Product Manual

CytoSelect™ 96- Well Phagocytosis Assay (*E. coli* Substrate)

Catalog Number

CBA- 222 **96 assays**

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

Introduction

In mammals, phagocytosis by phagocytes (e.g. macrophages, dendritic cells, and neutrophils) is essential for a variety of biological events, including tissue remodeling and the continuous clearance of dying cells. Furthermore, phagocytosis represents an early and crucial event in triggering host defenses against invading pathogens. Phagocytosis comprises a series of events, starting with the binding and recognition of particles by cell surface receptors, followed by the formation of actin-rich membrane extensions around the particle. Fusion of the membrane extensions results in phagosome formation, which precedes phagosome maturation into a phagolysosome. Pathogens inside the phagolysosome are destroyed by lowered pH, hydrolysis, and radical attack (Figure 1). These early events that are mediated by the innate immune system are critical for host survival. As a result of this process, pathogen-derived molecules can be presented at the cell surface (antigen presentation), allowing the induction of acquired immunity.

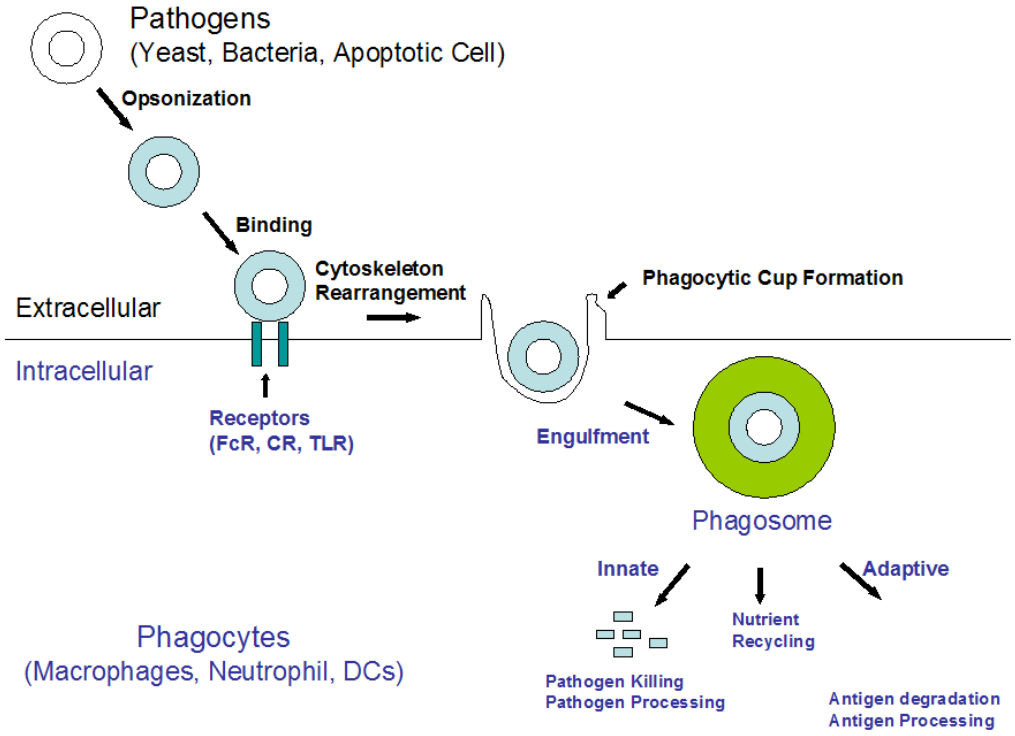
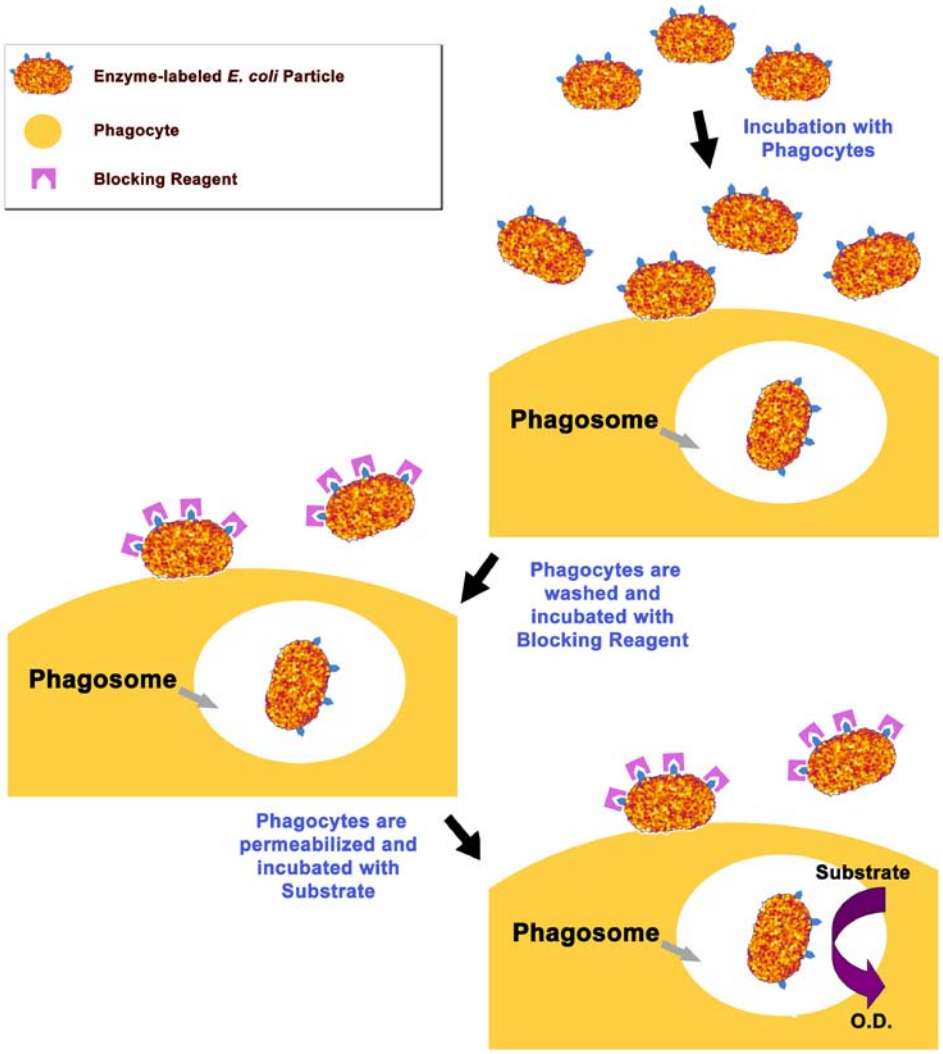


