7040 Virginia Manor Road
Beltsville, MD 20705, USA
Web: www.abpbio.com;
Email: info@abpbio.com

Cell-Check™ Viability/Cytotoxicity Kit for Bacteria Cells

Catalog Number: A018

Table 1. Kit Components and Storage

Material	Amount	Concentration	Storage	Stability
SYTO 9 dye (Component A)	300 µL	3.34 mM	-20 °C	The product is stable for one year when stored as directed.
Propidium Iodide (Component B)	300 µL	20 mM	-20 °C	

Number of assays: 1,000 tests using a fluorescence microscope or 200 tests using a flow cytometer.
Approximate fluorescence excitation/emission maxima, in nm: SYTO 9 dye**:** 480/500, bound to DNA; Propidium Iodide: 528/617, bound to DNA.

Introduction

The Cell-Check™ Viability/Cytotoxicity Kit for Bacteria Cells provides two-color fluorescence staining on both live (green) and dead (red) bacteria using two probes, SYTO 9 dye and Propidium iodide. SYTO 9 dye is a green-fluorescent nucleic acid dye that stains both live and dead bacteria with intact and damaged cell membranes. Propidium iodide is a red-fluorescent nucleic acid dye that stains only dead bacteria with damaged cell membranes. With an appropriate mixture of SYTO 9 and Propidium iodide, bacteria with intact cell membranes is stained fluorescent green, whereas bacteria with damaged cell membranes is stained fluorescent red. The kit is suitable for use with fluorescence microscopes and flow cytometers. The assay principles are general and applicable to most bacteria types.

A common criterion for bacterial viability is the ability of a bacterium to reproduce in suitable nutrient media that is referred to as growth assays. This kit yields results that correlate well with growth assays in liquid or solid media. Under certain conditions, however, bacteria having damaged membranes may be able to recover and reproduce - such bacteria may be scored as “dead” in this assay. Conversely, some bacteria with intact membranes may be unable to reproduce in nutrient medium, and yet these may be scored as “alive”. Therefore, these situations need to be considered if a vast difference of live and dead bacteria counts is observed between this assay and growth assays.

Experimental Protocols

Note: The following protocols are provided as examples to guide researchers in the development of their own bacterial staining procedures. Both gram-positive and gram-negative bacteria have been validated using the following procedures.

Preparation of Live and Dead Bacterial Suspensions as Controls

Note: Care must be taken to remove traces of growth medium before staining bacteria with these kit reagents. Phosphate wash buffers are not recommended because they appear to decrease staining efficiency.

1.1 Grow 4 mL cultures of your bacteria to late log phase in nutrient broth.

1.2 Prepare two tubes of 1 mL of the bacteria culture in Eppendorf tubes and centrifuge at 10,000 × g for 10-15 minutes.

1.3 Remove the supernatant and resuspend the pellet of one tube in 0.3 mL of 0.85% NaCl solution and another tube in 1 mL of 0.85% NaCl.

1.4 Add 0.7 mL isopropyl alcohol into the tube with 0.3 mL of 0.85% NaCl and mix well (final concentration of isopropyl alcohol: 70%) for preparing dead bacteria.

1.5 Incubate both samples at room temperature for 1 hour, mixing every 15 minutes.

1.6 Pellet both samples by centrifugation at 10,000 × g for 10-15 minutes.

1.7 Resuspend the pellets in 1 mL of 0.85% NaCl and centrifuge again as in step 1.6.

1.8 Determine the optical density at 670 nm (OD_{670}) for a 3 mL aliquot of the bacterial suspensions in glass or acrylic absorption cuvettes (1 cm pathlength).

1.9 Use live and dead bacteria at your desired concentration for staining experiments shown below.

Fluorescence Microscopy Protocol

Staining Bacteria in Suspension

Note: Best results are obtained by adjusting the dye concentrations to achieve distinct labeling of live cells with SYTO 9 and of dead cells with Propidium Iodide. The optimal concentrations are likely to vary depending on the bacteria types. In general it is best to use the lowest dye concentration that gives sufficient signal. The following condition is optimal for *E. coli* live and dead cell staining.

2.1 Combine 1.5 μL of SYTO 9 stock solution and 1.5 μL of Propidium Iodide stock solution in a microcentrifuge tube, and then add 7 μL of 0.85% NaCl solution, mix thoroughly to derive 100X dye mixture solution.

2.2 For each 100 μL of your bacteria sample and live and dead bacteria control suspensions, add 1 μL of 100X dye mixture (prepared in step 2.1).

2.3 Mix thoroughly and incubate at room temperature in the dark for 15 minutes.

2.4 Trap 5 μL of the stained bacterial suspension between a slide and an 18 mm square coverslip.

2.5 Observe under a fluorescence microscope equipped with any of the filter sets as below.

Selection of Optical Filters

The fluorescence from both live and dead bacteria may be viewed simultaneously with any standard fluorescein longpass filter set. Alternatively, the live (green fluorescent) and dead (red fluorescent) cells may be viewed separately with fluorescein and Texas Red bandpass filter sets. Typical characteristics of some appropriate filters are summarized in Table 1.

Table 1. Characteristics of common filters suitable for use with SYTO 9 and Propidium Iodide

Omega Filters	Chroma Filters	Notes
XF25, XF26, XF115	11001, 41012, 71010	Longpass and dual emission filters useful for simultaneous viewing of SYTO 9 and Propidium Iodide
XF22, XF23	31001, 41001	Bandpass filters for viewing SYTO 9 alone
XF32, XF43, XF102, XF108	31002, 31004, 41002, 41004	Bandpass filters for viewing Propidium Iodide alone

Fluorescence Microplate Protocol

3.1 Adjust the *E. coli* suspensions (live and killed) to 2×10^8 bacteria/mL (~0.06 OD_{670}) or the *S. aureus* suspensions (live and killed) to 2×10^7 bacteria/mL (~0.30 OD_{670}). *S. aureus* suspensions typically should be 10-fold less concentrated than *E. coli* when using a fluorescence microplate reader.

