Fluorometric Assay Kit for Measuring Chitotriosidase Activity

CycLex Chitotriosidase Fluorometric Assay Kit

For 100 Assays

Cat# CY-1249

Intended Use

The MBL Research Product **CycLex Chitotriosidase Fluorometric Assay Kit** is used for the quantitative measurement of chitotriosidase activity in serum/plasma, culture supernatant of activated macrophages and other biological samples.

Applications for this kit include:

1) Measuring chitotriosidase activity in serum/plasma and other biological samples.
2) Evaluating the effects of pharmacological compounds on chitotriosidase activity.
3) Screening inhibitors or activators of chitotriosidase.

This assay kit is for research use only and not for use in diagnostic or therapeutic procedures.

Storage

- Upon receipt store all components at -70°C.
- Don't expose reagents to excessive light.

Introduction

Chitotriosidase was discovered in plasma of patients suffering from Gaucher's disease; it was found that the 1,000-fold-elevated enzyme originates from lipid-laden macrophages that accumulate in various tissues of Gaucher's patients (1). Chitotriosidase has subsequently been purified from the spleen of a Gaucher's patient and its cDNA was cloned from a human macrophage cDNA library (2, 3). This enzyme is a human chitinase member of family 18 glycosyl hydrolases (3–5), and has the capability to hydrolyze chitin. This enzyme is selectively expressed in activated tissue macrophages that accumulate in various tissues of several lysosomal diseases (6). Therefore its activity has been proposed as a biochemical marker of macrophage accumulation in Gaucher's disease (1, 7). In some other inherited lysosomal storage disorders, especially sphingolipidoses such as Niemann Pick, GM1-gangliosidosis, and Krabbe disease, which involve accumulation of different lipids, more modest elevations in plasma chitotriosidase have been noted (7). Chitotriosidase is the only biomarker identified up to date for the monitoring the efficacy of the extremely costly enzyme-replacement therapy of Gaucher patients and male Fabry patients (8).

Elevated levels of serum chitotriosidase were also found in disorders caused by the abnormal activation of immune system, including sarcoidosis (9) and atherosclerosis (10, 11). It has been shown that chitotriosidase activity was elevated up to 55-fold in extracts of atherosclerotic tissue, showing a clear connection between chitotriosidase expression and lipid-laden macrophages inside human atherosclerotic vessel wall (10). Human chitotriosidase also associates with pathogen-driven diseases, and in particular with fungal infections, suggesting the role of this enzyme in host defense against chitin-containing pathogens (12, 13). Other clinical data for instance show that chitotriosidase activity is raised in plasma of African children infected with acute Plasmodium falciparum malaria (14). Additional evidence for a role of chitotriosidase during immunological responses is the observation that the enzyme is shortly and acutely up-regulated both at the level of mRNA and activity following stimulation with prolactin, IFN-γ, TNF α and LPS, but not with IL-10 (15, 16).

In the blood stream, tissue macrophages largely secrete newly synthesized 50-kDa chitotriosidase, but about one-third is directly routed to lysosomes and proteolytically processed to the 39-kDa unit that remains catalytically active (17). A common chitotriosidase gene polymorphism leads to a null allele and therefore a defective enzyme activity. In white populations, 30% to 40% of individuals are carriers of this abnormal chitotriosidase allele and approximately 6% are homozygous (1, 18).

Principle of the Assay

The **CycLex Chitotriosidase Fluorometric Assay Kit** is based on an exclusive fluorescence substrate, 4-Methylumbelliferyl-β-D-N,N',N"-triacetylchitotriose. This homogenous assay kit is sensitive and convenient.

Summary of Procedure

Dispense 5 µL of sample or Recombinant chitotriosidase in the well.

↓

Add 95 µL of 1X Fluoro-Substrate solution.

↓

Measure velocity of fluorescence intensity with excitation at 340-380 nm and emission at 440-460 nm for 30-60 minutes at 30°C.

Materials Provided

Each kit contains

Materials	Quantity	Storage
#1. 10X Chitotriosidase Assay Buffer	**1.0 mL x 2**	**Below -20°C**
#2. 50X Fluoro-Substrate (1 mM 4 MU-chitotrioside *)	**220 µL x 1**	**Below -20°C**
#3. Recombinant Chitotriosidase ** (ca. 5 µg/ml)	**50 µL x 1**	**-70°C**
#4. 4-Methylumbelliferone standard (100 µM)	**200 µL x 1**	**Below -20°C**
Instruction manual	**1**	**Room temp.**

* 4-Methylumbelliferyl-β-D-N,N',N"-triacetylchitotriose
** Recombinant human chitotriosidase expressed in HEK293 cells.

