LIVE/DEAD® Bac*Light™ Bacterial Viability and Counting Kit (L34856)*

Quick Facts

Storage upon receipt:

- 2–6°C
- Do not freeze Component C
- Protect from light

Introduction

The LIVE/DEAD® *Bac*Light™ Bacterial Viability and Counting Kit allows researchers to reliably distinguish and quantitate live and dead bacteria with the aid of a flow cytometer, even in a mixed population containing a range of bacterial types. This kit utilizes a mixture of two nucleic acid stains — green-fluorescent SYTO® 9 dye and red-fluorescent propidium iodide — for viability determinations, and a calibrated suspension of microspheres for accurate sample volume measurements. With the appropriate mixture of the SYTO 9 and propidium iodide stains, bacteria with intact cell membranes fluoresce bright green, whereas bacteria with damaged membranes exhibit significantly less green fluorescence and often also fluoresce red. The cell type and the gram character influence the amount of red-fluorescent staining exhibited by dead bacteria. Both the SYTO 9 and propidium iodide stains are efficiently excited by the 488 nm–spectral line of the argon-ion laser found in many flow cytometers, and their nucleic acid complexes can be detected in the green and red channels, respectively; the background remains virtually nonfluorescent.

The calibrated suspension of microspheres serves as a reference standard for sample volume. The size and fluorescence of the microspheres have been carefully chosen to ensure that they will be clearly distinguishable from any stained bacteria population in a fluorescence-versus-side scatter cytogram. A bacterial culture is simply stained with the optimal mixture of SYTO 9 dye and propidium iodide, and then a fixed number of microspheres are added before analyzing the sample on a flow cytometer. Live and dead bacteria and the microspheres are all easily distinguished in a plot of fluorescence versus side scatter (Figure 1). The concentration of both the live bacteria and the dead bacteria can then be determined from the ratio of bacterial events to microsphere events in the cytogram (Figure 2).

Materials

Contents

- **SYTO 9 nucleic acid stain** (Component A), 200 µL of a 3.34 mM solution in DMSO
- **Propidium iodide** (Component B), 200 µL of a 20 mM solution in DMSO
- **Microsphere standard** (Component C), 1.0 mL of 6.0 µm diameter microspheres at a concentration of 1.0×10^8 beads/mL in deionized water containing 2 mM sodium azide

Number of Tests Possible

At the recommended reagent dilutions and volumes, this kit contains sufficient material to perform approximately 100 individual assays by flow cytometry.

Storage and Handling

Store the DMSO stock solutions (Components A and B) at ≤6°C protected from light. Allow reagents to warm to room temperature and centrifuge briefly before opening the vials. After sampling, reseal the vials tightly. Store the microsphere standard (Component C) at 2–6°C. DO NOT FREEZE THE MICROSPHERE STANDARD. When stored properly, these kit components are stable for at least 1 year.

Caution: Propidium iodide and SYTO 9 stain bind to nucleic acids. Propidium iodide is a potential mutagen; we have no data addressing the mutagenicity or toxicity of the SYTO 9 stain. The DMSO stock solutions should be handled with particular care, as DMSO is known to facilitate the entry of organic molecules into tissues. Please dispose of the stains and microspheres in compliance with all pertinent local regulations.

General Considerations

Tested Bacterial Species

The LIVE/DEAD *Bac*Light bacterial viability assay has been tested at Molecular Probes on logarithmically growing cultures of the following bacterial species: *Bacillus cereus*, *B. subtilis*, *Clostridium perfringens*, *Escherichia coli*, *Klebsiella pneumoniae*, *Micrococcus luteus*, *Mycobacterium phlei*, *Pseudomonas aeruginosa*, *P. syringae*, *Salmonella oranienburg*, *Serratia marcescens*, *Shigella sonnei*, *Staphylococcus aureus*, and *Streptococcus pyogenes*. All of these species have shown a good correlation between

