pHrodo™ Phagocytosis Particle Labeling Kit for Flow Cytometry

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
Lysis Buffer A, Component A	10 mL	• 2–6˚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™, succinimidyl ester (MW = ~650), Component D	1 mg lyophilized product	• 2–6˚C • Desiccate • Protect from light	
DMSO, Component E	0.5 mL	• 2–6˚C • Do not freeze	
0.1 M Sodium bicarbonate, pH 9.3, Component F	50 mL	• 2–6˚C • Do not freeze	
Number of assays: 100 assays when using 100 µL whole blood sample volume per assay.			
Approximate pHrodo™ dye fluorescence excitation and emission maxima: 560/585 nm. Compatible with 488 nm argon-ion laser excitation.			

Introduction

The pHrodo™ Phagocytosis Particle Labeling Kit for flow cytometry allows rapid labeling of biological particles, such as bacteria and assessment of phagocytic activity in whole blood samples by flow cytometry. The pHrodo™ dye-based assay provides 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 kit includes reagents required for labeling the bacteria with the pHrodo™ dye, 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.

pHrodo™ dye is a novel, fluorogenic dye that dramatically increases in fluorescence as the pH of its surroundings becomes more acidic (Figure 1). This amine-reactive succinimidyl ester (SE) form of the dye has a pKa of ~7.3 in solution, which shifts to about ~6.5 upon conjugation to the K-12 strain of *E. coli*. pHrodo™ dye is extremely sensitive to its local environment, therefore the pH response in your system needs to be determined empirically. 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] The optimal absorption and

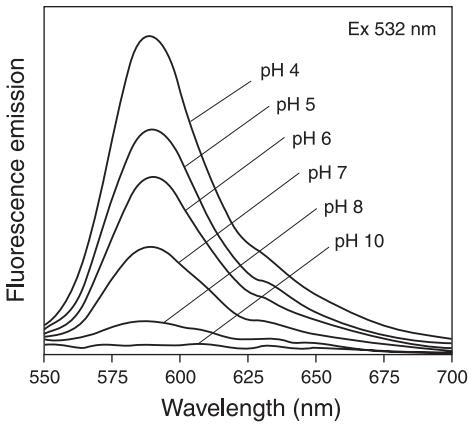

Figure 1. The fluorescence emission spectra of pHrodo™ dye-labeled *E. coli* were measured in a series of 50 mM potassium phosphate buffers ranging in pH from 4 to 10. The *E. coli* were at a concentration of 0.1 mg/mL, and the readings were made on a Hitachi F4500 fluorometer using an excitation wavelength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

fluorescence emission maxima of the pHrodo™ dye-labeled particles is approximately 560 nm and 585 nm, respectively (Figure 1), however, the fluorophore is readily excited with the 488 nm argon-ion laser installed on most flow cytometers.

A general protocol for using the amine-reactive, succinimidyl ester form of the pHrodo™ dye, to label your own sample of lyophilized or freshly prepared bacteria, or to label other particles with available amine groups is described below. We recommend using this procedure as a starting point. Based on your initial results, you may need to optimize the procedure by varying the incubation time or concentration ratio of fluorophore to particle.

A phagocytosis assay using whole blood samples with the pHrodo™ dye-labeled bacteria and the reagents included with the kit is also described. The Lysis Buffer A (Component A) supplied with the kit is a proprietary formulation that stops phagocytic uptake and selectively eliminates red blood cells from the samples. Finally the phagocytic cells are resuspended in Wash Buffer (Component C) which allows direct analysis using flow cytometry or further DNA staining and antibody labeling (e.g., anti-CD45) using protocols described in this manual.

Before You Begin

Materials Required but Not Provided

- *E. coli* lyophilized powder, or freshly prepared bacteria at a known concentration (mg/mL or particles/mL). A labeling reaction using *E. coli* (in mg/mL) is described in the protocol below which can also be used with any bacteria or other particles with available amine groups
- 10 mL 100% methanol
- Particle counting kit if working in particles/mL (Invitrogen Cat. no. B7277, C36950)
- 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

Collecting the Whole Blood Sample

1.1. 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. Do not use any other anticoagulants except heparin for blood collection.

