



Lipolysis Assay Kit for 3T3-L1 Cells Non-Esterified Fatty Acids Detection

100 point assay kit

Cat# LIP-2-L1; LIP-2-NC-L1

INSTRUCTION MANUAL ZBM0041.00

STORAGE CONDITIONS

- **96-well plate cultured 3T3-L1 preadipocytes (LIP-2-L1)** 37°C incubator
- **Reagents & Buffers:** 4°C
- **Vehicle & Controls:** -20°C

For in vitro Use Only

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.

ORDERING INFORMATION AND TECHNICAL SERVICES

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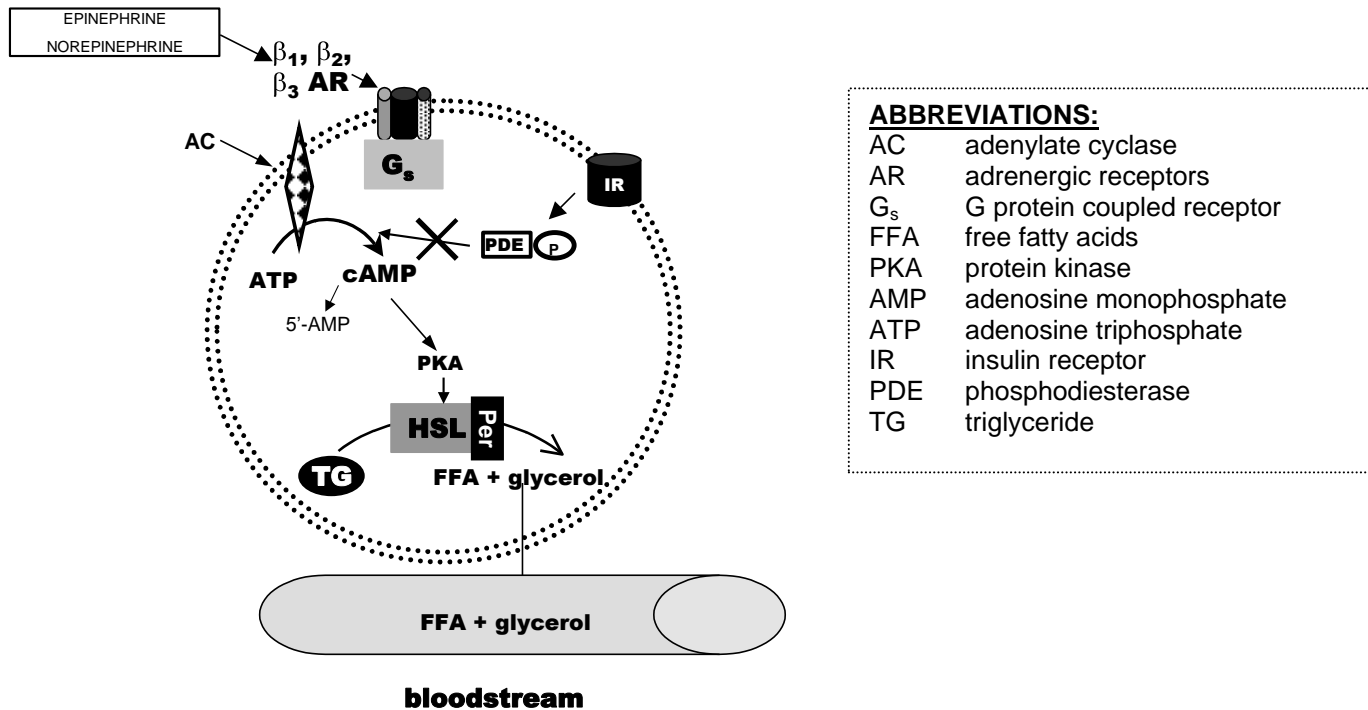
INTRODUCTION

Lipolysis plays a central role in the regulation of energy balance. Lipolysis is the process in which triglycerides are hydrolyzed into glycerol and free fatty acids. This process releases free fatty acids (FFA) into the bloodstream where they may be either re-esterified by the adipocyte or travel to other tissues and exert other effects throughout the body. Elevated adipocyte lipolysis has been observed in obese and diabetic individuals (Arner 1996). Alterations in lipolytic capacity have also been implicated in the susceptibility to obesity of African-American individuals versus their Caucasian cohorts (Danadian *et al.* 2001).

