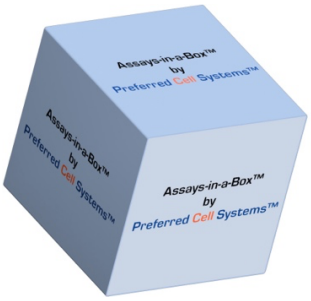

Assays-in-a-Box™
by
Preferred Cell Systems™

HemoLIGHT™ PCA

Absorbance Hematopoietic Progenitor Cell Assays for Bone Marrow, Umbilical Cord Blood and Peripheral Blood Cellular Therapy Testing

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

HemoLIGHT™ PCA is a methylcellulose-free, cell viability and proliferation assay platform that detects and quantitatively measures progenitor cells present in umbilical cord blood, bone marrow or mobilized peripheral blood products used for hematopoietic cellular therapy.

HemoLIGHT™ PCA can completely replace the traditional colony-forming unit (CFU) assay, by providing non-subjective, results within 5 days, instead of the 14 days normally required when using a CFU assay.

HemoLIGHT™-96 PCA incorporates a colorimetric/absorbance readout using the ability of living cells to reduce a novel tetrazolium compound (MTS), in the presence of an electron coupling reagent (PES), to produce a soluble yellow formazan product that is detected in an absorbance plate reader or multiparameter plate reader with an absorbance filter at 490nm. The quantity of formazan produce is proportional to the number of cells in the culture. Unlike an MTT assay which produces a insoluble product, which then has to be solubilized requiring a 2-step process, HemoLIGHT™ PCA is a 1-step reagent addition.

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 used in MethoCult™ products and combined them with Suspension Expansion Culture™ (SEC™) Technology, to provide a highly advanced cell culture system for cellular therapy products. The Master Mixes provided in HemoLIGHT™ PCA assay kits are available either as serum-free or low serum formulations.

HemoLIGHT™ PCA is just one of a large family of highly advanced hematopoietic stem and progenitor cell assays that can be used to enhance and expand the science of cellular therapy.

3. Use and Availability

HemoLIGHT™ PCA is an instrument-based, quantitative, non-subjective alternative to 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. HemoLIGHT™ PCA can be used on fresh or frozen cells. If testing cells prior to cryopreservation, it should be emphasized that results produced using HemoLIGHT™ PCA or any other PCA assay, will not reflect the actual viability or proliferation ability after thawing; proliferation ability will be lower after thawing a cryopreserved sample. HemoLIGHT™ PCA is available only for human cell use with the following tissues:

- Bone marrow
- Peripheral blood
- Umbilical cord blood

HemoLIGHT™ 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.

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If possible, it is highly recommended to use mononuclear cells or 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 HemoLIGHT™ PCA assays incorporate Suspension Expansion Culture (SEC) Technology. No methylcellulose is used and therefore no colonies are produced. 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%.

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

Since colonies of cells are not produced in HemoLIGHT™ PCA cultures, the following equivalent cell populations can be detected using HemoLIGHT™ PCA.

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

HemoLIGHT™ PCA	Growth Factor/Cytokine Cocktail	Equivalent HemoLIGHT™ 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" or MethoCult™ Express	CFC-GEM, 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® product, but is included in the HemoLIGHT™-96 PCA^{EQ} for the PCA5 products. This means the H4435 stimulates a more mature stem cell population than the HemoLIGHT™ product and will not stimulate cells of the megakaryopoietic lineage.

