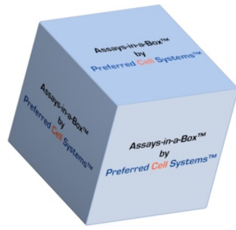

Assays-in-a-Box™
by
Preferred Cell Systems™

HemoFLUOR™

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

HemoFLUOR™ is a proliferation assay platform that detects and quantitatively measures stem and progenitor cells of the blood-forming (lympho-hematopoietic) system.

HemoFLUOR™ incorporates an alamarBlue™ resazurin, blue, non-fluorescence reagent. This enters viable cells and is reduced to red fluorescent resorufin. Fluorescence of viable and/or proliferating cells is measured in a fluorescence plate reader with excitation between 530nm and 560nm and an emission at 590nm. The HemoFLUOR™ Reagent is ready-to-use.

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.

HemoFLUOR™ 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.

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

3. Use and Availability

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

HemoFLUOR™ can be used for the following tissues:

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

HemoFLUOR™ is available for the following species:

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

- Rat
- Mouse

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

HemoFLUOR™ 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 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 HemoFLUOR™ 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 HemoFLUOR™ cultures, the following equivalent cell populations can be detected using HemoFLUOR™

Cell Populations Detected using HemoFLUOR™

- 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

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

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

HemoFLUOR™ “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. Principle of the HemoFLUOR™ Assay

HemoFLUOR™ is a fluorescence *in vitro* cell viability/proliferation assay. It incorporates an alamarBlue™, ready-to-use resazurin non-fluorogenic reagent. Resazurin is a blue-colored reagent that can enter viable cells. It is then reduced within the cell to highly fluorescent red resorufin that is excited between 530- and 560nm and emits at 590nm. After adding 0.1ml of the prepared HemoFLUOR™ reagent to the wells and mixing briefly, the plates are incubated at 37°C for at least 30 minutes, but no longer than 3 hours. Optimal fluorescence is usually achieved after about 2 hours incubation. The plate can be removed from the incubator at different times to follow the development of the fluorescence signal in real-time. It is recommended to include a background control without cells and subtract the background fluorescence from the sample being measured.

