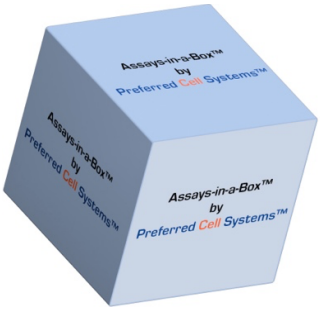

Assays-in-a-Box™
by
Preferred Cell Systems™

RenalGLO™-Tox HT

An *In Vitro* Toxicity Assay for Renal Cells

Technical Manual

(Version 02-24)

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

For *In Vitro* Research Use Only.

Not for clinical diagnostic use.

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1. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

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

RenalGLO™-Tox HT is a standardized and validated cytotoxicity / cell proliferation assay platform that detects and quantitatively measures the response of kidney cells to pharmaceutical drugs, environmental agents, and other perturbations.

All mammalian cells require chemical energy in the form of intracellular adenosine triphosphate (iATP), which is also a biochemical indicator of viability, functionality, and cytotoxicity/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 functionality is iATP using a luciferin/luciferase bioluminescence signal detection system. This concept is used RenalGLO™-Tox HT.

Cells can be obtained from different parts of the kidney, e.g. medulla, cortex, tubules. When primary cells from these sources are cultured under appropriate conditions, they will proliferate and expand for a limited period. During this time, they can be used as target cells for determining potential cytotoxicity to compounds.

Cells are not included with the RenalGLO™-Tox HT assay kit. In addition, renal cells from different sources may require different medium and culture conditions. For this reason, specific culture medium for different renal cell types is not included in the assay kit. However, RenalGLO™-Tox HT assay kits do include the ability to calibrate the luminescence plate reader required for measuring iATP, and to standardize the luminescence readout. Assay standardization, in turn, allows the establishment of measurement assurance parameters (Section 12) that indicate to the user that the assay is functioning correctly prior to measuring any samples. This also allows the investigator to compare results over time.

3. Use and Availability

RenalGLO™-Tox HT can be used for virtually any cytotoxicity application that requires the detection and measurement of renal cells in culture.

RenalGLO™-Tox HT is available as a 4 x 96-well plate kit. Upon request, RenalGLO™-Tox HT is available as bulk order and with 384-well plates for true high throughput screening of compounds.

RenalGLO™-Tox HT can be used with the following kidney sources and cell types:

- Renal medulla
- Renal cortex
- Renal tubules
- Epithelial cells from the above locations
- Renal transformed cell lines

RenalGLO™-Tox HT can be used for kidney cells derived from different species:

4. The Concept of ATP Bioluminescence Assays

RenalGLO™-Tox HT is an ATP bioluminescence assay. The fundamental concept underlying the assay is the measurement of the cell's chemical energy in the form of intracellular adenosine triphosphate (iATP). If a cell is producing iATP, it is demonstrating cellular and

mitochondrial integrity and is therefore viable. When renal cells are allowed to proliferate in culture, 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 concentration of the growth factor(s) and/or cytokine(s) used to stimulate the cells.
- The plated cell concentration.

Renal cells are usually cultured under adherent conditions. Once established, cells can proliferate for a defined period or a defined number of passages. It is during this phase that compounds, with potential renal toxicity, can be added to the culture at different doses. The cells are then further cultured, usually for 1 to 5 days. After the culture period has elapsed, a single-step addition of an ATP Enumeration Reagent 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 of a luciferin/luciferase reaction to produce bioluminescence in the form of light according to the following equation:

$$\text{ATP} + \text{Luciferin} + \text{O2} \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{LIGHT}$$

The bioluminescence emitted is detected and measured in a luminescence plate reader as relative luminescence unit (RLU). The assay can be calibrated and standardize, and controls and standards are included for this purpose. Performing an ATP standard curve and controls has the following advantages:

1. The controls calibrate the instrument and ensure that the reagents are working correctly.
2. The ATP standard curve also ensures 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.
5. The results obtained from controls and standard curve should be compared with those provided in Section 12. These are the measurement assurance parameters that allow the investigator to ensure that the assay is working correctly prior to measuring samples. When the values from the controls and ATP standard curve are within the ranges provided in Section 12, the investigator can consider the results trustworthy.

