

Polyamine and Paraquat Transport Assays in *Arabidopsis* Seedling and Callus

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[Abstract] Polyamines (PAs) are polycationic compounds found in all living organisms and play crucial roles in growth and survival. We here show the 'Polyamine and paraquat (PQ) transport assay' protocol, which can be used to examine the uptake activity of PA/PQ transporters. We have used this protocol to demonstrate that PUT3 in *Arabidopsis* is a polyamine transporter and is able to take up spermidine and its analog paraquat.

Keywords: *Arabidopsis thaliana*, Polyamine, Paraquat, Transport, Uptake

[Background] PAs are involved in gene regulation by interacting with and modulating the functions of anionic macromolecules such as DNA, RNA and proteins. In living cells, PAs' contents must be regulated to maintain the cellular hemostasis. In higher plants, three major polyamines, putrescine (Put), spermidine (Spd) and spermine (Spm), are present in either free form or conjugated forms with other molecules (Gill and Tuteja, 2010). In yeast, four plasma membrane polyamine transporters, DUR3, SAM3, GAP1 and AGP2 were identified (Uemura *et al.*, 2007). In *Arabidopsis*, five putative polyamine uptake transporters (PUT1-PUT5) were identified and PUT1-3 have been experimentally validated as polyamine transporters (Mulangi *et al.*, 2012; Li *et al.*, 2013). Our protocol described below has successfully confirmed that PUT3 is an influx transporter for polyamines and paraquat, and PQ/Spd uptake is impaired in the *put3* mutant (Shen *et al.*, 2016).

Materials and Reagents

1. Pipette tips
2. Plastic Petri dish (VWR, catalog number: 25384-326)
3. Parafilm
4. 1.5 ml Eppendorf centrifuge tube (VWR, catalog number: 20170-355)
5. Filter paper
6. Blue pestle (DWK Life Sciences, Kimble, catalog number: 749521-1500)
7. Cuvette (VWR, catalog number: 414004-051)
8. Syringe filters, 0.2 μ m pore size (VWR, catalog number: 28145-475)
9. Syringe
10. *Arabidopsis thaliana* ecotype Columbia (Col-0) and mutant line *lhr1 (put3)*
11. ScintiVerse™ BD Cocktail (Fisher Scientific, catalog number: SX18-4)
12. Clorox Bleach

13. Triton X-100 (Sigma-Aldrich, catalog number: T8787)
14. (2,4-Dichlorophenoxy) acetic acid sodium salt monohydrate (Sigma-Aldrich, catalog number: D6679)
15. Kinetin (Duchefa Biochemie, catalog number: K0905)
16. Sodium hydroxide (NaOH)
17. Murashige & Skoog Basal Salt Mixture (PhytoTechnology Laboratories, catalog number: M524)
18. Sucrose (Sigma-Aldrich, catalog number: S0389)
19. Agar (Sigma-Aldrich, catalog number: A1296)
20. Low melting point agarose (Sigma-Aldrich, catalog number: A9414)
21. Methyl viologen dichloride hydrate (Sigma-Aldrich, catalog number: 856177)
22. Spermidine (MP Biomedicals, catalog number: 02152068)
23. Paraquat-methyl-¹⁴C dichloride hydrate (Sigma-Aldrich, catalog number: 313947)
Note: This product has been discontinued.
24. Spermidine trihydrochloride [Terminal Methylene-3H (N)] (PerkinElmer, catalog number: NET522001MC)
25. Trizma[®] base (Sigma-Aldrich, catalog number: T1503)
26. Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) (Sigma-Aldrich, catalog number: E5134)
27. 2-Mercaptoethanol (Sigma-Aldrich, catalog number: M7522)
Note: This product has been discontinued.
28. Seed sterilization solution (see Recipes)
29. 2,000x 2,4-D (9.05 mM) (see Recipes)
30. 2,000x kinetin (1.86 mM) (see Recipes)
31. Callus induction solid medium (see Recipes)
32. Callus induction liquid medium (see Recipes)
33. ½ MS solid medium (see Recipes)
34. ½ MS liquid medium (see Recipes)
35. 200 µM non-¹⁴C-labeled PQ solution (see Recipes)
36. 2.04 mM non-³H-labeled Spd solution (see Recipes)
37. 40.37 µM non-³H-labeled Spd solution (see Recipes)
38. Solution 1: ¹⁴C-labeled PQ solution (see Recipes)
39. Solution 2: ¹⁴C-labeled PQ solution (see Recipes)
40. Solution 3: ³H-labeled Spd solution (see Recipes)
41. Solution 4: ³H-labeled Spd solution (see Recipes)
42. Solution 5: ³H-labeled Spd solution (see Recipes)
43. Solution 6: ³H-labeled Spd solution (see Recipes)
44. 1 M Tris-HCl, pH 7.5 (see Recipes)
45. 0.5 M EDTA, pH 8.0 (see Recipes)
46. Crude protein extraction buffer (see Recipes)

