Studying Phagocytosis using the GloMax® Discover System

Promega Corporation

Materials Required

- Vybrant™ Phagocytosis Assay Kit (Molecular Probes Cat.# V-6694)
- CytoSelect™ 96-well Phagocytosis Assay (*E.coli* Substrate) (Cell BioLabs Cat. CBA-222)
- GloMax® Discover System (Cat.# GM3000)
- Cytochalasin D (Millipore Cat.# 25-025-51MG)
- Black, 96-well assay plates (Corning Cat.# 3903)
- PMA (Cat.# V1171)
- U937 Cells (ATCC Cat.# CRL-1593.2)

Caution: We recommend the use of gloves, lab coats and eye protection when working with these or any chemical reagents.

Protocols: *GloMax Discover System Technical Manual* #TM397 available at: **www.promega.com/protocols/**

Acquiring and removing material from cells is a vital process to maintain healthy, viable cells. Phagocytosis is an engulfment process that serves this function in different cell types. For example, phagocytosis processes in mammalian immune cells are important in the removal of pathogens and cellular debris through macrophages. Defective phagocytosis pathways in this cell type are thought to be involved in various autoimmune diseases, and therefore are useful targets during drug development.

Studying phagocytosis can be achieved by several methods. In this application note we examined two commercially available phagocytosis assays which provide the ability to study phagocytosis in multi-well plate formats. The Vybrant™ Phagocytosis Assay (Molecular Probes) monitors a fluorogenic substrate, and the CytoSelect™ 96-well Phagocytosis Assay (Cell BioLabs) uses absorbance to monitor substrate. Both assays can be measured on the GloMax® Discover System, which provides extended dynamic range, superior sensitivity and minimal well-to-well cross talk. This application note describes the protocols to measure phagocytosis using these two commercially available assay kits.

Cell Differentiation Into Macrophages

Preparation of cells in to macrophages for use in phagocytosis assays requires stimulation of cell differentiation. In this testing we used lymphoma U937 cells due to their adoption of macrophage morphology and characteristics upon differentiation.

1. Plate U937 cells at 5×105 cells/ml. Stimulated differentiation by adding PMA (also known as TPA,12-O-tetradecanoylphorbol-13-acetate) to a final concentration of 10ng/ml diluted in ethanol (1% final ethanol concentration).
2. Incubate with PMA for 60 hours prior to phagocytosis assay.
3. Cells are differentiated when they adhere to the bottom of the plate wells instead of remaining in suspension. Prior to phagocytosis assay remove any cells which remain in suspension.

Vybrant™ Phagocytosis Assay

1. Thaw fluorescent-labeled *E.coli* Bioparticles and 10X concentrated HBSS (Hanks' Balanced Salt Solution).
2. Add a vial of HBSS to a vial of *E.coli* Bioparticles and briefly sonicate the suspension.
3. Transfer the suspension into a clean glass tube containing 4.5ml of deionized water and homogenize until suspended uniformly.
4. The recommended negative control for this assay is a no-cell control. Plate 150µl of media into negative control wells in a 96-well black tissue culture plate.
5. Plate 100µl of cell suspension (described above) into positive control and experimental wells.
6. Add 50µl of compound (dilutions of Cytochalasin D or DMSO).
7. Incubate the plate for 1 hour at 37°C.
8. Remove the media and 100µl of fluorescent Bioparticle suspension to all wells.
9. Incubate the plate for 2 hours at 37°C.
10. During the incubation step, prepare 1X trypan blue by transferring the contents of a vial into a clean glass tube containing 4ml of deionized water. If any precipitate is visible, sonicate briefly.
11. Remove Bioparticls gently and add 100µl of 1X trypan blue to each well.
12. After 1 minute incubation remove the excess trypan blue.
13. Measure fluorescence on the GloMax® Discover instrument using the Blue filter (Ex 475, Em 500-505).

