

Drug Sensitivity Assay of *Xanthomonas citri* subsp. *citri* Using REMA Plate Method

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[Abstract] Resazurin Microtiter Assay (REMA) is a simple, rapid, reliable, sensitive, safe and cost-effective measurement of cell viability. Resazurin detects cell viability by converting from a nonfluorescent dye to the highly red fluorescent dye resorufin in response to chemical reduction of growth medium resulting from cell growth (Palomino *et al.*, 2002). The REMA assay can be used as a fluorogenic oxidation-reduction indicator in a variety of cells, including bacteria, yeast and eukaryotes (Silva *et al.*, 2013).

Materials and Reagents

1. Chemicals: Synthetic esters of gallic acids (Ximenes *et al.*, 2010)
2. Bacterial strain: Wild type *Xanthomonas citri* subsp *citri* strain 306 (Schaad *et al.*, 2005)
3. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D8418)
4. Kanamycin (Sigma-Aldrich, catalog number: K4000)
5. Luria-Bertani broth (LB) culture medium
6. Resazurin sodium salt (Sigma-Aldrich, catalog number: R7017)

Equipment

1. 96-well plate, polystyrene, with clear flat bottom wells (Greiner Bio-one, catalog number: 655101)
2. SPECTRAfluor Plus (Tecan) microfluorimeter
3. Multichannel pipetman (Eppendorf)

Procedure

- A. Prepare stock solutions of chemicals (dried-powder samples) dissolving in 10% in DMSO (diluted in sterile water).
- B. Add 100 µl of water to columns 1 and 12 to avoid evaporation (Table 1).

- C. Dilute the stock solutions in LB medium directly in a 96-well plates using a 2-fold scheme (final volume of 100 μ l per a well); after serial dilution, the most concentrated sample should have maximum 1% DMSO.
- D. Cells were grown in LB medium at 30 °C under rotation (200 rpm) until OD₆₀₀ 0.6 (log phase).
- E. Add 10 μ l of bacterial inoculum (standardized to 10⁵ CFU/well).
 - a. Negative control: 1% DMSO dissolved in LB.
 - b. Positive control: Kanamycin at 15.6 μ g/ml.

Table 1. Example for setup of REMA 96-well assay plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	200 μ l H ₂ O	200 μ l drug 1	200 μ l drug 2	200 μ l drug 3	200 μ l drug 4	200 μ l drug 5	200 μ l drug 6	200 μ l drug 7	200 μ l drug 8	200 μ l drug 9	100 μ l negative control	200 μ l H ₂ O
B	200 μ l H ₂ O	100 μ l 2A	100 μ l 3A	100 μ l 4A	100 μ l 5A	100 μ l 6A	100 μ l 7A	100 μ l 8A	100 μ l 9A	100 μ l 10A	100 μ l negative control	200 μ l H ₂ O
C	200 μ l H ₂ O	100 μ l 2B	100 μ l 3B	100 μ l 4B	100 μ l 5B	100 μ l 6B	100 μ l 7B	100 μ l 8B	100 μ l 9B	100 μ l 10B	100 μ l negative control	200 μ l H ₂ O
D	200 μ l H ₂ O	100 μ l 2C	100 μ l 3C	100 μ l 4C	100 μ l 5C	100 μ l 6C	100 μ l 7C	100 μ l 8C	100 μ l 9C	100 μ l 10C	100 μ l negative control	200 μ l H ₂ O
E	200 μ l H ₂ O	100 μ l 2D	100 μ l 3D	100 μ l 4D	100 μ l 5D	100 μ l 6D	100 μ l 7D	100 μ l 8D	100 μ l 9D	100 μ l 10D	100 μ l positive control	200 μ l H ₂ O
F	200 μ l H ₂ O	100 μ l 2E	100 μ l 3E	100 μ l 4E	100 μ l 5E	100 μ l 6E	100 μ l 7E	100 μ l 8E	100 μ l 9E	100 μ l 10E	100 μ l positive control	200 μ l H ₂ O
G	200 μ l H ₂ O	100 μ l 2F	100 μ l 3F	100 μ l 4F	100 μ l 5F	100 μ l 6F	100 μ l 7F	100 μ l 8F	100 μ l 9F	100 μ l 10F	100 μ l positive control	200 μ l H ₂ O
H	200 μ l H ₂ O	100 μ l 2G	100 μ l 3G	100 μ l 4G	100 μ l 5G	100 μ l 6G	100 μ l 7G	100 μ l 8G	100 μ l 9G	100 μ l 10G	100 μ l positive control	200 μ l H ₂ O

- F. Incubate the test plates at 30 °C for 6 h.
- G. Add 15 μ l of a 0.01% (w/v) resazurin solution, and incubate at 30 °C for 2 h.
- H. Measure fluorescence at 530 nm (excitation) and 590 nm (emission) using a fluorescence scanning.
- I. Percentage of inhibition is defined as:

$$\frac{[(\text{average FU negative control}) - (\text{average FU test})]}{(\text{average FU negative control})} \times 100$$
 FU: Fluorescence Units

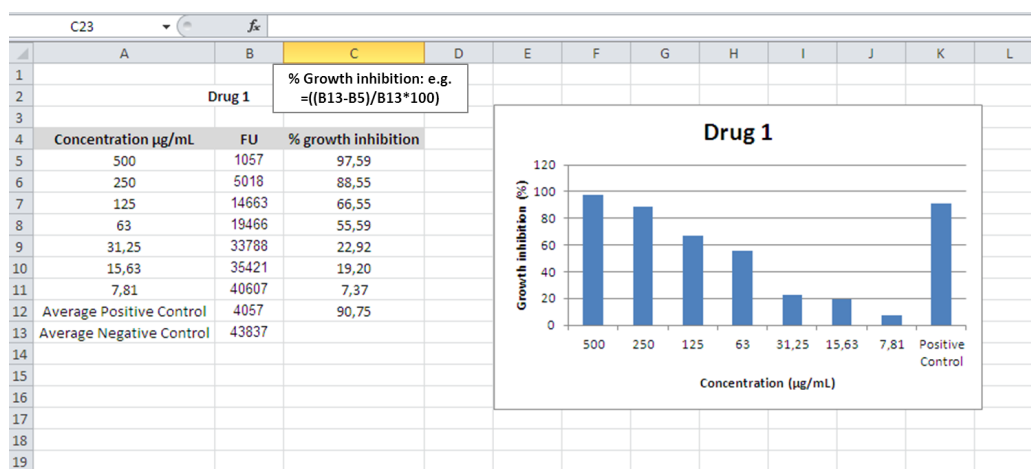


Figure 1. Example for calculation of growth inhibition

Note: Three independent experiments should be conducted, and the data is used to construct plots of chemical concentration versus cell growth inhibition in order to determine the MIC* (Figure 1).

*The minimum inhibitory concentration (MIC) is defined as the lowest concentration of the antibiotic able to inhibit the growth of 90% of organisms.

Acknowledgments

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- [*alfalfae* \(ex Riker and Jones, 1935\) dye 1978 as *X. alfalfae* subsp. *alfalfae* \(ex Riker et al., 1935\) sp. nov. nom. rev.; and "var. *fuscans*" of *X. campestris* pv. *phaseoli* \(ex Smith, 1987\) Dye 1978 as *X. fuscans* subsp. *fuscans* sp. nov. Syst Appl Microbiol 28\(6\): 494-518.](#)
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