Bac*Light™ RedoxSensor™ CTC Vitality Kit*

Quick Facts

Storage upon receipt:

- ≤–20ºC
- Desiccate
- Protect from light

Ex/Em:

- 5-Cyano-2,3-ditolyl tetrazolium chloride (CTC): 450/630 nm
- SYTO® 24 green fluorescent nucleic acid stain: 490/515 nm
- 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI): 344/450 nm

Introduction

The *Bac*Light™ RedoxSensor™ CTC Vitality Kit provides effective reagents for evaluating bacterial health and vitality that can withstand fixation procedures. The kit contains 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), which has been used by researchers to evaluate the respiratory activity of many bacterial populations derived from environmental sources including food,[1,2] soil,[3] stone,[4] marine and fresh water,[5–8] as well as populations undergoing drug efficacy evaluations. Briefly, healthy cells respiring via the electron transport chain will absorb and reduce CTC into an insoluble, red fluorescent formazan product. Cells not respiring or respiring at slower rates will reduce less CTC, and consequently produce less fluorescent product, giving a semi-quantitative estimate of healthy vs. unhealthy bacteria. SYTO® 24 green-fluorescent nucleic acid stain and DAPI are offered as counterstains to assist the researcher in differentiating cells from debris and in calculating total cell numbers. Bacteria labeled with CTC and either counterstain may be evaluated immediately or after storage, with or without fixation using a flow cytometer equipped with appropriate excitation sources (CTC in combination with SYTO® 24 green fluorescent nucleic acid stain: single 488 nm argon-ion laser; CTC in combination with DAPI: dual UV and 488 nm lasers), or by microscopy using the optimal filter sets described below.

Materials

Kit Contents

- 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), (Component A), 5 vials each containing 15 mg
- SYTO® 24 green fluorescent nucleic acid stain (Component B), 100 µL of 1 mM solution in DMSO
- 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Component C), 100 µL of 5 mg/mL solution in water

Number of Tests Possible

Using the protocols recommended below, each kit contains sufficient material to perform at least 50 tests by flow cytometry or microscopy.

Storage and Handling

Store all components ≤–20ºC. Allow reagents to warm to room temperature before opening the vials. When stored properly, these reagents are stable for at least one year. Stock CTC solutions made in water should be stored at 4˚C and used within 24 hours. Caution: DAPI is a known mutagen. The hazards posed by SYTO® 24 stain have not been fully investigated, but SYTO® 24 is known to bind DNA. DMSO stock solutions of SYTO® 24 stain should be handled with particular care, as DMSO is known to facilitate the entry of organic molecules into tissues. Reagents should be handled using equipment and practices appropriate for the hazards posed by such materials. Please dispose of the reagents in compliance with all pertaining local regulations.

Experimental Protocols

General Considerations

Organisms: The *Bac*Light™ RedoxSensor™ CTC staining reagent has been tested by Molecular Probes on logarithmically growing cultures of the following bacterial species: *Micrococcus luteus*, *Staphylococcus aureus*, *Bacillus cereus*, *B. subtilis*, *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella cholerasuis*. In addition to the above, CTC has been used to investigate a wide variety of organisms including, but not limited to: *Helicobacter pylori*, *Listeria monocytogenes*, *Campylobacter jejuni*, *Vibrio cholerae*, *Salmonella typhurmurium*, and *Thiothrix nivea*.[8–17] The response of each bacterial system should be investigated and optimized. The experimental protocols below are provided as examples to guide researchers in the development of their own bacterial staining procedures.

Staining environment: The removal of an organism from its growth medium and placement in a different environment may enhance or inhibit the organism's reductase activity and overall response to CTC. Therefore, choices of staining buffer and conditions need to be considered carefully in the context of the desired end results, assays, instrumentation, and analyses.

Staining concentrations and incubation times: Protocols described in published scientific literature recommend staining with concentrations of CTC ranging from 2–6 mM, with 5 mM being the more common concentration. Incubation times range from 5 minutes to 24 hours. The optimal incubation condition may depend upon the organism being investigated and the experimental system.

