Product Manual

Adipogenesis Assay Kit

Catalog #: ENZ-KIT103-0005

5 x 24 Well or 225 cm^2 Tissue Culture Plate Surface Area

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Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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DESCRIPTION

The Adipogenesis Assay Kit is a complete kit for the qualitative determination of differentiation of 3T3L1 cells into adipocytes.

All of reagents needed to induce and detect differentiation are provided. This kit can be used to either test agonists that induce adipogenesis or antagonists that inhibit it from occurring. The protocol provided is for use with 24 well plates, but can be adapted for use with other plate sizes. The stain provided allows for visual confirmation and can also be easily extracted allowing measurement using a spectrophotometer or microplate reader. Please read the complete kit insert before performing this assay.

BACKGROUND

Obesity is a serious health problem that leads to elevated triglyceride levels, which can lead to cardiovascular disease, diabetes, hypertension, and hypercholesterolemia[5,13]. It is characterized by the enlargement of adipose tissue to store excess triglyceride intake[5]. This can occur through two different mechanisms, known as hyperplasia (increase in the number of adipocytes) and hypertrophy (increase in the volume of adipocytes)[3]. Triglycerides accumulate in lipid droplets that are embedded in the cytoplasm of the adipocyte[13]. Lipid droplets are intracellular organelles that are involved in the regulation of the storage and hydrolysis of triglycerides and cholesterol esters, which are both neutral lipids[13]. Lipid droplets are found in two types of adipose tissue. White adipose tissue is made up of white adipocytes, which are composed of a single, large lipid droplet that takes up most of the cell volume and functions in neutral lipid storage[9,10]. Brown adipose tissue is made up of brown adipocytes, which are composed of several small lipid droplets with increased mitochondria in the cytoplasm[9,10]. This tissue functions in maintaining body temperature during long periods of cold exposure[10].

Essential to understanding and treating disease, is the identification of drugs that modulate differentiation and adipogenesis. Agonists of PPARγ play an important role in decreasing insulin resistance[6]. Rosiglitazone, a thiazolidinedione that binds to PPARγ and sensitizes fat cells to insulin, is used clinically as an anti-diabetic drug in the treatment of type 2 diabetes[6,12]. It enhances adipogenesis and stimulates hyperplasia by speeding up differentiation and increasing the degree of differentiation[7,8]. Antagonists also play an important role in

treating obesity. Pro-inflammatory cytokines, such as TNF-α and IL-1 inhibit adipogenesis by decreasing the expression of PPARγ and CEBPα[9,11]. Inhibitors of PPARγ have been identified as a potential target of anti-obesity drugs[2].

One of the most utilized models for the study of differentiation of fibroblast into adipocytes is the 3T3-L1 cell line[1,5,9]. Differentiation requires three components, which include insulin or insulin-like growth factor, dexamethasone (DXM), and 3-isobutyl-1-methylxanthine (IBMX)[5,7,9]. Insulin induces proliferation and differentiation of preadipocytes[8,9]. DXM is a glucocorticoid, which activates CEBPβ (CCAAT/enhancer binding protein-β)[5,8]. IBMX is a competitive, nonselective phosphodiesterase inhibitor that increases intracellular cAMP levels and activates CEBPδ[5,8]. It also increases intracellular protein kinase A levels, which is important for the transcriptional activation of PPARγ (peroxisome proliferator-activated receptor-γ)[8]. The combination of DXM and IBMX regulates PPARγ, which is a nuclear hormone receptor that is highly expressed in adipose tissue and promotes adipogenesis[2,3,12]. CEBPβ and CEBPδ induce transcription of CEBPα and PPAR, which in turn activates the expression of adipocyte-specific genes[11]. During the differentiation process, 3T3-L1 cells undergo a post-confluent mitosis, which occurs 24 hours after induction with insulin, DXM, and IBMX, followed by growth arrest[5]. After growth arrest occurs, the cells are committed to becoming adipocytes and express late markers of differentiation at day 3, after induction[5]. Growth arrest of the cells is a requirement for terminal adipocyte differentiation[5,9]. After 5-7 days post-induction, the cell morphology changes from the elongated, fibroblastic cells to rounded cells, and lipid droplets begin to accumulate[4,5].

