COSMO BIO CO., LTD.

2-Deoxyglucose (2DG) Uptake Measurement Kit

Cat. No. CSR-OKP-PMG-K01E

Last Updated on 3rd Aug, 2016

【1】Introduction

Measurement of 2-deoxyglucose (2DG) uptake in tissues and cells is a reliable approach for estimating glucose uptake and thereby to explore the regulation of glucose metabolism and mechanisms of insulin resistance. While assays employing radioisotope-labeled 2DG are often used to measure 2DG uptake in vivo and in vitro, the use of radioisotopes is not possible without problems including permitting, handling, and disposal. Furthermore, in actual practice, radioactive assay protocols for 2DG uptake require a corrective separation step to account for labeled 2DG that remains in extracellular fluids and can lead to substantial variation. Such problems are obviated by the enzymatic method of 2DG detection employed by this kit, based on published methods (Saito K and Minokoshi Y, et al. Analytical Biochem 412: 9-17, 2011).

【2】Advantages

1. Does not use radioisotope (RI) or require radiation counting instrumentation.
2. Photometric readout on standard microplate readers.
3. Direct measurement of 2DG6P accumulated in cells.
4. High sensitivity through a recycling enzymatic amplification reaction.

【3】Assay Principle

This assay has five key steps:

1. Oxidation of glucose-6-phosphate (G6P) with a low concentration of G6P dehydrogenase (G6PDH) with NAD+ to eliminate endogenous G6P in target cells.
2. Elimination of NAD(P)H with HCl, which removes endogenous NAD(P)H as well as NADH produced in Step 1 in Figure 1.
3. Generation of NADPH through oxidation of 2DG6P in the cells with a high concentration of G6PDH, with the generated NADPH being used for quantification of 2DG6P.
4. Elimination of NAD(P)+ and G6PDH that remains after Step 2 in Figure 1 with NaOH.
5. Recycling amplification reaction for the small amount of NADPH generated, and quantification of 2DG6P using a photometric microplate reader at 420 nanometers wavelength.

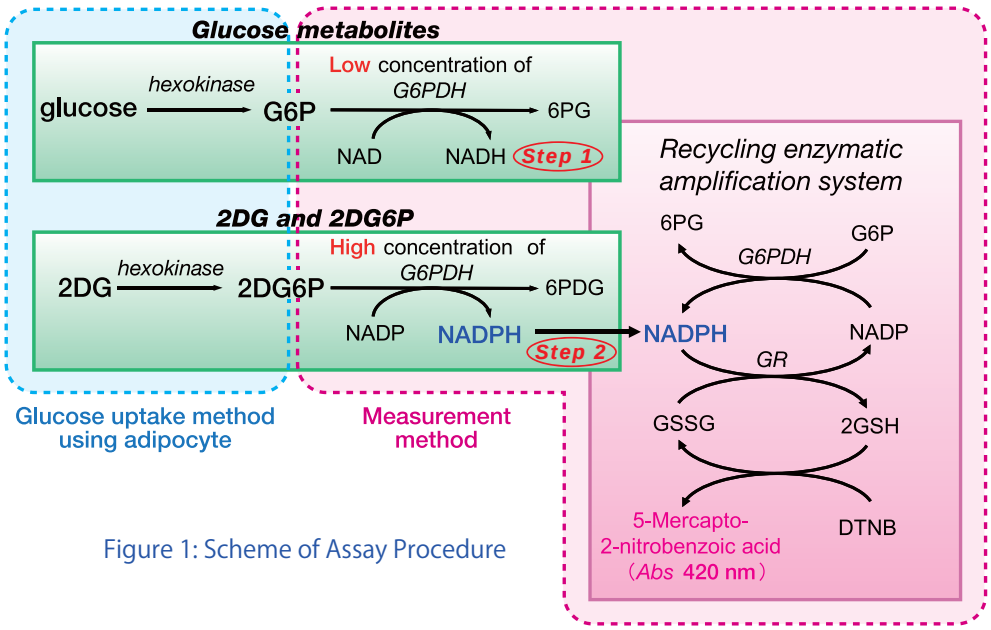

Figure 1: Scheme of Assay Procedure

【4】Kit Components

Reagents for 50 reactions.

Unopened kit is stable at -20℃ until expiration date printed on the label.