Counterstaining: SYTO® 24 green-fluorescent nucleic acid stain and DAPI are offered as counterstains to assist the researcher in differentiating cells from debris and in calculating total cell numbers. The staining concentrations and conditions will vary with live/dead status and analysis platform (flow cytometry or microscopy). SYTO® 24 green fluorescent nucleic acid stain may be used on live or fixed samples at a concentration of 10 nM for flow cytometry or 1 µM for microscopy; DAPI may be used at 10 µg/mL for flow cytometry (UV-laser required) on live or fixed samples, but the dimly stained live population may not be detectable using a microscope.

Calculating percent active and determining total numbers: A number of factors can impact the determination of vitality and total cell numbers with the *Bac*Light™ RedoxSensor™ CTC Vitality Kit, including metabolic status, membrane integrity, and gram character. In most cases, the counterstains will stain all bacteria and the CTC will stain those with active dehydrogenases. However in some cases, fluorescence resonance energy transfer may interfere with counterstain fluorescence in CTC-positive cells. In these situations, the total population would be the sum of cells positive for either CTC or the counterstain. The active cell index can be calculated as the percentage of total cells that are CTC-positive.

Preparation of Working Solutions

1.1 CTC. Each vial of CTC (Component A) contains 15 mg. Dissolve the contents of one vial with 1 mL ultrapure water for a final concentration of 50 mM. Most protocols recommend staining with a 5 mM staining solution (final concentration).

1.2 Sterile buffers. Phosphate-buffered saline (PBS) or PBS + 10 mM glucose (PBSG) have been shown to be adequate staining buffers for both gram-positive and gram-negative bacteria. Additives that reduce respiration activity (e.g., sodium azide) may also inhibit CTC reduction. Sterilize staining buffers using 0.2 µm filtration.

1.3 SYTO® 24 green fluorescent nucleic acid stain. For flow cytometry use, prepare a 10 µM working solution with a 1:100 dilution of the 1 mM stock solution of SYTO® 24 green fluorescent nucleic acid stain using a high-quality, anhydrous DMSO. Adding 1 µL of the working solution to 1 mL cell samples will give a final staining solution of 10 nM.

Staining the Bacteria

2.1 Grow cultures according to necessary growth conditions. Cells in log-phase and showing optimal vitality will reduce the highest level of CTC, resulting in a greater percentage of the population producing high fluorescence signal.

2.2 Dilute cells either washed in warm buffer (PBS or PBSG are suitable) or directly from the culture into warm buffer to ~10^6 cells/mL. Aliquot ~1 mL to flow cytometry tubes. If desired, prepare an inactivated control by treating with 10% alcohol or 1–4% formaldahyde.

2.3 Add 100 µL of the 50 mM *Bac*Light™ RedoxSensor™ CTC working solution (prepared in step 1.1). Vortex gently to mix. Incubate 30 minutes at 37°C, protected from light. **Note:** Reserve a sample without dye addition as an unstained bacteria control.

2.4 Analyze by flow cytometry without washing. If desired, CTC-stained cells may be counterstained (protocol below). Formaldehyde treatment (1–4% final concentration) can be employed to fix CTC-stained cells, and this step can be carried out before counterstaining.

Counterstaining (Optional)

Cells may be counterstained with SYTO® 24 nucleic acid stain or DAPI at either room temperature or 37˚C for 15 minutes at recommended final concentration (see Table 1) and then analyzed immediately. Cells may also be fixed with 1–4% formaldehyde and prior to counterstaining. Samples may be analyzed without washing.

3.1 For counterstaining with SYTO® 24 green fluorescent nucleic acid stain:

Flow cytometry: Transfer 1 µL of the 1:100 dilution of SYTO® 24 nucleic acid stain working solution (prepared in step 1.3) to 1 mL of CTC-labeled cells.

Microscopy: Transfer 1 µL of the undiluted stock solution of SYTO® 24 nucleic acid stain (1 mM) to 1 mL of CTC-labeled cells.

3.2 For counterstaining with DAPI:

Flow cytometry and microscopy: Transfer 2 µL of the 5 mg/mL stock solution of DAPI to 1 mL of CTC-labeled cells.

Table 1. Concentrations of SYTO® 24 nucleic acid stain and DAPI for counterstaining.

