A simple dot blot assay to measure hygromycin B phosphotransferase activity in whole cell extracts of Neurospora crassa

Michael Freitag
Oregon Graduate Institute of Science & Technology

Matthew S. Sachs
Oregon Graduate Institute of Science & Technology

Follow this and additional works at: https://newprairiepress.org/fgr

This work is licensed under a Creative Commons Attribution-Share Alike 4.0 License.

Recommended Citation

This Regular Paper is brought to you for free and open access by New Prairie Press. It has been accepted for inclusion in Fungal Genetics Reports by an authorized administrator of New Prairie Press. For more information, please contact cads@k-state.edu.
A simple dot blot assay to measure hygromycin B phosphotransferase activity in whole cell extracts of Neurospora crassa

Abstract

A simple dot blot assay to measure hygromycin B phosphotransferase activity in whole cell extracts of *Neurospora crassa*

Michael Freitag and Matthew S. Sachs - Department of Chemistry, Biochemistry and Molecular Biology, Oregon Graduate Institute of Science & Technology, Portland OR


We have used *hph* as a reporter gene in studies on arginine (Arg)-specific negative regulation of the *N. crassa* arg-2 gene. *N. crassa* host strains were transformed (Selitrennikoff and Sachs 1991 Fungal Genet. Newsl. 38:90-91) with a plasmid containing a translational fusion of 5' regulatory arg-2 sequences to hph (pGV4, Sachs and Freitag, unpublished) or a plasmid containing a human cytomegalovirus IE94 promoter-driven hph-tk fusion gene (plasmid tgCMV/HyTK; Lupton et al. 1991 Mol. Cell Biol. 11:3374-3378; tk specifies herpes simplex virus type 1 thymidine kinase). Single copy ectopic integrants were selected for further analyses. Transformed strains were resistant to high levels (>2 mg/ml) of Hyg on minimal growth medium (1X Vogel's N medium with 2% sucrose and 2% agar). An arg-12s pyr-3 *N. crassa* host strain transformed with the arg-2-hph fusion gene exhibited a phenotype indicating Arg-dependent Hyg resistance; this strain grows on minimal medium supplemented with uridine (Uri, 0.5 mg/ml) and Hyg (2 mg/ml), but not on minimal medium supplemented with Arg (0.5 mg/ml), Uri and Hyg.

We desired to quantify Hph activity, but found that the previously published assay procedures for aminoglycoside-modifying enzymes were too cumbersome and time-consuming (Haas and Dowding 1975 Meth.
Enzymol. 43:611-627). Therefore we adapted a previously described, simple dot blot assay for measuring Hph activity from *N. crassa* whole cell extracts (Duch et al. 1990 Gene 95:285-288, Sørensen et al. 1992 Gene 112:257-260). Our results show that the *hph* gene can be used as a combined selectable dominant marker/reporter gene in *N. crassa* strains transformed with plasmids carrying *hph* fusion genes.

To analyze Hph activity in *N. crassa*, aliquots of whole cell extracts are incubated with of Hyg and [gamma-32P]-labeled ATP. The samples are filtered through successive layers of nitrocellulose membrane, P81 phosphocellulose paper and filter paper. Proteins present in whole cell extracts, some of which are radioactively labeled by the activity of cellular protein kinases, are retained on the nitrocellulose membrane, while the weakly positively charged [gamma-32P]-labeled Hyg passes through the nitrocellulose and binds to the negatively charged P81 phosphocellulose paper. The filter paper below the phosphocellulose traps most of the unincorporated [gamma-32P]-labeled ATP. The amount of [gamma-32P]-labeled Hyg in a dot on the phosphocellulose paper, quantified by autoradiography followed by densitometry or by phosphoimager analyses, is used as a measure of Hph activity.

**Experimental.** *N. crassa* conidia (2 x 10^7 conidia/ml as inoculum) were germinated at 34°C as shaking cultures (200 rpm on an orbital shaker) for 6.5 h in 125 ml Erlenmeyer flasks containing 30 ml of 1X Vogel's minimal medium with 2% sucrose, supplemented with 0.5 mg/ml Uri (U) or 0.5 mg/ml Arg and 0.5 mg/ml Uri (R), as indicated in Figure 1. The cultures were harvested through Millipore filter assemblies onto Whatman filter paper (#1). In the cold room, mycelial pads (ca. 0.5 g wet weight) were added to 0.8 g of acid-washed glass beads (0.5 mm) in 2 ml Sarstedt screw cap tubes containing 1 ml of breaking buffer (20 mM HEPES-OH, pH 7.9, 100 mM KCl, 2 mM EDTA, 10 mM DTT, 20% glycerol; Sachs and Ebbole 1990 Fungal Genet. Newslet. 36:35-37). Tubes were filled completely with breaking buffer and cells broken by bead-beating in a Mini Beadbeater (Biospec, Bartlesville, OK) for two 1 min cycles, interrupted by a 1 min rest on ice. Whole cell extracts were clarified by centrifugation at 4°C for 10 min at 16,000g. Extracts were transferred to fresh Eppendorf tubes and either used immediately or quick frozen in liquid nitrogen and stored at -80°C. In our experience, extracts retained Hph activity levels comparable to fresh extracts after 1 year of storage at -80°C.

