Revised: 22–March–2011

ViaGram™ Red⁺ Bacterial Gram Stain and Viability Kit (V-7023)

Quick Facts

Storage upon receipt:
- –20°C
- Desiccate
- Protect from light

Ex/Em: See Table 2

Introduction

Molecular Probes' ViaGram™ Red⁺ Bacterial Gram Stain and Viability Kit provides a simple fluorescent staining protocol that differentially stains many gram-positive and gram-negative bacterial species and, at the same time, discriminates live from dead cells on the basis of plasma membrane integrity. The kit contains three reagents: two nucleic acid stains for viability determination and a fluorescently labeled wheat germ agglutinin (WGA) for gram-sign determination. The nucleic acid stains differ from one another in their spectral characteristics and in their ability to penetrate bacterial cell membranes. Bacteria with intact cell membranes stain fluorescent blue with 4′,6-diamidino-2-phenylindole (DAPI), whereas bacteria with damaged membranes stain fluorescent green with SYTOX® Green nucleic acid stain. The background remains virtually nonfluorescent. The Texas Red®-X dye–labeled WGA component selectively binds to the surface of gram-positive bacteria[1] and stains them fluorescent red, effectively distinguishing them from gram-negative bacteria, even in the presence of the viability stains. Thus, with three fluorescent colors, the four possible combinations — live vs. dead cells and gram-positive vs. gram-negative cells — are discriminated. Live bacteria are stained fluorescent blue; dead cells, fluorescent green; and gram-positive cells, fluorescent red on the surface (Table 1).

Table 1. *Staining pattern for the ViaGram Red⁺ Kit.*

	Gram-Negative	Gram-Positive
Live Cells	Blue interior	Blue interior, Red surface
Dead Cells	Green interior	Green interior, Red surface

Materials

Contents

- **DAPI solution** (in water) (Component A), 40 µL, blue-fluorescent live-cell stain
- **SYTOX Green solution in anhydrous DMSO** (Component B), 40 µL, green-fluorescent dead-cell stain
- **Texas Red-X conjugate of wheat germ agglutinin**, lyophilized (Component C), 1.0 mg, red-fluorescent gram-positive stain
- **Reconstitution buffer** (Component D), 1.0 mL of 0.1 M sodium bicarbonate, pH 8.3, for use with Component C
- ***Bac*Light™ mounting oil** (Component E), 10 mL, for bacteria immobilized on membrane filters. Refractive index at 25°C is 1.517 ± 0.003. DO NOT USE FOR IMMERSION OIL.

At the recommended reagent dilutions and volumes, the ViaGram Red⁺ Gram Stain and Viability Kit contains sufficient reagents for 200 individual slide preparations.

Materials Required but Not Provided

- BSA–saline solution: 0.25% bovine serum albumin (BSA), 0.15 M NaCl; sterilized by filtration
- Spin filters, 0.2 µm–pore size (such as Molecular Probes' catalog number E-6606)

Storage and Handling

Upon receipt, the kit should be stored at -20°C, upright and protected from light. Allow reagents to warm to room temperature and centrifuge briefly before opening the vials. Before refreezing, tightly seal all vials. The *Bac*Light mounting oil may be stored at room temperature. When stored properly, the components of this kit are stable for at least one year. The wheat germ agglutinin conjugate (Component C) is supplied as a lyophilized powder. Once reconstituted (see below), store the solution at 4°C or, for longer storage, divide into aliquots and freeze at -20°C. PROTECT FROM LIGHT. AVOID REPEATED FREEZING AND THAWING. It is a good practice to centrifuge the protein conjugate solution briefly in a microcentrifuge before use; only the supernatant solution should be used in experiments. This step will eliminate any protein aggregates that may have formed in solution, thereby reducing nonspecific background staining.

Caution: DAPI and SYTOX Green stains (Components A and B) bind to nucleic acids. DAPI is a known mutagen, and we have no data addressing the mutagenicity or toxicity of SYTOX Green nucleic acid stain. Both stains should be used with appro-

priate care. The DMSO stock solution of SYTOX Green stain should be handled with particular caution as DMSO is known to facilitate the entry of organic molecules into tissues. We strongly recommend using double gloves when handling the DMSO stock solutions. As with all nucleic acid stains, solutions containing these reagents should be poured through activated charcoal before disposal. The charcoal must then be incinerated to destroy the dyes.

Experimental Protocol

The following protocol is provided to guide researchers in the development of their own bacterial staining procedures. Molecular Probes has used this procedure and found it effective in discriminating bacteria with respect to viability and gram sign. The reliability of WGA staining for assessing gram sign in fixed bacteria has been discussed by Sizemore and co-workers.[1,2] Care should be taken when comparing bacterial viability determined by plasma membrane integrity with determinations made by other techniques. For example, formaldehyde or glutaraldehyde fixation will usually kill bacteria; however, when aldehyde-fixed bacteria are stained with the ViaGram Red$^+$ Kit, the staining pattern for viability is frequently identical to that of unfixed cells. In contrast, all bacteria in a suspension treated with 70% isopropyl alcohol are likely to stain as dead cells. It is therefore very important to evaluate the efficacy of the kit's viability discrimination in reference to a specific experimental procedure.

Care must be taken to remove traces of growth medium before staining bacteria with these reagents. Nucleic acids and other media components can bind the DAPI and SYTOX Green stains in unpredictable ways, resulting in unacceptable variations in staining. A single wash step is usually sufficient to remove traces of inter-fering media components from the bacterial suspension. Phosphate wash buffers are not recommended because they may decrease staining efficiency.

