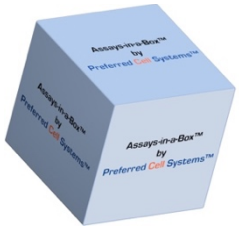

Assays-in-a-Box™
by
Preferred Cell Systems™

# HALO®

## Proliferation Assays for Stem and Progenitor Cells of the Lympho-Hematopoietic System

For Individual Populations and "Global" Assay Kits

Technical Manual

(Version 10-23)

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

HALO® or Hematopoietic Assays via Luminescence Output is a proliferation assay platform that detects and quantitatively measures stem and progenitor cells of the blood-forming (lympho-hematopoietic) system.

All mammalian cells require chemical energy in the form of intracellular adenosine triphosphate (iATP), which is also a biochemical indicator of viability, functionality and cell proliferation. The amount of iATP produced by a cell correlates directly with its functional status. The most sensitive non-radioactive readout to measure cell proliferation is iATP using a luciferin/luciferase bioluminescence signal detection system. This concept is used for HALO®.

To detect and measure stem and progenitor cells, growth factors are required to stimulate the cells in culture. The growth factor cocktail used to stimulate the cells defines which cell populations can be detected. When blood-forming stem and progenitor cells are stimulated to proliferate, the iATP concentration varies proportionately. The amount of iATP produced can be used to distinguish the primitiveness of different stem cell populations. In addition, it can also help to distinguish between stem and progenitor cells.

Besides ATP bioluminescence technology, HALO® incorporates growth factor master mixes that use Suspension Expansion Culture™ (SEC™) Technology that provide far superior accuracy, sensitivity and reliability than colony-forming unit (CFU) or colony-forming cell (CFC) assays. In addition, all HALO™ assay kits 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.

HALO® is presently available to detect multiple stem cell and progenitor cell populations from up to 9 different species. In addition, HALO® “Global” Assays are also available than can be used for to detect and measure 4-, 5- or 7-cell populations simultaneously.

## 3. Use and Availability

HALO® can be used for virtually any research application that requires the detection and measurement of stem and progenitor cells of the lympho-hematopoietic system. As such, HALO® can be used instead of the colony-forming cell (CFC) or Unit (CFU) assay. HALO® can also be used for hematopoietic cell therapy applications. However, specialized HALO® assays have been designed for this purpose. Another specialized HALO® platform is HALO®-Tox HT for bone marrow toxicity/stem cell hematotoxicity screening and testing.

HALO® can be used for the following tissues:

- Bone marrow
- Peripheral blood
- Umbilical cord blood
- Spleen
- Fetal liver
- Embryonic tissue (e.g. yolk sac)

HALO® is available for the following species:

- Human

- Non-human primate
- Horse
- Pig
- Minipig
- Sheep
- Dog
- Rat
- Mouse

HALO® Serum-Free Assays Kits are available for human, primate and mouse cells.

HALO® can be used with cells that have the following degrees of purity:

- Total nucleated cells (TNC) containing about 30% red blood cells
- Mononuclear cell (MNC) fractions
- Purified stem or progenitor cells obtained by flow cytometry or magnetic bead separation.

If possible, it is recommended to use mononuclear cells or cells of greater purity to reduce the effect of cell dilution that will underestimate the presence of primitive cells due to large numbers of cell impurities.

Suspension Expansion Culture™ (SEC™) Technology

All HALO® assays incorporate Suspension Expansion Culture (SEC) Technology. No methylcellulose is used. This has the following advantages over the traditional CFU/CFC assay methodology:

- All reagents can be dispensed using normal pipettes.
- Allows cell-cell interaction.
- Produces greater assay sensitivity.
- Shorter cell incubation times; cell proliferation is measured on the exponential part of the growth curve.
- Coefficients of variation ≤15%.

Since colonies of cells are not produced in HALO® cultures, the following equivalent cell populations can be detected using HALO®

Cell Populations Detected using HALO®

- No growth factors included. Used for background control or to add growth factors/cytokines .
- SC-HPP 1 equivalent to HPP-CFC 1
- SC-HPP 2 equivalent to CFC-HPP 2
- SC-GEMM 1 equivalent to CFC-GEMM 1
- SC-GEMM 2 equivalent to CFC-GEMM 2
- SC-GEM 1 equivalent to CFC-GEM 1
- SC-GEM 2 equivalent to CFC-GEM 2
- P-BFU 1 equivalent to BFU-E 1
- P-BFU 2 equivalent to BFU-E 2
- P-GM 1 equivalent to GM-CFC 1
- P-GM 2 equivalent to GM-CFC 2
- P-Mk 1 equivalent to Mk-CFC
- P-Tcell 1 (see also ImmunoGlo™ PCP)

- P-Tcell 2 (see also ImmunoGlo™ PCP)
- P-Tcell 3 (see also ImmunoGlo™ PCP)
- P-Bcell 1 (pre-B)
- P-Bcell 2 (human only)
- P-Bcell 3 (human only)

HALO® "Global" 4-Population Assays include reagents for:
SC-GEMM 1, P-BFU 1, P-GM 1 and P-Mk 1, plus a background control.

