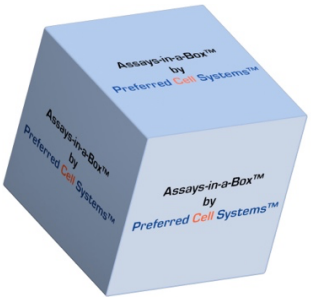

Assays-in-a-Box™
by
Preferred Cell Systems™

ImmunoGlo™-Tox HT

Immunotoxicity Assays for T- and B-Lymphocytes

Technical Manual

(Version 03/24)

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

For *In Vitro* Research Use Only

Not for Clinical Diagnostic Use

KM026.002

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1. Limitations of the Assay and Precautions

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

2. Introduction

Immunotoxicity is one of the most important decision-making aspects in drug development and environmental toxicology. Immunotoxicity can involve a multitude of complex cellular interactions that are often difficult to prize apart into their individual components. Under normal, steady-state conditions, lymphocytes demonstrate little or no proliferation. When stimulated with mitogens, cytokines or co-factors, 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.

For this reason, Preferred Cell Systems™ has developed several toxicity assays that can be used to study general immunotoxicity or toxicity to the T-lymphocyte or B-lymphocyte compartment.

All of these assays include the same common signal detection system, namely the measurement of changes in chemical energy in the form of intracellular adenosine triphosphate (iATP), which is also a biochemical indicator of metabolic viability, functionality and cell proliferation. The amount of iATP produced by a cell correlates directly with its functional status. The most sensitive non-radioactive readout to measure cell proliferation is iATP using a luciferin/luciferase bioluminescence signal detection system. This concept is used for all ImmunoGlo™-Tox HT assays. However, reliability and reproducibility are key to comparing results from different agents over time and to do this, all ImmunoGlo™-Tox HT assays are standardized prior to measuring samples. The standards and controls included with the kits also allow the assay to be validated in-house, even through Preferred Cell Systems™ has validated the assay for you (see Page 14).

ImmunoGlo™-Tox HT assays are easy to learn and rapid to use and can replace may other methods (e.g. CSFE, BudR etc) for measuring lymphocyte proliferation/cytotoxicity.

3. Use and Availability

ImmunoGlo™-Tox HT is intended as a research tool to measure potential immunotoxicity in vitro to virtually any agent.

It is recommended that to study any immunotoxicity response using ImmunoGlo™-Tox HT assays, the cell source e.g. peripheral blood, bone marrow, spleen, thymus etc. should be fractionated to at least a mononuclear cell (MNC) fraction or purified using magnetic bead separation or other technology.

ImmunoGlo™-Tox HT Assays Available

Assay	Additions	Specificity	Species	No. of Plates/Kit
ImmunoGlo™-Tox HT	No growth factors, mitogens or co-stimulators included	General	Human. primate. dog, rat, mouse	2 or 4 plate kits available
ImmunoGlo™-Tox HT TCP	IL-2 only	T-cells	Human, primate, rat, mouse	2 plate kits*
ImmunoGlo™-Tox HT TCP	CD3 + CD28 co-stimulators only	T-cells	Human only	2 plate kits*
ImmunoGlo™-Tox HT TCP	IL-2 + CD3 + CD28	T-cells	Human only	2 plate kits*
ImmunoGlo™-Tox HT BCP	Cocktail contains cytokines only	B-cells	Human only	2 plate kits*
ImmunoGlo™-Tox HT BCP	Cocktail include cytokines	B-cells	Human only	2 plate kits*

* Please note that ImmunoGlo™-Tox HT can also be obtained as 4 plate kits and in bulk. Please contact Preferred Cell Systems™ for more information. In addition, ImmunoGlo™-Tox HT TCP assay kits are also available with a serum-free formulation.

4. Principle of ATP Bioluminescence Assays

ImmunoGlo™-Tox HT is an ATP bioluminescence assay. The fundamental concept underlying this assay 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 controls are provided. **Performing the ATP standard curve and controls prior to measuring samples is highly recommended for the assay, since it provides the user with the information that the assay is functioning correctly. Calibrating and standardizing the assay has the following important advantages:**

