molecular probes®
by life technologies™

pHrodo™ BioParticles® Phagocytosis Kits for Flow Cytometry

Table 1 Contents and storage

Material	Amount	Storage	Stability
Lysis Buffer A (Component A)	10 mL	• 2–8°C • Do not freeze	When stored as directed, the product is stable for at least 6 months.
Buffer B (Component B)	200 mL		
Wash Buffer (Component C)	200 mL		
pHrodo™ Red *E. coli* BioParticles® (Cat. no. A10025), pHrodo™ Green *E. coli* BioParticles® (Cat. no. P35381), or pHrodo™ Green *S. aureus* BioParticles® (Cat. no. P35382) (Component D)	1 vial of lyophilized product	• 2–8°C • Desiccate • Protect from light	
Number of assays: 100 assays when using 100 µL whole blood sample volume per assay.			
Approximate fluorescence excitation and emission maxima: pHrodo™ Red BioParticles® conjugate: 560/585 nm; pHrodo™ Green BioParticles® conjugate: 509/533 nm. Both pHrodo™ Red and pHrodo™ Green conjugates are compatible with 488 nm argon-ion laser excitation.			

Introduction

pHrodo™ BioParticles® Phagocytosis Kits for flow cytometry offer an outstanding approach for assessing phagocytic activity in whole blood samples by flow cytometry. The pHrodo™ BioParticles® assays provide sensitive detection without the need for quenching reagents and extra wash steps, saving time and eliminating the uncertainty of whether particle signals derive from internalized particles. The kits include all the reagents required for assessing particle ingestion and red blood cell lysis. Sufficient reagents are provided in the kit for performing approximately 100 assays using 100 µL of whole blood per assay.

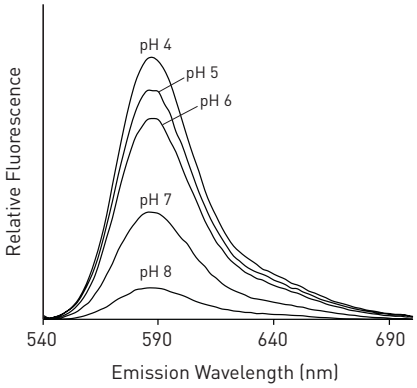
The pHrodo™ BioParticles® are inactivated, unopsonized *E. coli* or *S. aureus* reagents, which are highly sensitive, fluorogenic particles for the detection of phagocytic ingestion. The unique pHrodo™ dye-based system measures phagocytic activity based on acidification of particles as they are ingested, 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 the pHrodo™ dyes, novel, fluorogenic reagents that dramatically increase in fluorescence as the pH of their surroundings becomes more acidic (Figure 1, page 2). The optimal absorption and fluorescence emission maxima of the pHrodo™ dye is approximately 560 nm and 585 nm for the pHrodo™ Red BioParticles® conjugate, and approximately 509 nm and 533 nm for the pHrodo™ Green BioParticles® conjugate, respectively (Figure 1, page 2). However, the fluorophores are readily excited with the 488-nm argon-ion laser installed on most flow cytometers.

For Research Use Only. Not for use in diagnostic procedures.

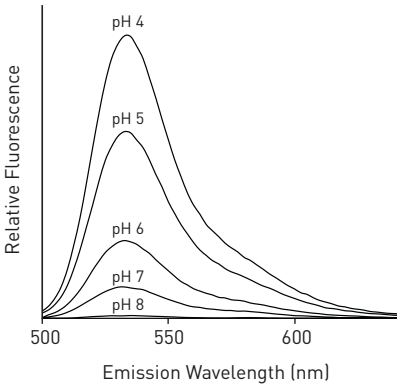
The methodology for these reagents' use was developed using whole blood cells and it also works well with adherent RAW and MMM (J774A.1) murine macrophage cells.[2] However, the methodology can also be adapted for use with other adherent cells, primary cells, or even cells in suspension.[3] Cells assayed for phagocytic activity with pHrodo™ BioParticles® conjugates 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 for up to 48 hours. pHrodo™ BioParticles® conjugate preparations are also amenable to opsonization (Cat. nos. E2870, S2860), which can greatly enhance their uptake and signal strength in the assay.

