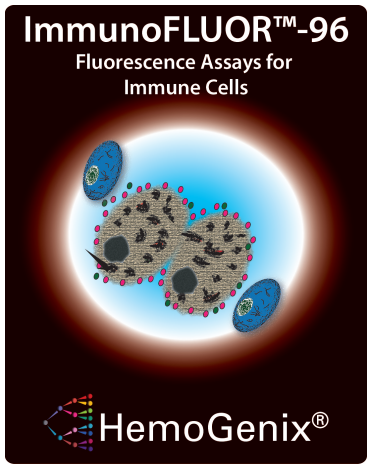
Ordering Information
Tel: (719) 264-6250
Fax: (719) 264-6253
Email: info@hemogenix.com
Order online at www.hemogenix.com

Technical Support
Tel: (719) 264-6250
Email: info@hemogenix.com

HemoGenix®, Inc
1485 Garden of the Gods Road
Suite 152
Colorado Springs, CO 80907
U.S.A.
Tel: (719) 264-6250
Fax: (719) 264-6253
Web: www.hemogenix.com

FOR *IN VITRO* RESEARCH USE ONLY (RUO)
NOT FOR CLINICAL DIAGNOSTIC USE



ImmunoFLUOR™-96
Fluorescence Assays for Immune Cells

Instruction Manual

(Version 5-2013)

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

HemoGenix®, Inc

HemoGenix®
Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

TABLE OF CONTENTS

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.

HemoGenix®
Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

1. INTRODUCTION

ImmunoFLUOR™-96 is a viability, cell functionality, proliferation and cell number assay for cells of the immune system. ImmunoFLUOR™-96 can be used for primed and unprimed T-cells, to test the activity of lymphocytes used for donor lymphocyte infusions (DLI), to study the effect of accessory cells and co-stimulators on T-cell induction and many other T-cell responses involving the stimulation of resting T-cells. ImmunoFLUOR™-96 can also be used to study potential toxicity to cells of the immune system.

ImmunoFLUOR™-96 allows the investigator to use their own reagents and culture protocols. Although ImmunoFLUOR™-96 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. ImmunoFLUOR™-96 can also used to monitor cell growth kinetics and single cell expansion during cloning studies (e.g. hybridoma production).

ImmunoFLUOR™-96 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.

ImmunoFLUOR™-96 is one of three signal detection systems available for use with immune cells. The other are ImmunoLIGHT™-96 and ImmunoGLO™-96. The former uses an absorbance readout, while the latter incorporates Bioluminomics™ technology and offers the greatest sensitivity. Bioluminomics™ is the instrument-based, non-subjective measurement of intracellular ATP concentrations using a luciferin/luciferase bioluminescence signal detection system. Each of the three signal detection systems have also been incorporated into a mixed lymphocyte culture (MLC) or reaction (MLR) assay. For high throughput *in vitro* immunotoxicity studies, ImmunoGLO™-Tox HT is recommended.

2. LIMITATIONS OF THE ASSAY AND PRECAUTIONS

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

HemoGenix® Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

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

(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 HemoGenix® if required.

(v) Humidity Chamber

If cell incubation time 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.

6. Using the non-sterile, 96-well plate provided with the kit, dispense 0.1ml 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 fluorescence.
7. Accurately dispense 0.1ml of the ImmunoFLUOR™ 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 ImmunoFLUOR™ 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 ImmunoFLUOR™ 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 both the background and sample plates at 37°C for a minimum of 30 minutes and a maximum of 3 hours. The plates can be removed from the incubator to measure the fluorescence at any time during this period and then replaced back into the incubator. A 2 hours incubation is usually sufficient to obtain optimum sensitivity.
15. Measure the background fluorescence at 505nm 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.

HemoGenix® Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

3. INTENDED USE

ImmunoFLUOR™-96 is intended for measuring the viability, cellular functionality, proliferation and/or cell number of immune cells. Specific applications include, but are not limited to:

- Testing unprimed T-cells in the presence of antibodies, enterotoxins, mitogens etc.
- Cellular immune response studies.
- Testing DLI (Donor Lymphocyte Infusion) samples for stimulation/induction ability prior to use.
- Effect of accessory (non T-cells) on T cell induction.
- Effect of co-stimulators on T-cell induction.
- Effect of epitope sequences and novel peptides or proteins.
- Test the response of primed T-cells *in vitro*.
- Single-cell, T-cell cloning studies.
- *In vitro* immunotoxicity studies.

Species
ImmunoFLUOR™-96 has been tested for human, non-human primate and mouse, but could be used for many other species.

ImmunoFLUOR™-96 Assays Available

Catalog Nos.	Additions	No. of Plates/Kit
KM2-LPA-1	No growth factors, mitogens or co-stimulators included	1
KM2-LPA-2	No growth factors, mitogens or co-stimulators included	2
KM2-LPA-4	No growth factors, mitogens or co-stimulators included	4

ImmunoFLUOR™-MLC (1- and 2-Way Mixed Lymphocyte Culture) Assays Available

Catalog Nos.	Additions	No. of Plates/Kit
KM2-MLC-1	No growth factors, mitogens or co-stimulators included	1
KM2-MLC-2	No growth factors, mitogens or co-stimulators included	2
KM2-MLC-4	No growth factors, mitogens or co-stimulators included	4

IMPORTANT:
ImmunoFLUOR™-96 is for research use only and has not been approved for clinical diagnostic use.

