H_2O_2 Assay (Amplex Red)

2011年12月12日
15:48


0000_00_00 _Amplex_...


Amplex Red Kit manual


2010_Rhee et al._Met...


Amplex Red Third Party...


Sigma - Catalase

Chris Harvey
2013_03_01

General Comments

This protocol has been optimized for *Arabidopsis thaliana* samples collected using the #8 (1.49cm diameter) leaf punch. A catalase labile signal has been difficult to obtain when applying this protocol to *Phaseolus vulgaris* samples. Use of 10kD filters during the centrifugation step was needed in the case of *P. vulgaris* samples. This method has been fairly robust for me, but does require haste downstream of the tissue grinding step to minimize H_2O_2 degradation. Upstream of this step, one may take one's time. The analysis template assumes that you'll be following this scheme pretty closely. It uses macros to fit the standard curve, and will give a warning when opened because of this. It is still a bit rough around the edges too… However, it can be referenced to get a visual sense of the plate layout for this protocol.

Starting Materials

- Invitrogen Amplex Red H_2O_2 Assay Kit
- 96 well "mixing" plate (Sarstedt 82.1581 plates work)
- 96 well "reaction" plate (Costar 3912 white, opaque plates)
- .1% (w/v) Trichloro Acetic Acid (TCA) solution

Prepare Amplex Red Kit components

1. Retrieve/thaw out components
   a. Amplex Red (-80°C)
   b. HRP (-80°C)
   c. 3% (.88M) H_2O_2 (-80°C)
   d. 250mM $NaPO_4$ (4°C)
   e. DMSO (ambient)
2. Dissolve Amplex Red in 60µL DMSO
3. Prepare at least 40mL of 50mM $NaPO_4$ working solution

*If using the kit for the first time dissolve the lyophilized HRP (yellow cap) in 1mL of 50mM $NaPO_4$ buffer. Prepare 5 aliquots of 200µL each. Store unused aliquots in -80°C
**DMSO can be stored at ambient
***$NaPO_4$ can be stored at 4°C

Prepare 2x Reaction Buffer

1. A 15mL falcon tube works well
2. Store on ice

2x Reaction Buffer	
50mM $NaPO_4$	4750 µL
10mM A. Red	50 µL
10U/mL HRP	200 µL
Total	5000 µL

Prepare 20mg/mL catalase solution

1. A 500µL microfuge tube is sufficient
2. Catalase is in the chest freezer in the "other enzymes" box
3. Weigh out ~1mg of catalase using the enclosed balance
4. Add 50µL of 50mM $NaPO_4$ buffer per mg of catalase

5. Store on ice

Prepare 5mM/20µM H_2O_2 solutions

1. 4µL 3% H_2O_2 into 700µL 50mM $NaPO_4$ (5mM)
2. 4µL 5mM H_2O_2 into 996µL 50mM $NaPO_4$ (20µM)

Start Sample Preparation (After this is started, time is limited)

1. Grind samples in Retsch Mill at 30Hz for 30sec
2. Add 400µL .1% (w/v) TCA solution to each sample
3. Mix sample thoroughly
4. Centrifuge at 12,000xg for 15 minutes at 4°C

While samples are centrifuging…..

1. Add 50µL of the 2x reaction buffer to each well of the "reaction" plate that will be used (multichannel helps)
2. Add 2µL catalase solution to the appropriate wells of the "mixing" plate
3. Prepare H_2O_2 standard curve on the "mixing" plate
    a. Add 120µL 50mM $NaPO_4$ to each well of the standard
    b. Add 120µL 20µM H_2O_2 to well containing 10µM standard
    c. Successively mix/transfer 120µL aliquots down the entirety of the standard curve to make a curve with 2x dilution steps

After samples are done centrifuging…..

1. Aliquot 70µL from each sample into 4 separate wells (allows for two technical replicates of both the w/ and w/o catalase condition) of the "mixing" plate
2. Shake the "mixing" plate on the plate reader for a few seconds to mix in the catalase in the respective cells
3. Wait ~5 minutes for catalase to react
4. Use the multichannel pipette to transfer 50µL from each well of the "mixing" plate to the corresponding well of the "reaction" plate

After the assay is mixed…..

1. Mix the "reaction" plate using the plate reader
2. Incubate the reactions for 30 minutes in the dark
3. Read the reaction plate using the plate reader in flourescence mode with 550/590nm excitation/emission.
4. 590nm flourescence data can be analyzed using the excel spreadsheet attached to this page.