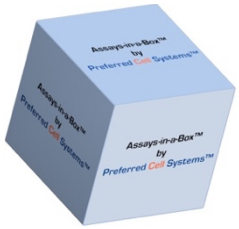

Assays-in-a-Box™
by
Preferred Cell Systems™

The Keystone Assay™

A 1-Day In Vitro Quality Assay for Hematopoietic Cellular Therapy Products

Technical Manual

(Version 11-25)

Please read this manual 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. *The Keystone Assay™ is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)*
2. *The Keystone Assay™ 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

In 1966, Robert Paine observed that the ecosystem declined in intertidal pools as the number of predators in those pools decreased. His research led to the theory of a single species in an ecosystem that exerted a down influence to prevent lower species from monopolizing critical resources. This single species he designed as the “keystone” species. This theory has been supported by numerous publications since Paine’s seminal publication in 1969.

After 3 years of research, the scientists at Preferred Cell Systems™ identified a rare cell population that could be used to indicate whether a human bone marrow, cord blood or peripheral blood sample demonstrates high or low quality. The term “quality” is defined as a quantitative measure of cell proliferation at a specific cell density. The cell population demonstrating this “quality” could be identified within 24hrs of culture and has been designated the Keystone Cell Population™ since, in the absence of this population, lympho-hematopoiesis would decline dramatically. The 24hr assay developed by Preferred Cell Systems™ to measure this population is now The Keystone Assay.

The ability to measure the quality of the rare, lympho-hematopoietic Keystone Cell Population™ is dependent upon using the most sensitive signal detection system available. Cell suspensions from a mononuclear cell (MNC) fractionated sample are cultured for 24 hours in a proprietary Keystone Reagent™ and the results compared to the same sample cultured in a Keystone Control Reagent™. After 24 hours incubation, a luminescence plate reader is calibrated and The Keystone Assay™ standardized prior to measuring the intracellular adenosine triphosphate (iATP), which acts as a limiting substrate for a luciferin-luciferase reaction. This produces bioluminescence, which is measured in a luminescence plate reader. All of the culture and measuring reagents, as well as the 96-well plate, are included in the assay kit.

The ability of the Keystone Cell Population™ to initiate proliferation within 24 hours is an indication not only of its presence in the sample, but also of whether the sample will exhibit the necessary characteristics of a high quality cellular therapy product.

3. Benefits, Use and Availability

There are multiple benefits of using The Keystone Assay™ over other cellular therapy product test. These include, but are not limited to:

- Simple, easy-to-use, 1-day, quantitative functional assay.
- The kit comes complete with everything needed to perform the assay. Just prepare and add cells.
- No colony counting and far less expensive instrumentation, allowing anyone in the laboratory to perform the assay.
- Requires only a very small sample of fractionated cells (see below).
- Can be used for cord blood, bone marrow and peripheral blood.
- Incorporates the most sensitive readout technology, presently available, namely ATP bioluminescence.
- Exceptionally high precision, reliability and reproducibility.
- Fully standardized and validated.
- Incorporates proficiency testing technology and measurement assurance parameters to indicate that the assay is functioning correctly and that the results are trustworthy.
- Can be used in high-throughput mode for large numbers of samples using either 96- or 384-well plates.

The Keystone Assay™ can be used for the following human tissues:

- Bone marrow
- Peripheral blood

- Umbilical cord blood

The Keystone Assay™ is only available for testing human lympho-hematopoietic cells.

4. The Concept of ATP Bioluminescence Assays

The Keystone Assay™ relies on the ability to measure intracellular ATP concentrations. 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 the Keystone Population™ is stimulated with the proprietary Keystone Reagent™, the iATP concentration begins to increase with 24 hours in culture.

After culture, a single-step addition of an 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 of a luciferin/luciferase reaction to produce bioluminescence in the form of light according to the following equation:

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

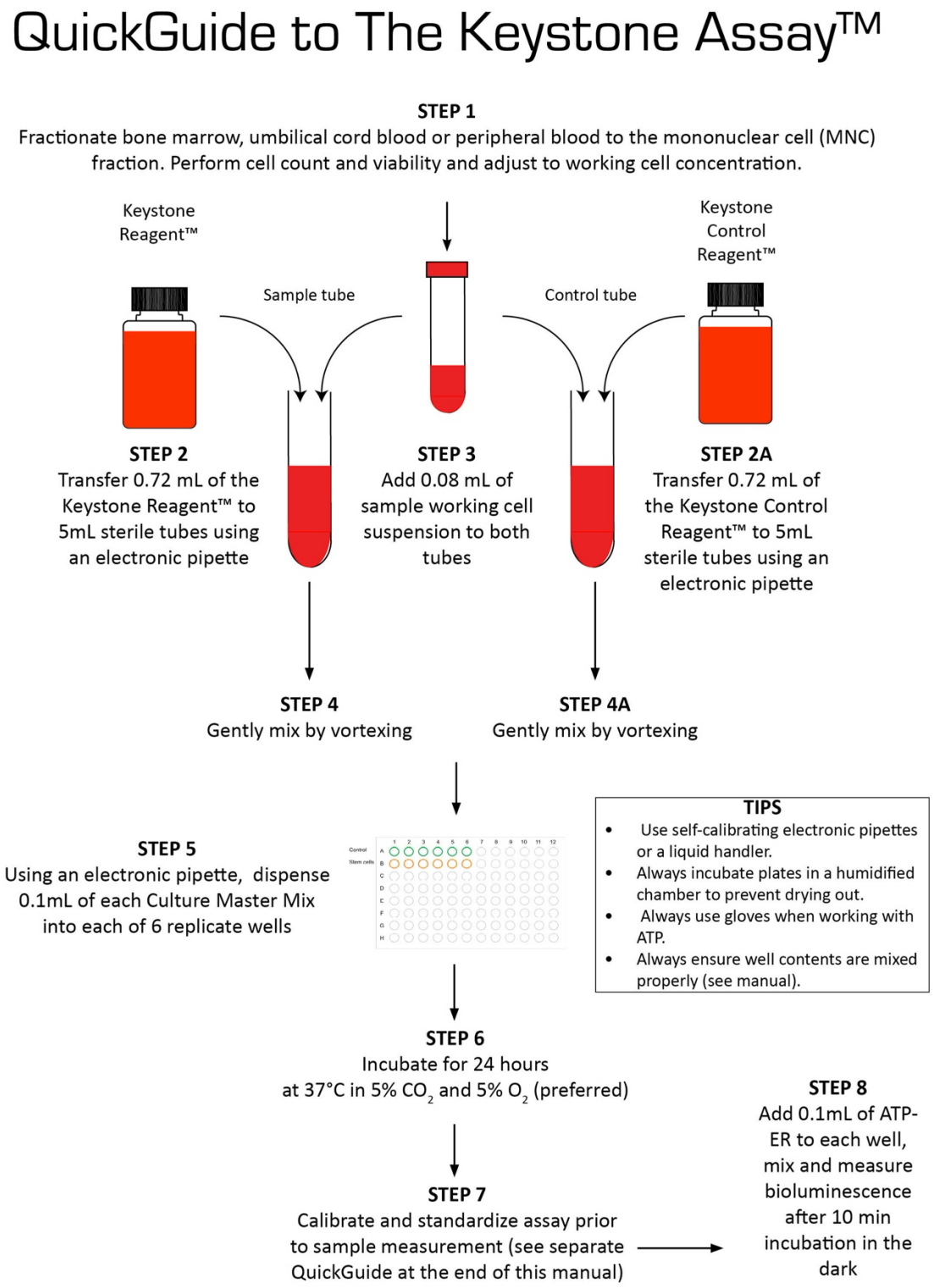
The bioluminescence emitted is detected and measured in a luminescence plate reader as relative luminescence units (RLU). The instrument is calibrated and the assay standardized using ATP controls and standards that are included for this purpose. Performing an ATP standard curve and controls has the following advantages:

1. The controls calibrate the instrument and also 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 the 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 has performed a proficiency test and may also 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 The Keystone Assay™ (Figure 1)



6. Kit Contents and Storage Conditions

The Keystone Assay™ contains 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	Amount	Storage
1	Keystone Reagent™	7.5mL (for 8 samples)	-20°C until used
2	Keystone Control Reagent™	7.5mL (for 8 controls)	-20°C until used
3	Medium (IMDM) for dilution of the ATP standard.	3 x 14mL	-20°C until used
4	ATP standard.	3 vials of 325uL	-20°C until used
5	High and low ATP controls	9 sets of 2 vials for high and low controls	-20°C until used
6	ATP Enumeration Reagent (ATP-ER)*	3 x 15mL	-20°C in the dark until used
7	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	1	Can be kept with other kit components
8	Sterile, wrapped, 96-well plates for cell culture	1	Can be kept with other kit components
9	Non-sterile 96-well plate(s) for ATP standard curve determination.	3	Can be kept with other kit components
10	Technical manual	Included with kit. Also available for download on The Keystone Assay™ webpage	

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. Berthold LB962 CentroLIA/pc; Molecular Devices, SpectraMaxL).
3. Sterile plastic tubes (5ml, 10ml, 50ml).
4. Single channel pipettes, preferably electronic (e.g. Integra Viaflo electronic pipettes for variable volumes between 1µl and 1000µl).
5. 8 or 12-channel pipette, preferably electronic (e.g. Integra Viaflo electronic 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.

Reagents

1. HemoGro™ Hematopoietic Growth Medium for cell suspensions and dilutions (Preferred Cell Systems™)
2. Iscove's Modified Dulbecco's Medium (IMDM)
3. Density-gradient medium (e.g. NycoPrep 1.077, Axis-Shield).
4. 7-AAD, propidium iodide or trypan blue for viability assay.

8. The Keystone Assay™ Protocol

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

Performing The Keystone Assay™ is a 3-step process.

Step 1 – Cell preparation.
Step 2A – Setup of The Keystone Assay™: 1-day methodology.
Step 2B – Setup of The Keystone Assay™: 3-day methodology.
Step 3 – Luminescence measurement. An ATP dose response is required prior to sample luminescence measurements with conversion of RLUs to µM ATP.

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

STEP 1 – Cell Preparation

IMPORTANT. The Keystone Assay™ should not be used with a red blood cell reduced, total nucleated cell (TNC) fraction. This is often used for other human bone marrow and umbilical cord blood assays, but cannot be used for The Keystone Assay™. This is because (a), the rare, primitive Keystone Cell Population™ would be diluted so that it would not be detectable, (b) quantitation of the

Keystone Cell Population™ would be underestimated and, (c) high red blood cell concentrations and other cells would interfere with the ATP readout resulting in a false, high ATP readout.

