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AdipoRed™ Assay Reagent

Instructions for Use

Receiving Instructions

Protect from light; Store at room temperature.

Introduction

The differentiation of stem cells (such as Poietics™ Primary Human Mesenchymal Stem Cells, product # PT-2501, Poietics™ Primary Human Visceral Preadipocytes, product # PT-5005 and Poietics™ Primary Human Subcutaneous Preadipocytes, product # PT-5001 or PT-5020) or adipogenic cell lines such as the mouse fibroblast 3T3 cell line, is accompanied by the accumulation of intracellular droplets of triglyceride. The accumulation of intracellular triglycerides is often used as a marker of adipocyte differentiation [(1)]. Depending on the nature of the differentiation protocol, primary human preadipocytes begin to accumulate lipid droplets after 4 to 5 days of culture. The number and the size of the lipid droplets continue to increase for an additional 1 to 2 weeks. Inhibitors of adipocyte differentiation such as TNF-α and mifepristone, a glucocorticoid receptor antagonist, significantly reduce accumulation of intracellular lipids[(2, 3)]. Quantitative measurement of intracellular lipids has utilized hydrophobic stains such as Oil Red O that partition into intracellular lipid droplets[(4)]. Quantification of Oil Red O requires the manual counting of stained cells viewed in a microscope.

AdipoRed™ is a Reagent that enables the quantification of intracellular lipid droplets in a high-throughput manner. AdipoRed™ is a solution of the Hydrophilic Stain Nile Red. Nile Red, when partitioned in a hydrophobic environment, becomes fluorescent. The emission maximum of the fluorescent signal is dependent on the nature of the hydrophobic environment. Nile Red Fluorescence, when bound to protein or the phospholipids of the plasmalemma of a cell, is distinct from that produced when the stain is partitioned in droplets of triglyceride[(5)]. The AdipoRed™ Reagent has been optimized relative to its solvent, concentration and use in a 96-well based assay.

AdipoRed™ can be used to determine the effects of test compounds on the differentiation of adipocyte precursors as characterized by the accumulation of intracellular triglycerides. AdipoRed™ can also be used to determine the effects of test samples on the depletion of intracellular triglycerides from terminally differentiated adipocytes.

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Safety Statements

THESE PRODUCTS ARE FOR RESEARCH USE ONLY. Not approved for human or veterinary use, for application to humans or animals, or for use *in vitro* diagnostic or clinical procedures. See MSDS for product safety information.

Protocol Details

NOTE:

- For maximum throughput, the AdipoRed™ Assay will require a fluorimeter capable of reading multiwell plates. The fluorimeter should ideally be equipped with an injector capable of delivering 1 µl, 5 µl or 12 µl of reagent to each well of 384-well, 96-well and 48-well plates respectively. The fluorimeter should also have the ability to mix or shake the plate immediately upon reagent addition. For 24-well, 12-well and 6-well plates, mixing of the AdipoRed™ Reagent is most efficiently done by hand (See below).
- If the fluorimeter being used has the ability to make multiple readings per well (at different points in each well) of a plate, it is highly recommended to do so. Taking the average of multiple readings per well will reduce variation between replicates.
- White plates are recommended for assays using AdipoRed™, however clear or black plates can be used.
- AdipoRed™ Assay Reagent is not sterile. It is not intended to be used as a vital stain.

Protocol for 96-well plates:

1. Following the instructions for the fluorimeter to be used, load the injector with AdipoRed™ Reagent. Program the instrument to inject 5 µl of AdipoRed™ Reagent/well. Each vial of AdipoRed™ contains sufficient reagent to process a minimum of five 96-well plates depending on the injector tubing volume. Program the instrument to mix/shake the assay plate for 1 second immediately after addition of AdipoRed™ Reagent to each individual well.

2. When the cells are ready to be assayed for intracellular triglyceride content, the tissue culture plate should be removed from the cell culture incubator and allowed to cool to room temperature. The culture supernatant should be removed and each well carefully rinsed with 200 µl of phosphate-buffered saline (PBS). **Be extremely careful not to remove cells from the wells during this rinse step.** Each well should then be filled with 200 µl of room temperature PBS.
3. Place the assay plate in the fluorimeter and initiate the AdipoRed™ Reagent Addition Program. Make sure that the plate's lid/cover has been removed! Upon completion of reagent addition, the assay plate should be incubated at room temperature for a minimum of 10 minutes. The plate can be removed from the fluorimeter or remain in the instrument during the incubation period. If the fluorimeter is not equipped with an injector, the AdipoRed™ Reagent can be added with a multichannel pipette if the following protocol is used: Add the AdipoRed™ Reagent with a multichannel pipette that can accurately deliver 5 µl of reagent. Mix the plate by rapping the edge of the plate against the lab bench several times immediately upon addition of reagent to each row of wells – do not wait until the reagent has been added to all wells of the plate before mixing the plate's contents.
4. After 10 minutes, place the plate in the fluorimeter and measure the fluorescence with excitation at 485 nm and emission at 572 nm. If the fluorimeter does not have the appropriate filters, the settings used for the common fluorophore fluorescein (excitation 485 nm; emission 535) can be used.