The sympathetic nervous system plays a key role in the regulation of lipid mobilization. The main lipolytic pathway involves beta-agonists (β -agonists), which activate β -adrenergic receptors via the intracellular G_s proteins in adipocytes. This leads to the activation of adenylate cyclase (AC), which then increases cyclic AMP (cAMP) levels. Elevated cAMP acts as a second messenger to activate hormone sensitive lipase (HSL). HSL, the rate-limiting enzyme regulating adipocyte lipolysis, then catalyzes the hydrolysis of triglycerides and results in the release of glycerol and FFA (increased lipolysis). Phosphodiesterases (PDE) are enzymes that hydrolyze cAMP to 5'-AMP (5 prime adenosine monophosphate). This action results in a decrease in lipolysis. PDE inhibitors increase intracellular cAMP levels. 3-isobutyl-1-methylxanthine (IBMX), a non-specific inhibitor of cAMP phosphodiesterases (PDE), is used as the positive control if your test compounds are suspected PDE inhibitors. Isoproterenol, a non-specific β -adrenergic agonist is used as the positive control if your test compounds affect lipolysis via β -adrenergic receptors.

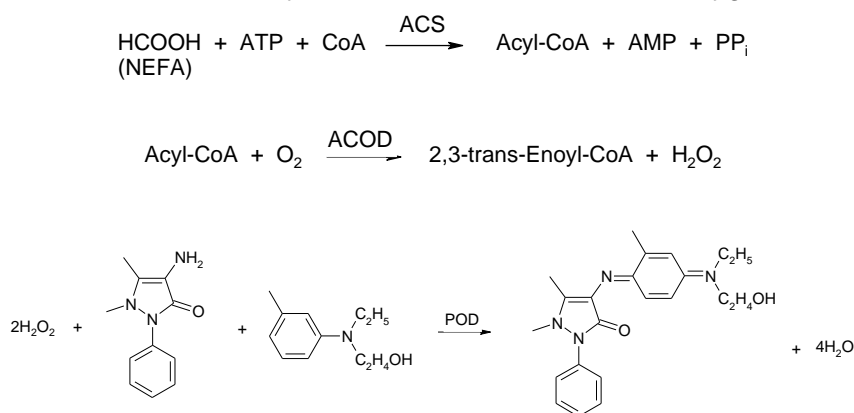
This lipolysis assay kit provides the tool to study chemical compounds that may influence lipolysis in cultured human adipocytes.

Figure 1. Overview of adipocyte lipolysis



PRINCIPLE OF THE ASSAY

Assessment of lipolytic activity is through a coupled reaction to measure non-Esterified fatty acids (NEFA) released by adipocytes. The initial step, carried out by acyl-CoA synthetase (ACS), produces fatty acyl-CoA thiol esters from the NEFA, ATP, Mg, and CoA in the reaction. The acyl-CoA derivatives react with oxygen in the presence of acyl-CoA oxidase (ACOD) to produce hydrogen peroxide. Hydrogen peroxide in the presence of peroxidase (POD) allows the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline with 4-aminoantipyrine which forms a purple product that absorbs light at 550nm. This allows the concentration of NEFA to be determined from the optical density measured at 540 - 550nm.