Materials Required but not Provided

- **(Optional) Allosamidin:** Chitinase inhibitor, available from 2A PharmaChem, Cat# 2A-300198 (CAS number 103782-08-7). Make 500 µM solution in water.
- **Microplate suitable for use with a fluorometric plate reader** (black microplates provide better signal to noise ratio)
- **Microplate reading fluorometer:** capable of excitation at a wavelength in the range 340-380 nm and detection of emitted light in the range 440-460 nm
- **Pipettors:** 2-20 µL, 20-200 µL and 200-1,000 µL precision pipettors with disposable tips.
- **Multi-channel pipette**
- **Microplate shaker**
- **Deionized water of the highest quality**
- **Reagent reservoirs**
- **(Optional) Stop Solution:** Add 23.6 ml of deionized water to 2 g of sodium carbonate (Cat#: S2127) and mix well until completely dissolved. Store the Stop Solution (c.a. 1 M Na_2CO_3) at room temperature.

Precautions and Recommendations

- **Although we suggest to conduct experiments as outlined below, the optimal experimental conditions will vary depending on the parameters being investigated, and must be determined by the individual user.**
- Please avoid repeated freezing and thawing of the Recombinant chitotriosidase in this kit. There is a possibility that the enzyme activity may be inactivated. Aliquot to 10 µL and store at –70°C.
- Do not use kit components beyond the indicated kit expiration date.
- Rinse all detergent residue from glassware.
- Use deionized water of the highest quality (ddH_2O).
- Do not mix reagents from different kits.
- Do not mouth pipette or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- **Biological samples may be contaminated with infectious agents. Do not ingest, expose to open wounds or breathe aerosols. Wear protective gloves and dispose of biological samples properly.**

Detailed Protocol

The MBL Research Product **CycLex Chitotriosidase Fluorometric Assay Kit** is provided with concentrated reagents. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

I. Preparation of Reagents

Thaw the reagents at room temperature except **#3. Recombinant Chitotriosidase** and keep all reagents on ice until use. Use them only after they are completely thawed and mixed.

A. Assay Buffer (Quantity required: 60 µL/assay)
- Dilute the **#1. 10X Assay Buffer** (provided) 1 : 10 in distilled (deionized) water.
- Unused buffer should be stored at -20°C.

B. Reaction Buffer (Quantity required: 100 µL/assay)
- Mix following reagents well.
- Discard any unused "B. Reaction Buffer".

Assay reagents	1 assay	8 assays	16 assays	32 assays	48 assays
#1. 10X Assay Buffer	**10 µL**	**80 µL**	**160 µL**	**320 µL**	**480 µL**
#2. 50X Fluoro-Substrate	**2 µL**	**16 µL**	**32 µL**	**64 µL**	**96 µL**
Distilled water	**88 µL**	**704 µL**	**1,408 µL**	**2,816 µL**	**4,224 µL**
Total Volume	**100 µL**	**800 µL**	**1,600 µL**	**3,200 µL**	**4,800 µL**

C. Diluted Chitotriosidase (Quantity required: 10 µL/assay)
- Dilute the **3. Recombinant Chitotriosidase** (provided) 1 : 10 in "A. Assay Buffer".
- Discard any unused "C. Diluted Recombinant Chitotriosidase".

Assay reagents	1 assay	8 assays	16 assays	32 assays	48 assays
A. Assay Buffer	**9 µL**	**45 µL**	**81 µL**	**153 µL**	**225 µL**
#3. Recombinant Chitotriosidase	**1 µL**	**5 µL**	**9 µL**	**17 µL**	**25 µL**
Total Volume	**10 µL**	**50 µL**	**90 µL**	**170 µL**	**250 µL**

II. Assay Procedure of Chitotriosidase Activity

1. Following Table 1 below, first, add "**Test Sample**" or "**C. Diluted Chitotriosidase**" or "**A. Assay Buffer**" to each well of a microplate.

2. Finally, initiate the reaction by adding **95 µL** of "**B. Reaction Buffer**" to each well and mixing thoroughly.

Table 1: Reaction Mixture of Chitotriosidase Activity Assay

Assay Reagents	Test Assay	Positive Control	No enzyme Control
Test Sample *	**5 µL**	**-**	**-**
C. Diluted Chitotriosidase **	**-**	**5 µL**	**-**
A. Assay Buffer **	**-**	**-**	**5 µL**
B. Reaction Buffer **	**95 µL**	**95 µL**	**95 µL**
Total Volume	**100 µL**	**100 µL**	**100 µL**

** Test sample: Human serum/plasma sample or culture supernatant of activated macrophages. If sample might contain high concentration of chitotriosidase such as serum/plasma from Gaucher's disease patients, samples should be diluted 25-50 times in "A. Assay Buffer" before incubation.*

*** See the "I. Preparation of Reagents" of this section above.*

3. Read fluorescence intensity for 30-60 minutes or desired length of time at 2 to 5 minutes intervals using a microplate fluorometer with excitation at 340-380 nm and emission at 440-460 nm at 30°C *.