Reagent	Volume	Quantity	Storage
Solution A (Including NAD)	3,400 µL	1 tube	Opened kit stable at 4° C
Solution B (Acid Solution)	1,000 µL	1 tube	
Solution C (Acid Neutralizing Solution)	1,000 µL	1 tube	
Solution D (Including NADH)	1,600 µL	1 tube	
Solution E (Alkali Solution)	1,000 µL	1 tube	
Solution F (Alkali Neutralizing Solution)	1,000 µL	1 tube	
Solution G (Including GSSG and G6P)	2,000 µL	1 tube	
1mM 2DG6P Solution	500 µL	1 tube	
Sample Diluent Buffer Concentrate (100-fold Concentrated Solution)	3 mL	1 tube	
Substrate Buffer	11 mL	1 vial	
DTNB Substrate (Powder)	Reconstitute to 2 mL/vial	5 vials	
Low G6PDH	25 µL	1 tube *Red Cap Tube	Opened kit stable at -20° C
High G6PDH	250 µL	1tube *Black Cap Tube	
GR	20 µL	1tube *Blue Cap Tube	

*G6PDH: Glucose-6-phosphate dehydrogenase

*GR: Glutathione Reductase

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【5】Preparation of Reaction Solutions

Preparation of 1x Sample Diluent Buffer

- Dilute 1mL of Sample Diluent Buffer Concentrate with 99 mL of ultrapure water, and mix completely.
- 1x sample diluent buffer can be stored in a refrigerator for 3 months.

Preparation of 2DG6P standard

- Prepare a 2DG6P standard dilution series by diluting the 1mM 2DG6P solution in 1x sample diluent buffer in the range of 0 to 5 µM 2DG6P.
- 2DG6P standard can be stored at 4° C for one week.

Preparation of Reaction Mix A (assay uses 60 µL Reaction Mix A per sample)

- Prepare Reaction Mix A for 10 samples as shown in Table 1. Mix completely by gentle vortex. (Prepare Reaction Mix A before use and hold on ice until use).

TABLE 1

Reagent	Total volume required for 10 assay points (Includes overage to compensate for volume loss)
Solution A	650 µL
Low G6PDH Red Cap Tube	3 µL

* Before opening the Low G6PDH tube, spin briefly to collect all contents.
* Pipette the Low G6PDH solution carefully and slowly as it contains 50% glycerol and
* is very viscous.
* Keep the Low G6PDH tube at -20° C until just before use. Return to -20° C promptly after use.

Preparation of Reaction Mix D (assay uses 30 µL Reaction Mix D per sample)

- Prepare Reaction Mix D for 10 samples as shown in Table 2, and mix completely by gentle vortex. (Prepare just before use. Hold on ice until use. → Refer to【6】Measurement Method, step (7) on page 4).

TABLE 2

Reagent	Total volume required for 10 assay points (Includes overage to compensate for volume loss)
Solution D	290 µL
High G6PDH Black Cap Tube	30 µL

* Before opening the High G6PDH tube, spin briefly to collect all contents.
* Pipette the High G6PDH solution very carefully and slowly as it contains 50% glycerol and is very viscous.
* Keep the Low G6PDH tube at -20° C until just before use. Return to -20° C promptly after use.

Preparation of Chromogenic Solution

- To one vial of DTNB Substrate, add 2mL of Substrate Buffer and mix thoroughly for complete dissolution.
- Reconstituted Chromogenic Solution can be stored in the dark at 4° C for one week.

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Preparation of Enzyme Cycling Solution (assay uses 70 µL Enzyme Cycling Solution per sample)

- Prepare an Enzyme Cycling Solution for 10 samples as shown in Table 3 and mix completely by gentle vortex.
  (Prepare Enzyme Cycling Solution just before use and hold on ice use. → Refer to 【 6 】 Measurement Method, Step (13) on page 4.)

TABLE 3

Reagent	Total volume required for 10 assay points (Includes overage to compensate for volume loss)
Solution G	360 µL
Chromogenic Solution	360 µL
High G6PDH Black Cap Tube	10 µL
GR Blue Cap Tube	2 µL

* Before opening High G6PDH and GR tubes, spin briefly to collect all contents.

* Piipette the High G6PDH and GR solutions carefully and slowly as they contain 50% glycerol and are very viscous.

* Keep the High G6PDH and GR tubes at -20° C until just before use. Return to -20° C promptly after use.

【 6 】 Measurement Method

Read the following precautions prior to measurement.

- Make sure liquid adhering to the inner surface of reagent tubes is all collected at the tube bottom by spinning briefly before adding each reagent (before opening the tubes, etc.) and after adding and mixing each reagent. Please note that measurement results may have a major error if operation is done with droplets of reagent attached to the inner surface.
- Cap the tubes or seal the plate with an adhesive cover at each incubation step.
- Refer to【7】An example of unknown sample preparation.

