ColorFlux
Protocol

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The following protocol has been validated and optimized with MH II culture medium. If using another medium, the ColorFlux concentration and incubation time might need to be adjusted. As a general rule, we do not recommend the use of a rich medium, that leads to a rapid efflux of the ColorFlux below 15 minutes.
When you start an experiment, we recommend that you follow it through to the end at a steady pace. Efflux is an active process, thus overwashing, storing live cells in the buffer for an extended period of time would lead to misinterpretation.
At the end of the experiment, you can either look at the color of the pellet (chapter 4), or read the fluorescence (chapter 5) to evaluate the strength of the efflux.

1. The material you need

Reagents

- 1 mg/mL ColorFlux stock solution (provided)
- Muller Hilton Broth II (MH-II) culture medium
- Potassium Phosphate Buffer (PPB) or Phosphate Buffered Saline (PBS), pH 7.0-7.4
- Gram+ bacteria of your choice

Consumables

- Conical flasks
- 15 mL or 50 mL centrifuge tubes

2. Storing ColorFlux

The 1 mg/mL ColorFlux stock solution is stable for at least 8 months when stored at 4°C.

! Do not freeze.

3. The staining of bacteria with ColorFlux

- Plate the bacteria on freshly prepared MH-II agar plates with or without antibiotics. Incubate overnight at 37°C.

Incubation time and temperature might be adjusted depending on the bacteria used for the experiment.

- On the next day, pick up one or two colonies from the overnight culture and inoculate a conical flask containing 20-30 mL of MH-II medium.
- Incubate the conical flasks on an orbital shaker incubator set at 37°C and 160 rpm for a few hours to reach a mid-exponential phase.

You will obtain optimal results using bacteria in the mid-log exponential phase. The incubation time might be adjusted depending on the bacterial species used and its doubling time.

- Transfer the cells into a 50 mL or 15 mL centrifuge tube and centrifuge at 2 600-3 500 g for 10 min to obtain a cell pellet.
- Discard the supernatant.
- Add fresh MH-II medium to the centrifuge tube so that OD600 is comprised between 1.5 and 4.

- Transfer 5 mL of OD adjusted cell suspension into clean 50 mL or 15 mL centrifuge tubes.
- Add 5 µL of 1 mg/mL ColorFlux stock solution to reach a final concentration of 1 µg/mL.

We recommend using 50 mL tubes to facilitate their shaking. Alternatively, this step can be performed in smaller tubes or conical flasks.

• Screw cap the tubes and shake them thoroughly to mix the compound. Incubate at room temperature for 15 min.

! Do not incubate for less than 15 min or more than 30 min.

Then wash the cells as follow:

• Centrifuge the tubes at 2600-3500 g for 10-15 min to get a cell pellet.
• Remove the supernatant.
• Resuspend the cell pellet in 5 mL of PPB or PBS.
• Centrifuge the tubes at 2600-3500 g for 10 min.
• Resuspend the cell pellet in 1 mL of PPB or PBS.

Check that the resulting cell suspension is free from lumps.

4. Assessing colorimetry

• Quickly transfer an aliquot (800 µL) of cell suspension in an 1.5 mL tube
• Centrifuge the tube (2600 g, 10 min)
• Proceed to naked eyes evaluation of the coloration.

We recommend checking your pellets immediately after the second wash as this is when colors will look the brightest and different efflux levels will be the easiest to discriminate. They will progressively fade afterwards. If needed, store your cell pellets at 4°C to extend their coloration for few hours.

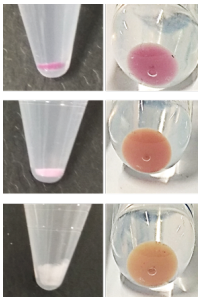
5. Using a microplate reader

• Take the remaining 200 µL of the cell suspension obtained at the end of the 3rd chapter.
• Adjust OD600= 1.
• Proceed to fluorescence reading in a black 96 well plate (Ex = 530nm / Em = 645-650nm).

Alternatively, you can scan the plate for Emission = 560 to 820nm to get fluorescence curves.

Visually assessing the colorimetry

Efflux level	Bacterial pellet color
Low efflux activity (i.e., lack functional efflux pumps)	Dark pink
Moderate efflux activity	Pale pink to orange (depending on inherent bacterial color)
High efflux activity (i.e., have efflux-mediated antibiotic resistance)	White/off-white to yellow (depending on inherent bacterial color)



How to use ColorFlux

A

Plate the bacteria on an MH II agar plate

Grow overnight at 37°C

B

Suspend 2-3 colonies into 20 mL of MH-II

Grow the cells to mid-exponential phase

Transfer the cell suspension into a falcon tube

C

Centrifuge at 3500 g for 10 min to get a cell pellet

Discard the supernatant and resuspend the cell pellet in fresh MH-II medium so that $1.5 < OD_{600} < 4$

Transfer 5 mL of the cell suspension into a new falcon tube

D

Add ColorFlux at a final concentration 1 µg/mL

Gently shake the falcon tubes and keep it on table for **15** min, no more than 30.

E

Centrifuge the tubes at 3500 g for 10 min to get a cell pellet

Discard the supernatant and resuspend the cell pellet in 5 mL of PBS or PPB.

F

Centrifuge the tubes at 3500 g for 10 min to get a cell pellet

Discard the supernatant and resuspend the cell pellet in 1 mL of PBS or PPB.

G Gi

Quickly transfer to a 1.5 mL tube

Centrifuge the tube at 3500 g for 10 min to get a cell pellet

Assess color of the pellet by naked eyes

Gii

Take a 200 µL aliquot
Adjust OD_{600} to 1

Transfer in a black 96-well plate and proceed to fluorescence reading (λ.exc = 530 nm; λ.em = 645/650 nm)

Check protocol, examples of results and much more on:
idylle-labs.com/colorflux

Credits: Icons from Noun Project
Agar plate: Amethyst Studio
Flask: Atif Arshad
Centrifuge tube: Julie Ko
1.5 mL tube: ims.icon
Centrifugation: Carl Holderness

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