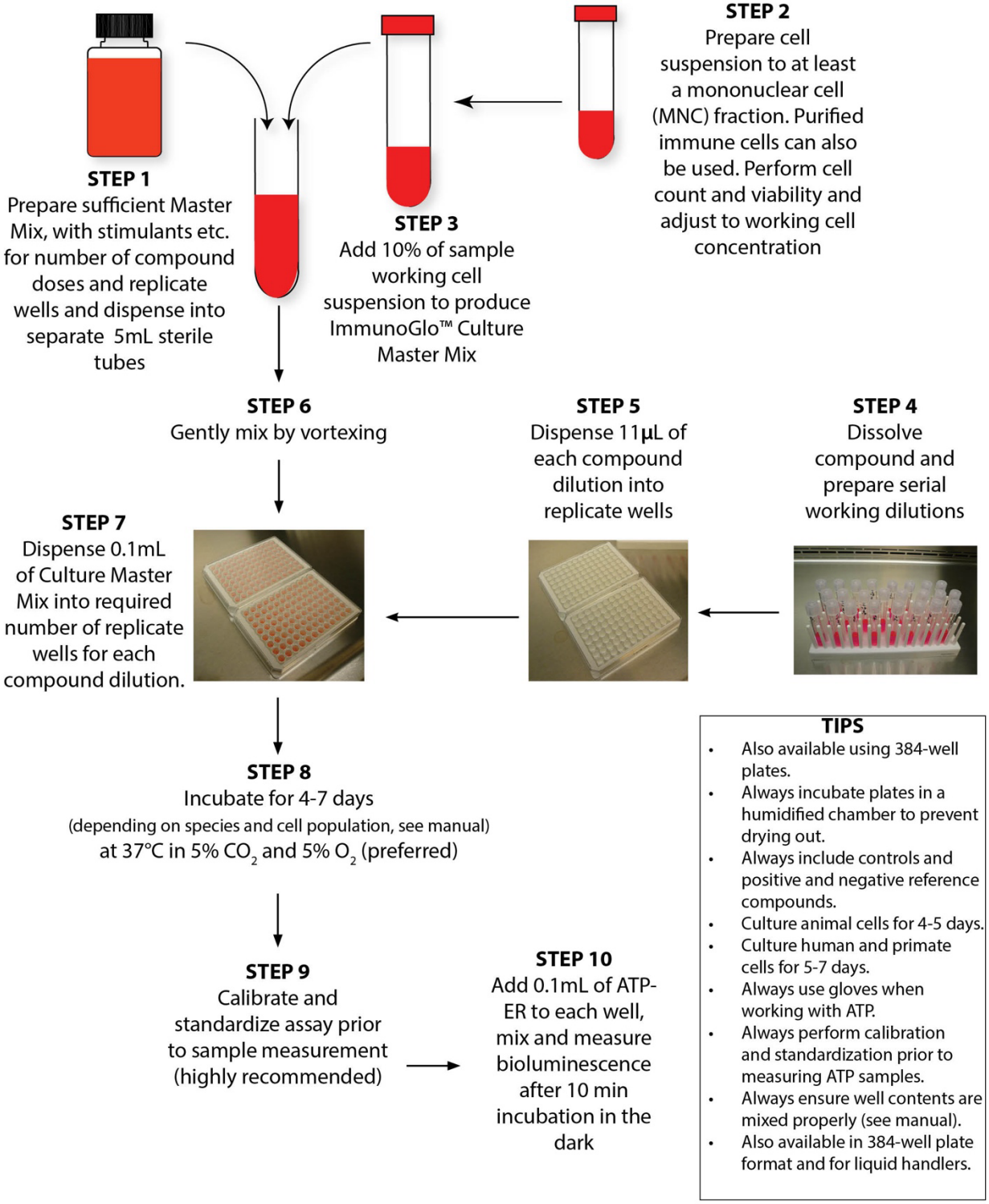
1. Performing an ATP standard curve calibrates and standardizes the assay.
2. The controls ensure that the instrument is working correctly as well as the reagents.
3. Allows the investigator to compare measurement assurance parameters with those in Section 12 of this technical manual. All results for both the ATP standard curve and controls must lie within the ranges stated in Section 12. This will allow the investigator to proceed with sample measurement and have the confidence that the results will be trustworthy.
4. The ATP standard curve allows the luminometer output in Relative Luminescence Units (RLU) to be converted to standardized ATP concentrations (µM).
5. Performing the ATP standard curve allows results to be compared over time.

The ATP standard curve and controls need only to be 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.

To convert non-standardized RLU values to standardized ATP concentrations, please see Section 11 of this Technical Manual.

5. QuickGuide™ to ImmunoGlo™-Tox HT (Figure 1)

QuickGuide™ to ImmunoGlo™-Tox HT



6. Kit Contents and Storage

ImmunoGlo™-Tox HT 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	Base ImmunoGro™ Medium for ImmunoGlo™-Tox HT cell culture without growth factors and cell dilutions (if needed) and containing antibiotics (gentamicin, streptomycin, penicillin and neomycin)	-20°C until used
1a	ImmunoGlo™-Tox HT TCP Master Mix included (ImmunoGro Medium not included in these assay kits)	-20°C until used
1b	ImmunoGlo™-Tox HT BCP Master Mix included (ImmunoGro™ Medium not included with these assay kits)	-20°C until used
2	Iscove's Modified Dulbecco's Medium (IMDM) for dilution of the ATP standard only. NOT FOR CELL CULTURE.	-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, solid white, 96-well plates for cell culture	Can be kept with other kit components
8	Non-sterile, solid white, 96-well plates for ATP standard curve determination.	Can be kept with other kit components
	Technical Manual	Download from website

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 Preferred Cell Systems™.