ITEMS INCLUDED IN THE KIT

ITEM	DESCRIPTION	Cap Color	UNIT	QTY	STORAGE
Plate A	96 well plate 3T3-L1 preadipocytes (LIP-2-L1 ONLY)	---	PLATE	1	37°C
Assay Plate	96-well assay plate, blank (for samples & standards)	---	PLATE	2	-----
Preadipocyte Medium	3T3-L1 Preadipocyte Medium (cat# PM-1-L1); 50ml (LIP-2-L1 ONLY)		BOTTLE	1	4°C
Differentiation Medium	3T3-L1 Adipocyte Differentiation Medium (cat# DM-2-L1); 15ml (LIP-2-L1 ONLY)		BOTTLE	1	4°C
Adipocyte Medium	3T3-L1 Adipocyte Maintenance Medium (cat# AM-1-L1); 100ml (LIP-2-L1 ONLY)		BOTTLE	1	4°C
LIP-2/3 Assay Buffer	100 ml	---	BOTTLE	1	4°C
Wash Buffer	50 ml	---	BOTTLE	1	4°C
Vehicle	0.1% DMSO in LIP-2/3 Assay Buffer	PURPLE	1 ml / VIAL	1	-20°C
Positive control	Isoproterenol, 10 mM in DMSO. <u>Dilute to 1 μM in Assay Buffer before use!</u> (i.e.1 μl in 10 ml Assay Buffer)	BLUE	10 μl / VIAL	1	-20°C
FFA Standard	1mM Stock. See page 5 for standard curve preparation	AMBER	100 μl / VIAL	1	4°C
FFA Diluent A		YELLOW	10.5ML	1	4°C
FFA Diluent B		PINK	5.5ML	1	4°C
FFA Reagent A	Reconstitute using 10.5 ml FFA Diluent A. Discard remainder after 10 days	YELLOW	BOTTLE	1	4°C
FFA Reagent B	Reconstitute using 5.5 ml FFA Diluent B. Discard remainder after 10 days	PINK	BOTTLE	1	4°C
Tray	For multi-channel pipetters, clear polyvinyl	CLEAR	EACH	2	-----

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

- Multi-channel Pipet , single channel pipet and pipet tips
- Sterile trays for multi-channel pipetters during differentiation of cells
- Plate reader with a filter of 540 nm
- Incubator at 37°C
- Large gauge needle
- Tubes for dilution of standards

ASSAY PROCEDURE

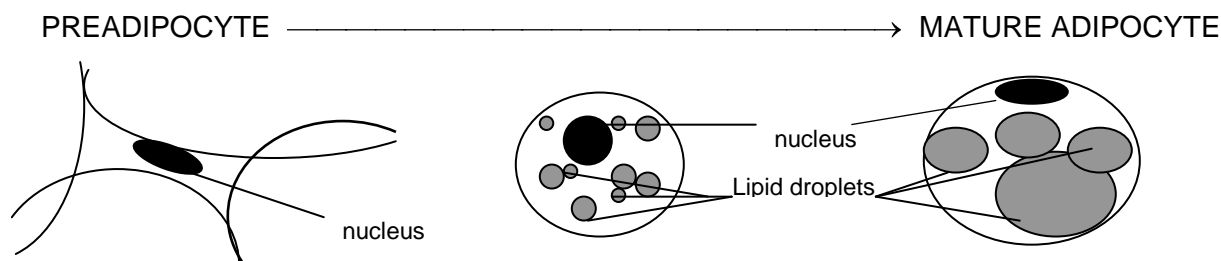
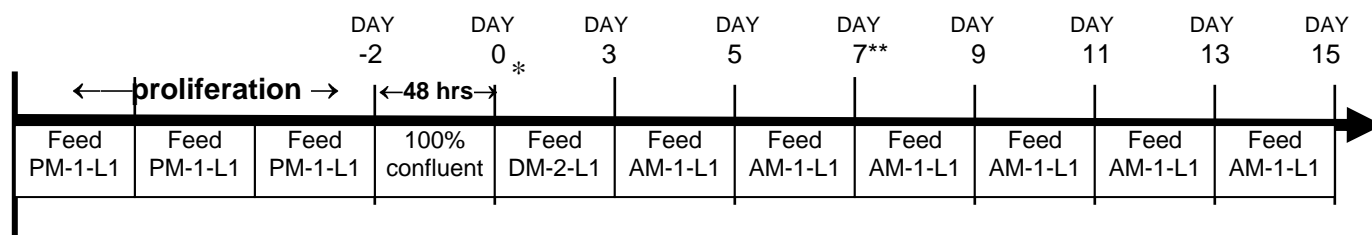
A. DIFFERENTIATION PROCEDURE

1. Preadipocytes are plated sub-confluent in 3T3-L1 Preadipocyte Medium (cat# PM-1-L1) and shipped the next day via overnight delivery.
2. Incubate cells until they are 100% confluent (in about 4-5 days). Cells will need to be fed every other day with PM-1-L1 during this time. See Table 1 for feeding volumes.
3. Once the cells are confluent, incubate an additional 48 hours before initiating differentiation.
4. Two days after the cells have been confluent, remove the Preadipocyte Medium (cat# PM-1-L1) and replace with an appropriate volume 3T3-L1 Differentiation Medium (cat# DM-2-L1; see table 1 below for recommended volumes). Incubate for 3 days.
5. Remove the 3T3-L1 Differentiation Medium and replace with 3T3-L1 Adipocyte Maintenance Medium. Incubate for 2-3 days.
6. Feed cells every 2-3 days using 3T3-L1 Adipocyte Maintenance Medium until ready for assay. 3T3-L1 adipocytes are suitable for most assays 7-14 days post differentiation (see Table 1 and 3T3-L1 Growth and Differentiation Feeding Schedule)

