HemoGLO™

The Fast and Easy Viability and Proliferation Assays for Stem and Progenitor Cells of the Lympho-Hematopoietic System

For Individual Populations and "Global" Assay Kits

Technical Manual

(Version 8-21)

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

For *In Vitro* Research Use Only.
Not for clinical diagnostic use.

Preferred Cell Systems™

KM030.001

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1. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

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

HemoGLO™ is a viability and 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 HemoGLO™.

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, HemoGLO™ incorporates growth factor master mixes that use Suspension Expansion Culture™ (SEC™) Technology that provide far superior accuracy, sensitivity, reliability and reproducibility than colony-forming unit (CFU) or colony-forming cell (CFC) assays.

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

HemoGLO™ 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, HemoGLO™ can completely replace the colony-forming cell (CFC) or Unit (CFU) assay. Specialized HemoGLO™ assay kits are also available hematopoietic cell therapy applications. If required, HemoGLO™ can be standardized and validated by purchasing the HemoGLO™ Standardization Assay Kit from Preferred Cell Systems™.

HemoGLO™ can be used with the following tissues:

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

HemoGLO™ is available for the following species:

- Human
- Non-human primate
- Horse
- Pig
- Minipig (upon request)
- Sheep
- Dog
- Rat
- Mouse

HemoGLO™ Serum-Free Assays Kits are available for human, primate and mouse cells.

HemoGLO™ 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 (highly recommended)
- Purified stem or progenitor cells obtained by flow cytometry or magnetic bead separation (highly recommended).

If possible, it is recommended to use MNCs 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 HemoGLO™ 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 HemoGLO™ cultures, the following equivalent cell populations can be detected using HemoGLO™

Cell Populations Detected using HemoGLO™

- No growth factors included. Used for background control or to add growth factors/cytokines .
- SC-HPP 1 equivalent to HPP-CFC 1 (human and mouse only)
- SC-HPP 2 equivalent to CFC-HPP 2
- SC-GEMM 1 equivalent to CFC-GEMM 1
- SC-GEMM 2 equivalent to CFC-GEMM 2 (human and mouse only)
- SC-GEM 1 equivalent to CFC-GEM 1
- SC-GEM 2 equivalent to CFC-GEM 2 (human and mouse only)
- 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 3 equivalent to GM-CFC 3
- P-Mk 1 equivalent to Mk-CFC
- P-Tcell 1 (see also ImmunoGlo™ TCP)
- P-Tcell 2 (see also ImmunoGlo™ TCP)
- P-Tcell 3 (human only; see also ImmunoGlo™ TCP)
- P-Bcell 1 (pre-B)
- P-Bcell 2 (human only)
- P-Bcell 3 (human only)

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

HemoGLO™ "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.

HemoGLO™ "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.

HemoGLO™ "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

HemoGLO™ is a 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 cultures 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 HemoGLO™ 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:

