2-Deoxyglucose (2DG) Uptake Measurement kit

Introduction

Measurement of 2-deoxyglucose (2DG) uptake in tissues and cells is a reliable approach with which to estimate glucose uptake and thereby to explore the regulation of glucose metabolism and mechanism of insulin resistance. Radioisotope-labeled 2DG is usually used for the measurement of 2DG uptake both *in vivo* and *in vitro*. However the radioisotope (RI) method is required a specialized facility for RI in strict limitation and cannot be handled in ordinal laboratories. Furthermore, radioactive 2DG administered into cultured cells remains in the extracellular space, and therefore the results must be corrected by separating the extracellular 2DG and intracellular 2DG/2DG-6-phosphate (2DG6P) in the cells.

This kit is based on the enzymatic method for the direct measurement of 2DG6P amount without any use of radioisotope materials (Saito K and Minokoshi Y, et al. Analytical Biochem 412: 9-17, 2011). The kit includes following advantages.

< Advantages >

1. No RI materials are required, and 2DG uptake can be measured in any ordinal laboratories.
2. Direct measurement of 2DG6P amount accumulated in target cells.
3. High sensitivity with the use of enzyme-recycling amplification reaction.

< Principle of Assay >

The kit has five key steps.

1) Oxidation of glucose-6-phosphate (G6P) with a low concentration of G6P dehydrogenase (G6PDH) plus NAD^+ to eliminate endogenous G6P in target cells. Elimination of NAD(P)H with HCl, which removes endogenous NAD(P)H as well as NADH produced in step 1.
2) Production of NADPH through oxidation of 2DG6P in the cells with a high concentration of G6PDH, with the NADPH being used for quantification of 2DG6P. Elimination of $NAD(P)^+$ and G6PDH remains after step 2 with NaOH.
3) Recycling amplification reaction for the small amount of NADPH produced, and quantification of 2DG6P with a (96-well microplate) photometric reader.

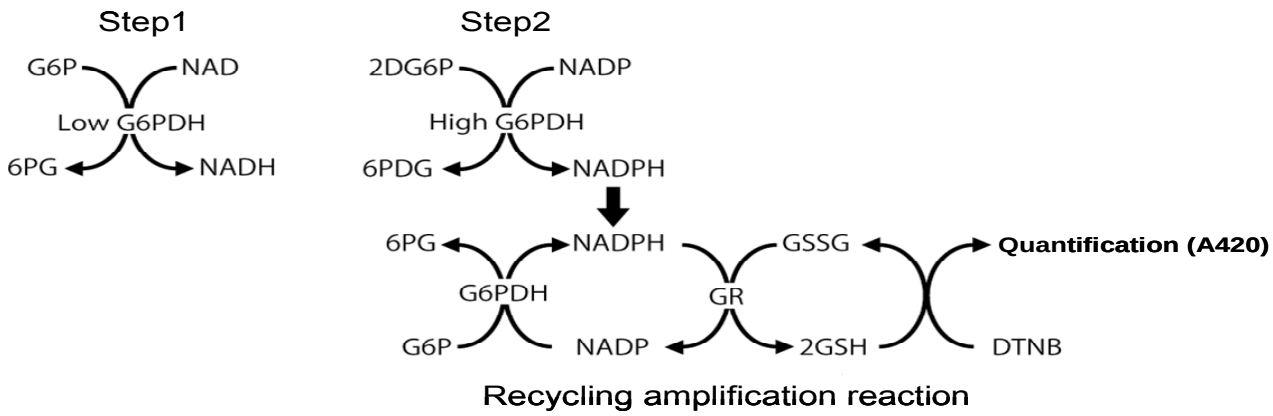

Figure 1: Scheme of Assay Procedure

I. Kit Components

Reagents for 50 reactions (5 assays) are supplied.
Unopened kit: Stable at -20°C until expiration date printed on the label.

