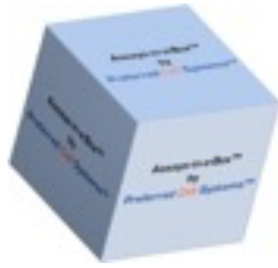

Assays-in-a-Box™
by
Preferred Cell Systems™

HemoLIGHT™

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

For Individual and "Global" Assay Kits

Technical Manual

(Version 10-23)

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

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

No part of this instruction manual may be copied, duplicated or used without the express consent of Preferred Cell Systems™

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1. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. *HemoLIGHT™ is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA)*
2. *HemoLIGHT™ 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™ is a proliferation assay platform that detects and quantitatively measures stem and progenitor cells of the blood-forming (lympho-hematopoietic) system.

HemoLIGHT™ incorporates a fluorescence readout that measures the activity of a constitutive live-cell protease that is present in intact viable cells. The fluorogenic peptide substrate enters cells and is cleaved by the protease activity to generate a fluorescence signal at 505nm after excitation between 380 and 400nm.

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.

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

HemoLIGHT™ is presently available to detect multiple stem cell and progenitor cell populations from up to 8 different species. In addition, HemoLIGHT™ "Global" Assays are also available than can be used to detect and measure 4-, 5- or 7-cell populations simultaneously.

3. Use and Availability

HemoLIGHT™ 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, HemoLIGHT™ can be used instead of the colony-forming cell (CFC) or Unit (CFU) assay. HemoLIGHT™ can also be used for hematopoietic cell therapy applications. However, specialized HemoLIGHT™ assays have been designed for this purpose.

HemoLIGHT™ can be used for the following tissues:

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

HemoLIGHT™ is available for the following species:

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

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

HemoLIGHT™ can be used with cells that have the following degrees of purity:

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

If possible, it is recommended to use mononuclear cells 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 HemoLIGHT™ 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 HemoLIGHT™ cultures, the following equivalent cell populations can be detected using HemoLIGHT™

Cell Populations Detected using HemoLIGHT™

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

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

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

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

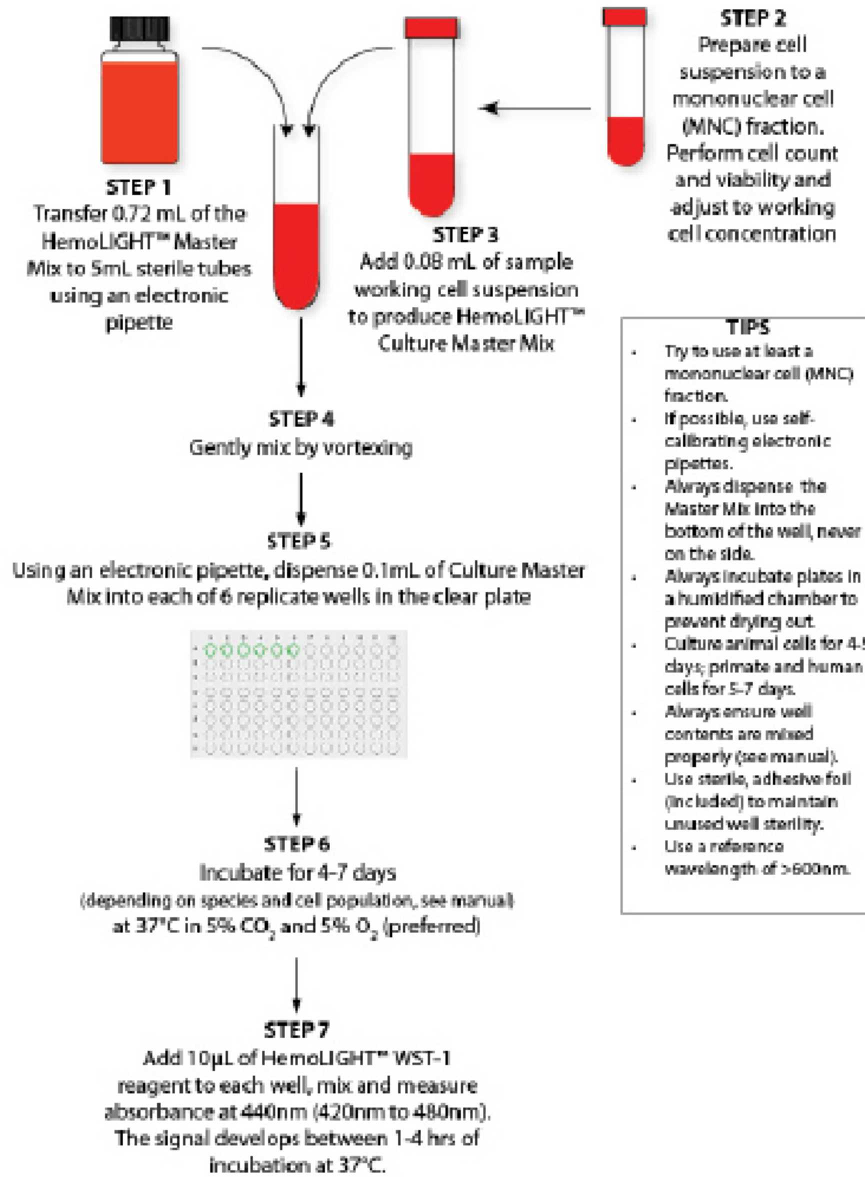
4. The Concept of HemoLIGHT™ Assays

HemoLIGHT™ 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™ 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 occurs at about 3 hours after addition of the MTS reagent.