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

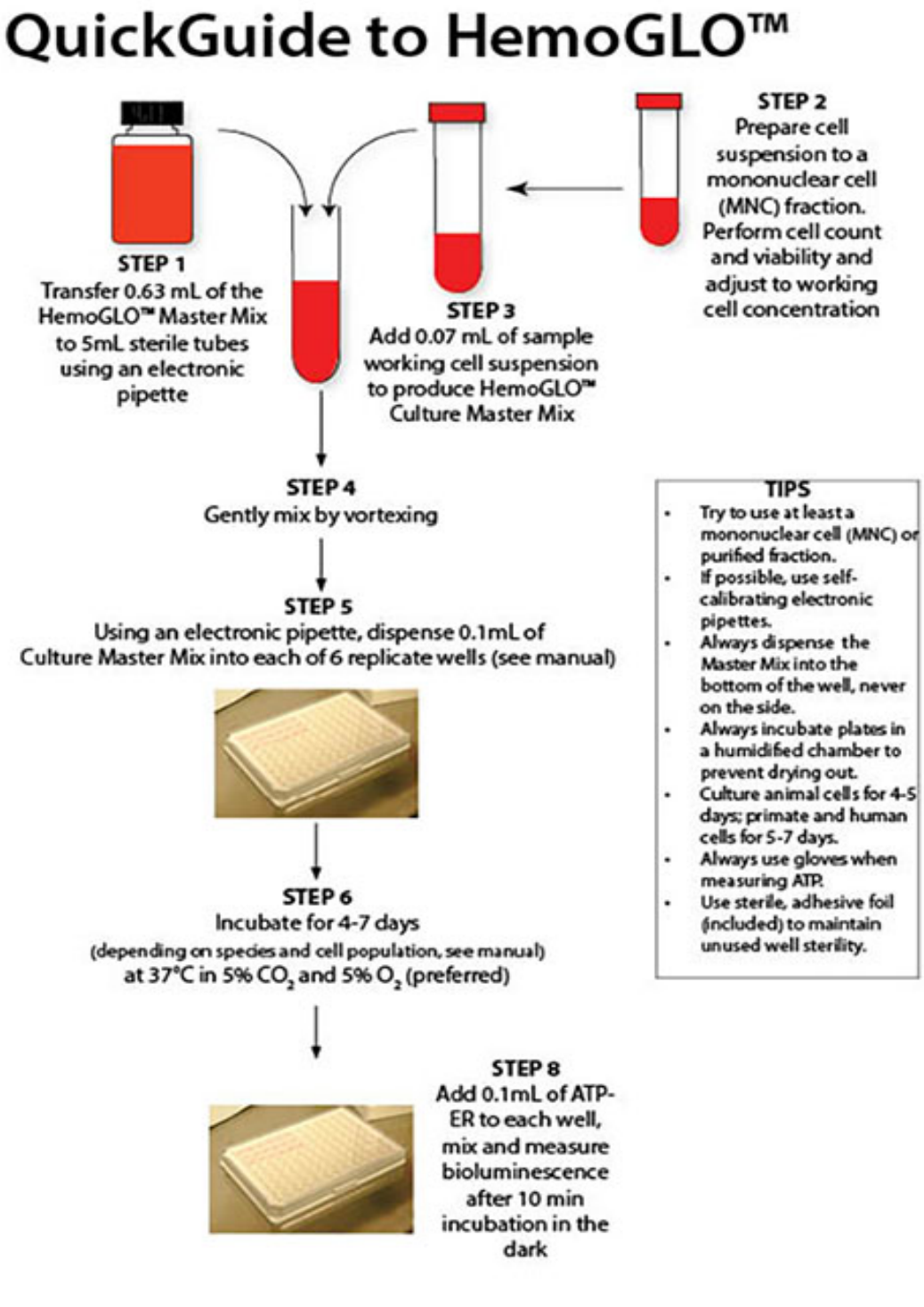
The bioluminescence emitted is detected and measured in a luminescence plate reader as relative luminescence units (RLU).

PLEASE NOTE. If required, HemoGLO™ can be calibrated and standardize (see Section 7). This can be performed by purchasing the HemoGLO™ Assay Standardization Kit from Preferred Cell Systems (Cat No. K6-ASK-1). Alternatively, the corresponding HALO® Assay can be used instead. 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. QuickGuide to HemoGLO™ 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

HemoGLO™ 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	HemoGLO™ Master Mix for an individual cell population	-20°C until used
1a	HemoGLO™ "Global" 4-Population Kit contains 4 individual Master Mixes plus a Background with no growth factor cocktail	-20°C until used
1b	HemoGLO™ "Global" 5-Population Kit contains 5 individual Master Mixes plus a Background with no growth factor cocktail	-20°C until used
1c	HemoGLO™ "Global" 7-Population Kit contains 8 individual Master Mixes plus a Background with no growth factor cocktail	-20°C until used
2	Medium (IMDM) for dilution of the ATP standard.	-20°C until used
3	ATP Enumeration Reagent (ATP-ER)*	-20°C in the dark until used
4	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
5	Sterile, wrapped, 96-well plates for cell culture	Can be kept with other kit components
5a	HemoGLO™ "Global" 4-Population kits contain 5 sterile, wrapped, 96-well plates for cell culture	Can be kept with other kit components
5b	HemoGLO™ "Global" 5-Population kits contain 6 sterile, wrapped, 96-well plates for cell culture	Can be kept with other kit components
5c	HemoGLO™ "Global" 7-Population kits contain 8 sterile, wrapped, 96-well plates for cell culture	Can be kept with other kit components
	Technical manual can be downloaded from the HemoGLO™ TE page on the Preferred Cell Systems™ web page	

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. HemoGLO™ Assay Standardization Kit (Preferred Cell Systems™ Cat. No.: K6-ASK-1).
2. HemoGro™ Hematopoietic Growth Medium for cell suspensions and dilutions (Preferred Cell Systems™)
3. Iscove's Modified Dulbecco's Medium (IMDM).
4. Density-gradient medium (e.g. NycoPrep 1.077, Axis-Shield).
5. 7-AAD, propidium iodide or trypan blue for viability assay.
6. LIVEGlo™ metabolic viability assay (Preferred Cell Systems™ Cat. No. PS-96-1).