4. The Concept of HemoLIGHT™ Assays

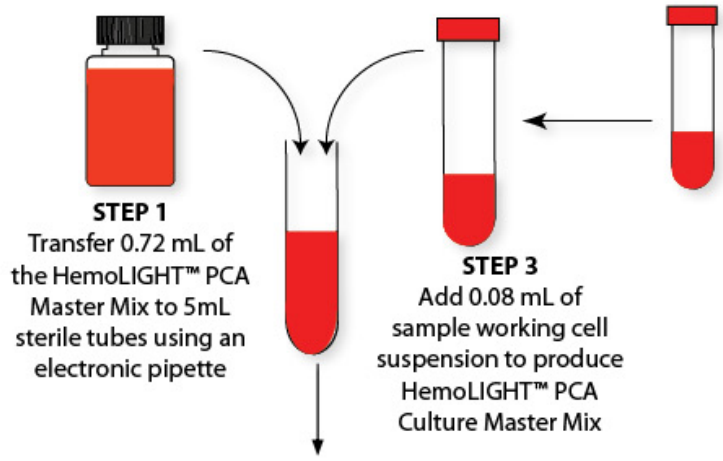
HemoLIGHT™-96 PCA^{EQ} is a colorimetric/absorbance *in vitro* assay. It contains the tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt] or MTS. In the presence of an electron coupling reagent (phenazine ethosulphate, PES), a stable MTS reagent is produced. When added to viable and proliferating hematopoietic cell cultures, the metabolically active cells reduce MTS, presumably in the presence of NADPH or NADP, into a soluble, yellow formazan product that develops in the cultures when the cells are incubated with the reagent for 1-4 hours. The absorbance is measured at 490nm in a 96-well plate reader. The plate can be removed from the incubator at different times to measure the optimal absorbance. Preferred Cell Systems™ recommends incubating the cells for a minimum of 3 hours to develop a sensitive signal. This is especially important for cells exhibiting low growth. The amount of absorbance 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 HemoLIGHT™ PCA Master Mix provided with this kit for 5-7 days. When the culture period has elapsed, a single-step addition of the MTS Reagent is dispensed into each culture well and the contents mixed. The plate is incubated at room temperature in the dark for 1-4 hours. During this time, the yellow formazan color will develop. Optimal absorbance usually occur at about 3 hours after addition of the MTS reagent.

5. QuickGuide to HemoLIGHT™ PCA (Figure 1)

Quick Guide to HemoLIGHT™ PCA



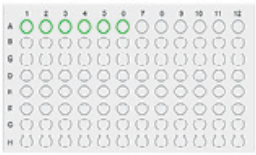
STEP 1
Transfer 0.72 mL of the HemoLIGHT™ PCA Master Mix to 5mL sterile tubes using an electronic pipette

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 HemoLIGHT™ 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 10µL of HemoLIGHT™ WST-1 reagent to each well, mix and measure absorbance at 440nm (420nm to 480nm). The signal develops between 1-4 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.
- Use a reference wavelength of >600nm.

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6. Overview of the HemoLIGHT™ PCA Procedure

There are 3 steps to use HemoLIGHT™ PCA.

Step 1 – Cell Preparation
Cells are not provided with HemoLIGHT™ PCA assay kits. Cells are prepared either with a user-defined, pre-validated protocol to obtain a single cell suspension or procedures that are suggested in this manual. A dye exclusion viability and/or metabolic viability and nucleated cell count should be performed on all samples.

Step 2 – Cell Culture
Each HemoLIGHT™ PCA assay contains a Master Mix to stimulate the cell populations shown in Table 2. However, the assay does not detect individual cells or differentiate between cell populations. It measures the proliferation of all the cells in culture (to measure proliferation of individual cell populations, separate assays must be used). Sufficient HemoLIGHT™ Master Mix volumes are provided to culture as many samples at the required number of replicate wells that will fit on to a single 96-well plate. Depending a the number of replicates required, a specific volume of HemoLIGHT™ Master Mix is dispensed into a sterile plastic tube followed by a 10% volume of the cell suspension adjusted to the correct working concentrating. The contents of the tube(s) are mixed and 0.1ml dispensed into replicate wells. The plates are incubated for 5-7 days. However, once a cell incubation time has been chosen and optimized, it should not be changed, since it will be difficult to compare results.

Step 3 – Absorbance measurement
To measure absorbance, the MTS reagent is first thawed, gently mixed and 20µl dispensed into each replicate well. The plate is returned to a 37°C incubator for 1-4 hours. A 3 hour incubation time is usually sufficient. After incubation the absorbance is measured in a plate reader with an absorbance filter of 490nm. A HemoLIGHT™ PCA Base Medium is provided together with a non-sterile, 96-well plate in order to measure the background absorbance, which is subtracted from absorbance of the sample.