	Flow Cytometry	Microscopy
SYTO® 24 nucleic acid stain	10 nM	1 µM
DAPI	10 µg/mL	10 µg/mL

Adjusting the Flow Cytometer and Analyzing the Samples

Instrument capabilities may vary considerably, but the techniques and parameters established here should aid in setting up analyses in the majority of flow cytometers now in use. In the flow cytometer, bacteria are identified solely on the basis of their size and staining. It is best to inspect each sample by fluorescence microscopy to confirm that the particles detected are indeed bacteria (see *Analyzing the Stained Bacteria in Suspension by Fluorescence Microscopy*). Unstained and single-color controls should be used to locate cell populations and determine compensation settings.

4.1 Instrument configuration. Use appropriate excitation sources and emission filters for stained bacteria samples (see *Quick Facts*). If bacteria are costained with SYTO® 24 nucleic acid stain and CTC, a single 488 nm excitation source is appropriate. If bacteria are stained with DAPI and CTC, a dual excitation (UV and 488 nm) instrument configuration is required.

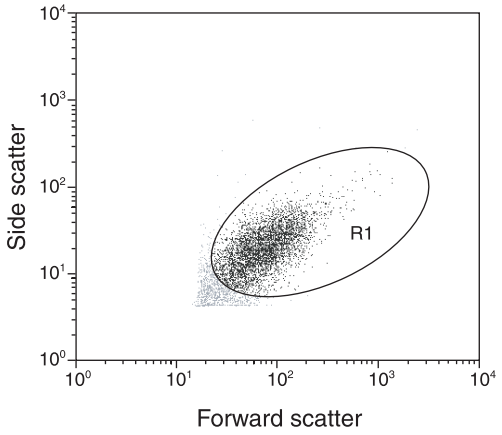

Figure 1. Dot plot of forward scatter vs. side scatter obtained on a sample of unstained bacteria analyzed using a BD FACSCalibur™ flow cytometer with thresholds set on forward and side scatter. The region R1 contains particles of the appropriate size for bacterial cells and is used to set the instrument to exclude debris in the sample.

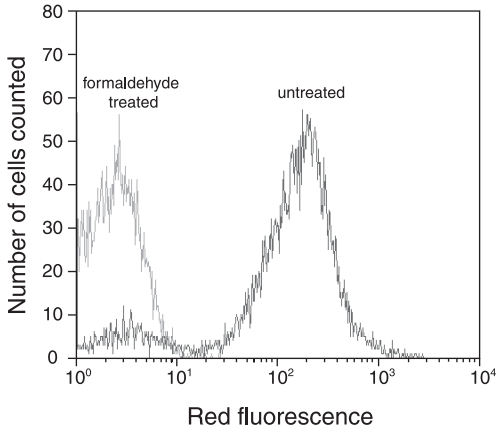

Figure 2. Untreated or formaldehyde-treated *E. coli* were stained with 5 mM CTC (30 minutes at 37°C) and analyzed on a flow cytometer using 488 nm excitation and a 670 nm longpass filter

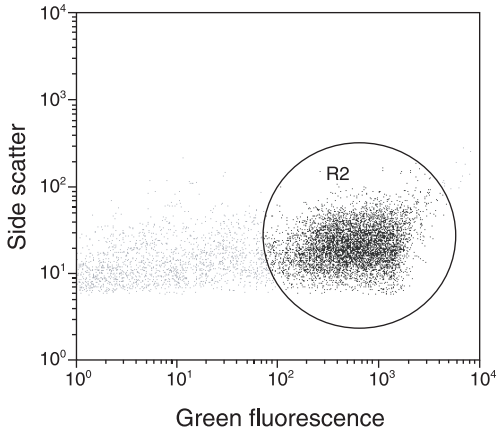

Figure 3. Dot plot of green fluorescence vs. side scatter. The region R2 contains events stained with SYTO® 24 nucleic acid stain.

4.2 Instrument set-up

4.2.1 Set amplifiers to logarithmic amplification. Use side-scatter and/or forward scatter to set threshold parameter.

4.2.2 With an unstained sample of bacteria cells, set the amplification of the signals from forward and side scatter so that the bacteria are in the middle of the data space. Adjust threshold to minimize electronic noise appearing on the monitor. To avoid coincidence error, maintain flow rate ≤1000 events/second. Set a gate on the bacterial population (Figure 1).

