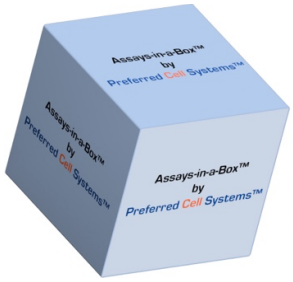

Assays-in-a-Box™
by
Preferred Cell Systems™

HemoGLO™ PCA

Fast and Easy Progenitor Cell Assays for Umbilical Cord Blood, Bone Marrow and Peripheral Blood in the Cell Processing Laboratory

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. *HemoGLO™ PCA is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)*
2. *HemoGLO™ PCA 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™ PCA is a fast and easy progenitor cell assay for cord blood, bone marrow or peripheral blood and can completely replace the traditional methylcellulose colony-forming unit (CFU) assay. HemoGLO™ PCA assays are methylcellulose-free, non-subjective and can be completed in 5 days instead of 14 days normally required when using a CFU assay.

HemoGLO™ PCA is based on a simple concept. 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. It is this concept that is used in HemoGLO™ PCA.

To detect and measure 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. Preferred Cell Systems™ has taken the growth factor cocktails normally used and combined them with Suspension Expansion Culture™ (SEC™) Technology, to provide an easy to use, fast, accurate, sensitive, reliable and reproducible assay for the cell processing laboratory. The Master Mixes provided in HemoGLO™ PCA assay kits are available either as serum-free or low serum formulations.

3. Use and Availability

HemoGLO™ PCA is an instrument-based, quantitative, non-subjective replacement for the colony-forming unit (CFU) assay used in hematopoietic cellular therapy processing laboratories. It measures both the viability and total proliferation ability of stem cells and progenitor cells from the tissues noted below. HemoGLO™ PCA can be used on fresh or frozen cells. If testing cells prior to cryopreservation, it should be emphasized that results produced using HemoGLO™ PCA or will not reflect the actual viability or proliferation ability after thawing; proliferation ability will be 2-3 times lower after thawing a cryopreserved sample. HemoGLO™ PCA is available only for human cell use with the following tissues:

- Bone marrow
- Peripheral blood
- Umbilical cord blood

HemoGLO™ PCA 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.

It is highly recommended to use mononuclear cells or a cell preparation 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™ PCA 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%.

HemoGLO™ PCA can be obtained with serum-free or low serum SEC™ Master Mix formulations.

Since cells are grown in suspension culture and no colonies are not produced, the following equivalent cell populations can be detected using HemoGLO™ PCA.

HemoGLO™ PCA CFU-Equivalent Assays Available as Serum-Free and Low Serum Formulations

HemoGLO™ PCA	Growth Factor/Cytokine Cocktail	Equivalent HemoGLO™ PCA Populations	Equivalent MethoCult® Product	CFU Populations Detected
PCA1	EPO, GM-CSF, IL-3, SCF	P-BFU, P-E, P-GM, P-G, P-M	H4434 "Classic"	BFU-E, CFU-E, GM-CFC, G-CFC, M-CFC
PCA2	EPO, GM-CSF, G-CSF. IL-3, SCF	P-BFU, P-E, P-GM, P-G, P-M	H4034 "Optimum"	BFU-E, CFU-E, GM-CFC, G-CFC, M-CFC
PCA3	GM-CSF, IL-3, SCF	P-GM, P-G, P-M	H4534 "Classic"	GM-CFC, G-CFC, M-CFC
PCA4	GM-CSF, G-CSF, IL-3, SCF	P-GM, P-G, P-M	H4035 "Optimum"	GM-CFC, G-CFC, M-CFC
PCA5	EPO, GM-CSF, G-CSF, IL-3, IL-6, SCF + TPO(#)	SC-GEM, P-BFU, P-GM, P-G, P-M	H4435 "Enriched"	CFC-GEM, BFU-E, GM-CFC, G-CFC, M-CFC, Mk-CFC
PCA6	EPO, GM-CSF, IL-3, IL_6, SCF, Flt3-L, TPO	SC-GEMM, P-BFU, P-GM, P-Mk	MethoCult "Express"	CFC-GEMM, BFU-E, GM-CFC, G-CFC, M-CFC, Mk-CFC

P = Progenitor cell. SC = Stem cell.

