Chitinase Assay from Cultured Bone Marrow Derived Macrophages

Danielle Worth, J. Philip Nance, Emma H. Wilson*

Division of Biomedical Sciences, University of California, Riverside, USA
*For correspondence: emmaw@ucr.edu

[Abstract] Chitinases are chitin-degrading enzymes. Chitinases play essential roles in combating chitin-containing pathogens as well as established roles in asthmatic inflammation. This assay is designed to detect chitinase activity in macrophage cell lysates. The chitin substrate is labeled with 4-methylumbelliferone. Hydrolysis of chitin releases 4-methylumbelliferone, and is measured fluorometrically to determine chitinase activity.

Materials and Reagents

1. Cells to be analyzed. *e.g.* Bone Marrow-derived macrophages from C57BL/6 mice
2. L-cell
3. cDMEM/F12
4. 4-Mehtylumbelliferyl labeled substrates:
   a. 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide (exochitinase activity; β–N-acetylglucosaminidase activity) (Sigma-Aldrich, catalog number: M2133)
   b. 4-Methylumbelliferyl N,N′-diacetyl-β-D-chitobioside (exochitinase activity; chitobiosidase activity) (Sigma-Aldrich, catalog number: M9763)
   c. 4-Methylumbelliferyl β-D-N,N′,N″-triacetylchitotriose (endochitinase activity) (Sigma-Aldrich, catalog number: M5639)
5. Chitinase from Trichoderma viride (Sigma-Aldrich, catalog number: C6242)
6. Methylumbelliferone Standard (50 mg/ml) (Sigma-Aldrich, catalog number: M1381)
7. PBS
8. DMSO
9. Plate reader
10. Diabasic Sodium Phosphate
11. Citric Acid
12. Glycine
13. DTT
14. Assay buffer (see Recipes)
15. Stop buffer (see Recipes)
16. Protein lysis buffer (see Recipes)

17. Substrate Stock (see Recipes)
18. Positive control (see Recipes)

Equipment

1. Flat bottom black 96-well plates
2. Fluorescent plate reader (Molecular Devices)

Procedure

1. Cells to be analyzed. We have used Bone Marrow-derived macrophages from C57BL/6 mice.
   a. In brief, bone marrow is flushed from tibias and femurs of 6-8 week old mice with 10 ml complete DMEM/F12 supplemented with 20% L-cell supernatant (day 0).
   b. Bone marrow cells are plated in non tissue culture treated 10 cm petri dishes ~10 ml cells per dish. After 4 days, add 10 ml additional media.
   c. Cells are harvested by gentle scraping on day 7.
   d. Macrophages are plated at 50,000 cells/well in 96 well plates in cDMEM/F12 in 10% L-cell supernatant and allowed to rest for 3 days.
   e. On day 10, media is changed to cDMEM/F12 without L-cell supernatant and rested overnight.
   f. Cells are ready for use on day 11.
2. Aspirate media in each well from the culture plates, and add lysis buffer to each (50 µl for 96 well plate, and 200 µl for a 24 well plate). Place the plate on the rocker for 15 min at room temperature.
3. Dilute an aliquot of 4-Methylumbelliferyl substrate stock solution 40-fold in assay buffer, such that the final concentration of the substrate is 0.5 mg/ml. This will be termed the “working solution”.
   a. Approximately 100 µl of working solution will be needed per sample. Allow solution to equilibrate in 37 °C water bath.
   b. For each form of chitinase being tested there is a unique substrate. Separate assay plates will needed for each substrate.
4. Prepare Methylumbelliferone standards by diluting the top Methylumbelliferone standard 1:100 (500 µg/ml), 1:1,000 (50 µg/ml), and 1:10,000 (5 µg/ml) in stop buffer.
   a. For best resolution, add 2 µl top Methylumbelliferone standard to 198 µl of stop buffer for the 1:100 dilution, followed by a 1:10 dilution series.

b. Samples will be diluted further in the assay plate to yield 1,000 ng, 500 ng, 100 ng, and 10 ng (Figure 1).