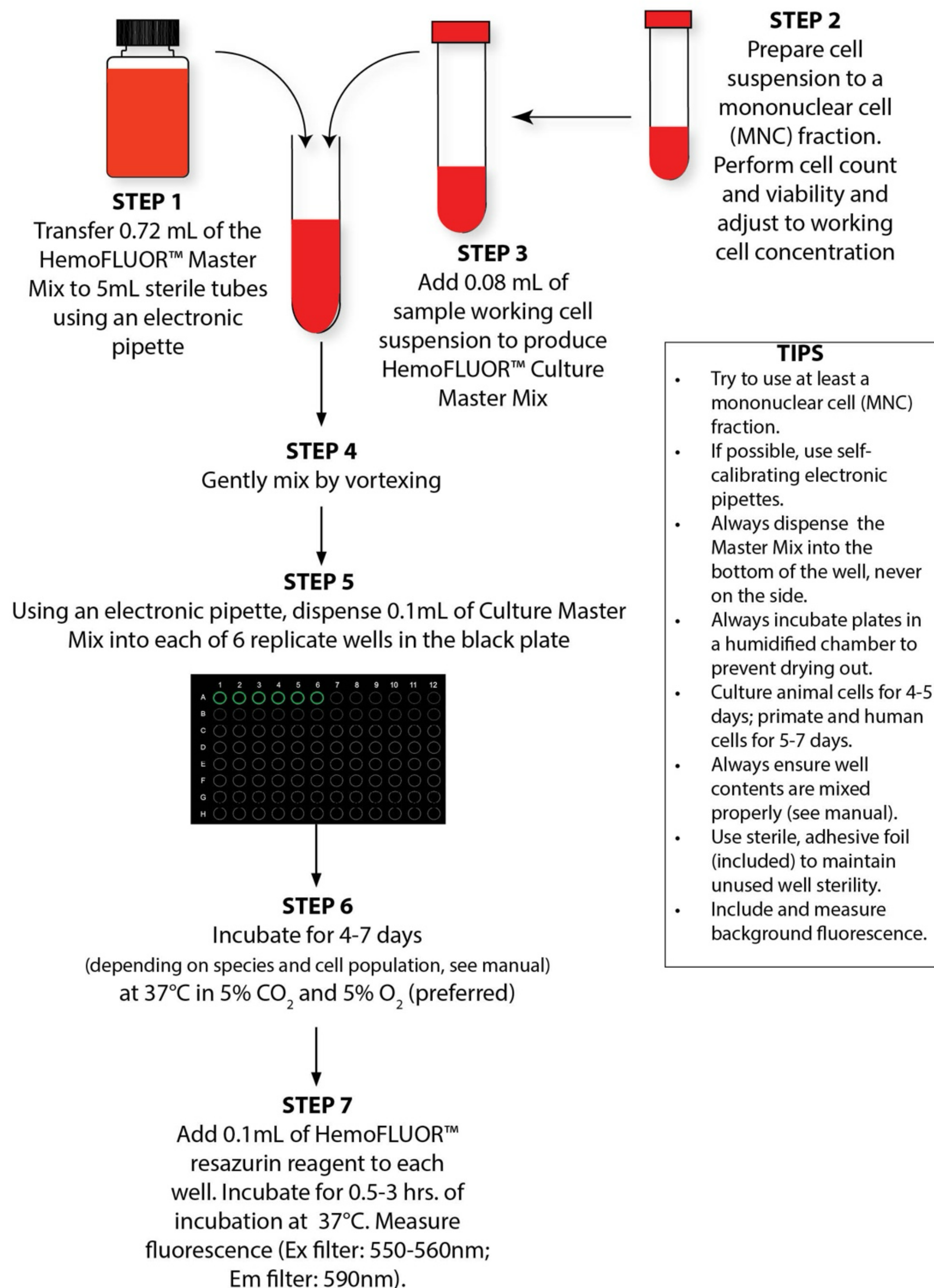
The HemoFLUOR™ reagent is non-destructive, i.e. it does not lyse cells. Therefore, cells can also be labeled with fluorophore-conjugated antibodies that excite and emit fluorescence at different wavelengths in order to detect specific cell populations. In addition, HemoFLUOR™ can also be multiplex with other Preferred Cell Systems™ ATP bioluminescence assays (e.g. HemoFLUOR™, ImmunoGlo™, MSCGlo™) to provide an extremely powerful and informative assay system.

Advantages of using HemoFLUOR™

- The resazurin-resorufin reaction produces a large, dynamic range that allows for high sensitivity.
- Single-addition reagent with no mixing, no washing and no lysis.
- Non-subjective, instrument-based and quantitative.
- Determines cell proliferation, viability and cell number.
- Multiplexes with flow cytometric protocols using other fluorescent labels.
- Multiplexes with Preferred Cell Systems™ ATP bioluminescence assays.
- Greater sensitivity than colorimetric/absorbance assays.
- Learn in 1 day and easy to use.

5. QuickGuide to HemoFLUOR™ and "Global" Assays

Please note that for HemoFLUOR™ 4-, 5- or 7-Population "Global" Assays, the same procedure is used except that each population uses a separate sterile, 96-well plate.



