Bac*Light*™ Bacterial Membrane Potential Kit (B34950)

Quick Facts

Storage upon receipt:

- ≤ 6°C

Introduction

The *Bac*Light™ Bacterial Membrane Potential Kit provides solutions of the carbocyanine dye $DiOC_2(3)$ (3,3′-diethyloxacarbocyanine iodide, Component A; Figure 1) and CCCP (carbonyl cyanide 3-chlorophenylhydrazone, Component B), both in DMSO, and a 1X phosphate-buffered saline solution (Component C). $DiOC_2(3)$ exhibits green fluorescence in all bacterial cells, but the fluorescence shifts toward red emission as the dye molecules self-associate at the higher cytosolic concentrations caused by larger membrane potentials. Proton ionophores such as CCCP destroy membrane potential by eliminating the proton gradient.[1,2]

Membrane potentials have been detected in all bacteria tested with $DiOC_2(3)$, although the magnitude varies with species (Figure 2). For many gram-positive species, including *Staphylococcus aureus* and *Micrococcus luteus*, the $DiOC_2(3)$ red:green ratio has been shown to vary with the intensity of the proton gradient (Figure 3). In gram-negative bacteria such as *Escherichia coli* and *Salmonella choleraesuis*, a $DiOC_2(3)$ response is observed in the presence of a membrane potential, but the response does not appear to be proportional to proton gradient intensity.

This kit is designed to assay bacterial concentrations in the range of 10^5–10^7 organisms per mL. Stained cells should be analyzed using 488-nm excitation and emission filters suitable for fluorescein and the Texas Red® dye. $DiOC_2(3)$ staining can be combined with the impermeant DNA-binding dye TO-PRO®-3 (T3605) at 500 nM to distinguish depolarized cells from cells with damaged membranes.[2] The TO-PRO-3 dye must be excited with a red laser, such as the 633 nm He–Ne laser. **Caution:** Carbocyanine dyes, including $DiOC_2(3)$ and CCCP, are inhibitors of respiration.[3] While these dyes do not alter assay results over the recommended staining periods, both $DiOC_2(3)$ and CCCP are toxic to bacterial cells and the cells will not be culturable after even brief exposure.

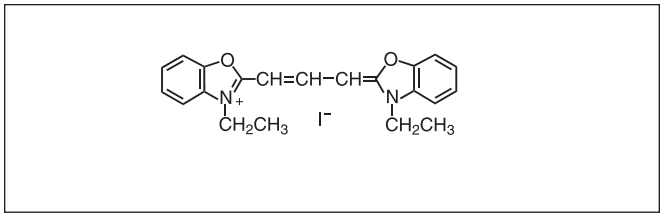

Figure 1. Structure of $DiOC_2(3)$

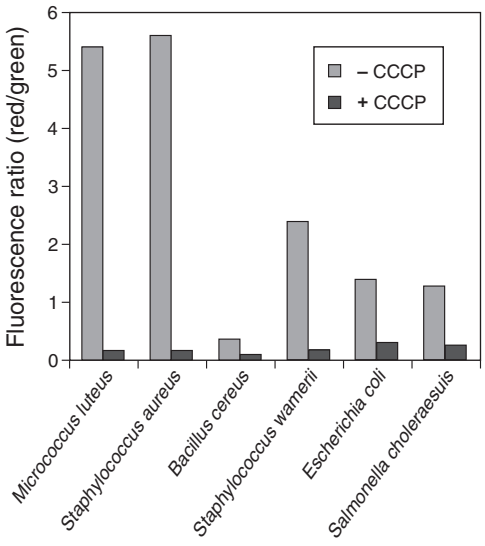

Figure 2. Detection of membrane potential in various bacteria. Red/green ratios were calculated using population mean fluorescence intensities for gram-positive (*M. luteus*, *S. aureus*, *B. cereus*, *S. warneri*) and gram-negative (*E. coli, S. choleraesuis*) bacteria, incubated with 30 µM $DiOC_2(3)$ for 30 minutes in either the presence or absence of 5 µM CCCP.

Materials

Content

- $DiOC_2(3)$ (Component A), 1.2 mL of a 3 mM solution in DMSO
- CCCP (Component B), 300 µL of a 500 µM solution in DMSO
- Phosphate-buffered saline (PBS, Component C), 200 mL of 10 mM sodium phosphate, 145 mM sodium chloride, pH 7.4

Number of Tests Possible

Using the recommended reagent dilutions and volumes, this kit provides sufficient $DiOC_2(3)$ to perform approximately 100 individual assays by flow cytometry; sufficient 500 µM CCCP is provided for 30 depolarized control samples.