Protocol for 384-well plates:

1. Sterile 384-well cell culture plates can be seeded with 1,000 to 3,000 primary human preadipocytes or other cell types (e.g. the murine cell line 3T3-L1)/well in 40 µl of preadipocyte growth medium and cultured until confluent (1 to 3 days).
2. When the cultures are confluent, add 40 µl of preadipocyte growth medium to those cells designated as "undifferentiated controls". Add 40 µl of 2X adipocyte differentiation medium to all other wells. Test samples, at a 2X concentration, should be added with the differentiation medium.
3. Differentiate the cells for 14 days and then aspirate the cell culture medium. Be sure not to touch the cell monolayer with the pipette tip or aspirate cells from the bottom of the wells. Rinse each well with 100 µl of PBS and then add 50 µl of PBS to each well. If a robotic injector is used, program the injector to inject 1 µl of AdipoRed™ into each well and mix the plate upon completion of reagent addition. Incubate the plate for 10 to 15 minutes and read the plate as in Step 4 above.

Protocol for 6-, 12-, 24- and 48-well plates:

1. Seed cells at 30,000/cm^2 and culture and differentiate the cells as described above, using appropriate volumes of cell culture media.
2. Immediately prior to the assay, rinse each plate with PBS, and add AdipoRed™, using the volumes in Table 1.

TABLE 1

	Rinse volume/well	Final volume of PBS/well	Volume of AdipoRed™/well
6-well plate	2 ml	5 ml	140 µl
12-well plate	1 ml	2 ml	60 µl
24-wll plate	1 ml	1 ml	30 µl
48-well plate	0.4 ml	0.4 ml	12 µl
96-well plate	0.2 ml	0.2 ml	5 µl

3. After addition of the AdipoRed™, the best mixing of the reagent is obtained by pipetting 50% of the contents of each well up and down two times (three times for 6-well plates). It is important to obtain a homogeneous dispersion of the blue AdipoRed™ Reagent. Be very careful not to touch the tip of the pipette to the cell monolayer or remove cells from the well surface.
4. Incubate the plate for 10 to 15 minutes and read the plate as in Step 4 above.

Expected results

The actual readout in relative fluorescence units (RFU) will vary with the fluorimeter used. However, after 10 days of differentiation, the ratio of RFU (differentiated cells) to RFU (undifferentiated cells) should exceed 10. See Figures 1 and 2 for representative graphs.

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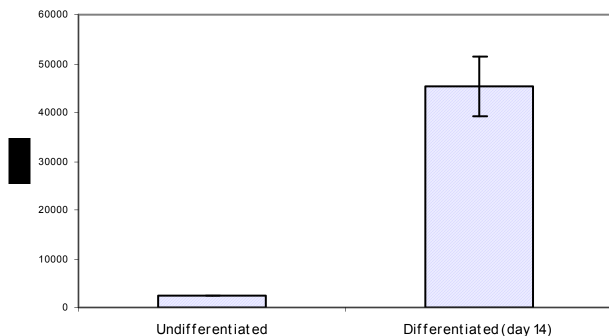

Figure 1

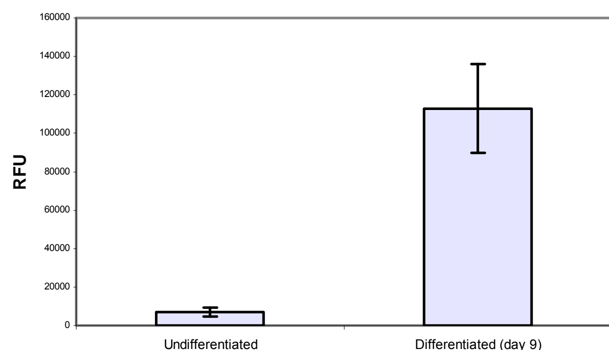

Figure 2

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2. Petruschke, T. and H. Hauner. 1993. Tumor necrosis factor-alpha prevents the differentiation of human adipocyte precursor cells and causes delipidation of newly developed fat cells. J. Clin. Endocrinol. Metab. 76:742-747.
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4. Memon RA, Tecott LH, Nonogaki K, Beigneux A, Moser AH, Grunfeld C, Feingold KR. Up-regulation of peroxisome proliferator-activated receptors (PPAR-alpha) and PPAR-gamma messenger ribonucleic acid expression in the liver in murine obesity: troglitazone induces expression of PPAR-gamma-responsive adipose tissue-specific genes in the liver of obese diabetic mice. Endocrinology (2000) 141: 4021-31.
5. Greenspan P, Mayer EP and Fowler SD. Nile Red: A selective fluorescent stain for intracellular lipid droplets. Journal Cell Biology (1985) 100: 965-973.

Ordering Information

PT-7009	AdipoRed™ Assay Reagent	Five vials, each containing 4.0 ml

Related Products

PT-2501	hMSC, Human Mesenchymal Stem Cells, Cryopreserved	≥750,000 cells
PT-5005	Human Visceral Preadipocytes	≥1 million cells
PT-5001	Human Subcutaneous Preadipocytes	≥4 million cells
PT-5020	Human Subcutaneous Preadipocytes	≥1 million cells
PT-5023	Human Visceral Preadipocytes (Diabetes Type I)	≥1 million cells
PT-5024	Human Visceral Preadipocytes (Diabetes Type II)	≥1 million cells
PT-5021	Human Subcutaneous Preadipocytes (Diabetes Type I)	≥1 million cells
PT-5022	Human Subcutaneous Preadipocytes (Diabetes Type II)	≥1 million cells

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