(#) **Please note** that thrombopoietin (TPO) is not included in the H4435 "Enriched" MethoCult® or MethoCult "Express" products, but is included in the HemoGLO™-96 PCA for the PCA5 and PCA6 products. This means the H4435 and "Express" do not stimulate cells of the megakaryopoietic lineage.

4. The Concept of ATP Bioluminescence Assays

HemoGLO™ PCA 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.

Hematopoietic cells are incubated in the HemoGLO™ PCA 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).
If required, any HemoGLO™ PCA assay can be calibrated and standardize, by purchasing the HemoGLO™ Standardization Kit from Preferred Cell Systems™ (see Section 7). Performing an ATP standard curve and controls has the following advantages:

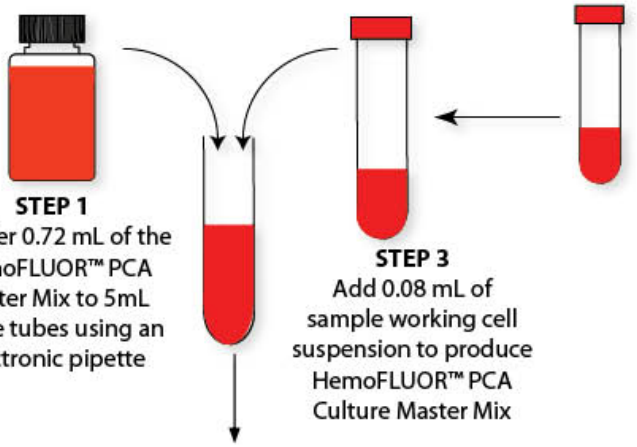
1. The controls calibrate the instrument and also ensure that the reagents are working correctly.
2. The ATP standard curve also ensures that the reagents are working correctly.
3. The ATP standard curve allows the luminometer output in Relative Luminescence Units (RLU) to be converted to standardized ATP concentrations (µM).
4. Performing the ATP standard curve allows results to be compared over time.
5. Proficiency testing is incorporated into the assay. No additional proficiency testing is needed.
6. Allows calibration and standardization parameters to be compared to measurement assurance parameters that confirm the assay is performing correctly and can provide trustworthy 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.

NOTES

5. QuickGuide to HemoGLO™ PCA (Figure 1)

QuickGuide to HemoFLUOR™ PCA



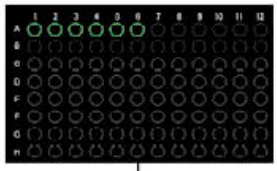
STEP 1
Transfer 0.72 mL of the HemoFLUOR™ PCA Master Mix to 5mL sterile tubes using an electronic pipette

STEP 2
Prepare cell suspension to a mononuclear cell (MNC) fraction. Perform cell count and viability and adjust to working cell concentration

STEP 3
Add 0.08 mL of sample working cell suspension to produce HemoFLUOR™ PCA Culture Master Mix

STEP 6
Gently mix by vortexing

STEP 5
Using an electronic pipette, dispense 0.1mL of Culture Master Mix into each of 6 replicate wells



STEP 6
Incubate for 5-7 days at 37°C in 5% CO_2 and 5% O_2 (preferred)

STEP 7
Add 0.1mL of HemoFLUOR™ reagent to each well, mix and measure fluorescence (Ex filter: 380-400nm; Em filter: 505nm). The signal develops between 0.5-3 hrs. of incubation at 37°C.