4.2.3 On the red fluorescence histogram used for CTC analysis, set the fluorescence amplification so that the unstained bacteria are in the lowest decade of the plot. Refer to the position of the formaldehyde-tested control in Figure 2.

4.2.4 Using the active bacteria control stained only with the RedoxSensor™ CTC reagent, verify that the signal appears above the level of the unstained or inactivated control in the red fluorescence channel (Figure 2).

4.2.5 When using a nucleic acid counterstain, use a single color control to set the voltage of the PMT so that the bacterial signal appears above background (Figure 3). To exclude acellular fluorescence, set a gate on the population stained with the nucleic acid counterstain.
Note: The cytometer may also be configured to trigger on fluorescence, capturing events that are labeled with a nucleic acid stain, thereby reducing the level of background noise.

4.3 After adjusting the flow cytometer as described above, apply a control or experimental sample containing stained bacteria, gated as in Figures 1 and 3. Analyze in green versus red fluorescence, marking populations as in Figure 4. If necessary, adjust the compensation settings with single color controls.

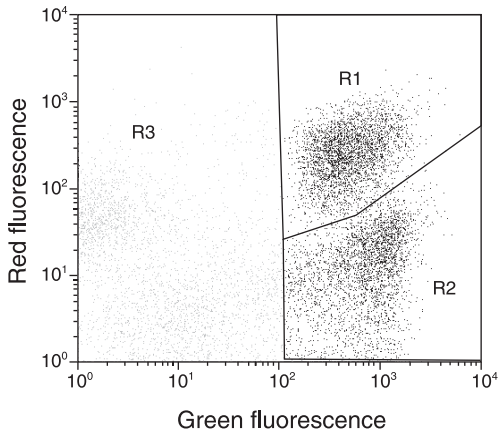

Figure 4. *E. coli* were stained with 5 mM CTC and 10 nM SYTO® 24 nucleic acid stain, then analyzed on a flow cytometer with 488 nm excitation and with 530 bandpass and 670 longpass filters. Events are not gated as in Figures 1 and 3 to demonstrate the presence of debris in the sample.

Analyzing the Stained Bacteria in Suspension by Fluorescence Microscopy

Bacteria stained using *Bac*Light™ RedoxSensor™ CTC Vitality Kit may be viewed using most standard epifluorescence microscopes with the appropriate filters. For the dyes CTC, SYTO® 24 nucleic acid stain, and DAPI, standard Texas Red® dye, FITC (Alexa Fluor® 488 dye), and DAPI filters, respectively, are appropriate. Filter sets are available for simultaneous viewing of dual-stained populations, as well. More information on appropriate filter sets can be found at www.semrock.com, www.omegafilters.com, and www.chroma.com.

To analyze any of the samples in suspension using fluorescence microscopy, trap 5 µL of the stained bacteria suspension between a slide and an 18 mm square coverslip and observe on a fluorescence microscope equipped with the appropriate filters.

References

1. Cytometry 54A, 27 (2003); **2.** Appl Environ Microbiol 69, 2857 (2003); **3.** Biodegradation 13, 285 (2002); **4.** J Microbiol Methods 52, 75 (2003); **5.** Int J Food Microbiol 92, 327 (2004); **6.** J Microbiol Methods 52, 47 (2003); **7.** Huan Jing Ke Zue (article in Chinese) 25, 167 (2004); **8.** Appl Environ Microbiol 69, 7462 (2003); **9.** APMIS 106, 571 (1998); **10.** Vet Res 33, 359 (2002); **11.** J Appl Bacteriol 77, 353 (1994); **12.** Appl Environ Microbiol 69, 711 (2003); **13.** Int J Food Microbiol 89, 31 (2003); **14.** Syst Appl Microbiol 24, 331 (2001); **15.** Cytometry 29, 298 (1997); **16.** FEMS Microbiol Lett 179, 265 (1999); **17.** Appl Environ Microbiol 69, 641 (2003).

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

Cat #	Product Name	Unit Size
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