Comparing the Triglyceride-Glo™ Assay to Oil Red O Staining to Assess Adipogenesis

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Kit: Triglyceride-Glo™ Assay

Abstract

The Triglyceride-Glo™ Assay provides a simple and accurate method for assessing adipogenesis utilizing luminescence-based chemistry, which allows better sensitivity and extended quantitative range in comparison to absorbance- and fluorescence-based methods. Here, we compare the performance of the Triglyceride-Glo™ Assay to the Cayman Chemical Adipogenesis Assay in terms of quantitative range and usability.

Introduction

Research into the process of adipogenesis, the mechanism by which stem cells differentiate into mature adipocytes (fat cells), is vital to understanding the pathogenesis of, and discovering potential treatments for, such diseases as diabetes, obesity, cardiovascular disease, liver diseases and cancer. The ability to measure the extent of adipogenesis within a culture of adipocyte progenitor cells (subjected to certain growth conditions or chemical compounds for instance) is a fundamental aspect of such research and, therefore, of primary interest to those studying within this area. Traditionally, the extent of adipogenesis has been evaluated using absorbance-based chemistries involving lipid-soluble dyes, such as Oil Red O. However, there are several limitations to this method, including low sensitivity, low quantitative range and increased hands-on time in relation to newer methods (1). Conversely, the Triglyceride-Glo™ Assay utilizes a luminescence-based chemistry, which allows better sensitivity and extended quantitative range in comparison to absorbance- and fluorescence-based methods, while employing a simple, timesaving and user-friendly protocol. In this experiment, the Triglyceride-Glo™ Assay was compared to the Cayman Chemical Adipogenesis Assay in terms of quantitative range and usability by assaying completely differentiated (mature adipocytes), partially differentiated and undifferentiated 3T3L1-MBX fibroblast cells.

Assay Principles

In the Triglyceride-Glo™ Assay, luminescent signal is directly proportional to the amount of glycerol present. Free glycerol is quantified by lysing cells without lipase, and total glycerol is quantified by lysing separate cells in the

presence of lipase (which liberates glycerol from triglycerides). A detection reagent is then added to the cell lysates and glycerol is utilized in a coupled chemical reaction to generate light. Luminescent signal is converted to concentration values using a glycerol standard curve, with the difference between free and total glycerol concentrations determining the concentration of triglyceride (constituting about 95% of mature adipocytes by weight [2]), which is used to assess adipogenesis. The Cayman Chemical Adipogenesis Assay utilizes lipid-soluble Oil Red O stain to evaluate the lipid content (mostly triglycerides) of a sample. Following stain and wash steps, lipid-bound dye is extracted from cells, transferred to an assay plate, and absorbance is measured. Absorbance is thus proportional to the lipid content of the sample and used to assess adipogenesis; however, lipids cannot be directly quantified using this method.

Methods

Growth/Differentiation of 3T3L1-MBX Fibroblasts

3T3L1-MBX fibroblast cells were seeded in a 96-well plate at a density of 20,000 cells per well in 100µl of DMEM + 10% FBS on day 1, 10 and 20 of the experiment. Cells were maintained in a humidity-controlled, 37°C/5% CO_2 incubator and treated with the necessary compounds to induce differentiation (according to *Glucose Uptake-Glo™ Assay Technical Manual* TM467). By day 22 of the experiment, cells plated on day 1 completely differentiated into mature adipocytes, cells plated on day 10 partially differentiated, and cells plated on day 20 did not differentiate. Two identical plates of cells were prepared, one for use with each assay.

Triglyceride-Glo™ Assay

Cells were assayed for triglyceride content on day 22 of the experiment using the Triglyceride-Glo™ Assay according to the standard protocol outlined in *Triglyceride-Glo™ Assay Technical Manual* TM600. Briefly, growth media was removed from the wells, cells were washed twice with PBS, cells were lysed using Glycerol Lysis Solution (± lipase), cell lysates were diluted into white 96-well assay plates, Glycerol Detection Reagent was added, mixtures were incubated at room temperature for 1 hour, and luminescence measurements were taken using a GloMax® Discover Microplate Reader. To generate standard curves, the provided Glycerol Standard was diluted to 10, 20, 40, 60 and 80µM in Glycerol Lysis Solution (± lipase).