Equipment

1. Pipettes
2. pH meter
3. Weighing balance
4. Laminar flow hood
5. Stirring bar
6. Magnetic stirrer (VWR, model: 200 Mini-stirrer)
7. Vortex (Fisher Scientific, model: Vortex-Genie 2)
8. Scintillation counter (Beckman Coulter, model: LS-6500)
9. Centrifuge (Beckman Coulter, model: Microfuge[®] 22R, catalog number: 368831)
10. Spectrophotometer (Bio-Rad Laboratories, model: SmartSpec Plus, catalog number: 1702525)
Note: This product has been discontinued.
11. Autoclave

Procedure

A. Preparation of callus cells and plant seedlings

1. Callus cells
 - a. Put the *Arabidopsis* seeds in seed sterilization solution (see Recipes) for 15-20 min. Discard the sterilization solution and resuspend the seeds with sterilized ddH₂O in a laminar flow hood. Let the seeds settle down and discard the solution. Repeat the washing step 5 times.
 - b. Sow the surface-sterilized seeds in a row on callus induction medium (see Recipes) in a laminar flow hood, seal the Petri dishes with Parafilm and incubate the Petri dishes at 4 °C in the dark for 2 days.
 - c. Place the Petri dishes in a horizontal position and let the seeds germinate and grow under 16 h light/8 h dark cycle (lighting provided by fluorescent bulbs giving an average light intensity of ~150 μmol/m²/sec) at 22 °C for 3 weeks.
 - d. Transfer induced callus (Figure 1) to new callus induction medium in a laminar flow hood every 3 weeks until the transport assay (see Note 1).

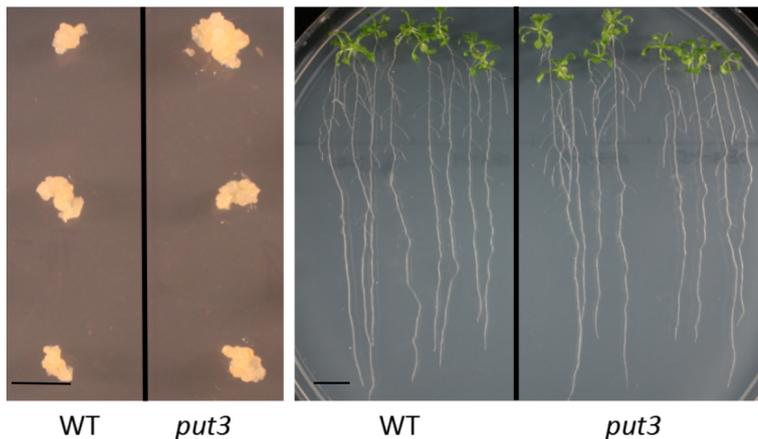


Figure 1. 3-week-old induced callus cells and 2-week-old *Arabidopsis* seedlings. Bars = 1 cm.

2. Plant seedlings

- a. Put the *Arabidopsis* seeds in seed sterilization solution for 15-20 min. Discard the sterilization solution and resuspend the seeds with sterilized ddH₂O in a laminar flow hood. Let the seeds settle down and discard the solution. Repeat the washing step 5 times.
- b. Sow the surface-sterilized seeds in a row on ½ MS medium (see Recipes) in a laminar flow hood, seal the Petri dishes with Parafilm and incubate the Petri dishes at 4 °C in the dark for 2 days.
- c. Place the Petri dish in a vertical position and let the seeds germinate and grow under 16 h light/8 h dark cycle (lighting provided by fluorescent bulbs giving an average light intensity of ~150 μmol/m²/sec) at 22 °C for 2 weeks (Figure 1).

B. Generation of standard curve

1. Standard curve for ¹⁴C-labeled PQ

- a. Make a 50x and 500x dilution of 10 μl solution 2 (see Recipes) with ddH₂O.
- b. In 1 ml of the ScintiVerse™ BD Cocktail, add 0 μl, 25 μl, 125 μl and 250 μl of 500x diluted solution to make ¹⁴C-labeled PQ solutions with 0 nCi, 0.1 nCi, 0.5 nCi, 1 nCi radioactivity, and add 125 μl and 250 μl of 50x diluted solution to make ¹⁴C-labeled PQ with 5 nCi and 10 nCi radioactivity, respectively.
- c. Measure the radioactivity by using Beckman LS-6500 scintillation counter to generate a standard curve for ¹⁴C-labeled PQ.
- d. Perform 3 sets of this test to ensure accuracy of the standard curve (Figure 2).

