

Polyamine Transport Assay Using Reconstituted Yeast Membranes

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[Abstract] ATP13A2/PARK9 is a late endo-/lysosomal P5B transport ATPase that is associated with several neurodegenerative disorders. We recently characterized ATP13A2 as a lysosomal polyamine exporter, which sheds light on the molecular identity of the unknown mammalian polyamine transport system. Here, we describe step by step a protocol to measure radiolabeled polyamine transport in reconstituted vesicles from yeast cells overexpressing human ATP13A2. This protocol was developed as part of our recent publication (van Veen *et al.*, 2020) and will be useful for characterizing the transport function of other putative polyamine transporters, such as isoforms of the P5B transport ATPases.

Keywords: Polyamine, Spermine, Transport assay, Reconstitution, Yeast membranes, P5 ATPase, ATP13A2

[Background] ATP13A2/PARK9 encodes a ubiquitously expressed late endo-/lysosomal membrane protein that is implicated in a spectrum of neurodegenerative disorders, like early-onset Parkinson's disease (Di Fonzo *et al.*, 2007; Lin *et al.*, 2008) and Kufor-Rakeb syndrome (an early-onset parkinsonism with dementia) (Ramirez *et al.*, 2006; Park *et al.*, 2011). ATP13A2 belongs to the P-type transport ATPases, a family of active transporters that transiently form a phospho-intermediate as a consequence of ATP hydrolysis (Kuhlbrandt, 2004). ATP13A2 is a member of the P5 subfamily, which was identified by genome sequencing more than twenty years ago (Axelsen and Palmgren, 1998) and contains five human isoforms (ATP13A1-5). However, the transported substrate of ATP13A2 remained unknown until recently, when we characterized ATP13A2 as a lysosomal polyamine exporter that shows the highest affinity for spermine (SPM) (van Veen *et al.*, 2020). ATP13A2 is activated by two regulatory lipids, phosphatidic acid and phosphatidylinositol(3,5)bisphosphate (Holemans *et al.*, 2015; van Veen *et al.*, 2020). To prove that ATP13A2 transports polyamines over the membrane, we developed a transport assay using tritium-labelled SPM ([³H]-SPM) and yeast membrane-derived vesicles recombinantly expressing human ATP13A2 with C-terminal BAD (biotin acceptor domain) tag (ATP13A2-BAD).

The polyamine transport assay starts with the production of reconstituted vesicles from solubilized yeast membranes that contain overexpressed ATP13A2-BAD, in the presence of the lipids phosphatidylcholine and phosphatidic acid. The generated proteoliposomes will contain both right-side-out and inside-out oriented reconstituted ATP13A2 (Figure 1), and we made use of this principle to set up a transport assay where we follow the luminal accumulation of [³H]-SPM in the vesicles. Since ATP13A2 is a lysosomal exporter, ATP13A2 should be inserted inside-out (cytosolic

domains facing the lumen) to allow luminal accumulation of [3 H]-SPM. In this orientation, the lumen of the proteoliposomes should be supplemented with ATP to ensure that ATP binding and ATPase activity can occur at the nucleotide-binding domain. Therefore, we reconstitute the proteoliposomes in the presence of ATP and an ATP regenerating system (phosphocreatine/creatine phosphokinase). In the inside-out orientation, ATP13A2 will promote the uptake of [3 H]-SPM in the proteoliposomes if ATP is present inside, in line with ATP13A2-mediated polyamine transport from the extra-cytosol to the cytosol in a cellular context (Figure 1).

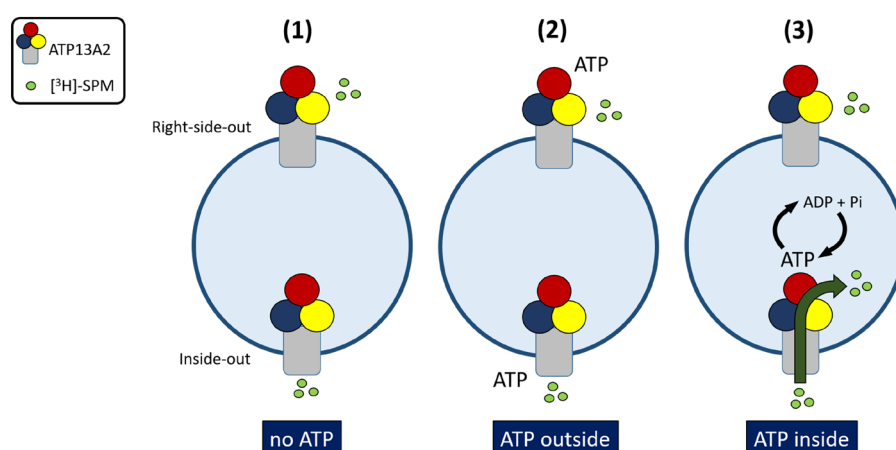


Figure 1. Graphical representation of the principle behind the polyamine transport assay. To assay polyamine transport, we use proteoliposome vesicles, reconstituted from yeast membranes that contain ATP13A2. The generated proteoliposomes will contain both right-side-out and inside-out oriented reconstituted ATP13A2. To allow luminal accumulation of [3 H]-SPM, ATP13A2 should be inserted inside-out (cytosolic domains facing the lumen) as ATP13A2 is a lysosomal exporter. In addition, the lumen of the proteoliposomes should be supplemented with ATP as in P-type ATPases, ATP binding occurs at a nucleotide-binding site located in one of the cytosolic domains. Only when ATP is present inside the proteoliposomes, [3 H]-SPM accumulates within the vesicles, in line with ATP13A2's cellular role as a lysosomal polyamine exporter.