TIPS

- Try to use at least a mononuclear cell (MNC) fraction for cord blood, mobilized peripheral blood or bone marrow samples.
- If possible, use self-calibrating electronic pipettes.
- Always dispense the Master Mix into the bottom of the well, never on the side.
- Always incubate plates in a humidified chamber to prevent drying out.
- Culture human cells for 5-7 days.
- Always ensure well contents are mixed properly (see manual).
- Use sterile, adhesive foil (included) to maintain unused well sterility.
- Include and measure background fluorescence.

6. Kit Contents and Storage Conditions

HemoGLO™ PCA 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™ PCA Master Mix for an individual cell population	-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, individually wrapped, 96-well plate for cell culture	Can be kept with other kit components
	Technical manual is NOT included with the kit. It must be downloaded from www.preferred-cell-systems.com	

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. Ficoll-Paque, Lymphoprep)
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™ PCA Protocol

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

Performing HemoGLO™ PCA is a 3-step process.

Step 1 – Cell preparation.
Step 2 – HemoGLO™ PCA cell culture master mix procedure, 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™ PCA 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. It is highly recommended to use a mononuclear cell fraction or higher for these assays.
2. Mononuclear cell (MNC) fraction is the cell preparation of choice. This fraction can be prepared by density gradient centrifugation using, for example, Ficoll-Paque or Lymphoprep.
3. Purified stem and progenitor cell populations prepared by cell sorting or magnetic bead separation.

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 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™. The volume of the adjusted cell suspension required will be 10% of the total volume of HemoGLO™ PCA Culture Master Mix prepared.

TABLE 1

Recommended Cell Doses for Cell Types, Cell Preparations and Cell States for HemoGLO™ PCA

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

STEP 2. HemoGLO™ PCA Cell Culture Procedure

- *HemoGLO™ PCA 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.*

1. Remove the HemoGLO™ PCA 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 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 HemoGLO™ PCA Master Mix minus 10%, that will allow 0.1mL to be dispensed into the required number of replicate wells. See Table 2.

TABLE 2

Number of Replicate Wells Required	Volume of HemoGLO™ PCA 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

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™ PCA Master Mix.
6. Mix the contents gently on a vortex mixer. Do not cause bubbles.
7. Remove the sterile, individually 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 or 4 replicates, dispense either in across the plate in rows in columns. If 6 replicates are prepared, these should be dispensed across the plate in rows.
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

Cell Type	Cell Source	Incubation Period (days)
All cell populations detected by HemoGLO™ PCA	Bone marrow, normal and mobilized peripheral blood, umbilical cord blood	5 days for all, but can be extended to 7 day for PCA1, PCA2, PCA5 and PCA 6 for greater sensitivity

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 "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.*
- *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℃ 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. 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 the plate in the reader for the last 2 min to stabilize the plate. Alternatively, place the 96-well plate in the reader, in the dark, for 10 minutes and then read 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.

9. Recommendations and Tips Prior To Using HemoGLO™ PCA.

(i) Cell Suspension

a) The preferred cell suspension is a mononuclear cell suspension (MNC).

b) Extraneous ATP, red blood cells (which have high concentrations of ATP) and hemoglobin interfere with the ATP analysis. The cell suspension must have a hematocrit of 10% or less.

c) If cells have been treated prior to cell culture, higher cell concentrations than those shown in Table 1 may be required.

(ii) Number of Replicates Performed

The recommended number of replicates is 6/sample, although a minimum of 4 is also possible. 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 or 6 replicate wells/sample, these 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 HemoGLO™ PCA 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

Please see Table 3. Assay sensitivity might improve with longer incubation times, but usually at the expense of higher variability between wells. Once an optimal incubation time has been found, the same time period should be maintained for all future experiments so that results can be directly compared.