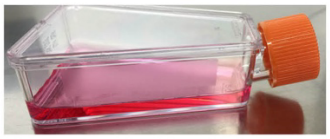
The ATP standard curve and controls need only be measured once on the day samples are to be processed. Do not use previous results from an ATP standard curve and controls performed on a different day. This will cause erroneous results.

The ATP standard curve is used to convert sample RLU results into ATP concentrations by interpolation. This procedure can often be performed automatically by the instrument software. If the software does not allow this, it will be necessary to use third-party software to perform this operation.

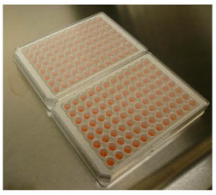
5. QuickGuide™ to RenalGLO™-Tox HT (Figure 1)

QuickGuide™ to RenalGLO™-Tox HT

STEP 1
Expand and/or passage renal cells on an adherent surface in a flask in your preferred groth medium

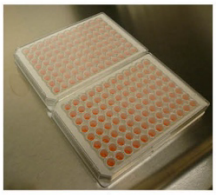


STEP 2
Once suffient cells have been obtained, remove cells from flask using trypsin/EDTA, count, adjust cell concentration. and dispense into white 96-well plate provided. Suspend cells in preferred renal medium and dispense into the supplied 96-well, sterile plate.



STEP 3
Allow cells to adhere at the required concentration.

STEP 4
Dispense test compounds and controls using a minimum of 6 doses and 6-8 replicates/dose



TIPS

- Renal cells from any source can be used in the assay.
- If possible, use self-calibrating electronic pipettes.
- Always dispense cell suspensions and reagents 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.
- Consider performing the calibration and standardization procedure since this has several advantages.
- Use sterile, adhesive foil (included) to maintain unused well sterility.

STEP 5
Culture cells for the desired amount of time at 37ºC in 5% CO_2

STEP 6
Prior to measuring potential cytotoxicity, it is recommended to calibrate the instrument and standardize the assay to provide measurement assurance.

STEP 7
Add 0.1mL of ATP-ER to each sample well, mix and measure bioluminescence after 10 min incubation in the dark

6. Kit Contents and Storage Conditions

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

Item	Component	Storage
1	Medium (IMDM) for dilution of the ATP standard.	-20°C until used
2	ATP standard.	-20°C until used
3	ATP extra high, high and low controls.	-20°C until used
4	ATP Enumeration Reagent (ATP-ER)*	-20°C in the dark until used
5	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
6	Sterile, wrapped, white-walled, clear bottom, 96-well plates for cell culture	Can be kept with other kit components
7	Non-sterile, white-walled, clear bottom, 96-well plate(s) for ATP standard curve determination.	Can be kept with other kit components
8	Technical manual. Available online at www.preferred-cell-systems.com	Included

Exact volumes of 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 cycles 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.

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. Preferred Cell Systems™ recommends 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 reagent can be obtained 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. Material safety data sheets (MSDS) are included in each literature packet.

7. Equipment, Supplies and Reagents Required, but not Provided

Equipment and Supplies

1. Laminar Flow Biohood.
2. Plate luminometer (e.g. Molecular Devices, SpectraMaxL; Berthold LB962 CentroLIA/pc).
3. Sterile plastic tubes (5ml, 10ml, 50ml).

4. Tissue culture flasks for expanding and passaging cells.
5. Single channel pipettes, preferably electronic (e.g. Viaflo pipettes for variable volumes between 1µl and 1000µl).
6. 8 or 12-channel pipette, preferably electronic (e.g. Viaflo pipettes for fixed or variable volumes between 10µl and 100µl).
7. Reservoir for 8- or 12 channel pipette.
8. Sterile pipette tips.
9. Vortex mixer.
10. Tissue culture incubator, humidified at 37°C with 5% CO_2.
11. 1.5ml plastic vials (5 for each ATP dose response).
12. Hemocytometer or electronic cell counter to determine cell concentration.
13. Flow cytometer or hemocytometer for determining viability.
14. Inverted microscope to view cell adherence and cell coverage.

