Transport Assays in *Aspergillus nidulans*

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[Abstract] Transport assays allow the direct kinetic analysis of a specific transporter by measuring apparent K_m and V_{max} values, and permit the characterization of substrate specificity profiles through competition assays. In this protocol, we describe a rapid and easy method for performing uptake assays in the model filamentous ascomycete *Aspergillus nidulans.* These assays make use of *A. nidulans* germinating conidiospores, thus avoiding technical difficulties associated with the use of mycelia. The ease of construction genetic null mutants in this model fungus permits the rigorous characterization of any transporter in the absence of similar transporters with overlapping specificities, a common problem in relevant studies.

Materials and Reagents

1. p-aminobenzoic acid (Sigma-Aldrich, catalog number: P5669)
2. d-Biotin (Sigma-Aldrich, catalog number: B4501)
3. Calcium-D-pantothenate (Sigma-Aldrich, catalog number: 21210)
4. Riboflavine (Sigma-Aldrich, catalog number: R4500)
5. Pyridoxine hydrochloride (Sigma-Aldrich, catalog number: P9755)
6. KCl
7. $MgSO_4 \cdot 7H_2O$
8. KH_2PO_4
9. $Na_2B_4O_7 \cdot 10H_2O$
10. $CuSO_4 \cdot 5H_2O$
11. $FeO_4P \cdot 4H_2O$
12. $MnSO_4 \cdot H_2O$
13. $Na_2MoO_4 \cdot 2H_2O$
14. $ZnSO_4 \cdot 7H_2O$
15. NaOH
16. Tween 80 (Sigma-Aldrich, catalog number: P1754)
17. Radiolabelled substrate
    e.g. [8-^{3}H]-xanthine, 22.8 Ci/mmol (Moravek Biochemicals, catalog number: MT537)
    [2,8-^{3}H]-hypoxanthine, 27.7 Ci/mmol (Moravek Biochemicals, catalog number: MT700)

[5-^{3}H]-uracil, 23 Ci/mmol (Moravek Biochemicals, catalog number: MT610)

18. Non radiolabelled substrate
    e.g. Xanthine (Sigma-Aldrich, catalog number: X7375)
    Hypoxanthine (Sigma-Aldrich, catalog number: H9377)
    Uracil (Sigma-Aldrich, catalog number: U0750)
19. Toluol (AppliChem GmbH, catalog number: A3393)
20. Triton X-100
21. 2,5-Diphenyloxazole (PPO) (Sigma-Aldrich, catalog number: D4630)
22. 1,4-bis (5-phenyloxazol-2-yl) benzene (POPOP) (Sigma-Aldrich, catalog number: P3754)
23. Complete Media (CM) (see Recipes)
24. Minimal Media (MM) (see Recipes)
25. Scintillation Fluid (see Recipes)

Equipment

1. Petri dishes
2. Neubauer counting-chamber slide
3. Spatula
4. Orbital shaking incubator
5. Incubator at 37 °C
6. Nylon net filter 60 µm (Merck KGaA, catalog number: NY60)
7. 50 ml Falcon tubes
8. 1.5 ml centifuge tubes
9. Centrifuge
10. Vortex
11. Scintillation vials
12. Scintillation counter
13. Heat block
14. Magnetic stirrer
15. Magnetic strirr bar
16. pH meter
17. Pasteur pipette