Our protocol using yeast-derived membranes offers several advantages for the polyamine transport analysis as compared to mammalian systems (e.g., Uemura and Gerner, 2011). Although the preparation of yeast crude extract is more laborious and time-consuming compared to mammalian cell lysis, the benefit lies in the fact that yeast cells grow fast and are easy to culture with low cost and high yield of biomass. Moreover, yeast is a malleable model organism that is easily genetically manipulated. Furthermore, our protocol represents a clean *in vitro* technique as opposed to a cellular polyamine uptake assay. Our polyamine transport assay is applicable for other candidate polyamine transporters, which will help to establish the molecular players of the mammalian polyamine transport system, which remain largely unknown. Based on the high conservation of the substrate binding domain in the transmembrane helix M4, it is very likely that other mammalian P5B ATPases (ATP13A3-5) also play a role in the polyamine transport system, possibly with a slightly different substrate specificity, subcellular

localization and/or tissue distribution. Therefore, our transport assay will also be valuable for characterizing the transport function of the other related P5B ATPases.

Materials and Reagents

Notes:

- a. *All materials and reagents are kept at room temperature unless otherwise described. For the shelf life and storage temperature of reagents, we refer directly to the manufacturer's instructions.*
- b. *Equivalent materials and reagents of other companies might also be suitable.*

1. Pipette tips

P10 tips (Sarstedt, catalog number: 70.1130.20)

P200 tips (Sarstedt, catalog number: 70.760.102)

P1000 tips (VWR, catalog number: 613-0738)

2. Filter pipette tips

P10 filter tips (Greiner, catalog number: 771288)

P200 filter tips (Greiner, catalog number: 739288)

P1000 filter tips (Greiner, catalog number: 740288)

3. Eppendorf tubes (Greiner, catalog number: 616201)

4. Falcon tubes (Greiner, catalog numbers: 188271 [15 ml], 227261 [50 ml])

5. Nitrile gloves (VWR, catalog number: 112-2371)

6. Duran glass bottle (VWR, catalog numbers: 215-1516 [500 ml], 215-1517 [1,000 ml])

7. Culture flasks (DWK Life Sciences, catalog number: 217715407)

8. Syringe-driven filter unit (Merck, Millex, catalog number: SLGS033SB)

9. Petri dishes (60 mm) (ThermoFisher, catalog number: 123-17)

10. Bottle top vacuum filtration system (VWR, complete filtration unit with 0.2 µm pore size, catalog number: 514-0334)

11. Acid-washed glass beads (Sigma-Aldrich, catalog number: G8772)

12. Test tubes Soda glass (VWR, catalog number: 212-0013)

13. Membrane filters, 0.45 µm pore size (Millipore, catalog number: HAWP02500)

14. *Saccharomyces cerevisiae* strain W303-1B/Gal4-ΔPep4 (leu2-3, his3-11,15, trp1-1::TRP1-GAL10-GAL4, ura3-1, ade2-1, canr, cir+, ΔPep4 MATα)

Note: Strain available from corresponding author upon request.

15. Plasmid DNA

We use a pYeDP60 vector (with 2-µm circle replication origin, *URA3* and *ADE2* selection markers, and a galactose inducible promoter) containing a yeast codon-optimized version of human ATP13A2 variant 2 cDNA followed by a thrombin cleavage site and a C-terminal BAD tag (Jidenko *et al.*, 2006; Azouaoui *et al.*, 2014) (Figure 2). In our experiments, we use a

catalytically inactive ATP13A2 variant, namely the E343A mutant (van Veen *et al.*, 2020), as a negative transport control. E343 is positioned in the catalytic site for dephosphorylation, which is highly conserved among P-type ATPases (³⁴¹TGES motif in human ATP13A2 isoform 2).

Note: Plasmids available from corresponding author upon request.

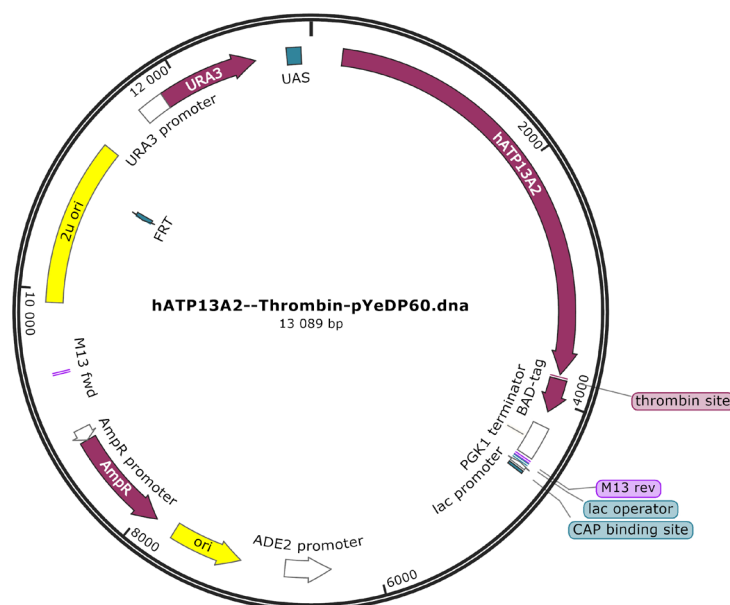


Figure 2. Schematic representation of the ATP13A2 expression plasmid map. The expression plasmid for ATP13A2 is a modified pYeDP60 plasmid. The pYeDP60 vector contains the 2-μm circle replication origin, the *URA3* and *ADE2* selection markers, a galactose inducible *GAL10/CYC1* promoter, multiple cloning sites, and the *PGK1* terminator. The human ATP13A2 gene was cloned into the plasmid with a thrombin cleavable BAD (biotin acceptor domain)-tag (Jidenko *et al.*, 2006; Azouaoui *et al.*, 2014).

