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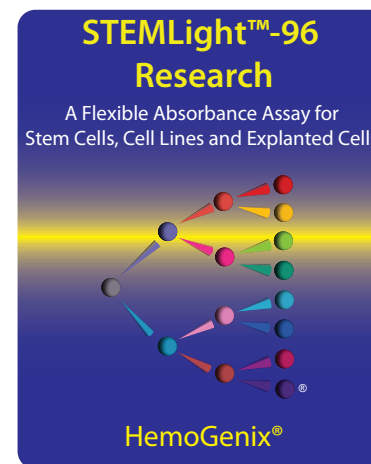
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STEMLight™-96 Research

An proliferation, viability and functionality absorbance assay for stem cells, cell lines, and *ex vivo* primary cells

Assay Manual

(Version 8-2014)

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

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10. TROUBLESHOOTING

High Coefficients of Variation (%CV)

Coefficients of variation (%CV) should be $\leq 15\%$. The percent coefficient of variation is calculated as standard deviation/mean $\times 100$. High %CVs are usually an indication of incorrect dilutions or pipetting error. Although outliers can be obtained, 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 been 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 master mix. 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 (iv) 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 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 RLU 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.

1. INTRODUCTION

STEMLight™-96 Research is a flexible, proliferation, functionality, metabolic viability and cell number assay for primary stem cells, cell lines, tumor cells and explanted cells types from various organs and tissues.

STEMLight™-96 Research allows the investigator to use their own reagents and culture protocols. Although STEMLight™-96 Research include sterile 96-well plates for culturing cells, the investigator can grow and expand cells in other culture vessels and use the 96-well plates to monitor viability, cell functionality and proliferation status. STEMLight™-96 Research can also be used to monitor cell growth kinetics and single cell expansion during limiting dilution studies.

STEMLight™-96 Research 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.

STEMLight™-96 Research is one of three signal detection systems available for use with immune cells. The others are STEMFluor™-96 Research and STEMGlo™-96 Research. The former uses an fluorescence readout, while the latter incorporates Bioluminomics™ technology and offers the greatest sensitivity. Bioluminomics™ is the instrument-based, non-subjective measurement of iATP concentrations using a luciferin/luciferase bioluminescence signal detection system.

2. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

1. **STEMLight™ is not approved by either the U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMA).**
2. **STEMLight™ is for research use only and has not been approved for clinical diagnostic use.**
3. **This kit should not be used beyond the expiration date on the kit label.**
4. **Do not mix or substitute reagents or other kit components from other kit lots or sources.**
5. **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.**
6. **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.**

9. RECOMMENDATIONS and TIPS PRIOR to USING the STEMLight™-96 Research ASSAY KIT.

- (i) **Number of Replicates Performed**
The number of replicates/sample is arbitrary. For statistical purposes, 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 can be the case especially with primary cells, using a small number of replicate wells may result in loss of statistical significance. As a consequence, additional experiments might be needed resulting in extra time and costs.
- (ii) **Plate Configuration**
Performing 6 replicates/well means that the samples can be plated across the plate, for example from A1 to A6, A7 to A12 or B1 to B6. In this manner 16 samples can be tested on a single plate.
- (iii) **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.
- (iv) **Humidity Chamber**
If cell incubation times are greater than 3 days, a humidity chamber is recommended due 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.

- 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.
- Using the non-sterile, 96-well plate provided with the kit, dispense 0.1 ml of the same base culture medium used to culture the cells into replicate wells. 4-6 wells should be sufficient. This will provide the background absorbance.
 - Accurately dispense 20 μ l (0.02ml) of the MTS Reagent into each of the background replicate wells.
 - Dispense 20 μ l 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 20 μ l of the MTS Reagent into each well.
 - After dispensing the MTS Reagent for each sample, change the pipette tip(s).
 - Repeat this procedure for each column or row using new tips.
 - When the MTS Reagent has been dispensed into all sample wells, replace the plastic lid.
 - 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.
 - Unused MTS Reagent can be refrozen up to 10 times without significant loss of activity.
 - Measure the background absorbance at 490nm prior to measuring the absorbance of the sample(s).
 - Subtract the background absorbance from the absorbance obtained from the samples to yield the corrected absorbance.