To determine the effect that freeze-thawing the cell lysates would have on the assay, cell lysates (lysed using Glycerol Lysis Solution) were frozen at –20°C for 5 days. Cell lysates were then thawed, diluted and assayed following the same protocol as stated previously.

Cayman Chemical Adipogenesis Assay

An identical plate of cells was stained and assayed using the Cayman Chemical Adipogenesis Assay Kit, following standard protocol (3). Briefly, growth media was removed from the wells, cells were fixed, washed twice and dried. Cells were then stained with Oil Red O, washed 5–6 times to remove excess dye and dried. Microscopic images were then taken, dye was extracted from the cells, transferred to a UV-transparent microplate, and absorbance was read at 490nm using a GloMax® Discover Microplate Reader.

Results

In this experiment, completely differentiated (mature adipocytes), partially differentiated and undifferentiated 3T3L1-MBX fibroblast cells (Figure 1) were assayed using the Triglyceride-Glo™ Assay and the Cayman Chemical Adipogenesis Assay. The quantitative range and usability of each assay was assessed.

The extent of adipogenesis was estimated by calculating triglyceride concentrations based on free and total glycerol concentrations determined using the Triglyceride-Glo™ Assay (using the standard curve displayed in Figure 2) or by measuring the amount of light absorbed by Oil Red O dye bound to lipids using the Adipogenesis Assay. These values are displayed in Figure 3, with higher values indicating more complete adipogenesis. Both assays displayed a similar trend, with completely differentiated cells producing higher values compared to partially differentiated and undifferentiated cells. While both assays assess adipogenesis based on triglyceride content, the Triglyceride-Glo™ Assay does so quantitatively by