Serial dilutions of 5, 2.5, 1.25 and 0.63 µg of total protein (determined by Bradford assay with BSA as standard) from whole cell extracts in a 10 ul total volume were added to wells of non-sterile 96-well microtiter dishes. The reactions were started by the addition of 50 ul of reaction buffer (13.4 mM Tris-maleate, pH 7.1, 8.4 mM MgCl₂, 80 mM NH₄Cl, 60 uM hygromycin B, 15 uM ATP and 25 uCi/ml of [gamma-32P]-ATP) and incubated at room temperature for 1 hr. During this time, filter paper (Micro Filtration Systems, #1514A6X57CM), phosphocellulose paper (P81 cation exchanger, Whatman #3698 915) and nitrocellulose membrane (Nitrobind, 0.22 micron, MSI #EP2HY00010) were placed in this order onto a Biorad BioDot filtration apparatus (two successive vacuum traps were attached to the apparatus to trap radioactive efflux). The wells were sealed by application of a light vacuum and washed with 150 ul water.

The completed reactions were mixed with 150 ul water and heat- inactivated for 10 min at 70°C. Reactions were loaded into wells of the Biorad BioDot filtration apparatus and filtered by immediate application of a light vacuum. Wells were washed three times with 250 ul water. The apparatus was disassembled and immediately rinsed until no counts were detected by Geiger counter and wipe tests. Both nitrocellulose membrane and phosphocellulose paper were washed three times with water (15 min per wash) at 65°C, air-dried and exposed to Kodak XAR-5 film (12 h to 3 days with an intensifying screen at -80°C) or to phosphoimager storage plates. The filter paper, on which most of the unincorporated label was detected, was discarded. Amounts of radioactivity in dots on the phosphocellulose paper were determined with a Molecular Dynamics Phosphorimager system.
Results and Discussion. As expected, Hph activity was not detected in the *N. crassa* wild type strain 74A- OR23-1VA, while strains transformed with *hph*-containing plasmids exhibited readily detectable Hph activity (Figure 1). A wild type strain transformed with plasmid tgCMV/HyTK showed Hph activity that was independent of the presence of Arg in the growth medium (Figure 1), as anticipated. In contrast, an *N. crassa* arg-12s pyr-3 double mutant transformed with a construct containing *N. crassa* arg-2 5' regulatory sequences fused to *hph* showed Arg-specific regulation (Figure 1). The magnitude of regulation, approximately 3-fold, is similar to that due to regulation of the *arg-2* gene in a wild type strain (Davis and Ristow 1987 J. Biol. Chem. 262:7109-7117).

Hph activity was proportional to the amount of total protein assayed (data not shown). We found that stopping reactions by incubation at 70 C for 10 min and processing the reactions quickly by applying a light vacuum improved the reliability of the assay when compared with the previously published procedure (Sørensen et al. 1992), in which reactions were not stopped and in which reactions were filtered by gravity flow alone. In our experience, filtering by gravity flow leads to weaker signals.

In summary, we show that the Hph activity dot assay first described by Sørensen et al. can be adapted for use with filamentous fungi. Extracts from positive and negative control strains should be used in each experiment to allow comparisons between assays performed on different days. This requirement is easily fulfilled because Hph activity in extracts appears to be stable for at least one year when extracts are stored at -80 C. By using the assay described here, the *hph* gene can be used in filamentous fungi not only as a selectable dominant marker, but also as a reporter gene.

Figure 1: Hygromycin B phosphotransferase dot blot assay on whole cell extracts of *N. crassa*. Conidia from wild type (wt) and *hph*-containing strains were germinated for 6.5 hrs in minimal medium containing 0.5 mg/ml uridine (U) or minimal medium containing 0.5 mg/ml arginine and 0.5 mg/ml uridine (R). Hph activity assays were performed using the amounts of total protein indicated as described in the text.