N-acetyl-D-glucosamine Assay Manual v1.0

Cat. No. 0210

500 Microplate Assays per Kit or

*100 Cuvette Assays per Kit**

**Assay volumes are 0.2 and 1.0 ml, respectively.*

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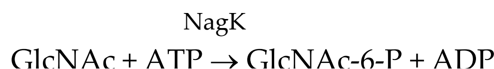
INTRODUCTION

N-acetyl-D-glucosamine ($C_8H_{15}NO_6$) is a monosaccharide derivative of glucose, a secondary amide between glucosamine and acetic acid. GlcNAc is found in bacterial and fungi cell walls, and is the monomeric unit of chitin, forming the exoskeleton of many arthropods.

GlcNAc is also involved in post-translational cell protein processing. N-acetyl-D-glucosamine is added to the serine or threonine of a protein during O-GlcNAcylation. Glycosylation is critical to protein structure and host immune response.

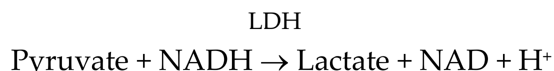
PRINCIPLE:

N-acetyl-D-glucosamine (GlcNAc) is phosphorylated by the enzyme GlcNAc Kinase (NagK) using adenosine-5′-triphosphate (ATP) to form GlcNAc-6-phosphate with the simultaneous formation of adenosine-5′-diphosphate (ADP) (1).



In the presence of the enzyme pyruvate kinase (PK) and phosphoenolpyruvate, ADP is converted to ATP and pyruvate.

In the presence of lactate dehydrogenase (LDH) and pyruvate, NADH is oxidized to NAD yielding lactate.



The amount of NAD formed in this reaction is stoichiometric with the amount of GlcNAc phosphorylated and is measured by a decrease in absorbance at 340 nm.

DYNAMIC RANGE AND DETECTION LIMIT:

The assay is for quantifying N-acetyl-D-glucosamine. The assay can be performed as a kinetic assay (broader detection range) or as an endpoint assay. The detection limit of the kinetic assay is 3 µM GlcNAc. The detection limit of the endpoint assay is 5 µM GlcNAc.

The dynamic range of the assays are:

	1 mL Cuvette Assay	200 µL Microplate Assay
Endpoint	6 µM to 200 µM 1.3 mg/L – 44.3 mg/L 252 ng – 8.86 ug Lower LoD 4 µM	10 µM to 125 µM 2.1 mg/L - 27.7 mg/L 420 ng - 5.54 ug Lower LoD 4 µM
Kinetic	6.9 µM to 5,000 µM 1.5 mg/L - 1.1 g/L 1.5 ug - 1.1 mg Lower LoD 3 µM	6.9 µM to 5,000 µM 1.5 mg/L - 1.1 g/L 300 ng - 220 ug Lower LoD 5 µM

SPECIFICITY AND INTERFERENCE:

The assay is specific for N-acetyl-D-glucosamine. Glucosamine, glucose, galactose, and mannose up to 500 mM are not phosphorylated by NagK-8. Glucosamine is a competitive inhibitor. Dilute the samples to give GlcN levels below 10 mM or include the appropriate amount of GlcN in the GlcNAc calibrator curve. Interference by a sample component can be identified by spiking the sample with a known amount of GlcNAc. If the amount added is measured as expected, then there is no interference. If the amount added is not fully measured, then the sample may have an interfering component (e.g., an enzyme inhibitor). Buffers containing phosphate, citrate, EDTA, or EGTA will inhibit the assay. Dilute to below 0.1 mM if present.

SAFETY:

Follow generally accepted laboratory safety practices when using the kit. The assay is for research use only and should not be used for human or veterinary diagnoses.

KITS:

Kits are suitable for performing 100 assays in the manual format (1 mL assay) or 500 assays in microplate format (200 µL). The kits contain the full assay method plus:

Vial 1 (yellow): 10x Buffer Reaction Mix (5 x 2 mL, pH 7.5) plus sodium azide (0.02% w/v). Stable for 1 year at 4°C.