10. Recommendations and Tips Prior To Measuring Bioluminescence

- *Always wear laboratory (e.g. latex) gloves during this operation to avoid ATP contamination from skin.*
- *DO NOT wipe the pipette tip with tissue etc as this will wick the reagent from the tip and cause an erroneous ATP standard curve and false sample results.*
- *Always change pipette tips after each use.*
- *HemoGLO™ PCA 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

- 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

- ☐ 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% for overage).

Adhesive Plate Covering Film

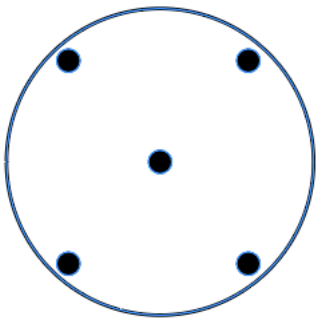
To help keep the plate(s) sterile, adhesive, air permeable, sterile films are provided so that the part of the plate that is not being used can be covered and kept sterile until required. If using the adhesive film provided, the plate cover should be removed in a laminar air-flow hood and replaced with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

Mixing the Contents of 96-well Plate

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

1. Take up the required amount of reagent and add it to the well without inserting the tip into the well contents.
2. Starting from the center of the well, aspirate and dispense the contents twice without removing the pipette tip from the contents of the well.
3. Move the pipette tip to one corner of the well and aspirate and dispense the contents twice without removing the tip from the contents of the well.
4. Repeat this operation as shown in Figure 4 for each corner of the well.
5. Try not to cause excessive bubbles in the culture and DO NOT over mix since this can result in drastically reduced luminescence values.
6. This procedure effectively and optimally mixes the contents well.

Figure 2. Positions of pipette tip for mixing the well contents



11. Luminescence Plate Reader Setup

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™ PCA provides an instrument-based, non-subjective, quantitative readout of viability and the total proliferation ability of the cells being tested. HemoGLO™ PCA is a replacement for the colony-forming unit (CFU) assay for hematopoietic cellular therapy processing laboratories. Since HemoGLO™ PCA measures cell proliferation and proliferation occurs prior to differentiation, HemoGLO™ PCA can not only be completed more rapidly, but actually predicts results obtained with the CFU differentiation assay. This is because there is a direct correlation between HemoGLO™ PCA and the CFU assay.

HemoGLO™ PCA can be used on fresh or frozen cells. If testing cells prior to cryopreservation, it should be emphasized that results produced using HemoGLO™ PCA or any other PCA assay 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. If this does not occur, then either cellular impurities are hindering progenitor cell proliferation (see Troubleshooting) or the cell source has a low proliferation ability (also referred to as cell quality).

13. 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 calibrated to avoid pipetting error.
- Insufficient mixing of components prior to cell plating and insufficient mixing during the addition of luminescence reagents to cultures in the 96-well plate can also lead to high CVs. Use repeater pipettes. Use calibrated or self-calibrating electronic pipettes or dispensers to add and mix the luminescence reagents.
- If the luminometer requires determining the “gain” empirically, it is possible that this parameter has not been optimally set and will result in an incorrect signal to noise ratio. Once the optimal “gain” has been set for the instrument, it should not be changed.

Low RLU Values

Performing an ATP dose response prior to sample measurement can help detect problems prior to sample measurement. If low RLU values occur, this could be due to the following reasons.

- *Cell Starting Preparation:* If starting with a TNC fraction, RLU values may be close to zero or not significantly different from a background control. If this is the case, it is highly recommended to fractionate the sample to a mononuclear cell (MNC) preparation, since this will remove cell impurities that can hinder progenitor cell proliferation. It is also possible that the starting cell source did not have cells with high proliferation ability or potential. This is an indication that the starting cell source did not exhibit a high viability and, therefore, was not of high quality.
- *Reagent decay:* The ATP-ER decays with time, even when frozen. This can lead to low bioluminescence. Once thawed the reagent can be refrozen up to 11 cycles without significant loss of sensitivity. Do not use the reagent after expiry date has elapsed. As a rule of thumb, the RLU value for the lowest ATP standard should be 10 times greater than that of the background value.
- *Inadequate cell growth:* Cells did not exhibit sufficiently high viability. Measure cell viability prior to using cells. A cell viability lower than 85% should not be used. Viabilities lower than 85% can be an indication that the sample was not processed in a time-sensitive manner or that the processing procedures were not standardized and controlled.