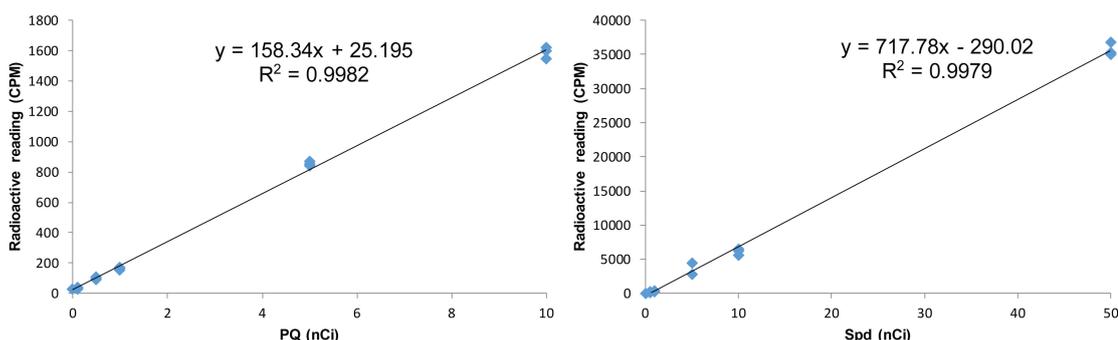


Figure 2. Representative of standard curves for radioactive labeled PQ and Spd

2. Standard curve for ³H-labeled Spd

- a. Make a 100x dilution of 10 μl solution 3 (see Recipes) with ddH₂O.
- b. In 1 ml of the ScintiVerse™ BD Cocktail, add 0 μl, 2.5 μl, 5 μl, 25 μl, 50 μl and 250 μl 100x diluted solution to make ³H-labeled Spd solutions with 0 nCi, 0.5 nCi, 1 nCi, 5 nCi, 10 nCi and 50 nCi radioactivity, respectively.
- c. Measure the radioactivity by using Beckman LS-6500 scintillation counter to generate a standard curve for ³H-labeled Spd.
- d. Perform 3 sets of this test to ensure accuracy of the standard curve (Figure 2).

C. Transport assays

1. Transport assays with callus (Table 1)

Table 1. Designed temperatures and Spd or PQ concentrations used in transport assay of callus cells

Treatment	Temperature	Chemical and final concentration	Radioactive labeled Material
Background (Non-specific binding)	0 °C	10 μM PQ	10 nCi/nmol PQ
	0 °C	2 μM Spd	500 nCi/nmol Spd
	0 °C	100 μM Spd	10 nCi/nmol Spd
Normal condition	25 °C	10 μM PQ	10 nCi/nmol PQ
	25 °C	2 μM Spd	500 nCi/nmol Spd
	25 °C	100 μM Spd	10 nCi/nmol Spd

- a. Transfer 100 mg fresh callus cells into a 1.5 ml Eppendorf centrifuge tube containing 950 μl liquid callus-inducing medium and vortex for 30 sec.
- b. Pre-treat samples at designed temperature for 30 min to reach equilibrium (Shen *et al.*, 2016).
- c. Add 50 μl solution 2 (final PQ concentration at 10 μM), solution 3 (final Spd concentration at 2 μM) or solution 4 (final Spd concentration at 100 μM) (see Recipes) into the tubes according to the treatment conditions (see Note 2).
- d. Briefly vortex for mixing and return the tubes back to the designed temperature.

- e. Incubate for 1 h, gently invert each tube by hand every 15 min to resuspend the cells and ensure the equilibrium of PQ or Spd in the solution.
 - f. Place tubes on ice immediately.
 - g. Let the callus cells settle down and remove the PQ- or Spd-containing liquid callus inducing medium by using pipette.
 - h. Wash callus cells five times on ice each with 1.5 ml pre-chilled liquid callus inducing medium. For each treatment, three replicate samples should be used at the same time.
2. Transport assays with seedlings (Table 2)

Table 2. Designed temperatures and Spd or PQ concentrations used in transport assay of plant seedlings

Treatment	Temperature	Chemical and final concentration	Radioactive labeled Material
Background (Non-specific binding)	0 °C	10 µM PQ	10 nCi/nmol PQ
	0 °C	2 µM Spd	250 nCi/nmol Spd
	0 °C	100 µM Spd	5 nCi/nmol Spd
Normal condition	25 °C	10 µM PQ	10 nCi/nmol PQ
	25 °C	2 µM Spd	250 nCi/nmol Spd
	25 °C	100 µM Spd	5 nCi/nmol Spd