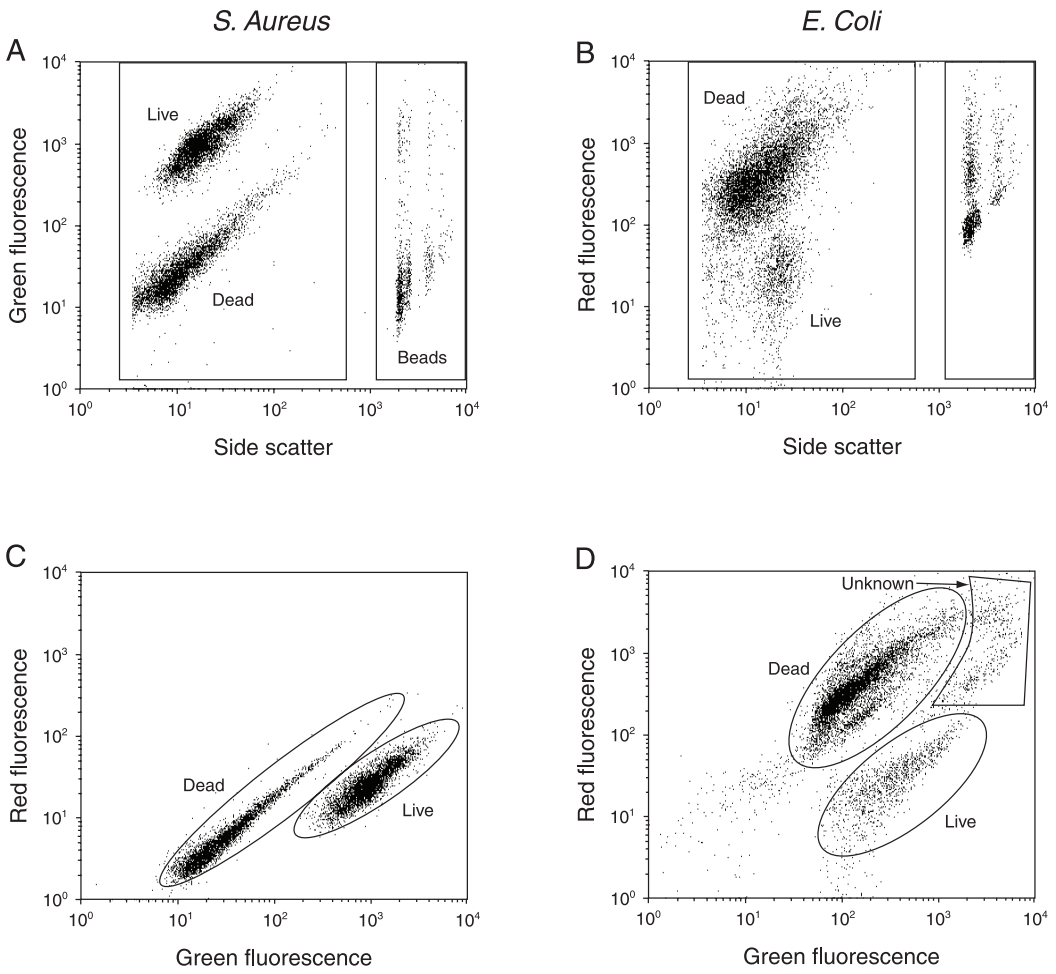

Figure 1. Analysis of bacterial cultures using the LIVE/DEAD *Bac*Light Bacterial Viability and Counting Kit. Suspensions of live (untreated) and dead (alcohol-treated) *Staphylococcus aureus* (Panels A and C) and *Escherichia coli* (Panels B and D) were stained and analyzed by flow cytometry according to the kit protocol. Using green or red fluorescence versus side scatter cytogram (Panels A and B), the bacterial populations and the bead populations were gated (left and right boxes, respectively). Events in the bacteria region of each cytogram are also displayed in red fluorescence versus green fluorescence cytograms (Panels C and D). Live and dead bacteria/mL can be calculated from either the fluorescence versus side scatter cytogram or the green fluorescence versus red fluorescence cytogram, depending on which one shows the best separation of the live and dead populations. The position of the live and dead populations in these cytograms may be dependent on cell type and gram character. Some samples may exhibit events that fall outside the defined regions and should be evaluated appropriately (e.g., see Panel D).

the results obtained with the LIVE/DEAD *Bac*Light bacterial viability assay and those obtained with standard plate counts. In addition, we have received favorable reports from researchers who have used this assay with *Agrobacterium tumefaciens*, *Edwardsiella ictaluri*, *Eurioplasma eurilytica*, *Lactobacillus* sp., *Mycoplasma hominus*, *Propionibacterium* sp., *Proteus mirabilis*, and *Zymomonas* sp.

