GTP Binding Assays in *Arabidopsis thaliana*

Ana Romina Fox and Maria Agustina Mazzella*

Plant Molecular Biology, INBEBI, Buenos Aires, Argentina
*For correspondence: mazzella@dna.uba.ar

[Abstract] Signaling through G proteins constitutes an ancient mechanism that functions in the transduction of extracellular signals into intracellular responses. Activation of a proper receptor by a stimuli leads to an exchange of GDP for GTP, the activation of G proteins and the dissociation of G*α*-GTP from the G*βγ* dimer for the heterotrimeric G proteins. The G protein subunits remain active until the intrinsic GTPase activity or/and a GTPase activating protein result in the hydrolysis of GTP to GDP and the inactivation of the protein. Here we describe a protocol for measuring GTP binding activity from Arabidopsis plant protein extracts using GTP γ^{35}S. GTP γ^{35}S assay measures the level of G protein activation following a stimuli, by determining the binding activity of the non-hydrolysable analog GTP γ^{35}S. To determine specific G protein activities specific mutants and/or overexpressors extracts should be included and measured as controls.

Materials and Reagents

1. GTP γ^{35}S 1,200 Ci/mmol (PerkinElmer, catalogue number: NEG030H250UC).
2. 0.45 µm nitrocellulose filters (Bio-Rad Laboratories, catalogue number: 162-0115)
3. Scintillation solution (Optiphase Hisafe 3, PerkinElmer, catalogue number: 1200-437)
4. Bradford reagent
5. Triton X-100
6. Tris HCl (pH 8)
7. NaCl
8. EDTA
9. DTT (USB Corporation, Cleaveland OH USA, cataslogue number: 15397)
10. Roche complete protease inhibitor (Roche, Molecular Biochemicals)
11. Extraction buffer (see Recipes)
12. Reaction buffer (see Recipes)
13. Initiation buffer (see Recipes)
14. Stop buffer (see Recipes)

Equipment

1. Liquid scintillation counter Wallac 1214 RackBeta (Pharmacia, Turku, Finland)

Procedure

A. Protein extraction protocol (keep all steps at 4 °C)
   1. Harvest approximately 300 mg of Arabidopsis tissue in liquid nitrogen
   2. Homogenize with mortar and pestle to a fine powder.
   3. Resuspend the powder in 500 µl of extraction buffer (you can use manual homogeneizer).
   4. Centrifuge 15 min at 10,000 rpm at 4 °C and discard the pellet.
   5. Measure protein concentration in the supernatant as described (Bradford, 1976).

B. GTP binding assay (reaction: final volume 200 µl)
   1. Mix 100 µg of proteins in 100 µl extraction buffer with 80 µl of reaction buffer.
   2. Add 20 µl of initiation buffer.
   3. Expose to the corresponding stimuli (Light, agonist etc).
      (You should adjust the specific stimuli, time of exposition, concentration, intensity etc where you can detect optimum binding).
   4. Incubate the reaction 10 min at room temperature.
   5. Add 2 ml of ice-cold stop buffer to finish the reaction.
   6. Filter the reaction through 0.45 µm nitrocellulose filters using vacuum. We use 3 cm diameter disks.
   7. Wash the disk filters 5 times with 2 ml of cold Stop Buffer using vacuum. This step is important to wash away unbound GTP $\gamma^{35}S$.
   8. Dry disk filters containing the membranes for 15 min at 75 °C.
   9. Put the disks in plastic vials containing 2 ml of scintillation solution.
   10. Record the cpm in the ^{35}S energy range with a liquid scintillation counter.
   11. Important: Unspecific binding must be estimated using blank reactions. We recommend three replicates of two possible blanks: reactions without proteins (reaction buffer plus initiation buffer) or 100 µg of protein samples preheated to 95 °C for 10 min. We did not find differences in GTP binding between both blanks.
   12. Subtract the average cpm obtained with the blank samples from the cpm obtained for each measured sample to obtain the correct value.

Recipes

1. Extraction buffer
   50 mM Tris HCl (pH 8)
   100 mM NaCl
   1 mM EDTA
   1 mM DTT
   0.1% Triton X-100
   1x Roche complete protease inhibitor
   Reaction buffer (use 80 µl from the stock per reaction)

Stock	Final concentration in 200 µl reaction
50 mM Tris HCl (pH 8)	20 mM Tris HCl (pH 8)
250 mM NaCl	100 mM NaCl
2.5 mM EDTA	1 mM EDTA
2.5 mM DTT	1 mM DTT
0.25% Triton X-100	0.1% Triton X-100

2. Initiation buffer (se 20 µl from the Stock per reaction) (pH 8)

Stock	Final concentration in 200 µl reaction
100 mM $MgCl_2$	10 mM $MgCl_2$
1% Triton X-100	0.1% Triton X-100

   Add GTP $\gamma^{35}S$, 300,000 cpm/reaction.
3. Stop buffer (use 2 ml per reaction)
   20 mM Tris-HCl (pH 8)
   100 mM NaCl
   25 mM $MgCl_2$
   1 mM phosphate buffer

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References

1. Fox, A. R., Soto, G. C., Jones, A. M., Casal, J. J., Muschietti, J. P. and Mazzella, M. A. (2012). Cry1 and GPA1 signaling genetically interact in hook opening and anthocyanin synthesis in *Arabidopsis*. *Plant Mol Biol* 80(3): 315-324.

