



Human Adipogenesis Assay Kit

PPAR γ Agonists

Cat# DIF-AG: Cells + Reagents

Cat# DIF-AG-NC: Reagents Only

96 well format 100 point kit

INSTRUCTION MANUAL ZBM0005.07

STORAGE CONDITIONS

- **Frozen subcutaneous preadipocytes [DIF-AG only]**
Store in vapor phase liquid nitrogen IMMEDIATELY upon receipt. Any other storage negates the warranty. No expiration date is applicable when stored as indicated. The cells must be plated within 1 week of receiving the kit to account for the 30 day expiration of the kit media components.
- **Media, Reagent A, Buffers:**
Store at +2 - 8°C.
Use reconstituted Glycerol Reagent A within 7 days.
- **Glycerol Standard & Reagent B Solution**
Remove from kit box and store at -20°C IMMEDIATELY UPON ARRIVAL.

ALL ZEN-BIO INC PRODUCTS ARE FOR RESEARCH USE ONLY. NOT APPROVED FOR HUMAN OR VETERINARY USE OR FOR USE IN DIAGNOSTIC OR CLINICAL PROCEDURES.

ORDERING INFORMATION AND TECHNICAL SERVICES

- Zen-Bio, Inc.
- 3920 S. Alston Avenue
- PO Box 13888
- Durham, NC 27713, United States
- Telephone (919) 547-0692
- Facsimile (FAX) (919) 547-0693
- Toll Free 1-866-ADIPOSE (866)-234-7673
- Electronic mail (e-mail) information@zen-bio.com
- World Wide Web <http://www.zenbio.com>

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, expressed or implied, including without limitation implied warranties of merchantability or fitness for a particular purpose, are provided by Zen-Bio, Inc. Zen-Bio, Inc. shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

Zen-Bio, Inc warrants its cells only if Zen-Bio media, storage conditions and recommended protocols are followed. Cryopreserved human cells are assured to be viable when stored and thawed according to Zen-Bio protocols without amendment or substitution.

Contact ZenBio, Inc. within no more than 24 hours after receipt of products for all claims regarding shipment damage, incorrect ordering or other delivery issues. Delivery claims received after 7 days of receipt of products are not subject to replacement or refund.

INTRODUCTION

ZenBio human primary subcutaneous preadipocytes were isolated from human subcutaneous adipose tissue obtained from a competent volunteer adult donor undergoing elective surgery in the United States. Each donor has signed an Institutional Review board (IRB) validated donor consent form that specifically lists both the intended uses for the donation for non-clinical research and confirms the procedures for processing the samples are Standard Operating Procedure (SOP) managed GLP protocols in compliance with ethical regulations. All samples are collected and processed in the United States.

Samples from each donor are tested and found non-reactive to viral DNA from HIV and hepatitis B and viral RNA from Hepatitis C using US Food and Drug Administration (FDA) licensed tests. However, no known test can offer complete assurance that these viruses are not present. Since we cannot test all pathogens, always treat the culture as a potentially infectious reagent. We recommend using the US Centers for Disease Control (CDC) Universal Precautions for prevention of blood-borne pathogens as a minimum guideline for standards of practice at Biosafety Level 1 (BSL-1) or higher.

ZenBio differentiation assay kits provide the tools to study the compounds that stimulate cultured human adipocyte differentiation or lipogenesis. Such compounds may be PPAR γ (gamma) agonists or a combination of thiazolidinediones and glucocorticoids that are potentially useful in the treatment of diabetes.

This kit, DIF-AG and DIF-AG-NC (Reagents only) is designed to test compounds as potential PPAR γ agonists. It is our experience that the PPAR γ agonist used as the Positive Control sufficiently stimulates human adipocyte differentiation after 7 days of treatment. The protocol is designed so that the test compounds are also used in a 7-day treatment regimen. This kit contains sufficient reagents to assay 100 assay points in a 96 well format.