8. The HemoGLO™ Protocol

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

Performing HemoGLO™ is a 3-step process.

Step 1 – Cell preparation.
Step 2 – HemoGLO™ cell culture master mix preparation, plating and incubation in the 96-well plate.
Step 3 – Luminescence measurement.

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

STEP 1 – Cell Preparation

HemoGLO™ 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 HemoGLO™ Assay Kit or HemoGLO™ "Global" 7-Population Assay Kit. See Section 9.

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. HemoGLO™ Cell Culture Preparation

- ***HemoGLO™ 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 HemoGLO™ Methodology

1. Remove the HemoGLO™ 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 37°C water bather or incubator.
2. Label sufficient 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. A minimum of 4 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 (minimum)	0.45mL	0.05mL	0.5mL
6 (recommended)	0.63mL	0.07mL	0.7mL
8 (recommended for "Global" assays	0.9mL	0.1mL	1.0mL

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 (5×10^5) cells/mL.
5. Dispense the required volume of cells into each tube containing the HemoGLO™ Master Mix.
6. Mix the contents gently on a vortex mixer. Do not cause bubbles.
7. Remove the sterile, wrapped, 96-well plate from the assay kit box. If using a HemoGLO™ "Global" Kit, each cell population will be plated in its own 96-well plate.
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 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.
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

HemoGLO™ Assay Kits for 4-, 5- or 7-Populations

- These HemoGLO™ assay kits include 5, 6 or 8 sterile, wrapped, 96-well plates, including 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 HemoGLO™ 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 HemoGLO™ "Global" 7-Population Assay Kits. See Section 9 vii.
- In preparing the tubes, use the same HemoGLO™ Methodology as above.

STEP 3 – Sample 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 37°C incubator.***
- ***If using a HemoGLO™ Assay Standardization Kit (Cat. No. K6-ASK-1), follow the instructions for thawing and using the reagents.***

Sample Measurement

1. If possible, place the sample plate(s) in a humidified incubator set at 22-23˚C gassed with 5% CO_2 for 30min. Otherwise, allow the plate to come to room temperature for 30 min.
2. **IMPORTANT FOR ALL HemoGLO™ and HemoGLO™ "Global" Assays.** If only part of the plate has been used, transfer the plate to a bio-safety hood and remove the lid under sterile conditions. Take a sterile adhesive plate coverfoil from the kit box, remove the backing and layer it over the top of the plate. Using a sharp knife or scalpel, cut away the foil that covers the wells to be processed. The unused, empty wells will now remain sterile for the next samples. (See Section 10, Adhesive Plate Covering Film).
3. Although a single channel pipette can be used, a multichannel pipette (8- or 12-channel depending on the plate configuration), is recommended. Add 0.1mL of ATP-ER to each well of the first column (A1-H1) or row (A1-12). Mix the contents as described in Section 10.
4. Repeat this procedure for each column or row **using new tips**.
5. When ATP-ER has been added to all wells, replace the plastic cover and incubate for 10 min at room temperature in the dark to lyse the cells and stabilize the luminescence signal. Incubate in the reader for the last 2 min to stabilize the plate and then read the plate. Alternatively, incubate the plate (without the lid) in the plate reader, in the dark, for 10 minutes, prior to reading the plate.
6. Unused ATP-ER may be returned to the bottle and refrozen. See section 10 for ATP reagent storage conditions and

stability.

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

E. Using a liquid handler
HemoGLO™ can be performed in high throughput mode. If you intend to perform any part of the HemoGLO™ 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 HemoGLO™.

(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. If detecting 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 a minimum of 4 replicates/sample, although 6 replicate wells/sample will provide better statistical relevance. Using 4 replicates/sample, a total of 24 samples can be performed. For HemoGLO™ "Global", 8 replicates/samples is recommended, allowing 12 samples/plate to be perfomed. 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

For 4 and 6 replicate wells/sample cultures should be plated in rows across the plate. For 8 replicates/sample, plate in columns.

(iv) 96-Well Plates Provided

The reagents have been optimized to work with the 96-well plate(s) provided in the HemoGLO™ 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.***
- ***HemoGLO™ includes solid white plates for cell culture. Do not use different plates for the assay. Doing so will result in inaccurate results and invalidation of the assay kit warranty. Extra plates can be purchased from Preferred Cell Systems™.***

Bioluminescence Assay Kit Components

- 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˚C, in the dark, for 48h or is stable at -20ºC for 20 weeks.