HALO® "Global" 5-Population Assays include reagents for:
SC-HPP 2, SC-GEMM 1, P-BFU 1, P-GM 1 and P-Mk 1, plus a background control.

HALO® "Global" 7-Populations Assays include reagents for:
SC-HPP 2, SC-GEMM 1, P-BFU 1, P-GM 1, P-Mk 1, P-Tcell and P-Bcell, plus a background control.

**PLEASE NOTE:** P-Tcell progenitor assay kits are now available with 3 different growth factor cocktails that contain co-stimulators. P-Bcell progenitor assay kits are also now available with 3 different growth factor cocktails for specific species. Please refer to the Preferred Cell Systems website for growth factor cocktails: http://preferred-cell-systems.com/HALO.php. To reliably measure T and B-cell progenitor cells in culture, it is highly recommended to purify these cells from a mononuclear cell fraction using magnetic bead separation, e.g. Miltenyi Biotec, Inc.

HALO® "Global" 7-Population Assay Kits for human cells include P-Tcell 3 and P-Bcell 2 growth factor cocktails. Assay kits for primate, canine, rat or mouse, include P-Tcell 1 and P-Bcell 1, http://preferred-cell-systems.com/HALO.php.

## 4. The Concept of ATP Bioluminescence Assays

HALO® 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 hematopoietic cells are stimulated to proliferate, in culture with growth factors and/or cytokines, 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.

Lympho-hematopoietic cells are incubated in the HALO® Master Mix provided with this kit for a specific period of time. When 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 units (RLU). The assay can be calibrated and standardized; controls and standards are included for this purpose. Performing an ATP standard curve and controls has the following advantages:

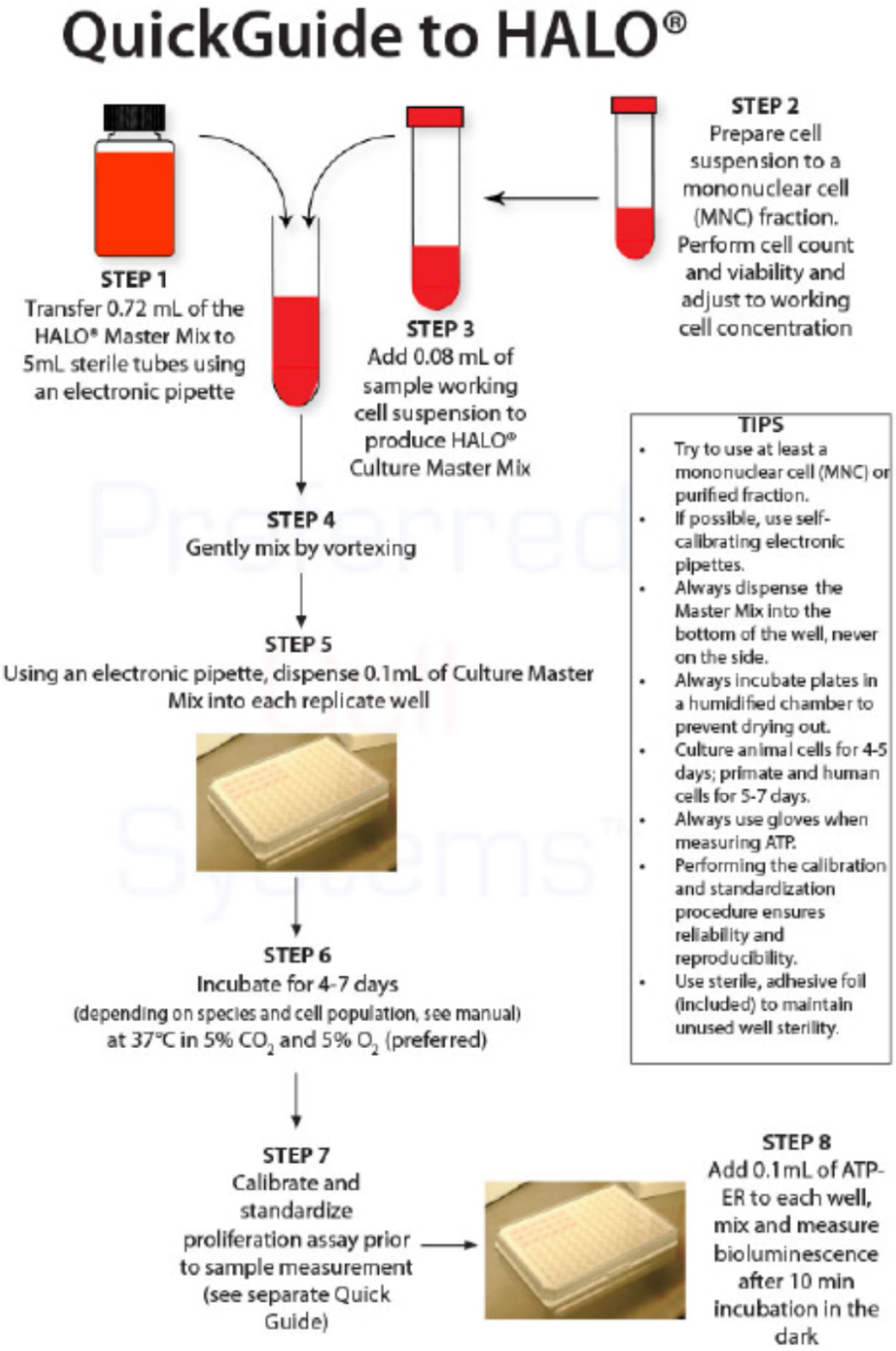
1. The controls calibrate the instrument and also ensure that the reagents are working correctly.
2. The ATP standard curve also ensures that the reagents are working correctly.
3. The ATP standard curve allows the luminometer output in Relative Luminescence Units (RLU) to be converted to standardized ATP concentrations (µM).
4. Performing the ATP standard curve allows results to be compared over time.
5. The results obtained from 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 HALO® and Multi-Population "Global" Assays (Figure 1)

