

Product Information

Chitinase Assay Kit, Fluorimetric

Catalog Number **CS1030**

Storage Temperature -20 °C

TECHNICAL BULLETIN

Product Description

Chitinase catalyzes the hydrolytic cleavage of the $\beta(1\rightarrow4)$ -glycoside bond present in biopolymers of N-acetylglucosamine, primarily in chitin. Chitinases are widely distributed in living organisms and are found in fungi, bacteria, parasites, plants, and animals.¹ They are classified in families based on amino acid sequence similarities.²

The chitinolytic enzymes are also categorized based on their enzymatic action on chitin substrates.

Endochitinases are defined as the enzymes catalyzing the random cleavage at internal points in the chitin chain. Exochitinases catalyze the progressive release of acetylchitobiose or N-acetylglucosamine from the non-reducing end of chitin, and thus, are referred to as chitobiosidase and β -N-acetylglucosaminidase, respectively.^{3,4}

Chitinases perform different functions in different organisms. In bacteria they are mainly involved in nutritional processes, while in yeast and various fungi, these enzymes participate in morphogenesis. In animals and plants, chitinases primarily play a role in the defense of the organism against pathogen attack.

Human chitotriosidase (Chit), a chitinolytic enzyme, is a member of the chitinase family. In human plasma, Chit activity has been proposed as a biological marker of macrophage activity in several lysosomal diseases, and was found at higher levels in patients with *Plasmodium falciparum* malarial infection. This suggests that Chit induction may reflect an immunological response.⁵

Another member of the chitinase family is the acid mammalian chitinase (AMCase), thought to play a role in inflammatory disorders. Elevated levels of AMCase were observed in lung tissue of asthmatic patients, suggesting a role for this enzyme in asthma pathophysiology.⁶

The kit assay is based on the enzymatic hydrolysis of chitinase substrates. This enzymatic hydrolysis releases 4-methylumbelliferone (4MU), which upon ionization in basic pH, can be measured fluorimetrically at an excitation wavelength of 360 nm and an emission wavelength of 450 nm.⁷ The use of fluorimetric substrates provides a very sensitive detection system.

The Chitinase Assay Kit provides all the reagents required for efficient and sensitive detection of chitinase activity in fungal and bacterial growth media, macrophage lysates, and purified enzyme preparations. In addition, the kit provides three different substrates for the detection of the various types of the chitinolytic activity:^{8,9}

4-Methylumbelliferyl N,N'-diacetyl- β -D-chitobioside - a substrate suitable for exochitinase activity detection (chitobiosidase activity)

4-Methylumbelliferyl N-acetyl- β -D-glucosaminide - a substrate suitable for exochitinase activity detection (β -N-acetylglucosaminidase activity)

4-Methylumbelliferyl β -D-N,N',N'-triacylchitotriose - a substrate suitable for endochitinase activity detection

The kit was tested on *Trichoderma viride*, as well as HeLa, Jurkat, CHO, NIH-3T3, U-937 mammalian cell lines, human macrophages, and rat lung, kidney, liver, and brain tissues.

Components

The kit is sufficient for 200 multiwell plate reactions (including standard curve).

Assay Buffer Catalog Number A8730	25 ml
4-Methylumbelliferyl N-acetyl- β -D-glucosaminide Catalog Number M2133	5 mg
4-Methylumbelliferyl β -D-N,N'-diacetylchitobioside hydrate Catalog Number M9763	5 mg
4-Methylumbelliferyl β -D-N,N',N'-triacetylchitotriose Catalog Number M5639	5 mg
Chitinase from <i>Trichoderma viride</i> Catalog Number C6242	1 mg
4-Methylumbelliferone Standard Solution, 50 mg/ml Catalog Number M3570	1 ml
Sodium Carbonate Catalog Number S2127	2 g
Dimethyl Sulfoxide (DMSO) Catalog Number D8418	1 ml

Reagents and Equipment Required but Not Provided.

- Dulbecco's Phosphate Buffered Saline (PBS, Catalog Number D8537)
- Ultrapure water
- A plate reader (Fluorimeter)
- Nunc-Immuno™ MicroWell™96 well black plates (flat bottom, Catalog Number P8741)
- Water bath, 37 °C
- For cell lysis: CelLytic™M Cell Lysis Reagent (Catalog Number C2978).
- For tissue extraction: Homogenizer and CelLytic MT Cell Lysis Reagent (Catalog Number C3228)

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Use ultrapure water (17 M Ω -cm or equivalent) for preparation of reagents.

