Phagocytosis Assay Kit (Red E. coli) (#BN01124)

(Catalog # BN01124; 100 assays; Store at 4°C)

I. Introduction:

Phagocytosis in mammals serves as an important first line defense mechanism against invading pathogens. It is also essential for continuous clearance of dying cells, tissue remodeling, and acquisition of nutrients for some cells. Phagocytosis is a specific form of endocytosis initiated by recognition and binding of foreign particles by cell surface receptors, followed by their engulfment, and formation of phagosomes. Maturing phagosomes transform to phagolysosomes which destroy the pathogen through enzymes and toxic peroxides. *E. coli* and other bacterial strains are often used as a pathogen in phagocytosis assays. Assay Genie's Phagocytosis Assay Kit (Red *E. coli*) utilizes heat-killed, fluorescently pre-labeled *E. coli* particles as a tool for rapid and accurate detection and quantification of *in vitro* phagocytosis by fluorescent microscope, spectrophotometer or flow cytometry. The kit provides a robust screening system for activators and/or inhibitors of phagocytosis and Toll-like receptors (TLR) ligands.

II. Applications:

- Rapid detection, quantification and validation of phagocytosis in convenient 96-well format
- Tracking ligand internalization and screening for effectors of phagocytosis

III. Sample Type:

- Phagocytic cell culture: adherent or suspension cells capable of phagocytosis

IV. Kit Contents:

Components	BN01124	Cap Code	Part Number
Phagocytosis Assay Buffer	2 X 100 ml	NM	BN01124-1
Buffer Additive	2 X 1 ml	Blue	BN01124-2
Red *E. coli*	600 µl	Red	BN01124-3
10X Quenching Solution	500 µl	Yellow	BN01124-4

V. User Supplied Reagents and Equipment:

- A 6-, 12-, 24-, or 96-well clear plates should be used only for cell culturing. The measurement of fluorescence should be performed in opaque plates with clear bottoms. Alternatively, sterile opaque plates with clear bottoms can be used for both, culturing and measurements.
- Adherent or suspension cells capable of phagocytosis and appropriate media (e.g., JM774 or U937)
- Stock solutions of effectors of interest (for example, Cytochalasin D, inhibitor of actin cytoskeletal rearrangement)
- Multi-well spectrophotometer measuring excitation and emission at 540 and 570 nm, respectively
- Fluorescent microscope (optional) for observation or flow cytometer equipped with laser capable of excitation at 550 nm

VI. Storage Conditions and Reagent Preparation:

Store the entire kit at 4°C protected from light. Read the protocol before performing the assay.

- **Phagocytosis Assay Buffer**: Upon arrival, combine one entire vial of Buffer Additive with one Phagocytosis Assay Buffer, mix well. Use sterile pipetting technique throughout the assay.
- **Red *E. coli***: Before each use, equilibrate the suspension to room temperature and vortex gently for 5 seconds.
- **Quenching Solution:** Dilute the content of the vial into 4.5 ml of 1X Phagocytosis Assay Buffer.

VII. Phagocytosis Assay Protocol:

1. **Preparation of control and experimental wells:** Subculture cells capable of phagocytosis in appropriate medium. Day prior to the experiment obtain a culture of 1 - 5 x 10^6 viable cells/ml. Aliquot 100 µl of the cell culture per well omitting the negative control wells and incubate the plate overnight at 37 °C, 5 % CO_2. Next day, change the media and proceed to the phagocytosis effector assay. Your experiment should always consist of parallel negative, positive and experimental wells respectively.
2. **Phagocytosis effector assay:** Add 100 µl of cell culture media containing your effector of interest (not provided in the kit) at desired concentration (e.g. 20 µM Cytochalasin D) to each of the experimental wells. Aliquot 100 µl of media to each of the positive and 200 µl media to each of the negative control wells respectively. Incubate for 1 hour at 37 °C, 5 % CO_2.
3. **Phagocytosis of Red *E.coli*:** Add 5 µl of *E. coli* slurry to all the wells. Immediately transfer the plate back to the incubator for 2 - 3 hours. The incubation time may be adjusted according to your protocol.
4. **Red *E. coli* Standard Curve:** Add 0, 1, 2, 3 and 4 µl of Red *E.coli* slurry into a series of wells in 96-well plate. Adjust the volume to 100 µl with Phagocytosis Assay Buffer. Mix well. Immediately measure fluorescence using plate reader at Ex/Em at 540/570 nm respectively. Subtract 0 Standard reading from all the readings and plot the Standard Curve.
5. **Sample preparation:** Harvest the cells by centrifugation for 5 minutes at 400 X g. Carefully aspirate off the media and gently re-suspend the cell pellets in 300 µl of ice cold Phagocytosis Assay Buffer containing the effector of interest at the same concentration as in the assay media. Centrifuge for 5 minutes at 400 X g and repeat the washing step 3 more times. Finally, suspend the cells in 200 µl of ice cold Phagocytosis Assay Buffer and proceed to the preferred method of detection.
6. **Detection:** Cells can be analyzed by FACS, fluorescent microscopy or by scanning of all experimental and control wells in the plate reader at Ex/Em at 540/570 nm, respectively.