*Please note that for HALO® 4-, 5- or 7-Population "Global" Assays, the same procedure is used except that each population uses a separate sterile, 96-well plate.*



## 6. Kit Contents and Storage Conditions

HALO® 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 | HALO® Master Mix for an individual cell population | -20°C until used |
| 1a | HALO® "Global" 4-Population Kit contains 4 individual Master Mixes plus a Background with no growth factor cocktail | -20°C until used |
| 1b | HALO® "Global" 5-Population Kit contains 5 individual Master Mixes plus a Background with no growth factor cocktail | -20°C until used |
| 1c | HALO® "Global" 7-Population Kit contains 8 individual Master Mixes plus a Background with no growth factor cocktail | -20°C until used |
| 2 | HALO® Growth Factor Mix - contains the growth factor cocktail formulated to support the specific cell population of interest. | -20°C until used |
| 3 | Medium (IMDM) for dilution of the ATP standard. | -20°C until used |
| 4 | ATP standard. | -20°C until used |
| 5 | ATP extra high, high and low controls. | -20°C until used |
| 6 | ATP Enumeration Reagent (ATP-ER)* | -20°C in the dark until used |
| 7 | Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile. | Can be kept with other kit components |
| 8 | Sterile, wrapped, 96-well plates for cell culture | Can be kept with other kit components |
| 8a | HALO® "Global" 4-Population kits contain 5 sterile, wrapped, 96-well plates for cell culture | Can be kept with other kit components |
| 8b | HALO® "Global" 5-Population kits contain 6 sterile, wrapped, 96-well plates for cell culture | Can be kept with other kit components |
| 8c | HALO® "Global" 7-Population kits contain 8 sterile, wrapped, 96-well plates for cell culture | Can be kept with other kit components |
| 9 | Non-sterile 96-well plate(s) for ATP standard curve determination. | Can be kept with other kit components |
| 10 | Technical manual | Can be kept with other kit components |

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. Rainin EDP pipettes for variable volumes between 1µl and 1000µl).
5. 8 or 12-channel pipette, preferably electronic (e.g. Rainin EDP pipettes for fixed or variable volumes between 10µl and 100µl).
6. Reservoir for 8- or 12 channel pipette
7. Sterile pipette tips.
8. Vortex mixer.
9. Tissue culture incubator, humidified at 37°C with 5% $CO_2$ (minimum requirement) and 5% $O_2$ (preferable).
10. 1.5ml plastic vials (5 for each ATP dose response).
11. Hemocytometer or electronic cell counter to determine cell concentration.
12. Flow cytometer or hemocytometer for determining viability.

**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.
5. LIVEGlo™ metabolic viability assay (Preferred Cell Systems™)

## 8. The HALO® Protocol

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

Performing HALO® is a 3-step process.