16. Yeast extract granulated (Merck, catalog number: 1.03753.0500)
17. Peptone from casein (Tryptone) (Merck, catalog number: 1.07213.2500)
18. D-(+)-Glucose (Sigma-Aldrich, catalog number: G8270)
19. Cuvettes (VWR, catalog number: 634-0676)
20. Tris base (Sigma-Aldrich, Trizma base, catalog number: T1503)
21. Ethylenediaminetetra-acetic acid (EDTA) (BDH Laboratory Supplies, catalog number: 280214S)
22. Hydrochloric acid (HCl) fuming 37% (Merck, catalog number: 1.00317.2501)
23. HCl 0.5 N (Reagecon, catalog number: H20501)
24. Lithium acetate dihydrate (LiAc) (Sigma-Aldrich, catalog number: L6883)
25. Acetic acid (Sigma-Aldrich, catalog number: 27225)
26. DNA from herring sperm (Sigma-Aldrich, catalog number: D7290)
27. Polyethylene glycol (PEG) (Sigma-Aldrich, catalog number: P4338)

28. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: 276855)
29. Bacto Agar (BD, catalog number: 214010)
30. Yeast dropout mix without uracil (Sigma-Aldrich, catalog number: Y1501)
31. Yeast nitrogen base without amino acids (Sigma-Aldrich, catalog number: Y0626)
32. Ethanol absolute (VWR, catalog number: 20821.296)
33. D-(+)-Galactose (Sigma-Aldrich, catalog number: G0625)
34. KCl (Sigma-Aldrich, catalog number: P9541)
35. Sorbitol (Sigma-Aldrich, catalog number: S1876)
36. NaOH (Merck, catalog number: 6498.1000)
37. Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: 93482)
38. Protease inhibitor (Sigma-Aldrich, SIGMAFAST Protease Inhibitor Cocktail Tablets, catalog number: S8830)
39. HEPES (Sigma-Aldrich, catalog number: H3375)
40. Sucrose (Sigma-Aldrich, catalog number: S7903)
41. CaCl₂ (Sigma-Aldrich, catalog number: C3881)
42. n-Dodecyl-β-D-Maltopyranoside (DDM) (Inalco, catalog number: 1758-1350)
43. Bradford reagent (Sigma-Aldrich, catalog number: B6916)
44. Egg phosphatidylcholine (Avanti Polar Lipids, L-α-phosphatidylcholine [Egg, Chicken], catalog number: 840051C)
45. 18:1 phosphatidic acid (Avanti Polar Lipids, 1,2-dioleoyl-sn-glycero-3-phosphate (sodium salt), catalog number: 840875C)
46. Bio-Beads SM-2 Adsorbents (BioRad, catalog number: 1523920)
47. ATP disodium salt (Roche Diagnostics, catalog number: 10127531001)
48. MgCl₂ hexahydrate (Sigma-Aldrich, catalog number: M2670)
49. Phosphocreatine disodium salt hydrate (Sigma-Aldrich, catalog number: P7936)
50. Creatine phosphokinase (Sigma-Aldrich, catalog number: C3755)
51. Anti-ATP13A2 antibody (Sigma-Aldrich, catalog number: A3361)
52. Anti-rabbit IgG, HRP-linked antibody (Bioké, catalog number: 7074S)
53. γ-(N-Morpholino)propanesulphonic acid (MOPS) (VWR, catalog number: A1076.1000)
54. KOH (Sigma-Aldrich, catalog number: 221473)
55. Dithiothreitol (DTT) (VWR, catalog number: A2948.0025)
56. SPM (Sigma-Aldrich, catalog number: 85590)
57. [³H]-SPM, 1 mCi/ml, 20 μM (American Radiolabeled Chemicals, Inc.; catalog number: ART0471)
58. Liquid scintillation cocktail (MP Biomedicals, Ecolite(+)TM Liquid Scintillation Fluid, catalog number: 882475)
59. Chloroform stock (Sigma-Aldrich, catalog number: C2432)
60. 20% glucose (see Recipe 1)
61. YPD-agar plates (see Recipe 2)

62. YPD medium (see Recipe 3)
63. 10x TE stock (see Recipe 4)
64. 10x LiAc stock (see Recipe 5)
65. 1x TE/LiAc solution (see Recipe 6)
66. Single-stranded herring sperm DNA (see Recipe 7)
67. 50% PEG w/v (see Recipe 8)
68. PEG solution (see Recipe 9)
69. SD-uracil agar plates (see Recipe 10)
70. SD-uracil medium (see Recipe 11)
71. YPGE2x medium (see Recipe 12)
72. 20% galactose (see Recipe 13)
73. TEKS buffer (see Recipe 14)
74. TESin buffer (see Recipe 15)
75. HS buffer (see Recipe 16)
76. Buffer T (see Recipe 17)
77. Buffer T/DDM (see Recipe 18)
78. Buffer T/lipid mix (see Recipe 19)
79. 0.1 M ATP (see Recipe 20)
80. 0.1 M MgCl₂ (see Recipe 21)
81. 0.2 M phosphocreatine (see Recipe 22)
82. ATP-regenerating system (see Recipe 23)
83. 500 mM DTT (see Recipe 24)
84. 4x reaction buffer (see Recipe 25)
85. 1x reaction buffer (see Recipe 26)
86. 0.1 M MOPS (pH 7.0) (see Recipe 27)
87. 10 mM SPM (see Recipe 28)
88. 100 μ l [³H]-SPM/unlabeled SPM mix (see Recipe 29)