Software

1. GraphPad Prism software (Amillis *et al.*, 2004)

Procedure

1. Inoculate a petri dish of CM with the strain of interest and let it reach full growth at 37 °C for 96 hours.
2. Using a spatula transfer one quarter of the fully grown colony (~4 cm) in a 50 ml Falcon tube containing 2 ml of 0.01% v/v Tween 80 in water. This amount usually corresponds to 10^8 conidiospores. The accurate amount of conidiospores can be estimated using a Neubauer counting-chamber slide or by measuring viable conidiospores after standard serial dilutions and plating on CM.
3. Vortex well the sample for separating the conidiospores.
4. Inoculate a 100 ml flask containing 25 ml MM supplemented with appropriate carbon (C) (*e.g.* Glucose 1% w/v) and nitrogen (N) (*e.g.* $NaNO_3$ 10 mM) sources and necessary vitamins (*e.g.* D-biotin 0.02 µg/ml) with the conidiospores filtered through a Nylon net filter 60 µM. (All necessary supplements and concentrations for *A. nidulans* strains can be found at www.fgsc.net).
5. Incubate for 3-5 h at 37 °C, shaking with 140 rpm, for the germinating conidiospores to reach a stage just before germ tube emergence. The time and temperature of incubation can change depending on the expression profile of the transporter of interest and the auxotrophic requirements of the strain. All transporters studied up to date in our lab (for example the purine/pyrimidine transporters UapA, UapC, AzgA, FcyB, FurD) reach maximum expression before germ tube emergence, driven by an unknown developmental control, irrespectively of the presence or absence of their substrates or other physiological conditions (Amillis *et al*., 2004; Vlanti and Diallinas, 2008; Amillis *et al*., 2007). In mycelia, transporter expression is very much dependent on physiological conditions (*e.g.* induction by substrates or/and N or C catabolite repression).
6. While conidiospores germinate, prepare the stock solution of radiolabeled (usually ^3H or ^{14}C) substrate of interest in water or MM so that for each assay 25 µl of the stock will be used.
7. Collect the conidiospores by centrifuging the culture in a 50 ml Falcon tube for 5 min, at 3,000 *x g*, room temperature.
8. Discard the supernatant and resuspend the pellet in 5 ml standard MM.
9. Distribute the spores in 75 µl aliquots in eppendorf tubes and use as many as needed. Conidiospore suspensions can be kept at 4 °C for at least 24 h without loss of transport activity.
10. Incubate conidiospore aliquots at 37 °C in a heat block for 5 min before addition of radiolabeled substrate.

11. Radiolabeled substrate is added for different periods of time. Most transporters show linearly increased activities for at least 1 min. For measuring initial uptake rates, which are necessary for determining K_m and apparent V_{max} values, the proper time of incubation must be defined for each transporter through a time-course experiment. For steady state substrate accumulation a period of 5 min is used. Usual time points are 10, 20, 30, 60 and 120 sec. For each time point, measurements are performed in triplicate. The temperature used for the incubation with the radiolabeled substrate depends on the transporter being studied at each experiment and the experiment being held, temperature for most experiments is 37 °C. The transport reaction is stopped by adding an equal volume (100 µl) of cold unlabeled substrate at 100-1,000 fold excess concentration, related to radiolabelled substrate, and direct transfer of the assay/eppendorf tube in an ice bucket.
12. Centrifuge the samples at 11,000 *x g* for 3 min at 4 °C.
13. Remove the supernatant through aspiration under vacuum using a Pasteur pipette. It is important to remove all the supernatant without losing any cells.
14. Wash the pellet of cells once with 1 ml ice cold MM and centrifuge at 11,000 *x g* for 3 min. Remove the supernatant as before.
15. Resuspend the pellet in 1 ml of scintillation fluid and put the eppendorf tubes into scintillation vials. Use a scintillation counter to measure substrate accumulation in the cells.
16. Analysis of transport measurements is performed using GraphPad Prism software. Radioactive counts should be converted to substrate concentration/min/conidiospores, based on the concentration and specific activity of the stock of radioactive substrate used.
17. For K_m determination of a transporter, different substrate concentrations should be used, for a fixed incubation time, previously determined to reflect initial uptake rates. This is usually 1 min. The range of concentrations used is determined at first empirically. In the final experiment, at least three concentration points below and above the apparent K_m value should be used. For each concentration point measurements are performed in triplicate.
18. The stock solutions are prepared using a mixture of fixed radiolabeled substrate and increasing concentrations of non-radiolabeled substrate, so that for each assay 25 µl of the stock will be used.
19. Terminate transport assays and perform measurements as described above.
20. For K_i determination of a transporter, the method is identical to the one for K_m determination, but stock solutions are prepared using a mixture of fixed radiolabeled

substrate and increasing concentrations of non-radiolabeled putative inhibitors. For each concentration point measurements are performed in triplicate.

21. K_m and V_{max} determination is carried out using typical Lineweaver-Burk plot analysis that is based on the Michaelis-Menten equation for enzyme kinetics $V = V_{max}[S]/(K_m + [S])$, where V is the reaction velocity (the reaction rate), K_m is the Michaelis–Menten constant, V_{max} is the maximum reaction velocity, and [S] is the substrate concentration. The Lineweaver-Burk plot depicts the linear expression of the previous equation which is transformed to the following: $1/V = (K_m/V_{max})\cdot(1/[S]) + 1/V_{max}$. The data obtained by this experiment correspond to the apparent velocity of the transporter for each substrate concentration. K_i measurements are determined by estimating IC_{50} values (inhibitor concentration for obtaining 50% inhibition) of given substrate/inhibitor, using the formula $K_i = IC_{50}/1 + [S]/K_m$, where [S] is the fixed concentration of radiolabeled substrate used. Another way to analyse the data is by using the GraphPad Prism Software through a non linear regression curve fit and sigmoidal dose response analysis. The IC_{50} value corresponds to the $K_{m/i}$ of the transporter. Quality factors for the analysis result are: R^2 which should be > 0.99 and the Hill co-efficient which should be approximately -1 for a transporter with one binding site.
22. The method described can be modified and adapted for most filamentous fungi that produce asexual spores, *e.g. A. fumigatus.* (Goudela *et al*., 2008)

