Protocol for the determination of white clover (*Trifolium repens*) HCN cyanotype using Feigl-Anger Assay

Note: This protocol is adapted from:

Gleadow, R., N. Bjarnholt, K. Jørgensen, J. Fox, and R. Miller. 2011. Cyanogenic glycosides *In* S. Narwal, L. Szajdak, and D. Sampietro, editors. Research Methods in Plant Sciences, Volume 1: Soil Allelochemicals. Studium Press, Houston, TX.

A video of this protocol can be viewed on www.globalurbanevolution.com. For questions, please email us at *globalurbanevolution@gmail.com*

List of Materials:

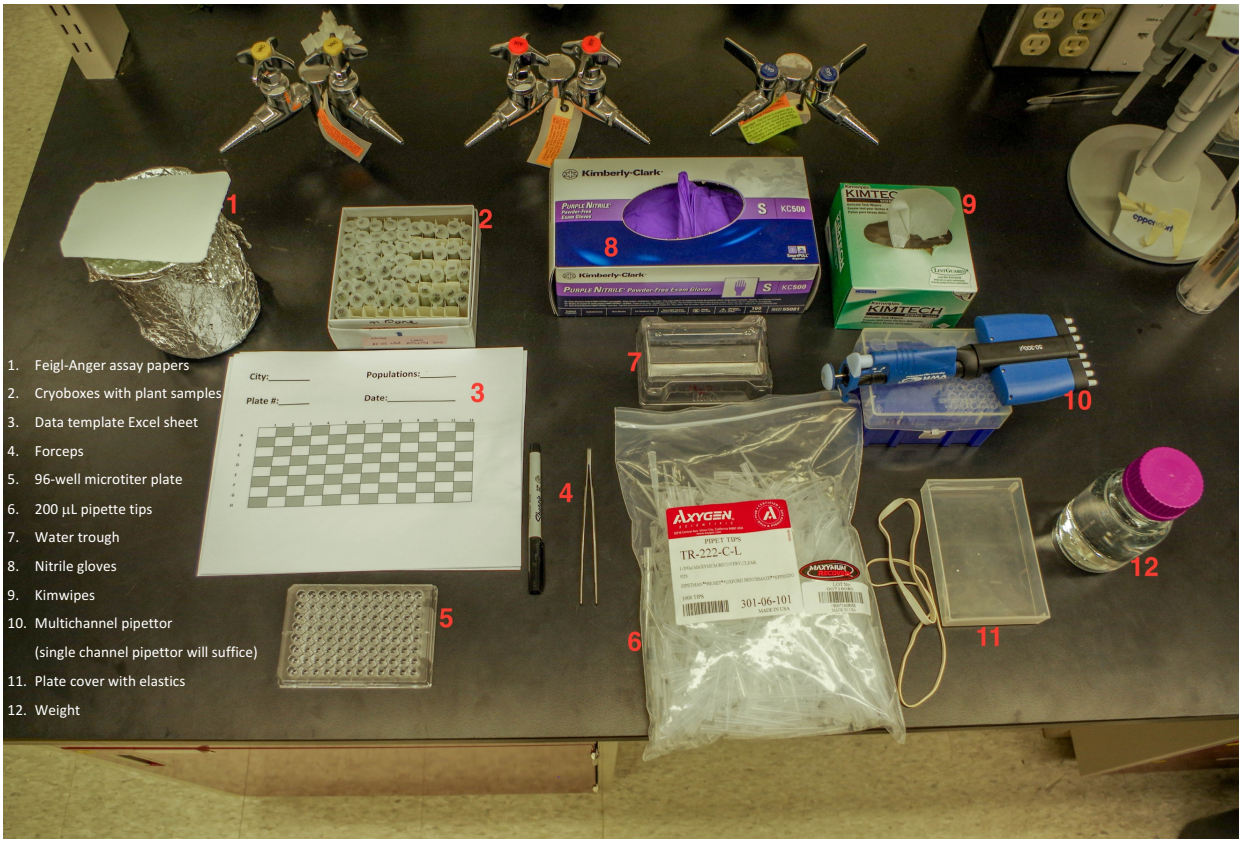
Provided by the Global Urban Evolution (GLUE) Team:

- Feigl-Anger assay papers
- Data template Excel sheet (downloaded from www.globalurbanevolution.com)
- 96-well microtiter plate (e.g. here)
- Elastic bands
- A multichannel trough

Provided by you:

- Samples in -80°C cryofreezer
- Heavy weight (e.g., water bottle, brick, etc.)
- Incubator set to 37°C
- 600 Pipette tips (200 µL)
- Cryoboxes (e.g. here)
- Distilled water
- Forceps
- Kimwipes
- Multichannel or single channel pipettor capable of dispensing 80µL of water

Note: See next page for photo of materials

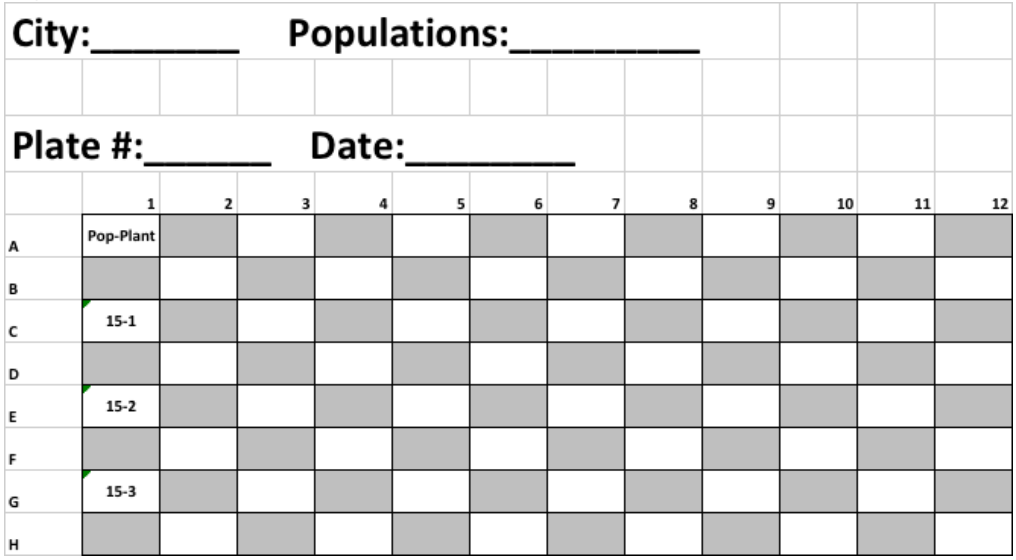


1. Feigl-Anger assay papers
2. Cryoboxes with plant samples
3. Data template Excel sheet
4. Forceps
5. 96-well microtiter plate
6. 200 µL pipette tips
7. Water trough
8. Nitrile gloves
9. Kimwipes
10. Multichannel pipettor
    (single channel pipettor will suffice)
11. Plate cover with elastics
12. Weight

Procedure:

1) Remove cryobox containing samples to be tested from –80°C freezer and let stand for 20-30 minutes to defrost.
2) While tissue is defrosting, download data template Excel sheet from www.globalurbanevolution.com. This sheet will be used to record and track which plant is in which well of the 96-well microtiter plate.
    - The assay uses 48 of the 96 wells; empty wells between samples containing tissue reduces contamination and false positives in the detection of HCN.
    - Begin in the top left of the plate (cell A1), working your way down the 8 wells (towards cell H1) before moving to column 2 (i.e., cell B2).
    - Avoid splitting populations across multiple plates. If not all plants from a population fit on the same plate, begin a new plate. This reduces errors.
    - Each unshaded cell in the data template Excel sheet should represent a single sample (refer to table 1). For each plate, record:
        i. In the header:
            a **city** name
            b **population** number (1 through 50 [minimum 40])
            c **plate number** (e.g. 1 of x, 2 of x, 3 of x, etc.)
            d **date** assayed
        ii. In the spreadsheet:
            a **population** number (1 through 50)
            b **plant sample** number within a population (1 through 20[minimum 15])

Table 1: Data template Excel sheet that will be used to keep track of samples and populations being tested. As in cell A1, this will represent the first sample used. The first number (left of the dash) refers to the population number (e.g., 15), and the second number (right of the dash) is the individual plant sample number (i.e., 1 through 20).