Equipment

1. Pipettes
 - P2 pipette (Gilson, catalog number: FA10001M)
 - P10 pipette (Gilson, catalog number: FA10002M)
 - P100 pipette (Gilson, catalog number: FA10004M)
 - P200 pipette (Gilson, catalog number: FA10005M)
 - P1000 pipette (Gilson, catalog number: FA10006M)
2. Serological pipettor (Sigma, BRAND® accu-jet® pro pipette controller, catalog number: Z637637)
3. Serological pipettes
 - 5 ml (Sarstedt, catalog number: 86.1253.001)

- 10 ml (Sarstedt, catalog number: 86.1254.001)
- 25 ml (Sarstedt, catalog number: 86.1685.001)
- 4. Ultracentrifuge tubes for Ti45 rotor (Beckman Coulter, catalog number: 355655)
- 5. Ultracentrifuge tubes for Ti70 rotor (Beckman Coulter, catalog number: 355630)
- 6. Glass Büchner filter funnel (Millipore, catalog number: XX1014700)
- 7. Magnetic stirrer (Heidolph Instruments, MR Hei-Standard, catalog number: 505-20000-00)
- 8. Refrigerated incubator with shaker (New Brunswick Scientific, model: Innova 4230)
- 9. Ice bucket (*e.g.*, styrofoam box)
- 10. Autoclave (LTE Scientific Ltd, Series 100 Autoclave)
- 11. MilliQ (MQ) water system (Sartorius, Arium Pro)
- 12. Spectrophotometer (Beckman Coulter, model number: DU-640B)
- 13. Centrifuge (Eppendorf, model: 5804 R) with rotor (Eppendorf, model: A-4-44)
- 14. Microcentrifuge (Eppendorf, Centrifuge 5417 R) with rotor (Eppendorf, model: F45-30-11)
- 15. Vortex (VWR, model: Vortex-Genie[®] 2, catalog number: 444-5900)
- 16. Thermomixer (Eppendorf, model: Thermomixer Comfort)
- 17. Water bath (Mettler)
- 18. Homogenizer (BioSpec products, BeadBeater, catalog number: 1107900EUR)
- 19. Pressure vacuum pump (Gelman Sciences, Gelman Little Giant, model: 13156)
- 20. Hamilton syringes (1 ml) (VWR, 1001 LTN, catalog number: 613-1300)
- 21. Nitrogen gas blow-down system (made in-house)
- 22. Ultracentrifuge (Beckman Coulter, model: Optima XPN-90)
- 23. Ultracentrifuge rotor (Beckman Coulter, model: Type 45 Ti)
- 24. Ultracentrifuge rotor (Beckman Coulter, model: Type 90 Ti)
- 25. Head-over-head rotator (Labinco BV, L28 Test-Tube Rotator, catalog number: 28000)
- 26. Vacuum filtration manifold (Millipore, catalog number: XX2702550)
- 27. Liquid scintillation analyzer (Perkin Elmer, TRI-CARB 2900TR)

Procedure

A. Yeast transformation

We transformed the yeast strain W303-1B/Gal4- Δ Pep4 (*leu2-3*, *his3-11,15*, *trp1-1::TRP1-GAL10-GAL4*, *ura3-1*, *ade2-1*, *canr*, *cir+*, Δ Pep4 MAT α) with the pYeDP60 vector containing hATP13A2 WT or the catalytically dead E343A mutant according to the lithium acetate/single-stranded carrier DNA/polyethylene glycol method with minor modifications (Gietz and Woods, 2002).

1. Plate the yeast from the glycerol stock on a YPD-agar plate and incubate for 48 h at 30 °C.
2. Inoculate 20 ml YPD medium from the fresh plate and incubate overnight at 30 °C, 230 rpm in a shaking incubator.
3. Measure the OD of the overnight culture at 600 nm (OD₆₀₀).

Note: 1 OD₆₀₀ equals approximately 10⁷ cells/ml.

4. Dilute the culture to 0.1 OD₆₀₀ in 10 ml YPD and incubate for 4-6 h at 30 °C, 230 rpm in a shaking incubator until the OD₆₀₀ of the culture lies between 0.4 and 0.8.
5. Spin down the cell amount equivalent to 2 OD₆₀₀ (= ± 2 x 10⁷ cells) in swinging buckets (700 x g; 5 min).
6. Wash the pellet in 1 ml sterile MQ and resuspend with a pipet tip. Transfer to an Eppendorf tube.
7. Spin down the cells (2,500 x g; 2 min) using a microcentrifuge.
8. Resuspend pellet in 1 ml 1x TE/LiAc.
9. Spin down the cells (2,500 x g; 2 min) using a microcentrifuge.
10. Resuspend pellet in 100 µl 1x TE/LiAc.
11. Add 10 µl of single-stranded herring sperm DNA.
12. Add 1 to 5 µg of plasmid DNA.
13. Vortex.
14. Add 600 µl of PEG solution.
15. Vortex.
16. Incubate for 45 min at 30 °C (volume = approximately 730 µl).
17. Add DMSO to a final concentration of 10% (approximately 73 µl).
18. Heat shock at 42 °C for 15 min.
19. Cool on ice for 1-2 min.
20. Spin down the cells (2,500 x g; 2 min) using a microcentrifuge.
21. Resuspend pellet in 200 µl of YPD and incubate for 2 h at 30 °C.
22. Spin down the cells (2,500 x g; 2 min) using a microcentrifuge.
23. Resuspend the cell pellet in sterile MQ and plate onto an SD-uracil agar plate.
24. Incubate the plate for 48 h at 30 °C to recover transformants.

