

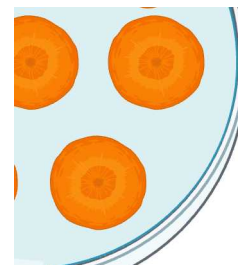
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# A Quantitative Bioassay for Crown-Gall Tumourigenesis using Carrot Disks V.1

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**Protocol status:** Working

**We routinely use this protocol and it is reproducibility working across multiple research institutes.**

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## Disclaimer

The authors declare no conflict of interest.

## Abstract

A number of bioassays have been used to study crown gall tumour induction by the bacterium *Agrobacterium tumefaciens*, *Rhizobium rhizogenes* and *Allorhizobium rhizogenes*. Here we describe in detail a quantitative, bioassay protocol to assess the tumour forming capacity of bacterial isolates on carrot disks. Furthermore, we include details to test the biological control capacity of non-tumourgenic bacteria on pathogenic tumourgenic bacteria. Visual results from the bioassay are possible after 14 days and quantitative results after 28 days.

## Materials

1.

## Protocol materials

 Ethanol 100%

## Preparation of *Allorhizobium* spp., *Agrobacterium* spp. cells & plates

- 1 Quantitative bioassays of crown gall disease causing pathogens on heterologous species was previously utilised by Klein and Tenenbaum in 1955 and this protocol outlines in detail the bioassay (1). The pathogenic strain used in this protocol is *Allorhizobium vitis* strain K377 (2, 3). A range of other crown gall or cane gall pathogens belonging to *Agrobacterium tumefaciens*, *Rhizobium rhizogenes*, *A. vitis* and related species such as *A. vitis* K306 (4, 5) strains or species can be tested in the assay.

Co-inoculation with a non-pathogenic biological control strain (for example *A. vitis* strain F2/5; 6, 7) to test for effective control against pathogenic strains can also be performed using the protocol below (8). If performing co-inoculation with a second strain, undertake the same steps as outlined for the pathogenic strain unless otherwise stated.

### Reagents and materials required

- Bacterial strains
- Fresh carrot taproots
- Sterile 1.5 mL and 2 mL plastic tubes
- Plastic sterile 90 mm Petri dishes
- Borosilicate petri dishes (150 mm x 30 mm)
- Borosilicate bottle 1 L
- Agar (such as Merck Sigma-Aldrich, A1296)
- Sodium salicylate (Biochemicals, BIOSB0885, MW 160.1)
- Milli-Q water
- Retractable, box cutting knife (Stanley 18 mm Snap knife); sharp kitchen knife (eg 15 cm)
- Plastic cutting board (36 cm x 25 cm)
- P200 micropipette and disposable plastic tips
- Parafilm® M
- 70 and 100 % ethanol in spray bottles
- Paper towel
- Aluminium foil (30 cm x 50 m)
- Safety matches
- Yeast mannitol agar (YMA) plates

### Optional reagents

- Cycloheximide (Merck 239763-M, MW 281.35)
- Amphotericin B (also known as Fungizone™)
- Dimethyl sulfoxide (Sigma-Aldrich D8418, MW 78.13)
- 95 % ethanol



## Equipment

- Laminar flow hood with UV germicidal lamp (Thomas Scientific, 1139C95, Mfr. No.VLF-36)
- Bench top vortex (Scientific Instruments, Vortex-Genie 2 Vortex Mixer)
- Autoclave (Steris, AMSCO® C Series Remanufactured Small Steam Sterilizer)
- Microbiology plate incubator (Thermo Scientific™ 50125590)
- Analytical Balance (METTLER TOLEDO, Balance XPR105)

2d

- Day 1:** Grow the test *A. vitis* pathogenic strain on a freshly-made YMA 90 mm plate by 16-streaking cells from a glycerol stock, seal the plate with Parafilm® M and incubate the plate at 26-28 °C for 48:00:00
- Day 1:** Sterilise the required number of 150 mm borosilicate plates by wrapping them in aluminium foil and placing in an autoclave at 15 PSI (104 kPa) 121°C for 15 mins.
- Day 1:** Prepare 1 litre of 1.5% water agar by dissolving 15 g of agar in 1 L of distilled or tap water in a borosilicate bottle. Sterilize the water agar in an autoclave at 15 PSI (104 kPa) 121 °C for 15 mins.
- Day 2:** Prepare the water agar plates in a sterile laminar by pouring about 90 mL of the cooled, molten agar water into the sterilised plates to a depth of approximately 5 mm. After the water agar solidifies, cover and store the water agar plates at 4 °C until use.
- Note 1.** Optional water agar supplementation: To inhibit the growth of fungi, cycloheximide can be added to the cooled water agar to a final concentration of 50 ug/mL before pouring the plates. Cycloheximide 50 mg/mL stocks can be prepared by dissolving 50 mg of cycloheximide in either 95 % ethanol or dimethyl sulfoxide (DMSO).