Recipes

1. Complete Media (1 L)

Vitamin solution from 100x stock solution*	10 ml
Salt solution from 50x stock solution**	20 ml
Glucose	10 g
Peptone	2 g
Yeast Extract	1 g
Cas-amino- acids	1 g
Agar	10 g

   Add water to 1 L final volume
   Adjust the pH to 6.8 using NaOH
   Autoclave for 20 min
   *Vitamin stock solution
   p-aminobenzoic acid 20 mg
   d-biotin 1 mg
   Calcium-D-pantothenate 50 mg

Riboflavin 50 mg

Pyridoxine 50 mg

Add water to 1 L final volume

**Salt stock solution

KCl 26 g

$MgSO_4 \cdot 7H_2O$ 26 g

KH_2PO_4 76 g

Trace elements 20x stock solution*** 50 ml

Add water to 1 L final volume

***Trace elements stock solution

$Na_2B_4O_7 \cdot 10H_2O$ 40 mg

$CuSO_4 \cdot 5H_2O$ 400 mg

$FeO_4P \cdot 4H_2O$ 714 mg

$MnSO_4 \cdot 1H_2O$ 728 mg

$Na_2MoO_4 \cdot 2H_2O$ 800 mg

$ZnSO_4 \cdot 7H_2O$ 8 mg

Add water to 1 L final volume

2. Minimal Media

   Salt solution from 50x stock solution* 20 ml

   Add water until 1 L final volume

   Adjust the pH to 6.8 using NaOH

   Autoclave for 20 min

3. Scintillation Fluid (1 L)

   Toluol 666 ml

   PPO 2.66 g

   POPOP 0.0066 g

   2 h stirring in RT

   Add Triton-X 100 333 ml

   Overnight stirring

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References

1. Amillis, S., Cecchetto, G., Sophianopoulou, V., Koukaki, M., Scazzocchio, C. and Diallinas, G. (2004). Transcription of purine transporter genes is activated during the isotropic growth phase of *Aspergillus nidulans* conidia. *Mol Microbiol* 52(1): 205-216.
2. Amillis, S., Hamari, Z., Roumelioti, K., Scazzocchio, C. and Diallinas, G. (2007). Regulation of expression and kinetic modeling of substrate interactions of a uracil transporter in *Aspergillus nidulans*. *Mol Membr Biol* 24(3): 206-214.
3. Diallinas, G., Gorfinkiel, L., Arst, H. N., Jr., Cecchetto, G. and Scazzocchio, C. (1995). Genetic and molecular characterization of a gene encoding a wide specificity purine permease of *Aspergillus nidulans* reveals a novel family of transporters conserved in prokaryotes and eukaryotes. *J Biol Chem* 270(15): 8610-8622.
4. Goudela, S., Reichard, U., Amillis, S. and Diallinas, G. (2008). Characterization and kinetics of the major purine transporters in *Aspergillus fumigatus*. *Fungal Genet Biol* 45(4): 459-472.
5. GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA. www.graphpad.com.
6. Koukaki, M., Vlanti, A., Goudela, S., Pantazopoulou, A., Gioule, H., Tournaviti, S. and Diallinas, G. (2005). The nucleobase-ascorbate transporter (NAT) signature motif in UapA defines the function of the purine translocation pathway. *J Mol Biol* 350(3): 499-513.
7. Meintanis, C., Karagouni, A. D. and Diallinas, G. (2000). Amino acid residues N450 and Q449 are critical for the uptake capacity and specificity of UapA, a prototype of a nucleobase-ascorbate transporter family. *Mol Membr Biol* 17(1): 47-57.
8. Tazebay, U. H., Sophianopoulou, V., Scazzocchio, C. and Diallinas, G. (1997). The gene encoding the major proline transporter of *Aspergillus nidulans* is upregulated during conidiospore germination and in response to proline induction and amino acid starvation. *Mol Microbiol* 24(1): 105-117.
9. Vlanti, A. and Diallinas, G. (2008). The *Aspergillus nidulans* FcyB cytosine-purine scavenger is highly expressed during germination and in reproductive compartments and is downregulated by endocytosis. *Mol Microbiol* 68(4): 959-977.