6. Kit Contents and Storage Conditions

HemoFLUOR™ 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	HemoFLUOR™ Master Mix for an individual cell population	-20°C until used
1a	HemoFLUOR™ "Global" 4-Population Kit contains 4 individual Master Mixes plus a Background with no growth factor cocktail	-20°C until used
1b	HemoFLUOR™ "Global" 5-Population Kit contains 5 individual Master Mixes plus a Background with no growth factor cocktail	-20°C until used
1c	HemoFLUOR™ "Global" 7-Population Kit contains 8 individual Master Mixes plus a Background with no growth factor cocktail	-20°C until used
2	Base Master Mix, used to subtract background fluorescence	-20°C until used
3	Background Control Master Mix, only included in 4-, 5- or 7-population assay kits	-20°C until used
4	HemoFLUOR™ Resazuring Reagent	2°C-8°C. Protect from light.
5	Adhesive Plate Covering: a sterile foil to protect and keep unused wells sterile.	Can be kept with other kit components
6	Sterile, wrapped, 96-well plate for cell culture	Can be kept with other kit components
7a	HemoFLUOR™ "Global" 4-Population kits contain 5 sterile, wrapped, 96-well plates for cell culture	Can be kept with other kit components
7b	HemoFLUOR™ "Global" 5-Population kits contain 6 sterile, wrapped, 96-well plates for cell culture	Can be kept with other kit components
7c	HemoFLUOR™ "Global" 7-Population kits contain 8 sterile, wrapped, 96-well plates for cell culture	Can be kept with other kit components
8	Technical manual	Can be kept with other kit components or download from website

Exact volumes of kit reagents and supplies are provided on a separate sheet included with this assay kit.

A Note on Master Mixes. The assay kit is shipped on dry ice (solid carbon dioxide). It is possible that when the kit arrives, bottles of Master Mix may be yellow or orange in color. This is not an indication that contamination has occurred. Carbon dioxide can leak into the bottles causing the pH to change. Prior to thawing the Master Mix, gently unscrew the lid, but do not remove the lid. Unscrewing the lid will release the carbon dioxide. When thawed, the Master Mix should be at the correct pH.

IMPORTANT
All kit components are quality controlled and optimized so that they work together. Please do not replace kit components with those of a different product. This will invalidate the warranty provided by Preferred Cell Systems™.

This kit contains a reagent for measuring fluorescence that decays with time. Preferred Cell Systems™ recommends that this kit be used before the expiry date of this reagent. Preferred Cell Systems™ does not take responsibility for the quality of reagents beyond their expiry date. If the kit cannot be used prior to the expiry date of this reagent, fresh reagent can be obtained from Preferred Cell Systems™.

Good laboratory practices and universal protective precautions should be undertaken at all times when handling the kit components as well as cells and tissues. Material safety data sheets (MSDS) are included in each literature packet.

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

Equipment and Supplies

1. Laminar Flow Biohood
2. Fluorescence plate reader
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. LymphoPrep 1.077).
4. 7-AAD, propidium iodide or trypan blue for viability assay.
5. LIVEGlo™ metabolic viability assay (Preferred Cell Systems™)

8. The HemoFLUOR™ Protocol

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

Performing HemoFLUOR™ is a 3-step process.

Step 1 – Cell preparation.
Step 2 – HemoFLUOR™ 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

HemoFLUOR™ 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 LymphoPrep 1.077. 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 HemoFLUOR™ Culture Master Mix prepared.

TABLE 1

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

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
	Mobilized peripheral blood	MNC	Fresh/Frozen	$0.5\text{-}0.75 \times 10^6$	5,000-7,500
	Umbilical cord blood	MNC	Fresh/Frozen	$0.5\text{-}0.75 \times 10^6$	5,000-7,500
	Umbilical cord blood	MNC	Frozen	$0.5\text{-}0.75 \times 10^6$	5,000-7,500
	Bone marrow	$CD34^+$	Fresh	$0.1\text{-}1 \times 10^5$	100-1,000
	Mobilized peripheral blood*	$CD34^+$	Fresh/Frozen	$0.1\text{-}5 \times 10^5$	100-5,000
	Umbilical cord blood	$CD34^+$	Fresh/Frozen	$0.1\text{-}5 \times 10^5$	100-5,000
Non-human primate	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
Horse, Pig, Sheep	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
Dog	Bone marrow	MNC	Fresh/Frozen	$0.5\text{-}0.75 \times 10^6$	5,000-7,500
Rat	Bone Marrow	MNC	Fresh	$0.5\text{-}0.75 \times 10^6$	5,000-7,500
	Peripheral blood	MNC	Fresh	$0.5\text{-}0.75 \times 10^6$	5,000-7,500
Mouse	Bone Marrow	MNC	Fresh	$0.5\text{-}0.75 \times 10^6$	5,000-7,500
	Spleen	MNC	Fresh	$0.5\text{-}1 \times 10^6$	5,000-10,000
	Fetal liver	MNC	Fresh	$0.5\text{-}0.75 \times 10^6$	5,000-7,500