1. The mononuclear cell (MNC) fraction is the preparation of choice for all human tissues. This fraction can be prepared by density gradient centrifugation.
2. Further purification of the MNC fraction using CD133, but not CD34, can also be used.

Cell Viability, Cell Counting and Cell Culture Suspension Preparation

1. For dye exclusion viability, use flow cytometry and 7-aminoactinomycin D (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.
   The viability, using dye exclusion, of 85% or greater should be obtained. It is not recommended 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 TNC count or the number of viable cells as this will give erroneous results.
3. Adjust the cell suspension concentration to 500,000 (0.5×10^6) cells/mL. This will be the working cell concentration and is 100 fold greater than the final concentration in the well. The working cell concentration should not be greater than 750,000 cells/mL.
4. Prepare all cell suspensions and dilutions in either HemoGro™ or IMDM. The working cell concentration will be added at 10% of The Keystone Assay™ cell culture master mix.

STEP 2. Setup of The Keystone Assay™

- *The Keystone Assay™ Reagent is supplied complete and ready-to-use.*
- *The Keystone Assay™ Control Reagent is supplied complete and ready-to-use.*
- *Perform all procedures under a laminar flow, bio-hazard hood.*
- *Wear protective clothing, including gloves for all operations.*

The 96-well, individually wrapped, sterile plate included with the kit can accommodate the following assay configurations:

1. 8 single sample and control 1- day assays
2. 5 single sample and control 1-day assays plus 1 sample and control 3-day assay.
3. 2 single sample and control 1-day assays plus 2 sample and control 3-day assays.

STEP 2A. The Keystone Assay™: 1-Day Methodology

1. Remove The Keystone Assay™ Reagent and Control Reagent from the freezer and thaw either at room temperature or in a beaker of cold water. Do not thaw in a 37°C water bather or incubator.
2. Label one 5mL tube for one sample and another 5mL tube for its control. Repeat for all samples to be tested and the respective controls.
3. Using a calibrated pipette, preferably an electronic pipette with repeat function, dispense 0.72mL of The Keystone Assay™ Reagent into the sample tubes.
4. Now dispense 0.72mL of The Keystone Assay™ Control Reagent into the respective control tubes.
5. Dispense 0.08mL of the sample working concentration cell suspension into the sample tube and 0.08mL of the same suspension into the control tube. The total volume of each tube will be 0.80mL. This procedure will reduce the cell suspension concentration 10 fold. The cell concentration will be 50,000 cells/tube.
6. Mix the contents gently on a vortex mixer. Do not cause bubbles.
7. Under the hood, remove the sterile, wrapped, 96-well plate from the assay kit box and place the lid to one side.
8. Using a calibrated pipette, preferably an electronic pipette with repeat function, dispense 0.1mL into each of 6 replicate wells of the 96-well plate. Starting at position A1, dispense the contents of the sample tube into wells A1 to A6.

9. Now repeat the sample procedure for the control culture master mix by dispensing 0.1mL into wells B1 to B6.
10. Repeat the process for each sample and its respective control.
11. Replace the lid of the 96-well plate.
12. Transfer the plate to a humidified container (see Section 9) and then to the incubator. The incubator should be fully humidified at 37°C and gassed with 5% CO_2 and, if possible, 5% O_2. This reduced oxygen tension improves plating efficiency by reducing oxygen toxicity cause by the producing of free radicals.
13. Incubate for exactly 24 hours.
14. Proceed to Step 3.

Step 2B. Setup of The Keystone Assay™: 3-Day Methodology

The Keystone Assay™ can be extended to 3 day, if required. In some cases, the statistical difference between the sample and the control after 24 hours incubation may not be apparent. By extending the incubation time to 3 days, a quantitative and statistical difference between the sample and the control should be demonstrated. If a difference, even after 3 days incubation, is not shown, then the sample can be considered to have low quality potential and should not be used.

To perform a 3-day Keystone Assay™, the culture is setup is setup in a slightly modified manner.

1. Follow steps 1 and 2 for the 1-day assay setup.
2. Using a calibrated pipette, preferably an electronic pipette with repeat function, dispense 1.8mL of The Keystone Assay™ Reagent into the sample tube.
3. Now dispense 1.8mL of The Keystone Assay™ Control Reagent into the respective control tube.
4. Dispense 0.2mL of the sample working concentration cell suspension into the sample tube and 0.2mL of the same suspension into the control tube. The total volume in each tube will be 2.0mL. As with the 1-day assay, this procedure will reduce the cell suspension concentration 10 fold. The cell concentration will be 50,000 cells/tube.
5. Mix the contents gently on a vortex mixer. Do not cause bubbles.
6. Under the hood, remove the sterile, wrapped, 96-well plate from the assay kit box and place the lid to one side.
7. Using a calibrated pipette, preferably an electronic pipette with repeat function, dispense 0.1mL of the sample culture master mix into each of 6 replicate wells of the 96-well plate, starting at position A1 and ending in A6.
8. Now dispense 0.1mL of the same sample culture master mix into wells C1 to C6 and E1 to E6.
9. Now repeat the sample procedure for the control culture master mix by dispensing 0.1mL first into wells B1 to B6, then into wells D1 to D6 and finally F1 to F6.
10. Replace the lid of the 96-well plate.
11. Transfer the plate to a humidified container (see Section 9) and then to the incubator. The incubator should be fully humidified at 37°C and gassed with 5% CO_2 and, if possible, 5% O_2. This reduced oxygen tension improves plating efficiency by reducing oxygen toxicity cause by the production of free radicals.
12. Prior to measuring the day 1, 2 and 3 samples and controls, calibrate and standardize the assay as described in Step 3.
13. After exactly 24hrs incubation, remove the lid and cover all wells of the plate with the sterile plastic foil included with the kit. Make the sure the foil adheres to all parts of the plate.
14. Using a scalpel or sharp knife, cut away and remove the foil from wells A1 to B6. Proceed to Step 3.
15. On day 2 of culture, cut away the foil from wells C1 to D6. Proceed again to Step 3.
16. On day 3 of culture, cut away the foil from wells E1 to F6. Proceed to Step 3.
17. Leave the sterile foil on the plate covering the unused wells. This will maintain sterility of the wells until they are used.

