pHrodo™ BioParticles® Conjugates for Phagocytosis

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
pHrodo™ *E. coli* BioParticles® Conjugate	5 vials each containing 2 mg lyophilized product	• ≤–20˚C • Desiccate • Protect from light	When stored as directed the product is stable for at least 6 months.
pHrodo™ *S. aureus* BioParticles® Conjugate			
Number of assays: 100 assays when using 100 µg per assay			
Approximate fluorescence excitation and emission maxima: 560/585 nm.			

Introduction

pHrodo™ *E. coli* and *S. aureus* BioParticles® Conjugates are novel, no-wash fluorogenic reagents developed for quantitative measurements of phagocytosis and its regulation by drugs and/or environmental factors. With a simple no-cell background subtraction method, a large and specific signal is obtained from cells that ingest the particles, providing a specific index of phagocytosis with a variety of pretreatments or conditions. The unique pHrodo™-based system measures phagocytic activity based on acidification of the particles as they are ingested, eliminating the wash and quenching steps that are necessary with nonfluorogenic indicators of bacterial uptake.[1–2] To achieve this, the particles are conjugated to pHrodo™ dye, a novel, fluorogenic dye that dramatically increases in fluorescence as the pH of its surroundings becomes more acidic (Figure 1). The amine-reactive succinimidyl ester form of this dye (pHrodo™ SE, Invitrogen Cat. no. P36600) has a pKa of ~7.3 in solution, which shifts to about ~6.5 upon conjugation to the K-12 strain of *E. coli* or the protein A-free Wood strain of *S. aureus* used in this product line. We have included sufficient pHrodo™ BioParticles® Conjugate for ~100 wells in a 96-well format, with step-by-step instructions for performing this assay in a fluorescence microplate reader.

The methodology for this reagent's use has been developed using adherent J774A.1 murine macrophage cells,[2] but can be adapted for use with other adherent cells, primary cells, or even cells in suspension.[3] pHrodo™ BioParticles® Conjugate is completely compatible with the cell culture system used for our popular Vybrant® Phagocytosis Assay Kit (Invitrogen Cat. no. V6694), with the exception of the wash and quench steps needed at the end of the Vybrant® assay. pHrodo™ BioParticles® Conjugate is also amenable to imaging and flow cytometry studies, allowing the visualization of phagocytosis in living cultures with no need for removing the particles for study. Cells assayed for phagocytic activity with pHrodo™ BioParticles® conjugate may also be fixed with standard 2-4% paraformaldehyde solutions for later analysis, preserving differences in signal between control and experimental samples with high fidelity. pHrodo™ BioParticles® conjugate preparations are also amenable to opsonization (Invitrogen Cat. nos E2870, S2860), which can greatly enhance their uptake and signal strength in the assay.

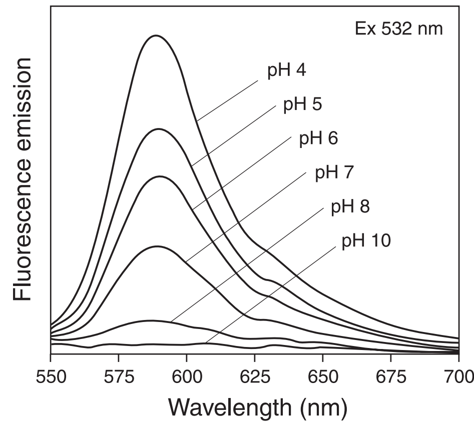

Figure 1. The fluorescence emission spectra of pHrodo™ dye–labeled *E. coli* were measured in a series of 50 mM potassium phosphate buffers ranging in pH from 4 to 10. The *E. coli* were at a concentration of 0.1 mg/mL, and the readings were made on a Hitachi F4500 fluorometer, using an excitation wavelength of 532 nm.

Before You Begin

Materials Recommended but Not Provided

- Murine macrophage J774A.1 cells (ATCC®, Rockville, MD) cultured in Dulbecco's Modified Eagle Medium (D-MEM, Invitrogen Cat. no. 11995) supplemented with 7.5% fetal calf serum, 450 U/mL penicillin and 420 µg/mL streptomycin. Other cell lines may be used as preferred.
- Opti-MEM® culture medium (Invitrogen Cat. no. 31985-062)
- Hemocytometer (cell-number counter)
- Uptake Buffer, we recommend using Hanks' Balanced Salt Solution (HBSS, Invitrogen Cat. no. 14025) with an additional 20 mM HEPES. Adjust the pH to 7.4 with NaOH to minimize background signal from uningested particles.
- Water-bath sonicator
- 96-well microplate with cover and fluorescence microplate reader capable of measuring fluorescence emission at ~600 nm with an excitation at ~550 nm
- Cell viability assay reagents
- Stock solutions of experimental effector compounds for testing their influence on phagocytosis (for example, cytochalasin D inhibits phagocytosis by inhibiting actin cytoskeletal rearrangements)

Experimental Protocol

Phagocytosis Assay Protocol

The following protocol describes an experimental test of phagocytic function with appropriate controls. Cellular auto fluorescence background is determined with cells plated free of pHrodo™ BioParticles® Conjugates (but otherwise under control and experimental conditions), and reagent background fluorescence is determined using wells that contain the pHrodo™ BioParticles™ Conjugates but no cells. This protocol describes the use of one vial of particles, prepared at 1 mg/mL in the buffered saline solution of your choice. To minimize background fluorescence from non-ingested pHrodo™ BioParticles® Conjugate, we strongly recommend controlling the extracellular pH by adding 20 mM additional HEPES to the assay buffer, adjusting the pH to a final value of 7.4 with NaOH.