Before You Begin

Figure 1 The fluorescence emission spectra of pHrodo™ Red and pHrodo™ Green BioParticles® conjugates.



The fluorescence emission spectra of the pHrodo™ Red conjugate



The fluorescence emission spectra of the pHrodo™ Green conjugate

Materials Recommended but Not Provided

- Whole blood sample collected in sodium heparin collection tube
- Water bath or incubator set to 37°C
- Ice bath/bucket
- Analysis tubes for your flow cytometer
- Water bath sonicator
- Centrifuge
- Flow cytometer with 488-nm excitation wavelength (argon-ion laser)
- Biohazard wipes

Caution

Be sure to take the appropriate precautions (wear a laboratory coat, disposable gloves, and eye protection) when handling whole blood samples. Dispose of blood samples as biohazardous waste.

Buffers

Bring the Lysis Buffer A (Component A) and Buffer B (Component B) to room temperature before use.

Preparing the BioParticles® Solution

1.1 Add 2.2 mL Buffer B (Component B) to the vial containing the lyophilized product to resuspend the pHrodo™ BioParticles® conjugate.

This provides sufficient pHrodo™ BioParticles® conjugate in a 20 µL aliquot for a 20:1 particle-to-phagocyte ratio.[3]

1.2 Vortex for 1 minute. Sonicate for 5 minutes until all the fluorescent particles are homogenously dispersed.

1.3 Store the pHrodo™ BioParticles® solution on ice for ~10 minutes prior to use.

Collecting the Whole Blood Sample

2.1 Collect whole blood samples in blood collection tubes containing heparin anticoagulant.

Note: You can collect and store whole blood samples on ice or at 4°C for up to 24 hours prior to using the blood samples in the phagocytosis assay. Anticoagulants other than heparin have not been tested with the pHrodo™ BioParticles® conjugates; therefore we do not recommend their use for blood collection.

2.2 Place the blood sample tubes on ice to cool the samples for 10 minutes before use.

Experimental Protocols

Overview of Phagocytosis Assay

The pHrodo™ BioParticles® phagocytosis assays investigate particle attachment and internalization, and measures phagocyte activity as described below.

The phagocytic function of polymorphonuclear leukocytes (PMNs) and macrophages is evaluated in the whole blood sample by exposing the heparinized whole blood sample to pHrodo™ BioParticles® for at least 15 minutes at 37°C. A control sample containing all reagents is incubated on ice. The phagocytosis is stopped by transferring the samples to ice. The red blood cells or erythrocytes are lysed using the proprietary Lysis Buffer A (Component A) and Buffer B (Component B), followed by centrifugation and washing. The final cell pellet containing white blood cells is resuspended in Wash Buffer (Component C) and is ready for analysis using a flow cytometer equipped with a 488-nm argon-ion laser.

The nucleated phagocytes are distinguished from debris by gating on the granulocyte and monocyte populations using forward and scatter properties. The percentage of active phagocytes is determined by further gating on orange fluorescence signals collected with an R-phycoerythrin emission filter, such as a 585/26 nm band pass filter for the pHrodo™ Red *E. coli* BioParticles® conjugate, or a FITC emission filter such as 530/30 nm band pass filter for the pHrodo™ Green *E. coli* or the pHrodo™ Green *S. aureus* BioParticles® conjugates. pHrodo™ BioParticles® conjugates are novel, no-wash fluorogenic reagents that are non- or weakly fluorogenic when attached to the outer surface of the phagocyte, but are highly fluorescent in the acidic environment of the phagosome upon internalization. This property of pHrodo™ dyes eliminates the wash and quenching steps associated with other phagocytosis assay protocols.

