COSMO BIO CO., LTD.

Glucose Cellular Uptake Measurement Kit (Broad Range, Fluorometric)

Cat. MBR-PMG-K01E

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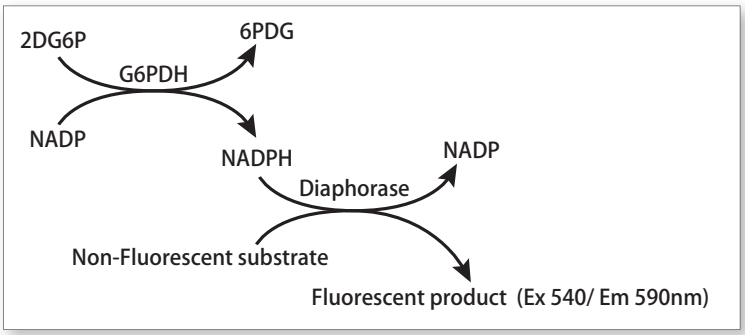
【I - 1.】Background and measurement principle

Insulin is a hormone that lowers the blood glucose level and is known to help glucose enter cells through an effect that enhances the activity of glucose transport, primarily in insulin-sensitive cells, such as adipocytes and skeletal muscles. The effect of insulin on cells is often assessed in the laboratory by measuring glucose uptake activity. Typically, glucose uptake is measured using 3-*o*-Methyl-D-Glucose or 2-deoxyglucose (2DG) labeled with radioactive ^{3}H or other isotope. However, the use of radioactive isotopes is not available to all labs and is subject to many restrictions.

This assay kit was developed to provide a simple, rapid and convenient means to measure cellular glucose uptake without the use of radioisotope.

This assay utilizes the glucose analog 2-deoxyglucose in place of glucose. Like glucose, 2DG taken up by cells is rapidly phosphorylated by hexokinase to 2-deoxyglucose-6-phosphate (2DG6P). However, unlike glucose, 2DG6P is not further metabolized and accumulates in cells. Using the provided reagents, cell lysate are then assayed for 2DG6P leveles in a coupled enzymatic re-dox reaction that produces a fluorescent signal whose intensity is proportional to the amount of accumulated 2DG6P. 2DG levels in cell lysate samples are thus calculated by comparing their fluorescence intensity to a standard curve produced with known amounts of 2DG6P.

Figure 1: Scheme of Assay Procedure



【I - 2.】Kit Features

	Glucose Cellular Uptake Measurement Kit (Broad Range, Fluorometric) (Cat.No. MBR-PMG-K01E)	Related product: 2-Deoxyglucose (2DG) Uptake Measurement kit (Cat.No.OKP-PMG-K01E)
Assay Format	non-radioactive	non-radioactive
Detection Method	Fluorometric (Ex 540nm/Em 590nm)	Chromogenic (420nm)
Operatation Time	3 hours	5 ～ 7 hours (Assay is a 2 day, as methods require an overnight incubation)
Measurement range	Broad (0 to 50μM 2DG6P)	High sensitivity (0 to 5μM 2DG6P)
Features	Fast, convenient, single step suitable for high sample through put.	Sensitivity comparable with radioactive assays. High accuracy. High precision.

【I- 3.】Kit Components

Reagents for 100 reactions are supplied.
Store kit at -20° C until expiration date printed on the label, protected from light.

Reagent	Volume	Quantity	Storage condition for opened vials
Substrate buffer	9 mL	3vials (3mL/vial) *Pink Cap Vial	-20° C (protected from light)
2DG6P solution (1mM)	500 μL	1tube	
Sample Diluent Buffer Concentrate (100x)	5 mL	1vial	
Fluorescent Substrate	120 μL	1tube *Yellow Cap Tube	
Enzyme solution	270 μL	1tube *Green Cap Tube	

Materials required but not supplied

- Ultrapure water (distilled water)
- 96-well Fluorescent plate reader (540 nm excitation/ 590nm emission)
- 96-well Black microplate

【II - 1.】Preparation of 1x sample diluent buffer

・Add 1mL of Sample Diluent Buffer Concentrate (100x) to 99mL of ultrapure water and mix thoroughly. If refrigerated, 1x sample diluent buffer can be used for up to 3months.

【II - 2.】Preparation of 2DG6P standard

・Prepare a series of 2DG6P standards in the range of 0-50μM 2DG6P by diluting the 1mM 2DG6P Solution with 1x Sample Diluent Buffer. Diluted 2DG6P standards can be stored in a refrigerator for up to one week.

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【 II - 3. 】Preparation of Reaction Mix (200µL Reaction Mix per sample)

・Prepare Reaction Mix for 10 samples as shown in Table 1. Mix thoroughly by vortexing gently. Reaction Mix should be prepared just prior to use and stored on ice and protected from light until used.

Table 1

Reagent	Volume per 10 samples (Include overage for pipetting loss)
Substrate Buffer *Pink Cap Vial	2500 µL
Enzyme Solution *Green Cap Tube	25 µL
Fluorescent Substrate *Yellow Cap Tube	10 µL

・Before opening the Enzyme Solution tube and Fluorescent Substrate tube, briefly spin down this tube to collect contents.
・Enzyme Solution contains 50% glycerol and is very viscous. Pipet slowly and carefully.
・Keep Enzyme Solution at -20° C until just before Reaction Mix preparation. Return into the freezer promptly after use.
・Protect fluorescent substrate from light.