ITEMS INCLUDED IN THE KIT

Item	Description	Unit	QTY	Item	Storage
Human SQ Preadipocytes	Human subcutaneous (SQ) preadipocytes, 2.0 million cells/vial, cryopreserved [DIF-AG only]	VIAL	1	1	Liquid nitrogen
PM-1	Preadipocyte Medium (read Appendix B page 11)	BOTTLE	50ml	2	4°C
NC	Negative control: (read Appendix B page 11)	VIAL	500 µl	2	+4°C
PC	Positive control: (read Appendix B page 11)	VIAL	500 µl	2	+4°C
VC	Vehicle control (read Appendix B page 11)	VIAL	500 µl	2	+4°C
IM	Initiation Medium (read Appendix B page 11)	BOTTLE	300ml	3	+4°C
MM	Maintenance Medium (read Appendix B page 11)	BOTTLE	100ml	2	+4°C
Wash buffer	Wash Buffer	BOTTLE	50ml	2	+4°C
Lysis buffer	Lysis Buffer	BOTTLE	15ml	2	+4°C
Glycerol Reagent A	Reconstitute using 11.0 ml deionized water prior to use. Use reconstituted reagent within 7 days.	BOTTLE	11.0ml	2	+4°C
Reagent B	2.5 ml Reagent B Solution. New Storage Condition.	BOTTLE	2.5 ml	2	-20°C
Glycerol standard	Glycerol; 1mM [Reconstitute with 400 µl Standards Diluent to make the 200 µM glycerol standard; read page 5 for recommended dilution scheme]	VIAL	100 µl	2	-20°C
Standards Diluent	Standards Diluent	BOTTLE	2 ml		+4°C
Tray	Clear polyvinyl tray for multi-channel pipetters	EACH	3	-----	-----
Data sheet	Certificate of Analysis and protocol	EACH	1	-----	-----
Assay Plates, blank	96-well assay plate, flat bottom, blank	PLATE	3	---	-----

Other equipment/reagents required but not provided with the kit:

- ▶ Additional Maintenance Medium, if necessary (read background information)
- ▶ Single-channel pipetter
- ▶ Multi-channel pipetter
- ▶ Plate reader with a filter of 540 nm
- ▶ Tubes to dilute glycerol standards

ASSAY PROCEDURE

A. DIFFERENTIATION PROCEDURE

On each day of the procedure, the appropriate medium must be warmed to 37°C prior to use.

Note: This protocol is designed to accommodate a weekday work schedule if started on a Monday-Thursday. Any deviation may require weekend work. We strongly recommend testing all compounds in triplicate.

Day 1: This is the day the cells are plated.

1. Remove cells from liquid nitrogen and place immediately into a 37° C water bath and agitate while in bath. Be careful not to submerge the cap of the vial into water. Do not leave the vials in water bath after most of the content has thawed. Rinse the vials with 70% ethanol before taking them to the culture hood.
2. Upon thawing, transfer the cells to a sterile conical bottom centrifuge tube containing 10 ml of Preadipocyte Medium (cat # PM-1).
3. Centrifuge: 1,200 rpm (282Xg) / 20°C / 5 minutes. Aspirate the supernatant. TAKE CARE TO NOT ASPIRATE ANY OF THE CELL PELLET.
4. The cell vial contains a minimum of 2 million viable cells; however, we recommend performing a cell count to determine a more exact number of cells. Resuspend the cell pellet in 2 ml Preadipocyte medium; dilute an aliquot in 0.4% trypan blue solution. We suggest withdrawing an aliquot of 50 µl of cells and mixing with 100 µl of the trypan blue solution, resulting in a dilution factor of 3. Count live (unstained) cells on a hemacytometer. The cell concentration required for approximately 40,000 cells / cm² in the 96 well format with 150µl /well is 1.3×10^6 cells in 15 ml Preadipocyte medium.
5. Plate cells in one of the 96 well plates provided in the kit. Do not agitate the plate, as cells will not plate evenly.
6. Place plate in 37°C incubator, 5% CO₂, 97% humidity. The cells will be maintained in the incubator after each manipulation until Day 14.

NOTE: This kit contains a sufficient volume of Initiation Medium (IM) to use 10 ml of medium per compound dilution for a maximum of 29 compounds tested in triplicate (87 wells) on the 96-well plate, leaving 9 wells for controls. If a compound stock is too concentrated to accomplish the desired dilution, use an appropriate solution (not supplied) to prepare an intermediate concentration that would allow for a final volume of 10 ml.

Also the positive control in this kit, the PPAR γ agonist, has a final solvent concentration of 0.1% DMSO. This concentration is included in the vehicle control (VC). If the concentration of any solvent for the compounds used is high enough to potentially alter differentiation, please include that solvent concentration as an additional treatment. We do not recommend treating the cells with solutions exceeding 1% of any solvent, as higher concentrations may be toxic to the cells.