B. Yeast culture

Here, we followed a similar strategy as described before (Jidenko *et al.*, 2006; Azouaoui *et al.*, 2014) with minor modifications.

1. Inoculate 20 ml of SD-uracil medium from the fresh plate (Step A24) to further select for yeast cells that carry the plasmid with URA3 selection marker and incubate for 24 h at 28 °C and 200 rpm in a shaking incubator.
2. Measure the OD₆₀₀ of the culture.
3. Inoculate 100 ml of SD-uracil medium to a final OD₆₀₀ of 0.2 and incubate for 12 h at 28 °C and 200 rpm in a shaking incubator.
4. Measure the OD₆₀₀ of the culture.
5. Inoculate 3.6 L of YPGE2X medium to a final OD₆₀₀ of 0.05 and incubate for 36 h at 28 °C and 175 rpm in a shaking incubator.

Note: Nine 1,000 ml culture flasks containing 400 ml of yeast culture.

6. Induce ATP13A2-BAD expression with 2% galactose (50 ml of 20% galactose solution per 500 ml culture) and incubate for 12 h at 18 °C and 175 rpm in a shaking incubator.

Note: The temperature downshift from 28 °C to 18 °C will reduce the rate of protein synthesis and therefore, facilitate proper protein folding, increasing the yield of properly folded and functional overexpressed protein. In addition, the lower temperature will decrease protein degradation.

7. Repeat galactose induction to ensure high overexpression levels of ATP13A2-BAD and incubate for another 12 h at 18 °C and 175 rpm in a shaking incubator.
8. Spin down the yeast cells (1,000 x g; 10 min; 4 °C).
9. Weigh the pellet.

Note: The yield is typically 20-30 g/L yeast culture. The cell pellet can be processed directly or stored at -20 °C for a maximum of 2 weeks.

C. Yeast membrane preparation

1. In case yeast cell pellet was frozen, thaw on ice.
2. Resuspend cell pellet in TEKS buffer (volume (ml) = approximately 2 x the weight of the cell pellet (g) as determined in step B9) and incubate for 15 min at 4 °C while mixing with a magnetic stirrer.
3. Spin down the cells (1,000 x g; 10 min; 4 °C).
4. Resuspend the pellet in TESin buffer (volume (ml) = approximately 1 x the weight of the cell pellet (g) as determined in Step B9).
5. Break the yeast cells using a BeadBeater.
 - a. Fill the chamber with 200 ml yeast suspension (add TES in buffer if not enough) and add 200 ml cold glass beads. Chamber should be as full as possible.
 - b. Fill ice water jacket with crushed ice and water.
 - c. Bead-beat in cold room for 5 min. Pause for 3 min after every min to reduce heating of the sample and BeadBeater.
 - d. Recover crude extract using Büchner funnel and vacuum pump.
6. Test the pH of the lysate by using pH paper and, if necessary, adjust the pH of the crude extract to pH 7.5 with saturated NaOH solution.
7. Centrifuge the crude extract at 2,000 x g for 20 min (4 °C).
8. Centrifuge the resulting supernatant at 20,000 x g for 20 min using ultracentrifuge rotor Type 45 Ti (4 °C).
9. Centrifuge the resulting supernatant at 200,000 x g for 1 h using ultracentrifuge rotor Type 45 Ti (4 °C).
10. Resuspend the resulting pellet (that is, the light membrane fraction, P3) in HS buffer.

Note: The volume for resuspension should be determined by eye and be the minimal amount required to homogeneously dissolve the pellet.

11. Determine the protein concentration of the P3 fraction using a classical Bradford assay (with known BSA concentrations as a protein standard).

Note: The measured protein concentration typically ranges between 35-50 mg/ml. For downstream reconstitution purposes, a minimum yield of 70 mg total protein is required per experiment.

12. Aliquot the P3 membranes per 2 ml and freeze in liquid N₂.

13. Store at -80 °C.

Note: The membranes can be stored at -80 °C for a maximum of 6 months.

D. Reconstitution of yeast membranes

To reconstitute yeast membranes, we followed a similar strategy as described before (Papadopoulos *et al.*, 2007) with some modifications.

- a. Dilute 2 ml of the P3 membranes (thawed on ice) to 10 µg/µl in buffer T/DDM.

Note: The amount of total protein should be at least 70 mg.