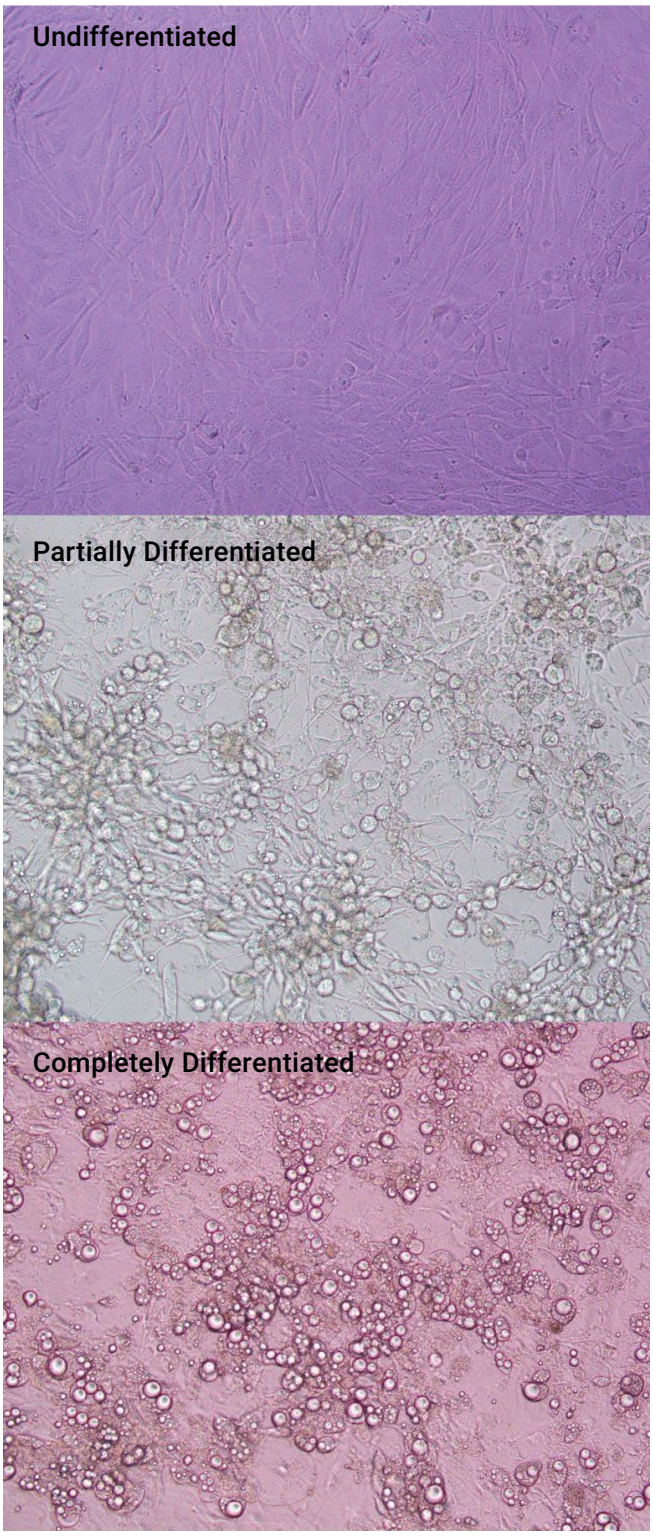

Figure 1. Images of 3T3L1-MBX cells at different stages of differentiation (20X magnification). Images (taken on day 22 of the experiment) of undifferentiated (top, plated day 20), partially differentiated (middle, plated day 10) and completely differentiated (bottom, plated day 1) 3T3L1-MBX fibroblast cells are displayed. Cells were assayed at each of these growth stages to determine the extent of adipogenesis using both the Triglyceride-Glo™ Assay and Cayman Chemical Adipogenesis Assay.

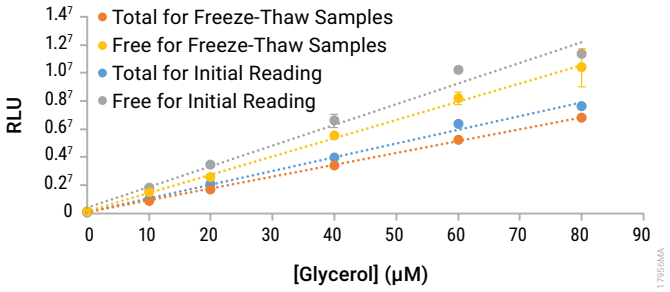

Figure 2. Glycerol standard curves for the Triglyceride-Glo™ Assay. 10, 20, 40, 60 and 80µM glycerol standards were prepared in lysis buffer (for free glycerol measurements) and lysis buffer + lipase (for total glycerol measurements). Fifty microliters of each standard were assayed in duplicate. The resulting standard curves are displayed, plotting mean relative luminescence units (RLU) ± standard deviation against glycerol concentration.

directly measuring glycerol and triglyceride levels, in contrast to the Adipogenesis Assay, which can only qualitatively assess the lipid content of cells.

To compare the quantitative range of the two assays, we examined signal-to-background (S/B) ratios determined using the raw RLU and absorbance values produced by the Triglyceride-Glo™ Assay and Adipogenesis Assay, respectively. Following dilution of total glycerol cell lysates to within the linear range of the assay (<80µM glycerol), the Triglyceride-Glo™ Assay displayed a maximum S/B ratio of about 50 compared to a maximum S/B ratio of about 5 for the Adipogenesis Assay, when assaying mature adipocytes (Figure 4). However, as S/B ratios vary based on the degree of cell lysate dilution for the Triglyceride-Glo™ Assay, total glycerol standards may better represent the quantitative range. Based on these standards, the maximum obtainable S/B ratio was about 70 at the maximum glycerol concentration of 80µM (Figure 5). For the Adipogenesis Assay, according to the standard protocol, typical absorbance readings taken from wells containing completely differentiated cells range from 0.2–0.4, while readings from wells containing undifferentiated cells are usually around 0.05. Considering this information, the maximum S/B ratio obtainable using this kit would be about 8. Therefore, using maximum S/B ratios as a measure of quantitative range, the Triglyceride-Glo™ Assay outperformed the Adipogenesis Assay.