** Any assay temperature from room temperature to 37°C may be used.*

4. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Alternatively

3'. After incubation at 30°C for 20-30 minutes, stop the reaction by addition of **100 µL** of **Stop Solution** *.

** Not provided in this kit. See the Section "Materials Required but not Provided" above.*

4'. Read fluorescence on a microplate fluorometer with excitation at 340-380 nm and emission at 440-460 nm.

III. Assay Procedure for Inhibitor Screening

In order to estimate the inhibitory effect on chitotriosidase activity by test compounds correctly, it is necessary to conduct the control experiment of "**Vehicle Control**" at least once for every experiment and "**Inhibitor Control**" at least once for the first experiment, in addition to "**Test Assay**" as indicated in the Table 2 (below). When test chemicals cause an inhibitory effect on Chitotriosidase activity, the level of increase of fluorescence intensity is weakened as compared with "**Vehicle Control**". The increase in fluorescence intensity is not observed in "**Inhibitor Control**".

1. Following Table 2 below, first, add "**C. Diluted Chitotriosidase**" or "**A. Assay Buffer**" to microplate wells. Second, add "**Test Compound**" or "**Vehicle of Test Compound**" or "**10X Allosamidin**" to each well of the microplate and mix well*.

   ** Optional: For best accuracy, it is advisable to pre-incubate the plate for 5-10 minutes. at assay temperature.*

2. Initiate reactions by adding **90 µL** of "**B. Reaction Buffer**" to each well and mixing thoroughly.

Table 2: Reaction Mixture for Inhibitor Screening

Assay Reagents	Test Assay	Vehicle Control	Inhibitor Control	No Enzyme Control
C. Diluted Chitotriosidase *	**5 µL**	**5 µL**	**5 µL**	**-**
A. Assay Buffer *	**-**	**-**	**-**	**5 µL**
Test Compound	**5 µL**	**-**	**-**	**-**
Vehicle of Test Compound	**-**	**5 µL**	**-**	**5 µL**
10X Allosamidin (500 µM) **	**-**	**-**	**5 µL**	**-**
B. Reaction Buffer *	**90 µL**	**90 µL**	**90 µL**	**90 µL**
Total Volume	**100 µL**	**100 µL**	**100 µL**	**100 µL**

** See the "I. Preparation of Reagents" in this section above.*
*** See the section "Materials Required but not Provided" above.*

3. Read fluorescence intensity for 30-60 minutes or desired length of time at 2 to 5 minutes intervals using microtiter plate fluorometer with excitation at 340-380 nm and emission at 440-460 nm at 30°C*.

   ** Any assay temperature from room temperature to 37°C may be used.*

4. Measure and calculate the rate of reaction while the reaction velocity remains constant.

Caution and Significance

- *All assays should be assayed in duplicate.*
- *Use of a microplate shaker is recommended for complete mixing.*
- *If the test compounds or samples themselves emit fluorescence at excitation wavelength: 340-380 nm and fluorescence wavelength: 440-460 nm, the test assay cannot be evaluated correctly.*

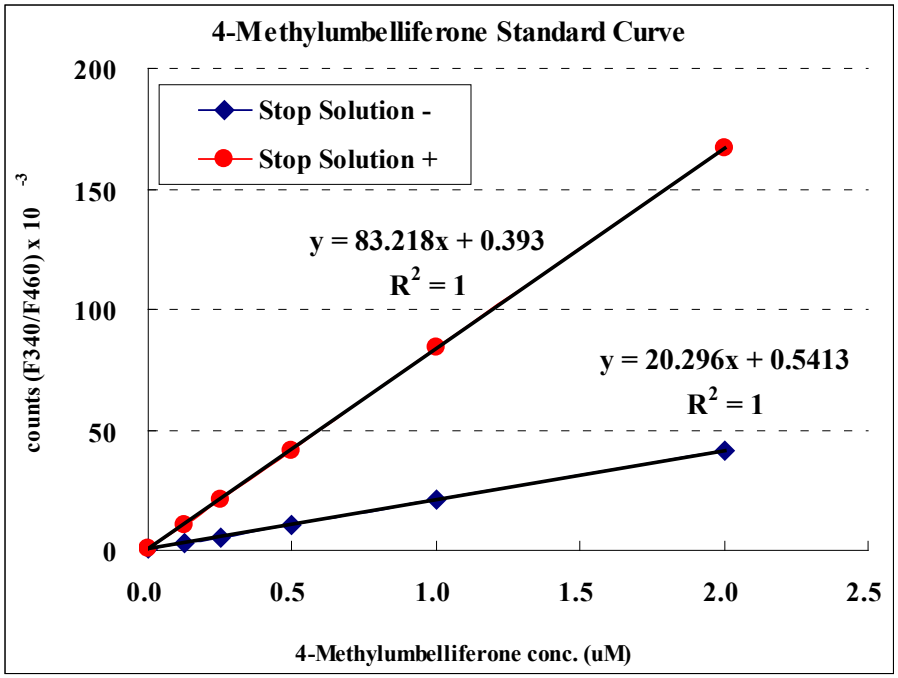
IV. Determine microplate reader conversion factor for 4-Methylumbelliferone