3. INTENDED USE

STEMLight™-96 Research is intended for measuring cell proliferation and/or cell number of numerous cell types. In addition, since the assay measures a reaction specific to the mitochondria, it is also a metabolic viability and cell functionality assay. Specific applications include, but are not limited to:

- Growth factor requirement and concentrations for growth different cell types.
- Cell proliferation kinetics.
- Stem cell self-renewal and expansion.
- Limiting dilution analysis.
- Cell-based, gene inclusion assays.
- Method procedure evaluation.

Cell Types and Source Used with STEMLight™-96 Research

Virtually any cell type (stem cells, including primary, ES and iPS, cell lines, primary cells, cryopreserved cells) capable of proliferation or having high metabolic activity can be detected and measured using STEMLight™-96 Research. Assay kits are available with sterile tissue culture-treated 96-well plates for adherent cells or sterile, 96-well plates for non-adherent cell types.

STEMLight™-96 Research can be used for different cell types from the following organs and tissues derived from different species:

- Bladder
- Brain
- Breast
- Eye
- Gut
- Heart
- Kidney
- Lung
- Liver
- Ovary
- Prostate
- Skin
- Embryonic tissue
- Fetal tissue

Other cell types include:

- Proliferating cell lines
- Tumor cells
- Embryonic stem (ES) cells
- Induce pluripotent stem (iPS) cells

IMPORTANT:

STEMLight™-96 Research is for research use only and has not been approved for clinical diagnostic use.

4. The CONCEPT and PRINCIPLE of the STEMLight™-96 Research ASSAY

STEMLight™-96 Research is a colorimetric/absorbance *in vitro* assay. It incorporates a reagent that 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. HemoGenix® 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 metabolic activity of the cells.
- The proliferation potential (or primitiveness) of the cell population being detected.
- The growth factor(s) and/or cytokine(s) used to stimulate the cells (if any).
- The plated cell concentration.

The cell sample can be cultured in the sterile 96-well plate(s) provided with the kit. When the culture period has elapsed, the cells are processed by adding just 20µl of the MTS reagent and further incubating the cells for 1-4 hours to develop the color reaction.

STEP 2. STEMLight™-96 Research Cell Culture

Please refer to Section 9 for recommendations and tips prior to beginning this stage of the procedure.

Perform all cell culture under sterile conditions in a biosafety cabinet.

STEMLight™-96 Research assay kits are available with either adherent or non-adherent, sterile 96-well plates. Please ensure that the correct plates are used for type of cells being investigated.

Cell culture should be performed according to the investigator's own protocols. The following is a general procedure for culturing cells in the 96-well plates provided.

1. Prepare a master mix of culture components without the cells. Ensure that the concentrations of all components are increased such that when dispensed into the wells of a 96-well plate, the correct final concentrations and doses are attained. Depending on the number of replicate wells to be assayed, prepare the master mix with approx. 10-20% increase in volume.
2. Prepare the cell suspension as required and adjust the cell concentration to a working cell concentration.
3. Prepare and label one 5ml tube for each sample to be assayed.
4. Dispense the master mix of culture components into the tube followed by the appropriate cell volume. For example, if 8 replicates are to be assayed, dispense 0.9ml of the culture component master mix and 0.1 ml of the cell suspension.
5. Mix the contents of each tube thoroughly using a vortex mixer.
6. Remove the sterile, clear 96-well plate from plastic covering under the hood.
7. Dispense 0.1ml of the Cell Culture Master Mix into each of the replicate wells.
8. Place the lid on the 96-well plate and transfer the culture plate to a humidity chamber to ensure high humidity during incubation (See section 9(iv)).
9. Place the humidity chamber into a fully humidified incubator set at 37°C and gassed with 5% CO₂ and, if available, 5% O₂. Culturing cells under low oxygen tension is usually advantageous because it reduces the production of dangerous free radicals.
10. Incubate the cells for the required period of time.

STEP 3 – ABSORBANCE MEASUREMENT

1. Remove the MTS reagent from the freezer and thaw at room temperature or in a 37°C water bath. **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°C gassed with 5% CO₂ 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

8. The STEMLight™-96 Research PROTOCOL

**PLEASE READ THE FOLLOWING PROTOCOL CAREFULLY.
SEE SECTION 8 BEFORE PERFORMING AN ASSAY**

Performing STEMLight™-96 Research is a 3 step process.

- Step 1** – Cell preparation.
- Step 2** – Cell culture
- Step 3** – Absorbance measurement.

