Megazyme

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D-GLUCOSE - HK

ASSAY PROCEDURE
(HK/G6P-DH Format)

K-GLUHK-110A/K-GLUHK-220A 03/20

(*110/220 Manual Assays per Kit) or
(1000/2000 Auto-Analyser Assays per Kit) or
(1100/2200 Microplate Assays per Kit)

** The number of tests per kit can be doubled if all volumes are halved*

INTRODUCTION:

D-Glucose is found in most plant and food products. In plant materials, it occurs as a free sugar or in a range of di-, oligo- and poly-saccharides such as starch, 1,3:1,4-β-D-glucans and cellulose. It is present in significant quantities in honey, wine and beer, and a range of solid foodstuffs such as bread and pastries, chocolate and candies.

PRINCIPLE:

D-Glucose is phosphorylated by the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) to glucose-6-phosphate (G-6-P) with the simultaneous formation of adenosine-5'-diphosphate (ADP) (1).

$$\text{(1)}\quad \text{D-Glucose} + \text{ATP} \xrightarrow{\text{(HK)}} \text{G-6-P} + \text{ADP}$$

In the presence of the enzyme glucose-6-phosphate dehydrogenase (G6P-DH), G-6-P is oxidised by nicotinamide-adenine dinucleotide phosphate ($NADP^+$) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (2).

$$\text{(2)}\quad \text{G-6-P} + NADP^+ \xrightarrow{\text{(G6P-DH)}} \text{gluconate-6-phosphate} + \text{NADPH} + H^+$$

The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucose. It is the NADPH which is measured by the increase in absorbance at 340 nm (Figure 1, page 11).

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for D-glucose.

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 0.332 mg/L of sample solution at the maximum sample volume of 2.00 mL. The detection limit is 0.663 mg/L, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 2.00 mL.

The assay is linear over the range of 4 to 80 µg of D-glucose per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 2.00 mL, this corresponds to a D-glucose concentration of approx. 0.166 to 0.332 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If, in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

INTERFERENCE:

If the conversion of D-glucose has been completed within the time specified in the assay (approx. 5 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding D-glucose (approx. 40 µg in 0.1 mL) to the cuvette on completion of the reaction. A significant increase in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments, i.e. by adding D-glucose to the sample in the initial extraction steps.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.

For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 110/220 assays in manual format (or 1000/2000 assays in auto-analyser format or 1100/2200 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

110 Assays per Kit (cat. no. K-GLUHK-110A)

Bottle 1:	Buffer (25 mL, pH 7.6) plus sodium azide (0.02% w/v) as a preservative. Stable for > 2 years at 4°C.
Bottle 2:	$NADP^+$ plus ATP. Stable for > 5 years below -10°C.
Bottle 3:	Hexokinase plus glucose-6-phosphate dehydrogenase suspension, 2.25 mL. Stable for > 2 years at 4°C.
Bottle 4:	D-Glucose standard solution (5 mL, 0.4 mg/mL). Stable for > 2 years; store sealed at 4°C.

220 Assays per Kit (cat. no. K-GLUHK-220A)
This has the same components as the 110 determinations kit, except that it contains two of bottle 2 and two of bottle 3.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Use the contents of bottle 1 as supplied.
   Stable for > 2 years at 4°C.

2. Dissolve the contents of bottle 2 in 12 mL of distilled water. **Stable for > 1 year at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes). **Do not** dissolve the contents of the second bottle (220 Assays per kit only) until required.

3. Use the contents of bottle 3 as supplied. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottle in an upright position. **Swirl the bottle to mix contents before use.** Stable for > 2 years at 4°C.

4. Use the contents of bottle 4 as supplied.
   Stable for > 2 years; store sealed at 4°C.

NOTE: The D-glucose standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. The concentration of D-glucose is determined directly from the extinction coefficient of NADPH (page 5).