Reagent	Volume	Quantity	Storage
Solution A	3,400µL	1tube	Opened kit stable at 4°C
Solution B (Acid solution)	1,000µL	1tube	
Solution C (Acid neutralizing solution)	1,000µL	1tube	
Solution D	1,600µL	1tube	
Solution E (Alkali solution)	1,000µL	1tube	
Solution F (Alkali Neutralizing solution)	1,000µL	1tube	
Solution G	2,000µL	1tube	
1mM 2DG6P solution	500µL	1tube	
Sample diluent buffer Concentrate (100-fold concentrated solution)	3mL	1tube	
Substrate buffer	11mL	1vial	
DTNB Substrate (powder)	2mL use	5vials	
Low G6PDH	25µL	1tube *Red Cap Tube	Opened kit stable at -20°C
High G6PDH	250µL	1tube *Black Cap Tube	
GR	20µL	1tube *Blue Cap Tube	

*G6PDH: Glucose-6-phosphate dehydrogenase
*GR: Glutathione Reductase

II. Preparation of reaction solutions

Preparation of 1x sample diluent buffer

- Combine 1mL of Sample diluent buffer Concentrate with 99mL of ultrapure water, and mix it thoroughly.
- 1x sample diluent buffer can be stored in a refrigerator for 3 mouths.

Preparation of 2DG6P standard

- Prepare a 2DG6P standard dilution series by diluting the 1mM 2DG6P solution in 1x sample diluent buffer, and 2DG6P standard with different concentrations in the range of 0 to 5μM 2DG6P.
- 2DG6P standard can be stored in a refrigerator for one week.

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

- Prepare a Reaction Mix A for 10 samples as shown in Table 1, and mix it thoroughly by gently vortexing (to be prepared before use and stored on ice until the time of use).

Table 1

Reagent	Volume per 10 samples (Include overage to compensate for volume loss)
Solution A	650μL
Low G6PDH ***Red Cap Tube**	3μL

*Before opening the enzyme tube (Low G6PDH), briefly spin down this tube to collect contents.
*Pipetting the enzyme carefully and slowly as the enzyme contains 50% glycerol and is very viscous.
*Keep the enzyme at -20°C until just before use and return into the freezer promptly after use.

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

- Prepare a Reaction Mix D for 10 samples as shown in Table 2, and mix it thoroughly by gently vortexing (to be prepared before use and stored on ice until the time of use).

Table 2

Reagent	Volume Per 10 samples (Include overage to compensate for volume loss)
Solution D	290μL
HighG6PDH ***Black Cap Tube**	30μL

*Before opening the enzyme tube (Hight G6PDH), briefly spin down this tube to collect contents.
*Pipetting the enzyme carefully and slowly as the enzyme contains 50% glycerol and is very viscous.
*Keep the enzyme at -20°C until just before use and return into the freezer promptly after use.

Preparation of Chromogenic solution.

- Label a vial “DTNB substrate”. Add 2mL of Substrate buffer to the vial, and mix thoroughly for complete dissolution.
- Chromogenic solution can be stored in the dark at refrigerator for one week.

Preparation of Enzyme cycling solution (70μL Enzyme Cycling solution per sample)

- Prepare an Enzyme cycling solution for 10 samples as shown in Table 3, and mix it thoroughly by gently vortexing (to be prepared before use and stored on ice until the time of use).

Table 3

Reagent	Volume Per 10 samples (Include overage to compensate for volume loss)
Solution G	360μL
Chromogenic solution	360μL
High G6PDH ***Black Cap Tube**	10μL
GR ***Blue Cap Tube**	2μL

*Before opening the enzyme tubes (Hight G6PDH and GR), briefly spin down this tube to collect contents.
*Pipetting the enzyme carefully and slowly as the enzyme contains 50% glycerol and is very viscous.
*Keep the enzyme at -20°C until just before use and return into the freezer promptly after use.

III. Measurement method

Read the following precautions prior to measurement.

- Make sure solution adhered to the inner surface sinks at the bottom with flash centrifuge etc., 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 for each step of incubation.

