Amyloglucosidase Suppresses Interference by Glycogen in the Quantification of DNA Using the Hoechst 33258 Dye

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The Hoechst dye fluorescence technique, which is used for the quantitative determination of DNA (12), requires high saline concentrations at neutral pH to reach maximal fluorescence of the DNA-dye complex and to render the DNA fully accessible to the dye by dissociating foreign molecules (3,12). In conventional assays, the DNA is alcohol-precipitated and then resuspended in buffer containing 2 M NaCl (12). The procedure has been modified for low DNA concentrations (<1 µg/mL) by adding a co-precipitating macromolecule such as glycogen, linear polyacrylamide, tRNA, or spermidine before the alcohol step to improve precipitation and recovery of DNA (8,10). A combination of the co-precipitation and fluorescence techniques is convenient for quantitative DNA determinations in samples containing low DNA concentrations.

However, the Hoechst 33258 dye can bind to compounds other than nucleic acids (2,4) and hence can cause nonspecific fluorescence. When proteins or polysaccharides such as glycogen are mixed with the dye at a weight ratio equivalent to that of DNA in cellular homogenates (4), the nonspecific fluorescence represents less than 1% of the fluorescence attributable to the DNA-dye complex. A substantially higher error is to be expected when proteins or polysaccharides are present at concentrations higher than that of DNA, for example, when a carrier is used for DNA precipitation. Between 10 and 30 µg carrier are required per milliliter to precipitate picogram or nanogram quantities of DNA (8,10,14). Here we investigated the effect of carrier on the DNA-Hoechst 33258 dye fluorescence, which was measured when DNA was precipitated in the presence of glycogen. We then developed a method to degrade the glycogen enzymatically (13) after the precipitation of DNA and before the fluorescence measurement.

We used the Hoechst 33258 dye (2′-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2′-bi-1H-benzimidazole), low-grade glycogen type VII (from mussel, approximately 10⁸ Da) (5) and genomic E. coli DNA type VIII (all purchased from Sigma-Aldrich, Taufkirchen, Germany). Glycogen for molecular biology (from mussel) and amyloglucosidase (approximately 10⁵ Da) were obtained from Roche Applied Science (Mannheim, Germany). All other chemicals were of analytical grade. The dye was dissolved at 0.2 µg/µL in water, passed through a 0.2-µm filter, aliquoted, and stored in the dark at -20°C. Amyloglucosidase (amylo-α-1,4-α-1,6-glucosidase) was prepared at 1 µg/µL in 0.1 M sodium acetate, 2.5 mM Tris, pH 4.8 (13), and stored at -20°C. The DNA was dissolved in filtered TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored at 4°C. The DNA stock solution contained fragments of less than 25 kb, their bulk ranging in size from 10 to 25 kb. Fragment sizes were determined by ultracentrifugation in 5%–25% sucrose density gradients or by electrophoresis on 0.7% agarose gels. The DNA of bacteriophage λ cleaved with EcoRI (Styl) and MluI was co-electrophoresed as size marker.

Fluorometric assays were carried out on resuspended DNA (Table 1), diluted in 700 µL PBS (50 mM NaH₂PO₄, 2 M NaCl, pH 7.4), and then mixed with 7.5 µL freshly prepared Hoechst 33258 dye solution at 0.02 µg/µL in water. Fluorescence was measured in a model LS 50B luminescence spectrophotometer (Perkin Elmer Ltd., Buckinghamshire, UK), adjusting excitation at 350 nm and emission at 460 nm. The excitation slit width was set to 2.5 nm and the emission slit to 7.0 nm. Mean values and simple regressions were calculated with a confidence interval of 95% for three replicate measurements. In addition, fluorescence was measured in the presence of increasing concentrations of glycogen type VII, which was thought to contain traces of contaminating DNA (10), and of glycogen for molecular biology, which was free of nucleic acids. As
both preparations of glycogen were routinely used as carrier, a comparison between them in terms of fluorescence was carried out. In both cases, glycogen at 20 μg/μL water was serially diluted and fluorescence was measured at each dilution step, after mixing 20 μL of an appropriate glycogen solution with PBS and dye as described earlier. Fluorescence was then measured in the presence of increasing DNA concentrations. A DNA stock solution was prepared at 1 μg/μL by determining the concentration of DNA at 260 nm in a model DU-600 spectrophotometer (Beckman Coulter, Munich, Germany): 1 mL DNA solution of A_260 = 1.0 contained 50 μg DNA (11). The stock solution was diluted in TE at 0.1 μg/μL and used to establish a standard curve.