Step 1 – Cell preparation.
Step 2 – HALO® cell culture master mix preparation, plating and incubation in the 96-well plate.
Step 3 – Luminescence measurement. An ATP dose response is performed 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**
HALO® can be performed using tissues with the following purity:

1. Total nucleated cell (TNC) fraction usually produced by red blood cell reduction. The TNC fraction is often used for human bone marrow and umbilical cord blood. The concentration of red blood cells in this preparation may be 30% or higher. Although the TNC fraction can be used, it is NOT recommended due to (a), dilution of primitive stem and progenitor cells, (b) underestimation of primitive stem and progenitor cells, and (c) interference of high red blood cell concentrations with the ATP readout resulting in an unsatisfactory high ATP readout.
2. Mononuclear cell (MNC) fraction is the preparation of choice for human, large animals and rats. This fraction can be prepared by density gradient centrifugation.
3. Purified stem and progenitor cell populations prepared by cell sorting or magnetic bead separation. This is highly recommended when testing human, rat or mouse P-Tcell or P-Bcell progenitor cells using the individual HALO® Assay Kit or HALO® "Global" 7-Population Assay Kit. See Section 9.
4. If using assay kits K2-B3-1H, K2-B3-4HH or HALO® 7-Population "Global" for human P-Bcell progenitor cells, it is required to purify human B-cells from the MNC fraction using the Human B-Cell Isolation Kit II (Catalog #: 130-091-151). This produces a highly purified P-Bcell population, which can be induced into proliferation.

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. Use LIVEGlo™ (Preferred Cell Systems™) as a metabolic viability assay.
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 that recommended in Table 1.
   *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.

4. Prepare the total volume of cell suspension required using HemoGro™ or IMDM. The volume of the adjusted cell suspension required will be 10% of the total volume of HALO® Culture Master Mix prepared.

**TABLE 1**

Recommended Cell Doses for Different Species, Cell Types, Cell Preparations and Cell States for HALO®

| Species | Cell Type | Cell Preparation | Cell State | Working Cell Concentration Required (100 x Final Cells/Well) | Final Cell Dose / Well |
|---|---|---|---|---|---|
| Human | Bone marrow | MNC | Fresh/Frozen | 0.5-0.75 x $10^6$ | 5,000-7,500 |
| | Peripheral blood | MNC | Fresh/Frozen | 0.5-0.75 x $10^6$ | 5,000-7,500 |
| | Mobilized peripheral blood | MNC | Fresh/Frozen | 0.5-0.75 x $10^6$ | 5,000-7,500 |
| | Umbilical cord blood | MNC | Fresh/Frozen | 0.5-0.75 x $10^6$ | 5,000-7,500 |
| | Umbilical cord blood | MNC | Frozen | 0.5-0.75 x $10^6$ | 5,000-7,500 |
| | Bone marrow | $CD34^+$ | Fresh | 0.1-1 x $10^5$ | 100-1,000 |
| | Mobilized peripheral blood* | $CD34^+$ | Fresh/Frozen | 0.1-5 x $10^5$ | 100-5,000 |
| | Umbilical cord blood | $CD34^+$ | Fresh/Frozen | 0.1-5 x $10^5$ | 100-5,000 |
| | Purified B-lymphocytes from bone marrow | Purified from MNC | Fresh/Frozen | 1 x $10^6$ | 10,000 |
| Non-human primate | Bone marrow | MNC | Fresh/frozen | 0.5-0.75 x $10^6$ | 5,000-7,500 |
| | Peripheral blood | MNC | Fresh/Frozen | 0.5-0.75 x $10^6$ | 5,000-7,500 |
| Horse, Pig, Minipig, Sheep | Bone marrow | MNC | Fresh/Frozen | 0.5-0.75 x $10^6$ | 5,000-7,500 |
| | Peripheral blood | MNC | Fresh/frozen | 0.5-0.75 x $10^6$ | 5,000-7,500 |
| Dog | Bone marrow | MNC | Fresh/Frozen | 0.5-0.75 x $10^6$ | 5,000-7,500 |
| Rat | Bone Marrow | MNC | Fresh | 0.5-0.75 x $10^6$ | 5,000-7,500 |
| | Peripheral blood | MNC | Fresh | 0.5-0.75 x $10^6$ | 5,000-7,500 |
| Mouse | Bone Marrow | MNC | Fresh | 0.5-0.75 x $10^6$ | 5,000-7,500 |
| | Spleen | MNC | Fresh | 0.5-1 x $10^6$ | 5,000-10,000 |
| | Fetal liver | MNC | Fresh | 0.5-0.75 x $10^6$ | 5,000-7,500 |
| | B-lymphocytes from bone marrow | MNC | Fresh/Frozen | 1-2 x $10^6$ | 10,000 - 20,000 |