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

1. Add 60 µL of Reaction Mix A to each well of a 96-well microplate.
2. Add 20 µL of 2DG6P standard or the unknown sample 20µL to each well and mix completely.
3. Incubate for 16 hours or more at room temperature (20-25° C).
4. Add 5 µL of Solution B to each well and mix completely.
5. Incubate at 38° C for one hour.
6. Add 5 µL of Solution C to each well and mix completely.
7. Let stand 5-10minutes at room temperature while preparing Reaction Mix D.
8. Add 30 µL of Reaction Mix D to each well and mix completely.
9. Incubate for 1 hour at 38° C.
10. Add 5 µL of Solution E to each well and mix completely.
11. Incubate at 70° C for one hour, and immediately chill on ice for 5 minutes.
12. Add 5µL of the Solution F to each well, and mix completely.
13. Let stand for 5-15 minutes at room temperature while preparing Enzyme Cycling Solution.
14. Add 70µL of Enzyme cycling solution to each well at the same time and mix completely.

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15. Immediately, read the optical density (OD) of each well using a microplate reader. Set the microplate reader at 420 nm(within the range of 420-430 nm) and preheated to 25-30℃ . On a kinetic program: read every 1-5 minutes over a period of 30 minutes. Determine the 2DG6P uptake concentration in an unknown sample solution using the calibration curve prepared with the 2DG6P standard solutions. In case it is unavoidable to use spectrophotometer with micro cuvette for measurement, add 50 µL of 5 M sodium chloride to color developing reaction to stop the reaction. Then, measure the absorbance at 420 nm immediately.

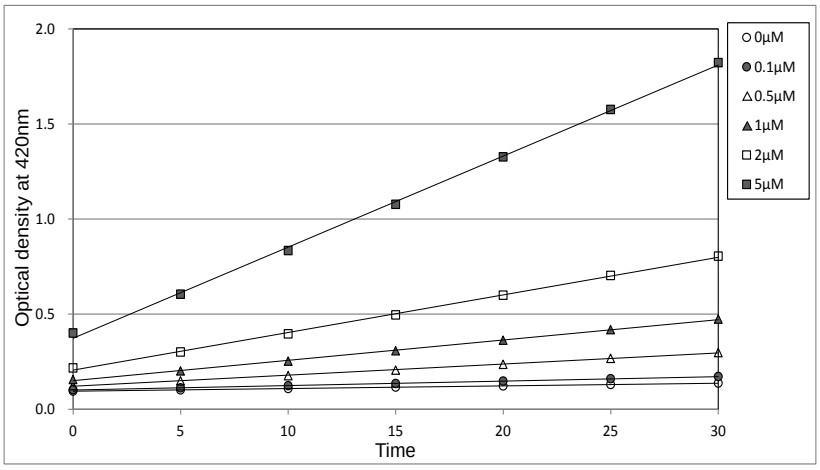

Figure 2: Temporal change of O.D. for different concentrations of 2DG6P

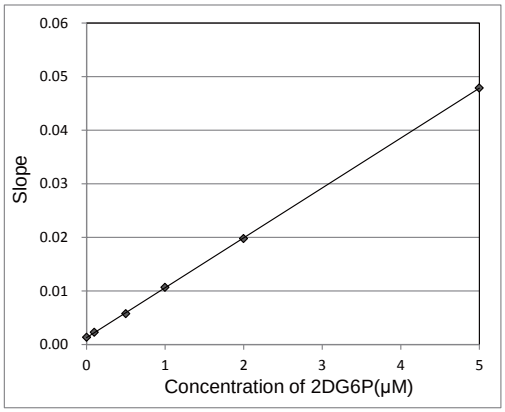

Figure 3: Calibration curve by kinetic method (30 minutes incubation)

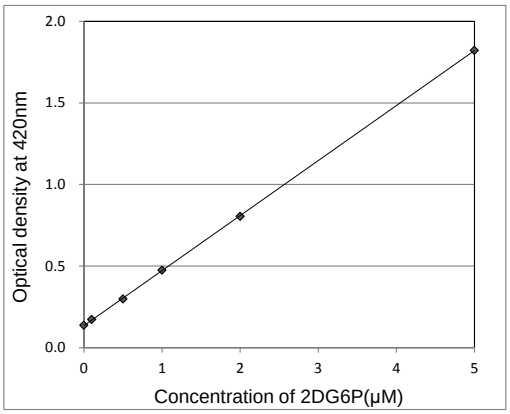

Figure 4: Calibration curve by end point method (30 minutes incubation)

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【7】An Example of Glucose Uptake Method - Glucose uptake method using adipocyte

Types of reagents required:

- 6-well culture plate for adipocytes, such as 3T3-L1 cell.
- Serum-free medium
- Krebs Ringer Phosphate Hepes (KRPH) buffer kept at 37° C temperature (1.2 mM KH2PO4, 1.2 mM MgSO4, 1.3 mM CaCl2, 118 mM NaCl, 5 mM KCl, 30 mM Hepes, pH7.5)
- BSA (essentially fatty acid free and globulin free grade, e.g. use an item equal to Sigma Cat. No. A0281)
- 2-Deoxy-D-glucose (2DG) solution
- Insulin solution
- PBS(-)
- Phloretin (or glucose uptake inhibitor, such as Cytochalasin B)
- 10 mM Tris-HCl buffer (pH8.0)

An example of method (This procedure is for a 6-well culture plate)

Each measurement should be stared with an optimization of reagent concentration and reaction time, depending on the type of cells and differentiation level of cells.