Staining Protocol

The following protocol describes the preparation of enough staining solutions for up to 20 slide preparations.

1.1 Prepare a 2 mg/mL stock solution of Texas Red-X conjugate of WGA (Component C) by adding 500 µL of buffer from the sodium bicarbonate solution (Component D). For 20 preparations, 50 µL will be used; the remainder can be stored in aliquots at -20°C.

1.2 Prepare a working solution of the viability indicators by adding 3 µL of DAPI stain (Component A) and 3 µL of SYTOX Green nucleic acid stain (Component B) to 54 µL of water for a 60 µL final volume.

1.3 Centrifuge 50 µL of a bacterial suspension (about 5×10^7 cells) in a 0.2 µm–pore size spin filter at 2000 rpm for 1–2 minutes.

1.4 Wash the cells in 50 µL BSA–saline solution by pipetting up and down several times.

1.5 Recentrifuge as in step 1.3 and resuspend in 50 µL BSA–saline.

1.6 Add 2.5 µL of the WGA conjugate stock solution (for a final concentration of 100 µg/mL) and mix by pipetting up and down several times. If there is a problem observing a difference between gram-negative and gram-positive cells, then it may be necessary to reduce the amount of WGA conjugate added to the cells.

1.7 Incubate for 5–15 minutes at room temperature.

1.8 Centrifuge at 2000 rpm for 1–2 minutes to remove the WGA staining solution.

1.9 Resuspend in 50 µL BSA–saline.

1.10 Add 2.5 µL of the DAPI stain/SYTOX Green working solution from step 1.2, mix and incubate for 10 minutes at room temperature.

1.11 Transfer about 10 µL of the sample to a slide, apply a glass coverslip, seal and observe immediately in the fluorescence microscope.

Bacteria Immobilized on Membranes

If desired, the stained bacteria may be immobilized on membrane filters and mounted in *Bac*Light mounting oil (Component E) before examination in the fluorescence microscopy. Filters with low dye binding and superior flatness should be used. Blackened polycarbonate filter membranes with a 13 mm diameter and 0.2 µm pores (e.g., Poretics cat. #10532) are typically used in conjunction with drain disc–support membranes, which are placed beneath the filters to promote uniform distribution of bacteria on the filter surface (e.g., Poretics cat. #87481).

2.1 Prepare and stain bacteria as in steps 1.1–1.10. After staining, increase the volume to 1 mL by adding 0.9 mL of BSA–saline.

2.2 For vacuum filtration, filter bacteria onto a 13 mm–diameter membrane under low vacuum using a stainless steel vacuum filtration apparatus. For pressure filtration, filter bacteria using a 13 mm–diameter filter membrane secured in a stainless steel Swinney filter holder that is attached to a syringe apparatus.

2.3 Place 4 µL of sterile water on a glass microscope slide.

2.4 Remove filter and drain disc together and place both, bacteria side up, on top of the water droplet.

2.5 Add 6–10 µL of *Bac*Light mounting oil to the top of the filter.

Table 2. *Optical filters recommended for use with the ViaGram Red$^+$ Kit.*

Kit Component	Optical Filters *	
	Omega	Chroma
A. DAPI	XF06, XF35	31000, 31013
B. SYTOX Green	XF22, XF23	31001, 41001
C. Texas Red-X–WGA	XF43, XF102	31004, 41004

* Catalog numbers for recommended bandpass filter sets for fluorescence microscopy. Omega® filters are supplied by Omega Optical Inc. (www.omegafilters.com). Chroma filters are supplied by Chroma Technology Corp. (www.chroma.com).

2.6 Place an oversized 22 mm square coverslip on top of the mounting oil and apply gentle pressure to spread the fluid over the filter. Do not spread the mounting oil past the edge of the filter.

2.7 Seal the coverslip with melted paraffin or other suitable sealant.

2.8 Observe in a fluorescence microscope equipped with appropriate filter sets, as described below.

Interpretation of Results

The ViaGram Red$^+$ staining reaction is designed to discriminate live from dead cells and, at the same time, gram-negative from gram-positive cells. The four possible staining patterns that may occur with the ViaGram Red$^+$ Kit are described in Table 1. The actual appearance of bacteria in stained preparations depends on several factors, including the optical filters used in the fluorescence microscope, differences in the relative brightness of the fluorescent reagents, the abundance of target sites for dye-binding and the spatial localization of the fluorophores.

For fluorescence microscopy, it is best to use three separate bandpass optical filter sets to independently view the blue, green and red fluorescent components of the stained preparation. An appropriate longpass optical filter can sometimes be used to simultaneously visualize the live- and dead-cell staining; however, the gram-positive stain must be viewed with a bandpass optical filter set. Likewise, a DAPI/fluorescein/Texas Red multiple-dye filter set, can be used to observe the live- and dead-cell staining, but the gram-positive surface staining is often obscured by the brighter interior stain. Table 2 presents filter sets recommended for visualizing the staining reactions of the ViaGram Red$^+$ Kit.

References

1. Appl Environ Microbiol 56, 2245 (1990).

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

Cat #	Product Name	Unit Size
E-6606	ELF® spin filters *20 filters*	1 box
V-7023	ViaGram™ Red$^+$ Bacterial Gram Stain and Viability Kit *200 assays*	1 kit

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.

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