**STEP 2. HALO® Cell Culture Preparation**

- ☐ *HALO® Master Mixes are complete and ready-.to-use.*
- ☐ *Perform all procedures under a laminar flow, bio-hazzard hood.*
- ☐ *Wear protective clothing, including gloves for all operations.*

The HALO® Methodology

1. Remove the HALO® Master Mix that came with the assay kit from the freezer and thaw either at room temperature or in a beaker of cold water. Do not thaw in a 37oC water bather or incubator.
2. Label a sufficient number of 5mL tubes for the number of samples to be tested.
3. Using a calibrated pipette, preferably an electronic pipette with repeat function, dispense a volume of the Master Mix minus 10%, that will allow 0.1mL to be dispensed into the required number of replicate wells. See Table 2. Preparation of 6 replicate wells/sample is recommended for statistical relevance.

**TABLE 2**

| Number of Replicate Wells Required | Volume of HALO® Master Mix | Volume of Cells (10% of final volume) | Total Volume |
|---|---|---|---|
| 4 | 0.45mL | 0.05mL | 0.5mL |
| 6 (recommended) | 0.63mL | 0.07mL | 0.7mL |

4. Adjust the cell concentration to the working cell concentration shown in Table 1. The working cell concentration should be 100 fold higher than the final cell concentration in the well. Thus, if the final cell concentration is to be 5,000 cells/well, the working cell concentration should be 100 times 5,000 or 500,000 ($5 \times 10^5$) cells/mL.
5. Dispense the required volume of cells into each tube containing the HALO® Master Mix.
6. Mix the contents gently on a vortex mixer. Do not cause bubbles.
7. Remove a sterile, wrapped, 96-well plate from the assay kit box.
8. Using a calibrated pipette, preferably an electronic pipette with repeat function, dispense 0.1mL into each of the required number of replicate wells of the 96-well plate.
   TIP: If preparing 2, 4 or 6 replicates, dispense across the plate in rows. If preparing 8 replicates, dispense in columns across the plate. This allows for the maximum number of samples/plate.
9. After replacing the lid, transfer the 96-well plate to a humidified container (see Section 9).
10. Transfer the humidified container to the incubator. The incubator should be fully humidified at 37oC 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.
11. Incubate the cells for the time shown in Table 3.

**TABLE 3**

| Species | Cell Type | Incubation Period (days) |
|---|---|---|
| Human | Bone marrow, normal and mobilized peripheral blood, umbilical cord blood | 5 |
| Non-human primate | Bone marrow, peripheral blood | 5 |
| Horse, Pig, Minipig, Sheep | Bone marrow, peripheral blood | 4 |
| Dog, Rat | Bone marrow | 4 |
| Mouse | All | 4 |

HALO® Assay Kits for 4-, 5- or 7-Populations

- These HALO® assay kits include 4, 5 or 7 sterile, wrapped, 96-well plates, plus an extra plate that is used for background controls.
- Each plate is used for a separate cell populations. For example, for a 4-populations assay kit, each plate will include HALO™ Master Mixes for SC-GEMM, P-BFU, P-GM and P-Mk.
- Prepare separate tubes for each cell population and for each sample to be tested.
- **IMPORTANT**: For HALO® "Global" 7-Population Assay Kits. See Section 9 vii.
- In preparing the tubes, use the same HALO® Methodology as above.

**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) 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*

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.

HALO® includes the following to calibrate and standardize the ATP bioluminescence part of the assay to measure cell proliferation occurring in the colonies.

- 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 25**. Human bone marrow and cord blood and all animal bone marrow, with the exception of mouse and sometime rat, will fall into this group.

**PROTOCOL 2:** For human mobilized peripheral blood, mouse bone marrow and purified cell populations, use the low and extra high calibration controls and perform an ATP standard curve from 0.03µM to 3µM. **See Page 26**.

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

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

1. If possible, place the sample plate(s) in a humidified incubator set at 22-23℃ gassed with 5% $CO_2$ for 30min. Otherwise, allow the plate to come to room temperature for 30 min.
2. If only part of the plate has been used, transfer the plate to a bio-safety hood and remove the lid under sterile conditions. Take a sterile adhesive plate coverfoil from the kit box, remove the backing and layer it over the top of the plate. Using a sharp knife or scalpel, cut away the foil that covers the wells to be processed. The unused, empty wells will now remain sterile for the next samples. (See Section 10, Adhesive Plate Covering Film).
3. Using a multichannel pipette (8- or 12-channel depending on the plate configuration), add 0.1mL of ATP-ER to each well of the first column (A1-H1) or row (A1-12). Mix the contents as described in Section 10.
4. Repeat this procedure for each column or row using new tips.
5. When ATP-ER has been added to all wells, replace the plastic cover and incubate for 10 min at room temperature in the dark to lyse the cells and stabilize the luminescence signal. Incubate the plate in the reader for the last 2 min to stabilize the plate.
6. Unused ATP-ER may be returned to the bottle and refrozen. See section 10 for ATP reagent storage conditions and stability.