5. QuickGuide to HemoLIGHT™ (Figure 1)



6. Overview of the HemoLIGHT™ Procedure

There are 3 steps to use HemoLIGHT™.

Step 1 – Cell Preparation
Cells are not provided with HemoLIGHT™ 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™ assay contains a Master Mix to stimulate the cell populations shown in Section 3. 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 on 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 concentration. 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 WST-1 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 3-4 hour incubation time is usually sufficient. After incubation the absorbance is measured in a plate reader with an absorbance filter of 490nm. A HemoLIGHT™ 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™ 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™ Master Mix for an individual cell population	-20°C until used
2	HemoLIGHT™ Base Medium for background absorbance measurement.	-20°C until used
3	WST-1 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™ Protocol

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

Performing HemoLIGHT™ is a 3-step process.

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

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

STEP 1 – Cell Preparation

HemoLIGHT™ 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 using NycoPrep 1.077 (Axis-Shield). Ficoll-Paque can also be used. However, if cells are not washed after Ficoll, the latter can be toxic to the cells.
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™ or IMDM. The volume of the adjusted cell suspension required will be 10% of the total volume of HemoLIGHT™ Culture Master Mix prepared.

TABLE 1

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

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	$0.5\text{-}0.75 \times 10^6$	5,000-7,500
	Peripheral blood	MNC	Fresh/Frozen	$0.5\text{-}0.75 \times 10^6$	5,000-7,500

Species	Cell Type	Cell Preparation	Cell State	Working Cell Concentration Required (100 x Final Cells/Well)	Final Cell Dose / Well
	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
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, 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

STEP 2. HemoLIGHT™ Cell Culture Preparation

- HemoLIGHT™ Master Mixes are complete and ready-to-use.
- Perform all procedures under a laminar flow, bio-hazard hood.
- Wear protective clothing, including gloves for all operations.

The HemoLIGHT™ Methodology

1. Remove the HemoLIGHT™ 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 a 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. Therefor, if 4 replicate wells are to be prepared, dispense 0.405mL of the HemoLIGHT™ 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™ 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 times 5,000 or 500,000 (5×10^5) cells/mL.
5. Dispense the required volume of cells into each tube containing the HemoLIGHT™ Master Mix.
6. Mix the contents gently on a vortex mixer. Do not cause bubbles.
7. Remove a sterile, wrapped, 96-well plate from the assay kit box.
8. Using a calibrated pipette, preferably an electronic pipette with repeat function, dispense 0.1mL into each of the required number of replicate wells of the 96-well plate.
   TIP: If preparing 2, 4 or 6 replicates, dispense across the plate in rows. If preparing 8 replicates, dispense in columns across the plate. This allows for the maximum number of samples/plate.
9. After replacing the lid, transfer the 96-well plate to a humidified container (see Section 9).
10. Transfer the humidified container to the incubator. The incubator should be fully humidified at 37oC and gassed with 5% CO_2 and, if possible, 5% O_2. This reduced oxygen tension improves plating efficiency by reducing oxygen toxicity cause by the producing of free radicals.
11. Incubate the cells for the time shown in Table 3.

TABLE 3

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

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

- These HemoLIGHT™ assay kits include 4, 5 or 7 sterile, wrapped, 96-well plates, plus an extra plate that is used for background controls.

- Each plate is used for a separate cell populations. Thus, for a 4-population assay kit, each plate will include HemoLIGHT™ Master Mixes for SC-GEMM, P-BFU, P-GM and P-Mk plus a Background Control Master Mix, which does not include any growth factors.
- Prepare separate tubes for each cell population and for each sample to be tested.
- In preparing the tubes, use the same HemoLIGHT™ methodology as above.

STEP 3 – Absorbance Measurement

1. Remove the MTS reagent and the HemoLIGHT™ Base Medium from the freezer and thaw at room temperature or in a beaker of water at room temperature. **IMPORTANT**: The WST-1 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™ Base Medium into each of 6 replicate wells. This will provide the background absorbance.
5. Accurately dispense 10µl (0.02ml) of the WST-1 Reagent into each of the 6 background replicate wells.
6. Dispense 10µl of the WST-1 Reagent into each of the sample wells. If a large number of samples are to be processed, pour the WST-1 Reagent into a reservoir and use a multichannel pipette to dispense 10µl of the WST-1 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 WST-1 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 WST-1 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 13ignificantly. This time period occurs at about 3 hours. For all future experiments using the same cells, this time period should be constant.
13. Unused WST-1 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 440nm 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.

Phenotypic Analysis by Flow Cytometry

HemoLIGHT™ is a non-destructive assay, i.e. it does not lyse the cells. As a result, the cells growing in culture can be analyzed phenotypically by flow cytometry after the fluorescence measurement has been performed.

The cells from replicate wells can be removed and pooled together prior to the addition of fluorochrome-conjugated antibodies. Alternatively, fluorochrome-conjugated antibodies can be added directly to each well so that phenotypic analysis can be performed on individual cell cultures using a mutli-well sampler.

10. Recommendations and Tips Prior To Using HemoLIGHT™.

12. 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™ 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 depends 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.

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11. 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.

NOTES

Ordering Information

Toll free: 1-888-436-6869
Tel: (719) 264-6251
Fax: (719) 264-6253
Email: info@preferred-cell-systems.com
Order online at preferred-cell-systems.com

Technical Support

Tel: (719) 264-6251
Email: info@preferred-cell-systems.com

Preferred Cell Systems™

1485 Garden of the Gods Road
Suite 152
Colorado Springs, CO 80907
U.S.A.
Website: www.preferred-cell-systems.com

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