The exact 4-Methylumbelliferone concentration range that will be useful for preparing a standard curve will vary depending on the fluorometer model, the gain setting, and the exact excitation and emission wavelengths used. Please dilute the "**#4. 4-Methylumbelliferone standard**" (provided; 100 µM) to 1.0 µM as the highest standard and make a 4-fold serial dilution with Assay Buffer and then measuring the fluorescence of 100 µl in a microtiter plate fluorometer with excitation at 340-380 nm and emission at 440-460 nm. The estimate of µM/RFU obtained with this measurement, together with the observed range of values obtained in the enzyme assays, can then be used to plan an appropriate series of dilutions for a standard curve. The slope of the standard curve can then be used as the µM/RFU conversion factor.

When Stop Solution* is added to samples after enzyme reaction**, it also should be added to this 4-Methylumbelliferone standard curve. The addition of Stop Solution will give 4-Methylumbelliferone approximately a 4-hold higher fluorescence intensity than no addition.

** See the section "Materials Required but not Provided" above.*
*** See "Alternatively" of "II. Chitotriosidase Activity Assay Procedure" in this section above.*

Typical 4-Methylumbelliferone Standard Curve



Evaluation of Results

I. Analysis of Kinetics

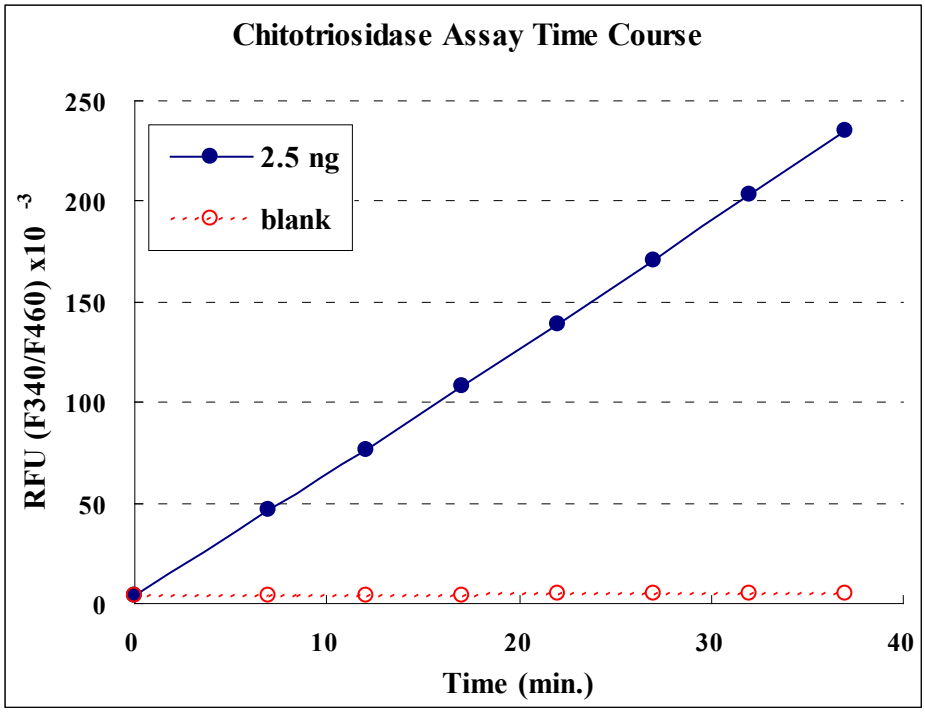
Time course curve

1. Run reactions as described in the *Detailed Protocol*.
2. Subtract fluorescence intensity at time 0 from all reaction time points.
3. Plot fluorescence intensity at 440-460 nm versus reaction time.
4. Determine the reaction time range in which the increase in fluorescence intensity at 440-460 nm is linear.
5. Calculate activity:

$$\text{Activity (reaction velocity)} = \frac{\text{Fluorescence Intensity of Test Assay}}{\text{Reaction time (min.)}}$$

NOTE: Usually, the linear range is from 0 to 30 minutes. This value is variable depending on reaction conditions and storage/handling of the Recombinant Chitotriosidase. Decreasing the amount of Recombinant Chitotriosidase in the assay may help to lengthen the time range.

