replicate wells. Addition of 0.05ml of the cell suspension to 0.05ml of culture medium will dilute the cells to the final cell concentration.
4. Dispense 0.05ml of the untreated stimulator cells into the next row of replicate wells.
5. Dispense 0.05ml of responder control cells treated with Mit-C into the third row of replicate wells.
6. Dispense 0.05ml of the untreated responder cells into the fourth row of replicate wells.
7. Finally, dispense 0.05ml of the Mit-C - treated stimulator cells into each replicate well followed by 0.05ml of the untreated responder cells into the same replicate wells. Each of these wells will now contain a total of 0.1ml diluted to the same concentration as the controls.
8. Transfer the 96-well plate to a 37°C fully humidified incubator containing an atmosphere of 5% CO_2 and, if possible, 5% O_2.
9. Culture the cells for 5 days. This time period may vary depending on the cells being studied and the species being used.

B. Two-Way MLR

Figure 2 shows the suggested 96-well plate configuration for a 2-way MLR and includes 2 controls.

FIGURE 2
ImmunoGlo™-MLC Plate Configuration* for a Two-Way MLR



* Suggested number of replicate wells = 6

A 2-way MLR does not use mitomycin-C - treated cells. The two donors are used to stimulate each other.

1. Prepare the stimulator and responder cells at the required working concentration, equivalent to twenty times (20 x) the final concentration/well. For example, if the final concentration is to be 1 x 10^5 cells/well, then the stimulator and responder cells will both have to be 2 x 10^6 cells/ml.
2. Dispense 0.05ml of culture medium into all control wells. See Fig. 2.
3. Dispense 0.05ml of Donor A into replicate wells. Fig. 2 shows that 6 replicate

- 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™-MLC 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.

Volumes of Luminescence Kit Components Required

wells are recommended, although this can be reduced to 4.
4. Dispense 0.05ml of Donor B into the next row of replicate wells.
5. Dispense 0.05ml of Donor A cells into the third row of replicate wells followed by 0.05ml of Donor B cells into the same replicate wells. The resulting dilution with a total volume of 0.1ml will produce the same final concentration as the controls.
6. Transfer the 96-well plate to a 37°C fully humidified incubator containing an atmosphere of 5% CO_2 and, if possible, 5% O_2.
7. Culture the cells for 5 days. This time period may vary depending on the cells being studied and the species being used.

STEP 4 – MEASUREMENT OF PROLIFERATION USING 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.

See Figure 3 for the ATP standard curve and control plate configuration.

ImmunoGlo™-MLC includes ATP standards for an "extra high" ATP standard curve (from 0.03µM to 3µM ATP and a "normal" ATP standard curve (from 0.01µM to 1µM 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. For non-compatible cells, the induced proliferation in a 1- or 2-way MLR will produce high ATP values. For this reason, it is recommended to perform the "extra high" ATP standard curve. If the cells from each donor are expected to be compatible, the "normal" ATP standard curve can be used. If ATP values from the samples are greater than the highest ATP standard curve value (3µM), it is recommended to dilute the sample with additional medium so that the values are within the ATP standard curve range. This may require removing an aliquot from the replicate wells, transferring the aliquot to a new well and diluting each aliquot with additional medium. The replicate wells would then be reread.

A. "Extra High" ATP Standard Curve Preparation.

Mobilized peripheral blood samples usually exhibit a high iATP concentration. For this reason, it is necessary to modify the ATP standard curve range so that sample ATP values fall within this range. If the ATP concentrations are found lower than 0.03µM, the ATP standard curve will have to be repeated using the dose range from 0.01µM to 1µM (see below). If a sample is found to exhibit an iATP concentration greater than 3µM, the sample will have to be diluted so that the iATP falls within the ATP standard curve dose range. The dilution would then be taken into account when calculating the ATP value of the sample.

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-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

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

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 HALO® 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

It is recommended to use 6 replicates/sample, although 4 replicates/sample can also be used. 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

Please refer to Figures 1 and 2 for the plate configuration. If using 4 replicates/sample, it might be easier to plate the samples in columns rather than rows.

(iv) 96-Well Plates Provided

The reagents have been optimized to work with the 96-well plate(s) provided in the ImmunoGlo™-MLC 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 normal incubation time is 5 days. However, this might vary depending on cell type and species. Assay sensitivity might improve with longer incubation times, but usually at the expense of higher variability between wells. Once

Vial #3: 0.1µM
Vial #4: 0.05µM
Vial #5: 0.01µM

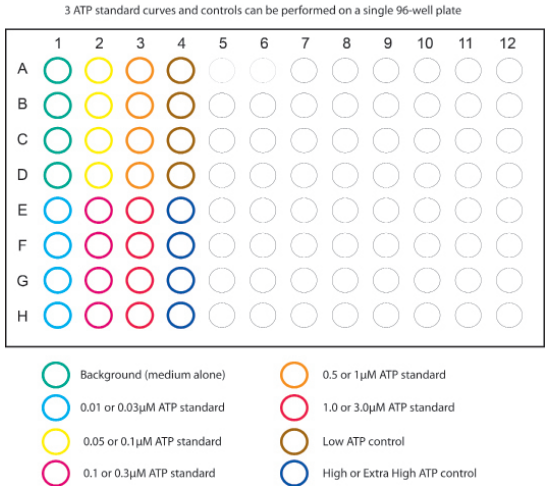
2. Now dispense:
   0.90ml of the medium to vial #1.
   0.35ml of the medium to vial #2
   0.90ml of the medium to vial #3
   0.90ml of the medium to vial #4
   0.90ml of the medium to vial #5
3. Remove 0.1ml of the supplied stock ATP solution (at 10µM) and transfer it to vial #1. Mix by vortexing. This 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 3
96-Well Plate Configuration for ATP Standard Curve and Controls



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 9, 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.
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.

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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™-MLC can be performed in high throughput mode. If you intend to perform any part of the HALO® 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.