**WARNING:** Cycloheximide is a potent inhibitor of protein biosynthesis in eukaryotic cells (e.g. humans) by effectively blocking translational elongation. Gloves and other personal protection should be worn while handling cycloheximide.

A good alternative to cycloheximide is Amphotericin B (also known as Fungizone™) for supplementation of the water agar. Amphotericin B can be added to the cooled water agar to a final concentration of 2 ug/mL before pouring the plates. Amphotericin B actively binds to membrane sterols, leads to the formation of pores thereby increasing fungal plasma membrane permeability.

- Day 3:** Resuspend the actively growing cells by using a sterile loop (3 mm diameter) to remove a loop full of cells from the YMA plate and placing them in 1 mL of water or 150 micromolar (μM) sodium salicylate solution in a 1.5 mL tube. Vigorously resuspend the cells by vortexing the tube for 10-15 seconds using a bench top vortex



until an even suspension of cells is observed. A final suspension of cells that gives  $5 \times 10^7$  to  $10^8$  CFU/mL is ideal.

Also prepare 100 uL of 150 uM sodium salicylate water solution as a negative control for step 21.

- 7.1 **Note 2.** Actively growing *A. vitis* K377 cells from a YMA plate resuspended in water to give an OD<sub>600</sub> absorbance of 1.0 produce about  $1 \times 10^9$  CFU/mL.

- 8 Incubate the cell suspension at  4 °C until use.

- 9 Sterilise the laminar flow cabinet by turning on the inbuilt ultraviolet (UV) lamp for 15 minutes.

- 10 **WARNING:** High intensity ultraviolet radiation emitted by the germicidal UV-C (~253 nm) lamp is extremely hazardous and short exposures can cause skin irritation or blisters and eye damage. Ensure the glass or metal shielding doors are in place while the UV lamp is activated.



## Carrot disc preparation

- 11 Remove any extraneous soil, lateral roots, root hairs and petioles and leaves from the fresh carrot taproot (*Daucus carota* L.). Use a sharp kitchen knife to cut the base of the leaf petiole and discard the leaves, scrub the carrot root with a scrubbing brush and soap, thoroughly rinse the root with non-sterile water, and dry the scrubbed and rinsed carrot root with paper towel.

- 12 **Note 3.** Carrots naturally vary in their response to inoculation with crown gall pathogens. Therefore it is necessary to use at least 4-6 replicates.



**Note 4.** We use a relatively, short and blunt tipped, broad shouldered variety such as Chantenay, as the taproot shape minimises the carrot disk volume variation.

- 13 Surface sterilise a bench near a sterile hood by applying 70 % ethanol and wiping the surface thoroughly with paper towel. Place the carrots on the sterile bench and using a spray bottle, spray 100%  Ethanol 100% on to the carrots. Carefully ignite the ethanol by using a match to flame the carrots for 3-5 seconds. Ensure the flame is completely extinguished.



- 14 **WARNING: Flaming the carrots produces a naked flame and is extremely hazardous.** Ensure appropriate personal protection is worn and the surrounding area is free from





flammable material.

15 Repeat step 13 two more times.

16 Place the flamed carrots in a sterile laminar flow and again surface sterilize the carrot by wiping the surface of carrot with 70 % ethanol.

17 Sterilize both sides of a plastic cutting board using 70 % ethanol. On the sterilised board, cut cross sections of the sterilised carrot into 5 to 7 mm thick discs using a surface sterilised retractable, box cutting knife or sharp kitchen knife.

Avoid the top and bottom 20 mm of the carrot as these sections give variable tumour formation.

18 **Note 5. CRITICAL** Note the basal (towards the shoot) and apical (towards the taproot meristem) sides of each carrot disc. Untransformed callus tissue will grow on the apical surface of an uninoculated disk and this is a normal process unrelated to crown gall tumour growth.



19 Using the retractable knife, remove the epidermis and the outer cortex and avoid damage to the vascular cambium ring. Trim the outer edges of each disk approx. halfway between the cambial ring and the outer edge. This minimises contamination of the disks during the incubation period.

20 Place the required number (eg 5 to 8) of trimmed carrot discs basal-side upwards on the sterile water agar (Figure 1).