- b. Incubate for 45 min at 4 °C in a head-over-head rotator.
- c. Centrifuge for 30 min at 200,000 x g to pellet the insoluble fraction using ultracentrifuge rotor Type 90 Ti (4 °C).
- d. Supplement 2 ml of the supernatant, *i.e.*, detergent extract, with an equal volume of buffer T/lipid mix and for the “ATP inside” condition, also an equal volume of ATP-regenerating system (Table 1):

Table 1. Sample preparation for reconstitution

CONDITION	“no ATP”	“ATP inside”
detergent extract	2 ml	2 ml
buffer T/lipid mix	2 ml	2 ml
ATP-regenerating system	-	2 ml

- e. Incubate the samples with 100 mg/ml Bio-Beads for 3 h at room temperature in a head-over-head rotator to remove the DDM and reconstitute proteoliposomes.
 - f. Add 200 mg/ml Bio-Beads and incubate overnight at 4 °C in a head-over-head rotator.
 - g. Centrifuge for 1 h at 200,000 x g using ultracentrifuge rotor Type 90 Ti (4 °C).
 - h. Recover vesicles by resuspending the pellet in 2 ml of buffer T.
- Note: The measured protein concentration typically ranges between 1-5 mg/ml.*
- i. Determine the protein concentration using a classical Bradford assay.
 - j. Check reconstituted protein levels via Western blot (Figure 3).

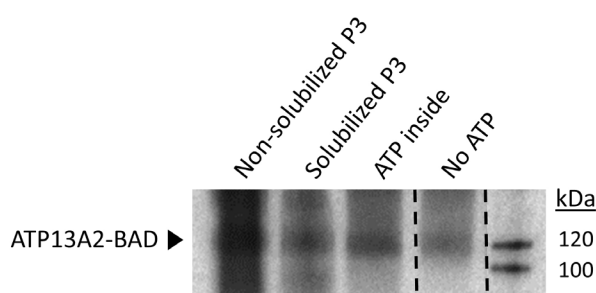


Figure 3. Immunoblot of yeast membranes and reconstituted vesicles. P3 membranes containing ATP13A2-BAD are solubilized by the detergent DDM. The detergent extract is supplemented with the lipids phosphatidylcholine and phosphatidic acid, and then treated with Bio-Beads to remove the DDM and reconstitute proteoliposomes ('no ATP'). To generate proteoliposomes that contained intraluminal ATP ('ATP inside'), we added ATP and an ATP-regenerating system before incubation with the Bio-Beads. Reconstituted ATP13A2-BAD protein levels are checked via Western blot analysis using primary anti-ATP13A2 antibody (1/1,000) and HRP-conjugated secondary antibodies (1/2,000). 12 µl of sample was loaded per lane.

E. Polyamine transport assay

Notes:

- a. *The polyamine transport assay should be performed in a designated radioactive area by authorized personnel and radioactive material handling precautions have to be undertaken. Gloves, lab goggles, lab coats and a dosimeter to monitor personal exposure should be worn at all times. The work should be performed behind Plexiglass screens and only pipette tips with filters should be used. The regulations of the institution should be followed for the storage of radioactive material, and disposal of solid and liquid radioactive waste.*
- b. *Measure [³H]-SPM uptake into freshly prepared vesicles within 60 min.*
 1. Dilute the freshly prepared vesicles ("no ATP" or "ATP inside") to 1 µg/µl in buffer T.
Note: We typically use 1 ml of vesicles. At least 300 µg ("ATP inside" vesicles) – 600 µg ("no ATP" vesicles) is required to proceed to the next steps.
 2. Place four 0.45 µm Millipore filters on filtration manifold (Figure 4).
Note: Filters are pre-wet in MQ. Use tweezers to hold them.
 3. Wash each filter with 4 ml 1x reaction buffer.
 4. Pipet the following into the reaction tubes for the different conditions (in duplo) (Table 2):

Table 2. Sample preparation for transport assay

CONDITION	"no ATP"	"ATP outside"	"ATP inside"
4x reaction buffer	250 µl	250 µl	250 µl
"no ATP" vesicles	150 µl	150 µl	-
"ATP inside" vesicles	-	-	150 µl
ATP-regenerating system	-	-	200 µl
MQ	500 µl	500 µl	300 µl

- Slightly vortex to thoroughly mix (avoid air bubble formation).
- Place the reaction tube in a 37 °C water bath for 5 min.
- Start the reaction by adding 100 µl [³H]-SPM/unlabeled SPM mix (final total SPM concentration: 1 mM).
- Take out 300 µl (45 µg vesicles) at chosen time points (0 min, 10 min) and pipet each aliquot on a different filter allowing to remove the liquid phase.
- Wash each filter with 4 ml 1x reaction buffer.
- Place the filters in the scintillation vials containing 7 ml scintillation liquid.
- Repeat steps for the remaining reaction tubes.
- Measure samples with a liquid scintillation counter.

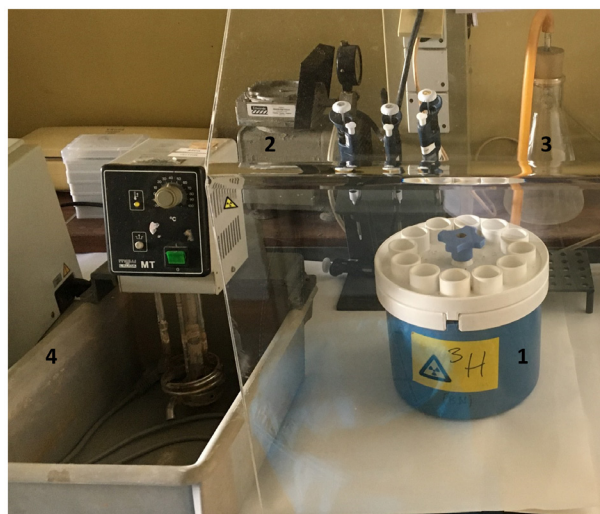


Figure 4. Experimental setup for polyamine transport assay. Filtration manifold (1) connected to a vacuum pump (2) with a collector for radioactive waste (3). Water bath (4).