D. Using a plate luminometer with automatic dispenser

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

E. Using a liquid handler

HALO® can be performed in high throughput mode. If you intend to perform any part of the HALO® 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 HALO®.

(i) Cell Suspension

a) The preferred cell suspension is a mononuclear cell suspension (MNC).
b) Extraneous ATP, red blood cells (which have high concentrations of ATP) and hemoglobin interfere with the ATP analysis. The cell suspension must have a hematocrit of 10% or less.
c) If cells have been treated prior to cell culture, higher cell concentrations than those shown in Table 1 may be required.
d) For human, rat or mouse T- or B-progenitor cell populations, a further purification step from the MNC fraction is highly recommended to improve specificity and selectivity.

(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 HALO® 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 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.

(vii) HALO® Individual Assay Kits for Human P-Bcell and "Global" 7-Population Assay Kits that Include P-Bcell

Preferred Cell Systems™ has introduced new growth factor formulations to detect and measure the human P-Bcell population. It is highly recommended that the starting cells used to detect a human P-Bcell response are purified by magnetic bead separation from a mononuclear cell fraction.

## 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.*
- *HALO® 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 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 2-8℃, in the dark, for 48h or is stable at -20°C for 20 weeks.

**Reconstitution of Lyophilized Monitoring Reagent (if included)**

- Thaw the ATP Enumeration Reagent Buffer at room temperature, in cold running water, or at 2-8°C overnight.
- Do not use any form of heat to thaw this reagent.
- Allow the lyophilized ATP-ER substrate (brown glass bottle) to come to room temperature.
- Remove the closures from both bottles.
- Carefully pour the entire contents of the buffer bottle into the lyophilized ATP-ER substrate bottle. Swirl gently or invert slowly to mix. Do not shake.

- Allow the ATP-ER mix to reconstitute for 10 minutes at room temperature.
- Reconstituted ATP-ER is stable for 8 hours at room temperature, 48 hours at 2-8°C, or 20 weeks at -20°C.
- ATP-ER can be refrozen up to 11 cycles without significant loss of sensitivity.

**Volumes of Luminescence Kit Components Required**

- Each vial of ATP standard contains enough volume to perform one or two ATP standard dose responses.
- The amount of ATP-ER added to each well is 0.10mL. Therefore:
  Total amount of ATP-ER (µl) required = 0.1mL x (number of wells used + 24 (background, ATP dose response wells and ATP controls)).

**ATP Standard Curve**

Depending on the size of the kit purchased, non-sterile, 96-well plates have been included to perform an ATP standard curve prior to processing the sample cultures. Performing an ATP standard curve and controls on each day samples are processed is an essential part of the assay because it has 4 functions:

- It tests whether the instrument is working properly and calibrates it.
- It ensures that the reagents are working correctly.
- It calibrates and standardizes the assay and allows the assay system to be validated, if required.
- It allows the output of the plate luminometer, in relative luminescence units (RLU), to be converted to ATP concentrations, thereby standardizing the procedure so that intra- and inter-laboratory experiments can be compared.

**Adhesive Plate Covering Film**

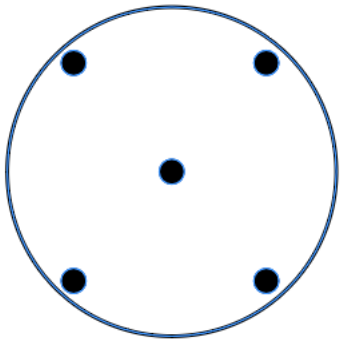
To help keep the plate(s) sterile, adhesive, air permeable, sterile films are provided so that the part of the plate that is not being used can be covered and kept sterile until required. If using the adhesive film provided, the plate cover should be removed in a laminar air-flow hood and replaced with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

**Mixing the Contents of 96-well Plate**

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

1. Take up the required amount of reagent and add it to the well without inserting the tip into the well contents.
2. Starting from the center of the well, aspirate and dispense the contents twice without removing the pipette tip from the contents of the well.
3. Move the pipette tip to one corner of the well and aspirate and dispense the contents twice without removing the tip from the contents of the well.
4. Repeat this operation as shown in Figure 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