7. Kit Contents and Storage Conditions

HemoLIGHT™ 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	HemoLIGHT™ PCA Master Mix for an individual cell population	-20°C until used
2	HemoLIGHT™ Base Medium for background absorbance measurement.	-20°C until used
3	MTS Reagent	-20°C, protected from light until used
4	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
5	Clear sterile, individually wrapped, 96-well plate for cell culture	Can be kept with other kit components
6	Clear, non-sterile 96-well plate(s) for background measurement.	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.

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™.

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.

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

Equipment and Supplies

1. Laminar Flow Biohood
2. Absorbance plate reader or multimode plate reader with the ability to measure absorbance at 490nm.
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. Ficoll-Paque, Lymphoprep).
4. 7-AAD, propidium iodide or trypan blue for viability assay.
5. LIVEGlo™ metabolic viability assay (Preferred Cell Systems™)

9. The HemoLIGHT™ PCA Protocol

PLEASE READ THE FOLLOWING PROTOCOL CAREFULLY.
SEE SECTION 10 BEFORE PERFORMING THE PROTOCOL.

Performing HemoLIGHT™ PCA is a 3-step process.

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

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

STEP 1 – Cell Preparation

HemoLIGHT™ 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) possible interference of high red blood cell concentrations with the absorbance readout resulting in false positive results.
2. Mononuclear cell (MNC) fraction is the cell preparation of choice. This fraction can be prepared by density gradient centrifugation, e.g. Ficoll-Paque, 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 HemoLIGHT™ PCA Culture Master Mix prepared.

TABLE 1

Recommended Cell Doses for Cell Types, Cell Preparations and Cell States for HemoLIGHT™ 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

Species	Cell Type	Cell Preparation	Cell State	Working Cell Concentration Required (100 x Final Cells/Well)	Final Cell Dose / Well
	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. HemoLIGHT™ PCA Cell Culture Procedure

- *HemoLIGHT™ 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.*

The HemoLIGHT™ PCA Methodology

1. Remove the HemoLIGHT™ 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 HemoLIGHT™ PCA Master Mix minus 10%, that will allow 0.1mL to be dispensed into the required number of replicate wells. See Table 2. For example, if 4 replicate wells are to be prepared, dispense 0.405mL of the HemoLIGHT™ PCA Master Mix into each tube. This would be followed by 0.045mL of cells to produce a total volume of 0.45mL.

TABLE 2

Number of Replicate Wells Required	Volume of HemoLIGHT™ PCA Master Mix	Volume of Cells (10% of final volume)	Total Volume
2	0.315mL	0.035mL	0.35mL
4	0.405mL	0.045mL	0.45mL
6	0.585mL	0.065mL	0.65mL

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 x 5,000 or 500,000 (5 x 10^5) cells/mL.
5. Dispense the required volume of cells into each tube containing the HemoLIGHT™ PCA Master Mix.
6. Mix the contents gently on a vortex mixer. Do not cause bubbles.
7. Remove the sterile, individually wrapped, clear 96-well plate from the assay kit box.
8. Using a calibrated pipette, preferably an electronic pipette with repeat function, dispense 0.1mL into the bottom of each of the required number of replicate wells of the 96-well plate. Do not dispense on the wall of the well.
   TIP: If preparing 2 or 4 replicates, dispense either 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 10).
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. Low oxygen tension improves plating efficiency by reducing oxygen toxicity cause by the production of free radicals.
11. Incubate the cells for the time shown in Table 3.

TABLE 3

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

STEP 3 – Absorbance Measurement

1. Remove the MTS reagent and the HemoLIGHT™ PCA Base Medium from the freezer and thaw at room temperature or in a beaker of water at room temperature. IMPORTANT: The MTS reagent is light sensitive and should be kept in an amber container. After several hours of light exposure, the reagent can discolor leading to a higher background absorbance at 490nm.
2. If possible, place the sample plate(s) in a humidified incubator set at 22-23℃ gassed with 5% CO_2 for 30min to equilibrate or allow to come to room temperature. Alternatively, place the sample plate(s) in the dark at room temperature for 30min.
3. If only part of the culture 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 provided with the kit and remove the backing foil. Layer the adhesive side on the plate to 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 experiment.
4. Using the non-sterile, 96-well plate provided with the kit, dispense 0.1ml of the HemoLIGHT™-96 Base Medium into each of 6 replicate wells. This will provide the background absorbance.
5. Accurately dispense 20µl (0.02ml) of the MTS Reagent into each of the 6 background replicate wells.
6. Dispense 20µl of the MTS Reagent into each of the sample wells. If a large number of samples are to be processed, pour the MTS Reagent into a reservoir and use a multichannel pipette to dispense 20µl of the MTS Reagent into each well.
7. Keeping the pipette tip(s) below the level of the liquid in the wells, gently mix the contents by repeated pipetting, making sure not to cause bubbles.