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

Buffers

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

Caution

Be sure to use appropriate techniques and personal protective equiment when handling whole blood samples. Dispose of blood samples as biohazardous waste.

Experimental Protocol

pHrodo™ Labeling Reaction

This protocol describes the labeling of 60 mg *E. coli* bacteria with one vial aliquot of amine-reactive pHrodo™ succinimidyl ester (SE) dye. Briefly, the bacteria are prepared at 20 mg/mL in the sodium bicarbonate (Compound F), and the dye is prepared at 10 mM in DMSO. The dye is then diluted into the bacterial suspension for a final dye concentration of 0.5 mM in the labeling reaction. The final dye concentration can be adjusted for your particular needs, but we have found 0.5 mM reactive dye to be optimal for *E. coli*. At higher concentrations of dye (>2 mM), we have observed that the pH dependent fluorescence response can be blunted by overlabeling. The amine-labeling reaction then proceeds for 45 minutes at room temperature. The bacteria are washed with a series of centrifugation steps using Wash Buffer (Component C) and methanol, and finally resuspended in Buffer B (Component B), or the buffer of your choice. If handled with clean techniques, the pHrodo™ dye-labeled particles may be stored in solution at 4°C for several months.

Phagocytosis Assay Protocol

pHrodo™ dye is a non- or weakly fluorogenic fluorophore when in a basic environment but is highly fluorescent in an acidic environment such as the phagosome.

The pHrodo™ dye-based phagocytosis assay investigates 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™ dye-labeled particles 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 and Buffer B followed by centrifugation and washing. The final cell pellet containing white blood cells is resuspended in Wash Buffer and is ready for analysis using a flow cytometer equipped with a 488 nm argon ion laser. The nucleated white blood cells 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 emission filter appropriate for R-phycoerythrin, such as a 585 nm band pass filter.

Preparing the Bacteria

2.1. Weigh out 60 mg of lyophilized *E. coli* or equivalent bacteria into a 15 mL screw-cap tube. Resuspend at 20 mg/mL using 3 mL 100 mM sodium bicarbonate buffer (Component F), and aliquot 750 µL each into four 1.5 mL microcentrifuge tubes.

Note: Use appropriate personal protection equipment to avoid inhalation hazards.

2.2. Centrifuge the bacteria at >15,000 × g in a microcentrifuge for 60 seconds.

2.3. Resuspend the sample in each tube in Component F at 20 mg/mL or 750 µL for each tube. The bacteria are now ready for the dye. Use the bacteria within 24 hours or within the time determined by your laboratory protocols.

Preparing the Dye and Labeling the Bacteria

3.1. Resuspend 1 mg amine-reactive pHrodo™ SE (Component D) in 150 µL DMSO (Component E) to obtain a solution of approximately 10 mM. Use the 10 mM dye stock solution as soon as possible on the day it is prepared. Discard any unused stock of reactive dye.

3.2. Dilute the DMSO stock solution into the *E. coli* for a final concentration of 0.5–1 mM, i.e., add 37.5 µL of dye to each of the four tubes of bacteria from step 2.3 for 0.5 mM labeling. Again, for individual use, more or less dye may be necessary, depending on the target.

3.3. Incubate the tubes at room temperature for 45 minutes, protected from light.

Removing Unincorporated Dye

4.1. After the 45-minute incubation, add 750 µL Wash Buffer (Component C) and centrifuge the tubes at >15,000 × g in a microcentrifuge for 60 seconds. Immediately remove the tubes and carefully aspirate the supernatant.

4.2. Carefully resuspend the bacteria in 1 mL 100% methanol.

We recommend disaggregating the pellet prior to adding the methanol by carefully flicking the tube, as the aggregates that normally form in this solution can clog the opening of an unmodified pipette tip. Minimize any loss of the particulate matter during this and subsequent steps, as losses have an impact on your final yield.