Disclaimer

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

beyond their expiry date. If the kit cannot be used prior to the expiry date of this reagent, fresh reagents can be purchased from HemoGenix®. Please contact Preferred Cell Systems™.

Good laboratory practices and universal protective precautions should be undertaken at all times when handling the kit components as well as cells and tissues. Certificates of Analysis (CoA) and Safety Data Sheets (SDS) can be downloaded from the Preferred Cell Systems™ website.

7. Equipment, Supplies and Reagents Required, But Not Provided

Equipment and Supplies

1. Laminar Flow Biohood
2. Luminescence plate reader (e.g SpectraMax L, Molecular Devices. LB962 CentroLIA/pc from Berthold Technologies and available from Preferred Cell Systems™).
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. For controls using ImmunoGlo™-Tox HT TCP and BCP assay kits, ImmunoGro™ Medium is available separately (Catalog Number: M-IG-100 for 100mL; M-IG-500 for 500mL).
2. Sterile Phosphate Buffered Saline (PBS)
3. DNase (Sigma-Aldrich, Catalog No. D4513-1VL)
4. Density-gradient medium (e.g. LymphoPrep).
5. ACK Lysis buffer (Cat. No. K-Lysis-100, Preferred Cell Systems™, Inc)
6. 7-AAD, propidium iodide, trypan blue or other dye exclusion viability assay.

8. The ImmunoGlo™-Tox HT Protocol

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

Performing ImmunoGlo™-Tox HT is a 4-step process.

Step 1 – Cell preparation.
Step 2 -- Controls and compound dose response preparation.
Step 3 – Cell culture and incubation in the 96-well plate.
Step 4 – Luminescence measurement. An ATP dose response can be performed prior to sample luminescence measurements for conversion of RLUs to µM ATP.

Steps 1, 2 and 3 must be performed in a laminar flow biohazard hood

STEP 1 – Cell Preparation

HALO®-Tox HT can be performed using tissues with the following purity:

1. Mononuclear cell (MNC) fraction is the preparation of choice. This fraction can be prepared by density gradient centrifugation.
2. Purified cell populations can be prepared by cell sorting or magnetic bead separation.
3. For B-cell assays, the cells should be purified prior to culture.

ImmunoGlo™-Tox HT assays are also metabolic viability assays, as opposed to dye exclusion viability assays.

It is <u>not</u> recommended to plate a cell concentration based on viability. This will result in a greater cell concentration (consisting of many dead cells) being plated.

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, propidium 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.

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.

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™ (Preferred Cell Systems™) 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. Controls and Compound Dose Response Preparation

A. Controls

Depending on whether the test compound is dissolved in aqueous medium or a solvent, up to 4 controls are recommended for each solvent used.

Control 1. Control contains cells, but no growth factors. (Control Master Mix provided).

Control 2. Vehicle-only control contains cells, but no growth factors. (Control Master Mix provided).

Control 3. Growth factor control contains cells with growth factors for cell populations being tested. (Master Mix bottles).

Control 4. Growth factor + vehicle control contains cells with growth factors for population being tested and the vehicle. (Master Mix bottle).

If studying the P-Tcell and/or P-Bcell populations, extra controls may be necessary.

The vehicle control should be prepared at the same concentration of vehicle used in the highest working test compound dose.

B. Test Compound Dose Response

The following points should be considered when preparing the test compound dose response.

1. It is recommended to perform a minimum of 6 compound doses to obtain a full dose response curve and estimation of IC or EC values.
2. It is also recommended to perform 8 replicate wells/dose configured in columns across the plate. If possible, include at least the growth factor+vehicle control on each plate. Otherwise, controls can use the extra 8th plate provided with the kit.
3. Alternative plate configurations can be performed depending on the number of compound doses, compounds, and replicates. However, for statistical purposes, 8 replicates are recommended, although 6 replicates can also be used.
4. If possible, it is best to configure the plate so that at least Controls 3 and 4 are on the same plate as the test compound.
5. If the test compound can be dissolved in water or aqueous medium, the vehicle controls (Controls 2 and 4) are not absolutely necessary.
6. If the test compound has to be dissolved in a solvent, such as dimethylsulphoxide (DMSO) or in the presence of fetal bovine serum (FBS), all controls should be included.
7. For 96-well plates, the test compound or vehicle is dispensed directly into each well before the addition of the Culture Master Mix containing the cell suspension.
8. For 384-well plates, the test compound is dispensed after the addition of the Culture Master Mix.
9. The original test compound stock concentration is defined as the concentration of the test compound after it is dissolved in water, aqueous medium, solvent or FBS.
10. The working concentration is defined as the test compound dose prepared prior to dispensing into a well, and should be 10 x the final concentration in culture.
11. The final concentration is defined as the test compound dose that is present in the culture well.
12. The volume of test compound dispensed into each well of a 96-well plate should be 11µL.
13. For 384-well plates, 2.8µL is dispensed using a liquid handler directly into the Culture Master Mix.
14. If using a solvent to dissolve the test compound, the final concentration of the solvent in the culture well should not exceed 0.1%.