PLEASE NOTE that it is necessary to calibrate and standardize the assay on each of the 3 days as described in Step 3 below. Failure to do so will result in inaccurate measurements and false results.

STEP 3 – 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), ATP controls and ATP standard 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 37°C incubator.*
- *Do not use ATP standard curves performed on previous days or for previous experiments or studies 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

The Keystone Assay™ is standardized and the instrument calibrated using the ATP standard and controls, respectively, prior to measuring the samples. This procedure is performed as a single step using the non-sterile, 96-well plate. The results obtained can be compared with the expected measurement assurance values shown in Section 12. The results from the standard curve and controls must be within the ranges shown in Section 12. If this is the case, proficiency has been attained and the samples and controls can be processed.

If the results are not with the ranges shown for the expected measurement assurance parameters, the sample and controls should not be processed. Repeat the procedure to ensure that the results are within the set ranges shown in Section 12.

The Keystone Assay™ kit 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 3µM: Serially diluted to produce the ATP standard curve.
- Low ATP Calibration Control.
- High ATP Calibration Control.
- Non-sterile, 96-well plate

B. Performing Calibration and Standardization

After just 24 hours in culture, high cell proliferation should not be expected. However, cells incubated with The Keystone Reagent™ should demonstrate a greater proliferation than the control cells. To ensure, this can be properly measured it is necessary to perform a calibration and assay standardization. The non-sterile, 96-well plate is used for this purpose.

To perform the calibration and assay standardization, follow the protocol provided at the end of this manual using the ATP standard curve from 0.001µM to 0.3µM. **See Section 15, Page 24**.

This is one of the most important steps of the assay, so care and accuracy are essential. Always use calibrated and, if possible, electronic pipettes, to dilute and dispense the controls and standards. **See Section 10 for Tips Prior to Measuring Bioluminescence**.

C. Sample Measurement

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

1. 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 cover foil 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 preferably a multichannel electronic pipette or a single pipette with repeat function, add 0.1mL of ATP-ER to each sample well of the first row.
4. Mix the contents as described in Section 10.
5. Using new tips, repeat this procedure for each well of the control row.
6. When ATP-ER has been added to all wells, replace the plastic cover and incubate for 10 min at room temperature in the dark to lyse the cells and stabilize the luminescence signal. Incubate the plate in the reader for the last 2 min to stabilize the plate. Alternatively, plate the plate in the luminescence plate reader draw, without the lid, for 10 minutes and then read the plate immediately.
7. Unused ATP-ER may be returned to the bottle and refrozen. See section 10 for ATP reagent storage conditions and stability.

D. Plate luminometer with automatic dispenser

Do not use the luminometer automatic dispenser and shaker. The contents of the well will not be mixed sufficiently and will lead to erroneous results.

E. Using a liquid handler

The Keystone Assay™ can be performed using a liquid handler and has high throughput capability. If it is intended to perform any part of the assay procedure using a liquid handler, please contact Preferred Cell Systems™ for information on setting up the instrument. Extra volumes of reagent and ATP-ER are required when using a liquid handler.

9. Recommendations and Tips Prior To Using The Keystone Assay™.

(i) Cell Suspension

a) A mononuclear cell suspension (MNC) is required for the assay. Using TNC or red blood cell reduced sample preparation will not provide the sensitivity and accuracy required for the assay.

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.

(ii) Number of Replicates Performed

Preferred Cell Systems™ recommends performing 6 replicate wells/sample to obtain good statistical relevance. 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 cultures should be plated in rows across the plate.

(iv) 96-Well Plates Provided

The reagents have been optimized to work with the 96-well plate(s) provided in The Keystone Assay™ 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 maintain 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 Keystone™ Assay has been designed to obtain results within 24 hours of cell culture. If required, the incubation time can be extended to up to 3 days (see Section 8, Step 2B).

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.*
- *Every time an assay is performed, calibration and standardization MUST also 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.*
- *The Keystone Assay™ 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 - 23℃.
- Sufficient ATP standard, controls and ATP-ER are supplied to perform up to 3 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 2-8℃, in the dark, for 48h or is stable at -20°C for 20 weeks.

Reconstitution of Lyophilized Monitoring Reagent (may be included on bulk orders)

- 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 up to 3 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 + 32 (background, ATP dose response wells and ATP controls)).