4.3. Add an additional 0.5 mL methanol to each tube and vortex the samples for 30 seconds.

4.4. Centrifuge the samples for 60 seconds at >15,000 × g. Carefully aspirate the supernatant from each and add 1 mL Wash Buffer (Component C) to each tube. Carefully resuspend the sample in each tube, add 0.5 mL Wash Buffer, and vortex for 30 seconds to evenly disperse the bacteria.

4.5. Repeat step 4.4 one more time.

4.6. Resuspend the bacteria in Buffer B (Component B) as described on the next page for the phagocytosis assay (step 6.1) or in the buffer of your choice. If handled with clean techniques, the pHrodo™ dye-labeled particles may be stored in solution at 4ºC for several months.

Determining the pH Response Range of the Conjugate

5.1. Resuspend some of your pHrodo™ dye-labeled particles at a final concentration of 1 mg/mL in 500 µL each of a series of buffers with pH adjusted between pH 4.0 and pH 8.0.

5.2. Pipette 100 µL of each in quadruplicate into a 96-well plate and measure the fluorescence in a plate reader.

5.3. Construct average fluorescence values for each pH data point, and plot the pH versus average fluorescence. You can also calculate the ratio of the average fluorescence measured at pH 4.0 versus the average fluorescence measured at pH 8.0. Fold increases of 8 or higher are favorable for cellular experiments.

Preparing the Solution of pHrodo™ labeled Bacteria

6.1. Add Buffer B (Component B) to the vial containing lyophilized pHrodo™ dye-labeled bacteria from step 4.6 to obtain a 5 mg/mL pHrodo™ labeled particles suspension.

Alternatively, perform a counting procedure (Invitrogen Cat. no. B7277 or C36950) to determine particles/mL. Prepare the particles to a final concentration such that 2 x 10^7 particles are added to 100 µL of a whole blood sample (e.g., 2 x 10^9 particles/mL, use 20 µL)

For optimal assay conditions, be sure to have >20:1 pHrodo™ particles to phagocytosing cell ratio.[3]

6.2. Vortex for 1 minute, then sonicate the vial of particles for 5 minutes until all the fluorescent particles are homogenously dispersed. Check under a microscope, if necessary, using a buffer with pH <4.0.

6.3. Store the pHrodo™ particles solution on ice for ~10 minutes prior to use.

Performing Phagocytosis Assay

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

7.1. Aliquot whole blood (step 1.2) and pHrodo™ dye-labeled particles (step 6.3) into flow cytometry tubes as described in the table below and vortex briefly:

Tube	Name	Whole Blood Sample	pHrodo™ dye-labeled Particles
1	Negative control on ice	100 µL	--
2	Negative control at 37°C	100 µL	--
3	Positive control on ice	100 µL	>2 x10^7 particles
4	Positive control at 37°C	100 µL	>2 x10^7 particles

For each experimental sample, prepare two tubes containing 100 µL whole blood sample and >2 x 10^7 pHrodo™ dye-labeled particles.

7.2. 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 at 37°C water bath for 15 minutes. After the incubation, remove all tubes from the water bath and place on ice.

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

7.4. Add 1 mL Buffer B (Component B) to samples, vortex briefly, and incubate at room temperature for 5 minutes.

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

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

7.7. Resuspend cell pellets with 1 mL Wash Buffer (Component C).

7.8. Repeat steps 7.5-7.6 one more time. Resuspend the cell pellets in 0.5 mL Wash Buffer for flow cytometry analysis.

At this point, you can proceed to analyzing results using flow cytometry (section 8) or perform additional staining reactions such as DNA staining (section 9) or antibody labeling (section 10).

Flow Cytometer Set-up and Analysis

8.1. Perform analysis using a flow cytometer equipped with a 488 nm argon-ion laser using an R-phycoerythrin emission filter. If additional staining is performed on the samples, ensure the emission spectra are appropriately separated, and use recommended emission filters.

