Megazyme

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D-GLUCOSAMINE

(INCLUDING *N*-ACETYL GLUCOSAMINE AND D-GLUCOSAMINE SULPHATE)

ASSAY PROCEDURE

K-GAMINE 04/18

(*50 Assays per Kit) or
(500 Auto-Analyser Assays per Kit) or
(500 Microplate Assays per Kit)

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

INTRODUCTION:

D-Glucosamine ($C_6H_{13}NO_5$) is an amino sugar and a prominent precursor in the biochemical synthesis of glycosylated proteins and lipids. It is produced commercially by the hydrolysis of crustacean exoskeletons or, less commonly, by fermentation. D-Glucosamine is commonly used as a treatment for osteoarthritis, although its acceptance as a medical therapy varies. Commercially available preparations contain D-glucosamine as the hydrochloride or sulphate forms. Analysis of such preparations indicates that the sulphate is not 2-, or 6-, linked to the D-glucosamine but rather occurs as free sulphate (possibly from preparation steps involving sulphuric acid hydrolysis).

PRINCIPLE:

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

$$\text{(1)}\quad \text{D-Glucosamine} + \text{ATP} \xrightarrow{\text{(HK)}} \text{D-glucosamine-6-P} + \text{ADP} + P_i$$

In the presence of the enzyme glucosamine-6-phospate deaminase (GNPDA) and water (H_2O), glucosamine-6-P is converted to fructose-6-phosphate (F-6-P) and ammonia (NH_4^+) (2).

$$\text{(2)}\quad \text{D-Glucosamine-6-P} + H_2O \xleftrightarrow{\text{(GNPDA)}} \text{F-6-P} + NH_4^+$$

On completion of reaction (2), F-6-P is converted into glucose-6-phospate (G-6-P) by phosphoglucose isomerase (PGI) (3).

$$\text{(3)}\quad \text{F-6-P} \xleftrightarrow{\text{(PGI)}} \text{G-6-P}$$

G-6-P is oxidised by the enzyme glucose-6-phosphate dehydrogenase (G6P-DH) in the presence of nicotinamide-adenine dinucleotide phosphate ($NADP^+$) to gluconate-6-phosphate (gluconate-6-P) with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (4).

$$\text{(4)}\quad \text{G-6-P} + NADP^+ \xrightarrow{\text{(G6P-DH)}} \text{gluconate-6-phosphate} + \text{NADPH} + H^+$$

The amount of NADPH formed in this reaction is stoichiometric with the amount of D-glucosamine. It is the NADPH which is measured by the increase in absorbance at 340nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for glucosamine. The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 0.666 mg/L of sample solution at the maximum sample volume of 1.00 mL. The detection limit is 1.33 mg/L, which is derived from an absorbance difference of 0.020 with a sample volume of 1.00 mL.

The assay is linear over the range of 4 to 80 µg of glucosamine 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 1.00 mL, this corresponds to a glucosamine concentration of approx. 0.333 to 0.666 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 glucosamine has been completed within the time specified in the assay, it can be generally concluded that no interference has occurred. However, this can be further checked by adding glucosamine standard (approx. 50 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.

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 50 assays in manual format (or 500 assays in auto-analyser format or 500 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: Buffer (6 mL, pH 7.6) plus sodium azide (0.02% w/v) as a preservative. Stable for > 2 years at 4°C.

Bottle 2: $NADP^+$, ATP and PVP.
Stable for > 5 years below -10°C.

Bottle 3: Hexokinase, glucose-6-phosphate dehydrogenase and phosphoglucose isomerase suspension (1.3 mL).
Stable for > 2 years at 4°C.

Bottle 4: Glucosamine-6-P deaminase suspension (1.3 mL).
Stable for > 2 years at 4°C.

Bottle 5: D-Glucosamine standard solution (5 mL, 0.5 mg/mL).
Stable for > 2 years at 4°C.

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 6 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).

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

5. Use the contents of bottle 5 as supplied.
Stable for > 2 years at 4°C.

NOTE: The D-glucosamine standard solution (bottle 5) 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 glucosamine is determined directly from the extinction coefficient of NADPH (page 5).