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

（1） Add 60μL of the Reaction Mix A to each well of a 96-well microplate.
（2） Add 20μL of the 2DG6P standard or the unknown sample to each well, and mix thoroughly.
See IV. An example of glucose uptake method for preparation of unknown sample
（3） Incubate at room temperature (20-25°C) for overnight (16hours or more)
（4） Add 5μL of the Solution B to each well, and mix thoroughly.
（5） Incubate at 38°C for one hour.
（6） Add 5μL of the Solution C to each well, and mix thoroughly.
（7） Leave to stand for 5-10minutes at room temperature.
*Prepare Reaction Mix D in the meantime.
（8） Add 30μL of the Reaction Mix D to each well, and mix thoroughly.
（9） Incubate for one hour at 38°C.
（10） Add 5μL of the Solution E to each well, and mix thoroughly.
（11） Incubate at 70°C for one hour, and immediately chill on ice for a few minutes.
（12） Add 5μL of the Solution F to each well, and mix thoroughly.
（13） Leave to stand for 5-15minutes at room temperature.
*Prepare Enzyme cycling solution in the meantime.
（14） Add 70μL of Enzyme cycling solution to each well at the same time, and mix thoroughly.
（15） Immediately, read the optical density (OD) of each well using a microplate reader.
Set the microplate reader at 420nm(within the range of 420-430nm) and preheated to 25-30°C.
On a kinetic program: read every 1-5minitues over a period of 30minutes.
Determine the 2DG6P uptake concentration in an unknown sample solution using a calibration curve prepared with the 2DG6P standard solutions.
In case it is unavoidable to use spectral photometer with micro cuvette for measurement, add 50μL of 5M sodium chloride to color developing reaction to stop the reaction. Then, measure the absorbance at 420nm immediately.

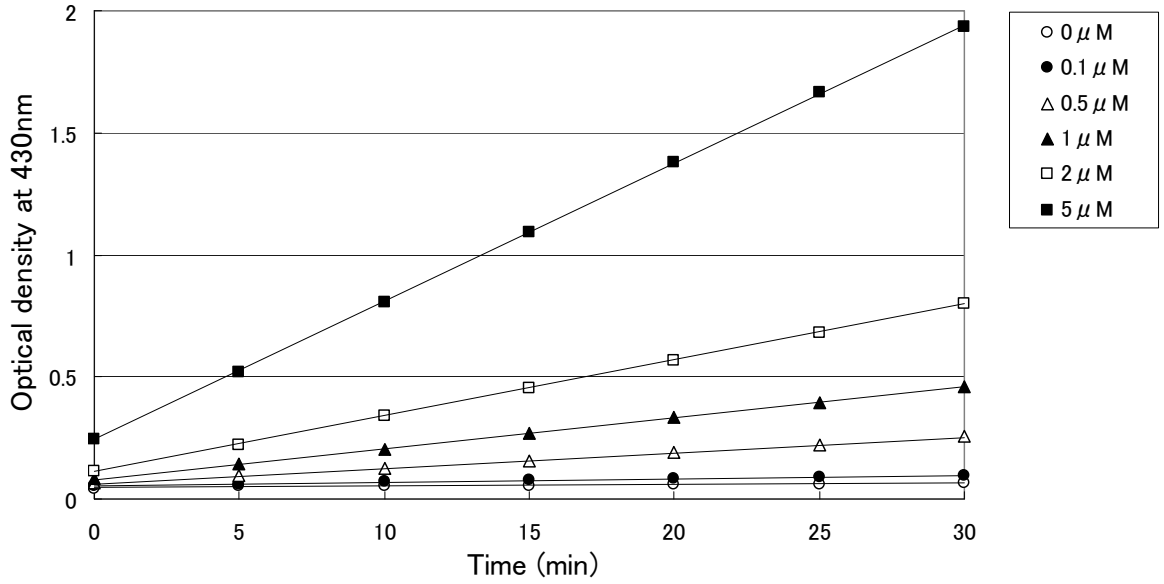

Figure 2 : Temporal change of O.D. for different concentrations of 2DG6P

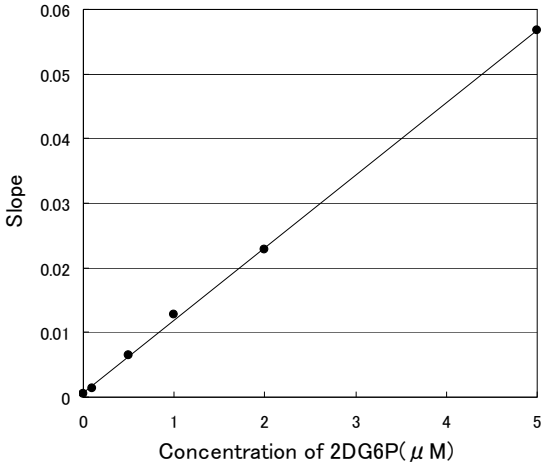

Figure 3 : Calibration curve by kinetic method (30 minutes incubation)