**Note 6.** It is important that at least a 5 mm gap is between the top of the carrot disk and the bottom on the closed lid. If there is insufficient space the tumour growth can be reduced.

## Carrot disc inoculation with pathogenic *A. vitis* cells

1h

21 Apply about  25-30  $\mu\text{L}$  of the bacterial cell suspension to the entire cambium region of the basal surface using a P200 micropipette to achieve  $3-6 \times 10^6$  CFU/disc. Ensure even distribution around the cambium ring. An important negative control is the same volume of water or 150  $\mu\text{M}$  aqueous sodium salicylate solution



- 22 If testing only for tumour formation proceed to step 24.

## Co-inoculation with a second non-pathogenic strain

- 23 Dry the inoculated cell suspension on the carrot disks (step 21) by leaving the uncovered plate in the sterile laminar flow for about 30 minutes before undertaking this step.

Add  25-30  $\mu\text{L}$  of the non-pathogenic bacterial cell suspension onto the pre-inoculated cambium region using a micropipette tip. Typically, a 1:1 of pathogenic:non-pathogenic cells is used.

**Note 7.** It is not essential to add the pathogenic strain first. The non-pathogenic strain can be added first and in our experience can be added up to several hours before the pathogenic strain.

## Carrot disc-bacterium co-culture

1h

- 24 Dry the inoculate carrot discs at room temperature in the laminar flow for about 30 minutes with the dish open.
- 25 Place the lid on the dish and seal the dish with Parafilm<sup>®</sup> M. Ensure the lid is completely sealed and that there are no gaps.

- 26 Cover the sealed plates with aluminium foil and incubate at room temperature  21 °C for 3-4 weeks.

Check the tumour growth every 5-7 days by removing the foil and observing the carrots through the glass lid. Do not remove the lid.

- 27 At the end of the growth period, shave the tumours off the carrot surface into a tared 2 mL tube and weigh.

### Notes

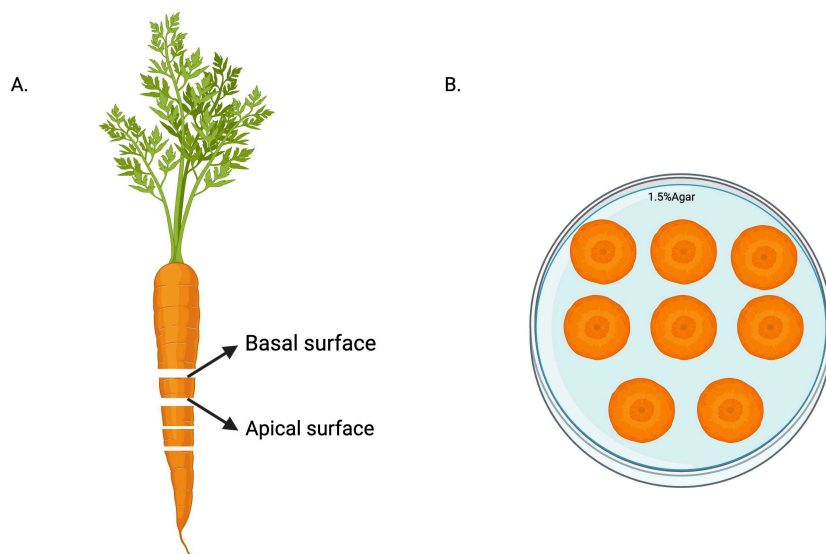
**8.** Sodium salicylate is not essential for tumour formation and does not have to be included in step 7. However addition at the inoculation stage accelerates tumour formation possibly by accelerating early cambium cell division. Serendipitously in a chemical genetic screen for inhibitors of tumour formation we discovered sodium salicylate promoted tumour formation on carrot discs.

9. We use at least four, preferably 6, replicate carrots (one carrot/dish).
10. For each carrot it is important to have a negative and positive control treatment to allow identification of weak tumour forming carrots. Depending of the variety and freshness of the carrots, approximately one in every 5 carrots weakly forms tumours. A positive control pathogenic strain is used to identify these weakly responding carrots.
11. Treatments/carrot: On a single water agar dish that has eight carrot discs, six pathogenic isolates plus positive and negative controls can be tested.

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**Overview of carrot taproot disks used during the protocol.**

(A) Dissection of a carrot taproot showing the basal and apical surfaces of the carrot disks relative to the root and shoot apices. (B) Plan view of sterilised carrot disks on agar medium. The basal surface of the carrot disk is shown including the inoculated cambium ring (pale orange inner ring).