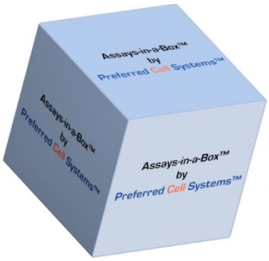


Assays-in-a-Box™
by
Preferred Cell Systems™

ImmunoGlo™

Lymphocyte Proliferation Assay

Technical Manual

(Version 3-24)

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

For *In Vitro* Research Use Only

Not for Clinical Diagnostic Use

KM024.002

TABLE OF CONTENTS

1. Limitations of the Assay and Precautions

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

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.

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), WST-1 or CSFE which can be detected using a colorimetric (absorbance) or fluorescence readout. Absorbance or fluorescence readouts are not as sensitive as radioactive readouts. The radioactive marker has usually been the method of choice because of the high sensitivity. However, use of any radioactive compound is a hazardous operation that also involves regulated waste removal.

All mammalian cells require chemical energy in the form of intracellular adenosine triphosphate (iATP), which is also a biochemical indicator of 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 ImmunoGlo™.

All ATP bioluminescence assays from Preferred Cell Systems™ include reagents to calibrate and standardize the assay so that results can be compared over time.

ImmunoGlo™ is easy to learn and rapid to use and can replace all other methods for measuring lymphocyte proliferation. To standardize the assay, use the optional ATP Bioluminescence Standardization and Calibration Kit (Cat. No. K6-ASP-1).

3. Use and Availability

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

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

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

ImmunoGlo™ Assays Available

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

Please note that ImmunoGlo™ can also be obtained is larger sized assay kits and in bulk. Please contact Preferred Cell Systems™ for more information.

4. Principle of ATP Bioluminescence Assays

ImmunoGlo™ 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:

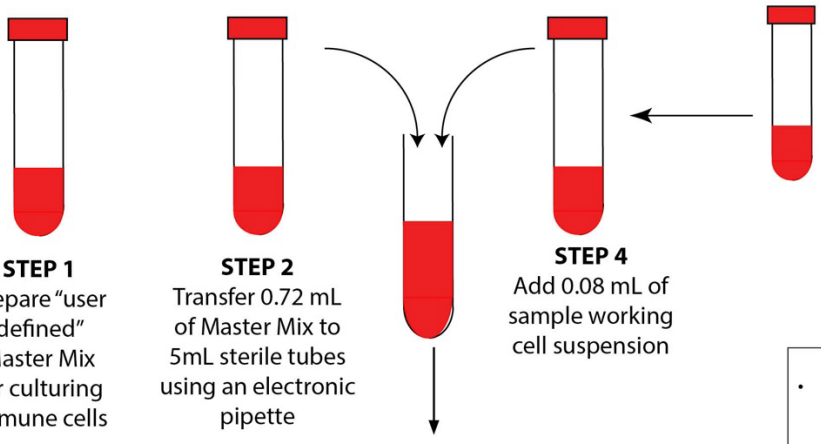
$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{LIGHT}$$

The bioluminescence emitted is detected and measured in a plate luminometer as Relative Luminescence Units (RLU).

To compares results day to day, between experiments or even between laboratories, it is necessary to standardize the assay. To do this, use the optional ATP Bioluminescence Standardization and Calibration Kit (Cat. No. K6-ASP-1).

5. QuickGuide to ImmunoGlo™ (Figure 1)

QuickGuide to ImmunoGlo™



STEP 1
Prepare "user defined" Master Mix for culturing immune cells

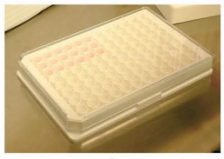
STEP 2
Transfer 0.72 mL of Master Mix to 5mL sterile tubes using an electronic pipette

STEP 3
Prepare cell suspension PBMNC or other immune cell fraction. Perform cell count and viability and adjust to working cell concentration

STEP 4
Add 0.08 mL of sample working cell suspension

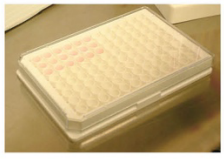
STEP 5
Gently mix by vortexing

STEP 6
Using an electronic pipette, dispense 0.1mL of Culture Master Mix into each of 6 replicate wells of the white culture plate



STEP 7
Incubate cells for specified time at 37°C in 5% CO_2 and 5% O_2 (preferred)

STEP 8
Calibrate and standardize proliferation assay prior to sample measurement (see separate QuickGuide)



STEP 9
Add 0.1mL of ATP-ER to each well, mix and measure bioluminescence after 10 min incubation in the dark

TIPS

- Always use at least a mononuclear cell (MNC) fraction as starting suspension.
- If possible, use self-calibrating electronic pipettes.
- Always dispense the Master Mix into the bottom of the well, never on the side.
- Always incubate plates in a humidified chamber to prevent drying out.
- Always use gloves when measuring ATP.
- Performing calibration and standardization provides measurement assurance for trustworth results.
- Always ensure well contents are mixed properly (see manual).
- Use sterile, adhesive foil (included) to maintain unused well sterility.