SAFETY WARNINGS & PRECAUTIONS

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

- Refer to the products MSDS before use.
- Reagents should be treated as though they are potentially toxic. They should be handled with care and disposed of properly.
- Observe good laboratory practices. The use of protective equipment including, nitrile gloves, lab coat, and protective eyewear is recommended.

Handle with care

- Solutions in DMSO can be readily absorbed through the skin.
- Adipogenesis Dye will stain clothing and exposed skin.
- Isopropanol is flammable. Keep away from open flames.

MATERIALS SUPPLIED

1. **Differentiation Solution, 65 µL, Catalog No. 80-2634:**
   A 1000X solution of dexamethasone and 3-Isobutyl-1-methylxanthine (IBMX) in DMSO.
2. **Insulin Solution, 130 µL, Catalog No. 80-2635:**
   A 1000X solution of insulin in 0.02N HCl.
3. **Enhancer Solution, 65 µL, Catalog No. 80-2639:**
   A 1000X solution of rosiglitazone in DMSO.
4. **Cell Fixative, 28 mL, Catalog No. 80-2636:**
   A 1X solution of 3.7% formaldehyde in phosphate buffered saline.
5. **Adipogenesis Dye, 28 mL, Catalog No. 80-2637:**
   A 1X solution of Oil Red O lipid dye in 60% isopropanol.
6. **Extraction Solution, 28 mL, Catalog No. 80-2638:**
   A 1X solution containing 60% isopropanol.

Reagents require separate storage conditions.

STORAGE

All components of this kit are shipped at -20°C. Upon receipt the Differentiation Solution, Insulin Solution, and Enhancer Solution should be stored at or below -20°C. The Cell Fixative, Adipogenesis Dye, and Extraction Solution can be stored at room temperature. If stored properly all reagents should be stable until the kits expiration date. Induction media is stable for 2 weeks at 4°C.

Note: The Cell Fixative and Adipogenesis Dye will form insoluble precipitates especially upon freezing. Removal of the precipitate from the Cell Fixative is recommended but not required for performance; however removal of the precipitate from the dye is required for assay performance. Filtering either reagent will have no effect on their performance in the assay.

OTHER MATERIALS NEEDED

1. Preadipocyte cell line (Ex. 3T3-L1, ATCC # CL-173)
2. 24 well or other size tissue culture treated plate.
3. Tissue culture media for growing 3T3-L1 cells (DMEM +10% FBS)
4. Deionized or distilled water.
5. Precision pipets for volumes between 5 µL and 1,000 µL.
6. Spectrophotometer or microplate reader capable of reading 96 well plates at 490-520nm.
7. 0.2 µM PES syringe filter

OPTIONAL MATERIAL

1. Collagen I, rat tail (thin layer coating), Enzo Catalog No. ALX-522-440-0050.

IMPORTANT TECHNICAL NOTES

1. The 3T3-L1 cell line must be maintained as a subconfluent monolayer prior to use in the assay. However, actively growing cells will not undergo adipogenesis.

2. Low passage cells are highly recommended. Higher passage number cells (≥10) have been shown to cause increased OD's in untreated cells and may dislodge from the plate when treated. Improperly maintained cells may adversely affect assay performance.

3. Other preadipocyte cell lines may be used but it is up the user to validate the results.

4. This kit was validated using 24 well plates, however if the use of smaller plates is required the user must determine the appropriate culture conditions. Use tremendous care when changing the media. As stated above the cells have a tendency to pull away from the edges and form a clump of cells. This may lead to variability in the data. The use of collagen coated plates may help.

5. Fetal bovine serum lots may vary potentially causing increased background. The use of calf serum may help to eliminate background in the assay.

6. The components of the kit are not sterile. They should be sterile filtered through a 0.2 μM PES filter before adding to cells to prevent contamination. The use of antibiotics in the media does not affect assay performance.