Reconstitution of Lyophilized Monitoring Reagent (if included)

The assay kit may include ATP-ER as a lyophilized powder and an ATP-ER Buffer. Perform the following steps.

- Thaw the ATP-ER (Enumeration Reagent) Buffer at room temperature, in cold running water, or at 2-8ºC 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 2-8ºC, or 20 weeks at -20ºC.
- ATP-ER can be refrozen up to 11 cycles without significant loss of sensitivity.

Volumes of Luminescence Kit Components Required

- 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 + 5-10%)).

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.

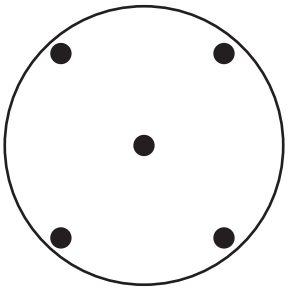
Mixing the Contents of 96-well Plate

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

Multimode instruments, i.e. those that can detect absorbance, fluorescence and luminescence, often need to be manually set for both the integration time and the "gain". Dedicated instruments, i.e. those that only detect luminescence, usually only have to be set for the "integration time". It is therefore necessary to first know whether the instrument is a multimode or multipurpose instrument and whether "integration time" and "gain" need to be set. The instrument instruction manual will provide this information. If the "gain" has to be set, the instruction manual will explain how the correct "gain" is established. Once the "integration time" and "gain" are set, they should not be changed.

a. First set the integration time to 2 seconds.
b. Next, set the "gain" (if required). The gain should be adjusted so that the percent coefficients of variation (%CV) for the mean of the replicates are the lowest value. These values should be about 5% or less.
c. The measurement temperature of the instrument should be set to between 22ºC and 24ºC or turned off.
d. Do not use plate shaking or the injectors if the instrument has this capability.
e. The output of the luminescence plate reader is in Relative Luminescence Units (RLU). To convert RLU values into iATP concentrations (µM), it is necessary to to calibrate the instrument with controls and perfom an ATP standard curve. If this is required, it will be necessary to purchase the HemoGLO™ Assay Standardization Kit from Preferred Cell Systems™ (Cat. No. K6-ASK-1). Alternatively, use the corresponding HALO® assay that includes standards and controls.

12. Results

HemoGLO™ provides an instrument-based, non-subjective, quantitative readout of viability and the total proliferation ability of the cells being tested. HemoGLO™ is a replacement for the colony-forming unit (CFU) assay. Since HemoGLO™ measures cell proliferation and, proliferation occurs prior to differentiation, HemoGLO™ ontaining results is not only more rapid, but also predicts results obtained with the CFU differentiation assay. This is because there is a direct correlation between HemoGLO™ and the CFU assay.

HemoGLO™ can be used on fresh or frozen cells. If testing cells prior to cryopreservation, it should be emphasized that results produced using HemoGLO™ or any other similar assays from Preferred Cell Systems™, will not reflect the actual viability or proliferation ability after thawing; proliferation ability will be 2-3 fold lower after thawing a cryopreserved

sample than a fresh sample.

It is strongly recommended to include a background control, i.e. cells cultured without growth factors. This will allow the user to compare growth and proliferation between the background and the cells cultured with the HemoGLO™ Master Mix. In general, cells cultured in HemoGLO™ Master Mix should produce RLU values at least 2 standard deviations greater than the background or demonstrate a statistical significance from the background RLU value.

12. Troubleshooting

Decay of ATP-ER
The ATP Enumeration Reagent (ER) decays with time, even when frozen. DO NOT use the ATP-ER past the expiration date. Doing so can result in very low RLU values or no bioluminescence at all (see also Low RLU Values, below).

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

- Accurate reagent dispensing and mixing are of prime importance. Since the volumes dispensed are small it is imperative to use instruments that have been properly calibrate to avoid pipetting error.
- Insufficient mixing of components prior to cell plating and insufficient mixing during the addition of luminescence reagents to cultures in the 96-well plate can also lead to high CVs. Use repeater pipettes. Use calibrated or self-calibrating electronic pipettes or dispensers to add and mix the luminescence reagents.
- If the luminometer requires determining the "gain" empirically, it is possible that this parameter has not been optimally set and will result in an incorrect signal to noise ratio. Once the optimal "gain" has been set for the instrument, it should not be changed.

Low RLU Values
Performing 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 at 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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