Figure 1: Phagocytosis Processes.

Cell Biolabs' CytoSelect™ 96-well Phagocytosis Assay (*E. coli*) uses enzyme-labeled, *E. coli* particles as a phagocytosis pathogen; however, it does **not** involve subjective manual counting of *E. coli* particles inside cells. Instead external particles are blocked before the colorimetric detection of engulfed *E. coli* particles (Figure 2). This format provides a quantitative, high-throughput method to accurately measure phagocytosis. The CytoSelect™ 96-well Phagocytosis Assay (*E. coli*) provides a robust system for screening TLR ligands, phagocytosis activators or inhibitors. Each kit provides sufficient quantities to perform 96, 48, 24 tests in a 96, 48, 24-well plate, respectively.

Assay Principle



Related Products

1. CBA-220: CytoSelect™ 96-Well Phagocytosis Assay (Red Blood Cell, Colorimetric Format)
2. CBA-224: CytoSelect™ 96-Well Phagocytosis Assay (Zymosan, Colorimetric Format)
3. CBA-210: CytoSelect™ Leukocyte-Endothelium Adhesion Assay
4. CBA-211: CytoSelect™ Leukocyte-Epithelium Adhesion Assay
5. CBA-212: CytoSelect™ Leukocyte Transmigration Assay

Kit Components

1. *E. coli* Suspension (Part No. 122201): One 1 mL tube of enzyme-labeled, fixative-inactivated *E. coli* in PBS/1% BSA
2. Fixation Solution (Part No. 122402): One 20 mL bottle of 3.2% Buffered Formaldehyde Solution
3. 10X Blocking Reagent (Part No. 122202): One 1.5 mL tube
4. 10X Permeabilization Solution (Part No. 122404): One 1.5 mL tube of PBS/1% Triton X-100
5. Substrate (Part No. 310807): One 12 mL amber bottle
6. Stop Solution (Part No. 310808): One 12 mL bottle
7. Phagocytosis Inhibitor (Part No. 122006): One 20 µL amber tube of 2 mM Cytochalasin D in DMSO

Materials Not Supplied

1. Phagocytes and Culture Medium
2. PBS
3. 37ºC Incubator, 5% CO_2 Atmosphere
4. Light Microscope
5. 96-well Mitrotiter Plate
6. 96-well Microtiter Plate Reader

Storage

Upon receipt, aliquot and store the *E. coli* Suspension at -20ºC to avoid multiple freeze/thaw cycles. Store all other kit components at 4ºC.