Day 2:

7. Twenty-four hours later, check the cells for confluence.
8. Using the Initiation Medium (IM), prepare treatments. Plan to do all treatments in triplicate. A blank plate map is included in these instructions to record the well treatments (Appendix C).
9. When all treatments are prepared, remove Preadipocyte medium from control wells. We recommend doing the treatments in small groups so the cells do not dry out.
10. Pipet 150 μ l Positive Control, (PC), 150 μ l Negative Control (NC), and 150 μ l Vehicle Control (VC) into appropriate wells.
11. Remove media from experimental wells and pipet 150 μ l each treatment media into appropriate wells. Incubate the plate for 7 days.

Day 8:

12. Using a multichannel pipetter remove 90 μ l media from all wells. Gently feed all wells with 120 μ l of the Maintenance Medium (MM) that is provided with this kit. Incubate the plate for 7 days.

Day 15:

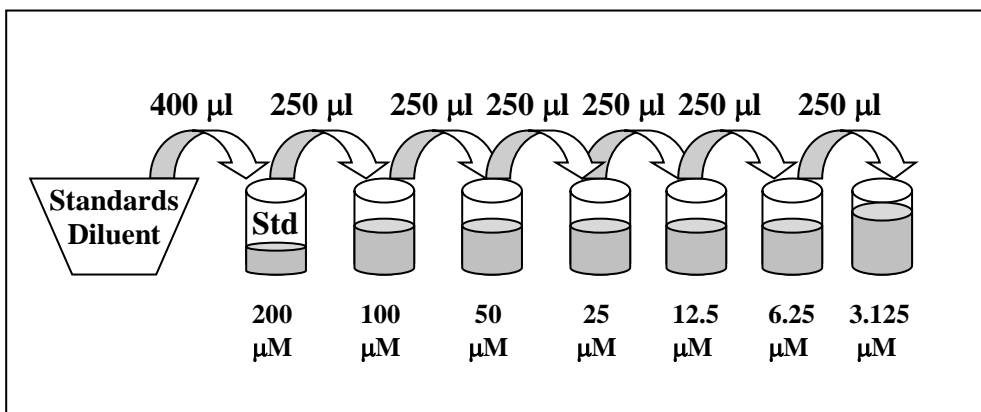
13. Cells are now mature. Example images are in Appendix A. Proceed to part B.
14. The positive control wells should exhibit significantly greater lipid accumulation than the negative control wells or the vehicle control wells. Refer to page 9 for a picture of a typical positive control when the adipocytes are mature.

B. TRIGLYCERIDE ASSAY

1. Warm the Wash buffer and Lysis buffer in a 37°C water bath.
2. Thaw Reagent B Solution to room temperature and gently invert. DO NOT VORTEX! Store in a light protected bottle. Reagent B solution can be refrozen and stored -20°C. Avoid repeat freeze/thaw cycles.
3. Remove all media. Using about 15 ml of the wash buffer, wash the cells one time with 150 μ l wash buffer. Label the disposable tray “wash buffer” and retain for later use.

4. Remove all Wash Buffer. Using a new tray, add 15 μ l Lysis buffer. Incubate at 37– 50°C for 20 minutes.
5. After the incubation is complete, visually confirm cell lysis by checking the wells under a microscope. If cells are not fully lysed, incubate another 10 minutes.
6. Add 135 μ l warm Wash Buffer and mix the lysates by pipetting up and down three times.
7. Add 20 μ l Reagent B to each well. It is not necessary to mix at this time, however, gently tap the plate to help mix the reagents. Incubate the plate at 37°C for 2 hours.
8. Bring Glycerol Reagent A and the glycerol standards to room temperature during this time. The Wash Buffer can also be kept at room temperature at this point. Warm the Standards Diluent to 37°C. Prepare the standard curve as follows:

Pipette 400 μ l of the Standards Diluent into the 1 mM glycerol standard tube provided and mix well by vortexing. This produces a diluted stock glycerol standard of 200 μ M. Pipette 250 μ l of diluent into 6 tubes (not provided). Using the newly diluted stock glycerol solution, prepare a dilution series as depicted below. Mix each new dilution thoroughly before proceeding to the next. The 200 μ M stock dilution serves as the highest standard, and the diluent serves as the zero standard (STD).