1. Prepare differentiated adipocytes to the 6-well culture plate.
2. Remove the medium from the culture plate wells, and incubate the cells in serum-free medium for 6 hours.
3. Gently wash the cells 3 times with 3 mL of warm KRPH buffer.
4. Gently add 3 mL of warm KRPH buffer containing 2% of BSA to each well.
   * Add insulin, Phloretin or 2DG as mentioned hereafter as necessary, based on the purpose of measurement.
5. Add insulin solution to a final concentration of 1 μM and incubate at 37° C.
6. Add phloretin solution to a final concentration of 200-1,000 μM 16 minutes after adding insulin solution.
7. Add 2DG solution to a final concentration of 1 mM 18 minutes after adding insulin solution, and incubate at 37° C for 20 minutes.
8. Remove medium and gently wash the cells 3 times with cooled PBS containing 200 μM phloretin.
9. Add 3 mL of 10 mM Tris-HCl buffer (pH8.0) to each well, cells are disrupted by microtip sonicator.
   * Do not use NaOH solubilization in cell lysate method which might destroy 2DG6P.
10. Collect cell lysate to tube and apply heat treatment at 80° C for 15 minutes.
11. Centrifuge at 4° C, at 15,000 x g for 20 minutes, and transfer the supernatant to a new tube.
12. A part of the supernatant diluted ≥1:4 with 1x sample diluent buffer (see II. Preparation of Reaction Solutions for 1x Sample Diluent Buffer) is used as unknown sample for III. Measurement Method.
    * Cell lysate (supernatant) should be stored at -20° C.
    * Do not add protease inhibitor or reducing agent such as 2-mercaptoethanol or dithiothreitol to the cell lysate.

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An example of actual measurement

See Figure 5 for the measurement result of 3T3-L1 cell extraction implemented according to the example of aforementioned method and addition schedule below (*Note that this is an example of measurement).

Additives	Sample A	Sample B	Sample C	Sample D
Insulin	-	-	-	+
2DG	-	+	+	+
Phloretin	-	-	+	-

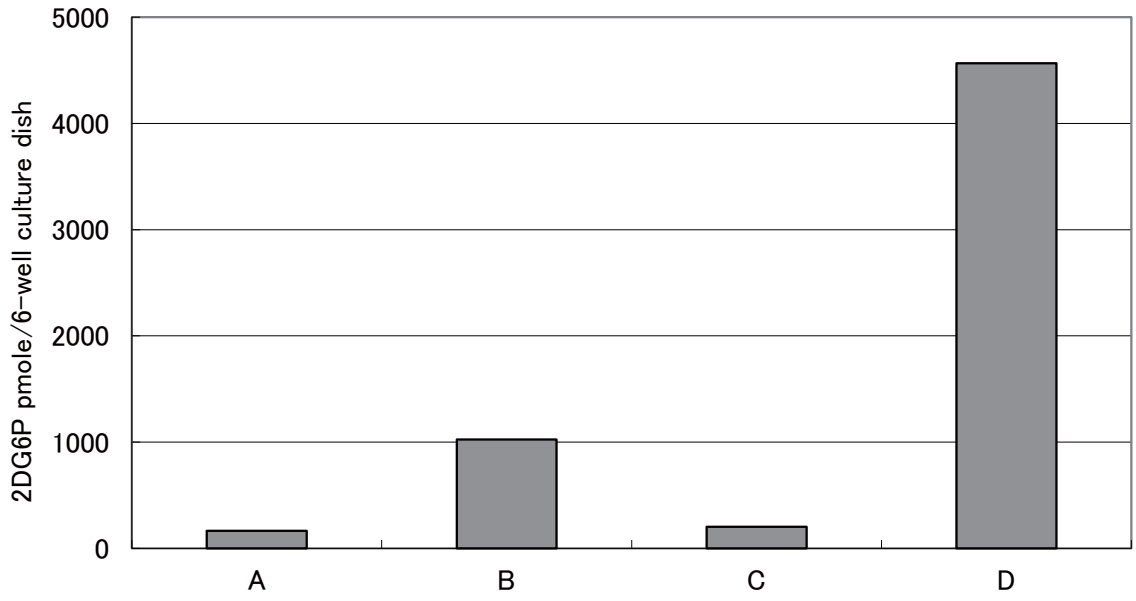

Figure 5: Measurement result

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