Preparation of Reagents

- *E. coli* Suspension: Thaw *E. coli* Suspension at 4ºC. Either nonopsonized or opsonized *E. coli* particles can be used in phagocytosis assay. **For opsonization, it is recommended to use the entire 1 mL suspension (due to the small particle pellet during centrifugation).** To opsonize *E. coli* particles, incubate particles with desired serum or IgG for 30 minutes at 37ºC, pellet particles by centrifugation at 10,000 xg for 1 minute, and wash a few times with sterile 1X PBS. Prior to using, resuspend the particles in the same volume of sterile 1X PBS. Store at 4ºC.
- 1X Blocking Reagent: Prepare the appropriate volume for the number of samples being tested. IMMEDIATELY prior to using, dilute the provided 10X Blocking Reagent 1:10 in 1X PBS. Store at 4ºC.
- 1X Permeabilization Solution: Prepare the appropriate volume for the number of samples being tested. Prior to using, dilute the provided 10X Permeabilization Solution 1:10 in 1X PBS. Store at 4ºC.

Assay Protocol: Adherent Phagocytes

The following assay protocol is written for a 96-well format. Refer to the below table for the appropriate dispensing volumes of other plate formats.

Culture Dish	96-well	48-well	24-well
Phagocyte Seeding Volume (µL/well)	100	200	400
E. coli Suspension (µL/well)	10	20	40
Fixation Solution (µL/well)	100	200	400
Permeabilization Solution (µL/well)	100	200	400

Table 1: Dispensing Volumes of Different Plate Formats

I. Phagocytosis of *E. coli*

1. Harvest and resuspend phagocytic cells in culture medium at 0.2 – 1 x 10^6 cells/mL or the appropriate concentration that yields >80% confluency after overnight incubation. Seed 100 µL in each well of a 96-well plate and incubate overnight at 37ºC, 5% CO_2.
2. Treat phagocytes with desired activators or inhibitors.
3. Add 10 µL of *E. coli* Suspension to each well. Mix well and immediately transfer the plate to a cell culture incubator for 3 – 6 hours, undisturbed. Each sample including a negative control without *E. coli* particles should be assayed in duplicate.

   Note: The E. coli particle is much smaller than other phagocytosis particles (e.g. Zymosan, RBC, latex bead), requiring longer incubation times to settle on phagocytic cells. Leave the plate undisturbed during this step.
4. Remove the culture medium by gently aspirating or inverting the plate and blotting on a paper towel. Gently tap several times.
5. Gently add 200 µL of cold, serum-free medium (e.g. DMEM, RPMI) to each well. Promptly remove the cold media by gently aspirating or inverting the plate and blotting on a paper towel. Gently tap several times. Repeat four times total.

   Note: For loosely attached cells, complete culture media is preferred to maintain cell attachment

II. Removal and blocking of external particles

Note: Perform steps with care, gently adding solutions as to not disrupt cell attachment

1. Add 100 µL of Fixation Solution to each well, incubating 5 minutes at room temperature.
2. Promptly remove the Fixation Solution by gently aspirating or inverting the plate and blotting on a paper towel. Gently tap several times.
3. Wash twice with 1X PBS.

4. Add 100 µL of prediluted 1X Blocking Reagent to each well (see Preparation of Reagents Section). Incubate the plate for 30 minutes at room temperature on an orbital shaker.
5. Promptly remove the Blocking Reagent by gently aspirating or inverting the plate and blotting on a paper towel. Gently tap several times. Wash three times with 1X PBS.

III. Detection of internalized particles

Note: Perform steps with care, gently adding solutions as to not disrupt cell attachment

1. Remove the PBS wash and add 100 µL of prediluted 1X Permeabilization Solution (see Preparation of Reagents Section) to each well, incubate 5 minutes at room temperature.
2. Promptly remove the Permeabilization Solution by gently aspirating or inverting the plate and blotting on a paper towel. Gently tap several times. Wash twice with 1X PBS.
3. Promptly remove the PBS by gently aspirating or inverting the plate and blotting on a paper towel. Gently tap several times.
4. Initiate the reaction by adding 100 µL of Substrate. Incubate for 10-30 minutes at room temperature.
5. Stop the reaction by adding 100 µL of the Stop Solution and mix by placing the plate on an orbital plate shaker for 30 seconds.
6. Read the absorbance of each well at 450 nm.

Assay Protocol: Suspension Phagocytes

1. Harvest and resuspend phagocytic cells in culture medium at 0.2 – 1 x 10^6 cells/mL. Seed 100 µL in each well of a 96-well plate.
2. Treat phagocytes with desired activators or inhibitors.
3. Add 10 µL of *E. coli* Suspension to each well. Mix well and immediately transfer the plate to a cell culture incubator for 3 – 6 hours, undisturbed. Each sample including a negative control without *E. coli* particles should be assayed in duplicate.