Data analysis

The polyamine transport assay described measures the accumulation of [³H]-SPM, following a 10 min incubation period, in reconstituted vesicles that contain inside-out ATP13A2 and intraluminal ATP ("ATP inside" condition). The experiment is performed in parallel with different control

conditions. Proteoliposomes with overexpressed ATP13A2 that only contain extraluminal ATP ("ATP outside" condition) or have no ATP at all ("no ATP" condition) serve as negative controls as these vesicles do not take up [³H]-SPM due to lack of ATP binding at the luminal nucleotide-binding domain of reconstituted inside-out ATP13A2 protein (Figure 1). In addition, reconstituted vesicles with overexpression of a catalytically inactive ATP13A2 variant (E343A) are included as a negative transport control. [³H]-SPM uptake is quantified by scintillation counting and values are normalized to 0 min for every condition. For each uptake time, duplicate determinations are made. A representative graph is shown in Figure 5.

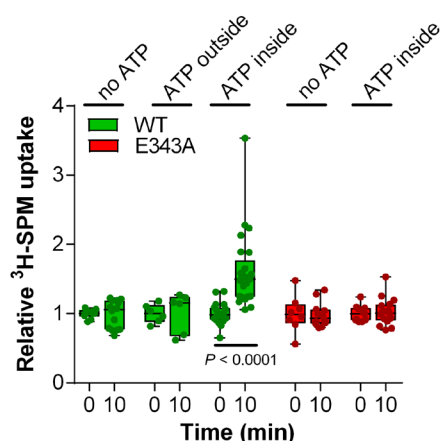


Figure 5. Polyamine transport assay. Uptake of [³H]-SPM in yeast membrane-derived vesicles with overexpression of ATP13A2 WT vs. the catalytically dead mutant E343A (negative control). The condition 'no ATP' stands for reconstituted vesicles without ATP, whereas the conditions 'ATP outside' and 'ATP inside' represent proteoliposomes that contain extra- or intraluminal ATP and ATP-regenerating system, respectively (van Veen *et al.*, 2020).

Notes

The here-described protocol can be expanded to perform the following types of measurements:

1. Time dependency should be linear, which can be verified at various time points in the range of 0-30 min. The uptake rate can be calculated from the slope.
2. A dose/response curve can be generated by testing a range of total SPM concentrations (the combination of cold and [³H]-SPM labeled together), in the physiological range of 0.01 μ M-10 mM. The concentration dependency allows the determination of the apparent affinity for SPM (K_m) and maximal turnover rate (V_{max}) of the transporter. The kinetic parameters are calculated from the Hill equation with non-linear regression analysis, as in Holemans *et al.* (2014).

Recipes

1. 20% glucose

- a. Put 500 ml MQ in a large beaker glass with a stir bar
 - b. Add 200 g glucose
 - c. Stir the solution for a few minutes
 - d. Finalize volume to 1,000 ml with MQ
 - e. Filter sterilize the solution
2. YPD-agar plates

Note: Plates should be stored at 4 °C in the dark.

 - a. Put 3 g yeast extract, 6 g peptone and 6 g agar in a Duran glass bottle
 - b. Add MQ up to 270 ml
 - c. Autoclave at 121 °C for at least 30 min
 - d. Add 30 ml 20% glucose
 - e. Pour into Petri dishes
3. YPD medium
 - a. Put 10 g yeast extract and 20 g peptone in a Duran glass bottle
 - b. Add MQ up to 900 ml
 - c. Autoclave at 121 °C for at least 30 min
 - d. Add 100 ml 20% glucose
4. 10x TE stock (0.1 M Tris-HCl, pH 7.5, 0.01 M EDTA)

0.606 g Tris
0.146 g EDTA
Add MQ up to 50 ml
Adjust pH to 7.5 with HCl
Filter sterilize the solution
5. 10x LiAc stock (1 M LiAc, pH 7.5)

5.101 g LiAc
Add MQ up to 50 ml
Adjust pH to 7.5 with diluted acetic acid
Filter sterilize the solution
6. 1x TE/LiAc solution

Mix together 120 µl 10x TE stock, 120 µl 10x LiAc stock and 900 µl sterile MQ
7. Single-stranded herring sperm DNA

DNA is boiled for 20 min in water bath and then immediately cooled on ice
8. 50% PEG w/v
 - a. Weigh off 25 g PEG
 - b. Add sterile MQ up to 50 ml
 - c. Filter sterilize the solution
9. PEG solution (40% PEG, 1x TE, 1x LiAc)

Mix together 800 µl 50% PEG, 100 µl 10x TE stock and 100 µl 10x LiAc stock
10. SD-uracil agar plates

Note: Plates should be stored at 4 °C in the dark.

- a. Put 5 g agar, 0.475 g yeast dropout mix without uracil and 1.675 g yeast nitrogen base without amino acids in a Duran glass bottle
- b. Add MQ up to 200 ml
- c. Autoclave at 121 °C for at least 30 min
- d. Add 50 ml 20% glucose
- e. Pour into Petri dishes

11. SD-uracil medium

Note: Medium should be stored in the dark.