Criteria for Bacterial Viability

Viability is not easily defined in terms of a single physiological or morphological parameter, and therefore a single viability assay may introduce specific biases into the experiment. Exponentially growing cultures of bacteria typically yield results with this LIVE/DEAD *Bac*Light bacterial viability assay that correlate well with growth assays in suitable liquid or solid nutrient media, a common criterion for bacterial viability. Under certain conditions, bacteria having compromised membranes may recover and reproduce, even though such bacteria may be scored as "dead" in this assay. Conversely, some bacteria with intact membranes may be unable to reproduce in nutrient medium, yet be scored as "live".[5] Combining several different measures of viability, such as membrane permeability, enzyme activity, and redox potential, offers a more thorough assessment of bacterial viability and eliminates the inherent limitations of any single viability assay.

Optimization of Staining

The two dye components provided with the LIVE/DEAD *Bac*Light Bacterial Viability and Counting Kit have been balanced so that a 1:1 mixture works well with a broad spectrum of bacterial types. Occasionally, however, the proportions of the two dyes must be adjusted for optimal discrimination of live and dead bacteria. This kit provides separate solutions of the SYTO 9 and propidium iodide stains to accommodate fine-tuning of the dye mixture so that optimal staining can be achieved under a variety of conditions. To optimize the dye ratio, see steps 4.1–4.8.

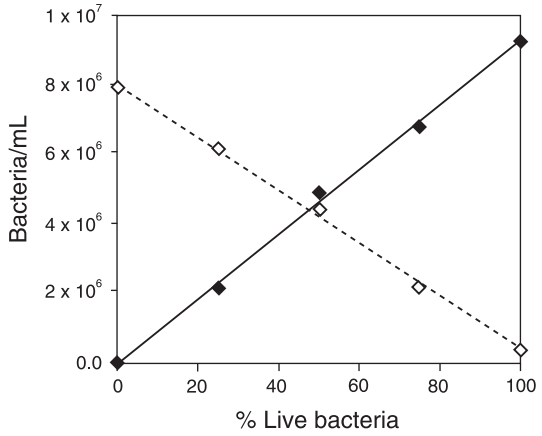

Figure 2. Best-fit linear regression analysis generated using the LIVE/DEAD *Bac*Light Bacterial Viability and Counting Kit. Live (untreated, solid line (r^2=0.9982)) and dead (alcohol-treated, dashed line (r^2=0.9974)) *Staphylococcus aureus* were mixed at various live:dead ratios (Table 1). Mixtures were stained according to the kit protocol and analyzed in triplicate by flow cytometry. Values of bacteria/mL were calculated according to the equation in step 3.4; the mean values are shown above. This experiment may be performed to determine optimal dye concentrations, to practice the cell-staining procedure, or to generate a standard curve for unknown samples.

Experimental Protocols

These experimental protocols are provided as examples to guide researchers in the development of their own bacterial staining procedures. Researchers at Molecular Probes have used these procedures and found them to be reliable for both gram-positive and gram-negative bacteria.

Preparing Bacterial Suspensions

The following protocol describes the steps for preparing live-cell (untreated) and dead-cell (alcohol-treated) bacterial suspensions, which will be used to adjust the flow cytometer as well as to optimize the dye ratio, if necessary. The bacterial cultures being assayed should be prepared exactly as described for the live-cell bacterial suspension in steps 1.1–1.6. Because a flow cytometer is used to detect the fluorescent signals from the stained bacteria, this counting assay can be used on a wide range of bacterial densities; it is generally not necessary to concentrate or dilute a log-phase culture or to take initial OD readings.

When growing bacterial cultures, use a low-phosphate growth medium and late–log phase cultures. The SYTO 9 and propidium iodide dyes can bind nucleic acids and other components from the medium in unpredictable ways, resulting in unacceptable variations in staining. A single wash step (see step 1.5) is usually sufficient to remove significant traces of interfering components from the bacterial suspension. Phosphate wash buffers are not recommended because they appear to decrease staining efficiency.

1.1 Centrifuge two ~1 mL samples of the bacterial culture in a microcentrifuge at 10,000 × g for 1–3 minutes to pellet the cells.

1.2 Remove the supernatants. Resuspend one pellet in 1 mL of 0.85% NaCl or appropriate buffer (for the live-cell suspension) and the second pellet in 1 mL of 70% isopropyl alcohol (for the dead-cell suspension).