15. The first or highest working concentration of a test compound dissolved in a solvent should be diluted 1:100 from the original test compound stock concentration so that when 11µl of the test compound working concentration is added to the well, the final concentration of the solvent is reduced 1:1000 or 0.1% in the final culture. Example: Test compound stock concentration dissolved in DMSO = 10mM. First working concentration diluted to 0.1mM of compound. DMSO diluted in this stage is 1:100. Final concentration of compound when 11µl is added to the well followed by 0.1ml of Culture Master Mix = 10µM. Final dilution of DMSO in culture is 1:1000 or 0.1%.
16. If the test compound is dissolved in a solvent, all further dilutions must be either in water, PBS, aqueous medium or FBS. The best diluent should be determined empirically prior to preparing the full dose response. If precipitation of the test compound occurs at the first dilution, a different diluent has to be used. If FBS has to be used in the diluent, try using a 10% FBS concentration in medium to determine if the compound, when diluted, will produce a clear solution. If precipitation still occurs, increase the concentration of the FBS in steps of 10%. Use the same diluent for all remaining serial dilutions.
17. It is possible that the test compound can only be dissolved at low pH. If this is the case, dilution to the first working concentration should include achieving a normal pH. The medium used in the HALO® Culture Master Mix contains HEPES buffer and therefore can accommodate a change in pH that will not harm the target cells.
18. Prepare enough vehicle control for the number of wells to be used.

STEP 3. ImmunoGlo™-Tox HT 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 prepared is arbitrary. However, a minimum of 4 replicate wells/sample is recommended for statistical purposes.***

1. If using ImmunoGlo™-Tox HT, prepare “user-defined Master Mixes” containing all the required culture components for the different treatments to be examined. Do not add cells at this time. ImmunoGro™ Medium (included with the kit) can be used for this step or other lymphocyte growth medium can be used. If using ImmunoGlo™-Tox HT TCP or BCP, the Master Mixes, requested by the user to stimulate T- or B-cells, are included with the kits. No additional ImmunoGro™ Medium is included with these kits.
2. The volume of “user-defined Master Mix or Master Mix for T- or B-cells included with the kits, for each treatment will depend on the number of replicate well required. For example:

Number of Replicate Wells Required	Volume of “User-Defined Master Mix”	Volume of Cells (10% of final volume)	Total Volume
2	0.315mL	0.035mL	0.35mL
4	0.405mL	0.045mL	0.45mL
6	0.585mL	0.065mL	0.65mL

3. Prepare and label 5mL sterile plastic tubes for each cell sample or treatment to be analyzed.
4. Using a calibrated pipette, preferably an electronic pipette, dispense the required amount of "user-defined Master Mix" to Master Mix for T- or B-cells into each tube.
5. Prepare the cell suspension as required and adjust the cell concentration to a working cell concentration. The working cell concentration will be 100 x the final cell concentration/well. Thus, if a final cell concentration of 10,000 cells/well is required, the working cell concentration will be 1 x 10^6 cells/mL. The final cell concentration/well can range from 1,000 to 20,000 cells/well. However, Preferred Cell Systems™ recommends using a cell dose that is between 2,500 and 10,000 cells/well. To determine the optimal cell concentration/well, it is recommended to perform a cell dose response.
6. Add the cell concentration to the Master Mix in each tube. Adding 10% cell suspension to make up the final volume will reduce the cell concentration 10 fold. The resulting master mix is now defined as the Culture Master Mix.
7. Mix the contents of each tube thoroughly using a vortex mixer.
8. Remove the sterile, wrapped, 96-well plate from plastic covering under the hood.
9. Dispense 0.1mL of the Culture Master Mix into each replicate well. This will again reduce the cell concentration 10 fold to achieve the final cell concentration required per well.
10. 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).
11. Place the humidity chamber into a fully humidified incubator set at 37℃ 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.
12. Incubate the cells for the required period of time.