Table 1. Feeding Volumes

Format	Change PM-1-L1 to PM-1-L1		Change PM-1-L1 to DM-2-L1		Change DM-2-L1 to AM-1-L1		Change AM-1-L1 to AM-1-L1	
	OUT	IN	OUT	IN	OUT	IN	OUT	IN
96 well plate	90µl/well	90µl/well	150µl/well	150 µl / well	90 µl /well	120µl /well	90 µl /well	120µl /well
48 well plate	300 µl /well	300 µl /well	500µl /well	500 µl /well	300 µl /well	400 µl /well	300 µl /well	400 µl /well
24 well plate	0.6 ml/well	0.6 ml/well	1.0 ml/well	1.0 ml/well	0.6 ml/well	0.8 ml/well	0.6 ml/well	0.8 ml/well
12 well plate	1.2 ml/well	1.2 ml/well	2.0 ml/well	2.0 ml/well	1.2 ml/well	1.6 ml/well	1.2 ml/well	1.6 ml/well
6 well plate	1.8 ml/well	1.8 ml/well	3.0 ml/well	3.0 ml/well	1.8 ml/well	2.4 ml/well	1.8 ml/well	2.4 ml/well
T-75 flask	12 ml/flask	12 ml/flask	20 ml/flask	20 ml/flask	12 ml/flask	16 ml/flask	12 ml/flask	16 ml/flask
T-25 flask	4.2 ml/flask	4.2 ml/flask	7 ml/flask	7 ml/flask	4.2 ml/flask	5.6 ml/flask	4.2 ml/flask	5.6 ml/flask

3T3-L1 Growth and Differentiation Feeding Schedule



* Once the cells are 100% confluent, incubate an additional 48 hours before initiating differentiation.

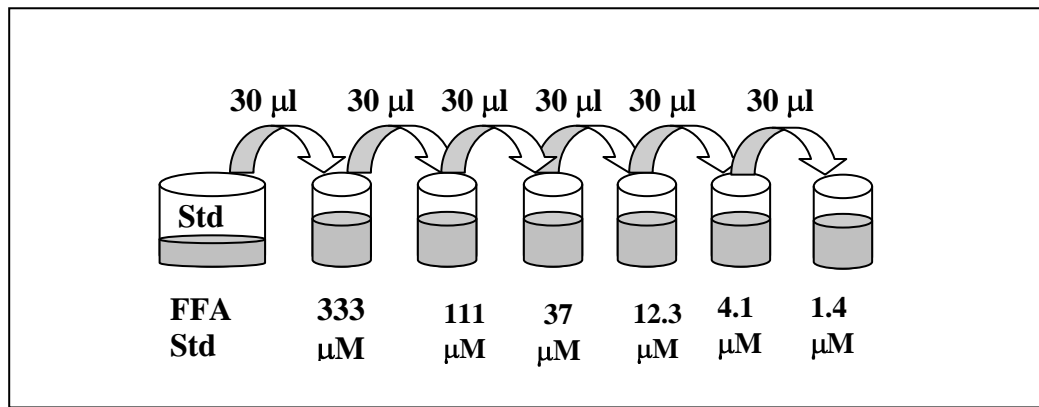
** 3T3-L1 adipocytes are suitable for most assays 7-14 days post differentiation