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

STEP 1 – Cell Preparation

Cells should be prepared according to the investigator's own protocol. Different organs and tissues usually require a specific protocol for preparing a single cell suspension. Regardless of the protocol, it will be necessary to measure the cell viability and cell number prior to culturing the cells. It is not recommended to plate a cell concentration based on viability. This will result in a greater cell concentration (consisting of many dead cells) being plated.

It should be emphasized that the type of viability method used can influence the result of the assay. There are essential two viability methods:

- Dye exclusion viability
- Cellular and metabolic integrity viability.

Dye exclusion viability uses dyes that can enter the cell and usually bind with DNA. The dyes enter the cell due to a leaky cell membrane or loss in membrane integrity due to apoptosis and cell death. Using dyes such as typan blue, propidium iodide, acridine orange and 7-aminoactinomycin D (7-AAD) are membrane integrity assays and do not detect loss of viability due to cellular and mitochondrial integrity.

STEMLight™-96 Research is essentially a cellular and metabolic integrity viability assay because it relies of the cell performing a biochemical reaction based on the reduction of a tetrazolium compound. However, when cells are stimulated or induced into proliferation or are inhibited from proliferating by cytotoxic agents, STEMLight™-96 Research can be used to detect and measure these responses.

Cell Counting and Cell Culture Suspension Preparation

1. Determine the cell concentration using either a hemocytometer or electronic cell/particle counter.
2. Adjust the cell suspension concentration to the desired working cell concentration. This will usually be 10-100 fold greater than the final cell concentration/well. For cell culture, the optimal cell concentration/well should be determined using a cell dose response.

5. OVERVIEW of the STEMLight™-96 Research PROCEDURE

There are 3 steps to use STEMLight™-96 Research.

Step 1 – Cell Preparation

Cells are not provided with STEMLight™-96 Research assay kits. Cells should be prepared with a user-defined, pre-validated protocol to obtain a single cell suspension. The viability of the cells and a nucleated cell count should be performed on all samples.

Step 2 – Cell Culture

The investigator determines how the cells should be cultured. Considerable pipetting error with high coefficients of variation (%CV) can result if individual culture components are added separately to the wells. It is recommended that all of the components required to grow or culture the cells be prepared as a Reagent Master Mix. The cells are then added to the Master Mix and the resulting complete Cell Culture Master Mix dispensed into each replicate well. Sufficient volumes should be prepared for both the Reagent Master Mix and cell suspension to accommodate all replicates wells and samples.

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. The medium used to prepare or culture the cells should be used to measure the background absorbance. This is performed in the non-sterile, 96-well plate provided with the kit. The background absorbance is usually subtracted from the absorbance measured for the sample.

6. KIT CONTENTS and STORAGE CONDITIONS

STEMLight™-96 Research kits contains the following components:

1. Base Iscove's Modified Dulbecco's Medium (IMDM). Store for 2 months at 2-4°C or 1 year at -20°C.
2. MTS Reagent: Store at -20°C, protected from light. If used frequently, the reagent may be stored at 4°C, protected from light, for up to 6 weeks. The reagent can undergo up to 10 freeze-thaw cycles without losing activity.
3. Sterile, clear, flat-bottom, 96-well plate(s) for cell culture.
4. Non sterile, clear, flat-bottom, 96-well plate(s) for background readings.
5. Adhesive Plate Covering—a sterile foil to protect and keep unused wells sterile.
6. Instruction manual.

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 HemoGenix®.

This kit has an expiry date on the box. HemoGenix® 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 HemoGenix®. Please contact HemoGenix®.

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 safety hood.
2. Any absorbance plate reader or multiparameter plate reader equipped with a 490nm absorbance filter can be used.
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. Multichannel pipette, preferably electronic (e.g. ViaFlow or Rainin EDP pipettes for fixed or variable volumes between 10µl and 100µl).
6. Reservoir for multichannel pipette.
7. Sterile pipette tips.
8. Vortex mixer.
9. Tissue culture incubator, humidified at 37°C with 5% CO₂ (minimum requirement) and 5% O₂ (preferable).
10. Hemocytometer or electronic cell counter to determine cell concentration.
11. Flow cytometer or hemocytometer for determining viability.

Reagents

1. Sterile Phosphate Buffered Saline (PBS)
2. Culture/growth medium of choice.
3. Trypsin-EDTA or Accutase (Innovative Cell Technologies, San Diego, CA) to remove adherent cells from the growth surface.
4. Reagent to measure cell viability.