

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

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Hygromycin B (Hyg) is an aminocyclitol antibiotic with broad spectrum activity against prokaryotes and eukaryotes (Pettinger *et al.* 1953 Antibiot. Chemother. **3**:1268- 1278). Hyg inhibits protein synthesis by blocking ribosomal translocation; it prevents polypeptide elongation by interfering with aminoacyl tRNA recognition and ribosomal A-site occupation (Cabanas *et al.* 1978 Euro. J. Biochem. **87**:21-27, Hausner *et al.* 1988 J. Biol. Chem. **263**:13103-13111). Hyg can lead to misreading during translation *in vitro* (Davies and Davies 1968 J. Biol. Chem. **243**:3312-3316, Gonzales *et al.* 1978 Biochim. Biophys. Acta **521**:459-469, Singh *et al.* 1979 Nature **277**:146-148); however, this effect was not duplicated *in vivo* (Bakker 1992 J. Gen. Microbiol. **138**:563-569).

Resistance to Hyg is conferred by hygromycin B phosphotransferase (Hph), first isolated from *Streptomyces hygroscopicus* (Leboul and Davies 1982 J. Antibiot. **35**:527-528). Hph catalyzes the phosphorylation of the 4-hydroxyl group on the hyosamine moiety, thereby inactivating Hyg (Rao *et al.* 1983 Antimicro. Agents Chemother. **24**:689-695). Plasmid-determined resistance to Hyg had been observed in *Escherichia coli* (Rao *et al.* 1983) and *Klebsiella pneumoniae* (Gritz and Davies 1983 Gene **25**:179-188). Three plasmid-borne genes encoding Hph were independently isolated and characterized (Kaster *et al.* 1983 Nucl. Acids Res. **11**:6895-6911, Gritz and Davies 1983, Malpartida *et al.* 1983 Biochem. Biophys. Res. Comm. **117**:6-12).

Plasmids carrying fusions of a variety of promoters to the *hph* gene have been used to transform eukaryotic cells to Hyg resistance, including *Saccharomyces cerevisiae* (Gritz and Davies 1983, Kaster *et al.* Curr. Genet. **8**:353-358), *Aspergillus nidulans*, *A. niger* (Punt *et al.* 1987 Gene **56**:117-124, Cullen *et al.* Gene **57**:21-26) and *Neurospora crassa* (Staben *et al.* 1989 Fungal Genet. Newsl. **36**:79- 81). At least twenty additional species of filamentous fungi have been successfully transformed to Hyg resistance using two plasmids, pAN7-1 and pAN8-1, which contain a fusion gene consisting of the *A. nidulans gpd* promoter, *E. coli hph* and the *A. nidulans trpC* terminator (van den Hondel and Punt, in: Peberdy *et al.* 1991 Applied Molecular Genetics of Fungi, Cambridge Univ. Press, pp 1-28). The use of Hyg resistance as a dominant selectable marker in filamentous fungi has been reviewed (Punt and van den Hondel 1992 Meth. Enzymol. **216**:447- 457).

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 [γ -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 [γ -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 [γ -32P]-labeled ATP. The amount of [γ -32P]-labeled Hyg in a dot on the phosphocellulose paper, quantified by autoradiography followed by densitometry or by phosphorimager analyses, is used as a measure of Hph activity.

Experimental. *N. crassa* conidia (2×10^7 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 16,000x g. 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 μ g of total protein (determined by Bradford assay with BSA as standard) from whole cell extracts in a 10 μ l total volume were added to wells of non-sterile 96-well microtiter dishes. The reactions were started by the addition of 50 μ l of reaction buffer (13.4 mM Tris-maleate, pH 7.1, 8.4 mM MgCl₂, 80 mM NH₄Cl, 60 μ M hygromycin B, 15 μ M ATP and 25 uCi/ml of [γ -32P]-ATP) and incubated at room temperature for 1 hr. During this time, filter paper (Micro Filtration Systems, #1514A46X57CM), 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 μ l water.

The completed reactions were mixed with 150 μ l 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 μ l 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 phosphorimager 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.

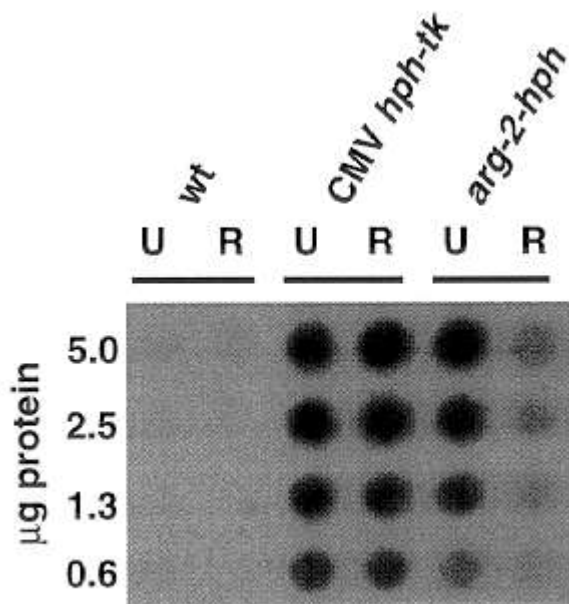


Figure 1: Hygromycin B phosphotransferase dot blot assay on whole cell extracts of *N. crassa*. Conidia from wild type (wt) and *hph*-containing strains were germinated for 6.5 hrs in minimal medium containing 0.5 mg/ml uridine (U) or minimal medium containing 0.5 mg/ml arginine and 0.5 mg/ml uridine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