ATP Standard Curve

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

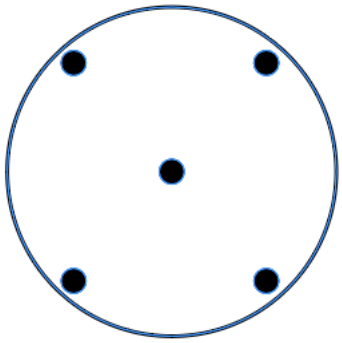
To help keep the plate(s) sterile, adhesive, air permeable, sterile films are provided so that the part of the plate that is not being used can be covered and kept sterile until required. If using the adhesive film provided, the plate cover should be removed in a laminar air-flow hood and replaced with 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 2 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

The readout from all luminescence plate readers or luminometers is Relative Luminescence Units (RLU). The term "relative" is used because luminometers from different manufacturers produce different RLU ranges. The RLU range may be from 0-100 for one instrument and 1-1,000,000 for another. A RLU value is a non-standardized unit of measurement. However, to compare results it is necessary to standardize the assay.

A. For Luminometers with Software Analysis Capabilities

Step 1. Luminescence Plate Reader Setup

Multimode instruments, i.e. those that can detect absorbance, fluorescence and luminescence, often need to be manually set for both the integration time and the "gain". Dedicated instruments, i.e. those that only detect luminescence, usually only have to be setup for the "integration time". If the "gain" has to be set, the instruction manual will explain how the correct "gain" is established. Once the "integration time" and "gain" are set, they should not be changed.

a. First set the integration time to 1 second.
b. Next, set the "gain". This must be determined empirically and is best performed when the ATP standard curve is measured. The gain should be adjusted so that the percent coefficient of variation (%CV) for the mean of the replicates is the lowest value. This value should be about 5% or less.
c. The measurement temperature of the instrument should be set to between 22ºC and 24ºC or turned off.
d. Do not use plate shaking or the injectors if the instrument has this capability.

Step 2. Software Setup

The luminometer is usually controlled by software installed on a computer using a serial or USB interface cable. The software for some luminometers comes with extensive analysis capabilities. This allows all the calculations to be programmed and performed by the luminometer software. If the software does not include analysis capabilities, the results are usually exported directly to a Microsoft Excel file for calculation and analysis.

Before using any luminometer, ensure that you are familiar with the software that controls the instrument. For luminometer software that has analysis capabilities, setting up the software properly prior to any measurements can save considerable time and produce an optimized report. It may be necessary to contact the instrument manufacturer to determine whether the software can provide the information below and whether it can perform the necessary calculations so that the procedure can be automated.

If using luminometer software that allows tabulation and calculation of results, setup as follows:

First Set of Measurements - Background

The first measurement to be performed will be to detect background (Bkg) luminescence in wells A1 – D1. Setup the software to produce the following results:

1. Well numbers
2. RLU/well
3. Mean RLU
4. RLU Standard Deviation (St. Dev)
5. RLU Percent Coefficient of Variation (%CV)

Second Set of Measurements – ATP Standard Curve and Controls

The second set of measurements to be performed will be the ATP standard curve. Setup the software to give the following information:

Group or sample designation

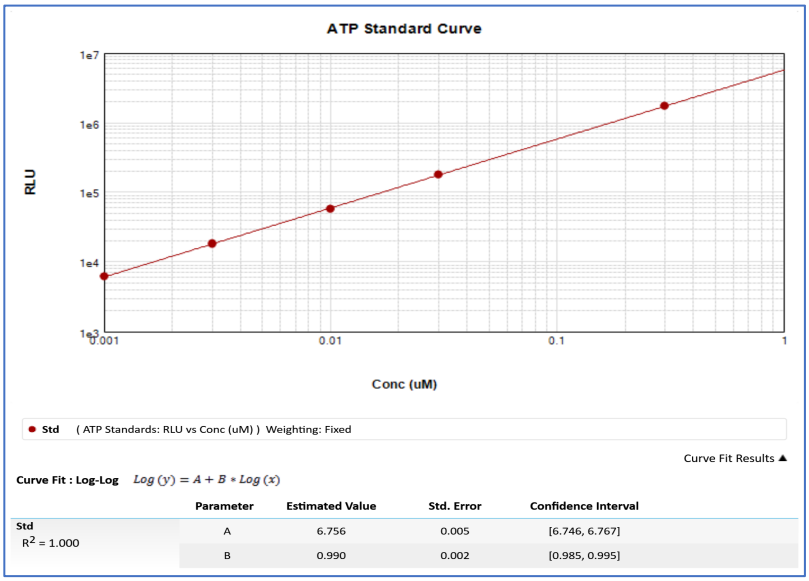
1. Well numbers
2. Expected ATP standard dose response values
3. RLU/well
4. Predicted (calculated) ATP concentration/well obtained by interpolating the RLU values from the ATP standard curve into ATP concentrations actually obtained. This should be performed automatically by the luminometer software. This is actually a back calculation of the ATP doses used to generate curve. The calculated ATP concentrations should correspond to the expected ATP values (see Table 1).
5. Mean predicted ATP
6. Standard deviation of mean predicted ATP
7. %CV of mean predicted ATP.

The software should be capable of performing a log-log linear regression curve fit according to the equation:

$$\log Y = A + B * \log X$$

where A is the Y-intercept and B is the slope of the dose response curve. A typical ATP standard curve should be similar to that shown in Figure 3

Figure 3 – Log-Log Linear Regression Curve Fit



Do not use the equation Y = A + B*X as this will normally produce negative values for the lowest ATP dose. In addition, converting the X- and Y-axes to log is not equivalent to the log-log linear regression curve fit above. The goodness of fit (r^2), Y-intercept and slope of the log-log linear regression curve fit should be similar to the results shown in Table 1 to continue with the third set of measurements, namely those of the samples.