Vial 2 (green): 200x NagK-8 (0.5 mL, 5.4 U/ml) in storage buffer plus sodium azide (0.02% w/v) as a preservative. Stable for 2 years at -20°C; 2 months at 4°C.

Vial 3 (blue): Lactate Dehydrogenase and Pyruvate Kinase enzyme mix in 3.2 M ammonium sulphate (0.180 mL, 2,222 U/mL each) Stable for 1 year at 4°C.

Vial 4 (white): 100 mM N-acetyl-D-glucosamine standard solution in sterile 18 Mohm H_2O (0.5 mL). Stable for > 2 years at 4°C.

PREPARATION OF REAGENTS:

Use the contents of all vials as supplied.

Store a 100 µL aliquot of vial 2, 200x NagK-8, at 4°C. Store rest of vial 2 at -20°C.

The N-acetyl-D-glucosamine standard solution (vial 4) is used to create a calibrator curve. As enzyme reaction rates are condition specific (e.g., incubation temperature, spectrophotometric instrumentation) and can vary from lab to lab, it is recommended that a calibrator set be run with each assay. The concentration of GlcNAc is determined from the calibrator curve.

EQUIPMENT (RECOMMENDED):

1. Cuvette Assay: Quartz or disposable plastic cuvettes (1 cm light path, 1.0 mL)

   Microplate Assay: 96-well low protein binding polystyrene plate or strip wells (e.g., Nunc PolySorp or Costar 9017).
2. Micro- and multichannel pipettors, e.g., Gilson Pipetman® (10 µL, 20 µL, 100 µL and 1 mL).
3. Spectrophotometer set at 340 nm or microplate reader with 340 nm filter5.
4. Vortex mixer.
5. Heated water bath (set at 30°C).
6. Disposable reagent reservoirs and vials.

SAMPLE PREPARATION:

Dilutions

If sample dilution is necessary, it is recommended to use deionized water or Tris-HCl pH 7.5. (See Specificity and Interference section for further information.)

1 mL Cuvette Assay:

The amount of N-acetyl-D-glucosamine present in the assay should be in the range between 1.5 to 1100 ug. The sample solution must therefore be diluted sufficiently to yield a GlcNAc concentration between 1.5 to 1100 mg/L.

200 µL Microplate Assay:

The amount of N-acetyl-D-glucosamine present in the assay should be in the range between 300 ng to 220 ug. The sample solution must therefore be diluted sufficiently to yield a GlcNAc concentration between 1.5 to 1100 mg/L.

General considerations

1. Clear or slightly colored liquid samples can be used directly in the assay.
2. The pH of acidic samples should be increased to pH 7.5 +/- 0.2 using 2 M NaOH or dilute into a Tris-based buffer, and the solution incubated at room temperature for 30 min to allow outgassing.
3. Samples containing carbon dioxide, such as beer, soda or other carbonated beverages, should be degassed by increasing the pH to 7.5 +/- 0.2 with 2 M NaOH, or Tris-based buffer and stirred until degassed.
4. Samples with chemicals that absorb at 340 nm should only be used in the kinetic assay. A suitable blank should be prepared to adjust for interference.
5. Strongly colored 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.
6. Solid samples should be homogenized or ground and dissolved in deionized water or Tris-based aqueous buffer. Undissolved particulate matter should be removed by filtration.
7. Samples containing fat should be extracted with 2 volumes of hot water at a temperature above the melting point of the fat. Lower to room temperature and add 5 volumes of deionized 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.
8. Samples containing protein should be deproteinized with Carrez reagents.