Reagents

1. Preferred renal growth medium.
2. Iscove's Modified Dulbecco's Medium (IMDM).
3. 7-AAD, propidium iodide or trypan blue for viability assay.

8. The RenalGLO™-Tox HT Protocol

PLEASE READ THE FOLLOWING PROTOCOL CAREFULLY
SEE SECTION 9 BEFORE PERFORMING THE PROTOCOL

Performing RenalGLO™-Tox HT is generally a 4-step process.

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

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

Step 1 – Cell preparation.
For fresh primary, cryopreserved primary renal cells and transformed renal cell lines, they will have to be expanded and passaged to obtain sufficient cells for the assay. Please refer to the vendor's instructions on the best methods for these procedures.

Cell Viability, Cell Counting and Cell Culture Suspension Preparation

1. For dye exclusion viability methods, use trypan blue and a hemocytometer or automated method, flow cytometry 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.

2. Determine the cell concentration using either a hemocytometer or electronic cell/particle counter. **NOTE**: Do not base the working concentration on the number of viable cells as this will give erroneous results.
3. Adjust the cell suspension concentration based on the vendor's instructions in the preferred renal medium. ***Please Note*** *that the working cell concentration per ml is 100 x the final cell concentration per well.* If cells have been treated prior to cell culture, higher cell concentrations may be required.

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 only. (Control Master Mix provided).

Control 2. Vehicle-only control contains cells. (Control Master Mix provided).

Control 3. If specific additions are made to the preferred renal medium used, there should also be an internal control.

Control 4. Vehicle + Control 4.

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. When using primary cells, it is also recommended to perform 8 replicate wells/dose configured in columns across the plate.
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 Controls 1 and 2 or Controls 3 and 4 are on the same plate as the test compound(s).
5. If the test compound can be dissolved in water or aqueous medium, the vehicle controls (Controls 2 and 4) are not necessary.
6. If the test compound must 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 <u>before</u> to the addition of the Cell Master Mix (cells in the preferred renal growth medium).
8. For 384-well plates, the test compound is dispensed <u>after</u> the addition of the Cell 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 containing the Cell Master Mix.
14. If using a solvent to dissolve the test compound, the <u>final</u> 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 Cell 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 must be used. If FBS must 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. If the preferred renal medium used for the Cell Master Mix contains HEPES buffer, it might 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. RenalGLO™-Tox HT Cell Culture Preparation

- ***Perform all procedures under a laminar flow, bio-hazard hood.***
- ***Wear protective clothing, including gloves for all operations.***
- ***Use calibrated pipettes and sterile tips throughout.***

1. Renal cells need to be cultured under adherent conditions. The cells should be plated and left to adhere prior to any additional or perturbations. The sterile, white-walled, clear-bottom (adherent surface), 96-well plates are used for this purpose.
2. Adjust the working cell concentration such that the required final cell concentration will be contained in 0.1mL/well.
3. Dispense the cell master mix into each well. A minimum of 6 replicate wells/compound dose or control, should be performed. If using 6 replicates, dispense across the plate, i.e. A1-A6. If using 8 replicates, dispense in columns, A1-H1.
4. Leave the cells to adhere for 6-24h. This will vary depending on the type of renal cells being used.
5. After adherence, carefully remove the medium and replace with 0.1mL of fresh renal growth medium.
6. 11µL of test compound or vehicle should be added at this point. Dispense using a calibrated (preferably electronic, not manual) pipette and change tips between each addition.
7. Ensure that the respective controls are also included.
8. After replacing the lid, transfer the 96-well plate(s) to a humidified container (see Section 9 (v)). Transfer the humidified container to the incubator.
9. Incubate for the desired time period, usually 24-48 hours, but may range from hours to several days.
10. Cells are usually incubated at 37°C in a humidified chamber in an atmosphere containing 5% CO_2.

STEP 4. Bioluminescence Measurement

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.*
- *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 not 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.*

A. Calibrating and Standardizing the Assay

Please also refer to the video tutorial on the Preferred Cell Systems website entitled "How to Calibrate and Standardize Any ATP Bioluminescence Assay".

It is highly recommended to calibrate the luminescence plate reader and standardize the assay prior to measuring samples. This will allow a comparison with the expected measurement assurance values (**see Section 12**) that should be obtained prior to measuring samples. Use the non-sterile, 96-well white plate provided with the assay kit for this purpose.