Third Set of Measurements - Samples

Only when the goodness of fit, Y-intercept and slope are within the ranges shown in Table 1 should the samples be measured using the software setup to perform the following:

1. Group or sample designation
2. Sample number
3. Well number
4. RLU/well
5. ATP values/well (calculated from the ATP standard dose response curve)
6. Calculated mean ATP values
7. Standard deviation of calculated ATP values
8. % CV of calculated ATP values.

B. For Luminometers without Software Analysis Capabilities

The luminescence plate reader may not come with built-in analysis software. Instead, the raw data will probably be exported directly to Microsoft Excel. If this is the case, then Excel has functions to perform the necessary calculations for interpolating RLU values into ATP concentrations using the ATP standard curve data. The basic Excell procedure is as follows:

1. Column 1: Make a column for the calculated ATP concentrations used for the ATP standard curve.
2. Column 2: Copy the RLU values for the standard curve.
3. Column 3. Transform the RLU values into log RLU values using the LOG function.
4. Column 4. Transform the ATP values in column 1 into log ATP values.
5. Column 5. Using the Excel TREND function, perform a Trend analysis for the log RLU values in Column 3.
6. Column 6. Transform the log values back into actual values using the Excel ANTILOG function.
7. Column 7. Perform a TREND function for the log ATP values.
8. Column 8. Transform the log trend ATP values back into actual ATP values using the Excel ANTILOG function.
9. Column 9. Copy the sample RLU values.
10. Column 10. Transform the sample RLU values into LOG RLU values.
11. Column 11. Using the Excel TREND function, perform a trend analysis for the sample.
12. Column 12. Convert the calculated sample values back into ATP concentrations.

C. Using Third-Party Software After Export to Microsoft Excel

Instead of using the analysis capabilities of Microsoft Excel, the raw data exported to an Excel file can be copied into third-party software that can perform a log-log linear regression analysis. The following software can perform this function:

- GraphPad Prism
- TableCurve 2D from Systat Software, Inc
- OriginLab from Origin Software

For technical assistance, please contact Preferred Cell Systems™.

12. The Keystone Assay™: Measurement Assurance, Proficiency Testing and Validation Parameters

Calibrating the instrument and standardizing the assay are integral parts of The Keystone Assay™. As described in the document provided in Section 11, the ATP standard curve must be a log-log transform of both the RLU values and ATP concentrations obtained. Simply converting the X and Y axes to log axes will not produce a linear, straight line, graph from which RLU values for samples can be converted to standardized ATP concentrations.

When the ATP standard curve and controls have been performed correctly, the slope and correlation coefficient (R) of the ATP standard curve parameters should lie within the measurement assurance parameters shown in Table 1. If this is the case, then the User has also performed a successful proficiency test.

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 1, contact Preferred Cell Systems for help.

By obtaining acceptable ATP standard curve and control data:

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.

Table 1: ATP Controls and Standard Curve Measurement Assurance Parameters

Expected Parameter	Observed Value(*)	Mean ± SD(**)	Min / Max	%CV(***)
0.001µM ATP	0.001µM ATP	0	0	0%
0.003µM ATP	0.003µM ATP	0	0	0
0.01µM ATP	0.01µM ATP	0.0003	0.009 – 0.01	2.937%
0.03µM ATP	0.031µM ATP	0.001	0.03 – 0.032	2.175%
0.3µM ATP	0.299µM ATP	0.003	0.29 – 0.302	1.038%
Y-Intercept	6.699	0.062	6.532 – 6.759	0.924%
Slope	0.977	0.017	0.934 – 0.993	1.740%
r^2 goodness of fit)	1	-	0.999 - 1	-
R (correlation coefficient)	1	-	-	-
Low control, (0.0025µM ATP	0.002	0.0004	0.002 – 0.003	18.682
High control 0.25µM ATP	0.255	0.0247	0.228 – 0.34	9.698

(*) All results obtained from 16 experiments performed on different days by 2 different users.
(**) 1 x standard deviation.
(**) For the Lower level of Quantification (LLQ) a %CV of 20% or less is allowed under FDA Bioanalytical Method Validation Guidelines

Assay Validation Parameters
The Keystone Assay™ exhibits the following validation parameters:

- Assay ATP linearity => 3 logs
- Assay ATP sensitivity: < 0.001µM
- Accuracy (% correct outcomes): ~95%
- Precision (Reliability and Reproducibility) =< 15%. At lower limit of quantification (LLOQ): 20%
- Robustness (intra- and inter-laboratory comparison): ~95%.

13. Examples and Interpretation of Results

Normally, it would only be necessary to perform a single, 24hr time-point measurement to determine the quality of a cell therapy sample. The results shown below depict typical results produced over a 3-day incubation period.

Figure 4 shows a comparison between the Keystone Cell Population™ used as a control or stimulated with the Keystone Cell Reagent. At 24hr incubation there should be a statistical difference between the two points. If measured over 3 days, the cells of the Keystone Cell Population™ should increase in ATP concentration and, therefore, the slope of the time course. In contrast, the slope of the time course for the control will usually decrease slightly.