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 100 mm).
2. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL and 100 µL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.1 mL aliquots of buffer and $NADP^+$/ATP solution).
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
5. Analytical balance.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Stop clock.
9. Whatman No. 1 (9 cm) filter papers.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.32 mL
Sample solution: 4-80 µg of D-glucose per cuvette (in 0.10-2.00 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C)	2.10 mL	2.00 mL
sample	-	0.10 mL
solution 1 (buffer)	0.10 mL	0.10 mL
solution 2 ($NADP^+$/ATP)	0.10 mL	0.10 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:		
suspension 3 (HK/G-6-PDH)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 2 min intervals until the absorbances remain the same over 2 min**.		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

** if the absorbance continues to increase, this may be due to effects of colour compounds or enzymes in the sample. These interfering substances may be removed during sample preparation.

CALCULATION:

Determine the absorbance difference (A_2-A_1) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{D\text{-glucose}}$. The value of $\Delta A_{D\text{-glucose}}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of D-glucose can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{D\text{-glucose}} \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of D-glucose [g/mol]
ε = extinction coefficient of NADPH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for D-glucose:

$$c = \frac{2.32 \times 180.16}{6300 \times 1.0 \times 0.1} \times \Delta A_{D\text{-glucose}} \quad [g/L]$$

$$= 0.6634 \times \Delta A_{D\text{-glucose}} \quad [g/L]$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of D-glucose

$$= \frac{c_{D\text{-glucose}}\ [g/L\ sample\ solution]}{weight_{sample}\ [g/L\ sample\ solution]} \times 100 \quad [g/100\ g]$$

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for D-glucose can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of D-glucose **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Reagent preparation is performed as follows:

Preparation of R1:

Component	Volume
distilled water solution 1 (buffer) solution 2 ($NADP^+$/ATP)	53.2 mL 3.0 mL 3.0 mL (after adding 12 mL of H_2O to bottle 2)
Total volume	59.2 mL

Preparation of R2:

Component	Volume
distilled water suspension 3 (HK/G-6-PDH)	6.25 mL 0.55 mL
Total volume	6.8 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 5 min at 37°C
Wavelength:	340 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 0.810 g/L of D-glucose using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for D-glucose can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of D-glucose **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength: 340 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C
Final volume: 0.232 mL
Linearity: 0.1-8 µg of D-glucose per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water	0.210 mL	0.200 mL	0.200 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.010 mL	0.010 mL	0.010 mL
solution 2 ($NADP^+$/ATP)	0.010 mL	0.010 mL	0.010 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:			
suspension 3 (HK/G-6-PDH)	0.002 mL	0.002 mL	0.002 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 5 min). If the reaction has not stopped after 5 min, continue to read the absorbances at 2 min intervals until the absorbances increase constantly over 2 min**.			

* for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

** if this "creep" rate is greater for the sample than for the blank, extrapolate the sample absorbances back to the time of addition of suspension 3.

CALCULATION (Microplate Assay Procedure):

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L\ standard \times F$$

If the sample is diluted during preparation, the result must be multiplied by the dilution factor, F.

SAMPLE PREPARATION:

1. Sample dilution.

The amount of D-glucose present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 4 and 80 µg. The sample solution must therefore be diluted sufficiently to yield a D-glucose concentration between 0.04 and 0.8 g/L.

Dilution Table

Estimated concentration of D-glucose (g/L)	Dilution with water	Dilution factor (F)
< 0.8	No dilution required	1
0.8-8.0	1 + 9	10
8.0-80	1 + 99	100
> 80	1 + 999	1000

If the value of $\Delta A_{D\text{-glucose}}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased up to 2.00 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.10 mL and using the new sample volume in the equation.

2. Sample clarification.

a. Solutions:

Carrez I solution. Dissolve 3.60 g of potassium hexacyanoferrate (II) $\{K_4[Fe(CN)_6].3H_2O\}$ (Sigma cat. no. P9387) in 100 mL of distilled water. Store at room temperature.

Carrez II solution. Dissolve 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (Sigma cat. no. Z4750) in 100 mL of distilled water. Store at room temperature.