   Optional: For plate reader and microscope detection, re-suspend the cell pellets in 50 µl of the diluted Quenching Solution and incubate for two minutes at room temperature. Centrifuge for 5 minutes at 400 X g and carefully remove the Quenching solution. Suspend the cells in 200 µl of ice cold Phagocytosis Assay Buffer.

a. For plate reader: Transfer 100 µl of each control and sample into a separate well and record the fluorescence.
b. For fluorescent microscope: Control and experimental wells can be imaged directly in the plate.
c. For flow cytometry: Transfer 100 µl of cell suspension into a 900 µl of the Phagocytosis Assay Buffer in the flow cytometry compatible vessel. Analyze immediately in the FL2 channel of flow cytometer equipped with laser capable of excitation at 550 nm.

7. Calculation: To calculate the net phagocytosis subtract the average RFU of the no-cell negative-control wells from all positive control and experimental wells. The phagocytosis response to the experimental effector (% Effect) can be expressed as follows:

$$\% \text{ Effect } = \frac{\text{Net experimental phagocytosis } \times 100\%}{\text{Net positive control phagocytosis}}$$

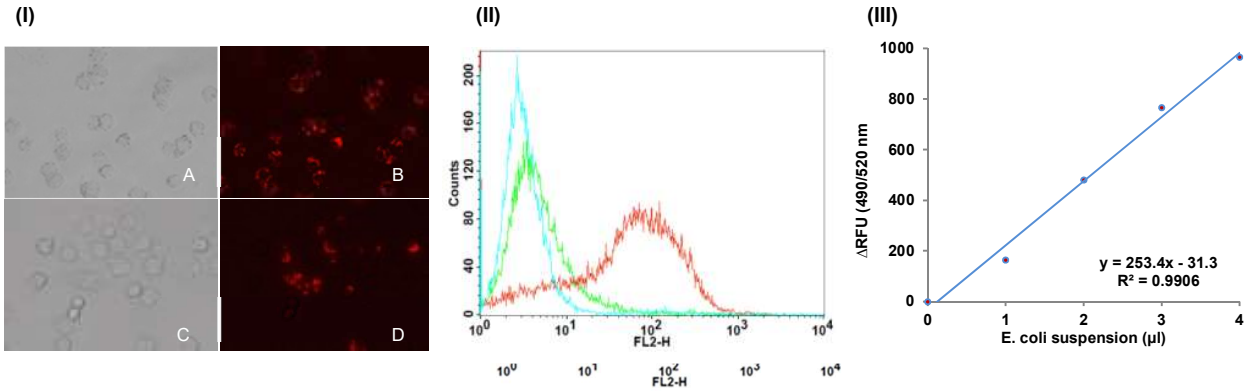

Figure: J774 macrophages were seeded overnight at 5 x 10^5 of viable cells/well. The next day the cells were pretreated with 20 µM Cytochalasin D for 1 h at 37ºC prior to addition of 5 µl of *E. coli* particles. Phagocytosis was conducted for 2 hours and the amount of engulfed *E. coli* was determined as described in the Assay Protocol. **(I) Inhibition of phagocytosis**. Panel A and B: images of non-treated cells. Panel C and D: treatment with Cytochalasin D. **(II) Flow cytometry plot.** Blue line: untreated control cells; red line: macrophages with engulfed *E. coli* particles; green line: inhibition of phagocytosis by Cytochalasin D. **(III) *E. coli* Standard curve**. The following figures demonstrate typical results with the Phagocytosis Assay Kit (Red *E. coli*). These data is for reference only and should not be used to interpret actual results. Your data will depend on the cell type and tested compound.

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