STEP 2. HemoFLUOR™ Cell Culture Preparation

- *HemoFLUOR™ 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 HemoFLUOR™ Methodology

1. Remove the HemoFLUOR™ 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 Master Mix minus 10%, that will allow 0.1mL to be dispensed into the required number of replicate wells. See Table 2. Therefore, if 4 replicate wells are to be prepared, dispense 0.405mL of the HemoFLUOR™ 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 HemoFLUOR™ 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 HemoFLUOR™ 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

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

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

- These HemoFLUOR™ 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 HemoFLUOR™ 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 HemoFLUOR™ methodology as above.

STEP 3 – Fluorescence Reagent Preparation and Measurement

1. Remove both the Base Master Mix, HemoFLUOR™ Reagent from the freezer and thaw at 37°C in a water bath.
2. Vortex the reagent gently to ensure homogeneity. The Reagent is ready to use.
3. Reagent should be stored at 2°C to 8°C.
4. If possible, place the sample plate(s) in a humidified incubator set at 22-23C̊ gassed with 5% CO_2 for 30min to equilibrate or allow the plate to come to room temperature.
5. 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.
6. Dispense 0.1ml of the Base Master Mix provided with the kit into empty replicate wells. 4-6 wells should be sufficient. This will provide the background fluorescence that is subtracted from the sample fluorescence.
7. Accurately dispense 0.1ml of the HemoFLUOR™ Reagent into each of the background replicate wells.
8. Briefly mix the contents of the plate on an orbital shaker.
9. Dispense 0.1ml of the HemoFLUOR™ Reagent into each of the sample wells. If a large number of samples are to be processed, pour the Reagent into a reservoir and use a multichannel pipette to dispense 0.1ml into each well.
10. After dispensing the Reagent for each sample, change the pipette tip(s).
11. Repeat this procedure for each column or row using new tips.
12. When the HemoFLUOR™ Reagent has been dispensed into all sample wells, replace the plastic lid.
13. Briefly mix the contents of the plate on an orbital shaker.
14. Incubate plate(s) at 37°C for a minimum of 30 minutes and a maximum of 4 hours. The plate(s) can be removed from the incubator to measure the fluorescence at any time during this period and then replace the plate(s) back into the incubator. A 2 hour incubation is usually sufficient to obtain optimum sensitivity.
15. Measure the background fluorescence at 590nm prior to measuring the fluorescence of the sample(s).
    Please note that it may be necessary to adjust the instrument gain. (The “gain” is the applied photomultiplier tube energy).

16. Subtract the background fluorescence from the fluorescence obtained from the samples to yield the corrected fluorescence.

Phenotypic Analysis by Flow Cytometry

HemoFLUOR™ 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 mutliwell sampler. The antibody markers should not emit fluorescence at the same wavelength as the HemoFLUOR™ reagent (505nm).

9. Recommendations and Tips Prior to Using the HemoFLUOR™

(i) Background Controls
If the culture medium contains serum, background fluorescence may result. It is recommended to always include a background control of the same culture medium, but without cells. Other controls may be needed depending on the type of experiment being conducted.

(ii) 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 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
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.

(iv) 96-Well Plates Provided
The reagents have been optimized to work with the black 96-well plate(s) provided. Other plates can be used. However, cell growth and fluorescence 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
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 Preferred Cell Systems™ 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 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 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 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.

Ordering Information

Toll free: 1-888-436-6869

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Email: info@preferred-cell-systems.com

Order online at preferred-cell-systems.com

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Website: www.preferred-cell-systems.com

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HALO® Patents: 7,354,729, 7,354,730, 7,666,615, 7,709,258, 7,883,861, 7,700,354.

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