STEP 4 – Measurement of Lymphocyte Proliferation using ATP Bioluminescence

Please note the following important points:

- ***FOR ALL OF THE FOLLOWING STEPS, WEAR LABORATORY GLOVES. ATP is present on the skin and can cause erroneous results.***
- ***PLEASE REFER TO SECTION 11 ON HOW TO SETUP THE PLATE LUMINOMETER. The instrument should be setup and prepared for use prior to any of the following steps being performed.***
- ***Please refer to Section 10 for recommendations and tips prior to starting this part of the procedure. In particular, please refer to Section 10 for important information on mixing components in the wells.***
- ***Remove the ATP Enumeration Reagent (ATP-ER) from the freezer and thaw at room temperature or in cold running water prior to analysis. Do not thaw the ATP-ER in a water bath or 37oC incubator.***
- ***If the assay is to be calibrated and standardized, remove the ATP standard, controls and reagents from the freezer and thaw to room temperature or in cold running water prior to analysis.***
- ***ATP standard curves performed on previous days or for previous experiments or studies must <u>not</u> be used since the ATP-ER intensity changes with time and lot number.***
- ***Use the unwrapped, non-sterile, 96-well plate provided with the kit to perform the ATP standard dose response curve.***

It is highly recommended to standardize the assay prior to measuring samples. Use the non-sterile, 96-well white plate provided with the assay kit for this purpose.

ImmunoGlo™ includes the following to calibrate and standardize the ATP bioluminescence part of the assay to measure cell proliferation.

- IMDM medium: Used only for ATP standard serial dilution.
- ATP Standard at 10µM. Serially diluted to produce the ATP standard curve.
- Low ATP Calibration Control. Used for normal and extra high cell proliferation.
- High ATP Calibration Control. Used for normal cell proliferation.
- Extra High ATP Calibration Control. Used for extra high cell proliferation.

B. Deciding Which Calibration Controls to Use and ATP Standard Curve Range

PROTOCOL 1: If it is expected that the cells have a low proliferation ability, use the low and high calibration controls and perform an ATP standard curve from 0.01µM to 1µM. **See Page 22**.

PROTOCOL 2: If it is expected that the cells have a high proliferation ability, use the low and extra high calibration controls and perform an ATP standard curve from 0.03µM to 3µM. **See Page 23**.

It is important that the sample ATP values are within the limits of the ATP standard curve, otherwise the interpolation of Relative Luminescence Unit (RLU) values from the luminescence plate reader into ATP concentrations will not be accurate. If Protocol 2 has been used and values are not as high as 0.03µM ATP, perform Protocol 1. In some cases, cell proliferation could be greater than 3µM ATP. If ATP values from the samples are greater than 3µM , it is recommended to dilute the sample with additional medium so that the values are within the ATP standard curve range. This may require removing an aliquot from the replicate wells, transferring the aliquot to a new wells and diluting each aliquot with additional medium. The replicate wells would then be reread.

C. Sample Measurement

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

1. If possible, place the sample plate(s) in a humidified incubator set at 22-23C 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, remove the backing and layer it over the top of the plate. Using a sharp knife or scalpel, cut away the foil that covers the wells to be processed. The unused, empty wells will now remain sterile for the next samples. (See Section 10, Adhesive Plate Covering Film).
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.

D. Using a plate luminometer with automatic dispenser

The user may have a plate luminometer that allows reagents to be dispensed automatically directly into the well. Preferred Cell Systems™ does not recommend using the automatic dispensers, since the contents of the well are not mixed sufficiently using this method.

E. Using a liquid handler

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

9. Recommendation and Tips Prior to Using ImmunoGlo™-Tox HT.

(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™ 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 Preferred Cell Systems™ 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 Preferred Cell Solutions™ for further information about assembling and using humidity chambers.

(vi) Incubation Times

The incubation time may 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 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.

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™-Tox HT includes solid white plates for both cell culture and the ATP standard curve and controls. Do not use different plates for the assay. Doing so will result in inaccurate results and invalidation of the assay kit warranty. Extra plates can be purchased from Preferred Cell Solutions™.***

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℃.
- Enough ATP standard and enumeration reagent is supplied to perform 2 standard curves and controls for each sterile plate provided. Additional ATP standards and controls can be obtained from Preferred Cell Solutions™.
- 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℃, in the dark, for 48h or is stable at -20°C 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 2-8°C 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 2-8°C, or 20 weeks at -20°C.
- ATP-ER can be refrozen up to 11 cycles without significant loss of sensitivity.

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 highly recommended.

- 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 results to be compared with the measurement assurance criteria in Section 12 of this Technical Manual. Results should be within the ranges provided. For technical support, please contact Preferred Cell Systems™.
- It allows the output of the plate luminometer, in relative luminescence units (RLU), to be converted to ATP concentrations, thereby standardizing the procedure so that intra- and inter-laboratory experiments can be compared.
- It allows results to be compared over time, thereby providing valuable historical data.