CUVETTE ASSAY PROCEDURE (1 ml cuvette):

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: 22-37°C
Final volume: 1 mL
Prepared reagent stability: Prepared fresh daily
Reaction direction: Decrease in absorbance relative to zero calibrator reaction
Kinetic:
Reaction time: 5 min at 30°C
Dynamic Range: 6.9 µM to 5,000 µM (1.5 mg/L – 1,100 mg/L)
Endpoint:
Reaction time: up to 45 min at 30°C

Dynamic Range: 6 μM to 200 μM (1.3 mg/L – 44.3 mg/L)

Assay Procedure:

1. It is recommended that each assay point be performed in triplicate.
2. Prepare the 2x calibrators at 2,000, 400, 80, 16 and 0 μM. **[Note: The calibrator set can be customized but should be within the stated dynamic range.]**
   2.1. Label five 5 mL tubes C1, C2, C3, C4 and C5.
   2.2. Dispense 2 mL water into tubes C2 to C5.
   2.3. Dispense 2.45 mL water into tube C1. Add 0.05 mL 100 mM GlcNAc stock and mix well.
   2.4. Remove 0.5 ml from C1 and add to C2, mix and serially dilute 0.5 mL through to C4.
3. Prepare enough 2x Reaction Cocktail for the number of assays to be performed. (See table.)
   3.1. For each reaction, mix 393.2 μL water, 100 μL 10x Buffer Reaction Mix, 5 μL 200x NagK-8 Mix and 1.85 μL LDH/PK Mix.
   3.2. It is recommended that the volume of the 2x cocktail is the number of assays plus one to provide a sufficient volume of reaction mix for all of the assays.
4. Prepare the samples to be tested by dilution in water so that the expected GlcNAc concentration is within the calibrator range.
5. Blank the spectrophotometer using water.
6. Perform each assay by rapidly mixing 0.5 ml of calibrator (prepared in Step 1) or sample with 0.5 ml of Reaction Cocktail.
7. Perform
   7.1. Kinetic Assay
      7.1.1. Record the change in absorbance at 340 nm over 2 min at 2 to 10 sec intervals.
      7.1.2. Determine the rate over the linear most part of the rate curves.
   7.2. Endpoint Assay
      7.2.1. The end of the reaction will occur within 45 min at 30°C, depending on the GlcNAc concentration. Take absorbance readings at 2 minute intervals until the absorbances remain the same over 2 minutes.

Pipette into preparation cuvette	Calibrator (μl)	Sample (μl)	Zero Calibrator (μl)
Reaction Mix (500 μL pre-made):			
Sterile 18 Mohm H_2O	393.1	393.1	393.1
10x Reaction Cocktail	100.0	100.0	100.0
200x NagK Mix	5.0	5.0	5.0
1,000x LDH/PK Mix	1.9	1.9	1.9
Sample		500.0	
Calibrator (Tubes C1 to C4)	500.0		
Sterile 18 Mohm H_2O (Tube C5)			500.0

MICROPLATE ASSAY PROCEDURE (200 µL microwell):

Wavelength: 340 nm
Microplate: 96-well clear flat-bottomed
Temperature: 22-37°C
Final volume: 0.2 mL
Prepared reagent stability: Prepared fresh daily
Reaction direction: Decrease in absorbance relative to zero calibrator reaction
Endpoint:
Reaction time: 5 - 45 min at 30°C, until minimum absorbance plateau is reached
Dynamic Range: 10 µM to 125 µM (2.1 mg/L - 27.7 mg/L)
Kinetic:
Reaction time: 15 min at 30°C
Dynamic Range: 6.9 µM to 5,000 µM (1.5 mg/L – 1,100 mg/L)

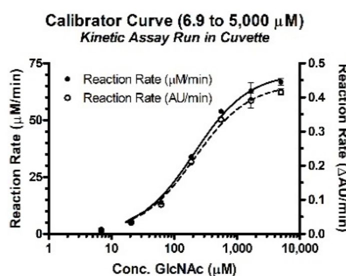
Assay Procedure:

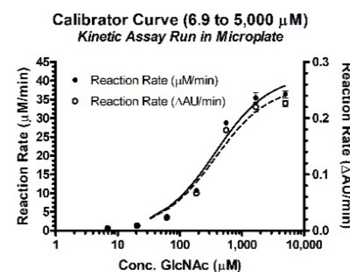
1. It is recommended that each assay point be performed in triplicate.
2. Prepared the 2x calibrators at 2,000, 400, 80, 16 and 0 µM. **[Note: The calibrator set can be customized but should be within the stated dynamic range.]**
   a. With a multichannel pipettor, dispense 100 µL water into microplate wells B, C, D, E, and F.
   b. Dispense 122.5 µL water into well A. Add 2.5 µL 100 mM GlcNAc stock and mix well.
      i. [Alternatively, prepare 400 µL of 2,000 µM GlcNAc, and dispense 125 µL into row A.]
   c. Remove 25 µL from well A and add to well B and mix. Continue to serially dilute 25 µL through to well E. Row F is the 0 calibrator.
3. Prepare the samples to be tested by dilution in water so that the expected GlcNAc concentration is within the calibrator range.
4. Prepare enough 2x Reaction Cocktail for the number of assays to be performed. (See table.)
   a. For each reaction (well), mix 78.6 µL water, 20 µL 10x Buffer Reaction Mix, 1 µL 200x NagK-8 Mix and 0.4 µL LDH/PK Mix.
   b. It is recommended that the volume of the 2x cocktail is in slight excess to the volume needed so there is a sufficient supply of reaction mix for all of the assays.
5. After the microplate wells contain 100 µL of each calibrator and sample (prepared in Step 1 and 2, respectively), quickly pipette with a multichannel pipettor, 100 µL of Reaction Cocktail into each well. **[Note: When performing the kinetic assay, the maximum number of wells assayed at a given time yielding reliable rate values is machine-dependent. Please adjust accordingly.]**
6. Kinetic Assay
   a. Record the decrease in absorbance at 340 nm over 15 min at ~30 sec intervals with shaking in between readings.
   b. Determine the reaction rate over the linear most part of the rate curves.
7. Endpoint Assay
   a. The end of the reaction will occur within 45 min at 30°C, depending on the GlcNAc concentration. Record absorbance readings at 2 minute intervals until the absorbances in all reactions remain the constant over 2 minutes.

Pipette into reagent reservoir	Calibrator (µl)	Sample (µl)	Zero Calibrator (µl)
Reaction Mix:			
Sterile 18 Mohm H_2O	78.63	78.63	78.63
10x Reaction Cocktail	20.00	20.00	20.00
200x NagK Mix	1.00	1.00	1.00
1,000x LDH/PK Mix	0.37	0.37	0.37
Sample		100.00	
Standard	100.00		
Sterile 18 Mohm H_2O			100.00

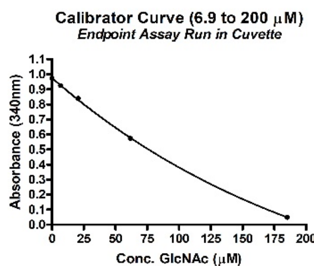
DETERMINATION OF SAMPLE GlcNAc CONCENTRATION:

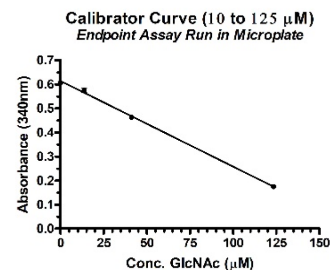
1. Kinetic Assay
   a. To generate the calibrator curve, plot the average rates against the log of the corresponding GlcNAc concentration (remember the final concentrations are ½ that in the starting solutions of Step 1). **[Note: Change in absorbance values are negative. Correct accordingly so all are positive.]**
   b. Use a Boltzmann sigmoidal curve fit.
   c. Use the calibrator curve to calculate the amount of GlcNAc in the sample corrected for the dilution factor.





2. Endpoint Assay
   a. To generate the calibrator curve, plot the average minimum absorbance against the log of the corresponding GlcNAc concentration (remember the final concentrations are ½ that in the starting solutions of Step 1).
   b. Use the calibrator curve to calculate the amount of GlcNAc in the sample corrected for the dilution factor.





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