

SIGMA QUALITY CONTROL TEST PROCEDURE

**Enzymatic Assay of CHITINASE
(EC 3.2.1.14)****PRINCIPLE:**

Chitin + H₂O $\xrightarrow{\text{Chitinase}}$ Chitobiose

Chitobiose + H₂O $\xrightarrow{\beta\text{-N-Acetylglucosaminidase}}$ N-Acetyl-D-Glucosamine

CONDITIONS: T = 25°C, pH = 6.0, A_{540nm}, Light path = 1 cm

METHOD: Colorimetric

REAGENTS:

- A. 200 mM Potassium Phosphate Buffer, pH 6.0 at 25°C with 2 mM Calcium Chloride.
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379, and Calcium Chloride, Dihydrate, Sigma Prod. No. C-3881. Adjust to pH 6.0 at 25°C with 1 M KOH.)
- B. 1.25% (w/v) Chitin Suspension (Chitin)
(Prepare 25 ml in Reagent A using Chitin, Sigma Prod. No. C-8908. Stir on a magnetic stirrer for approximately 15 minutes to ensure that large particles are broken apart.)
- C. 5.3 M Sodium Potassium Tartrate Solution
(Dissolve 12.0 grams of Sodium Potassium Tartrate, Tetrahydrate, Sigma Prod. No. S-2377, in 8.0 ml of 2 M NaOH. Heat in a boiling water bath to dissolve, but **DO NOT BOIL.**)
- D. 96 mM 3,5-Dinitrosalicylic Acid Solution
(Dissolve 438 mg of 3,5-Dinitrosalicylic Acid, Sigma Prod. No. D-0550, in 20 ml of deionized water. Heat in a boiling water bath to dissolve, but **DO NOT BOIL.**)

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REAGENTS: (continued)

- E. Color Reagent Solution (Clr Rgt Soln)
(With stirring, slowly add Reagent C solution to Reagent D. Dilute to 40 ml with deionized water. If not completely dissolved, the reagents should dissolve when mixed. The solution should be stored in an amber bottle at room temperature. The Color Reagent Solution is stable for approximately 6 months.)
- F. Chitinase Enzyme Solution
(Immediately before use, prepare a solution containing 1 unit/ml of Chitinase in cold Reagent A.)
- G. β -N-Acetylglucosaminidase (NAGase)
(Immediately before use, prepare a solution containing 35 units/ml in cold Reagent A using β -N-Acetylglucosaminidase, Sigma Prod. No. A-3189¹.)
- H. 0.1% (w/v) N-Acetyl-D-Glucosamine Standard Solution (NAG)
(Prepare 10 ml in deionized water using N-Acetyl-D-Glucosamine, Sigma Prod. No. A-8625.)

PROCEDURE:

Pipette (in milliliters) the following reagents into 4 dram vials:

	<u>Test</u>	<u>Blank</u>
Reagent B (Chitin)	2.00	2.00
Deionized Water	-----	0.50
Reagent F (Enzyme Solution)	0.50	-----

Close each container securely with a cap. The vials should be placed on their sides on a rotary platform at a speed sufficient to keep the chitin in suspension. Incubate the Test and Blank for two hours at 25°C.

After two hours, place the vials into a boiling water bath for 5 minutes. Cool to room temperature by placing the vials in a cold water bath.

Add 1 unit of Reagent G (NAGase) to each vial. Incubate the vials for 30 minutes at 25°C with mixing as before. Centrifuge the suspensions and retain the supernatant liquid.²

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COLORIMETRIC ASSAY:

Standard Curve:

A standard curve is prepared by pipetting (in milliliters) the following reagents into suitable containers:

	<u>Std 1</u>	<u>Std 2</u>	<u>Std 3</u>	<u>Std 4</u>	<u>Std 5</u>	<u>Std Blank</u>
Reagent H (NAG Std Soln)	0.1	0.2	0.3	0.4	0.5	0.0
Deionized Water	2.9	2.8	2.7	2.6	2.5	3.0
Reagent E (Clr Rgt Soln)	1.5	1.5	1.5	1.5	1.5	1.5

Sample:

Pipet the following reagents into suitable vials:

	<u>Test</u>	<u>Blank</u>
Test Supernatant	1.00	-----
Blank Supernatant	-----	2.00
Deionized water	2.00	1.00
Reagent E (Clr Rgt Soln)	1.50	1.50

Place all the containers in a boiling water bath for 5 minutes. Remove and allow the containers to cool to room temperature. Transfer the solutions to suitable cuvettes and record the A_{540nm} for each of the containers.

CALCULATIONS:

Standard Curve:

$$\Delta A_{540nm} \text{ Standard} = A_{540nm} \text{ Std} - A_{540nm} \text{ Std Blank}$$

Plot the ΔA_{540nm} of the standards versus milligrams of NAG.

Sample Determination:

$$\Delta A_{540nm} \text{ Sample} = A_{540nm} \text{ Test} - A_{540nm} \text{ Test Blank}$$

Determine the milligrams of NAG liberated using the Standard Curve.

$$\text{Units/ml enzyme} = \frac{(\text{mg NAG released})(2.5 + \text{Volume of NAGase}^3)}{(2)(1)(0.5)}$$

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CALCULATIONS: (continued)

2.5 = Initial reaction volume of assay
2 = Conversion factor for converting 2 hours to 1 hour as per the Unit Definition
1 = Volume (in milliliter) of supernatant used in colorimetric determination
0.5 = Volume (in milliliter) of enzyme used

$$\text{Units/g solid} = \frac{\text{units/ml enzyme}}{\text{g solid/ml enzyme}}$$

UNIT DEFINITION:

One unit will liberate 1.0 mg of N-acetyl-D-glucosamine from chitin per hour at pH 6.0 at 25°C in a two step reaction with β -N-acetylglucosaminidase from *Aspergillus niger*, (2 hour assay).

FINAL ASSAY CONCENTRATIONS:

In a 2.50 ml reaction mix, the final concentrations are 160 mM potassium phosphate, 2 mM calcium chloride, 1% (w/v) chitin, 1 unit of β -N-acetylglucosaminidase and 0.5 unit chitinase.

REFERENCE:

Monreal, J. and Reese, E.T. (1969) *Canadian Journal of Microbiology* **15**, 689-696

NOTES:

1. The color reaction is inhibited by ammonium sulfate. The enzyme is an ammonium sulfate suspension. Before being used in this assay β -N-Acetylglucosaminidase, Sigma Prod. No. A-3189, must be centrifuged and the supernatant liquid removed. The pellet must be resuspended in cold Reagent A immediately prior to use.
2. Centrifuge the 4-dram glass vials for 10 minutes at 5400 rpm. Remove 1.5 ml of supernatant and add it to the microcentrifuge tubes. Centrifuge for 10 minutes at 10,000 rpm. Remove 1.0 ml of supernatant for color development.

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NOTES: (continued)

3. The reaction volume needs to be adjusted according to the amount of Reagent E (NAGase used) in the assay.
4. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.
5. β -N-Acetylglucosaminidase Unit Definition: One unit will hydrolyze 1.0 μ mole of p-nitrophenyl N-acetyl- β -D-glucosaminide to p-nitrophenol and N-acetyl-D-glucosamine per minute at pH 4.0 at 25°C.

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