5. Load the standard wells in triplicate to the flat bottom black 96-well plate as demonstrated in Figure 1.

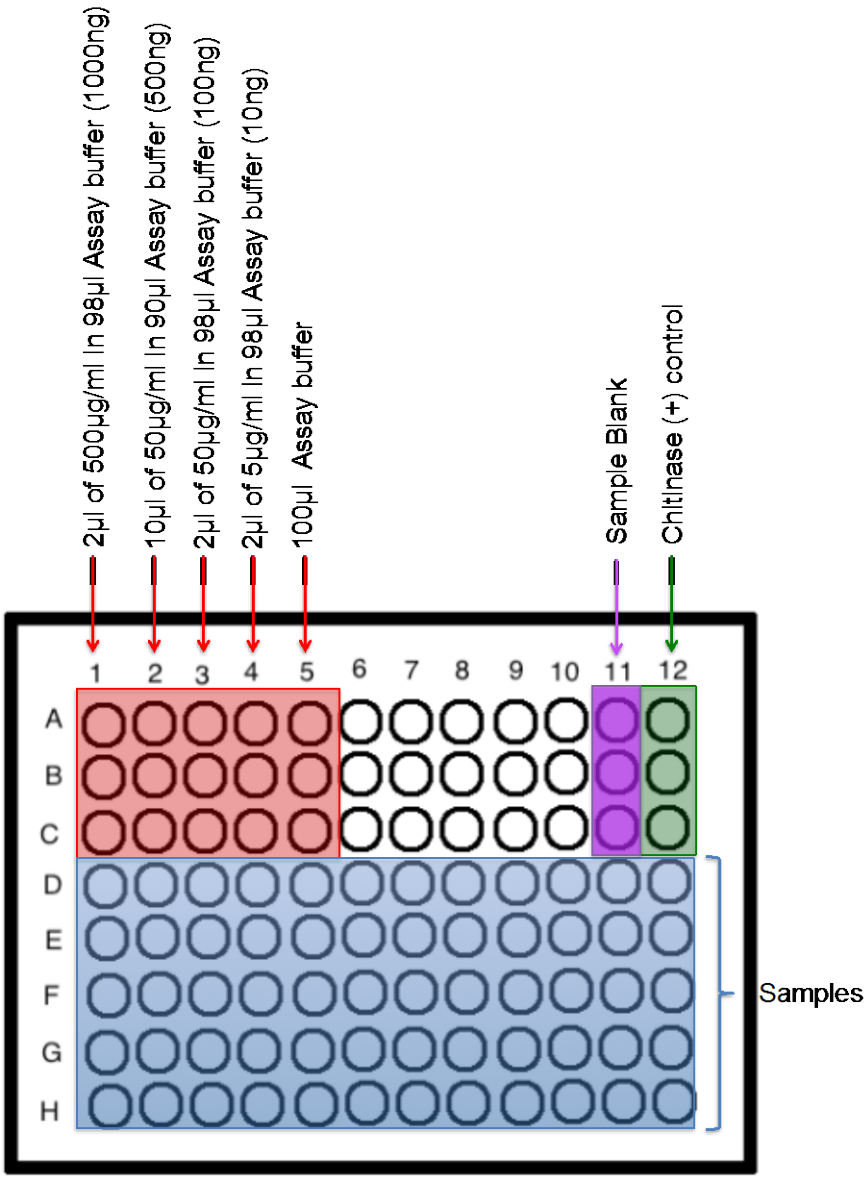

Figure 1. Plate set-up for chitinase assay. Red wells indicate 4-Methylumbelliferone standards, Purple indicates the sample blank (Working Solution), Green represents the positive control, and blue wells indicate sample wells.

6. Dilute the chitinase positive control 1:200 in PBS to yield a final concentration of 1 µg/ml and load positive control wells with 10 µl chitinase and 90 µl working solution

7. Use 100 µl working solution as the sample blank
8. Add 90-99 µl working solution to each sample well in triplicate followed by 1-10 µl of each sample (samples are in lysis buffer)
   a. The amount of sample used for this assay will need to be optimized. Some samples may contain so much chitinase activity that the fluorescence will be saturated. This will vary considerably depending on the amount of cells plated and the chitinase activity of those cells.
9. Wrap plate in foil and incubate at 37 °C for 30 min
   a. Incubation time may also need to be optimized. Cell lysates with high chitinase activity can be incubated for as little as 15 min. Alternatively, samples may be incubated for up to 1 h.
10. Add 200 µl of stop buffer to each well to stop the reaction.
11. Fluorescence can be measured on a plate reader at an excitation of 360 nm and emission of 450 nm within 30 min.
12. Chitinase activity is calculated from the standard curve. Alternatively, chitinase activity may be calculated using the following equation:

$$\text{Units/mL} = \frac{(\text{Fluorescence}_{\text{sample}} - \text{Fluorescence}_{\text{blank}}) \times 1.9 \times 0.3 \times \text{Dilution Factor}}{\text{Fluorescence}_{100\text{ ng standard}} \times \text{reaction time} \times \text{sample Volume}}$$

Recipes

1. Assay buffer
   Phosphate-Citrate Buffer pH=5.2 (26.7 ml of 0.2 M diabasic Sodium Phosphate, 23.3 ml of 0.1 M Citric Acid, top up to 100 ml DI water)
2. Stop buffer
   Glycine-NaOH buffer pH = 10.6 (combine 25 ml 0.2 M glycine stock solution with 22.75 ml 0.2 M NaOH, and dilute with DI water to make a 100 ml solution)
3. Protein lysis buffer
   50 mM Tris HCl (pH 7.5)
   200 mM NaCl
   10% Glycerol
   0.5% TX-100
   1 mM DTT (added to buffer fresh, just before adding to cultures)
4. Substrate Stock
   Prepare 20 mg/ml 4-Methylumbelliferyl substrate in DMSO.
5. Positive control
   Prepare 0.2 mg/ml chitinase from *Trichoderma* in PBS.

Acknowledgments

This protocol is adapted from Nance *et al*. (2012).

References

1. Nance, J. P., Vannella, K. M., Worth, D., David, C., Carter, D., Noor, S., Hubeau, C., Fitz, L., Lane, T. E., Wynn, T. A. and Wilson, E. H. (2012). Chitinase dependent control of protozoan cyst burden in the brain. *PLoS Pathog* 8(11): e1002990.

