Quantitative test kit for aidic mammalian chitinase activity

CycLex Acidic Mammalian Chitinase Fluorometric Assay Kit

For 100 Assays

Cat# CY-1248

Intended Use

The CycLex Research Product **CycLex Acidic Mammalian Chitinase Fluorometric Assay Kit** is used for the quantitative measurement of acidic mammalian chitinase (AMCase) activity in a culture supernatant of stimulated pulmonary epithelial cells, several tissue extracts and other biological samples.

Applications for this kit include:

1) Measuring AMCase activity in tissue extracts and other biological samples.
2) Evaluating the effects of pharmacological compounds on AMCase activity.
3) Screening inhibitors or activators of AMCase.

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

Storage

- Upon receipt store "**③Recombinant AMCase**" at -70°C and all other components below -20°C.
- Don't expose reagents to excessive light.

Introduction

The glycosyl hydrolase family is quite large, with over 100 families. Acidic mammalian chitinase (AMCase) and chitotriosidase-1 are enzymatically active true chitinases in a member of the glycosyl hydrolase-18 family, identified by EC 3.2.1.14. In mammals, only these two enzymes cleave N-acetyl-beta-D-glucosamine (1->4)-beta linkages in chitodextrins and chitin, the second most abundant biopolymer that can be found in the cell walls of fungi, micro-filarial sheaths of helminths, and exoskeletons of insects and crustaceans (1). Other member included in this family are chitinase-like molecules, e.g. YKL-40/Chitinase-3-like-1 and YKL-39/chitinase-3-like-2, that are thought to lack the ability to hydrolyze the chitin linkages, but retain chitin-binding activity (2, 3) and act more like connective tissue proteins, and immediate early gene products.

AMCase was cloned as an acid-stable active enzyme and is highly expressed in the gastrointestinal tract, and to a lesser extent, in the lung and alveolar macrophages in both humans and mice (4, 5), with an optimum activity at pH 4–5 (6). AMCase as well as chitotriosidase-1 is 50 kDa proteins, consisting of a 39 kDa catalytic region separated from a chitin-binding domain by a hinge region (7, 8). They both show considerable homology to chitinases from lower organisms.

AMCase is one of the important proteins involved in Th2-mediated inflammation and has been implicated in asthma and allergic diseases by affecting the IL-13 downstream pathway (9). Inhibition of AMCase results in decreased airway inflammation and airway hyper-responsiveness in a murine asthmatic model, suggesting that the AMCase activity is a part of the mechanism of Th2 cytokine-driven inflammatory response in asthma (9).

Principle of the Assay

The **CycLex Acidic Mammalian Chitinase 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

Make and dispense 80 µL of mixture of Fluoro-Substrate and AMCase Assay Buffer (pH 2.0) or Chitinase Assay Buffer (pH 4.0) in the wells.

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Dispense 10 µL of buffers of Test Samples or Recombinant AMCase in the wells.

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Add 10 µL of Test Samples or Recombinant AMCase in the wells.

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

Materials Provided

Components of Kit

Materials	Quantity	Storage
①10X AMCase Assay Buffer (pH 2.0)	1.0 mL x 2	Below -20°C
②10X Chitinase Assay Buffer (pH 4.0)	1.0 mL x 2	Below -20°C
③10X Fluoro-Substrate (200 µM 4-MU-chitotrioside*)	1.0 mL x 1	Below -20°C
④10X Recombinant AMCase **	1.0 mL x 1	-70°C
⑤Enzyme Dilution Buffer	1.0 mL x 2	Below -20°C
⑥4-Methylumbelliferone Standard (100 µM)	200 µL x 1	Below -20°C
Instruction Manual	1	room temp.

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

Materials Required but not Provided

- **Bisdionin F:** Bisdionin F is available from EMD Millipore; cat#. 112252.
- **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 200-1000 µL precision pipettors with disposable tips.
- **Multi-channel pipette**
- **Microplate shaker**
- **Deionized water of the highest quality**
- **Reagent reservoirs**
- **Stop Solution (Optional):** 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

- Please avoid repeated freezing and thawing of the Recombinant AMCase 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

All of the reagents included in this kit are supplied in a ready-to-use form. Disposable pipette tips and reagent troughs should be used for all liquid transfers to avoid cross-contamination of reagents or samples.

Assay Procedure of AMCase Activity in Tissue Extracts and Other Biological Samples

"**①10X AMCase Assay Buffer (pH 2.0)**" is recommend for measuring AMCase activity in several tissue extracts and other biological samples because the buffer makes specific pH condition for AMCase. Refer to Fig.1 and Fig.2 in Section "Evaluation of Results".

1. Following Table.1 below, first, add "**Distilled water**", "**①10X AMCase Assay Buffer (pH 2.0)**" and "**③10X Fluoro-Substrate**" to microtiter plate wells. Second, add "**Test Sample**" or "**Buffer of Test Sample**" to each well of the microtiter plate and mix well.