Adhesive Plate Covering Film

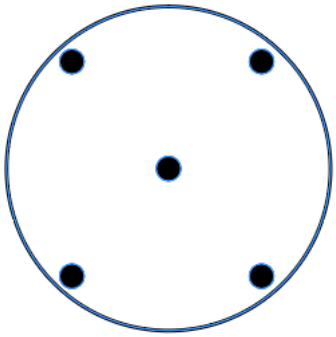
To help keep the plate(s) sterile, adhesive, air permeable, sterile films are provided so that the part of the plate that is not being used can be covered and kept sterile until required. If using the adhesive film provided, the plate cover should be removed in a laminar air-flow hood and replaced with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

Mixing the Contents of 96-well Plate

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

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

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



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

It is very important that the luminescence or multimode plate reader is setup correctly, otherwise false results could occur. Preferred Cell Systems™ has provided a separate document to help the investigator setup their instrument and perform the calculations in order to convert Relative Luminescence Units (RLU) into ATP concentrations using the ATP standard curve. It is strongly recommended that the investigator consult this document prior to performing any ATP bioluminescence assay. This document can be downloaded with this manual.

12. ImmunoGlo™-Tox HT Assay Measurement Assurance and Validation Parameters

If ImmunoGlo™-Tox HT has been calibrated and standardized, ATP bioluminescence technology allows the User's results to be compared to the measurement assurance parameters shown in the Table below. For each control, ATP standard dose and the log-log linear regression curve fit parameters provided, the User's results must lie within the ranges provided. If this is the case, then the following are applicable:

1. The User has performed and passed the integrated proficiency test.
2. The instrument and assay readout reagents are working correctly.
3. The User can continue to process and measure samples.
4. The User can trust results of the assay.

IMPORTANT. If the User's results DO NOT comply with those in the table, DO NOT measure the samples. Perform a repeat of the controls and ATP standard curve. If the results still do not comply with those in the Table, contact Preferred Cell Systems for help.

ATP Controls and Standard Curve Measurement Assurance Parameters

Expected Parameter	Observed Value	Mean ± 15%(*)	Min / Max	%CV (where applicable)
0.01µM ATP	0.0099µM ATP	0.00972 - 0.0114	0.009 - 0.01	2.34%
0.03µM ATP	0.029µM ATP	0.285 - 0.0336	0.028 - 0.03	1.67%
0.05µM ATP	0.0497µM ATP	0.0486 - 0.0571	0.048 - 0.051	1.57%
0.01µM ATP	0.1026µM ATP	0.1003 - 0.118	0.099 - 0.107	1.96%
0.3µM ATP	0.317µM ATP	0.310 - 0.364	0.302 - 0.325	1.51%
0.5µM ATP	0.5023µM ATP	0.491 - 0.578	0.491 - 0.515	1.19%
1.0µM ATP	1.048µM ATP	1.024 - 1.205	0.977 - 1.117	3.7%
3.0µM ATP	2.722µM ATP	2.661 - 3.130	2.633 - 2.934	2.09%
Intercept	6.533	6.386 - 7.513	5.86 - 6.7	1.84%
Slope	0.9656	0.944 - 1.110	0.947 - 0.988	1.21%
r^2 goodness of fit)	0.9993	-	0.998 - 1	0.05%

Expected Parameter	Observed Value	Mean ± 15%(*)	Min / Max	%CV (where applicable)
R (correlation coefficient)	1	-	0.999 - 1	0.02%
Low control, (0.05µM ATP	0.0487µM ATP	0.0476 - 0.0560	0.042 - 0.063	6.79%
High control 0.7µM ATP	0.725	0.710 - 0.836	0.655 - 0.904	5.35%
Extra high control (1.75µM ATP)	1.756	1.717 - 2.019	1.61 - 2.198	5.24%

(*) 15% represents the acceptable range of values for FDA Bioanalytical Method Validation Guidelines

Samples Values:

- Lowest ATP value indicating unsustainable cell proliferation for most immune cells: ~0.04µM.
- ATP value below which cells are not metabolically viable: ~0.01µM.
- All samples values must lie on the ATP standard curve for accurate RLU to ATP conversion. If ATP values are greater than 3µM, the replicate samples should be diluted with medium provided in the kit and re-measured. Take the dilution value into account when estimating the true ATP concentration. Alternatively, repeat the culture and ATP measurement using fewer cells.

Assay Validation Parameters

ImmunoGlo™-Tox HT exhibits the following validation parameters:

- Assay ATP linearity => 4 logs
- Assay ATP sensitivity: ~ 0.001µM
- Assay cell sensitivity: 20-25 cells/well (depending on cell type and purity)
- Accuracy (% correct outcomes): ~95%
- Sensitivity and specificity detected by Receiver Operator Characteristics (ROC) curve fit and detected as area under the curve (AUC): 0.73 - 0.752 (lowest possible value, 0.5; highest possible value, 1).
- Precision (Reliability and Reproducibility) =< 15%. At lower limit of quantification (LLOQ): 20%
- Robustness (intra- and inter-laboratory): ~95%.
- High throughput capability (Z-Factor): >0.76 (lowest possible value, 0.5; highest possible value, 1).

13. Troubleshooting

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

If the investigator has elected to calibrate and standardize the assay using the ATP controls and standard supplied with the kit, the results should be within the ranges provided in Section 12. If the values obtained conform to the measurement assurance parameters, the investigator can continue the assay and process and measure the samples with the assurance that the results can be trusted.