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

## 12. HALO® Assay Measurement Assurance and Validation Parameters

If HALO® has been calibrated and standardized, ATP bioluminescence technology allows the User's results to be compared to the measurement assurance parameters shown in Table 4. 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 4: ATP Controls and Standard Curve Measurement Assurance Parameters**

| Expected Parameter | Observed Value | Mean ± 15%(*) | Min / Max | %CV (where applicable) |
|---|---|---|---|---|
| 0.01µM ATP | 0.0099µM ATP | 0.00972 - 0.0114 | 0.009 - 0.01 | 2.34% |
| 0.03µM ATP | 0.029µM ATP | 0.285 - 0.0336 | 0.028 - 0.03 | 1.67% |
| 0.05µM ATP | 0.0497µM ATP | 0.0486 - 0.0571 | 0.048 - 0.051 | 1.57% |
| 0.01µM ATP | 0.1026µM ATP | 0.1003 - 0.118 | 0.099 - 0.107 | 1.96% |
| 0.3µM ATP | 0.317µM ATP | 0.310 - 0.364 | 0.302 - 0.325 | 1.51% |
| 0.5µM ATP | 0.5023µM ATP | 0.491 - 0.578 | 0.491 - 0.515 | 1.19% |
| 1.0µM ATP | 1.048µM ATP | 1.024 - 1.205 | 0.977 - 1.117 | 3.7% |
| 3.0µM ATP | 2.722µM ATP | 2.661 - 3.130 | 2.633 - 2.934 | 2.09% |
| Intercept | 6.533 | 6.386 - 7.513 | 5.86 - 6.7 | 1.84% |
| Slope | 0.9656 | 0.944 - 1.110 | 0.947 - 0.988 | 1.21% |
| $r^2$ goodness of fit) | 0.9993 | - | 0.998 - 1 | 0.05% |
| R (correlation coefficient) | 1 | - | 0.999 - 1 | 0.02% |
| Low control, (0.05µM ATP | 0.0487µM ATP | 0.0476 - 0.0560 | 0.042 - 0.063 | 6.79% |
| High control 0.7µM ATP | 0.725 | 0.710 - 0.836 | 0.655 - 0.904 | 5.35% |
| Extra high control (1.75µM ATP) | 1.756 | 1.717 - 2.019 | 1.61 - 2.198 | 5.24% |

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

**Samples Values**:

- Lowest ATP value indicating unsustainable cell proliferation for hematopoietic cells: ~0.04µM

  **Please note** that human B-cells, especially cryopreserved cells, may exhibit very low ATP values. It is important to compare the stimulated B-cells with their background (no growth factors added) to determine B-cell activity.
- 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**

HALO® 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 comparison): ~95%.
- High throughput capability (Z-Factor): >0.76 (lowest possible value, 0.5; highest possible value, 1).

## 13. Troubleshooting

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

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

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

High Coefficients of Variation (%CV)

Coefficients of variation (%CV) should be =< 15%. The percent coefficient of variation is calculated as standard deviation/mean x 100. High %CVs are usually an indication of incorrect dilutions or pipetting error. Although outliers can be obtained, these being observed for the more primitive stem cells than for the more mature proliferating cells, large variations between replicates should not be obtained. Please consider the following:

- Accurate reagent dispensing and mixing are of prime importance. Since the volumes dispensed are small it is imperative to use instruments that have been properly 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 the calibration and standardization procedure prior to sample measurement can help detect problems prior to sample measurement. If low RLU values occur, 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*: 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.

Low T- or B-Progenitor Cell Growth

The growth of P-Tcell or P-Bcell progenitors can demonstrate low proliferation. These cells are only meant to be stimulated under specific conditions. However, low proliferation ability is usually due to the cells not being purified sufficiently. If these populations are stimulated from a MNC fraction, it is highly possible that other cell populations will be stimulated and will result in an underestimation of true T- or B-progenitor cell proliferation ability. It is highly recommended to use magnetic bead separation to purify these populations from the MNC fraction.

Luminescence Reagent Mixing

The luminescence reagent has to be added and thoroughly mixed with the culture components. The ATP-ER lyses the cells and releases intracellular ATP. If mixing is not adequate, only a proportion of the cells will be lysed and the RLU values will be low. Conversely, too much mixing can lead to ATP degradation and low luminescence readings.

Culture Plates Drying Out

- Due to the relatively small culture volume (0.1mL), drying out of the culture wells, particularly around the outside of the plate can be a problem. These are called “edge effects”. An incubator with insufficient humidity will cause this problem. To ensure that this does not occur, the incubator water reservoir should be full and the humidity in the chamber checked using a hygrometer.
- If drying out continues, use of a humidity chamber is recommended. Please refer to Section 9 (v) for instructions on how to build a humidity chamber.