Table.1: Reaction mixture of AMCase activity assay

Assay reagents	Test Assay	Positive Control	No Enzyme Control
Distilled water (ddH_2O)	**60 µL**	**60 µL**	**60 µL**
①10X AMCase Assay Buffer (pH 2.0)	**10 µL**	**10 µL**	**10 µL**
③10X Fluoro-Substrate	**10 µL**	**10 µL**	**10 µL**
Test Sample*	**10 µL**	**-**	**-**
Buffer of Test Sample	**-**	**10 µL**	**10 µL**
④10X Recombinant AMCase	**-**	**10 µL**	**-**
⑤Enzyme Dilution Buffer	**10 µL**	**-**	**10 µL**
Total volume of the mixture	**100 µL**	**100 µL**	**100 µL**

** Test Samples: e.g. tissue extracts or culture supernatants of stimulated pulmonary epithelial cells. If samples might contain high concentration of AMCase, they should be diluted 5-25 fold with ⑤ Enzyme Dilution Buffer.*

2. Add **10 µL** of **Buffer of Test Sample**" or "**⑤Enzyme Dilution Buffer**" to each well and mix well.

3. Initiate reactions by adding **10 µL** of "**Test Sample**" or "**④10X Recombinant AMCase**" to each well and mixing thoroughly.

4. Read fluorescence intensity for 30-60 min or desired length of time at 2 to 5 minute 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.*

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

Alternatively (for Endpoint Assay)

4'. After incubation at 30°C for 50-60 min, the reaction was stopped by addition of **100 µL** of "**Stop**

Solution"*. Fluorescence was read on a microplate fluorometer with excitation at 340-380 nm and emission at 440-460 nm.

** Not provided in this kit. See page 3, Section "Materials Required but not Provided".*

Assay Procedure for Screening Inhibitors or Activators of AMCase

In order to estimate the inhibitory (or activatory) effect on AMCase 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 compounds cause an inhibitory effect on AMCase 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**".

"**②10X Chitinase Assay Buffer (pH 4.0)**" is recommend for screening inhibitors or activators of AMCase or for evaluating the effects of pharmacological compounds on AMCase activity.

1. Following Table.2 below, add "**Distilled water**", "**②10X Chitinase Assay Buffer (pH 4.0)**" and "**③ 10X Fluoro-Substrate**" to microtiter plate wells.

Table.2: Reaction mixture for screening inhibitors or activators

Assay reagents	Test Assay	Vehicle Control	Inhibitor Control	No Enzyme Control
Distilled water (ddH_2O)	**60 µL**	**60 µL**	**60 µL**	**60 µL**
②10X Chitinase Assay Buffer (pH 4.0)	**10 µL**	**10 µL**	**10 µL**	**10 µL**
③10X Fluoro-Substrate	**10 µL**	**10 µL**	**10 µL**	**10 µL**
Test compound *	**10 µL**	**-**	**-**	**-**
Vehicle of Test compound	**-**	**10 µL**	**-**	**10 µL**
10X Bisdionin F (50 µM) *	**-**	**-**	**10 µL**	**-**
④10X Recombinant AMCase	**10 µL**	**10 µL**	**10 µL**	**-**
⑤Enzyme Dilution Buffer	**-**	**-**	**-**	**10 µL**
Total volume of the mixture	**100 µL**	**100 µL**	**100 µL**	**100 µL**

** Not provided in this kit. See page 3, Section "Materials Required but not Provided".*

2. Add "**Test Compound**" or "**Vehicle of Test Compound**" or "**10X Bisdionin F**" to each well of the microtiter plate and mix well*.

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

3. Initiate reactions by adding **10 µL** of "**④10X Recombinant AMCase**" or "⑤Enzyme Dilution Buffer" to each well and mixing thoroughly.

4. Read fluorescence intensity for 30-60 min or desired length of time at 2 to 5 minute 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.*