The extended quantitative range of the Triglyceride-Glo™ Assay increases assay resolution, allowing for clearer, more accurate assessment of adipogenesis

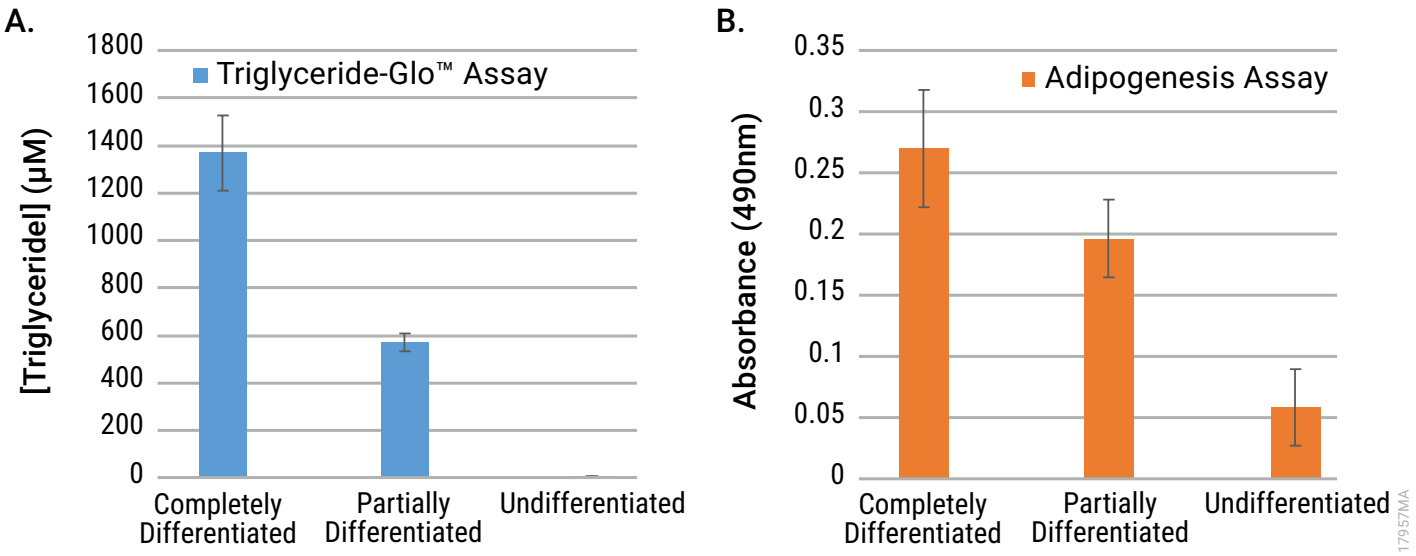

Figure 3. Assessing extent of adipogenesis using the Triglyceride-Glo™ Assay and Cayman Chemical Adipogenesis Assay. Completely differentiated (mature adipocytes), partially differentiated and undifferentiated 3T3L1-MBX fibroblast cells were analyzed using each assay. **Panel A.** Calculated triglyceride concentrations ± standard deviation for the Triglyceride-Glo™ Assay (n=9). RLU resulting from 1:25-diluted total glycerol lysates were converted to concentration values using a glycerol standard curve and correcting for dilution. As free glycerol concentrations were negligible, effectively, [total glycerol] = [triglyceride] (see Figure 5). **Panel B.** Mean absorbance ± standard deviation for the Adipogenesis Assay (n=9).

and making subtle changes more perceptible compared to the absorbance-based Adipogenesis Assay. In this experiment, for instance, the difference between partially and completely differentiated cells is much more apparent using the Triglyceride-Glo™ Assay. Increased assay resolution would also be of great importance in drug studies, for example, where the effects of small changes in drug concentrations on adipogenesis may need to be detected.

