

Protocol I: Staining of surface biofilm at an air-liquid interface

This protocol describes how to grow biofilm at the air-liquid interface using the inclined-coverslip method. As ECtracer™ doesn't influence biofilm formation when used in recommended concentrations, it can be present in growing cultures. If necessary for imaging, we recommend light fixation in ice-cold ethanol, but fixation in 4% paraformaldehyde works as well. We have tested this procedure with *Salmonella Enteritidis* and *Salmonella Typhimurium* strains. For these strains, we have not observed staining of intracellular or membrane components.

Solutions and Reagents:

ECtracer™ is provided as 1000-fold concentrated solution. The following common reagents are required (not supplied):

- Sterile glass coverslips (24x24 mm)
- 6-well plate with cover or adhesive seal
- Growth medium
- Phosphate buffered saline (PBS) pH 7.4
- Ethanol, 95% (-20°C) or 4% PFA
- Mounting medium

Assay Procedure:

- Place two sterile glass coverslips opposite to each other and inclined towards the walls of the wells in a 6-well plate.
- Prepare your bacterial culture at appropriate bacterial density. Then, add ECtracer™ (1:1000) and mix gently before pipetting 5-7 ml of the solution into each well fitted with glass coverslips.
- Seal the plate with a cover or adhesive seal and incubate at a temperature of choice for 24-48 h.
- Remove the glass coverslips from each well. Wipe the backside clean and fix biofilm formed at the air-liquid interface on the glass coverslip by immersing the coverslip in cold ethanol for 4 min or PFA for 30 min.
- Wash 3 x 1 min in PBS.
- Mount the samples using mounting medium and seal the coverslip onto the slide to prevent drying.

Fluorescence Microscopy:

- **ECtracer™ 480 (part of ECtracer™ Mix&Try Kit):** Excite at 405 nm (standard laser line) and detect emission using the DAPI or FITC filter sets. Optional: An excitation range of 405-458 nm and a detection range of 470–550 nm may be applied depending on available laser lines and filter sets.
- **ECtracer™ 520 (part of ECtracer™ Mix&Try Kit):** Excite at 458 or 488 nm (standard laser lines) and detect emission using a standard FITC filter set. Optional: An excitation range of 405-488 nm and a detection range of 500-600 nm may be applied depending on available laser lines and filter sets.
- **ECtracer™ 630 (part of ECtracer™ Mix&Try Kit):** Excite at 488 or 514 nm (standard laser lines) and detect emission using standard TRITC or TxRed filter set. Optional: An excitation range of 458-514 nm and a detection range of 600-650 nm may be applied depending on available laser lines and filter sets.
- **ECtracer™ 680:** Excite at 561 nm (standard laser line) and detect emission using a standard Cy5 filter set. Optional: An excitation range of 530-565 nm and a detection range of 600-800 nm may be applied depending on available laser lines and filter sets.

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