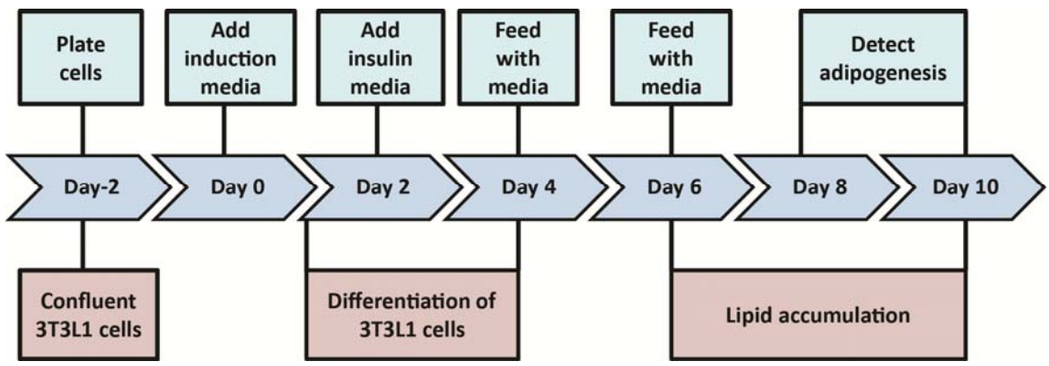
7. Absorbance values may vary due to plate reader differences and plate characteristics.

8. The Cell Fixative and Adipogenesis Dye will likely form insoluble precipitates which should be removed by centrifugation or filtering through a 0.2 μM PES filter.

9. Adipogenesis Dye can be read between 490nm - 520nm. The Adipogenesis Dye will stick to the plastic so it is recommended that non-specific background be subtracted from a blank plate.

10. It is recommended that only the amount of induction media or insulin media needed for each assay be made. However, if there is extra it may be stored at 4°C for up to two weeks without significant loss of activity. The stability of drugs that are being used as test conditions in the assay must be evaluated by the user.

WORKFLOW DIAGRAM



Basic Workflow of Adipogenesis Assay. Procedure on top row, cell characterization on bottom row.

ASSAY PROCEDURE

Cell Culture (volumes are for 1 x 24 well plate):

Note: For different size plates adjust the volumes accordingly.

1. Plate 1 mL of subconfluent 3T3L1 cells @ 0.25 - 0.5 x 10^6 / mL (≤ passage 10) onto the appropriate number of wells of a 24 well plate.

2. Grow 2 - 4 days at 37°C, 5% CO2.until they are 2 days past confluent (This is Day 0).

3. On Day 0 prepare Induction Media by adding 12 µL of differentiation solution, 12 µL enhancer solution, and 12 µL of insulin solution to 12 mL of DMEM + 10% FBS + 1X Penstrep.

4. Aspirate media from wells.

5. For induced wells: Add 0.5 mL Induction Media (from step 3) per well.

   For uninduced wells: Add 0.5 mL DMEM + 10% FBS + 1X PenStrep per well.

6. Incubate for 2 days at 37°C, 5% CO2.

7. Prepare Insulin Media by adding 12 µL of insulin solution to 12 mL of DMEM + 10% FBS + 1X penstrep.

8. Carefully aspirate media from wells. **(Note: Differentiated cells have a tendency to detach from wells).**

9. For induced wells: Add 0.5 mL Insulin Media (from step 7) per well.

   For uninduced wells: Add 0.5 mL DMEM + 10% FBS + 1X PenStrep per well.

10. Incubate for 2 days at 37°C, 5% CO_2.

11. Remove media from all wells and feed all cells with 1 mL of DMEM + 10% FBS + 1X penstrep.

12. Feed the cells every 2 days with 1mL of DMEM + 10% FBS + 1X penstrep for at least 4-6 more days. At 8-10 days post induction, full differentiation into adipocytes should be achieved. Fully differentiated cells have large oil droplets that are visible and often appear viscous when feeding. If cells do not appear to be fully differentiated, continue incubation and feed every 2 days with DMEM + 10 FBS + 1X penstrep.

Staining:

The Adipogenesis Dye can stick to the plastic causing non-specific background to occur. To correct for this perform the staining procedure on several empty well from an empty 24 well plate. The average value can then be subtracted from both uninduced and induced samples to remove non-specific background staining.