B. LIPOLYSIS PROCEDURE

1. Make your stock solution using whatever vehicle is appropriate for your test compounds. Dilute your stock solutions to their final concentration in LIP-2/3 Assay Buffer (100 ml is available). NOTE: if desired, maintain a constant concentration of solvent by preparing all compound dilutions in the highest concentration of that solvent. Dilute your controls in assay buffer. Prepare all vehicles as appropriate for your compounds, 0.1% DMSO has been included as the vehicle for the positive controls. Include the Assay Buffer alone as a vehicle control. PLEASE NOTE: ZEN-BIO DOES NOT RECOMMEND THE USE OF SOLVENTS AT CONCENTRATIONS ABOVE 1%.
2. Remove 120 μ l medium from each well. Gently add 200 μ l Wash Buffer to all wells. Remove 200 μ l of the media and Wash Buffer from each well and replace with another 200 μ l Wash Buffer.
3. Remove all the media and Wash Buffer from the cells from triplicate wells. Treat the cells with 100 μ l of the test compounds resuspended in Assay Buffer three (3) wells at a time. Treat with the diluted Isoproterenol as positive control. Use the Assay Buffer alone as one of the vehicle controls. Please be sure to include both the vehicle provided in the kit and your vehicle (if your test compounds are not dissolved in DMSO). The assay should be performed in triplicate.
4. Incubate the plates at 37°C-humidified incubator for 3 hours (for time course experiments the longest time point recommended is 5 hours). Note: Treatment times longer than 3 hours will result in significant fatty acid reutilization by the adipocytes and may decrease signal relative to total lipolysis activity.
5. Prepare the standard curve using the STANDARD SOLUTION as follows:

Briefly spin down the contents of the free fatty acid standard tube before reconstitution. Standards are: 0, 1.4, 4.1, 12.3, 37, 111, and 333 μ M fatty acid. Prepare as follows:

The kit standard solution is the 1.0 mM standard. Pipette 60 μ l of Dilution Buffer into 6 tubes (not provided). Pipette 30 μ l of the FFA Standard Stock into a tube labeled 333 μ M. Prepare a dilution series as depicted below. Mix each new dilution thoroughly before proceeding to the next. The Dilution Buffer alone serves as the zero standard.



6. Add 10.5ml FFA Diluent A to the FFA Reagent A bottle and gently invert. **DO NOT VORTEX!** Store any remaining solution at 2-8°C; it is stable for 10 days after reconstitution refrigerated (2-8°C).
7. At the end of the incubation, 50 µl of the conditioned media is removed and transferred to the corresponding well of a blank plate for assessment of non-esterified fatty acids. [This is most easily accomplished using a multi-channel pipet.] Add 50 µl of each standard to empty wells.
8. Add the reconstituted FFA Reagent A to one of the disposable trays provided in the kit. Add 100 µl of FFA Reagent A to each well. Gently shake the plate to ensure mixing. Place in a 37 °C incubator for 10 minutes.
9. Add 5.5 ml FFA Diluent B to the FFA Reagent bottle and gently invert. Store any remaining solution at 2-8°C; it is stable for 10 days after reconstitution refrigerated (2-8°C).
10. Add the reconstituted FFA Reagent B to the other disposable tray provided in the kit. Add 50 µl of FFA Reagent B to each well. Gently shake the plate to ensure mixing. Place in a 37 °C incubator for 10 minutes.
11. Allow the plate to equilibrate to room temperature for 5 minutes. During this time, ensure that there are no bubbles in the solution mixture. Use a large gauge needle or clean pipet tip to pop any bubbles as this will result in inaccurate absorbance readings.
12. The optical density of each well is then measured at 540 nm.

FATTY ACID STANDARD CURVE

Generate standard curve: see example below

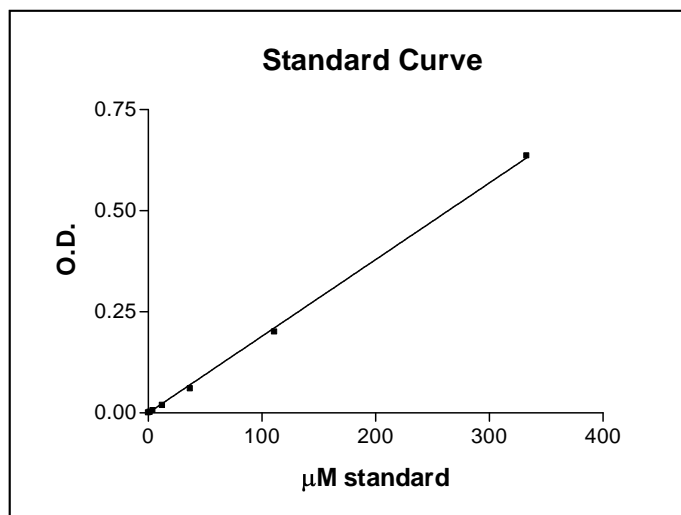
[DO NOT use this standard curve to generate your data. This is an example.]

