Assay Validation

Cocktail Preparation

1. Thaw the Extraction Cocktail. Swirl to mix well. Maintain on ice.

2. Thaw the Assay-Cocktail Pre-mix. Bring to room temperature. Mix well. Pour 15 ml of ACPM into a mixing cylinder. Prepare notebook for completion of the cocktail (i.e., make calculations, make entries, plan timing, secure spectrophometer, &c.)

Tissue Pretreatment

1. Harvest three punches (nominal 13 mm) from a leaf blade. Estimate the mass of the three punches at 150 mg.

2. Float the punches on water in a petri dish.

3. Place the dish in darkness for minimum activity or under illumination for maximum activity.

4. Incubate the tissue for 15 minutes.

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Tissue Extraction

1. Homogenize the pretreated leaf punches well in 2 ml of ice-cold extraction cocktail. It is important to minimize time between the end of pretreatment and tissue disruption (to prevent inadvertent changes in activation state).

2. Centrifuge the extract for 5 minutes at at least 500 x g.

3. Remove the clear, cell-free supernatant and retain it on ice in a clean test tube for subsequent assay. Discard the particulate pellet.

4. If necessary to clarify the extract, repeat the centrifugation steps.

Assay

Validation can be accomplished in several ways, but for an established assay, it is usually sufficient to show that the reaction indicator (in this case, diminution of A_{340}) is substrate-dependent, linear with time, and linear with extract amount. To this end:

1. Finish making the assay cocktail. Set aside.

2. Set up four spectrophotometer tubes, each containing 3 ml of assay cocktail. Amend the first two tubes to contain 2 mM PGA. Initiate the reaction by adding 25 µl of extract to Tubes 1 and 3. The result should be:

Tube 1: + PGA, + extract
Tube 2: + PGA, - extract
Tube 3: - PGA, + extract
Tube 4: - PGA, - extract

3. Using water to "blank" the spectrophotometer, determine the A_{340} of the four tubes, in order, and record the time of each reading. Repeat the readings, still in order, until one tube has declined by approximately 0.25 optical density (O.D.).

4. Plot the A_{340} of each tube against time. Calculate the slopes (A_{340}/ time).

5. Before proceeding, analyze your data: (a) Was the reaction rate in Tube 1 substantially higher than that in the controls, Tubes 2 to 4? (b) Was the reaction rate too high to monitor accurately (or too slow for convenience)? For subsequent experiments, alter the aliquot volume accordingly. (c) Was the reaction rate linear with time? (d) Was substrate-independent NADPH oxidation significant? (e) Calculate the specific activity