6. Kit Contents and Storage

ImmunoGlo™ 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 cell culture without growth factors and cell dilutions (if needed) and containing antibiotics (gentamicin, streptomycin, penicillin and neomycin)	-20°C until used
2	ATP Enumeration Reagent (ATP-ER)*	-20°C in the dark until used
3	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
4	Sterile, solid white, 96-well plates for cell culture	Can be kept with other kit components
	Technical Manual	Download from ImmunoGlo™ webpage

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 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 (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 and Kits

1. ATP Bioluminescence Standardization and Calibration Kit (Cat. No. K6-ASP-1 from Preferred Cell Systems™).
2. Additional Base ImmunoGro™ Low-Serum Medium. For cell dilutions and cell culture without growth factors, e.g. to measure background growth (Catalog Number: M-IG-100 for 100mL; M-IG-500 for 500mL).
3. Sterile Phosphate Buffered Saline (PBS)
4. Iscove's Modified Dulbecco's Medium (IMDM) or other growth medium. Used only for ATP standard dilution.
5. DNase (Sigma-Aldrich, Catalog No. D4513-1VL)
6. Density-gradient medium (e.g. NycoPrep 1.077, Axis-Shield).
7. ACK Lysis buffer (Cat. No. K-Lysis-100, Preferred Cell Systems™, Inc)
8. 7-AAD, propidium iodide, trypan blue or other dye exclusion viability assay.

8. The ImmunoGlo™ Protocol

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

Performing ImmunoGlo™ is a 3-step process.

Step 1 – Cell preparation.
Step 2 – Cell culture and incubation in the 96-well plate.
Step 3 – Luminescence measurement.

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

STEP 1 – Cell Preparation

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

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

- Dye exclusion viability
- Cellular and metabolic integrity viability.

Dye exclusion viability uses dyes that can enter the cell and usually bind with DNA. The dyes enter the cell due to a leaky cell membrane or loss in membrane integrity due to apoptosis and cell death. Using dyes such as typan blue, 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.

ImmunoGlo™ is a metabolic viability assay because if cells do not produce iATP, they are non-viable.

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 the assay. The RBC concentration should be reduced to below 10%. Otherwise, they will interfere with the ATP bioluminescence readout.

It is recommended to use a density gradient centrifugation procedure to prepare all mononuclear cell (MNC) preparations.

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. ImmunoGlo™ 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. 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.
2. The amount of "user-defined Master Mix for each treatment will depend on the number of replicate wells 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" 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 "user-defined Master Mix in each tube. Adding 10% cell suspension to make up the final volume will reduce the cell concentration 10 fold.
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 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 3 – 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 "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.*
- *If using an ATP Bioluminescence Standardization and Calibration Kit (Cat. No. K6-ASK-1), follow the instructions for thawing and using the reagents.*

Sample Measurement

1. If possible, place the sample plate(s) in a humidified incubator set at 22-23℃ 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. Although a single channel pipette can be used, a multichannel pipette (8- or 12-channel depending on the plate configuration), is recommended. 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. Alternatively, place the 96-well plate in the reader, in the dark, for 10 minutes and then read the plate.
6. Unused ATP-ER may be returned to the bottle and refrozen. See section 10 for ATP reagent storage conditions and stability.

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 <u>not</u> recommend using the automatic dispensers, since the contents of the well are not mixed sufficiently using this method.

9. Recommendation and Tips Prior to Using ImmunoGlo™.

(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.*
- *ImmunoGlo™ includes solid white plates for cell culture. 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 Enumeration Reagent (ATP-ER) from the freezer and thaw at room temperature or at 22 - 23℃.
- 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)).

Adhesive Plate Covering Film

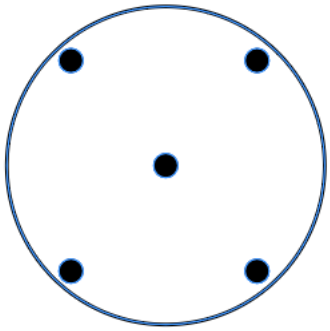
To help keep the plate(s) sterile, adhesive, air permeable, sterile films are provided so that the part of the plate that is not being used can be covered and kept sterile until required. If using the adhesive film provided, the plate cover should be removed in a laminar air-flow hood and replaced with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

Multimode 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 multimode 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" (if required). 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 22ºC and 24ºC or turned off.
d. Do not use plate shaking or the injectors if the instrument has this capability.
e. The output of the luminescence plate reader is in Relative Luminescence Units (RLU). To convert RLU values into iATP concentrations (µM), it is necessary to calibrate the instrument with controls and perform an ATP standard curve. If this is required, it will be necessary to purchase the ATP Standardization and Calibration Kit from Preferred Cell Systems™ (Cat. No. K6-ASK-1).

12. Results

ImmunoGlo™ provides an instrument-based, non-subjective, quantitative readout of viability and the total proliferation ability of the cells being tested.

ImmunoGlo™ can be used on fresh, frozen cells or fractionated cells (see ImmunoGlo™ TCP and ImmunoGlo™ BCP).

It is strongly recommended to include a background control, i.e. no stimulation. In general, stimulated immune cells should produce RLU values at least 2 standard deviations greater than the background or demonstrate a statistical significance from the background RLU value.

Do not use Relative Luminescence Units (RLU) to compare results from different experimental days. The assay needs to be standardized to perform this function. Use the optional ATP Bioluminescence Standardization and Calibration Kit (Catalog #: K-ATPSC-1).

13. Troubleshooting

Decay of ATP-ER

The ATP Enumeration Reagent (ER) decays with time, even when frozen. This is why results should not be compared when using Relative Luminescence Units (RLU) as the endpoint. To compare results over time, use the optional ATP Bioluminescence Standardization and Calibration Kit (Cat. No. K6-ASK-1).DO NOT use the ATP-ER past the expiration date. Doing so can result in very low RLU values or no bioluminescence at all (see also Low RLU Values, below).

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

Low RLU Values

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

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

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