1.3 Incubate the samples at room temperature for 30–60 minutes, mixing every 15 minutes.

1.4 Pellet both samples by centrifugation at 10,000 × g for 1–3 minutes.

1.5 Wash both samples in 1 mL of 0.85% NaCl or appropriate buffer and pellet again by centrifugation at 10,000 × g for 1–3 minutes.

1.6 Resuspend both samples in 1 mL of 0.85% NaCl or appropriate buffer.

1.7 Alternatively, if the untreated cells need to remain in optimal growing conditions, only prepare the dead-cell control as described in step 1.2. For the live-cell control, aliquots of bacteria may be washed before use or transferred directly from liquid cultures.

Staining the Bacteria

The following steps describe the method for staining one bacterial suspension as prepared in steps 1.1–1.6. The protocol should be scaled up as needed for additional samples.

2.1 Aliquot 977 µL of 0.85% NaCl or appropriate buffer into a flow cytometry analysis tube. Note: Aliquot 987 µL if the microsphere standard is not to be included.

2.2 Add 1.5 µL of 3.34 mM SYTO 9 nucleic acid stain (Component A) and 1.5 µL of 30 mM propidium iodide (Component B) to the flow cytometry tube from step 2.1.

2.3 Add 10 µL of a bacterial suspension from step 1.6 to the flow cytometry tube containing the staining solution. If the bacterial suspension is extremely turbid, perform a dilution prior to adding the bacteria to the staining solution.

2.4 Incubate the sample for 15 minutes at room temperature protected from light.

2.5 Thoroughly resuspend the microsphere standard (Component C) by vortexing the suspension, followed by sonication in a water-bath for 5–10 minutes. Add 10 µL of the microsphere suspension to the stained cell sample, mix well and analyze by flow cytometry. The total volume of the sample in the flow cytometry analysis tube should be 1000 µL for accurate counting.

Preparing Single-Color Controls for Instrument Adjustment

Four single-color controls — two live-cell and two dead-cell bacterial suspensions — are required for setting up the flow cytometer; the microsphere standard should be added to at least one of the four tubes. These samples are prepared exactly as described above in steps 2.1–2.5, except stain one live-cell and one dead-cell bacterial suspension with the SYTO 9 stain only and one live-cell and one dead-cell suspension with the propidium iodide stain only.

Analyzing Stained Bacteria by Flow Cytometry

Instrument capabilities vary considerably, but the techniques and parameters established here should aid considerably in setting up similar analyses in the majority of flow cytometers now in use in research and clinical environments.

3.1 Stained bacteria can be assayed in a flow cytometer equipped with a laser emitting at 488 nm. Fluorescence is collected in the

green and red channels; filters used for detecting fluorescein and the Texas Red® dye, respectively, are generally suitable. The forward scatter, side scatter, and fluorescence should be collected with logarithmic signal amplification.

3.2 Instrument adjustments are especially critical for detecting relatively small particles such as bacteria. To minimize electronic noise, use the following procedure for instrument setup. The single-color controls prepared as described above can be used to locate bacterial populations and determine compensation settings. Acquire signals with the amplifiers set to logarithmic amplification. Use the side scatter as the parameter for setting the acquisition trigger. Set the amplification of the signals from forward and side scatter so that the bacteria are in the middle of the data space. Adjust the trigger level (also named "threshold level" on some instruments) to minimize electronic noise appearing on the monitor. To check for exclusion of electronic noise, briefly interrupt the sample flow; if the instrument is correctly adjusted, the signal rate should drop to nearly zero. Set the amplification of the green-fluorescence channel so that the signals from the live bacteria stained with the SYTO 9 stain appear in the top range of the signal axis. If necessary, adjust the compensation settings so that the signal is in the lower range of the opposite axis. Next, set the amplification of the red-fluorescence channel so that the signals from the dead bacteria stained with propidium iodide appear in the top range of the signal axis. Adjust the compensation settings as necessary so that the signal is in the lower range of the opposite axis.

3.3 After adjusting the flow cytometer as described above, apply a control or experimental sample containing stained bacteria and microspheres, ensuring that both populations are on-scale (use fluorescence versus side-scatter parameters). Process the data by framing regions around the various populations in the fluorescence-versus-side scatter cytogram and/or the red fluorescence-versus-green fluorescence cytogram (Figure 1).