Storage and Handling

Store the components of the *Bac*Light Bacterial Membrane Potential Kit at ≤6°C. Allow Components A and B to warm to

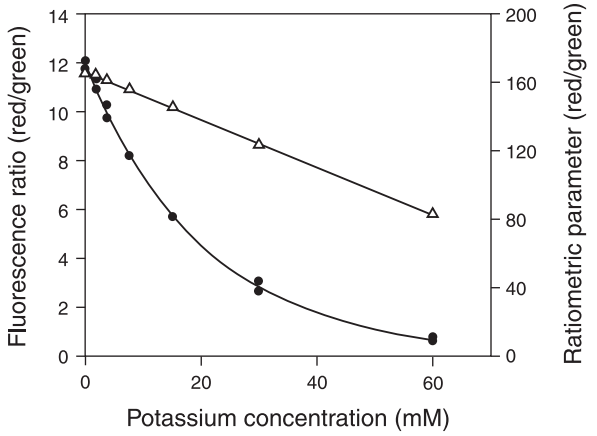

Figure 3. Response of *Staphylococcus aureus* to valinomycin and external potassium ions. Samples contained *S. aureus* treated with 5 µM valinomycin in different concentrations of potassium buffer, then stained using 30 µM $DiOC_2(3)$ for 30 minutes. Data are expressed using either a ratiometric parameter based on the formula in section 2.4 (triangles, right axis) or as the ratio of population red MFI/green MFI (circles, left axis).

room temperature before opening the vials. When stored properly, these kit components are stable for at least six months. **Caution**: 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 in compliance with all pertinent local regulations.

Experimental Protocols

General Considerations

The reagents in the *Bac*Light Bacterial Membrane Potential Kit have been tested at Molecular Probes on logarithmically growing cultures of the following bacterial species: *Micrococcus luteus, Staphylococcus aureus, S. warnerii, Bacillus cereus, Klebsiella pneumoniae, Escherichia coli,* and *Salmonella choleraesuis*. Many bacteria do not show a proportional response to partial membrane depolarization with $DiOC_2(3)$. The response of each bacterial system should be investigated and optimized. Occasionally the $DiOC_2(3)$ concentration and staining time must be adjusted for optimal detection of membrane potential. The experimental protocols below are provided as examples to guide researchers in the development of their own bacterial staining procedures.

Some common buffer components, such as Tween® 20, azide, and thimerosal, can alter membrane potential and should be avoided. Be sure to test buffer additives for their effect on membrane potential during optimization studies.

Staining the Bacteria

1.1 Filter the required volume of PBS (Component C) through a 0.22 µm pore size membrane, preparing enough for culture dilution and 1 mL per test.

1.2 Allow the 3 mM $DiOC_2(3)$ and 500 µM CCCP solutions (Components A and B) to come to room temperature before use.

1.3 Bacteria may be grown in any appropriate medium. Best results for healthy bacteria are obtained from log-phase cultures. Dilute the bacterial culture to approximately 1 x 10^6 cells per mL in filtered PBS (from step 1.1) or equivalent buffer. Bacteria may be diluted directly from the culture medium without washing. Prepare sufficient suspension to provide 1 mL for each test, including controls.

1.4 Aliquot 1 mL of the bacterial suspension into a flow cytometry tube for each staining experiment to be performed. Prepare two additional tubes for a depolarized control and an unstained control.

1.5 Add 10 µL of 500 µM CCCP (Component B) to the depolarized control sample and mix.

1.6 Add 10 µL of 3 mM $DiOC_2(3)$ (Component A) to each flow cytometry tube and mix (do not add stain to the unstained control sample). Incubate samples at room temperature for 15–30 minutes. Stained samples can be analyzed after 5 minutes, but signal intensity continues to increase until about 30 minutes.

Analyzing the Stained Bacteria by Flow Cytometry

Instrument capabilities vary considerably, but the techniques and parameters established here should aid considerably in setting up similar analyses using the majority of commercially available flow cytometers.

2.1 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 ("GC" and "RC"); 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.

2.2 Instrument adjustments are especially critical for detecting relatively small particles such as bacteria. To avoid contamination of the data by electronic noise, use the following procedure for instrument setup. Use the unstained control sample to locate bacterial populations in the forward and side scatter channels. 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 (called "threshold level" on some instruments) to minimize electronic noise appearing on the monitor.

2.3 After adjusting the flow cytometer as described above, apply the depolarized control sample. Gate on bacteria using forward versus side scatter and adjust fluorescence photomultiplier tube voltages such that the green and red MFI values are approximately equal. Do not set compensation.