【 III - 1. 】2DG Measurement Protocol

Quantity of solution to be added per sample is as shown below.

1. For each assay point, add 20µL 2DG6P standard dilutions or 20µL unknown sample to each well of the 96-well Black microplate.
2. Add 200µL of Reaction Mix to each well.
   Note: Reaction Mix should be added to all wells at the same time (or at least in rapid succession). Mix thoroughly.
3. Cover plate and Incubate at 37° C for 2 hours. Protected from light.
4. Read the fluorescence intensity of each well on a fluorescence plate reader set at Ex/Em=540/590 nm.
   Determine the 2DG uptake concentration of unknown samples by comparing unknown sample fluorescence intensity to a calibration curve plotted from the 2DG6P standard solutions readings.

【 III - 2. 】Experimental Example Results

2DG uptake by insulin-stimulated adipocytes following the differentiation of 3T3-L1 cells in culture.

Figure 2 : 2DG6P Calibration curve

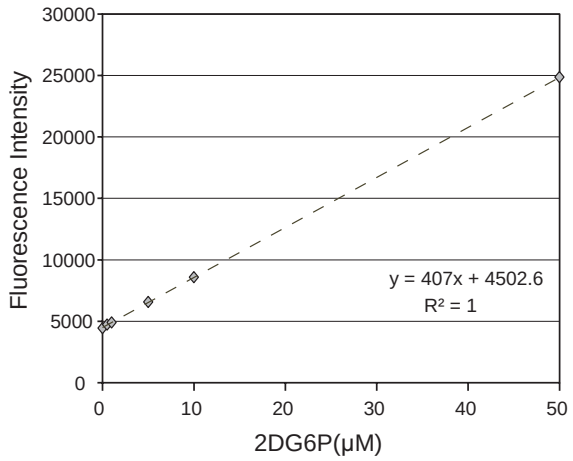
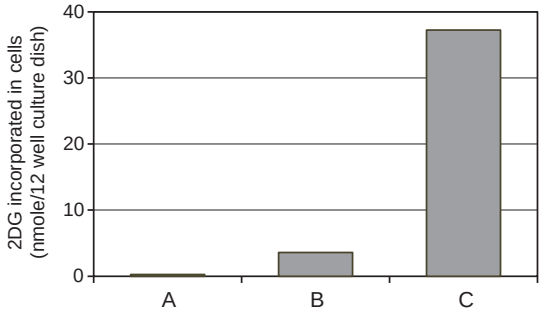


Figure 3 : Experimental Protocol and Result

Additives	Sample A	Sample B	Sample C
Insulin	−	−	+
2DG	−	+	+



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【IV.】Example of insulin stimulation and sample preparation using adipocytes differentiated from 3T3-L1 cells in culture

For your own experiments with your own cells, it will be necesssary to adjust and optimize all cell culture media and conditions, reagent concentrations, incubation times, and parameters according to your experimental needs.
The following procedure is for 3T3-L1 cells grown in one well of a standard 12-well culture plate.

Reagents and Media
- Serum-free culture medium
- Krebs Ringer Phosphate HEPES(KRPH) buffer at 37° C
  (1.2mM KH_2PO_4, 1.2mM $MgSO_4$, 1.3mM $CaCl_2$, 118mM NaCl, 5mM KCl, 30mM HEPES, pH7.5)
- BSA (essentially fatty acid free and globulin free grade, use Sigma Cat. No. A0281, or equivalent)
- 2-Deoxy-D-glucose (2DG) solution
- Insulin solution
- PBS(without calcium and Magnesium)
- Phloretin or Cytochalasin B (or glucose uptake inhibitor)

1	Differentiated 3T3-L1 cells to adipocytes in a 12-well culture plate.
2	Replace culture media with serum-free medium. Return to incubator for 6 hours.
3	Gently wash cells 3x with 1.5mL of warm KRPH buffer.
4	Gently add 3mL of warm KRPH buffer containing 2% BSA.
5	For this example, insulin is added to a final concentration of 1µM.
6	Incubate cells for 18min at 37° C .
7	For this example, 2DG is added to a final concentration of 1mM.
8	Incubate cell at 37° C for 20minutes.
9	Wash cells gently 3x with cooled PBS containing 200µM Phloretin to inhibit further 2DG uptake.
10	Add 1.5mL of 1x sample diluent buffer, and lyse cells by sonication with a microtip sonicator
11	Collect cell lysate to tube, and apply heat treatment at 80° C for 15 minutes. Note: Do not add protease inhibitors to cell lysates. Note: If an alternate method for cell lysis with be used, do not use NaOH as NaOH degrades 2DG6P.
12	Centrifuge lysates at 15000 x g for 20minutes at 4° C
13	Transfer cell lysate supernatants to a fresh tube. These cell lysate supernatants are your experimental samples. Note: Supernatants can be stored at -20° C for later analysis.
14	Follow the procedure of Section III-1 "2DG Measurement Protocol" above.

【V.】References

[1] Yamamoto N, *et al*,(2006). A nonradioisotope, enzymatic assay for 2-deoxyglucose uptake in L6 skeletal muscle cells cultured in a 96-well microplate. Anal. Biochem. 351: 139-145.

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