DNA in amounts of 0.1, 0.5, or 1.0 μg was precipitated with glycogen type VII as described in Table 1. We first determined the quantity of carrier needed to obtain maximal recovery of DNA. In this assay, DNA was precipitated in the presence of 20 or 200 μg glycogen, and resuspended in 50 μL water. Controls were carried out replacing DNA by TE or glycogen by water. In a second assay, DNA was precipitated with the defined quantity of carrier and resuspended in 40 μL 2 mM EDTA, pH 8.0, and then treated with 10 μL amylglucosidase (10 μg) for 1 h at 25°C. Degradation of glycogen by amylglucosidase was documented by thin layer chromatography on Merck analytical plates (20 × 20 cm) that were coated with silica gel. Samples were subjected to chromatography and eluted overnight at room temperature in a solvent system of n-butanol:acetic acid:water (2:1:1, by volume). Sugars were located on the chromatograms by reacting them with 0.2% orcinol in aqueous 20% H_2SO_4 at 100°C for 5 min. A control was carried out with 50 μg glucose.

At a constant concentration of Hoechst 33258 dye, enhanced fluorescence was observed with increasing amounts of glycogen type VII, as well as with glycogen for molecular biology (Figure 1). In either case, fluorescence increased as a linear function of glycogen concentration, and linear regressions were very close (Y = 0.6X + 157.7, r^2 = 0.99, for glycogen type VII). When DNA was precipitated in the presence of increasing amounts of carrier, the addition of 200 μg glycogen [i.e., a 10-fold higher amount than previously used (9,14)] in less than 1 mL solution containing less than 1 μg DNA improved precipitation and the recovery of DNA (Figure 2). However, when DNA was precipitated with 200 μg glycogen and resuspended in 2 mM EDTA, pH 8.0 (Table 1), the addition of amylglucosidase caused a decrease in fluorescence of about 100 U in all samples and in the control (Figure 3). The difference in fluorescence measured upon precipitating DNA in the presence of glycogen, and the fluorescence measured after the latter’s hydrolysis, indicated that the fluorescence due to glycogen would lead to an overestimation of the actual DNA concentration ranging between 10% and 40% of fluorescence (Figure 3).

Table 1. Outline of the Procedure to Precipitate DNA with Isopropanol and Glycogen

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
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<tbody>
<tr>
<td>1.</td>
<td>Add 85 μL 3 M sodium acetate, pH 5.2; final concentration, 0.3 M.</td>
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<tr>
<td>2.</td>
<td>Add 20 μL glycogen solution.</td>
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<tr>
<td>3.</td>
<td>Add 600 μL isopropanol.</td>
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<tr>
<td>4.</td>
<td>Cool to -20°C for 1 h.</td>
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<tr>
<td>5.</td>
<td>Centrifuge at approximately 13000× g for 30 min at 4°C and discard the supernatant.</td>
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<tr>
<td>6.</td>
<td>Add 1 mL 70% cold ethanol.</td>
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<tr>
<td>7.</td>
<td>Centrifuge at approximately 13000× g for 30 min at 4°C and discard the supernatant.</td>
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<tr>
<td>8.</td>
<td>Dry overnight at room temperature.</td>
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<tr>
<td>9.</td>
<td>Resuspend in 50 μL water or in 40 μL 2 mM EDTA, pH 8.0, for several hours at 37°C.</td>
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Addition of 10 μL DNA stock solution at 0.01, 0.05, or 0.10 μg/μL to 840 μL water

Glycogen type VII at 1 or 10 μg/μL

Figure 1. Fluorescence in the presence of glycogen. Increasing amounts of glycogen type VII are depicted by open circles, and glycogen for molecular biology are depicted by black squares. Insert shows measurements of fluorescence with increasing amounts of DNA (Y = 1044.6X + 159.5, r^2: 0.99).
Glycogen consists of linear chains of 1,4-α- and 1,6-α-linked glucose, which give rise to a highly branched structure (1,5) with negatively charged phosphate groups (7). The negative charges likely bind electrostatically to the NH$_3^+$ groups in the Hoechst 33258 dye molecule (2,6) and thus enhance fluorescence. Although pH 5.0 is recommended for the maximal activity of amylglucosidase (13), the addition of 10 μg enzyme in 2 mM EDTA at pH 8.0 degrades glycogen extensively (data not shown). The enzymatic degradation products migrated like glucose, while some larger polymers and the amylglucosidase enzyme remained at the bottom of the plate and were inert in terms of fluorescence. 