Table 1. Volumes of live- and dead-cell suspensions to mix to achieve various proportions of live:dead cells.

Ratio of Live:Dead Cells	mL Live-Cell Suspension	mL Dead-Cell Suspension
0:100	0	2.0
10:90	0.2	1.8
50:50	1.0	1.0
90:10	1.8	0.2
100:0	2.0	0

3.2 Mix five different proportions of *E. coli* or *S. aureus* (Table 1) in 16 × 125 mm borosilicate glass culture tubes. The total volume of each of the five samples will be 2 mL.

3.3 Mix 6 µL of Component A with 6 µL of Component B in a microfuge tube.

3.4 Prepare a 2X stain solution by adding the entire 12 µL of the above mixture to 2.0 mL of filter-sterilized dH_2O in a 16 × 125 mm borosilicate glass culture tube and mix well.

3.5 Pipet 100 µL of each of the bacterial cell suspension mixtures into separate wells of a 96-well flat-bottom microplate. We recommend that you prepare samples in triplicate. The outside wells (rows A and H and columns 1 and 12) are usually kept empty to avoid spurious readings.

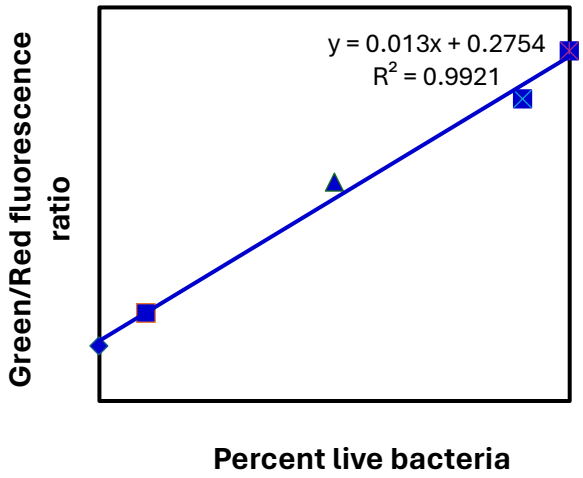
3.6 Using a new tip for each well, pipet 100 µL of the 2X staining solution (from step 3.4) to each well and mix thoroughly by pipetting up and down several times.

3.7 Incubate at room temperature in the dark for 15 minutes.

3.8 With the excitation wavelength centered at about 480 nm, measure the fluorescence intensity at a wavelength centered at about 510 nm (emission 1; green) for each well of the entire plate.
3.9 With the excitation wavelength still centered at about 480 nm, measure the fluorescence intensity at a wavelength centered about 630 nm (emission 2; red) for each well of the entire plate.
3.10 Analyze the data by dividing the fluorescence intensity of the stained bacterial suspensions (F_{cell}) at emission 1 by the fluorescence intensity at emission 2.

$$\text{Ratio}_{G/R} = \frac{F_{cell,em1}}{F_{cell,em2}}$$

3.11 Plot the $\text{Ratio}_{G/R}$ versus percentage of live cells in the *E. coli* suspension.



Analysis of relative viability of E. coli suspensions in a fluorescence microplate reader. Samples of E. coli were prepared and stained as outlined in the text. The integrated intensities of the green (510 ± 12.5 nm) and red (620 ± 20 nm) emission of suspensions excited at 480 ± 10 nm were acquired, and the green/red fluorescence ratios (RatioG/R) were calculated for each proportion of live/dead E. coli. Each point represents the mean of three measurements. The line is a least-squares fit of the relationship between % live bacteria (x) and RatioG/R (y).

Flow Cytometry Protocol

4.1 Adjust the *E. coli* suspensions (live and killed) to 1 × 10^8 bacteria/mL (~0.03 OD_{670}), then dilute them 1:100 in filter-sterilized dH_2O to reach a final density of 1 × 10^6 bacteria/mL if needed.

4.2 Mix 11 different proportions of *E. coli* in 16 × 125 mm borosilicate glass tubes according to Table 2. The volume of each of the 11 samples will be 1 mL.

4.3 Mix 20 µL of Component A with 20 µL of Component B in a microcentrifuge tube. Add 3 µL of the combined reagent mixture to each of the 11 samples and mix thoroughly by pipetting up and down several times.

Note: It may be desirable to prepare additional bacterial samples for staining with component A alone (stain both live and dead bacteria) and with Component B alone (stain dead bacteria only).

4.4 Incubate at room temperature in the dark for 15 minutes.

4.5 Analyze each bacterial sample by flow cytometry using the setting for fluorescein for NucView Green positive cells and propidium iodide for propidium iodide positive cells.

Table 2: Volume of Live- and dead-cell suspension to mix to achieve desired ratio of live:dead cell population

Ratio of Live:Dead Cells	mL Live-Cell Suspension	mL Dead-Cell Suspension
0:100	0	1.0
10:90	0.1	0.9
20:80	0.2	0.8
30:70	0.3	0.7
40:60	0.4	0.6
50:50	0.5	0.5
60:40	0.6	0.4
70:30	0.7	0.3
80:20	0.8	0.2
90:10	0.9	0.1
100:0	1.0	0