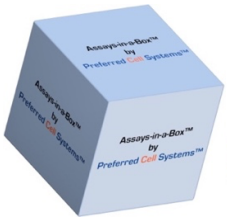

Assays-in-a-Box™
by
Preferred Cell Systems™

ImmunoGlo™ TCP

Human T-Lymphocyte Proliferation Assay with Co-Stimulators

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

KM019.002

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1. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. *ImmunoGlo™ TCP is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)*
2. *ImmunoGlo™ TCP 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, immune cells demonstrate little or no proliferation. When stimulated, however, different types of immune cells can exhibit different degrees of proliferation activity. The proliferation activity will be dependent upon the type of inducer, concentration and any co-stimulation that might be present.

ImmunoGlo™ TCP is a T-cell proliferation assay either of the following cocktails:

- IL-2 alone
- CD3 + CD28
- IL-2 + CD3 + CD28

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™ TCP.

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™ TCP is easy to learn and rapid to use and can replace all other methods for measuring lymphocyte proliferation.

3. USE and AVAILABILITY

ImmunoGlo™ TCP is a research tool to measure lymphocyte proliferation using either Interleukin-2 (IL-2), co-stimulators or both.

The present ImmunoGlo™ TCP technical manual is only for human cells that can be culture using a low serum or serum-free formulation (see Table on page 3). ImmunoGlo™ TCP is available for other species (primate, rat and mouse). A separate technical manual is available for these species, since they require a different protocol.

ImmunoGlo™ TCP can be used with the following human sources:

- Peripheral blood mononuclear cells
- Bone marrow
- Umbilical cord blood
- Purified lymphocyte populations from the above sources
- Other sources of T-lymphocytes.

ImmunoGlo™ TCP Assays Available

Catalog Nos.	Additions	Species	Serum Formulation	No. of Plates/Kit
KM1-T-1H	IL-2 alone (requires co-stimulators)	Human	Low serum	1
KM1-TCS1-1H	CD3 + CD28	Human	Low serum	1
KM1-TCS2-1H	IL-2 + CD3 + CD28	Human	Low serum	1
KM1SF-T-1H	IL-2 alone (requires co-stimulators)	Human	Serum-free	1
KM1SF-TCS1-1H	CD3 + CD28	Human	Serum-free	1
KM1SF-TCS2-1H	IL-2 + CD3 + CD28	Human	Serum-free	1
KM1-TCP1-1Pr	IL-2 alone (requires co-stimulators)*	Primate	Low serum	1
KM1-TCP1-1R	IL-2 alone (requires co-stimulators)*	Rat	Low serum	1
KM-TCP1-1M	IL-2 alone (requires co-stimulators)*	Mouse	Low serum	1

***** These assays require a different protocol to that described for human cells. Please see separate Instruction Manual.
ImmunoGlo™ TCP can also be obtained in larger sizes and in bulk upon request. Please contact Preferred Cell Systems™.

4. PRINCIPLE of ATP BIOLUMINESCENCE ASSAYS

ImmunoGlo™ TCP 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 not mandatory for the assay, but provides the user with the information that the assay is functioning correctly and has the following advantages:**

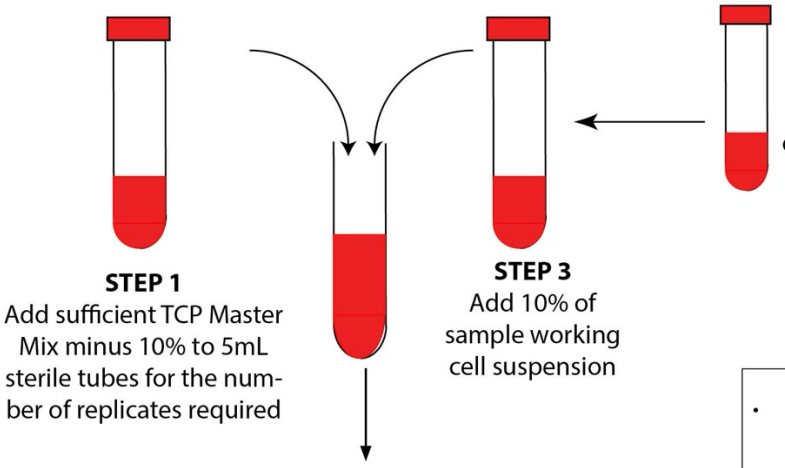
1. Performing an ATP standard curve calibrates and standardizes the assay.
2. The controls ensure that the reagents are working correctly.
3. The ATP standard curve allows the luminometer output in Relative Luminescence Units (RLU) to be converted to standardized ATP concentrations (µM).
4. Performing the ATP standard curve allows results to be compared over time.

