# A protocol for evaluation of the total number of pollen grains per anther and the pollen grain fertility in Citrus.

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**Abstract**

This protocol counts the number of pollen and evaluate their maturity by lactophenol blue staining. This protocol is adjusted for the flower of citrus.

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## Materials

Lactophenol blue solution 1.13741.0100 by Merck Millipore

## Protocol

### *Step 1.*

Three representative flowers of citrus per individual seedling were collected immediately after full bloom during anthesis. The flowers were stored in plastic containers with silica gel at −30°C until evaluation.

### *Step 2.*

The anthers were removed from each flower with forceps, and then immersed into 50 µl of lactophenol blue solution (Merck Millipore, Billerica, MA, USA) with shaking at 300 rpm for 1 h at room temperature. 10–15 anthers were used in an individual flower which has less than 500 of pollen grains per anther, and 3–5 anthers were used in that has more than 500 of pollen grains per anther.

### *Step 3.*

The anthers were dehisced with forceps, then shaken at the same speed for an additional two hours.

### *Step 4.*

An aliquot of the pollen grains dispersed in lactophenol blue solution was put on a Thoma hemocytometer (0.1 mm in depth, Nitirin, Tokyo, Japan), and then four photographs per flower were taken under a microscope (Eclipse 80iR, NIKON, Japan) that was equipped with a digital camera. Ten photographs were taken when pollen was less than 10 per microscope field.

### *Step 5.*

The number of observed pollen grains was counted from the digital images using ImageJ software (http://imagej.nih.gov/ij/) with a plug-in “cell counter”. The number of pollen grains per anther was

converted from the average number of grains in digital images in 4 or 10 photographs and determined from three flowers in an individual seedling.

***Step 6.***

The ratio of densely stained pollen grains to the total number of pollen grains (the sum of stained and unstained pollen grains) was regarded as the apparent pollen fertility. The pollen grains were counted up to 30–50 in an individual flower which has less than 500 of pollen grains per anther, and 50–200 in that has more than 500 of pollen grains per anther for calculating the apparent pollen fertility. The apparent fertility was determined from three flowers in an individual seedling.