EQUIPMENT (RECOMMENDED):

1. Volumetric flasks (50 mL, 100 mL and 1000 mL).
2. Disposable 1.5 mL polypropylene microfuge tubes, e.g. Sarstedt cat. no. 72.690 (www.sarstedt.com).
3. Disposable 13 mL polypropylene tubes, e.g. Sarstedt cat. no. 60.541.685 PP (www.sarstedt.com).
4. Disposable plastic cuvettes (1 cm light path, 3.0 mL).
5. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 100 µL and 1 mL).

6. Positive displacement pipettor, e.g. Eppendorf Multipette®
   - with 5.0 mL Combitip® (to dispense 0.1 mL aliquots of buffer 1 and $NADP^+$/ATP solution).
   - with 25 mL Combitip® (to dispense 2.0 mL aliquots of distilled water).
7. Stop clock.
8. Analytical balance.
9. Spectrophotometer set at 340 nm.
10. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
11. Heated water bath (set at 100°C).
12. Microfuge (required speed 13,000 rpm).

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: 40°C
Final volume: 2.34 mL
Sample solution: 4-80 µg of D-glucosamine per cuvette (in 0.10-1.00 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/PVP)	0.10 mL	0.10 mL
suspension 3 (HK/G6P-DH/PGI)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by the addition of:		
suspension 4 (GNPDA)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_2) at the end of the reaction (approx. 8-10 min). If the reaction has not stopped after 10 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 (A_2-A_1) of the blank from the absorbance difference (A_2-A_1) of the sample, thereby obtaining $\Delta A_{D\text{-glucosamine}}$.

The value of $\Delta A_{D\text{-glucosamine}}$ should as a rule be at least 0.100 absorbance units to achieve sufficiently accurate results.

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

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of D-glucosamine [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-glucosamine:

$$c = \frac{2.34 \times 179.17}{6300 \times 1 \times 0.1} \times \Delta A_{D\text{-glucosamine}} \quad [g/L]$$

$$= 0.6655 \times \Delta A_{D\text{-glucosamine}} \quad [g/L]$$

If the sample has been diluted in addition to the dilution during preparation, the result must be multiplied by the dilution factor, F. If the sample contains glucosamine in its HCl, sulphate or *N*-acetyl forms, the result must be multiplied by a factor of 1.203, 1.447 or 1.235 respectively.

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-glucosamine

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

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-glucosamine 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-glucosamine **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
bottle1 (buffer) bottle 2 ($NADP^+$/ATP/PVP) bottle 3 (HK/G6P-DH/PGI) distilled water	1.10 mL 1.10 mL (after adding 6 mL of H_2O to bottle 2) 0.22 mL 19.25 mL
Total volume	21.67 mL

Preparation of R2:

Component	Volume
bottle 4 (GNPDA) distilled water	0.24 mL 2.75 mL
Total volume	2.99 mL

EXAMPLE METHOD:

R1:	0.200 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 10 min at 37°C
Wavelength:	340 nm
Prepared reagent stability:	> 2 days when refrigerated
Calculation:	endpoint
Reaction direction:	increase
Linearity:	up to 0.8 g/L of D-glucosamine using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for D-glucosamine 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-glucosamine **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: 0.234 mL
Linearity: 0.1-8.0 µg of D-glucosamine 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/PVP)	0.010 mL	0.010 mL	0.010 mL
suspension 3 (HK/G6P-DH/PGI)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 3 min and start the reactions by addition of:			
suspension 4 (GNPDA)	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. 8-10 min). If the reaction has not stopped after 10 min, continue to read the absorbances at 2 min intervals until the absorbances either plateau or decrease 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).

CALCULATION (Microplate Assay Procedure):

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

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-glucosamine 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-glucosamine (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 $\Delta A_{D\text{-glucosamine}}$ 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 1.00 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 GNPDA, 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 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 distilled 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-glucosamine in neutraceutical preparations.