   Note: The E. coli particle is much smaller than other phagocytosis particles (e.g. Zymosan, RBC, latex bead), requiring longer incubation times to settle on phagocytic cells. Leave the plate undisturbed during this step.
4. Remove the culture medium by centrifugation at 300 x g for 5 minutes, and gently aspirate.
5. Add 200 µL of cold 1X PBS to each well. Promptly remove the PBS Solution by centrifugation and gentle aspiration.
6. Add 100 µL of Fixation Solution to each well, incubate 5 minutes at room temperature.
7. Promptly remove the Fixation Solution by centrifugation at 300 x g for 5 minutes, and gently aspirate.
8. Wash twice with 1X PBS.
9. Add 100 µL of prediluted 1X Blocking Solution to each well (see Preparation of Reagents Section). Incubate the plate for 30 minutes at room temperature on an orbital shaker.

10. Promptly remove the Blocking Solution by centrifugation at 300 x g for 5 minutes, and gently aspirate. Wash three times with 1X PBS.
11. Add 100 µL of prediluted 1X Permeabilization Solution (see Preparation of Reagents Section) to each well, incubate 5 minutes at room temperature.
12. Promptly remove the Permeabilization Solution by centrifugation at 300 x g for 5 minutes, and gently aspirate. Wash twice with 1X PBS.
13. Promptly remove the PBS by centrifugation at 300 x g for 5 minutes, and gently aspirate.
14. Initiate the reaction by adding 100 µL of Substrate. Incubate for 10-30 minutes at room temperature.
15. Stop the reaction by adding 100 µL of the Stop Solution and mix by placing the plate on an orbital plate shaker for 30 seconds.
16. Read the absorbance of each well at 450 nm.

Example of Results

The following figures demonstrate typical results with the CytoSelect™ 96-well Phagocytosis Assay Kit. Absorbance measurements were performed on a Microplate Autoreader EL311 (Bio-Tek Instruments Inc.) with a 450 nm filter. One should use the data below for reference only. This data should not be used to interpret actual results.

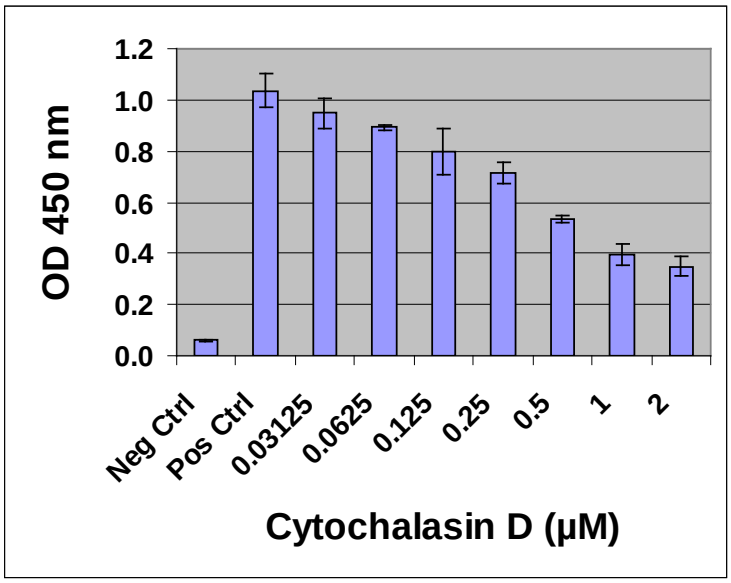


Figure 2. Inhibition of Raw 264.7 Macrophage Phagocytosis by Cytochalasin D. 50,000 cells/well of Raw 264.7 macrophages were seeded overnight in a 96-well plate. Cytochalasin D was used to pretreat Raw 264.7 cells for 1 hr at 37ºC. *E. coli* particles were then added for 4 hours before engulfed particles was determined as described in the Assay Protocol.

References

1. Hornef, M.W. et al. (2002) *Nat. Immunol.* **3**:1033-1040.
2. Lei, B. et al. (2001) *Nat. Med.* **7**:1298-1305.
3. Ojielo, C.I. et al. (2003) *J. Immunol.* **171**:4416-4424.
4. Foukas, L.C. et al. (1998) *J. Biol. Chem.* **273**:14813-14818.
5. Grunwald, U. et al. (1996) *J. Immunol.* **157**:4119-4125.

Recent Product Citations

1. Martin, I. et al. (2015). Fasciola hepatica fatty acid binding protein inhibits TLR4 activation and suppresses the inflammatory cytokines induced by lipopolysaccharide in vitro and in vivo. *J Immunol.* doi:10.4049/jimmunol.1401182.
2. Zhu, X. et al. (2014). Deletion of class A scavenger receptor deteriorates obesity-induced insulin resistance in adipose tissue. *Diabetes*. **63**:562-577.

Warranty

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