Substrate Stock Solutions (20 mg/ml) – Add 0.25 ml of DMSO (Catalog Number D8418) to the contents (5 mg) of the appropriate substrate bottle:

4-Methylumbelliferyl N-acetyl- β -D-glucosaminide
Catalog Number M2133, or
4-Methylumbelliferyl N,N'-diacetylchitobioside hydrate,
Catalog Number M9763, or
4-Methylumbelliferyl β -D-N,N',N'-triacetylchitotriose
Catalog Number M5639

Vortex until dissolved.

Note: Substrates M2133 and M5639 do not dissolve easily in DMSO. It may take up to two hours of vortexing or incubation at 37 °C until they completely dissolve. Aliquot the substrate solutions and keep at -20 °C.

Substrate Working Solutions - Just before the assay dilute an aliquot of the Substrate Stock Solution (20 mg/ml) 40-fold with Assay Buffer to a concentration of 0.5 mg/ml. Mix by vortexing. Approximately 100 μ l of Substrate Working Solution are required for each test (each well).

Note: If required, the substrate concentration in the Substrate Working Solution can be reduced to a concentration of 0.2 mg/ml.

Chitinase Control Enzyme – Add 5 ml of PBS to the contents of the chitinase bottle (Product Code C6242) to give a final chitinase concentration of 0.2 mg/ml. Vortex until dissolved. The chitinase dissolves immediately to give a slightly hazy solution. For long term storage, store in working aliquots at -20 °C (stable for at least 3 months at -20 °C). Just before use, dilute an aliquot of the chitinase 200-fold with PBS and use 1-10 μ l of the diluted enzyme per assay.

Stop Solution (sodium carbonate solution) - Add 47.2 ml of ultrapure water to the contents of the sodium carbonate bottle (Catalog Number S2127) and mix well with a magnetic stirrer until completely dissolved. Store the Stop Solution at room temperature.

Standard Solution - Before performing the assay, dilute an aliquot of the Standard Solution in Stop Solution. Perform 100, 1,000, and 10,000-fold dilutions to final concentrations of 500 μ g/ml, 50 μ g/ml and 5 μ g/ml, respectively. The final volume of the diluted Standard Solution in the assay should not exceed 10 μ l.

Sample preparation- *Trichoderma viride* and *Streptomyces griseus* growth medium can be sampled directly from the growing culture (since the chitinase is secreted into the growth medium) and can be used in the assay after a brief centrifugation to remove the organisms and debris particles from the medium. Mammalian cell proteins can be extracted with CellLytic M Cell Lysis Reagent (Catalog Number C2978). Mammalian tissue proteins can be extracted with CellLytic MT Cell Lysis Reagent (Catalog Number C3228).

Storage/Stability

The kit is shipped on wet ice and storage at -20 °C is recommended. Upon arrival it is recommended to store the Sodium Carbonate (Catalog Number S2127) at room temperature. The Assay Buffer (Catalog Number A8730) can be stored at 2-8 °C. However, in order to avoid contamination of this solution it can be stored at -20 °C, especially after opening the bottle.

Procedure

The chitinase hydrolysis is performed in an acidic environment (pH ~5.0) at 37 °C. The enzymatic hydrolysis liberates 4-methylumbelliferone (4MU). The fluorescence of liberated 4MU is measured in alkaline pH using a fluorimeter with excitation at 360 nm and emission at 450 nm.

In order to quantitate the total chitinolytic activity, separate reactions should be run with the three substrates supplied in the kit. Profiling of the chitinolytic enzymes can be determined after separation of the chitinolytic enzymes by SDS-PAGE, using an agarose overlay containing fluorescent substrates.^{3,10} Note that in crude preparations there may be additive/synergist activity of different chitinases (i.e., 4-Methylumbelliferyl N,N'-diacetylchitobioside can be cleaved by β -N-acetylglucosaminidase and also chitobiosidase).

It is recommended to perform the assays in duplicates. For each substrate, perform a separate activity assay according to the following instructions.

1. Equilibrate the Substrate Working Solution(s) and the Standard Solution(s) to 37 °C by incubating for several minutes in a water bath.
2. Set the fluorimeter at an excitation wavelength of 360 nm and an emission wavelength of 450 nm.
3. Add the reaction components to 96 well plates according to Table 1 and mix using a horizontal shaker or by pipetting. Prepare the standard samples first (samples No. 4-8). Then, prepare the enzyme samples: add the substrate solution to the appropriate wells first and then add the enzyme (positive control or test sample).

Table 1.