Subtract the OD value of the 0 μ M standard from all OD values including the standard curve. Note: 1mM standard is commonly omitted from analysis due to lack of linearity between 333 μ M and 1mM. Optionally, a 4-parameter fit may be used to calculate standard curve.

μ M std	OD	OD - zero
333	0.68	0.636
111	0.244	0.2
37	0.104	0.06
12.3	0.063	0.019
4.1	0.05	0.006
1.4	0.046	0.002
0	0.044	0

$$y = 0.0019x - 0.0045$$

$$R^2 = 0.9995$$



Data are expressed as μ M free fatty acids released.

OPTION: express data as Fold induction over appropriate vehicle

$$\text{Fold induction} = \frac{\mu\text{M free fatty acids SAMPLE}}{\mu\text{M free fatty acids VEHICLE}}$$

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

FREQUENTLY ASKED QUESTIONS

1. I do not have time to run the assay. Can I freeze the conditioned media in PLATE B? How long can I store the samples before I complete the assay? Yes. The conditioned media in PLATE B can be immediately stored at -80°C for a maximum of 7 days. Bring the conditioned media in PLATE B to room temperature BEFORE adding the FFA Reagents A and B and completing the assay.

APPENDIX A: PLATE LAYOUT

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

APPENDIX B: LIP-2-L1 PROCEDURE FLOWCHART

ON DAY OF ASSAY

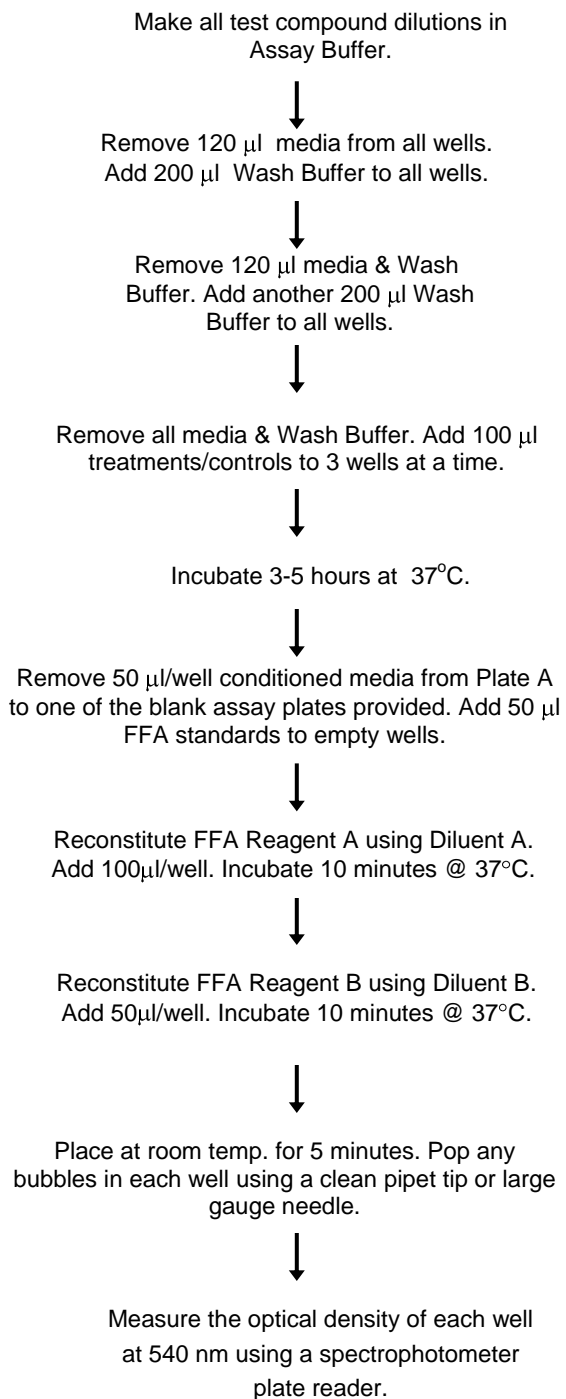


Plate A = plate of mature 3T3-L1 adipocytes