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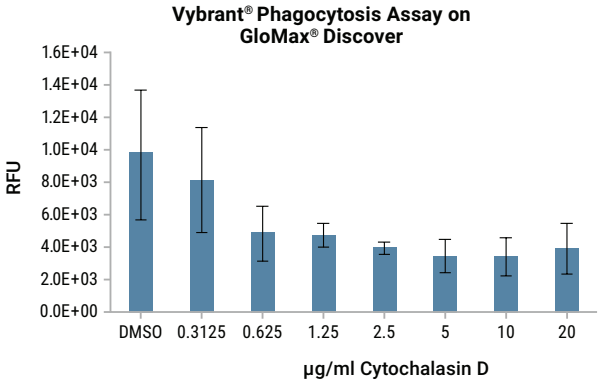

Figure 1. Inhibition of U937 cell phagocytosis by Cytochalasin D. Following treatment with Cytochalasin D and application of fluorescent Bioparticles®, fluorescence was measured on the GloMax® Discover System. Show is the average ± standard deviation (n=4).

CytoSelect™ Phagocytosis Assay

1. Thaw *E.coli* suspensions on ice. Prepare appropriate volume of 1X Blocking Reagent and 1X Permeabilization Solution by diluting 1:10 in PBS, store on ice.
2. Trypisinize cells (described above) and concentrate to 1X106 cells/ml.
3. Plate 100µl of cell suspension to positive and negative control wells of a 96-well black tissue culture plate. **Note:** The recommended negative control for this assay is a no-cell control.
4. Add 50µl of compound (Cytochalasin D or DMSO).
5. Incubate the plate for 1 hour at 37°C. Do not remove the media following the incubation.
6. Add 10µl of *E.coli* suspension to positive and negative control wells and mix well.
7. Incubate the plate for 3–6 hours at 37°C.
8. Gently remove media from the plate and add 200µl of cold media to each well.
9. Repeat Step 8 for a total of four washes.
10. Add 100µl of Fixation Solution to each well and incubate for 5 minutes at room temperature.
11. Immediately remove the Fixation Solution gently. Invert the plate and gently blot on a paper towel.

12. Wash twice with 1X PBS.
13. Add 100µl of 1X Blocking Solution and incubate for 30 minutes at room temperature on an orbital shaker.
14. Immediately remove the Blocking Solution gently. Invert the plate and gently blot on a paper towel.
15. Add 100µl of 1X Permeabilization Solution to each well and incubate for 5 minutes at room temperature.
16. Immediately remove the Blocking Solution gently. Invert the plate and gently blot on a paper towel.
17. Wash twice with 1X PBS.
18. Immediately remove the PBS gently. Invert the plate and gently blot on a paper towel
19. Begin the reaction by adding 100µl of Substrate, then incubate for 10–30 minutes at room temperature.
20. Stop the reaction by adding 100µl of Stop Solution, then shake for 30 seconds on an orbital shaker.
21. Measure absorbance on the GloMax® Discover instrument at 450nm.

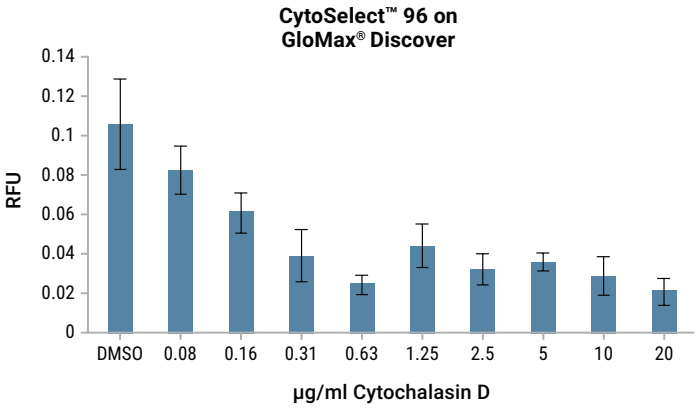

Figure 2. Inhibition of U937 cell phagocytosis by Cytochalasin D. Absorbance at 450nm and reference wavelength at 600nm were measured on the GloMax® Discover System. Shown is the average ± standard deviation (n=4) 450nm measurement minus 600nm reference measurement.

Conclusion

Fluorescence and absorbance substrates were shown to monitor phagocytosis using two commercially available assays, the Vybrant™ Phagocytosis Assay and the CytoSelect™ 96-well Phagocytosis Assay. To demonstrate detection of macrophage phagocytosis, differentiated cells induced to initiate phagocytosis were inhibited with Cytochalasin D, a compound that disrupts the phagocytosis pathway. Upon treatment with Cytochalasin D, both assays showed sequential reduction of signal across the treatment dilution series. GloMax® Discover was used to measure the fluorescence and absorbance to perform both of these assays and is a useful instrument to monitor phagocytosis.