Accurately weigh approx. 1.5 g of representative material into a 250 mL volumetric flask. After addition of approx. 100 mL of distilled water, stir the contents until fully dissolved or suspended, and fill up to the mark with distilled water. Mix and, if necessary, filter through Whatman No. 1 filter paper. Use the clear filtrate, with dilution according to the dilution table, if necessary, for the assay. *Typically for neutraceutical grade D-glucosamine, a further 1:4 dilution and sample volume of 0.1 mL are satisfactory.*

(b) Determination of D-glucosamine-6-sulphate or -2-sulphate in neutraceutical preparations.

Accurately weigh approx. 1.5 g of representative material into a 250 mL volumetric flask. After addition of approx. 100 mL of distilled water, stir the contents until fully dissolved or suspended. Make up to 250 mL with distilled water. Mix, and if then necessary, filter through Whatman No. 1 filter paper. Accurately transfer 1 mL of the clear filtrate to a glass, round bottomed test tube and add a further 1 mL of 4 M HCl to give a 2-fold dilution of both the acid and sample. Incubate in a boiling water bath for 1 h after which time the solution should be neutralised with 2 mL of 2 M NaOH. *Typically no further dilution is necessary for the assay and a sample volume of 0.1-1.0 mL is satisfactory.*

(c) Determination of total D-glucosamine, including D-glucosamine-6-sulphate, D-glucosamine-2-sulphate and *N*-acetyl D-glucosamine in pharmaceutical preparations.

Accurately weigh approx. 1.5 g of representative material into a 250 mL volumetric flask. After addition of approx. 100 mL of distilled water, stir the contents until fully dissolved or suspended. Make up to 250 mL with distilled water. Mix and, if then necessary, filter through Whatman No. 1 filter paper. Accurately transfer 1 mL of the clear filtrate to a glass, round bottomed test tube and add a further 1 mL of 4 M HCl to give a 2-fold dilution of both the acid and sample. Cover the tubes with foil or glass marbles and incubate in a boiling water bath for 2 h after which time the solution should be neutralised with 2 mL of 2 M NaOH. Solution should be left at room temperature for 10 min to effectively complete deacetylation of the *N*-acetyl glucosamine. *Typically no further dilution is necessary for the assay and a sample volume of 0.1-1.0 is satisfactory.*

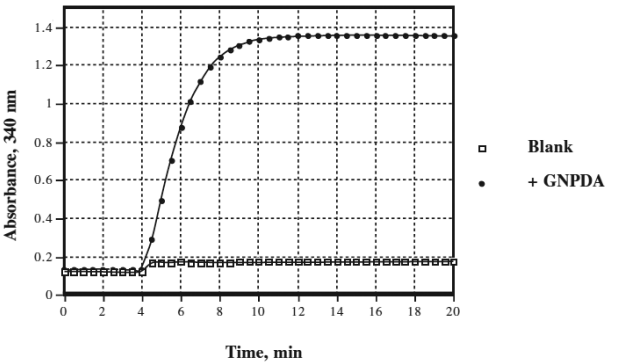

Figure 1. Increase in absorbance at 340 nm on incubation of 100 µg of D-glucosamine HCl with 1.4 U of glucosamine-6-P deaminase plus hexokinase and glucose 6-phosphate dehydrogenase in the presence of $NADP^+$ and ATP.

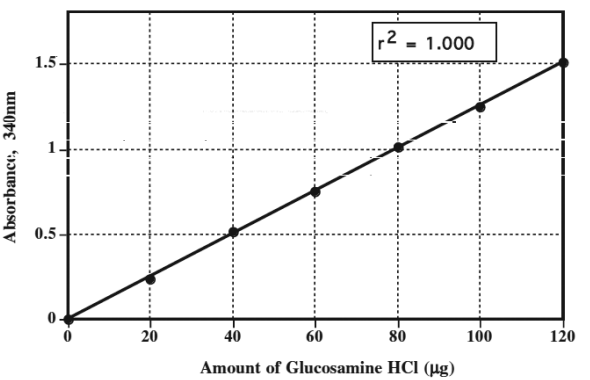

Figure 2. Standard curve relating final absorbance at 340 nm to concentration of D-glucosamine HCl on incubation of 0-120 µg of D-glucosamine HCl with 1.4 U of glucosamine-6-P deaminase plus hexokinase and glucose 6-phosphate dehydrogenase in the presence of $NADP^+$ and ATP.

