

***E. coli* Whole Cell Stain with BacLight Green and FM 4-64**

Derived from the Invitrogen/Molecular Probes BacLight manufacturer's protocol and lab FM 4-64 protocol.

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DAG, NSH

*Stain should be protected from light as much as possible.

Preparing Stain:

1. From original tube, make a **1mM stock solution** by adding DMSO (74uL for BacLight green, 69uL for BacLight red). Divide this stock solution into one-time-use aliquots of ~10uL in 1.5mL eppis. Store in a conical wrapped in foil desiccated at -20C.

2. Shortly before staining, prepare a **100uM working solution**. First, allow the tube of 1mM stock solution to come to room temperature before opening it to prevent condensation from forming in the stain. This is especially important if you plan to re-freeze the 1mM stock for later use. To protect the stain from light exposure, do this in a drawer or cover it with foil.

Dissolve 2uL of stain in 18uL of PBS (the original protocol suggests DMSO, but I saw no problems when staining with a PBS dilution). In PBS, the solution should turn a salmon-pink color.

Staining:

1. Grow culture in media of choice (*LB only media tested thus far*) to OD of choice.
2. Spin down 1mL culture, resuspend in 1mL PBS.
3. Add **1.5uL** of 100uM working stain solution to **1mL** PBS culture in eppi tube (stain ~**1:650**). Invert 6X to mix.
4. Incubate 30 minutes at room temp in the dark.
5. Add FM 4-64 (original dilution) at 1:500. Incubate for 1 min.

Mounting and Imaging

1. Adhere on Poly-L Lysine treated slide in the dark, appx. 5 min. It's helpful to do this in a Petri dish to make moving the slide around easier.
2. If you have problems with background, rinsing wells 2-3 times with PBS might reduce background without reducing cell signal.
3. Mount slide using SlowFade Antifade kit.
 - a. Add equilibration buffer, let incubate @ RT in the dark for 5 minutes.
 - b. Aspirate and mount using SlowFade Component A (contains glycerol).
4. Signal will bleach after 10-20 seconds of exposure to mercury light. For image capture, exposure ~2.0s.

Parameters Tested, Observations, and Conclusions:

STAIN

Stain Solvent: 100uM working dilution in PBS and DMSO appear to give same results. PBS nontoxic, preferred.

Stain Reuse & Freeze-Thaw: Multiple freeze-thaw cycles of 1mM stock are fine; that does not appear to affect signal fidelity.

Stain Age and Storage: All 1mM stocks stored desiccated at -20C. NSH stored 100uM PBS stock at 4C for reuse to no ill effect.

STAIN & CELLS

Stain Incubation Time: 10, 30, and 60min tested. 30min gives better results than 10min, but no significant improvement at >30min.

37C Incubation with Stain: Incubating stain @ 37C with growing culture produces no noticeable improvements, and in fact seems to have a weaker signal.

Fixing: Fixing *prior* to staining might help ~1 out of 3 times. Do not wash or fix the cells *after* staining. It removes most of the stain.

Stain Media: Cells resuspended in PBS probably stain slightly better than cells incubated with stain in original LB.

Lysozyme Treatment After Staining: Produces no noticeable improvements in staining or slide adhesion.

MOUNT FOR MICROSCOPY

Surface: Signal is weak in PBS mount on 1% agarose pads. Use slide.

Final Mounting Solution: Signal is weak if slide is mounted in PBS, and stronger in SlowFade. H₂O mount gave weak signal, and 50% glycerol gave intermediate signal. Each tested only once.

Placement of Mounting Solution: It's possible that adding SlowFade Component A directly to a well preserves that well's signal better. Not rigorously tested.

Rinse Slide to Reduce Background: After adhering cells to poly-L-lysine-treated slide, it is safe and can be helpful to rinse slide wells 2-3 times with PBS to mildly reduce background.

*OD has not been constant across all of this testing. As far as we can tell, this hasn't made a significant difference in signal fidelity.