

A reliable microplate assay for determination of B-galactosidase activity in *Neurospora crassa* strains bearing *lacZ* fusions

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We have been using *lacZ* as a reporter gene in *N. crassa*. The standard -galactosidase assay can be labor intensive and time consuming when large numbers of strains are assayed simultaneously. We sought a technique to simplify the pipetting steps involved in assay preparation and in optical density reading. High reproducibility and rapid processing was obtained by adapting the standard test tube method to a microassay performed in a 96-well microplate.

The protocol described below is a relatively straightforward adaptation of the standard assay to a microplate format.

1. Harvest mycelial samples in a Buchner funnel on 70 mm diameter Whatman filter paper. Conidial samples are harvested on 24 mm diameter glass fiber filters (Whatman). Briefly remove the excess water from the samples by pressing the sample between paper towels. Wrap the samples in aluminum foil, freeze the sample in liquid nitrogen and save until assay.
2. A 2 ml screw cap tube is filled with 1 g of glass beads (0.5 mm dia.) and 1 ml ice-cold Z buffer (Sambrook *et al.*, 1989, Molecular Cloning, page 17.35). Add 10 μ l of 100 mM PMSF (phenylmethyl sulfonyl fluoride; Sambrook *et al.*, 1989, Molecular Cloning, page 16.66) just before the first bead beating.
3. Place 70 to 100 mg of frozen cells into the tube and break in a mini bead beater (Biospec Products, Bartlesville, OK), twice for 30 sec each, with cooling on ice between bead-beating.
4. Microcentrifuge for 5 min at maximum rpm to pellet debris. Transfer the supernatant to new tubes. Centrifuge again if debris is present.
5. For protein assay, we adapted the manufacturer's instructions (Bio-Rad Protein Assay, Bio-rad, Richmond, CA) for the microassay using a microplate dish to measure the OD. 250 μ l of dye + extract was transferred to each well of a microplate. The microplate reader (EmaxTM Precision microplate reader, Molecular Devices Corporation, Menlo Park, CA) was set to read with wavelength of 595 nm.
6. Each well of a microplate is filled with Z buffer + extract (total of 150 μ l). We perform duplicate assays of two different amounts of extract. A control well is also included for each amount of extract (i.e., 6 wells for each mycelial extract is required). The amount of extract can be adjusted depending on the specific activity of the extract. Reaction times of 15-60 min are ideal.

7. Add 70 l of 1 M Na₂CO₃ and 30 l of ONPG (o-Nitrophenyl- -D-galactopyranoside) to each control well of a dish. It is important to add Na₂CO₃ first. Place the dish on a support (test tube rack) in a 28 C water bath.
8. Add 30 l of ONPG to the test wells at 10 sec intervals. Note the time of addition. Solutions are mixed with pipetting 2-3 times.
9. When a reaction shows a yellow color, the reaction can be stopped by adding 70 l of Na₂CO₃. Note the time of addition so that the total reaction time can be determined.
10. Water adhering to the bottom of the microplate dish is removed completely with a Kimwipe. The lid of the microplate is removed (if present) and transferred to the microplate reader.
11. The OD value of each sample is determined by subtracting the OD value of the control from that of the sample. The assay values are calculated by the formula below:

$$U = \{ OD \times 380 \} / Protein (mg) \times Time (min)$$

Table 1. Comparison of microplate assay and the standard assay.

Strains	Published activity 1	Specific Activity 2	Microplate Assay Value 3
DE3	not tested	2	8 (1)
DE1559	6 (1)	6	19 (1)
DE778	16 (1)	14	41 (2)
DE353	128 (1)	111	442 (6)
DE236	225 (36)	98	389 (5)

¹Published values from Corrachano et al., Dev. Biol. **167**:190-200, 1995. The numbers in parentheses show standard deviation.

²The values are means of two duplicate samples of each strain.

³The values are means of five duplicate samples of each strain. The numbers in parentheses show the standard deviation of the five assays.

Strains presented in Table 1 have a single *con-10/lacZ* translational fusion construct at the *his-3* locus. DE3 is a strain containing pDE3, which has no activation elements in the 5' end of *lacZ*. Other strains contain a translational fusion construct that is under control of different sized fragments of the *con-10* promoter (Corrochano et al., Dev. Biol. 167:190-200, 1995).

The microplate assay values correlate well with the specific activities determined by the standard assay. However, the microplate assay gives values for relative b-galactosidase activity that are 3 to 4-fold higher than the true specific activities (in nmole ONPG hydrolyzed/min/mg) of the extracts as determined by cuvette assay. In the microplate assay, the path length of the wells in a microplate dish differs from the path length of a standard cuvette. Also, the wavelenth used in

the plate reader is 405 nm rather than 420 nm, as used in the standard assay. This could account for the different values between the two assay methods. However, we found the method to be reproducible and it provides measurements that are consistent with specific activities determined using the standard cuvette assay.

We also noticed that strain DE236 behaved differently in our assays from the published value. The cultures reported by Corrachano et al. (ibid) were grown in constant darkness. In our experiments the cultures were grown in constant light. It is known that light influences the activity of the *con-10* promoter and this is the likely cause of the activity difference.

Last modified 7/25/96 KMC