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

ImmunoFLUOR™-96 is a fluorescence *in vitro* cell viability/proliferation assay. It incorporates a fluorogenic, glycyl-phenylalanyl-aminofluorocoumarin (GF-AFC) peptide substrate that can enter cells. In intact viable cells, a conserved and constitutive, live-cell protease cleaves the substrate and generates a fluorescence signal at 505nm that is proportional to the number of living cells. If cells loose their membrane integrity, the live-cell protease becomes inactive. The reaction is measured in a plate fluorometer using an excitation of 380-400nm. After adding 0.1ml of the prepared ImmunoFLUOR™ 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.

Cell can also be labeled with fluorophore-conjugated antibodies that excite and emit fluorescence at different wavelengths in order to detect specific immune cell populations. In addition, ImmunoFLUOR-96 can be multiplex with other HemoGenix® ATP bioluminescence assays (e.g. ImmunoGlo™-96, HALO®) to provide an extremely powerful and informative assay system.

Advantages of using ImmunoFLUOR™-96

- Non-subjective, instrument-based and quantitative.
- Determines cell proliferation, cytotoxicity, viability and cell number.
- Multiplexes with flow cytometric protocols using other fluorescent labels.
- Multiplexes with HemoGenix® ATP bioluminescence assays, e.g. HALO®, LUMENESC™.
- Greater sensitivity than colorimetric/absorbance assays.
- Easy to learn, fast to use.

HemoGenix® Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

STEP 2. ImmunoFLUOR™-96 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.

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 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 0.1ml 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% CO_2 and, if available, 5% O_2. 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 – REAGENT PREPARATION and FLUORESCENCE MEASUREMENT

1. Remove both the ImmunoFLOR™ buffer and GF-AFC substrate from the freezer and thaw at 37°C in a water bath.
2. Vortex the GF-AFC substrate to ensure homogeneity and briefly centrifuge to recove the complete volume.
3. Transfer all the GF-AFC substrate to the buffer container. This produces a 2 x ImmunoFLUOR™ Reagent. Mix by vortexing until thoroughly dissolved. The reagent may appear "milky" but this is normal and will dissolve with vortexing. The ImmunoFLUOR™ Reagent should be used within 24 hours if stored at room temperature. At 4°C, the ImmunoFLUOR™ Reagent can be stored for 7 days.
4. If possible, place the sample plate(s) in a humidified incubator set at 22-23˚C 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

8. The ImmunoFLUOR™-96 PROTOCOL

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

Performing ImmunoFLUOR™-96 is a 3 step process.

Step 1 – Cell preparation.
Step 2 – Cell culture
Step 3 – Fluorescence 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.

ImmunoFLUOR™-96 is essentially a cellular integrity viability assay because it relies on the ability of a live-cell protease to cleave a fluorogenic substrate. However, when cells are stimulated or induced into proliferation or are inhibited from proliferating by cytotoxic agents, ImmunoFLUOR™-96 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.

HemoGenix®
Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

5. OVERVIEW of the ImmunoFLUOR™-96 PROCEDURE

There are 3 steps to use ImmunoFLUOR™-96.

Step 1 – Cell Preparation
Cells are not provided with ImmunoFLUOR™-96 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 in a Culture Master Mix prior to adding the cells and dispensing the complete Cell Culture Master Mix into the wells of the sterile plate(s) provided. Sufficient volumes should be prepared for both the Culture Master Mix and cell suspension to accommodate all replicates wells and samples.

Step 3 – Fluorescence Measurement
To measure fluorescence, the buffer and GF-AFC substrate is first thawed and gently mixed. The substrate is then added to the buffer to produce the to produce the ImmunoFLUOR™ Reagent. After mixing, 0.1ml of the reagent is dispensed into each replicate well. The plate is returned to a 37°C incubator for a minimum of 30 minutes. A 2 hour incubation time is usually sufficient to provide sufficient sensitivity. After incubation the fluorescence is measured in a plate reader with an excitation filter of 380-400nm. Emission is measure at 505nm. The medium used to prepare or culture without the cells should be used to measure the background fluorescence. This is performed in the non-sterile, 96-well plate provided with the kit. The background fluorescence is usually subtracted from the fluorescence measured for the sample.

6. KIT CONTENTS and STORAGE CONDITIONS

ImmunoFLUOR™-96 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. ImmunoFLUOR™ Buffer: Store at -20°C, protected from light.
3. GF-AFC Substrate: Store at -20°C, protected from light.
4. Sterile, black, flat-bottom, 96-well plate(s) for cell culture.
5. Non sterile, black, flat-bottom, 96-well plate(s) for background readings.
6. Adhesive Plate Covering—a sterile foil to protect and keep unused wells sterile.
7. 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.

HemoGenix®
Changing the Paradigm
Assays You Can Trust
Innovative Expertise You Can Count On

7. EQUIPMENT, SUPPLIES and REAGENTS REQUIRED, but NOT PROVIDED

Equipment and Supplies

1. Laminar flow safety hood.
2. Any fluorescence plate reader or multiparameter plate reader equipped with an excitation filter at 380-400nm and an excitation filter at 505nm.
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. Orbital plate shaker.
10. Tissue culture incubator, humidified at 37°C with 5% CO_2 (minimum requirement) and 5% O_2 (preferable).
11. Hemocytometer or electronic cell counter to determine cell concentration.
12. 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 (if required).
4. Reagent to measure cell viability.