Note: The above dilution series generates enough volume to perform the standard curve in duplicate. If you wish to perform the standard curve in duplicate, please note that eight fewer data points can be assayed with this kit.

9. Also at this time prepare the Glycerol Reagent A by adding 11.0 ml deionized water per bottle and gently invert. DO NOT VORTEX Use a pipet to ensure that the powder is completely dissolved. Keep at room temperature. If using a Glycerol Reagent A solution previously prepared and stored at 2-8°C, also bring to room temperature. Make sure there is enough Glycerol Reagent A from one solution to treat all the points in the assay. It may be necessary to combine solutions. Store in a light protected bottle. Reconstituted Glycerol Reagent A is stable for 7 days refrigerated (2-8°C); store any remaining solution refrigerated (2-8°C).

10. To a blank 96 well plate, add 80 μ l wash buffer to each well needed for the assay (NOTE: do not add Wash Buffer to the wells used for the standard curve).
11. Working with one row or column at a time, mix the lysates very well using a multi-channel pipette. Immediately transfer 20 μ l per well of the lysates to the corresponding well of the plate containing the wash buffer. **This results in a Dilution Factor of 5.**
12. Prepare the standard curve. Pipet 100 μ l of each standard into a well. (NOTE: Eight wells are necessary for the curve or sixteen if performing the curve in duplicate. If there are remaining wells on the assay plate, you can utilize the remaining wells. If not, a second plate is included in this kit).
13. Using the third tray, add 100 μ l Glycerol Reagent A to samples and standards. Mix by pipetting up and down one time. Incubate at room temperature for 15 minutes.
14. Read at 540 nm using a microtiter plate reader.

GLYCEROL STANDARD CURVE

This kit is designed to show relative lipid accumulation of experimental treatments compared to controls. The assay is based on the equation

1 M Triglyceride yields 1M glycerol + Free Fatty Acids

The reagent measures the concentration of glycerol released after lysing the cells and hydrolyzing the triglyceride molecules. The triglyceride concentration can then be determined from the glycerol values.

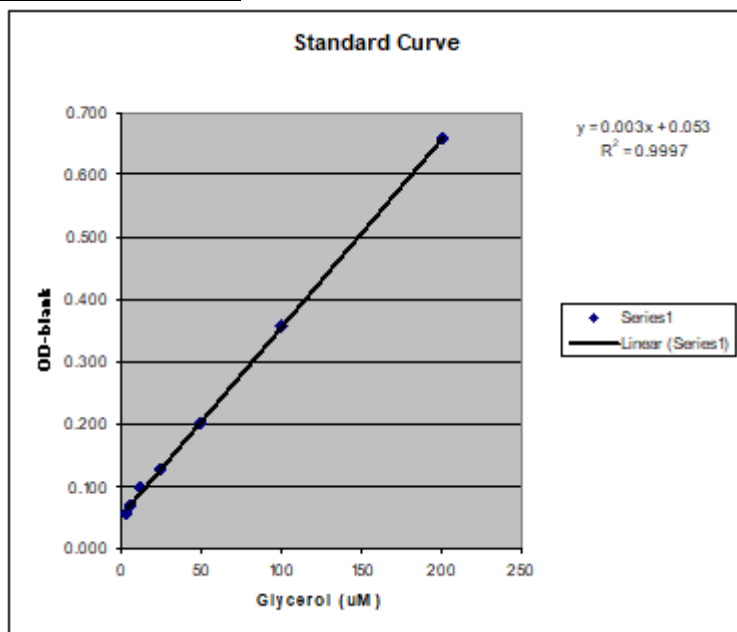
Generate standard curve: see example below

[DO NOT use this standard curve to generate your data. This is an example.]

- Subtract the OD value of the 0 μM standard from all OD values including the standard curve.

Glycerol (μM)	OD	OD	OD- blank	OD- blank	Avg OD- blank
0	0.048	0.048			0.048
3.125	0.059	0.058	0.011	0.01	0.059
6.25	0.07	0.07	0.022	0.022	0.070
12.5	0.098	0.098	0.05	0.05	0.098
25	0.127	0.13	0.079	0.082	0.129
50	0.2	0.205	0.152	0.157	0.203
100	0.353	0.362	0.305	0.314	0.358
200	0.649	0.667	0.601	0.619	0.658

slope	0.003
intercept	0.053
R^2	0.9997



$y = \text{observed O.D. minus the blank}$

x = concentration of glycerol in μM

To calculate x for each y, (i.e. to change the observed O.D. into glycerol concentration) use the following equation:

$y = (\text{slope}) \times (x) + \text{intercept}$

$y = mx + b$ so $x = (y - b)/m$

$x = (y - 0.0006)/0.0014$ where 0.0014 = slope of the line and 0.0006 = y intercept.