- a. Put 0.95 g yeast dropout mix without uracil and 3.35 g yeast nitrogen base without amino acids in a Duran glass bottle
- b. Add MQ up to 450 ml
- c. Autoclave at 121 °C for at least 30 min
- d. Add 50 ml 20% glucose

12. YPGE 2x medium

- a. Put 20 g yeast extract and 20 g peptone in a Duran glass bottle
- b. Add MQ up to 725 ml
- c. Autoclave at 121 °C for at least 30 min
- d. Add 50 ml 20% glucose
- e. Add 27 ml ethanol

13. 20% galactose

- a. Put 500 ml MQ in a large beaker glass with a stir bar
- b. Add 200 g galactose
- c. Heat up while mixing to get it into solution
- d. Finalize volume to 1,000 ml with MQ
- e. Filter sterilize the solution

14. TEKS buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M KCl, 0.6 M sorbitol)

Note: Buffer should be stored at 4 °C.

6.057 g Tris

0.292 g EDTA

7.456 g KCl

109.302 g sorbitol

Add MQ up to 1000 ml

Adjust pH to 7.5 with HCl

15. TESin buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.6 M sorbitol, 1 mM PMSF, protease inhibitor cocktail)

Note: Buffer should be stored at 4 °C.

6.057 g Tris

0.292 g EDTA

- 109.302 g sorbitol
Add MQ up to 1,000 ml
Adjust pH to 7.5 with HCl
Add Sigma Fast protease inhibitor cocktail on the day of the experiment. For 50 ml of buffer, add 1 tablet
PMSF is added just before use. For 50 ml of buffer, add 500 μ l of 100 mM PMSF
16. HS buffer (20 mM HEPES-Tris, pH 7.4, 0.3 M sucrose, 0.1 mM CaCl_2)
Note: Buffer should be stored at 4 °C.
0.477 g HEPES
10.269 g sucrose
0.0015 g CaCl_2
Add MQ up to 100 ml
Adjust pH to 7.4 with Tris
17. Buffer T (10 mM Tris-HCl, pH 7.4 and 1 mM EDTA)
Note: Buffer should be stored at 4 °C.
0.121 g Tris
0.029 g EDTA
Add MQ up to 100 ml
Adjust pH to 7.4 with HCl
18. Buffer T/DDM (buffer T supplemented with 1.4% DDM w/v)
20 ml buffer T
280 mg DDM
19. Buffer T/lipid mix (buffer T containing 0.7% DDM w/v, 4.5 mM egg phosphatidylcholine and 0.5 mM 18:1 phosphatidic acid (PA))
a. Add 173 mg egg phosphatidylcholine (6.92 ml of 25 mg/ml chloroform stock) to a glass tube and dry under nitrogen stream to make a lipid film
b. Add 18 mg 18:1 PA (1.8 ml of 10 mg/ml chloroform stock) to the lipid film and dry under nitrogen stream
c. Resolubilize the lipid film in 50 ml buffer T supplemented with 0.35 g DDM
20. 0.1 M ATP
0.551 g ATP
Add MQ up to 10 ml
Aliquot and store at -20 °C
21. 0.1 M MgCl_2
0.203 g MgCl_2
Add MQ up to 10 ml
22. 0.2 M phosphocreatine
1 g phosphocreatine
Add MQ up to 19.6 ml

Aliquot and store at -20 °C

23. ATP regenerating system

200 µl 0.1 M ATP

200 µl 0.1 M MgCl₂

200 µl 0.2 M phosphocreatine

200 µl creatine phosphokinase (1 U/µl)

1.2 ml MQ

24. 500 mM DTT

0.771 g DTT

Add MQ up to 10 ml

Aliquot and store at -20 °C

25. 4x reaction buffer (200 mM MOPS, 400 mM KCl, 44 mM MgCl₂, 4 mM DTT)

Note: Buffer should be stored at 4 °C.

4.184 g MOPS

2.982 g KCl

0.894 g MgCl₂

Add MQ up to 100 ml

Adjust pH to 7.0 with KOH

Add DTT just before use. For 5 ml of buffer, add 40 µl of 500 mM stock solution

26. 1x reaction buffer (50 mM MOPS, 100 mM KCl, 11 mM MgCl₂, 1 mM DTT)

100 ml 4x reaction buffer

300 ml MQ

27. 0.1 M MOPS (pH 7.0)

2.093 g MOPS

Add MQ up to 100 ml

Adjust pH to 7.0 with KOH

28. 10 mM SPM

0.020 g SPM

Add 0.1 M MOPS (pH 7.0) up to 10 ml

Aliquot and store under nitrogen gas at -80 °C. Once thawed, aliquots are not reused

29. [³H]-SPM/unlabeled SPM mix (10 µCi, 0.2 µM [³H]-SPM, 10 mM SPM, total SPM concentration: 9.9 mM)

10 µl [³H]-SPM

990 µl 10 mM SPM

Acknowledgments

The described protocol was originally published as a method in van Veen *et al.*, 2020. This work was funded by the Fonds Wetenschappelijk Onderzoek (FWO, Research Foundation Flanders)

(G094219N, SBO Neuro-TRAFFIC S006617N and 1503117N), the KU Leuven (LysoCaN C16/15/073) and the Queen Elisabeth Medical Foundation for Neurosciences, Valine de Spoelberch Award. S.v.V. is an aspirant FWO research fellow (11Y7518N). We thank Dr. J. Lyons, University of Aarhus, for his help with the generation of the ATP13A2 BAD-tag fusion construct.

Competing interests

The authors declare no financial or non-financial competing interests.

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