RenalGLO™-Tox HT includes the following to calibrate and standardize the ATP bioluminescence part of the assay to measure cell proliferation occurring in the colonies.

- Non-sterile, white-walled, clear bottom, 96-well plates. Only used for calibration and standardization.
- 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 20**.

PROTOCOL 2: If the cells are expected to 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 21**.

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 new wells, and diluting each aliquot with additional medium. The replicate wells would then be reread.

C. Sample Measurement

The addition of ATP-ER for the samples 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, transfer the plate to the plate reader and incubate with the plate door closed (i.e. in the dark) for 10 minutes. Alternatively, replace the plastic cover and incubate for 10 min at room temperature in the dark. During this time, the cells will lyse, and the luminescence signal will stabilize. 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 plate luminometer may be fitted with n automatic reagent dispenser and shaker to mix the contents of the wells. Preferred Cell Systems™ does not recommend using automatic dispensers or mixers, since the contents of the wells will not mix sufficiently using this method.

E. Using a liquid handler

RenalGLO™-Tox HT can be performed in high throughput mode. If you intend to perform any part of the RenalGLO™-Tox HT 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 RenalGLO™-Tox HT.

(i) Cell Suspension

a) If cells have been treated prior to cell culture, higher cell concentrations may be required.
b) When cryopreserved cells are thawed, it is possible that cells lyse releasing genetic material that can cause a clump in the suspension. To prevent this, it is recommended to add DNase to the thawing medium.

(ii) Number of Replicates Performed

The minimum recommended number of replicates/samples is 6 to obtain statistical relevance. For primary cells, consider using 8 replicates/sample. 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

When performing 6 replicate wells/sample, these should be plated in columns across the plate. When using 8 replicate well/sample, these should be dispensed in columns.

(iv) White-Walled, Clear Bottom, 96-Well Plates Provided

The reagents have been optimized to work with the 96-well plate(s) provided in the RenalGLO™-Tox HT 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 Systems™ for further information about assembling and using humidity chambers.

(vi) Incubation Times

The incubation time may vary depending on cell source, 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.*
- *RenalGLO™-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 Systems™.*

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.
- Sufficient ATP standard, controls and ATP-ER are supplied to perform 2 standard curves and controls/assay kit. Additional ATP standards and controls can be obtained from Preferred Cell Systems™.
- 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 2-8Ċ, in the dark, for 48h or is stable at -20^{o}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

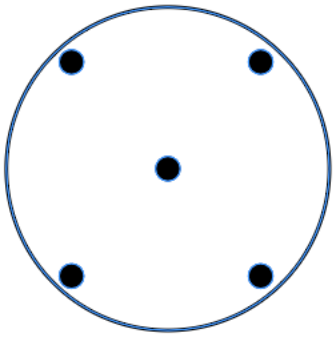
Please also refer to the video tutorial on the Preferred Cell Systems website entitled “How to Calibrate and Standardize Any ATP Bioluminescence Assay”.

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 should be downloaded with this manual, prior to starting the assay.

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

If RenalGLO™-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 Table 1. 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 4, contact Preferred Cell Systems for help.

Table 1: 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
PLEASE NOTE that values can differ slightly when using 96-well plates with a clear bottom.

Samples Values:

- Lowest ATP value indicating unsustainable cell proliferation: 0.01 - 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 IMDM 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

RenalGLO™-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 been 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 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 must 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: orders@preferred-cell-systems.com

Order online at preferred-cell-systems.com

Technical Support

Tel: (719) 264-6251

Email: info@preferred-cell-systems.com

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Colorado Springs, CO 80907

U.S.A.