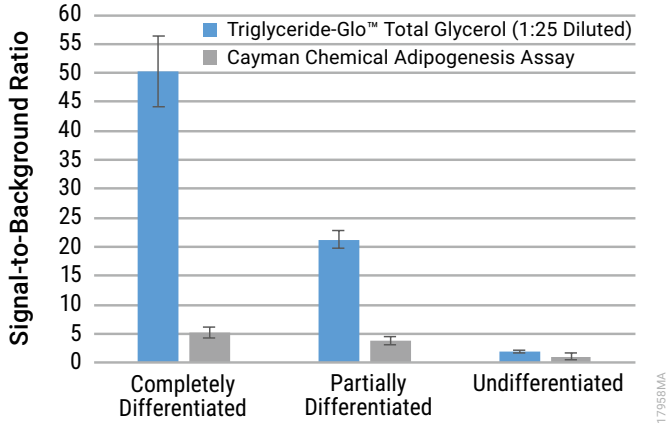

Figure 4. Signal-to-background (S/B) ratios resulting from the Triglyceride-Glo™ Assay and Cayman Chemical Adipogenesis Assay. Completely differentiated (mature adipocytes), partially differentiated and undifferentiated 3T3L1-MBX fibroblast cells were analyzed using each assay. For the Triglyceride-Glo™ Assay, cell lysates were diluted 1:25 before taking luminescence measurements to fit within the linear range of the assay (<80µM glycerol). Mean S/B ratios ± error for each assay are displayed (n=9).

In terms of usability, the Triglyceride-Glo™ Assay was also simpler to perform than the Adipogenesis Assay, with less hands-on time. The Triglyceride-Glo™ Assay follows the general protocol: wash cells twice, add lysis solution, dilute lysate if needed and add detection solution. The Adipogenesis Assay requires many more pipetting steps, as more reagents are added to the cells, and includes at least 6 washes. This is time-consuming, requires many pipette tips and increases the likelihood of dislodging or scraping cells off the surface of the wells, potentially affecting assay accuracy. Another

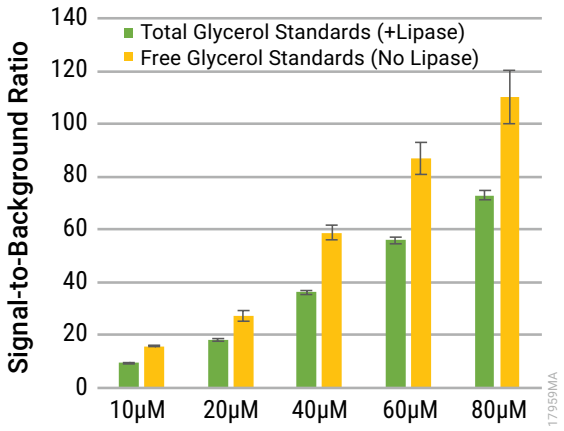

Figure 5. Signal-to-background (S/B) ratios of the Triglyceride-Glo™ Assay glycerol standards. Glycerol was diluted in Glycerol Lysis Solution ± lipase to generate total and free glycerol standards. Standards were then assayed following standard protocol. Mean S/B ratios ± error of the glycerol standards are displayed (n=2).

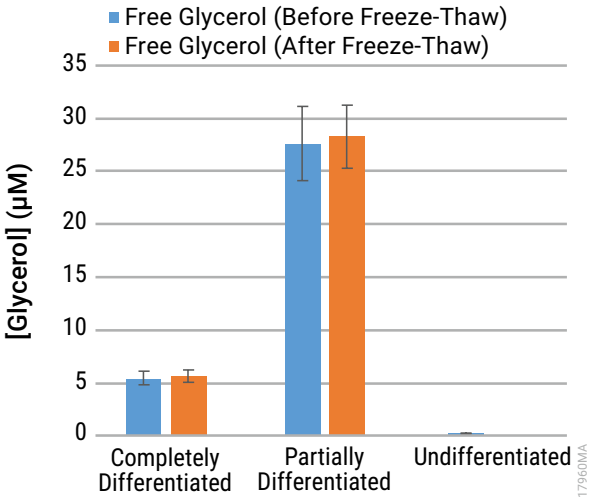

Figure 6. Calculated free glycerol concentrations resulting from the Triglyceride-Glo™ Assay. Completely differentiated (mature adipocytes), partially differentiated and undifferentiated 3T3L1-MBX fibroblast cells were analyzed for free glycerol content. RLU were converted to concentration values using a glycerol standard curve. Mean free glycerol concentrations before and after freeze-thawing cell lysates ± standard deviation are displayed (n=6 and n=9, respectively).

issue, was that the Oil Red O stain dried onto the sides of the wells and was not removed during the wash steps. This could lead to inaccurate measurements if the stain is extracted from the well walls.