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

Caution and Significance

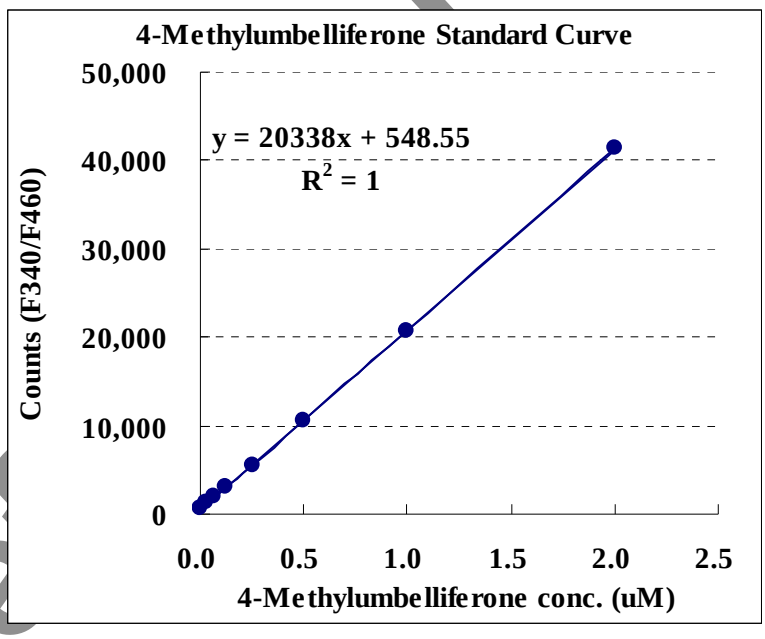
- All assays should be done in duplicate.
- Use of a microplate shaker is recommended for complete mixing.
- If the test compounds or samples themselves emit fluorescence at excitation wavelength: 340-380 nm and fluorescence wavelength: 440-460 nm, the test assay cannot be evaluated correctly.

Determination of microplate reader conversion factor for 4-MU fluorophore

The exact 4-Methylumbelliferone (4-MU) 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 "**⑥4-Methylumbelliferone Standard (100 µM)**" to 2.0 µM as the highest standard and make 4-fold serial dilution with 1X Assay Buffer*, and then measure 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.

** Dilute ①10X AMCase Assay Buffer (pH 2.0) or ②10X Chitinase Assay Buffer (pH 4.0) 10-fold with distilled water (ddH2O).*

Typical 4-Methylumbelliferone Standard Curve



Evaluation of Results

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 min (at pH 4.0). This value is variable depending on reaction conditions and storage/handling of the Recombinant AMCase. Decreasing the amount of Recombinant AMCase in the assay may help to lengthen the time range.

Fig.1 Typical time course curve and specificity of AMCase and chitotriosidase activities in AMCase Assay Buffer (pH 2.0)

3 ng of Recombinant AMCase (Cat# CY-E1248) was used per assay.
2.5 ng of Recombinant Chitotriosidase (Cat# CY-E1249) was used per assay.

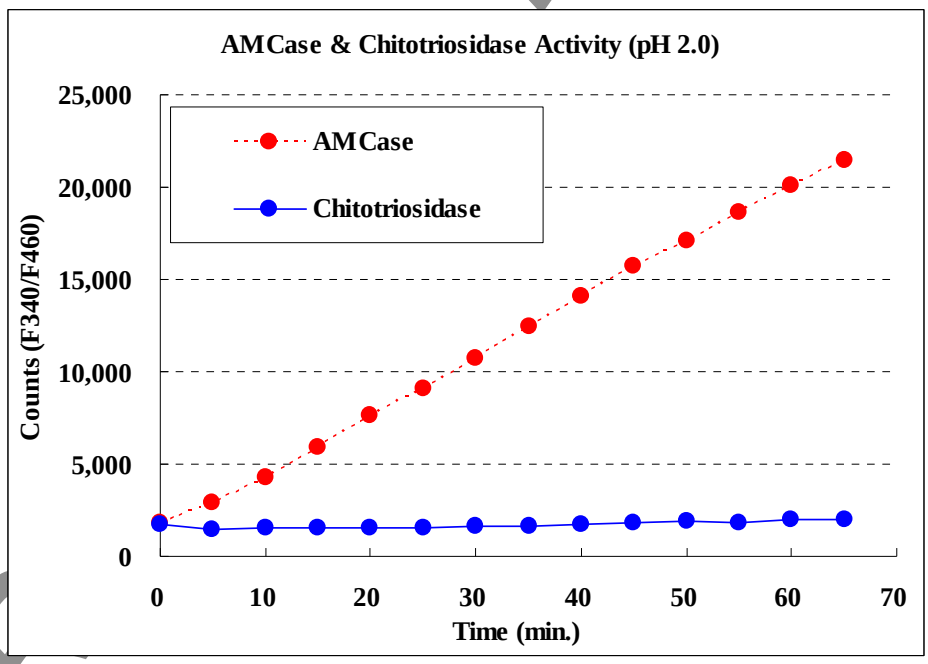
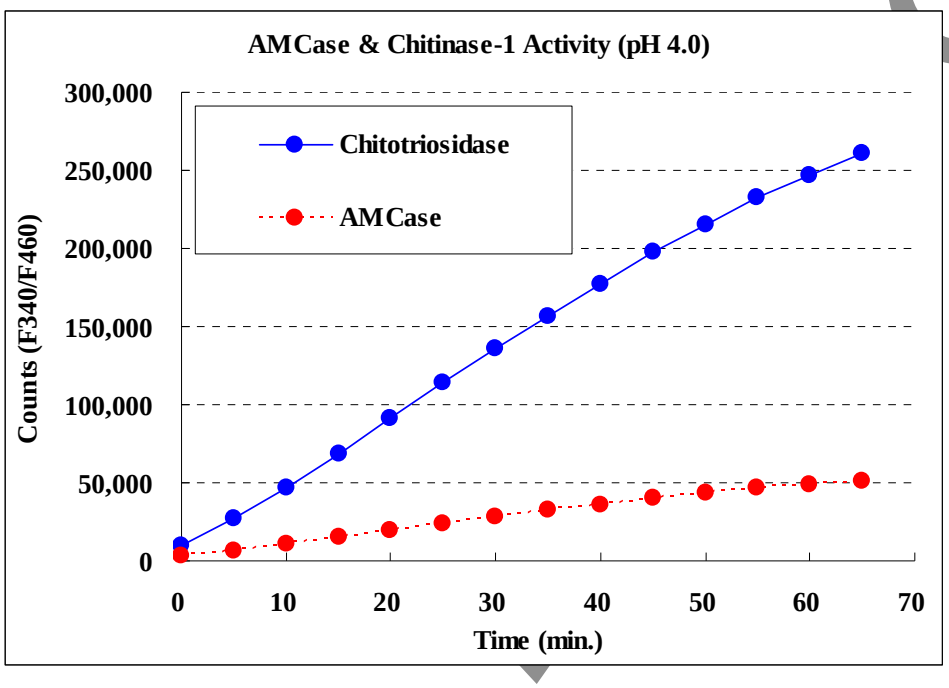