- a. Transfer 20 seedlings of both wild type Col-0 and *put3* mutant line with intact roots onto a filter paper saturated with 5 ml liquid ½ MS medium (see Recipes), and pre-incubate the seedlings at the designed temperature for 30 min (see Note 3).
 - b. Mix 250 µl solution 2 (final PQ concentration at 10 µM), solution 5 (final Spd concentration at 2 µM) (see Recipes), or solution 6 (final Spd concentration at 100 µM) (see Recipes) with 4.75 ml ½ MS medium to reach the final concentrations.
 - c. Saturate another piece of filter paper with the radioactive solution made in step C2b and pre-incubate it at the designed temperature for 30 min.
 - d. Transfer pre-incubated seedlings in the step C2a one by one to the filter paper containing radioactive-labeled PQ or Spd and pre-incubate at the same temperature as stated in the step C2c. Carefully arrange the seedlings to make sure all roots are directly in contact with the filter paper.
 - e. Incubate at designed temperature for 1 h.
 - f. Cut off roots of the seedlings from each treatment.
 - g. Transfer the roots from each treatment into a 1.5 ml Eppendorf centrifuge tube.
 - h. Wash the roots 5 times on ice by using 1.5 ml pre-chilled liquid ½ MS medium. For each treatment, three replicate samples should be used at the same time.
- D. Extraction of intracellular contents
1. Grind washed callus cells or seedling roots by using small blue pestle in 1 ml (for callus cells) or 500 µl (for seedling roots) crude protein extracting buffer (see Recipes) on ice until no clumps

remain to release all intracellular contents. The solution should be homogenized to an even mixture that can be pipetted without clogs.

2. Centrifuge the mixture at 15,500 x g for 20 min at 4 °C.
3. Transfer supernatant into a new tube for radioactive and protein concentration measurements.
4. Measure the UV absorbance of 300 µl (for callus cells) or 100 µl (for seedling roots) of the supernatant of each sample by using Bio-Rad SmartSpec Plus Spectrophotometer. The total protein concentration is then calculated based on the UV absorbance using the equation:

$$\text{Protein concentration (mg/ml)} = (1.55 \times A_{280}) - (0.76 \times A_{260})$$

E. Radioactive measurement

1. Add 300 µl (for callus cells) or 100 µl (for seedling roots) of the supernatant of each sample into 1 ml ScintiVerse™ BD Cocktail solution.
2. Measure the radioactivity of each sample by using Beckman LS-6500 scintillation counter.

Data analysis

The radioactivity (nCi) of each sample was calculated based on the scintillation counter reading (CPM) and the equation of the corresponding standard curve. The total amount of transported PQ/Spd in each sample was then calculated based on the radioactivity and the used nCi per nmol in the transport assay solution. The total protein of each sample was calculated based on the protein concentration and the volume. The PQ/Spd transport rates were presented in the unit of µmole PQ/Spd per gram protein per hour (µmole g⁻¹ h⁻¹). The PQ/Spd transport rate at 0 °C of each sample was considered as non-specific binding of PQ/Spd, and therefore was subtracted from the PQ/Spd transport rates at 25 °C to calculate the adjusted and accurate PQ/Spd transport rates at normal condition. Readers are referred to Shen *et al.* (2016) for examples of data graphs.

Notes

1. We maintained the callus cells by transferring fresh and fast-growing callus cells to a new callus induction medium every 3 weeks. By judging the color and growth of the callus cells, it is easy to select the callus cells for transfer. Fresh calli usually show light color whereas relative old calli show dark color.
2. 2 µM Spd was designed to test if the transporter is a high-affinity PA transporter whereas 100 µM Spd was designed to test if other transporters are also working at high Spd concentrations. In our test, the callus cells and seedlings of the *put3* mutant showed approximately 20-fold and 4-fold reduction, respectively, on 2 µM Spd uptake rate compared with the wild type. However, when a higher concentration (100 µM) of Spd was added into the medium, the transport rate was comparable in both *put3* mutant and wild type. This might suggest that PUT3 protein is a

high-affinity PA transporter and some low-affinity PA transporters may start to work at high PA concentrations.

3. In the transport assay with seedlings, we used 150 x 15 mm Petri dish and put a filter paper fitting into the Petri dish. Seedlings were arranged relatively separate.