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

8.3. Apply a negative control sample (whole blood aliquot with no pHrodo™ dye-labeled particles 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 PMT (photomultiplier tube) voltage on a log scale and set events in the lowest decade. Adjust threshold(s) to eliminate debris.

8.4. Draw a region around the granulocyte population as in Figure 2, and apply the gate as in Figure 3. Adjust the fluorescence PMT voltage, if necessary.

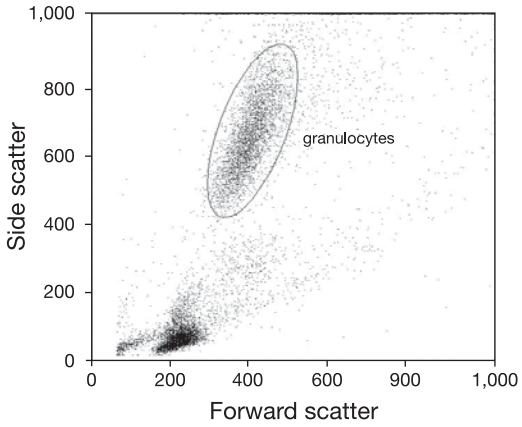

Figure 2. A whole blood sample was processed according to the basic protocol and applied to a FACSCalibur™ (BD Biosciences, San Jose, CA) equipped with a 488 nm argon-ion laser. The forward scatter (FSC) and side scatter (SSC) plot shows the selected granulocyte population. This region was used for gating in Figure 3B and Figure 4.

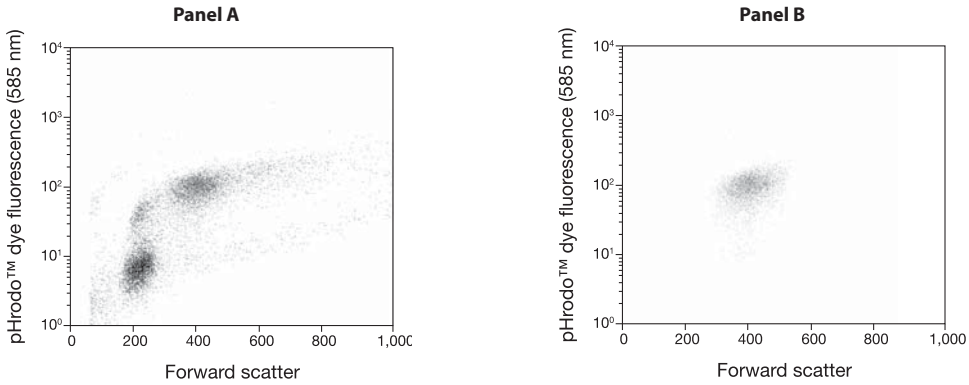

Figure 3. (A) The same sample as shown in Figure 2 with the pHrodo™ fluorescence signal on the Y-axis demonstrates the fluorescent intensities of the various white blood cell populations. (B) The fluorescence signal of the gated granulocyte population alone.

8.5. 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.

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

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

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

8.9. 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 granulocytes (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 for an example of expected results.