Be careful to enter the proper sign for the y intercept value as it may be a negative number.

Any OD values greater than the highest standard (200 μM) should be suspect. The compound should be re-assayed using a lower dose of the compound at treatment OR a dilute solution of the condition medium at the time of the assay.

The R^2 value should be equal or greater then 0.98 for the standard curve to be valid. Any R^2 values below 0.98, must have the standard curve run again.

Solve for the Total Glycerol concentration (i.e. total triglyceride concentration) for each OD.

Remember to include the Dilution Factor in the equation. Data is expressed as μM Glycerol.

NOTE: Any OD values that are negative after the blank is subtracted should be considered to be 0 for the OD value.

TROUBLESHOOTING

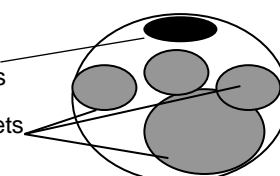
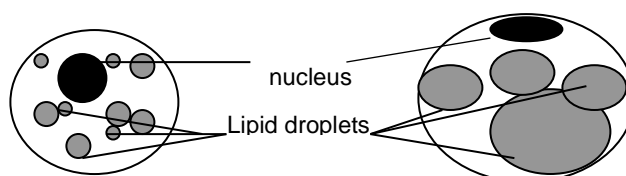
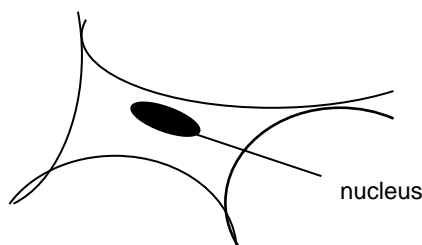
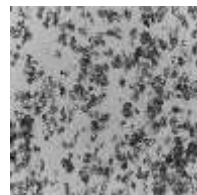
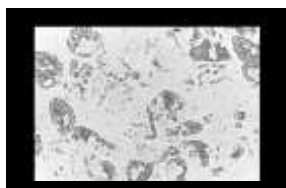
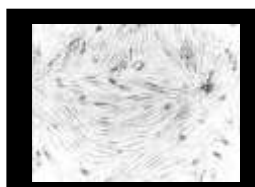
Problem	Suggestions
High background or the triglyceride reagent turns a darker color before the assay begins.	Use clean tray and tips Change pipet tips frequently
Edge effects	Ensure a saturated humidity in the incubator to prevent evaporation from the outside wells
Inconsistent OD reading	Be careful when pipetting to avoid bubbles. If bubbles persist, burst the bubbles using a large gauge needle prior to reading and read the plate again.
Cells appear dead after 7 days treatment with my compound	An acute treatment for 3 days in Initiation Medium followed by additional feedings each 7 days should yield suitable positive control signal to complete the assay.

FREQUENTLY ASKED QUESTIONS

1. **Can I buy the assay reagents separately?** The only reagents sold separately are Preadipocyte Medium (cat# PM-1), Glycerol Reagent A (cat# RGTA-10) and the glycerol standard for the Triglyceride Assay kit (cat# TG-GLYSTAN).
2. **Can I use another plate format besides 96 well?** This kit is designed for the assay of A 96 well plate (100 assay points). We do not have a protocol for other formats.
3. **Can I use this kit to measure total triglyceride in other cell lines and other human and non-human cells?** Yes. The assay is not species specific. As long as the sample concentration is in the linear range, this kit should be able to detect it.
4. **My cells did not lyse.** What can I do? If cells are not fully lysed, incubate for another 10 minutes at +37°C - 50°C. Sometimes mixing by pipetting up and down several times is necessary for full lysis.
5. **I do not have time to complete the assay. Can I freeze the samples?** Yes. The cell lysates can be stored at -80°C for a maximum of 7 days. Mix the thawed lysates in the plate by pipetting up and down several times. Allow all reagents and samples to reach room temperature BEFORE adding the Wash Buffer and Glycerol Reagent A to complete the assay.