Assay Controls

To minimize experimental errors, we recommend making measurements from a minimum

of three replicates of positive control, experimental, and no-cell control samples, though the numbers of experimental and control wells can be adjusted as required to meet the needs of the particular study.

Amount of BioParticles®

A single vial of pHrodo™ BioParticles® conjugate dilutes to 2 mL of 1 mg/mL that will be used in the assay, which is distributed across 20 wells. 100 µL of this suspension is used per sample well, including no-cell background controls. The average fluorescence value of these no-cell background control wells is subtracted from all cell-containing wells at the end of the assay to yield a cell-specific, net phagocytosis signal.

Preparing the Cells

1.1 Subculture the J774A.1 macrophage cells (or preferred cell type) in complete medium for 3–4 days in advance of performing the assay.

1.2 On the day of the assay, harvest the cells from the culture plates and centrifuge the suspension. Resuspend the pellet in Opti-MEM® medium or preferred culture medium at 10^6 cells/mL. Scale your culture to aim for ~2 x 10^6 cells per 2 mg vial of pHrodo™ BioParticles® Conjugate that will be used. Alternatively, cells can be plated into the 96 well plate a day or more in advance, with the aim of having 100,000 viable cells per well on the day of the assay.

If you are using cells other than J774A.1, you may need to determine optimal cell culture conditions and densities for your specific cell type. In general, better signals in the plate reader are obtained with maximal cell densities.

1.3 If desired, you can first determine the cell viability. The cell suspension should have >90% viability as determined using any conventional cell viability assay. Determine the living cell concentration using a hemocytometer (cell-number counter)[4] and adjust the final cell concentration to 10^6 cells/mL by adding Opti-MEM® or culture medium to the suspension.

1.4 Plate the cells into a 96 well plate at 100,000 cells/well, using 100 µL per well of the suspension prepared in step 1.3. Again, we recommend plating your positive control and experimental wells in triplicate or greater. Be sure to leave one well empty of cells for every positive control well, so that a no-cell control background subtraction may be performed.

For example, plate four columns of four wells, leaving the fifth column of four empty for the no-cell control. Note that higher background fluorescence levels may be seen with acidic poly-D-lysine coated microplates.

1.5 Add 100 µL of Opti-MEM® medium or complete culture medium to the wells left aside for the no-cell background determination.

1.6 Cover the loaded microplate and allow the cells to settle and adhere to the microplate for at least one hour in a humidified incubator with 5% CO_2 at 37˚C.

1.7 Prepare the experimental wells by adding the experimental phagocytosis effector at desired concentrations, taking care to add vehicle controls to untreated wells. Note that the time and concentration of experimental effector pretreatment may vary greatly with the agent or treatment under study.

Preparing the BioParticles® Conjugate

While the cells are settling onto the plate, prepare the pHrodo™ BioParticles® Conjugate as follows:

2.1 Thaw one vial each of the pHrodo™ BioParticles® fluorescent particles for every 20 wells to be

tested. This number includes the no-cell control wells that will receive fluorescent particles, but no cells. Pipette 2 mL Uptake Buffer into the vial containing 2 mg lyophilized product and briefly vortex the solution to completely resuspend the particles.

2.2 Transfer the suspension into a clean glass tube and sonicate for 5 minutes, until all the fluorescent particles are homogeneously dispersed. pHrodo™ BioParticles® Conjugate can be visually checked for even dispersion by placing a drop in buffer onto a glass slide and using fluorescence microscopy with a standard TRITC filter set. The dim fluorescence at pH 7.4 is normal and desired. It will increase greatly as the pH drops. A small amount of acid solution aids visualization.

Adding the Fluorescent Particles

3.1 After the cells have adhered and the phagocytosis effectors have been added, remove the culture medium from each of the microplate wells by vacuum aspiration.

3.2 Quickly replace the culture medium with 100 µL of the prepared pHrodo™ BioParticles® suspension from step 2.2, adding it to the positive control, experimental, and no-cell background subtraction wells. Experimental effector solutions may be prepared ahead of time with separate vials of pHrodo™ BioParticles® suspension to keep them present throughout the assay.

3.3 Cover and transfer the microplate to an incubator warmed to 37˚C for 2–3 hours. Do not use an elevated CO_2 cell culture incubator unless the Uptake Buffer in use has a bicarbonate buffering system, as this will artificially acidify the buffer and elevate the background fluorescence.

Fluorescence Measurements and Results

4.1 Scan all experimental, control, and no-cell control wells of the microplate in the fluorescence plate reader using ~550 nm excitation, ~600 nm emission with the appropriate cutoff values and sensitivity settings for your instrument.

4.2 Calculate the net phagocytosis and the response to the phagocytosis effector agent. Net phagocytosis is calculated by subtracting the average fluorescence intensity of the no-cell negative-control wells from all positive-control and experimental wells. The mean and standard deviation of the net positive control and net experimental wells should then be calculated. The phagocytosis response to the experimental effector (% Effect) can then be calculated as a fraction of the net positive control phagocytosis as follows:

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

References

1. J Immunol Methods 60, 115 (1983); **2.** J Immunol Methods 162, 1 (1993); **3.** J Biol Chem 273, 14813 (1998); **4.** Methods Enzymol 58, 141 (1979).

Product List Current prices may be obtained from our website or from our Customer Service Department.

Cat #	Product Name	Unit Size
A10010	pHrodo™ *S. aureus* BioParticles® conjugate for phagocytosis	5 x 2 mg
E2870	*Escherichia coli* BioParticles® opsonizing reagent	1 Unit
P35361	pHrodo™ *E. coli* BioParticles® conjugate for phagocytosis	5 x 2 mg
P36600	pHrodo™, Succinimidyl ester	1 mg
S2860	*Staphlyococcus aureus* BioParticles® opsonizing reagent	1 Unit

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