3) Once the tissue is defrosted (20-30 min), add leaf material from plants to wells in the 96-well plate, following the template created in step 2.
    - Use a single, large leaf (containing 3 leaflets) in each well. If plants have small leaves, use 2 full leaves. (see Fig. 1)
    - Please do not throw away unused tissue as we may use the tissue for subsequent genomic analyses. Place unused tissue back in the original cryoboxes and place it back in the -80°C freezer for storage.

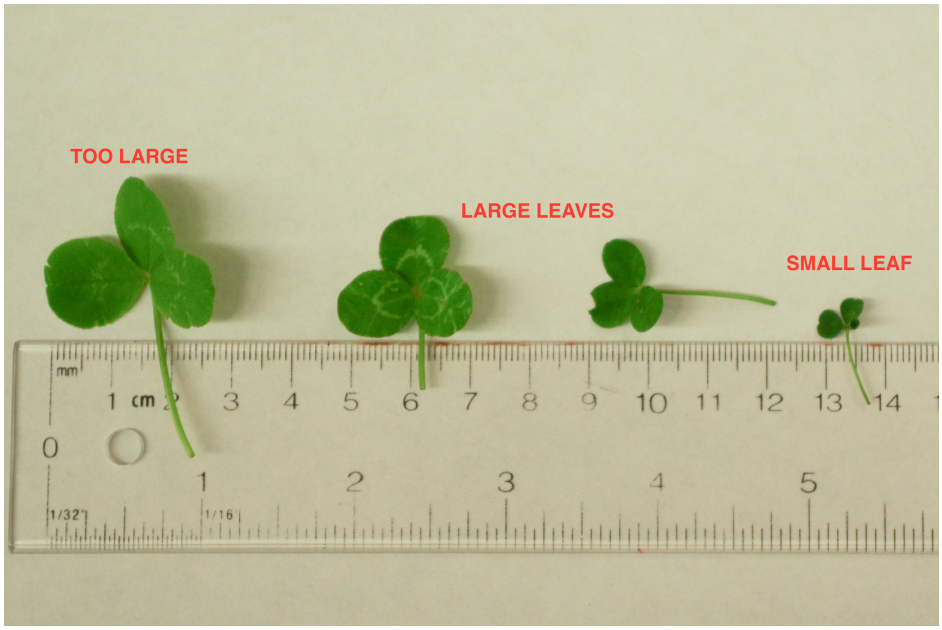


Figure 1: Appropriate leaf sizes to be used in the assay. The left most leaf is too large and should not be used. The middle two leaves are of good size and a single leaf of either is sufficient. The right most leaf is too small; use two leaves of this size per cell for the assay.

4) Pour water into the plastic multichannel trough provided and add 80 µL of distilled water to each well. It is easiest to use a multichannel pipette for this step, but a single channel pipette will work too. It is fine to have water in empty cells. The same pipette tip can be used to dispense all water across a single plate.
5) Using 200 µL pipette tips, macerate the tissue in the wells until well blended. The water will turn green and bubbles will form indicating the tissue is properly macerated (see fig. 2).

!!IMPORTANT!!: Use a separate pipette tip for each plant to avoid cross-contamination (i.e. 48 tips per plate)

6) Once all plants have been macerated, wipe down the top of the plate with Kimwipes to remove all liquid outside of wells. It may take multiple wipes to ensure there is no liquid on the surface of the plate that can interfere with the assay.

7) Use a fine-tipped Sharpie marker to label the top and left edges of the upper surface of the Feigl-Anger assay paper with corresponding column numbers and row letters, respectively; this will help minimize errors when scoring the plates once the assay is complete. Place the Feigl-Anger assay paper on top of the plate so that the paper covers all of the wells and the row and column labels are properly aligned. Add the 96-well plate cover on top of the plate and secure with elastic bands. The assay paper should be sandwiched between the plates and the cover (see fig. 3).

!!IMPORTANT!!: Make sure the plate and assay paper remain level when transferring them in and out of incubator. The assay paper will **not** react if it comes in contact with water.