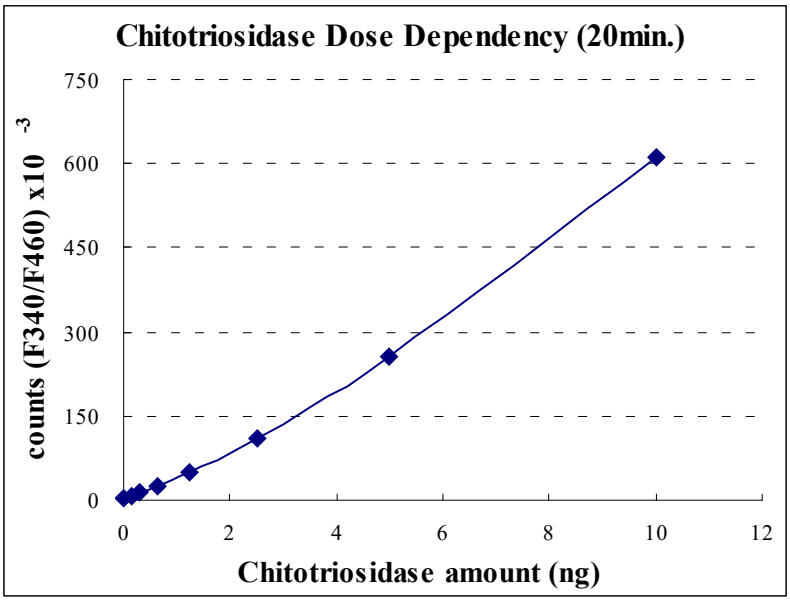
Fig.1 Typical Time Course Curve



Chitotriosidase Standard Curve and % Activity

1. Make serial dilutions of #3. X10 diluted Recombinant Chitotriosidase with #1. 1X Assay Buffer (ex. 100%, 50%, 25%, 12.5%, 6.25%, 3.13% and 0%).
2. Run reactions with Vehicle and serial dilutions of Recombinant Chitotriosidase as described in the Detailed Protocol.
3. Plot standard curve data as fluorescence intensity at 460 nm versus dose of Chitotriosidase (ng/assay).
4. Obtain a line-fit to the data using appropriate calculations.
5. Use the slope and Y-intercept to calculate the amount of Chitotriosidase activity for the experimental data.

Fig.2 Typical Dose Dependency Curve



II. Analysis of Inhibitor Effect

% Intensity

1. Run reactions with test compounds and Vehicle as described in the **Detailed Protocol**.
2. Subtract fluorescence intensity of "No Enzyme Control" from all other experimental samples (Test Assay, Vehicle Control and Inhibitor Control).
3. Calculate the % Intensity:

$$\%\ \text{Intensity} = \frac{\text{Reaction velocity of Test Assay}}{\text{Reaction velocity of Vehicle Control}} \text{ X } 100$$

NOTE: This % Intensity is a rough value of enzyme activity or inhibition. For greater accuracy, plot a standard curve of Chitotriosidase for each new set of reactions and estimate the % Activity (see above section "Chitotriosidase Standard Curve and % Activity)".

Troubleshooting

1. All assays should be run in duplicate using the protocol described in the *Detailed Protocol*. Incubation times or temperatures significantly different from those specified may give erroneous results.

2. The reaction curve is nearly a straight line if the kinetics of the assay is of the first order. Variations in the protocol can lead to non-linearity of the curve, as can assay kinetics of other than first order. For a non-linear curve, point to point or quadratic curve fit methods should be used.

3. Poor duplicates, accompanied by elevated values for wells containing no sample, indicate inaccurate dispensing of assay reagents. If all instructions in the *Detailed Protocol* were followed accurately, such results indicate a need for multi-channel pipette maintenance.

Reagent Stability

All of the reagents included in the **CycLex Chitotriosidase Fluorometric Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, all kit reagents should be stored at -70°C. Avoid repeated freeze-thaw cycles of "**#3. Recombinant Chitotriosidase**". After use, return kit reagents to -70°C as soon as possible.

References

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