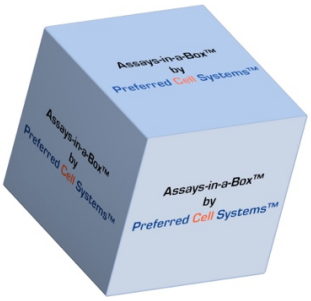


Assays-in-a-Box™
by
Preferred Cell Systems™

# HepatoLight™

## An In Vitro Assay for Liver Cells
## Incorporating a WST-1 Absorbance/Colorimetric Readout

Technical Manual

(Version 03/24)

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

## 2. Introduction

Under normal, steady-state conditions, hepatocytes demonstrate little or no proliferation. However, hepatocytes exhibit a high degree of metabolic activity that can be measured using different biochemical reagents.

HepatoLight™ incorporates a tetrazolium salt called WST-1, which is cleaved by a complex cellular mechanism primarily at the cell surface, to form a soluble formazan that is measured in an absorbance plate reader between 420nm and 480nm. The reaction is dependent on the metabolic activity and therefore the metabolic viability of the cells.

HepatoLight™ can be used with different sources of hepatocytes from different species. Since no single maintenance and growth medium is available for all sources of hepatocytes, HepatoLight™ provides the user with the flexibility to use their own maintenance and growth medium.

## 3. Use and Availability

HepatoLight™ is a research tool to measure changes in hepatocyte viability due to different conditions, e.g. compounds and perturbations, growth conditions etc..

HepatoLight™ can be used for research purposes to study metabolic/viability changes in the following types of hepatocytes from different species:

- Fresh heptocytes
- Cryopreserved hepatocytes
- iPS- or ES-derived hepatocytes
- Hepatocytes cell lines, e.g. HEP G2

**IMPORTANT:**

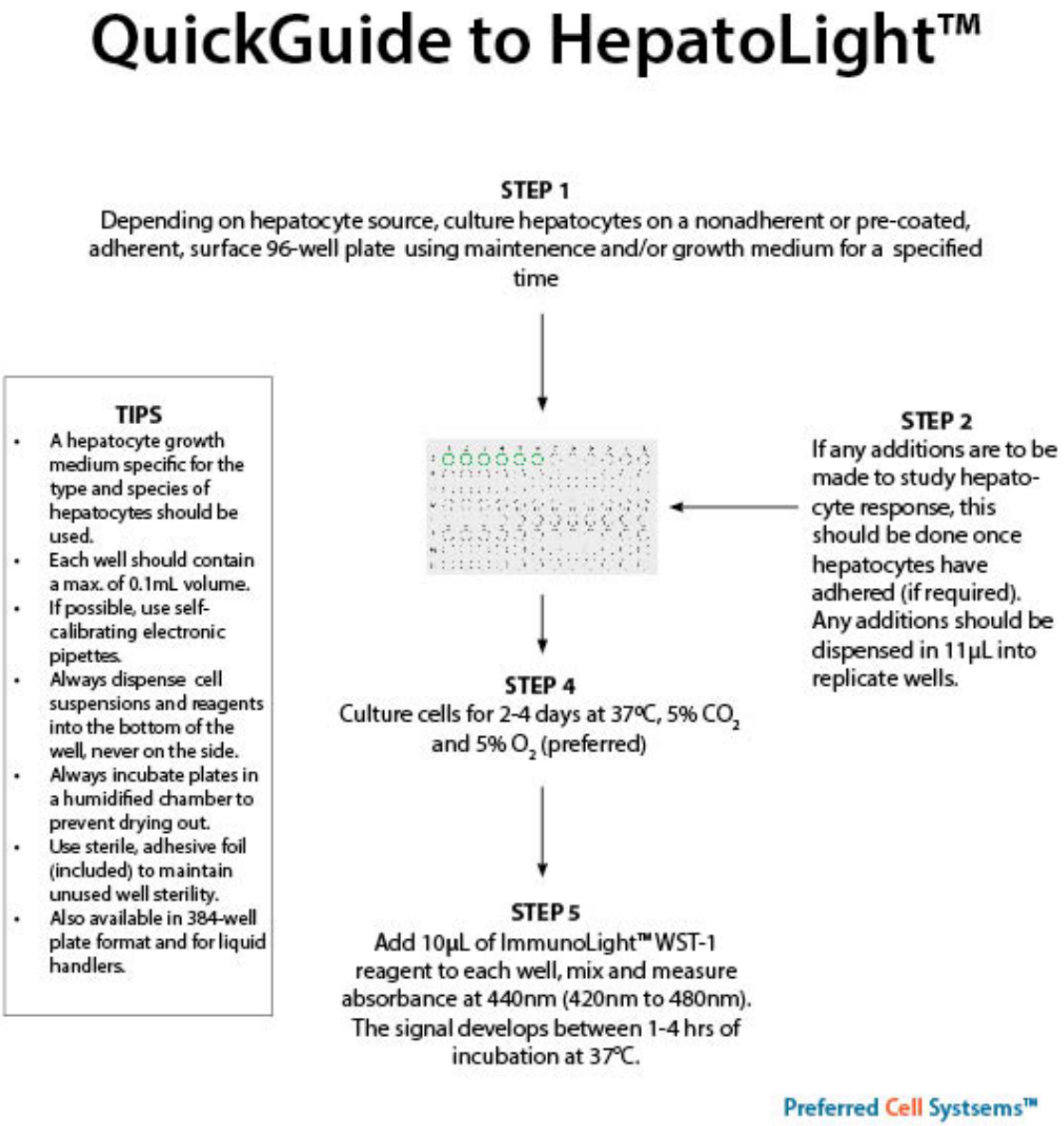
**HepatoLight™ is for research use only and has not been approved for any clinical diagnostic use.**