Phagocytosis Assay Protocol

To minimize experimental errors and allow proper interpretation of results, we recommend having four control tubes for each set of experimental samples as outlined in the table below. Each set of control tube and experimental tube is incubated at 4°C (ice) or 37ºC. We recommend performing the assay with replicates.

3.1 Aliquot whole blood and pHrodo™ BioParticles® conjugates from step 1.3 into flow cytometry tubes as described in the table below and vortex briefly.

Tube	Name	Whole blood sample	pHrodo™ BioParticle® conjugate
1	Negative control on ice	100 µL	—
2	Negative control at 37°C	100 µL	—
3	Positive control on ice	100 µL	20 µL
4	Positive control at 37°C	100 µL	20 µL

For each experimental sample, prepare two tubes containing 100 µL whole blood sample and 20 µL pHrodo™ BioParticles® conjugate.

3.2 Place tubes 1 and 3 on ice, and tubes 2 and 4 at 37°C water bath for 15 minutes.

For each experimental sample, place one experimental sample tube on ice and the other experimental sample tube in the 37°C water bath for 15 minutes. After the incubation, remove all tubes from the water bath and place on ice.

3.3 Add 100 µL of Lysis Buffer A (Component A) to all tubes, vortex briefly, and incubate at room temperature for 5 minutes.

3.4 Add 1 mL of Buffer B to samples, vortex briefly, and incubate at room temperature for 5 minutes.

3.5 Centrifuge the samples at 350 × *g* for 5 minutes at room temperature.

3.6 Discard the supernatant. If necessary, wick the tops of the tubes with biohazard wipes to absorb any residual liquid.

3.7 Resuspend the cell pellets with 1 mL of Wash Buffer (Component C).

3.8 Repeat steps 3.5–3.6 one more time. Resuspend the cell pellets in 0.5 mL of Wash Buffer (Component C) for flow cytometry analysis.

At this point, you may proceed to analyzing the results using flow cytometry (section 4) or perform additional staining reactions such as DNA staining (section 5) or antibody labeling (section 6).

4.1 Perform analysis using a flow cytometer equipped with a 488-nm argon-ion laser using a 585/26 nm emission filter for the pHrodo™ Red BioParticles® conjugates or 530/30 nm emission filter for the pHrodo™ Green BioParticles® conjugates.

If additional staining is performed on the samples as described, ensure the emission spectra are appropriately separated, and use recommended emission filters.

4.2 To analyze samples, set up two dot plots, one showing forward scatter (FSC) vs. side scatter (SSC) (see Figure 2, below) and another showing FSC vs. fluorescence (see Figure 3, below).

Figure 2 A whole blood sample was processed according to the basic protocol and applied to an Attune® Acoustic Focusing Cytometer (Life Technologies) equipped with a 488-nm argon-ion laser. The forward scatter (FSC) and side scatter (SSC) plot shows the selected granulocyte population. This region was used for gating in Figure 3B and Figure 4.