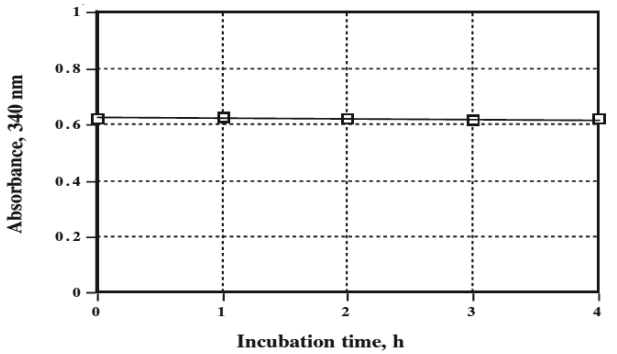

Figure 3. Stability of D-glucosamine in 2 M HCl on extended incubation at 100°C. On incubation of the D-glucosamine HCl (100 µg) in 2 M HCl for 1, 2 and 3 h at 100°C, samples were removed, neutralised and analysed using the method described here. Clearly, no degradation of D-glucosamine was observed under the described conditions.

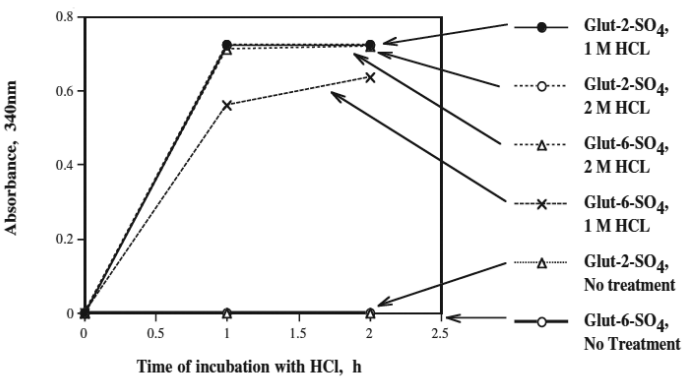

Figure 4. Hydrolysis of glucosamine-2-sulphate and glucosamine-6-sulphate to free D-glucosamine on incubation in 1 M or 2 M HCl at 100°C for 0, 1 and 2 h (measured using the assay procedure described here). Clearly, incubation in 2 M HCl for 1 h gives complete hydrolysis of both the -2- and -6-sulphate groups. The assay procedure for D-glucosamine does not measure either glucosamine-2-sulphate or glucosamine-6-sulphate.

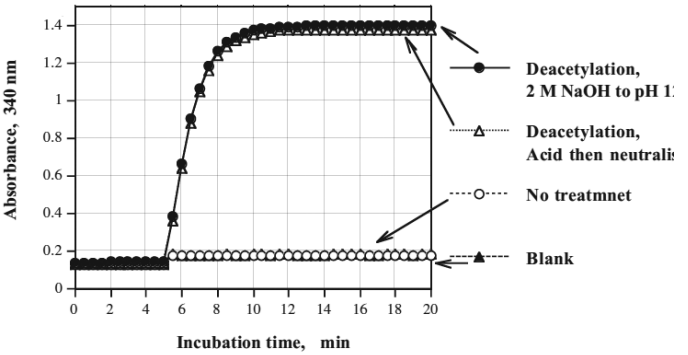

Figure 5. Increase in absorbance at 340 nm on incubation of 100 µg of *N*-acetyl-glucosamine before and after deacetylation (see example c, page 11) with 1.4 U of glucosamine-6-P deaminase plus hexokinase and glucose 6-phosphate dehydrogenase in the presence of $NADP^+$ and ATP. Deacetylation was effected by adjustment of pH to 12 with NaOH and incubation at room temperature for 10 min, or simply by adjusting the pH to approx. 7.6 after acid hydrolysis.

NOTES:

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