Figure 4

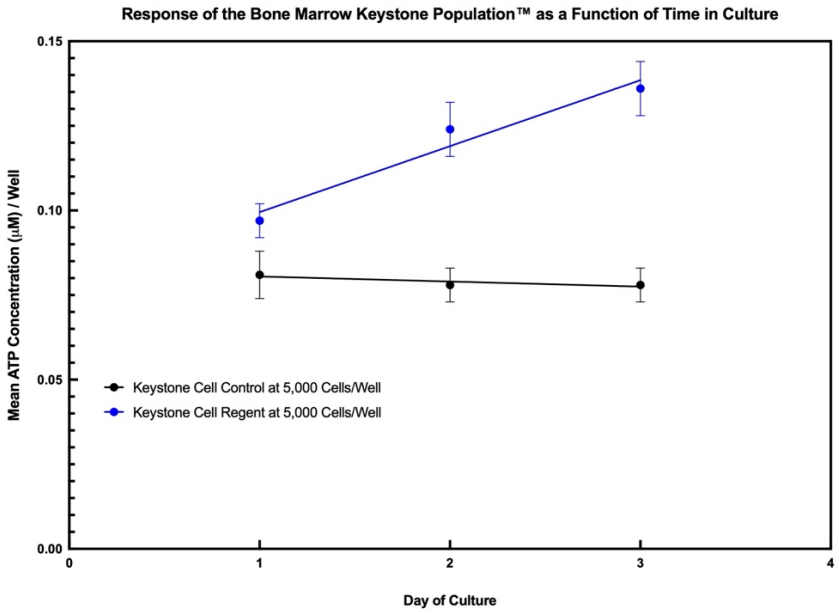


Figure 5 shows an example of two bone marrow samples, one of higher quality than the other. Notice the difference in slope of the two samples. However, in this case, a simple T-test indicates that there is no statistical difference between the day 1 ATP concentrations. This would indicate the need to perform a 3-day assay to ensure that the sample can indeed exhibit satisfactory proliferation.

Figure 5

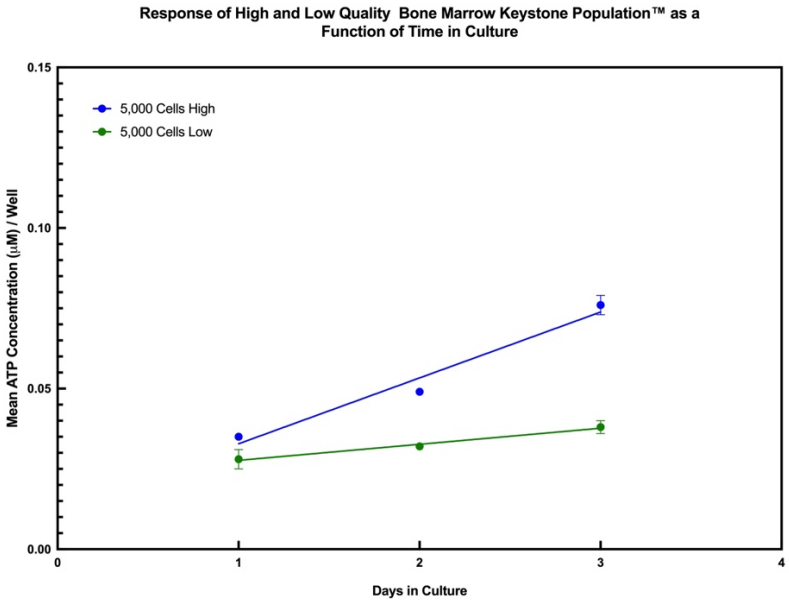


Figure 6 is similar to Figure 4, but using an umbilical cord blood sample. Notice that on Day 1, there is a statistical difference between the control and the Keystone Cell Reagent - stimulated population. In addition, whereas the slope of the control decreases, the slope of the Keystone Cell Population™ increases, indicating the potential of the cells to proliferate and, therefore, to demonstrate high quality. The greater the slope of the Keystone Cell Population™, the greater the quality and potency of the sample.

Figure 6

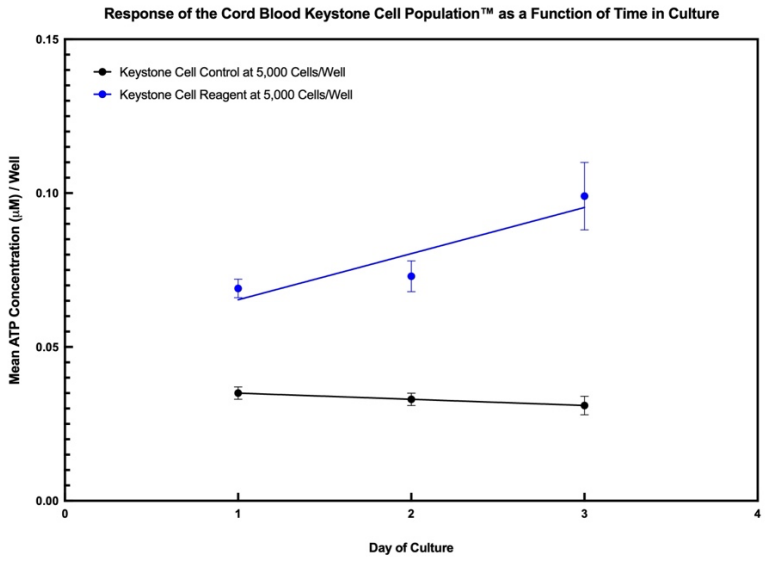
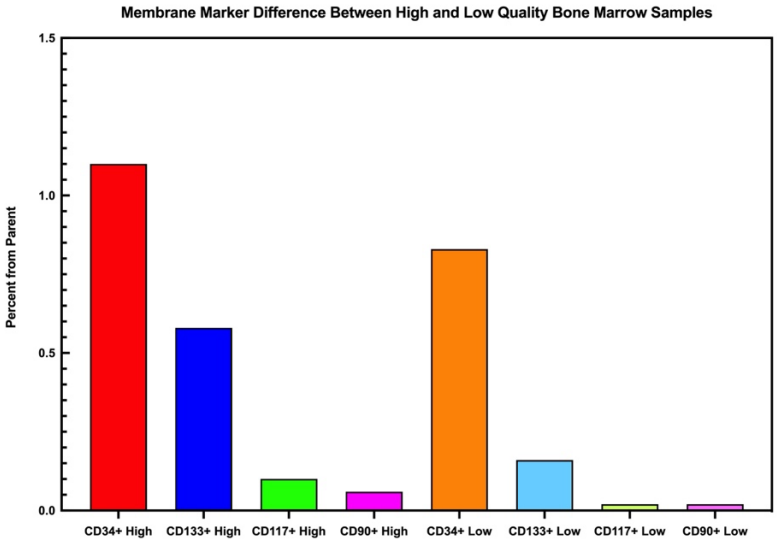