The ATP standard curve and controls 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™ TCP

Quick Guide to ImmunoGlo™ TCP



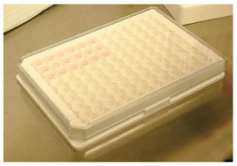
STEP 1
Add sufficient TCP Master Mix minus 10% to 5mL sterile tubes for the number of replicates required

STEP 2
Prepare cell suspension PBMNC or other immune cell fraction. Perform cell count and viability and adjust to working cell concentration

STEP 3
Add 10% of sample working cell suspension

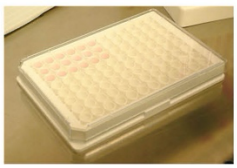
STEP 4
Gently mix by vortexing

STEP 5
Using an electronic pipette, dispense 0.1mL of Culture Master Mix into the required replicate wells of the white culture plate



STEP 6
Incubate cells for specified time at 37°C in 5% CO_2 and 5% O_2 (preferred)

STEP 7
Assay can be calibrated and standardized prior to sample measurement (see separate Quick Guide)



STEP 8
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 point.
- 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.
- Always perform calibration and standardization prior to measuring ATP samples.
- Always ensure well contents are mixed properly (see manual).
- Use sterile, adhesive foil (included) to maintain unused well sterility.

Preferred Cell Systems™
info@preferred-cell-systems.com

6. KIT CONTENTS and STORAGE

ImmunoGlo™ TCP 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	ImmunoGro™ Low Serum or Serum-Free Master Mix for cell culture containing antibiotics (gentamicin, streptomycin, penicillin and neomycin)	-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
2	ATP standard.	-20°C until used
3	ATP "extra high", high and low controls.	-20°C until used
4	ATP Enumeration Reagent (ATP-ER)*	-20°C in the dark until used
5	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
6	Sterile, 96-well plates for cell culture	Can be kept with other kit components
7	Non-sterile 96-well plates for ATP standard curve determination.	Can be kept with other kit components
	Technical manual.	Available on the Preferred Cell Systems™ 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™.

This kit contains a reagent for measuring luminescence (ATP-ER) that decays with time. This reagent should 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. Safety data sheets (SDS) are included in each literature packet.

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

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

8. The ImmunoGlo™ TCP PROTOCOL

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

Performing ImmunoGlo™ TCP is a 3-step process.

Step 1 – Cell preparation.

Step 2 – Cell culture and incubation in the 96-well plate.

Step 3 – Luminescence measurement. An ATP dose response can be performed prior to sample luminescence measurements for conversion of RLUs to µM ATP.

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

STEP 1 – Cell Preparation

Although ImmunoGlo™ TCP can accommodate cell preparations of different purities, it is highly recommended to start with a mononuclear cell (MNC) fraction from the human source, that contains 10% or less red blood cells, since these can interfere with the assay. It is also recommended not to use lysis for removing red blood cells, for the same reason. Further purification of T-cells should be performed using magnetic bead separation (Miltenyi Biotech) or single cell separation by flow cytometry.

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

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™ TCP Cell Culture

- ***Please refer to Section 9 for recommendations and tips prior to beginning this stage of the procedure.***
- ***Perform all cell cultures under sterile conditions in a biosafety cabinet.***
- ***Use calibrated pipettes and sterile tips throughout.***
- ***NOTE: The number of replicate wells prepare is arbitrary. However, a minimum of 4 replicate wells/sample is recommended for statistical purposes.***

1. Remove the ImmunoGlo™ TCP Master Mix from the box and either leave the contents thaw at room temperature or in normal cold running water. Do not thaw at 37oC in an incubator or water bath.
2. The amount of ImmunoGlo™ TCP Master Mix used for each experiment will depend on the number of replicates prepared. Preferred Cell Systems™ recommends preparing 6 replicates, but 4 replicates are also possible.

Number of Replicate Wells Required	Volume of ImmunoGlo™ TCP Master Mix	Volume of Cells (10% of final volume)	Total Volume
4	0.405mL	0.045mL	0.45mL
6 (recommended)	0.585mL	0.065mL	0.65mL

3. Prepare and label 5mL sterile plastic tubes for each cell sample to be analyzed.
4. Using a calibrated pipette, preferably an electronic pipette, dispense the required amount of ImmunoGlo™ TCP 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.
6. Add the cell concentration to the ImmunoGlo™ TCP Master Mix in the tube. Adding 10% cell suspension to make up the final volume will reduce the cell concentration by 10 fold.
7. Mix the contents of each tube thoroughly using a vortex mixer.
8. Remove the sterile, clear 96-well plate from plastic covering under the hood.
9. Dispense 0.1mL of the Cell Culture Master Mix into each replicate wells. 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 T-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 (Page 22): 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 (Page 23): 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 22-23℃ 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. 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™ TCP assays can be performed in high throughput mode. If you intend to perform any part of the ImmunoGlo™ TCP 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. RECOMMENDATIONS AND TIPS PRIOR TO USING THE ImmunoGlo™ TCP KIT CELL CULTURE MIXES.

(i) **Cell Suspension**

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

(ii) **Number of Replicates Performed**

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

(iii) **Plate Configuration**

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

(iv) **96-Well Plates Provided**

The reagents have been optimized to work with the 96-well plate(s) provided in the ImmunoGlo™ TCP 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™ TCP 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 - 23Ċ.
- 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 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 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.

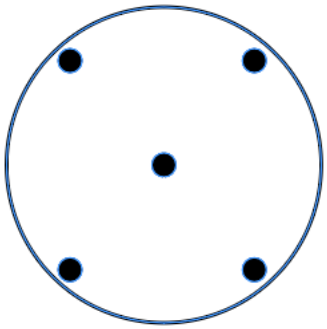
Mixing the Contents of 96-well Plate

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.

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™ Assay Measurement Assurance and Validation Parameters

If ImmunoGlo™ 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%
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™ TCP 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.

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