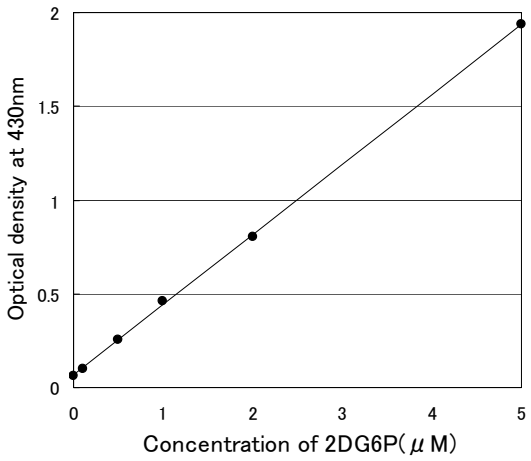

Figure 4 : Calibration curve by end point method (30 minutes incubation)

IV. 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
  (1.2mM KH2PO4, 1.2mM MgSO4, 1.3mM CaCl2, 118mM NaCl, 5mM KCl, 30mM Hepes, pH7.5)
- BSA (essentially fatty acid free and globulin free grade, use an item equal to Sigma Ca. No. A0281, for example)
- 2-Deoxy-D-glucose (2DG) solution
- Insulin solution
- PBS(-)
- Phloretin (or glucose uptake inhibitor, such as Cytochalasin B)
- 10mM Tris-HCl buffer (pH8.0)
  Exemplary

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 3times with 3mL of warm KRPH buffer.
（4） Gently add 3mL 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 1μM, and incubate at 37°C.
（6） Add Phloretin solution to a final concentration of 200-1000μM in 16 minutes after adding insulin
（7） Add 2DG solution to a final concentration of 1mM in 18 minutes after adding insulin, and incubate at 37°C for 20minutes.
（8） Remove medium and gently wash the cells 3times with cooled PBS containing 200μM Phloretin.
（9） Add 3mL of 10mM Tris-HCl buffer (pH8.0) to each well, cells are disrupted by microtip sonicator.
*Do not use NaOH solubilization in cell lysate method. (Destroy 2DG6P)
（10）Collect cell lysate to tube, and apply heat treatment at 80°C for 15 minutes.
（11）Centrifuge at 4°C, at 15000 x g for 20minutes, 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), and is used as unknown sample for **III. Measurement method**.
*Cell lysate (supernatant) should be store at -20°C.
*Do not add protease inhibitor or reducing agent such as 2-mercaptoethanol or dithiothreitol to the cell lysate.

< 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	SampleA	SampleB	SampleC	SampleD
Insulin	-	-	-	+
2DG	-	+	+	+
Phloretin	-	-	+	-

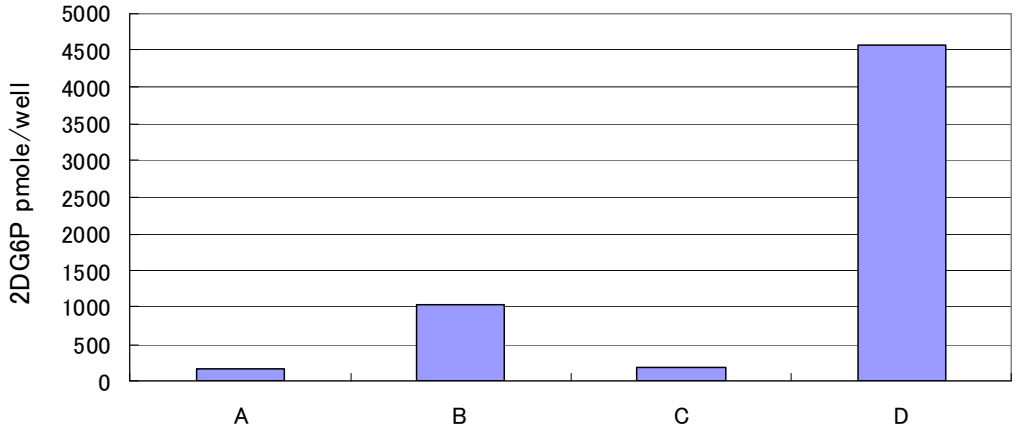

Figure 5 : Measurement result

References

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(3) Woo MS, et .al., Phytother Res. 2013 Jul;27(7):1102-5. PMID : 22991308
(4) Saito K, et.al., Anal Biochem. 2011 May 1;412(1):9-17. PMID : 21262191
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