## 4. Principle of HepatoLight™ and the WST-1 Absorbance Readout

HepatoLight™ is a colorimetric/absorbance *in vitro* assay. All cultures are setup in a total volume of 0.1mL/well. Background and other controls should always be included in any experiment.

Depending on the type of study, hepatocytes might be incubated either under non-adherent or adherent conditions. Hepatocytes should be left to adhere to a collagen surface. It is necessary to pre-treat the included 96-well plates with collagen under sterile conditions. Hepatocytes should be left to adhere for 6-24 hours prior to use for an experiment. Once the incubation period has elapsed, 10µL of the ready-to-use WST-1 reagent is added to each well, mixed and further incubated. WST-1 is a colorimetric/absorbance in vitro assay. It incorporates a customized reagent from Roche. The reagent contains a tetrazolium compound salt, WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate). In the presence of an electron coupling reagent (succinate-tetrazolium reductase system), WST-1 is cleaved to a soluble formazan by a complex cellular mechanism that occurs primarily at the cell surface. When added to metabolically viable cells WST-1 is reduced. This bioreduction is largely dependent on the glycolytic production of NADPH in viable cells. The cells are incubated with WST-1 for 1-4 hours. Longer incubation periods may be required. This is especially important for cells exhibiting low metabolism. The plate can be removed from the incubator at different times to measure optimal absorbance. The absorbance is measured between 420-480 nm (max. absorption at about 440 nm) in a 96-well plate reader. A background with medium, but no cells should be included and subtracted from the sample absorbance. A reference wavelength of more than 600nm can also be used.

## 5. QuickGuide™ to HepatoLight™ (Figure 1)



## 6. Kit Contents and Storage

HepatoLight™ 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 | WST-1 Reagent | Store at -20°C, protected from light. Once thawed, the reagent may be stored at 4°C, protected from light, for up to 4 weeks. The reagent may become viscous. Warm reagent for 2-10min at 370C. For longer storage periods, store at -200C. |
| 2 | Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile. | Can be kept with other kit components |
| 3 | 1 x Sterile, clear, 96-well plates for cell culture | Can be kept with other kit components |
| | Technical manual | Download from the Preferred Cell Systems, ImmunoLight™ MLC webpage |

Exact volumes of the 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™.**

**This kit has an expiry date on the box. 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 purchased from Preferred Cell Systems™.**

## 7. Equipment, Supplies and Reagents Required, But Not Provided

**Equipment and Supplies**

1. Laminar Flow Biohood
2. Plate reader for absorbance at between 420 and 480 nm with a reference wavelenth of >600nm.
3. Sterile, capped, plastic tubes (5mL, 10mL, 50mL)
4. Single channel pipettes, preferably electronic (e.g. ViaFow or Rainin EDP pipettes for variable volumes between 1µl and 1000µl).
5. 8 or 12-channel pipette, preferably electronic (e.g. ViaFlow or 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. Hemocytometer or electronic cell counter to determine cell concentration.