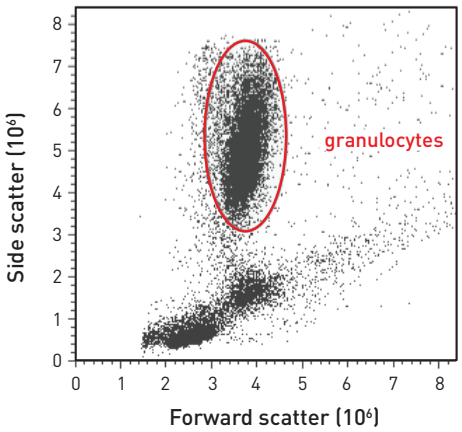
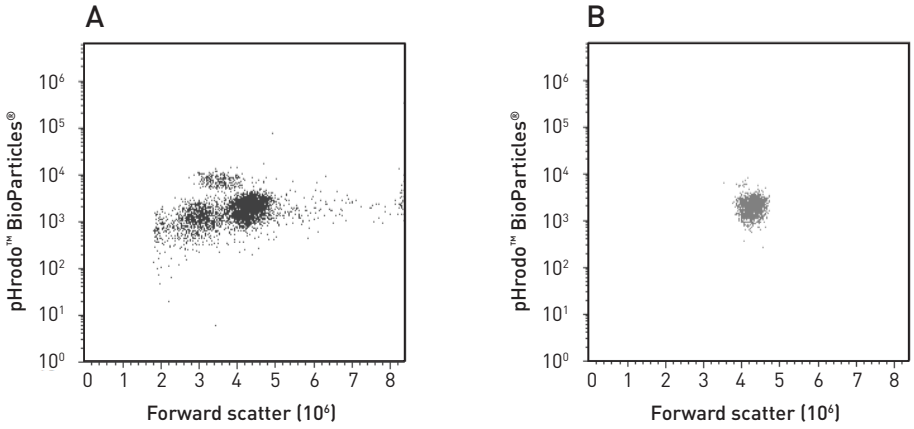


Figure 3 (A) The same sample as shown in Figure 2 with the pHrodo™ fluorescence signal on the Y-axis, demonstrating the fluorescent intensities of the various white blood cell populations. **(B)** The fluorescence signal of the gated granulocyte population alone.



4.3 Apply a negative control sample (whole blood aliquot with no pHrodo™ BioParticles® conjugates that went through the lysis procedure, tubes 1 or 2), and set linear FSC and SSC voltages to locate the white blood cell scatter pattern as in Figure 2. For FSC vs. fluorescence, set fluorescence on a log scale and set events in the lowest decade. Adjust threshold(s) to eliminate debris.

4.4 Draw a region around the granulocyte population as shown in Figure 2, and apply the gate as in Figure 3. Adjust the fluorescence PMT (photomultiplier tube) voltage, if necessary.

4.5 Apply the positive control sample (tube 3) or an experimental sample incubated at 37°C to locate the granulocyte population as in Figure 2, apply to Figure 3, and observe the difference in fluorescence between the negative control sample and the positive control or experimental sample. Adjust the fluorescence PMT voltage, if necessary.

4.6 Apply the positive control sample (tube 3, which includes all reagents, but is incubated on ice) and make sure the events for FSC vs. SSC are on scale.

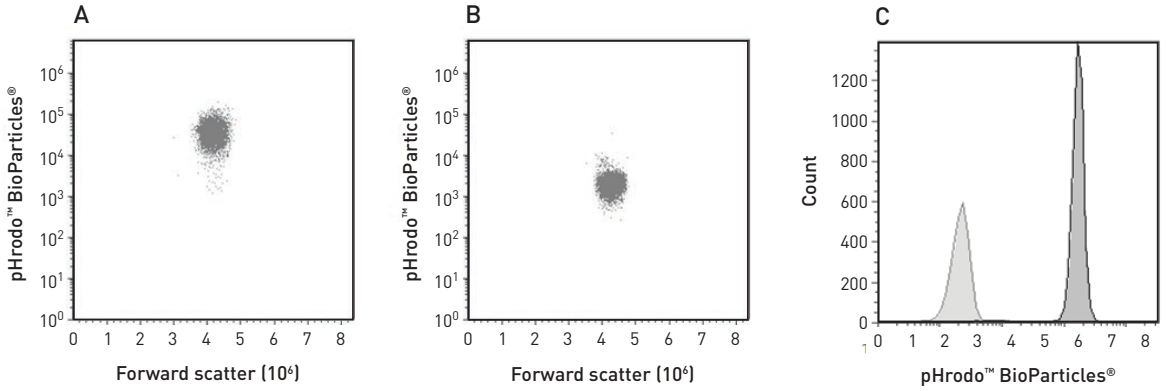
4.7 Once the settings are adjusted, apply experimental samples (incubated on ice and at 37°C), and collect the appropriate number of events.