8. After dispensing the MTS Reagent for each sample and mixing, change the pipette tip(s).
9. Repeat this procedure for each column or row using new tips.
10. When the MTS Reagent has been dispensed into all sample wells, replace the plastic lid.
11. Incubate both the background and sample plates at 37°C for 1-4 hours. The plates can be removed from the incubator to measure the absorbance at any time during this period and then replaced back into the incubator.
12. A time point is elected when the absorbance does not change signficantly. This time period occurs at about 3 hours. For all future experiments using the same cells, this time period be constant.
13. Unused MTS Reagent can be refrozen up to 10 times without significant loss of activity.
14. Unused Base Medium can be either stored frozen until the expiration date or kept at 4-8°C for one month.
15. Measure the background absorbance at 490nm prior to measuring the absorbance of the sample(s).
16. Subtract the background absorbance from the absorbance obtained from the samples to yield the corrected absorbance.

10. Recommendations and Tips Prior To Using HemoLIGHT™ PCA.

(i) Cell Suspension

a) The preferred cell suspension is a mononuclear cell suspension (MNC).
b) Extraneous ATP, red blood cells and hemoglobin can interfere with the results. 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

Any number of replicate wells can be used. Preferred Cell Systems™ recommends a minimum of 4 replicates/sample, although 6 replicates will provide better statistics. 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

Using 2 or 4 replicates/sample can be performed either in columns or rows across the plate. If 6 replicate wells/sample are used, 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 HemoLIGHT™ PCA kit. Please do not replace the plates included with the kit with those of another manufacturer. Cell growth and absorbance 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 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 depend on the cell population being tested. For progenitor cells, i.e. non-stem cell populations, a 5-day incubation is sufficient. For more primitive cell populations, i.e. stem cells, the incubation time can be extended to 7 days to increase sensitivity. However, this may be accompanied by increased coefficients of variation (%CVs).

(vii) 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.

11. Results

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

HemoLIGHT™ PCA can be used on fresh or frozen cells. If testing cells prior to cryopreservation, it should be emphasized that results, produced using HemoLIGHT™ 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 lower after thawing a cryopreserved sample than a fresh sample.

The ability of cells to reduce a tetrazolium substate such as MTS, is a direct indication of their metabolic viability. If cells after culture have low absorbance values or cannot be shown to reduce the tetrazolium substrate, they will not be able to proliferate sufficiently to result in engraftment after transplantation.

It is therefore recommended that sufficient cord blood, bone marrow or peripheral blood samples be tested (historical values) to obtain in-house acceptance/rejection criteria of cell units to be cryopreserved, so that upon eventual thawing, the cells will provide the proliferation ability necessary to be used for patients.

12. Troubleshooting

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.

Inadequate Cell Culture

- *Inadequate cell growth:* Cells did not exhibit sufficiently high viability. Measure cell viability prior to adding the cells to the master mix. 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 as indicated in Section 6 of this manual.
- *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 clear 96-well plates can be viewed under a microscope. If contamination occurs it will usually be seen by the difference in color of the cultures. Contaminated cultures will usually be bright yellow in color and probably cloudy in appearance. Cell cultures that demonstrate high proliferation will also 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 absorbance values.

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 10 (v) for instructions on how to build a humidity chamber.

Other Hematopoietic Cellular Therapy Assay Products from Preferred Cell Systems™

HemoGLO™ PCA an ATP bioluminescence assay version of HemoLIGHT™ PCS with the option to standardize the assay.

HemoFLUOR PCA is a 96-well, fluorescence readout version of HemoLIGHT™ PCS.

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 peripheral blood reference standards for HALO® 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, **HemoGLO™**, **HemoFLUOR™ TE** and **HemoLIGHT™ TE** are time to engraftment assays.

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

Ordering Information

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