**Reagents Needed, but Not Included with the HepatoLight™ Assay Kit**

1. Rat tail collagen 1 (Gibco). Only required for adherent plates.
2. Acetic acid. To dilute collagen. Only required for adherent plates.
3. Sterile, cell culture grade water. Only required for adherent plates.
4. Maintenance and/or growth medium for hepatocytes.
5. Sterile Phosphate Buffered Saline (PBS)
6. Trypan blue or other dye exclusion viability assay.
7. Hemocytometer.

## 8. The HepatoLight™ Protocol

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

Performing HepatoLight™ is a 4-step process.

Step 1 – Preparation of Collagen-Coated Plates (if required).
Step 2 -- Cell preparation.
Step 3 – Cell culture and incubation in the 96-well plate.
Step 4 – Absorbance measurement.

**Steps 1 to 3 must be performed in a laminar flow biohazard hood**

**STEP 1 - Preparation of Collagen-Coated Plates**

*This step is only required if hepatocytes are grown on an adherent surface. If non-adherent plates are used, go to Step 2.*

*THIS STEP MUST BE PERFORMED UNDER STERILE CONDITIONS.*

Rat tail collagen 1 (Gibco. 3mg/mL) should be used to coat the wells of the 96-well plate that is included with the assay kit.

The following is a general protocol:

1. Prepare a 0.02M solution of acetic acid and sterile water.
2. Sterile filter the solution through a PP, teflon or PVDF filter membrane.
3. Dilute 3mg/mL rat tail collagen 1 in 0.02M acetic acid to a stock concentration of 100µg/mL.
4. Dispense 40µL of the diluted collagen solution to each well of the 96-well plate provided.
5. Tap the plate to distribute the solution evenly in each well.
6. Incubate for 1 hours or more at room temperature.
7. Completely aspirate all the solution from each well.
8. Rinse each well 3 times with sterile water.
9. Air dry plate and store at 4°C for up to 7 days prior to use.
10. On day of use, equilibrate the plate to room temperature.

**NOTE: If using primary hepatocytes, it may be advantageous to overlay hepatocytes with Matrigel if culture incubation is to be in excess of 4-5 days.**

**STEP 2 – Cell Preparation**

Hepatocytes are not provided with the HepatoLight™ Assay Kit. It is recommended to procure hepatocyte target cells from a reputable vendor and follow the instructions provided with the cells.

Cell Viability, Cell Counting and Cell Culture Suspension Preparation

1. For dye exclusion viability methods, use trypan blue and a hemocytometer.
   *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%.
2. Determine the cell concentration using either a hemocytometer or electronic cell/particle counter.
3. Adjust the cell suspension concentration to the desired working cell concentration recommended by the vendor. The working cell contration may be 10-100 fold greater than the final cell concentration/well.

**STEP 3. HepatoLight™ Cell Culture Protocol**

**Perform all procedures under a laminar flow, bio-hazard hood.**
**Wear protective clothing, including gloves for all operations.**
**Use calibrated pipettes and sterile tips throughout.**

1. If hepatocytes need to be cultured under adherent conditions, the cells should be plated and left to adhere prior to any additional additions or perturbations.
2. Adjust the working cell concentration such that the final cell concentration will be contained in 0.1mL/well.
3. Leave the cells to adhere for 6-24h. This will vary depending on the source of hepatocytes.
4. After adherence, carefully remove the medium and replace with 0.1mL of fresh medium.
5. If any additions are to be added, use a calibrated (preferrably electronic) pipette. Any addition should be contained in 11µL to each replicate well.
6. Ensure that the respective controls are also included.
7. If required, Step 4.3 can also be performed here.
8. After replacing the lid, transfer the 96-well plate(s) to a humidified container (see Section 9 (v)). Transfer the humidified container to the incubator.
9. Incubate for the desired time period, usually 24-48 hours, but may range from hours to several days.
10. Cells are usually incubated at 37oC in a humidified chamber in an atmosphere containing 5% $CO_2$. However, depending on the location of the hepatocytes, they may be subjected to different concentrations of oxygen. To reduce potential oxygen toxicity and increase plating efficiency, culturing the cells under low oxygen tension (e.g. 5% $O_2$) may be beneficial.