Website: www.preferred-cell-systems.com

Preferred Cell Systems™

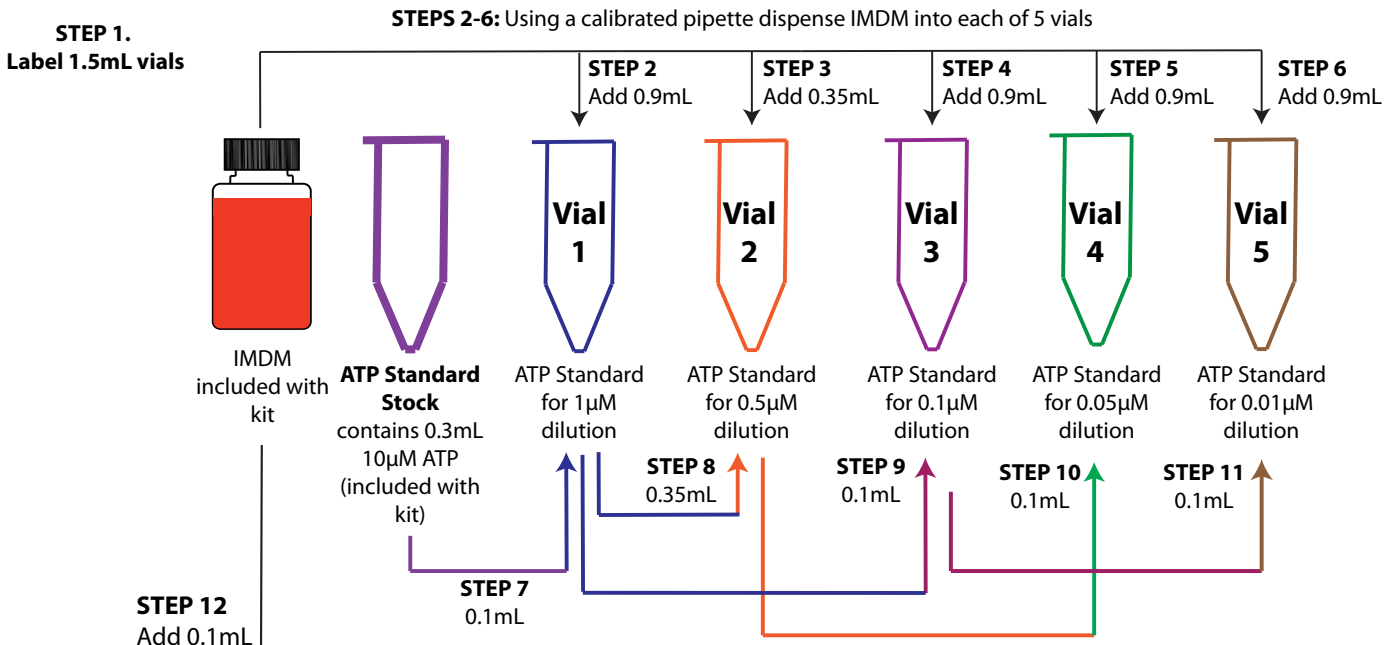
Calibration and Standardization Protocol of an ATP Bioluminescence Assay

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)

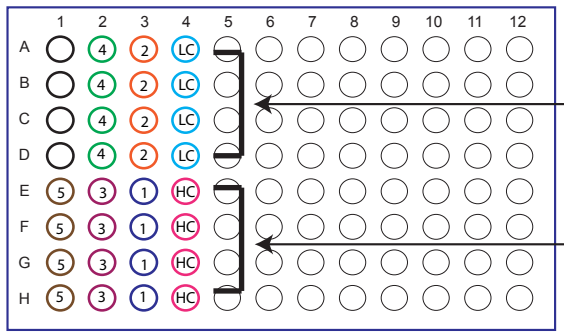
TIPS

> 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

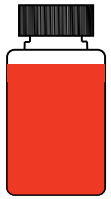
Calibration and Standardization Protocol of an ATP Bioluminescence Assay

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



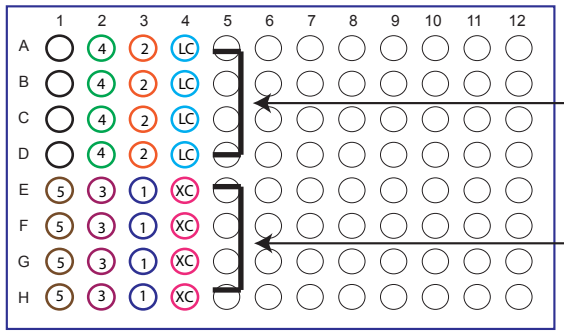
IMDM included with kit

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