If any of the values obtained during calibration and standardization do not conform or are not within the ranges provided in Section 12, the user should repeat the calibration and standardization. Often discrepancies occur due to pipetting and/or dilution errors. Accurate and careful dilution of the ATP stock solution is important. It is also possible that if pipettes have not been professionally calibrated, errors can occur. These will also be picked up during this phase of the assay. Finally, if the ATP-ER has not be handled or stored correctly, it will decay, leading to erroneous results. Please contact Preferred Cell Systems™ to obtain new ATP-ER.

High Coefficients of Variation (%CV)

Coefficients of variation (%CV) should be =< 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 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.

Ordering Information

Toll free: 1-888-436-6869

Tel: (719) 264-6251

Fax: (719) 264-6253

Email: info@preferred-cell-systems.com

Order online at preferred-cell-systems.com

Technical Support

Tel: (719) 264-6251

Email: info@preferred-cell-systems.com

Preferred Cell Systems™

1485 Garden of the Gods Road

Suite 152

Colorado Springs, CO 80907

U.S.A.

Website: www.preferred-cell-systems.com

ImmunoGlo™-Tox HT was designed and developed by Preferred Cell Systems™, Inc

The ATP readout used for ImmunoGlo™-Tox HT is protected by U.S. patents 7,354,729, 7,354,730, 7,666,615, 7,709,258, 7,883,861, 7,700,354.

Preferred Cell Systems™

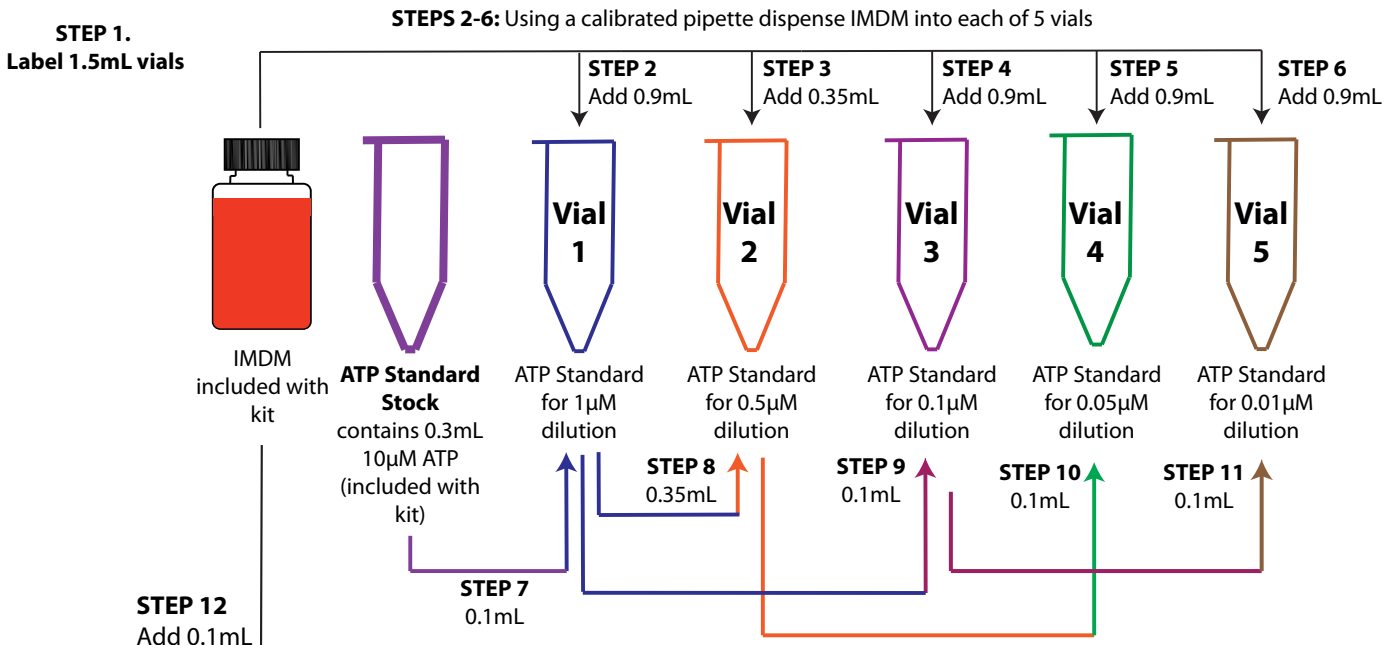
Calibration and Standardization Protocol of an ATP Bioluminescence Assay