**STEP 4 – Measurement of Hepatocyte Viability/Metabolism using WST-1**

Perform the following steps for sample measurement.

1. Remove the WST-1 reagent from the freezer and thaw at room temperature. If precipitation or turbidity is observed upon thawing, warm the reagent to 370C for 2-10 min and mix gently to dissolve the precipitates. Centrifugation is not recommended because the working concentration would decrease.
2. Remove the 96-well plate from the incubator.
3. If only part of the plate has been used, transfer the plate to a hood/laminar air flow bench and attach the sterile, adhesive film from the HepatoLight™ assay kit box as follows:
   (a) Remove the lid from the 96-well plate.
   (b) Remove the backing from the adhesive foil.
   (c) Carefully layer the foil over the plate so that it covers all of the wells and press down the foil over each well.
   (d) Using a sharp blade or knife, cut between the wells that contain the cultures.

   (e) Gently peel away the adhesive foil from the wells containing the cultures.
   (f) This will leave the remaining foil over the unused wells, which will remain sterile for later use.

4. Using a calibrated pipette, add 10µL of the WST-1 reagent to each well, including the wells used for the blanks. Mix gently using the same pipette tip. After each addition, make sure to change the pipette to avoid any carry-over from one well to another.
5. Once the WST-1 reagent has been added to all culture wells, replace the lid and transfer the 96-well plate to the incubator.
6. Incubate the cells for 1-4 hours. Longer incubation times may be necessary. At any time during the color development, the plate may be removed and the absorbance measured at about 440nm. NOTE: Make sure to measure the blank and subtract the value from the sample absorbance value (see below).
7. The absorbance can be plotted against incubation time to determine the optimized incubation time for future assays. NOTE: The absorbance for the blank will increase with time. At 0 hr, the absorbance will be about 0.1 absorbance units. After 4hr, the absorbance will be between 0.2 and 0.3 absorbance units.

## 9. Recommendations and Tips Prior to Using HepatoLight™.

(i) Always perform a cell dose response to determine the optimal cell concentration to use.

(ii) Number of Replicates Performed

The number of replicates/sample is arbitrary. However, for statistical purposes, 4-6 replicates/sample are recommended. 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

If performing 4 replicates/sample, plate the samples in vertical columns, e.g. A1, B1, C1, D1. If performing 6 replicates/well, the samples are be plated across the plate, for example from A1 to A6, A7 to A12 or B1 to B6.

(iv) 96-Well Plates Provided

The reagents have been optimized to work with the 96-well plate(s) provided. Other plates can be used. However, cell growth and absorbance output can be seriously affected and the assay kit warranty will be void. Additional plates can be purchased from HemoGenix® if required.

(v) Humidity Chamber

If cell incubation time are greater than 3 days, a humidity chamber is recommended due to sample volume evaporation. 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”. A humidity chamber

can be assembled using plastic lunch boxes or other plastic ware 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 HemoGenix® for further information about assembling and using humidity chambers.

## 10. 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 occur, large variations between replicates should not be obtained. Please consider the following:

- Accurate reagent dispensing and mixing are of prime importance. Small volumes are dispensed and the use of instruments that have not been calibrated correctly or have not be calibrated for a long period of time, can lead to high CVs.
- Insufficient mixing of components prior to and during plating should be performed. Use repeater pipettes where possible. Use calibrated or self-calibrating electronic pipettes or dispensers to add and mix reagents.
- Perform a minimum of 6 replicates per point.

Inadequate Cell Culture

- *Inadequate cell growth:* Cells did not exhibit sufficiently high viability. Measure cell viability prior to adding the cells to the culture media. Ensure that the viability is high prior to culture. If using dye exclusion viability, cells should exhibit approx. 85% viability.
- *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 Section 9 (v) and below).
- *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 about 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/or 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 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 (see Section 9 (v)).

**Ordering Information**

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