# Labeling Hepatic Markers:
## *Alpha-1 Antitrypsin Labeling with Flow Cytometry Analysis*

## Introduction

Flow cytometry is a straight-forward technique for assessing intracellular or cell surface markers. Several labeling methods are available depending on the biological sample, cell preparation, and antibody availability. The protocol presented here is specific for detecting alpha-1 antitrypsin in iCell® Hepatocytes and serves as a guide for detecting other markers via flow cytometry.

## Required Equipment and Consumables

The following equipment and consumables are required in addition to the materials specified in the iCell Hepatocytes User's Guide.

| Item | Vendor | Catalog Number |
|---|---|---|
| **Equipment** | | |
| Multichannel Pipettor | Multiple Vendors | N/A |
| Flow Cytometer | Multiple Vendors | N/A |
| **Consumables** | | |
| iCell Hepatocytes or iCell Hepatocytes 2.0 Kit (Hepatocytes) | Cellular Dynamics International (CDI) | HCC-100-010-001<br>PHC-100-020-001 |
| 96-well Cell Culture Plate | Multiple Vendors | N/A |
| 96-well Polystyrene V-bottom MicroWell Plate (96-well V-bottom Plate) | Nunc | 249570 |
| Dulbecco's Phosphate Buffered Saline without $Ca^{2+}$ and $Mg^{2+}$ (D-PBS) | Invitrogen | 14190 |
| Fetal Bovine Serum (FBS) | Multiple Vendors | N/A |
| Flow Cytometry Tubes | Multiple Vendors | N/A |
| Formaldehyde Solution, 37% | Sigma-Aldrich | F8775 |
| Live/Dead Fixable Far Red Dead Cell Stain Kit (Live/Dead Dye) | Life Technologies | L34973 |
| Saponin | MP Biomedicals | 02180622 |
| Trypsin-EDTA, 0.5%,10X, No Phenol Red | Life Technologies | 15400-054 |
| **Recommended Antibodies** | | |
| Goat Anti-human Alpha-1 Antitrypsin Antibody FITC Conjugated | Bethyl Laboratories | A80-122F |
| Purified Goat IgG-FITC Conjugated | Bethyl Laboratories | P50-100F |

## Methods

The following procedure details how to prepare the hepatocytes cultured in 96-well cell culture plates for flow cytometry analysis. Scale volumes appropriately for other well formats.

### Culturing Hepatocytes

Thaw and maintain the hepatocytes up to the point of analysis according to their User's Guide.

### Collecting Hepatocytes

1. Remove the 96-well cell culture plate containing hepatocytes from the incubator.
2. Aspirate or quickly decant the spent medium. Wash the cells with 200 µl/well of D-PBS using a multichannel pipettor.
3. Add 100 µl/well of 0.5% trypsin-EDTA.
4. Incubate at 37°C for 3 minutes.
5. Add 50 µl/well of FBS to a fresh 96-well V-bottom plate.
6. Remove the 96-well cell culture plate from the incubator. Triturate the cells 4 times using a multichannel pipettor.
7. Transfer the cell suspension into the corresponding wells of the 96-well V-bottom plate containing FBS.
8. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.
9. Aspirate or quickly decant the supernatant. Resuspend the cells in 100 µl/well of D-PBS.
10. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.

### Staining Live/Dead Hepatocytes

Stain the hepatocytes to distinguish live and dead populations before fixation for intracellular labeling for alpha-1 antitrypsin.

1. Dilute 10 µl of live/dead dye solution in 9.99 ml of D-PBS to make a 1:1000 dilution.

   ***Note:*** *Reconstitute live/dead dye according to the manufacturer's instructions.*
2. Aspirate or quickly decant the D-PBS from the plate. Resuspend the cells in 100 µl/well of diluted live/dead dye.
3. Incubate at room temperature for 15 minutes.
4. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.
5. Aspirate or quickly decant the D-PBS. Resuspend the cells in 200 µl/well of D-PBS.
6. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.
7. Prepare FACS wash buffer by diluting FBS to 2% (v/v) in D-PBS.
8. Aspirate or quickly decant the D-PBS. Resuspend the cells in 200 µl/well of FACS wash buffer.

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## Labeling Hepatocytes: Fixation, Permeabilization, and Antibody Incubation

1. Prepare the fixative solution by diluting a stock solution of formaldehyde solution to 4% (v/v) in D-PBS.
2. Aspirate or quickly decant the FACS wash buffer from the plate. Resuspend the cells in 100 µl/well of fixative solution.
3. Incubate at room temperature for 15 minutes.
4. Add 100 µl/well of FACS wash buffer and mix.
5. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.
6. Aspirate or quickly decant the D-PBS. Resuspend the cells in 100 µl/well of FACS wash buffer.
7. Repeat steps 5 and 6 three times to complete the wash.

   ***Note:*** *Fixed cells can be stored at 4°C in FACS wash buffer. The signal-to-noise ratio is improved with a 24 - 72 hour incubation at 4°C.*
8. Prepare the permeabilization buffer by diluting FBS to 2% (v/v) and saponin to 0.1% (w/v) in D-PBS.
9. Prepare the antibody solutions by diluting the detection antibody or isotype control antibody to 1:400 in permeabilization buffer.
10. Centrifuge the plate at 250 x g for 4 minutes.
11. Aspirate or quickly decant the FACS wash buffer. Resuspend the cells in 100 µl/well of permeabilization buffer.
12. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.
13. Aspirate or quickly decant the permeabilization buffer. Resuspend the cells in 50 µl/well of primary antibody (or isotype control) solution.
14. Cover the plate and incubate at room temperature for 1 hour, protected from light.
15. Centrifuge the plate at 250 x g for 4 minutes.
16. Aspirate or quickly decant the primary antibody (or isotype control) solution. Resuspend the cells in 100 µl/well of permeabilization buffer.
17. Cover the plate with a lid and centrifuge at 250 x g for 4 minutes.
18. Repeat steps 16 and 17 two times to complete the wash.
19. Aspirate or quickly decant the permeabilization buffer. Resuspend the cells in 100 µl/well of FACS wash buffer.
20. Transfer the cells to flow cytometry tubes for analysis.

## Data Analysis

Refer to the guide for the flow cytometry system for data analysis instructions.

1. Use the isotype control sample to set the negative population gates.
2. Use the live/dead signal (far red) and alpha-1 antitrypsin signal (green) to evaluate alpha-1 antitrypsin expression (Figure 1).

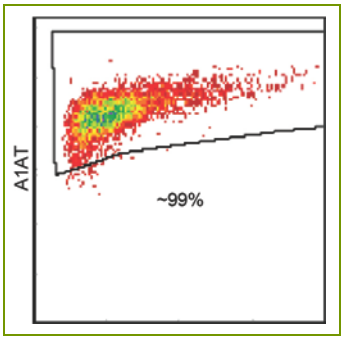


**Figure 1: Flow Cytometry Provides a Rapid and Easy Method for Assessing Marker Expression in iCell Hepatocytes**
*In this representative experiment, alpha-1 antitrypsin (A1AT) was used as a marker to determine hepatocyte identify and population purity. Acquisition and analysis were performed using a BD Accuri C6 Flow Cytometer (BD Biosciences).*

## Summary

iCell Hepatocytes provide a relevant in vitro test system that recapitulates native human hepatocyte physiology. This protocol provides guidance for easy detection of markers via flow cytometry and is one of a growing suite of CDI-developed protocols that enable meaningful interrogation of these hepatocytes.

Notes



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