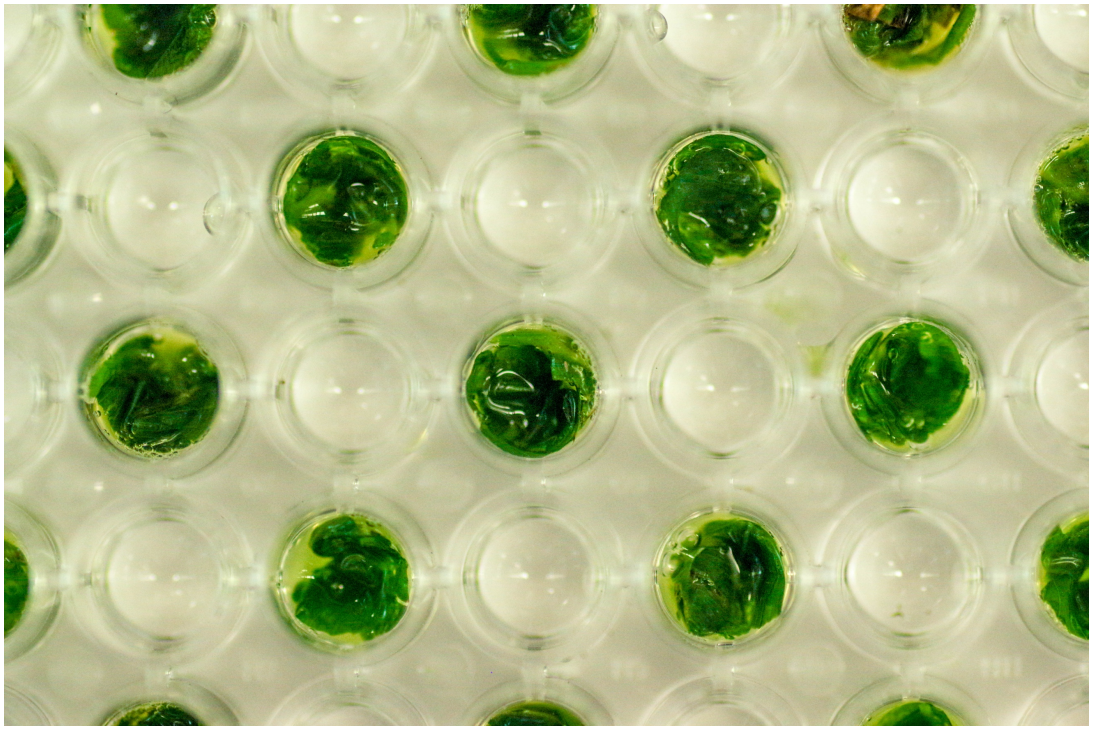


Figure 2: Sufficiently macerated tissue displaying a green hue to the water and/or the presence of bubbles.

8) Place the plate in an incubator set to 37°C for 3 hours. Add a weight on top of the plate to ensure the assay paper maintains a firm contact with the top of the plate. If you are adding more than one plate into the incubator at a time, make sure you label the plates with the population numbers and time to differentiate one plate from another.
   - While in the incubator, the water in the wells will begin to evaporate-this is normal. If there is HCN in the solution, it will react with the copper ethyl acetate on the assay paper and create a blue circle over top of the wells contains cyanogenic plant samples (HCN+, positive).