2.4 While the relative amount of red and green fluorescence intensity will vary with cell size and aggregation, the ratio of red to green fluorescence intensity can be used as a size-independent indicator of membrane potential. In flow cytometry, this measure

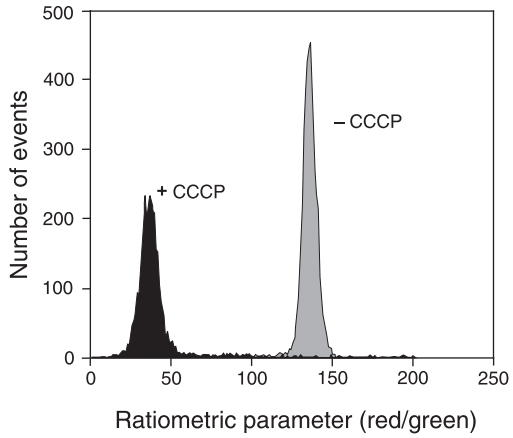

Figure 4. Analysis using the ratiometric parameter. The red/green ratiometric histogram shows *Staphylococcus aureus* incubated with 30 µM $DiOC_2(3)$ for 30 minutes in either the presence or absence of 5 µM CCCP. Flow cytometer data were collected with log amplification. The ratiometric parameter was calculated as [(red value) – (green value) + 384].

is best applied with a parameter that provides this ratio data for each event,[2,4] calculated as follows:

(RC mean) – (GC mean) + 1.5(# of channels per decade)

This calculation requires that red and green fluorescence are collected with logarithmic amplification and includes a 1.5 decade offset to assure that the ratiometric values are positive (Figure 4). If the analysis software cannot create a ratiometric parameter, useful information can also be obtained with a ratio of population red and green linear mean fluorescence intensity (MFI) values (Figure 5).

If your cytometer software has the feature, set up a ratiometric parameter. Otherwise, process data by gating on bacteria using forward versus side scatter, and analyze gated populations with a dot plot of red versus green fluorescence as in Figure 5, reporting MFI values as *linear* values, not as channels.

2.5 On a ratiometric histogram, set markers around the peaks of interest and record the mean ratio values. For a dot plot of red versus green fluorescence, set regions around the populations of interest and record red and green MFI values for each. To evaluate the data, divide the red population MFI by the green population MFI (Figure 4).

2.6 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.*

Calibration of Membrane Potential to Potassium Concentration

In the presence of the potassium ionophore valinomycin, bacteria are depolarized when the external potassium concentration exceeds the cytosolic concentration and are hyperpolarized when the external potassium concentration is less than the cytosolic concentration. By comparing the $DiOC_2(3)$ ratio measurement between untreated bacteria and bacteria exposed to valinomycin in the presence of various potassium concentrations, the membrane potential can be equated to a potassium concentration differential.[2]

3.1 Suspend bacteria in buffers with potassium ion concentrations varying from 0–60 mM.

3.2 Add valinomycin to a concentration of 5 µM.

3.3 Stain with $DiOC_2(3)$ and analyze as described above in steps 1.6 and 2.1–2.6.

Further Optimization of Assay

To increase the fluorescence intensity in stained bacteria, run the assay with a higher concentration of dye or for a longer period of time. Staining may also be performed at 37°C. The addition of 1 mM EDTA may facilitate dye uptake in some situations.

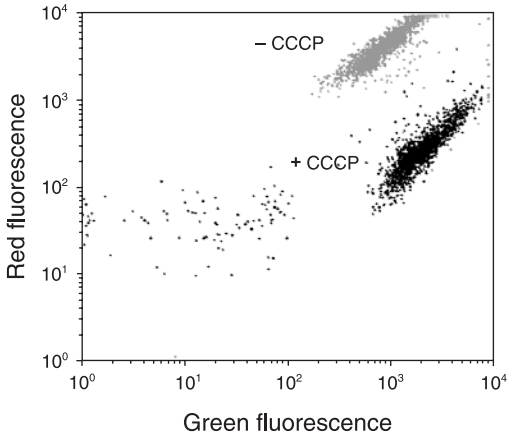

Figure 5. Analysis using red and green fluorescence parameters. The red-versus-green fluorescence dot plot show *Staphylococcus aureus* incubated with 30 µM $DiOC_2(3)$ for 30 minutes in either the presence or absence of 5 µM CCCP. Flow cytometer data were collected with log amplification.

References

1. Cytometry 35, 55 (1999); **2.** Antimicrobial Agents and Chemotherapy 44, 827 (2000); **3.** Glia 4, 611 (1991); **4.** Methods 21, 271 (2000).

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

Cat #	Product Name	Unit Size
B34950	*Bac*Light™ Bacterial Membrane Potential Kit	1 kit
T3605	TO-PRO®-3 iodide (642/661) *1 mM solution in DMSO*	1 mL

Contact Information

Further information on Molecular Probes' products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

Please visit our Web site — **www.probes.com** — for the most up-to-date information.

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