4.8 Perform gating on FSC vs. SSC and FSC vs. fluorescence as described above.

4.9 Generate and record statistics for experimental samples incubated on ice and at 37°C.

You should observe good white blood scatter pattern on FSC vs. SSC, greater than 96% phagocytosing neutrophils (for normal whole blood samples), and greater than 5-fold increase in fluorescence signals between the control and experimental samples. The desired statistics may be % phagocytosing neutrophils, granulocytes, or monocytes, or the fluorescence signal of various samples. See Figure 4, below, for an example of expected results.

Figure 4 (A) The same figure as Figure 3 showing a population of granulocytes that have phagocytosed pHrodo™ *E. coli* BioParticles® conjugates. **(B)** A control sample in which phagocytosis was inhibited by incubating on ice, demonstrating the lack of fluorescence because pHrodo™ BioParticles® conjugates were not in acidic phagosome compartments and any attached particles show very low fluorescence. **(C)** A histogram overlay of (A) and (B) showing the fluorescence separation between the experimental and negative control sample.



DNA Staining

The DNA staining of samples is performed just prior to flow cytometry analysis and may improve separating debris from events containing DNA.

5.1 Dilute the DNA stain of choice to 100 nM in Wash Buffer (Component C) for the appropriate number of samples (e.g., SYTO® 9, Cat. no. S34854) at a final concentration of 100 nM).

5.2 Resuspend the samples (from step 3.8, page 4) with 0.5 mL of DNA stain (e.g., SYTO® 9, Cat. no. S34854) in Wash Buffer (from step 5.1).

5.3 Incubate the samples for 15 minutes in the dark at room temperature.

5.4 Analyze the samples on the flow cytometer using the recommended emission filters and ensure that the emission spectra are appropriately separated.

Antibody Labeling

Antibody labeling may be performed just prior to flow cytometry analysis to identify subpopulations of white blood cells or label other relevant surface markers.

6.1 Resuspend the samples in antibody labeling buffer appropriate for the antibody (not provided).

6.2 Add the antibody to samples at the appropriate dilution.

6.3 Incubate the samples for the appropriate time and temperature as recommended by the antibody manufacturer.

6.4 Wash the samples with labeling buffer and centrifuge at 350 × *g*.

6.5 Resuspend the samples in 0.5 mL of Wash Buffer (Component C) or the prepared DNA staining solution (from step 5.1). Perform DNA staining as described above.

6.6 Analyze the samples on the flow cytometer using the recommended emission filters and ensure that the emission spectra are appropriately separated.

Opsonization

Our studies indicate that pHrodo™ *E. coli* and *S. aureus* BioParticles® conjugates do not require pre-opsonization for optimal uptake when used with normal whole blood samples. The serum in the whole blood sample is sufficient for opsonization. However, if you are using isolated white blood cells (WBC) or your sample requires the use of an opsonizing reagent, we recommend using the *Escherichia coli* BioParticles® opsonizing reagent (Cat. no. E2870) or *Staphyloccus aureus* BioParticles® opsonizing reagent (Cat. no. S2860). For instructions on using the BioParticles® opsonizing reagents, refer to the instructions supplied with the reagent.

Troubleshooting

Problem	Cause	Solution
Poor white blood cell scatter	Incomplete lysis	• Be sure to perform lysis for 5 minutes at room temperature. Lysis is complete when the solution is translucent. • Make sure the Lysis Buffer A and Buffer B are equilibrated to room temperature before use.
	Incorrect instrument set-up	Check the voltage and particle size setting on the flow cytometer.
Low fluorescence of particles or low percent phagocytosing cells	Incorrect assay conditions	It may be a normal result due to the condition of the serum or cells. If the uptake is expected to be high, then be sure to: • Perform the phagocytosis assay at 37ºC for at least 15 minutes. You may need to optimize incubation time for opsonized particles. • Resuspend the lyophilized product in 2.2 mL Buffer B to obtain sufficient particle concentration in a 20 µL aliquot for a minimum 20:1 particle-to-phagocytosing cell ratio.
	Phagosome pH maybe below the pKa of the pHrodo™ dye	The pKa of the pHrodo™ dye is ~7.3. If the pH of the intracellular compartment is higher than 7.3, there may not be sufficient fluorescence signal to detect.