Although fluorescence after DNA precipitation in the presence of 0, 20, or 200 μg glycogen as carrier. Results are for three replicates.

![Figure 2. Fluorescence after DNA precipitation](image)

**Figure 2. Fluorescence after DNA precipitation.** Fluorescence was measured after precipitation in the presence of 0, 20, or 200 μg glycogen as carrier. Results are for three replicates.

**REFERENCES**


**Figure 3. Fluorescence after DNA precipitation in the presence of 200 μg glycogen.** (A) No treatment, the pellet was resuspended in 40 μL 2 mM EDTA, pH 8.0, and then 10 μL enzyme buffer were added. (B) Glycogen was degraded after resuspension in 40 μL 2 mM EDTA, pH 8.0, with 10 μg amylglucosidase (AG; 10 μL at 1 μg/μL) for 1 h at 25°C. Insert shows fluorescence after precipitation without DNA. The composition of the blank was 40 μL 2 mM EDTA, pH 8.0, and 10 μg amylglucosidase. Results are for three replicates.

![Figure 3. Fluorescence after DNA precipitation](image)


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Address correspondence to Dr. Laurent Palka, National Museum of Natural History, Laboratory of General Ecology, 4, avenue du Petit Château, F-91800 Brunoy, France. e-mail: palka@worldnet.fr

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L. Palka, M.E. Palka-Santini, S. Chambon, D. Renz, P. Maurer, and W. Doerfler
University of Cologne
Cologne, Germany

Microscale Assay Monitors Algal Growth Characteristics

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Today’s agriculture and landscaping require effective and environmentally responsible herbicides. A detailed knowledge of the herbicide’s molecular mechanism of action would promote the design of new, safer, and more effective herbicides, but often their mode of action is unknown. As in other areas of research, a microorganism model provides advantages over the long generation time of many crop plants. The algae Chlamydomonas reinhardtii and higher plants share conserved photosynthetic machinery, and both organisms show similar responses to herbicides (6). C. reinhardtii presents unique opportunities to study photosynthesis-inhibiting herbicides since the addition of a carbon source allows the algae to grow without photosynthesis (4). Their facultative heterotrophism allows the identification and characterization of non-photosynthetic mutant strains with altered herbicide sensitivity. Further advantages lie in the fact that Chlamydomonas are unicellular, haploid eukaryotes with generation times measured in hours rather than weeks or months. Well-defined genetics and molecular tools such as the ability to transform the nuclear, mitochondrial, and chloroplast genomes make Chlamydomonas a very effective model in studies that require a photosynthetic organism.

The assay we describe overcomes previous technical hurdles that hinder growth analyses and provides an easy and efficient means to test multiple replicates of many herbicides simultaneously. Standard growth assays employ manual cell counts, making them quite labor intensive (2). As the combinations of time points, conditions, and/or replicates are reduced to manageable numbers, the statistical significance of the results decreases. Also, in addition to the universal premium on research space, assays for studying photosynthesis are best performed in small areas to protect against fluctuations in light intensity.

The assay employs samples in 96-well plates monitored by spectrophotometry. To protect against evaporation over an eight-day time course, sterile, covered microplates (FisherBrand® 0.45-mL, flat-bottom; Fisher Scientific, Pittsburgh, PA, USA) are used, and sterile water is placed in the outermost rows and columns. The 60 remaining wells are conveniently divided into six replicates of 10 different experimental conditions. Under these conditions, light variations are effectively eliminated, as the entire plate is only 8.5 × 13 cm.

Cultures for Chlamydomonas growth assays are usually grown in cultures greater than or equal to 6 mL, and cell counts determined using a hemocytometer. Unlike bacteria and yeast, the literature reports no standard wavelength for measuring Chlamydomonas growth. A spectrophotometric growth assay was tested by comparing the cell count versus absorbance readings at either wavelength 655 nm (triangles), or by manual cell counts (diamonds). Data points shown are the average of two separate trials.

Figure 1. Comparison of growth curves based on absorbance with growth curves based on manual cell counts. Standard (6-mL) cultures of wild-type C. reinhardtii were grown in the presence of acetate for eight days. Data were collected at 750 nm (squares), 655 nm (triangles), or by manual cell counts (diamonds). Data points shown are the average of two separate trials.