Another advantage of the Triglyceride-Glo™ Assay is the ability to measure free glycerol concentrations. As this molecule is metabolically important, acting as a substrate or product in various processes including lipolysis, measuring its levels in parallel with the assessment of adipogenesis may be of additional value depending on the user's area of study. In this experiment, free glycerol concentrations were found to be negligible compared to total glycerol concentrations, effectively indicating that the total glycerol concentration was equal to triglyceride concentration (Figure 6). However, free glycerol concentrations were noticeably different depending on the stage of adipogenesis, with higher free glycerol levels observed in actively differentiating cells compared to mature adipocytes. Additionally, freeze-thawing cell lysates (lysed using Glycerol Lysis Solution without lipase) did not appear to affect the measurement of free glycerol, as free glycerol concentrations were consistent before and after freeze-thawing. This may increase flexibility for the user. However, it is important to note that the intrinsic lipase activity of the model cell line used will determine whether freeze-thawing will affect measurements. The main advantage of the Adipogenesis Assay is the ability to identify differentiated cells visually via staining (Figure 7).

Summary

The Triglyceride-Glo™ Assay provides a simple method for accurate, quantitative assessment of adipogenesis. Overall, the Triglyceride-Glo™ Assay outperformed the Cayman Chemical Adipogenesis Assay both in terms of quantitative range, with a tenfold higher maximum S/B ratio, and usability, with less steps and less hands-on time. An additional advantage of the Triglyceride-Glo™ Assay is the ability to directly quantify triglyceride and glycerol levels in a sample of cells. These aspects ultimately make the Triglyceride-Glo™ Assay a more accurate method for assessing the extent of adipogenesis.

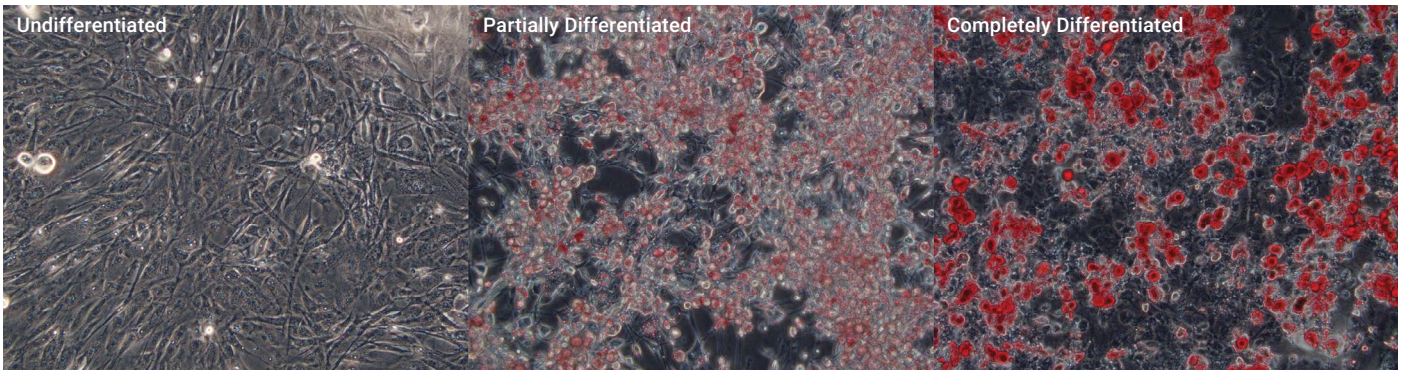

Figure 7. Images of 3T3L1-MBX cells stained at different stages of differentiation using Oil Red O lipid-soluble stain (20X magnification). 3T3L1-MBX fibroblast cells were fixated and stained on day 22 of the experiment using the Cayman Chemical Adipogenesis Assay Kit. Images of undifferentiated (top, plated day 20), partially differentiated (middle, plated day 10) and completely differentiated (bottom, plated day 1) cells following staining are displayed. Lipids (comprising ≥95% of mature adipocytes by weight) absorb the stain and appear red.

References

1. Haupt, K. and Valley, M. Homogeneous assays for triglyceride metabolism research. Promega.com. tpub_221, December 2020.
2. Cushman, S.W. (1970) Structure-function relationships in the adipose cell. I. Ultrastructure of the isolated adipose cell. *J Cell Biol.* **46(2)**, 326–341.
3. Cayman Chemical Adipogenesis Assay Kit Manual.

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