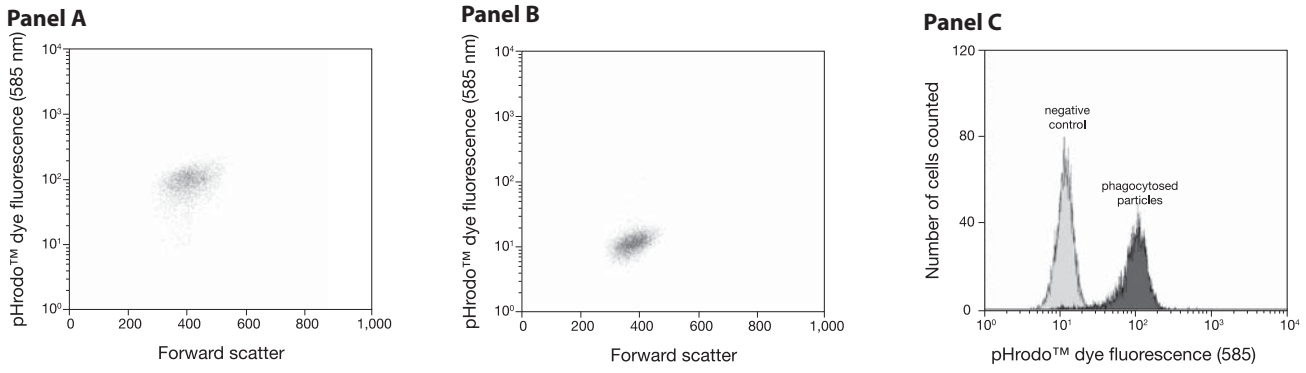

Figure 4. (A) The same figure as Figure 3 showing a population of granulocytes that have phagocytosed pHrodo™ *E. coli* BioParticles® conjugates. (B) A control sample in which phagocytosis was inhibited by incubating on ice, demonstrating the lack of fluorescence because pHrodo™ *E. coli* BioParticles® 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.

9.1. Dilute DNA stain of choice to 100 nM in Wash Buffer (Component C) for the appropriate number of samples (e.g., SYTO® 9 final concentration of 100 nM).

9.2. Resuspend the samples from step 7.8 with 0.5 mL DNA stain in Wash buffer from step 9.1.

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

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

Antibody Labeling

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

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

10.2. Add the antibody to samples at the appropriate dilution.

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

10.4. Wash samples with labeling buffer and centrifuge samples at 350 × g.

10.5. Resuspend samples in 0.5 mL Wash Buffer (Component C) or prepared DNA staining solution (step 9.1). Perform DNA staining as described above.

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

Opsonization

Our studies indicate that pHrodo™ dye-labeled particles may require pre-opsonization for optimal uptake when used with normal whole blood samples. The serum in the whole blood sample provides some level of opsonization and the assay can assess the efficacy of those serum factors. However, if you wish to evaluate the cellular phagocytic function as opposed to serum opsonizing efficacy, pre-opsonize the particles using the optimal opsonizing reagent for the particle.

Troubleshooting

Problem	Cause	Solution
Poor labeling efficiency	pHrodo™ dye-to-particle ratio too high or low	Perform labeling titration prior to finalizing the labeling concentration using the pHrodo™, succinimidyl ester available separately (Invitrogen Cat. no. P36600).
	Insufficient incubation time	Perform a time course for the labeling reaction to determine the optimal incubation time.
	Amine groups are not available for labeling	Most bacteria are suitable for labeling. If you are using latex beads for labeling, ensure that the latex beads have amine groups available for labeling. **Note:** We have observed that zymosan particles (carbohydrate from cell wall of yeast) are not suitable for labeling.
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 uptake incubation time for your labeled particles. • Check the particle count and maintain a minimum of 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 #	Product Name	Unit Size
A10026	pHrodo™ Phagocytosis Particle Labeling kit *for flow cytometry* *100 tests*	1 kit
A10025	pHrodo™ *E. coli* BioParticles® phagocytosis kit *for flow cytometry* *100 tests*	1 kit
A10010	pHrodo™ *S. aureus* BioParticles® conjugate for phagocytosis	5 x 2 mg
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
P36600	pHrodo™, Succinimidyl ester	1 mg
S2859	*Staphylococcus aureus* (Wood strain without protein A) BioParticles®, unlabeled	100 mg
S34854	SYTO® 9 Green Fluorescent nucleic acid stain *5 mM solution in DMSO*	100 µL
S7572	SYTO® Green Fluorescent Nucleic Acid Stain Sampler Kit #1 *SYTO® dyes 11-16* *50 µL each*	1 kit

A large variety of antibodies for flow cytometry is available from Invitrogen. For details, visit www.invitrogen.com/flowcytometry.

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