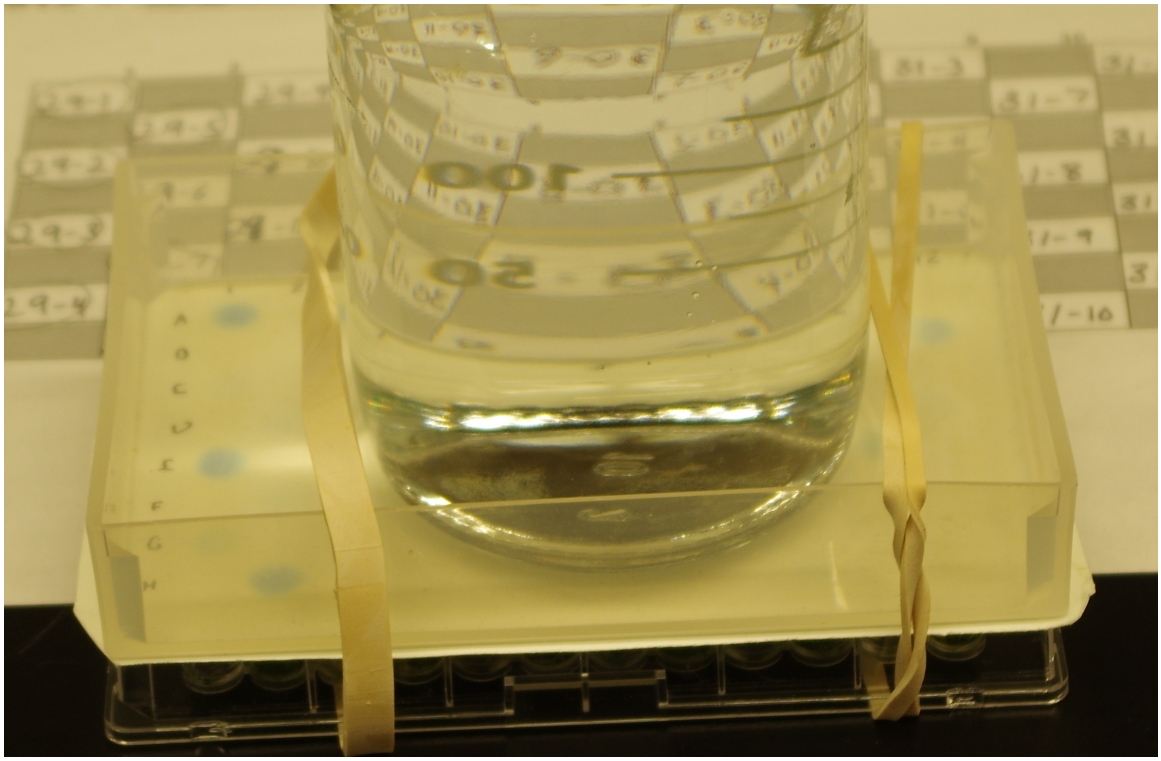


Figure 3: Feigl-Anger assay paper sandwiched between 96-well microtiter plate and plate cover with elastics securing cover to plate and weight ensuring assay paper is in firm contact with the plate. Note the lettering and numbering on the left and top edges of the upper surface of assay paper, respectively.

9) After 3 hours, gently remove the plate from the incubator. With the 96-well plate placed on top of the template paper in the correct orientation (see Fig. 4), take a high-resolution photo of the plate and assay paper from above. Turn the assay paper over, by turning it away from yourself, place the 96-well plate on top of the template paper again in the correct orientation, and take a second picture.
    - Photos will be used as a digital backup of cyanogenesis scores and will allow the GLUE Lead Team to help score wells should there be any concerns.
    - Photos are taken of the upper and lower surfaces because sometimes cyanogenic plants can only be distinguished from the lower-side of the paper (i.e. the side in contact with the microtiter plate).
10) Using the lower-side of the assay paper (i.e. side in contact with plate during assay), record which plants are cyanogenic based on the presence of a blue dot over the well. Circle on the assay paper samples that are positive for the presence of HCN so it can be referenced later when entering the data.

!!IMPORTANT!! If left too long, the reaction will bleed across the paper and it will be difficult to differentiate cyanogenic and acyanogenic plants. It is necessary to score the plants immediately upon removal from the incubator after 3 hours.

11) Immediately after scoring the presence (1) or absence (0) of HCN, add the HCN scores to the Excel data sheet in the column labeled “HCN (1=presence,0=absent)”. Cyanogenic plants (HCN+ = blue circle) should be coded as “1”; acyanogenic plants (HCN– = white) as “0”.
12) After entering the data for all samples and completing all data fields in the Excel data sheet, please go to globalurbanevolution.com and select “Upload Data”. This will take you to a page where you can upload the datasheet. Please follow the instructions on that page.
13) Please also make a zip file of all the Feigl-Anger assay photos and upload them on the same page.
14) Please email globalurbanevolution@gmail.com to indicate you have completed the upload of both the dataset and assay photo zip file.
15) Celebrate. You’ve just contributed an entire city’s worth of data to the GLUE Project!

Figure 4a (left): Example of digital backup photo displaying the up-side of Feigl-Anger assay paper along with associated template sheet. Note the lettering and numbering on the left and top sides of the assay paper respectively.
Figure 4b (right): Example of digital backup displaying the down-side of Feigl-Anger assay, after turning it away from yourself, along with the same associated template sheet as the up-side photo. Note the brown paper with “REV” indicating that this is now the down side of Feigl-anger assay paper.