3.4 Count the number of events in the bead region to accurately estimate the volume analyzed in the data file. The microsphere standard (Component C) contains approximately 1.0×10^8 beads/mL; thus, after ~100-fold dilution into the stained cell preparation, the microsphere density is 1.0×10^6 beads/mL, and one bead represents 10^{-6} mL. The number of signals in the bacteria region (bac region) divided by the number of signals in the bead region provides the total number of bacteria per 10^{-6} mL in the flow cytometry analysis tube. Likewise, the number of events in the live bacteria region (or dead bacteria region) divided by the number of events in the bead region provides the number of live bacteria (or dead bacteria) per 10^{-6} mL in the flow cytometry analysis tube. To calculate the density in the bacterial culture (step 1.6), the concentration in the flow cytometry tube must be multiplied by the dilution factors, including the 100-fold dilution at step 2.3 and any further dilutions made due to a turbid culture.

$$\frac{(\text{\# of events in bac. region}) \times (\text{dilution factors})}{(\text{\# of events in bead region}) \times 10^{-6}} = \text{bacteria/mL}$$

Table 1. Preparing mixed suspensions of live and dead cells.

Ratio of Live:Dead Cells	Live-Cell Suspension	Dead-Cell Suspension
0:100	0 mL	1.00 mL
25:75	0.25 mL	0.75 mL
50:50	0.50 mL	0.50 mL
75:25	0.75 mL	0.25 mL
100:0	1.00 mL	0 mL

3.5 In the flow cytometer, bacteria are identified solely on the basis of their size and stainability. *It is best to inspect each sample by fluorescence microscopy to confirm that the particles detected are indeed bacteria.*

3.6 Note that with the long data-acquisition times required for very dilute bacteria samples, the number of noise signals acquired in the bacteria frame may become significant. The results obtained from a control sample provide an estimate of the noise contribution to the experimental data files.

Optimizing the Dye Ratio in the Staining Mixture

4.1 Using OD measurements or the results from step 3.4, adjust the concentration of the live-cell and dead-cell bacterial suspensions prepared in step 1.6 to approximately 1×10^6 bacteria/mL or to the estimated concentration of the experimental samples by diluting them in filter-sterilized 0.85% NaCl or appropriate buffer.

4.2 Mix five different proportions of the bacterial suspension in flow cytometer analysis tubes according to Table 1. The volume of each of the five samples will be 1 mL.

4.3 Prepare a 1:1 mixture of the nucleic acid stains by mixing 8 µL of 3.34 mM SYTO 9 nucleic acid stain (Component A) with 8 µL of 20 mM propidium iodide (Component B) in a microcentrifuge tube.

4.4 Add 3 µL of the combined reagent mixture to each of the five samples and mix thoroughly.

4.5 Incubate the samples for 15 minutes at room temperature protected from light.

4.6 Analyze by flow cytometry as described in steps 3.1–3.6 (Figure 1).

4.7 Calculate bacteria/mL, generate the bacteria/mL-versus-percent live bacteria graph and then evaluate the linearity and r^2 value (Figure 2).

4.8 If the linearity and r^2 value of the bacteria/mL-versus-percent live bacteria graph are not acceptable using the 1:1 dye ratio prepared in step 4.3, then repeat steps 4.2–4.7 using different ratios of the SYTO 9 and propidium iodide stain solutions. For example,

if green fluorescence is too prominent in the preparation, try either lowering the concentration of SYTO 9 stain or raising the concentration of propidium iodide. To thoroughly optimize the dye ratio, we recommend staining the known proportions of live: dead cells prepared in step 4.2 with a range of concentrations of SYTO 9 dye, each in combination with a range of propidium iodide concentrations

References

1. J Appl Bacteriol 72, 410 (1992); **2.** Lett Appl Microbiol 13, 58 (1991); **3.** Curr Microbiol 4, 321 (1980); **4.** J Microbiol Methods 13, 87 (1991); **5.** Microbiol Rev 51, 365 (1987).

Product List *Current prices may be obtained from our Web site or from our Customer Service Department.*

Cat #	Product Name	Unit Size
L34856	LIVE/DEAD® *Bac*Light™ Bacterial Viability and Counting Kit *for flow cytometry* *100 assays*	1 kit

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