13. If needed centrifuge or filter the Adipogenesis Dye using a 0.2 µM filter if precipitate is observed. **Precipitated dye will lead to variability in the results.**

14. Aspirate media from cells.

15. Add 200 µL of Cell Fixative to all wells and allow the plate to sit at room temperature for 30 minutes. Remove cell fixative and add 200 µL of Adipogenesis Dye per well, and allow the plate to sit at room temperature for 30min.

16. Remove the stain and rinse each well with 3X with 1 mL distilled water.

17. Allow the wells to dry completely.

18. If desired the stained cells can be visualized using microscopy.

19. Once the wells are dry add 200 µL of Extraction Solution per well and incubate for 30min at room temperature with shaking (if possible).

20. Transfer contents of each well to a new 96 well plate and read at 490nm.

21. Remove non-specific background by subtracting the average OD 490nm readings obtained from empty wells from each sample well.

TYPICAL RESULTS

The results shown below are for illustration only.

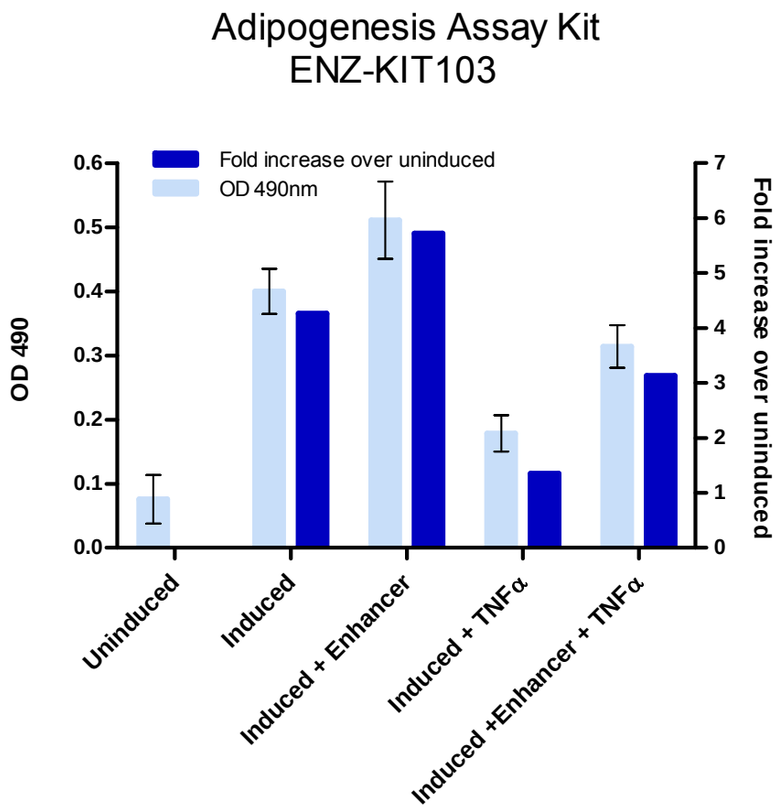


Figure 1. Induction Levels: OD 490nm values were obtained after extraction and the average background was subtracted from each well. The fold increase over the uninduced condition was determined using the following calculation: (Induced OD 490nm – Uninduced OD 490nm) / Uninduced OD 490nm

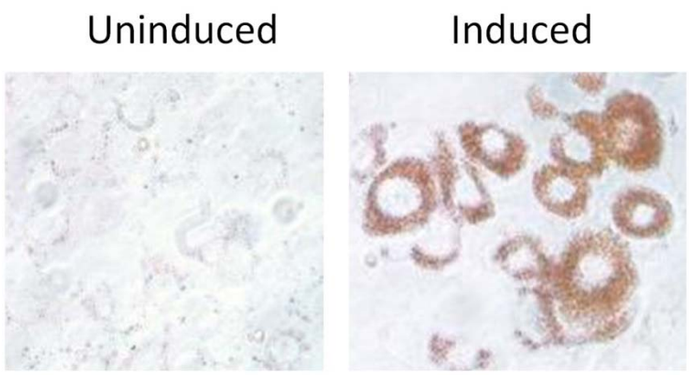


Figure 2. Microscopic Analysis of Induction: 3T3-L1 cells were treated with and without induction media. After 10 days they were stained and imaged at 40X magnification.

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NOTES

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