- *Reagent deterioration:* Reagents arrived thawed, at room temperature or greater or were not stored correctly.
- *Inadequate incubator conditions:* Maintaining a correct humidified gaseous atmosphere in the incubator is essential (See Culture Plate Drying Out).
- *Carbon dioxide concentration is inadequate:* Ensure that the carbon dioxide concentration in the incubator is correct using a Fyrite gas analyzer.
- *Use low oxygen tension:* Using an oxygen concentration of 5% reduces oxygen toxicity due to free radical production and increases plating efficiency. Check that the incubator oxygen concentration is correct using a Fyrite gas analyzer.
- *Low humidity:* Plates dry out (see below) and cell growth declines.

- *Contamination*: Cells cultured in 96-well plates cannot be view under a microscope. If contamination occurs it will usually be seen by the difference in color of the cultures, if the medium contains an indicator, e.g. phenol red. Contaminated cultures will usually be bright yellow in color and probably cloudy in appearance. Cell cultures that demonstrate high proliferation will usually appear orange to light orange, but will not be cloudy. If only "spot" contamination occurs, this is usually due to pipette or repeater tips coming in contact with materials other than the reagents. Contamination will usually lead to outlier RLU values.

Luminescence Reagent Mixing.

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

Culture Plates Drying Out

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

Additional Hematopoietic Cellular Therapy Assay Products from Preferred Cell Systems™

HemoFLUOR™ PCA similar to HemoGLO™ PCS, using a fluorescence readout. A fluorescence plate reader is required. HemoFLUOR™ PCS has excellent multiplexing capability with flow cytometry.

HemoLIGHT™ PCA similar to HemoGLO™ PCA and HemoFLUOR™ PCA, but using an absorbance/colorimetric readout. An absorbance plate reader is required. HemoLIGHT™ PCS has excellent multiplexing capability with flow cytometry.

HemoGLO™ TE, **HemoFLUOR™ TE** and **HemoLIGHT™ TE** are time to engraftment assays.

HemoGLO™ PMT "Global", **HemoFLUOR™ PMT "Global"** and **HemoLIGHT™ PMT "Global"** are 4-, 5- or 7-population assays to monitor patient "global" reconstitution after transplantation.

HALO® Cellular Therapy Assays from Preferred Cell Systems™
The HALO® Platform is for discerning scientists and the ultimate assay for testing cellular therapy products in the cell processing laboratory.

STEMpredict™ is a 3-day, fully standardized, ATP bioluminescence, stem cell quality assay designed primarily for cord blood banks to triage high from low quality cord blood units prior to cryopreservation. STEMpredict™ is the only assay for hematopoietic cellular therapy products designed for both high-throughput 96- and 384-well plate formats.

HALO® SPC-QC is a 5-day, 96-well, standardized, ATP bioluminescence stem cell quality control assay designed to optimized stem cell processing procedures. It is available to measure primitive hematopoietic stem cells as well as primitive lympho-hematopoietic tem cells.

HALO® RS is a 7 day, 96-well, standardized ATP bioluminescence assay to establish cord blood, bone marrow or pheripheral blood reference standards for HemoGLO™ SC-IPS assays.

HALO® SC-IPS is a 7-day, standardized, 96-well, ATP bioluminescence assay to measure the identity, purity and strength (potency) of 2 primitive stem cell populations in cord blood, bone marrow or peripheral blood samples, prior to use in patients.

HALO® TE are time to engraftment assays.

HALO® PMT "Global" are 4-, 5- or 7-population assays to monitor patient "global" reconstitution after transplantation.

Ordering Information

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