Fig.2 Typical time course curve and specificity of AMCase and chitotriosidase activities in Chitinase Assay Buffer (pH 4.0)

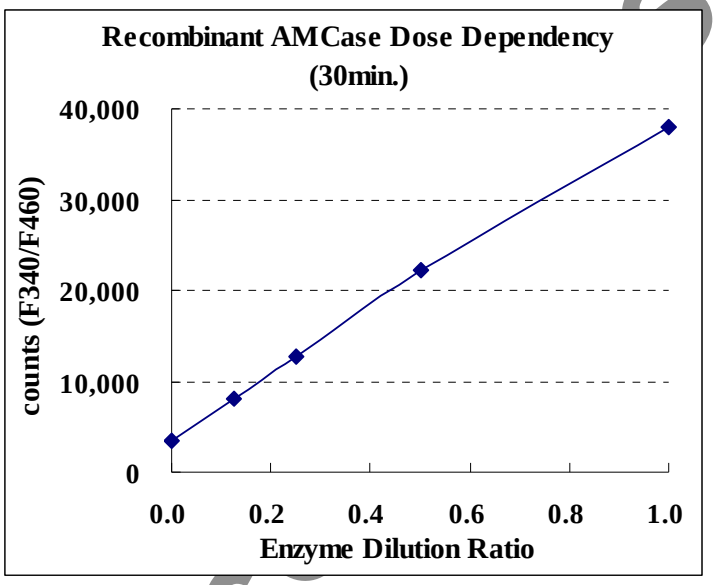
3 ng of Recombinant AMCase (Cat# CY-E1248) was used per assay.
2.5 ng of Recombinant Chitotriosidase (Cat# CY-E1249) was used per assay.



AMCase Standard Curve and % Activity

1. Make serial dilutions of ④Recombinant AMCase with ⑤Enzyme Dilution Buffer (e.g. 100%, 50%, 25%, 12.5%, 6.25 %, 3.13% and 0%).
2. Run reactions with Vehicle and serial dilutions of Recombinant AMCase as described in the Detailed Protocol.
3. Plot standard curve data as fluorescence intensity at 460 nm versus dose of AMCase (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 AMCase activity for the experimental data.

Fig.3 Typical dose dependency curve of AMCase in Chitinase Assay Buffer (pH 4.0)



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 AMCase for each new set of reactions and estimate the % Activity (see above section "AMCase 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 Acidic Mammalian Chitinase Fluorometric Assay Kit** have been tested for stability. Reagents should not be used beyond the stated expiration date. Upon receipt, "**③Recombinant AMCase**" should be stored at -70°C and all other components should be stored below -20°C. Avoid repeated freeze-thaw cycles of "**③Recombinant AMCase**".

For research use only, not for use in human, diagnostic or therapeutic procedures

References

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Related Products

*** CycLex Acidic Mammalian Chitinase Fluorometric Assay Kit: Cat# CY-1248**
*** CycLex Chitotriosidase Fluorometric Assay Kit: Cat# CY-1249**
*** CircuLex Human Chitotriosidase ELISA Kit: Cat# CY-8074**

*** CycLex Human Acidic Mammalian Chitinase: Cat# CY-E1248**
*** CycLex Human Chitotriosidase: Cat# CY-E1249**

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