APPENDIX A. DIFFERENTIATION PICTURES

PREADIPOCYTE —————→ MATURE ADIPOCYTE



APPENDIX B: COMPOSITIONS OF KIT REAGENTS

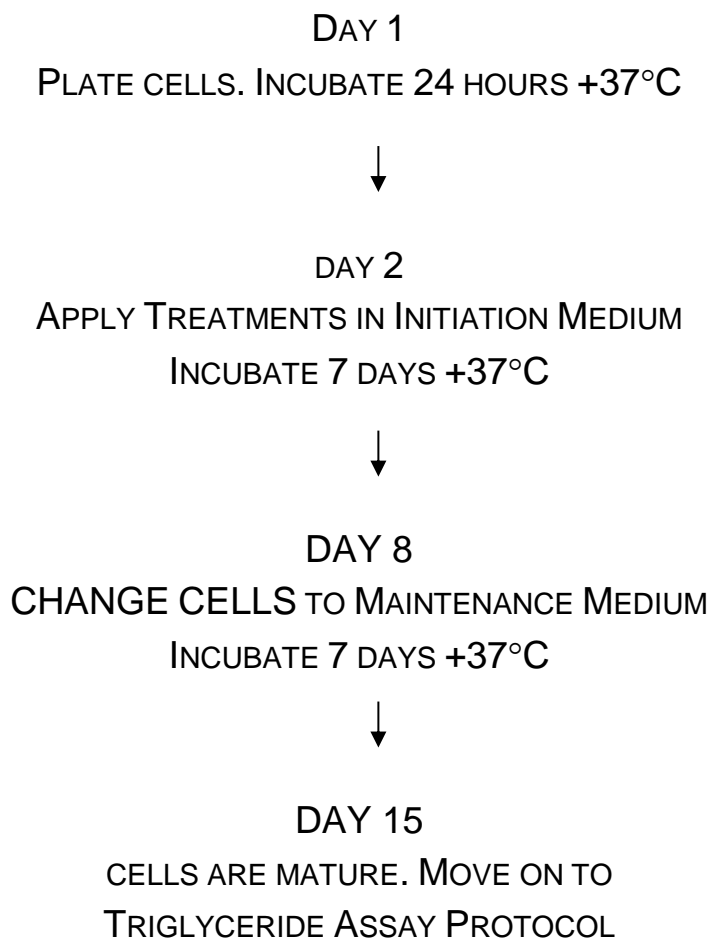
Negative Control (NC) IBMX, PPAR _γ agonist, TNF _α	Positive Control (PC) IBMX, PPAR _γ agonist
<ul style="list-style-type: none"> • DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Human insulin • Dexamethasone • Penicillin • Streptomycin • Amphotericin B • Isobutylmethylxanthine (IBMX) • PPAR_γ agonist • TNF_α 	<ul style="list-style-type: none"> • DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Human insulin • Dexamethasone • Penicillin • Streptomycin • Amphotericin B • Isobutylmethylxanthine (IBMX) • PPAR_γ agonist
Maintenance Medium (MM)	Initiation Medium (IM)
<ul style="list-style-type: none"> • DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Human insulin • Dexamethasone • Penicillin • Streptomycin • Amphotericin B 	<ul style="list-style-type: none"> • DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Human insulin • Dexamethasone • Penicillin • Streptomycin • Amphotericin B • Isobutylmethylxanthine (IBMX)

Reagents	Vehicle Control (VC) IBMX, 0.1% DMSO
Components	<ul style="list-style-type: none"> • DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Biotin • Pantothenate • Human insulin • Dexamethasone • Penicillin • Streptomycin • Amphotericin B • Isobutylmethylxanthine (IBMX) • DMSO
Reagents	Preadipocyte Medium
Components	<ul style="list-style-type: none"> • DMEM / Ham's F-12 medium • HEPES • Fetal bovine serum • Penicillin • Streptomycin • Amphotericin B

APPENDIX C: PLATE LAYOUT

H	G	F	E	D	C	B	A	
								1
								2
								3
								4
								5
								6
								7
								8
								9
								10
								11
								12

APPENDIX D: DIFFERENTIATION FLOWCHART ---



APPENDIX E: TRIGLYCERIDE ASSAY FLOWCHART