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

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

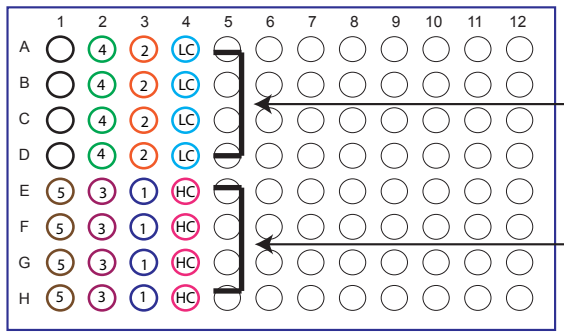
TIPS

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



STEP 12
Add 0.1mL into wells A1 - D1

Change pipette tips for each well



Follow Color Coding
STEP 13: Add 0.1ml from **Vial 5** into wells E1-H1
STEP 14: Add 0.1mL from **Vial 4** into wells A2-D2
STEP 15: Add 0.1mL from **Vial 3** into wells E2-H2
STEP 16: Add 0.1mL from **Vial 2** into wells A3-D3
STEP 17: Add 0.1mL from **Vial 1** into wells E3-H3

STEP 18: LOW CONTROL (LC, included with kit)
Vortex and lightly centrifuge to remove liquid from cap
Add 0.1mL from low control to wells A4-D4

STEP 19: HIGH CONTROL (HC, included with kit)
Vortex and lightly centrifuge to remove liquid from cap
Add 0.1mL from high control to wells E4-H4

STEP 20: Add ATP-ER to reserviour and using a multichannel pipette, dispense 0.1mL into each replicate well
STEP 21: Mix replicate wells as described for Figure 2 in this manual. Change tips for each new addition of ATP-ER
STEP 22: Transfer 96-well plate to luminescence plate reader
STEP 23: Incubate in the dark for 2 minutes and measure luminescence

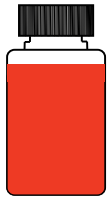
Calibration and Standardization Protocol of an ATP Bioluminescence Assay

PROTOCOL 2: ATP Standard Curve from 0.03µM - 3µM
For Samples with Known or Expected High Cell Proliferation

STEP 1.
Label 1.5mL vials

STEPS 2-6: Using a calibrated pipette dispense IMDM into each of 5 vials

STEP 6 Add 0.7mL
STEP 2 Add 0.4mL
STEP 3 Add 0.9mL
STEP 4 Add 0.9mL
STEP 5 Add 0.9mL



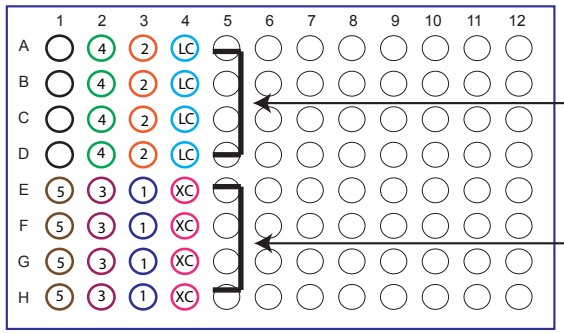
IMDM included with kit

Vial 1 — ATP Standard contains 0.3mL 10µM ATP (included with kit)
Vial 2 — ATP Standard for 1µM dilution
Vial 3 — ATP Standard for 0.3µM dilution
Vial 4 — ATP Standard for 0.1µM dilution
Vial 5 — ATP Standard for 0.03µM dilution

STEP 7 0.2mL
STEP 8 0.1mL
STEP 9 0.1mL
STEP 10 0.1mL

STEP 11
Add 0.1mL into wells A1 - D1

Change pipette tips for each well



Follow Color Coding
STEP 12: Add 0.1ml from **Vial 5** into wells E1-H1
STEP 13: Add 0.1mL from **Vial 4** into wells A2-D2
STEP 14: Add 0.1mL from **Vial 3** into wells E2-H2
STEP 15: Add 0.1mL from **Vial 2** into wells A3-D3
STEP 16: Add 0.1mL from **Vial 1** into wells E3-H3

LC

STEP 17: LOW CONTROL (LC, included with kit)
Vortex and lightly centrifuge to remove liquid from cap
Add 0.1mL from low control to wells A4-D4

XC

STEP 18: EXTRA HIGH CONTROL (XC, included with kit)
Vortex and lightly centrifuge to remove liquid from cap
Add 0.1mL from extra high control to wells E4-H4

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

TIPS

> Use calibrated pipettes throughout.
> Vortex thoroughly between each dilution.
> Change tips between each dilution.
> Follow color coding.
> A 0.01µM ATP Standard can be made from the Vial 4 and added to the plate.

STEP 19: Add ATP-ER to reserviour and using a multichannel pipette, dispense 0.1mL into each replicate well
STEP 20: Mix replicate wells as described for Figure 2 in this manual. Change tips for each new addition of ATP-ER
STEP 21: Transfer 96-well plate to luminescence plate reader
STEP 22: Incubate in the dark for 2 minutes and measure luminescence