Sodium hydroxide (NaOH, 100 mM). Dissolve 4 g of NaOH in 1 L of distilled water. Store at room temperature.

b. Procedure:

Pipette the liquid sample into a 100 mL volumetric flask which contains approx. 60 mL of distilled water, or weigh sufficient quantity of the sample into a 100 mL volumetric flask and add 60 mL of distilled water. Carefully add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

3. General considerations.

(a) Liquid samples: clear, slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

(b) Acidic samples: if > 0.1 mL of an acidic sample is to be used undiluted (such as wine or fruit juice), the pH of the solution should be increased to approx. 7.6 using 2 M NaOH, and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing a significant amount of carbon dioxide, such as beer, should be degassed by increasing the pH to approx. 7.6 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Coloured samples: an additional sample blank, i.e. sample with no HK/G6P-DH, may be necessary in the case of coloured samples.

(e) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 0.2 g of polyvinylpolypyrrolidone (PVPP)/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper.

(f) Solid samples: homogenise or crush solid samples in distilled water and filter if necessary.

(g) Samples containing fat: extract such samples with hot water at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask. Adjust to room temperature and fill the volumetric flask to the mark with water. Store on ice or in a refrigerator for 15-30 min and then filter. Discard the first few mL of filtrate and use the clear supernatant (which may be slightly opalescent) for assay. Alternatively, clarify with Carrez reagents.

(h) Samples containing protein: deproteinise samples containing protein with Carrez reagents.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of D-glucose in preserves and other vegetable and fruit products.

Homogenise approx. 10 g of sample in a mixer. Accurately weigh approx. 0.5 g into a 100 mL volumetric flask, mix with 50 mL of distilled water to dissolve, make up to the mark, mix and filter. Discard the first 5 mL of the filtrate. Use the clear, undiluted filtrate for the assay (0.1-2.0 mL).

(b) Determination of D-glucose in milk.

Pipette 20 mL of milk into a 100 mL volumetric flask, add the following solutions and mix after each addition: 10 mL of Carrez I solution, 10 mL of Carrez II solution and 20 mL of NaOH solution (100 mM). Fill up to the mark with distilled water, mix and filter. Use 1.00 mL of the filtrate for the assay.

(c) Determination of D-glucose in fermentation samples and cell culture medium.

Incubate an aliquot (approx. 10 mL) of the solution at approx. 90-95°C for 10 min to inactivate enzyme activity. Centrifuge or filter and use the supernatant or clear filtrate (diluted according to the dilution table, if necessary) for the assay. Alternatively, deproteinisation can be performed with Carrez reagents. Homogenise gelatinous agar media with water and treat further as described above.

(d) Solid foodstuffs.

Mill plant materials to pass a 0.5 mm screen. Homogenise solid foodstuffs such as bread, pastries, chocolate confectionery or candy in a mixer, meat grinder or mortar. Weigh out a representative sample and extract with water (heated to 60°C, if necessary). Quantitatively transfer to a volumetric flask and dilute to the mark with distilled water. Mix, filter and use the appropriately diluted, clear solution for the assay.

REFERENCE:

Kunst, A., Draeger, B. & Ziegenhorn, J. (1988). D-Glucose. "*Methods of Enzymatic Analysis*" (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VI**, pp. 163-172, VCH Publishers (UK) Ltd., Cambridge, UK.

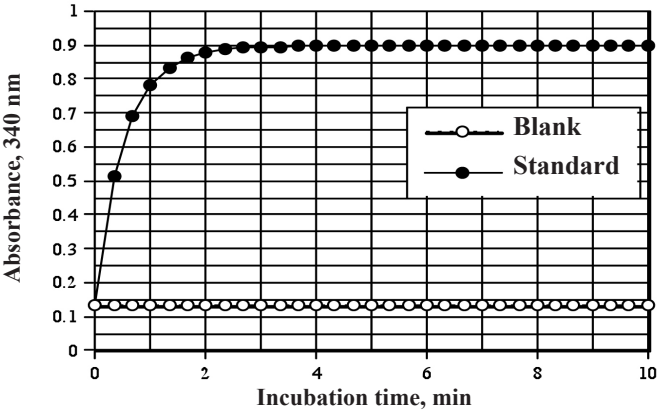

Figure 1. Increase in absorbance at 340 nm on incubation of 50 µg of D-glucose with hexokinase and glucose 6-phosphate dehydrogenase in the presence of $NADP^+$ and ATP.

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