Figure 7 shows the flow cytometric profile of high and low bone marrow Keystone Cell Populations™. The most important membrane markers of the Keystone Cell Population™ are CD133 and CD90.

Figure 7



14. Troubleshooting

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

Results from calibration and assay kit standardization 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 =20% or less after 24hrs and 15% or less after day 2 of incubation. 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 occur, these being observed for rare, primitive cells, large variations between replicates could 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 it is necessary to determine the luminometer “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

The ATP concentration results from The Keystone Assay™ will be low. However, performing the calibration and standardization procedure prior to sample measurement can help detect problems prior to sample measurement. If low RLU values occur during the calibration and/or standardization procedure, this could be due to several 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. **NOTE:** Even though the cell viability might be 85% or higher, this does not necessarily mean that the cells will proliferate and grow. This is because a dye exclusion viability measurement does not predict metabolic viability, e.g. intracellular ATP product. It is possible to have a high dye exclusion viability, but the metabolic viability, indicating the ability to proliferate, might be very low or zero, indicating that the cells will either not proliferate or are dead, respectively.
- *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*: Due to the short duration of the assay, it is unlikely that contamination will occur. However, cells cultured in 96-well solid white plates provided with the kit cannot be viewed under a microscope. If contamination occurs it will usually be seen by the difference in color of the cultures, since the medium contains an indicator, e.g. phenol red. Contaminated cultures will usually be bright yellow in color and possibly cloudy in appearance. 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.

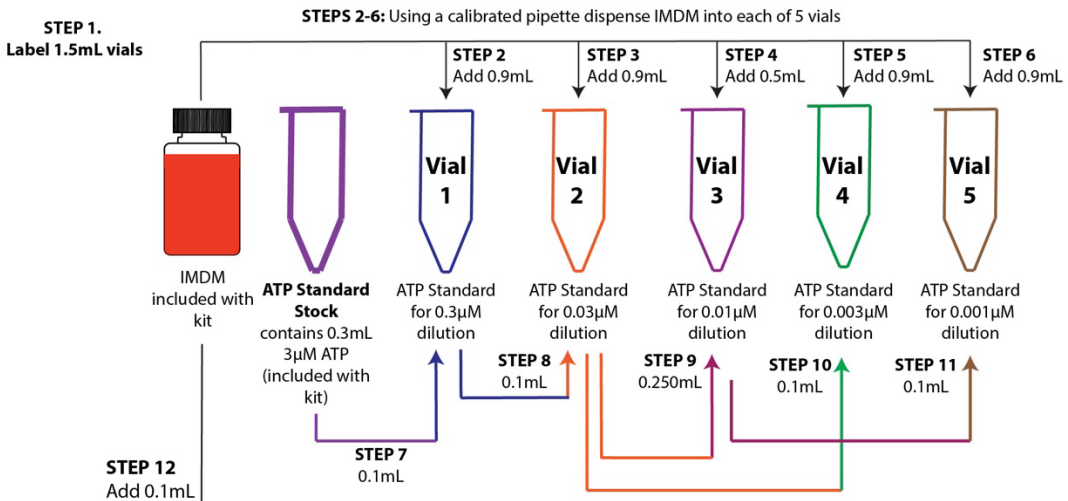
Calibration and Standardization Protocol for The Keystone Assay™

PROTOCOL: ATP Standard Curve from 0.001µM to 0.3µM

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)

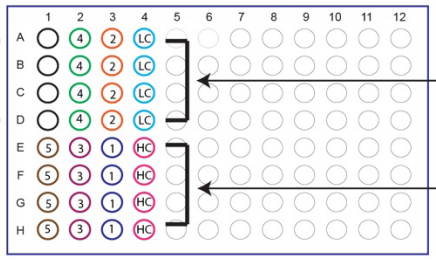
TIPS

> 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



STEP 18: LOW CONTROL (LC 0.0025µM, 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 0.25µM, included with kit)
Vortex and lightly centrifuge to remove liquid from cap
Add 0.1mL from high control to wells E4-H4

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 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

Ordering Information

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