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Preferred Cell Systems™

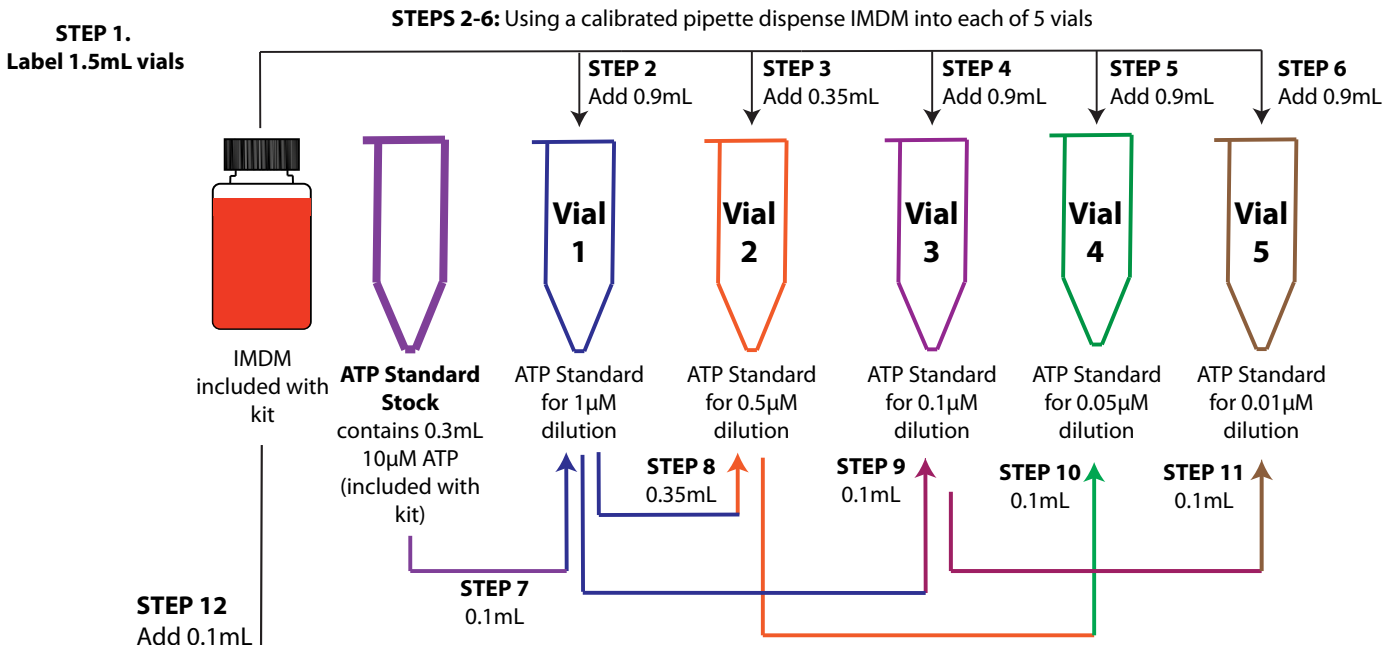
# Calibration and Standardization Protocol of an ATP Bioluminescence Assay

## 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)

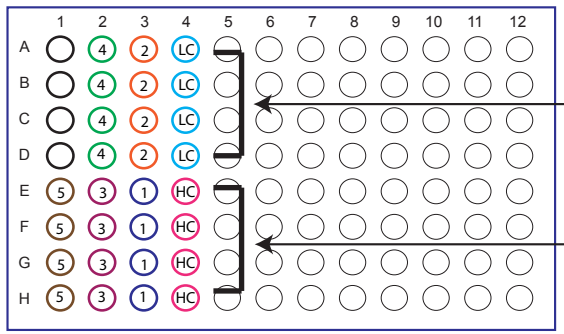
**TIPS**

**> 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

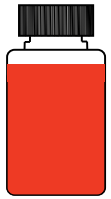
# Calibration and Standardization Protocol of an ATP Bioluminescence Assay

## 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



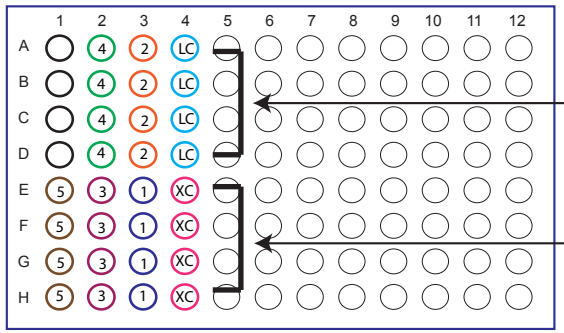
IMDM included with kit

**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