Reaction Scheme for 96 Well Plate Assays

No.	Assay	Substrate Working Solution	Sample or Standard Solution	Assay Buffer
1	Blank*	100 μ l	—	—
2	Positive Control**	90-99 μ l	1-10 μ l of Chitinase Control Enzyme	—
3	Sample	90-99 μ l	1-10 μ l of sample	—
4	Standard***	—	—	100 μ l
5	10 ng/assay Standard	—	2 μ l of 5 μ g/ml	98 μ l
6	100 ng/assay Standard	—	2 μ l of 50 μ g/ml	98 μ l
7	500 ng/assay Standard	—	10 μ l of 50 μ g/ml	90 μ l
8	1000 ng/assay Standard	—	2 μ l of 500 μ g/ml	98 μ l

* A Blank reaction (Substrate Solution without enzyme) should be run to account for the spontaneous hydrolysis of the substrate during the incubation time.

** The volume of the enzyme can range between 1-10 μ l, depending on the reaction duration (i.e., for shorter time a higher enzyme concentration is required).

*** Standards should be run when activity calculations are required (samples No. 4-8). A standard curve may be determined with the five Standard samples indicated in the table. It is also possible to use only one standard concentration within the range of 10-1,000 ng and use the equation in the Calculation Section.

4. Incubate the plate for 30 minutes at 37 °C. If required, the incubation time for highly active samples can be reduced to 15 minutes. On the other hand, in order to detect low levels of enzyme activity, the incubation time can be extended to 1 hour.
5. Stop the reactions by adding 200 μ l of Stop Solution to each well.
6. Measure the fluorescence at an excitation wavelength of 360 nm and an emission wavelength of 450 nm no later than 30 minutes after ending the reaction.

Results

Calculation

Unit definition: One unit of chitinase activity will release 1 μ mole of 4-methylumbelliferone from the appropriate substrate per minute at pH 5.0 at 37 °C.

The chitinase activity can be calculated using a standard curve prepared from the fluorescence readings of the 5 standard solutions (see Table 1).

Alternatively, the chitinase activity can be calculated using only a single standard concentration. It is recommended to measure the fluorescence of 100 ng (1.9 nmole/ml) Standard and then use the following equation:

Units/ml =

$$\frac{(FLU_{\text{sample}} - FLU_{\text{blank}}) \times 1.9 \times 0.3 \times DF}{FLU_{\text{standard}} \times \text{time} \times V_{\text{enz}} \times 1000}$$

Where:

FLU_{sample} - fluorescence of the sample

FLU_{blank} - fluorescence of the Blank (containing only Substrate Working Solution)

0.3 - final reaction volume in milliliters after addition of the stop solution

DF - enzyme dilution factor

FLU_{standard} - fluorescence of the Standard Solution minus the fluorescence of the Standard Blank.

time - minutes

V_{enz} - volume of the sample in milliliter

References

1. Kasprzewska, A., Plant Chitinases - Regulation and Function. *Cell. Mol. Biol. Lett.*, **8**, 809-824 (2003).
2. Henrrisat, B., New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.*, **280**, 309-316 (1991).
3. Tronsmo, A., and Harman, G.E., Detection and quantification of N-acetyl-beta-D-glucosaminidase, chitobiosidase, and endochitinase in solutions and on gels. *Anal. Biochem.*, **208**, 74-79 (1993).
4. Sahai, A.S., and Manocha, M.S., Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction. *FEMS Microbiol. Rev.*, **11**, 317-338 (1993).
5. Malaguarnera, L. *et al.*, Interferon- γ tumor necrosis factor- α , and lipopolysaccharide promote chitotriosidase gene expression in human macrophages. *J. Clin. Lab. Anal.*, **19**, 128-132 (2005).
6. Donnelly, L.E., and Barnes, P.J., Acidic mammalian chitinase - a potential target for asthma therapy. *Trends Pharmacol. Sci.*, **25**, 509-511 (2004).
7. Huang, C.J. *et al.*, Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus* 28-9. *J. Biochem. Mol. Biol.* **38**, 82-88 (2005).
8. Renkema, G.H. *et al.*, Purification and characterization of human chitotriosidase, a novel member of the chitinase family of proteins. *J. Biol. Chem.*, **270**, 2198-2202 (1995).
9. Barone, R. *et al.*, Plasma chitotriosidase activity in patients with β -thalassemia. *Blood Cells Mol. Dis.*, **25**, 1-8 (1999).
10. Chernin, L.S. *et al.*, Chitinolytic Activity in *Chromobacterium violaceum*: Substrate analysis and regulation by quorum sensing. *J. Bacteriol.*, **180**, 4435-4441 (1998).

CellLytic is a trademark of Sigma-Aldrich Co. LLC.
Nunc-Immuno and MicroWell are trademarks of Thermo Fisher Scientific or its subsidiaries.

EM,EB,MAM 11/13-1