Recipes

1. Seed sterilization solution
25% Clorox Bleach
0.05% Triton X-100
Store at room temperature
2. 2,000x 2,4-D (9.05 mM)
26 mg (2,4-dichlorophenoxy) acetic acid sodium salt monohydrate
Add ddH₂O to 11 ml and sterilize by filtration through a syringe filter (0.2 µm)
Store at -20 °C
3. 2,000x kinetin (1.86 mM)
10 mg kinetin
1 ml 1 N NaOH
Add ddH₂O to 25 ml and sterilize by filtration through a syringe filter (0.2 µm)
Store at -20 °C
4. Callus induction solid medium
4.33 g/L Murashige & Skoog basal salts
3% sucrose
Adjust pH to 5.7 with 0.1 N NaOH and add agar to 0.7% (w/v)
Autoclave
Cool medium to ~60 °C
Add 2,4-D to a final concentration at 4.52 µM
Add Kinetin to a final concentration at 0.93 µM
Store at room temperature
5. Callus induction liquid medium
4.33 g/L Murashige & Skoog basal salts
3% sucrose
Adjust pH with 0.1 N NaOH to 5.7 and autoclave
Cool medium to ~60 °C
Add 2,4-D to a final concentration at 4.52 µM
Add Kinetin to a final concentration at 0.93 µM
Store at room temperature

6. ½ MS solid medium
 - 2.17 g/L Murashige & Skoog basal salts
 - 1.5% sucrose
 - Adjust pH to 5.7 with 0.1 N NaOH and add agar to 1.2% (w/v)
 - Autoclave
 - Store at room temperature
7. ½ MS liquid medium
 - 2.17 g/L Murashige & Skoog basal salts
 - 1.5% sucrose
 - Adjust pH with 0.1 N NaOH to 5.7 and autoclave
 - Store at room temperature
8. 200 µM non-¹⁴C-labeled PQ solution
 - 5.14 mg methyl viologen dichloride hydrate
 - 100 ml ddH₂O
 - Store at -20 °C
9. 2.04 mM non-³H-labeled Spd solution
 - 3.20 µl spermidine
 - 10 ml ddH₂O
 - Store at -20 °C
10. 40.37 µM non-³H-labeled Spd solution
 - 198 µl 2.04 mM spermidine
 - 9.8 ml ddH₂O
 - Store at -20 °C
11. Solution 1: ¹⁴C-labeled PQ solution (200 µM, 32.3 nCi/nmol, 15.55 ml)
 - 0.8 mg paraquat-methyl-C¹⁴ dichloride
 - 15.55 ml ddH₂O
 - Store at -20 °C
12. Solution 2: ¹⁴C-labeled PQ solution (200 µM, 10 nCi/nmol, 1 ml)
 - 309.6 µl solution 1
 - 690.4 µl 200 µM non-¹⁴C-labeled PQ solution
 - Store at -20 °C
13. Solution 3: ³H-labeled Spd solution (40 µM, 500 nCi/nmol, 1 ml)
 - 20 µl spermidine trihydrochloride, [terminal methylenes-³H (N)] - solution
 - 980 µl 40.37 µM non-³H-labeled Spd solution
 - Store at -20 °C
14. Solution 4: ³H-labeled Spd solution (2 mM, 10 nCi/nmol, 1 ml)
 - 20 µl spermidine trihydrochloride, [terminal methylenes-³H (N)] - solution
 - 980 µl 2.04 mM non-³H-labeled Spd solution
 - Store at -20 °C

15. Solution 5: ³H-labeled Spd solution (40 μM, 250 nCi/nmol, 1 ml)
 - 500 μl solution 3
 - 500 μl 40 μM non-³H-labeled Spd solution
 - Store at -20 °C
16. Solution 6: ³H-labeled Spd solution (2 mM, 5 nCi/nmol, 1 ml)
 - 500 μl solution 4
 - 500 μl 2 mM non-³H-labeled Spd solution
 - Store at -20 °C
17. 1 M Tris-HCl, pH 7.5
 - 121.1 g Trizma[®] base
 - 800 ml ddH₂O
 - Adjust pH to 7.5 with concentrated HCl
 - Add ddH₂O to 1,000 ml, autoclave
 - Store at room temperature
18. 0.5 M EDTA, pH 8.0
 - 186.1 g ethylenediaminetetraacetic acid disodium salt dihydrate
 - 800 ml ddH₂O
 - Adjust pH to 8.0 with NaOH (about 20 g)
 - Add ddH₂O to 1,000 ml, autoclave
 - Store at room temperature
19. Crude protein extraction buffer
 - 50 mM Tris-HCl (pH 7.5)
 - 0.1 mM EDTA
 - 1 mM β-mercaptoethanol
 - Make fresh solution and store at room temperature

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