References

1. J Immunol Methods 60, 115 (1983); **2.** J Immunol Methods 162, 1 (1993); **3.** Current Protocols in Cytometry 9.19.1 (2002).

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

Cat. no.	Product Name	Unit Size
A10025	pHrodo™ Red *E. coli* BioParticles® phagocytosis kit *for flow cytometry* *100 tests*	1 kit
P35381	pHrodo™ Green *E. coli* BioParticles® phagocytosis kit *for flow cytometry* *100 tests*	1 kit
P35382	pHrodo™ Green *S. aureus* BioParticles® phagocytosis kit *for flow cytometry* *100 tests*	1 kit
Related Products		
A10010	pHrodo™ *S. aureus* BioParticles® conjugate for phagocytosis	5 x 2 mg
A10026	pHrodo™ phagocytosis Particle Labeling kit *for flow cytometry* *100 tests*	1 kit
B7277	Bacteria Counting Kit *for flow cytometry*	1 kit
C36950	CountBright™ absolute counting beads *for flow cytometry* *100 tests*	5 mL
E2870	*Escherichia coli* BioParticles® opsonizing reagent	1 Unit
F2902	Fc OxyBURST® Green assay reagent *25 assays* *3 mg/mL*	500 µL
L34856	LIVE/DEAD® BacLight™ Bacterial Viability and Counting Kit *for flow cytometry* *100 assays*	1 kit
O13291	OxyBURST® Green H_2HFF BSA *special packaging*	5 x 1 mg
P35361	pHrodo™ *E. coli* BioParticles® conjugate for phagocytosis	5 x 2 mg
P35364	pHrodo™ Red *Zymosan A* BioParticles® conjugate *for phagocytosis*	5 × 1 mg
P35365	pHrodo™ Green *Zymosan A* BioParticles® conjugate *for phagocytosis*	5 × 1 mg
P35366	pHrodo™ Green *E. coli* BioParticles® conjugate *for phagocytosis*	5 × 2 mg
P35367	pHrodo™ Green *S. aureus* BioParticles® conjugate *for phagocytosis*	5 × 2 mg
P36600	pHrodo™, Succinimidyl ester	1 mg
S2860	*Staphlyococcus aureus* BioParticles® opsonizing reagent	1 unit
S7572	SYTO® Green Fluorescent Nucleic Acid Stain Sampler Kit #1 *SYTO® dyes 11-16* *50 µL each*	1 kit
S34854	SYTO® 9 Green Fluorescent nucleic acid stain *5 mM solution in DMSO*	100 µL

A large variety of antibodies for flow cytometry is available from Life Technologies. For details, visit **www.lifetechnologies.com/flowcytometry**.

Purchaser Notification

Corporate Headquarters
5791 Van Allen Way
Carlsbad, CA 92008
USA
Phone: +1 760 603 7200
Fax: +1 760 602 6500
Email: techsupport@lifetech.com

European Headquarters
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF
UK
Phone: +44 141 814 6100
Toll-Free Phone: 0800 269 210
Toll-Free Tech: 0800 838 380
Fax: +44 141 814 6260
Tech Fax: +44 141 814 6117
Email: euroinfo@invitrogen.com
Email Tech: eurotech@invitrogen.com

Japanese Headquarters
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Japan
Phone: +81 3 5730 6509
Fax: +81 3 5730 6519
Email: jpinfo@invitrogen.com

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These high-quality reagents and materials must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Read the Safety Data Sheet provided for each product; other regulatory considerations may apply.

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