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Application Note

Fluorometric Quantitation of Hydrolyzed DNA

Hydrolysis of samples generally precludes the quantitation of DNA with intercalating fluorescent dyes. Here we describe a method to fluorometrically quantitate DNA from acid hydrolyzed samples.

One of the essential elements in conducting cell cycle studies or growth kinetics of cell cultures is the ability to quantitate DNA in large numbers of samples at a sensitivity that enables determination in small culture samples. The procedure described here is essentially that described by Janakidevi et al. (1) except that the assay was performed in microplates and read using a fluorescent plate reader. While many different enzymatic reactions can be standardized by DNA content, this method is particularly adapted for use in determining DNA synthetic rates in cultures labeled with [^{3}H]-thymidine.

Introduction

Thymidine incorporation into nucleic acids has been used for several decades as a means to quantitate DNA synthesis. Unlike the other nucleotides, thymidine is not incorporated into ribonucleic acids and is therefore a direct indicator of only DNA synthesis. Because of the presence of unincorporated [3H]-thymidine, as well as several salvage pathways available for the labeled thymidine to be recycled, it is important that only cold acid precipitable materials be quantitated. Following subsequent washes to remove unincorporated label, the DNA is solublized by acid hydrolysis and quantitated by scintillation counting. Because specific activity rather than the total amount of radioactivity is the most important determinant, total DNA must also be quantitated in order to normalize each sample.

There are several different fluorometric methods to quantitate DNA from tissue culture cells (2-3). Most of these methods rely on the intercalation of a fluorescent dye into intact double stranded DNA. The hydrolysis of the sample generally precludes the use of these methods on those samples, thus the investigator must use parallel samples for DNA quantitation. The ability to directly quantitate DNA from hydrolyzed samples alleviates the need to run parallel samples and reduces the variability inherent in such determinations.

Materials and Methods

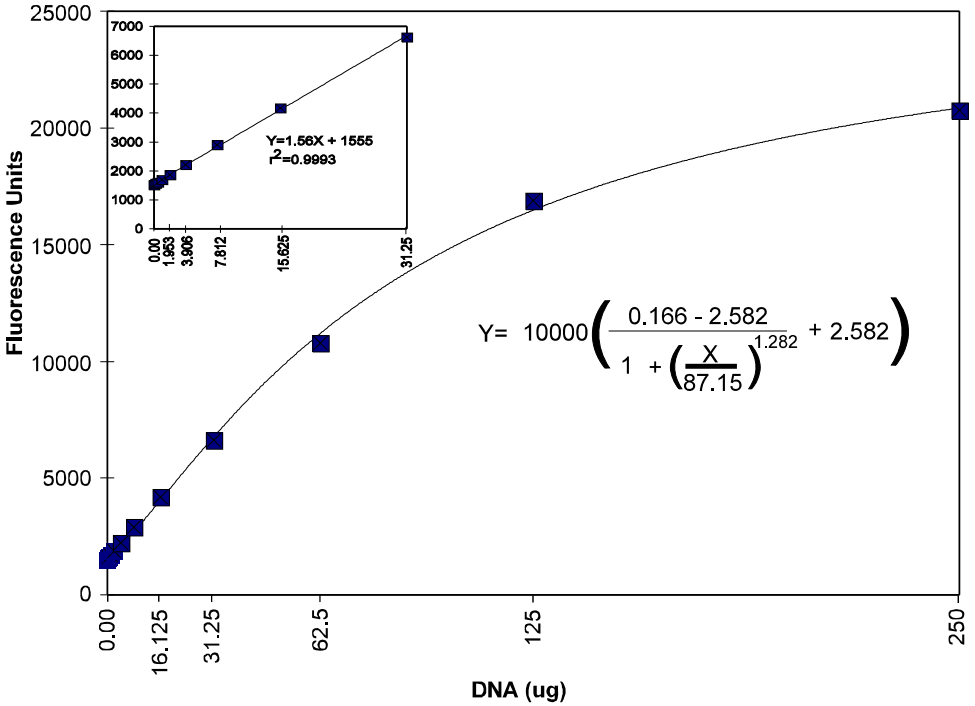
Diaminobenzoic acid dihydrochloride (DABA·2HCl), catalogue number D-1891 was purchased from Sigma Chemical Co., St. Louis, Missouri. Alternatively free diaminobenzoic acid (Sigma cat # D8251) can be converted to the dihydrochloride salt as described by Janakidevi et. al. much less expensively (1). The clear bottom FluoroNunc microplates used were purchased from Nunc and perchloric acid was purchased form Fisher Scientific.

Purified DNA (herring sperm) previously quantified by spectrophotometry was hydrolyzed in 0.5 N perchloric acid (PCA) at 70°C for 15 minutes. A series of 1:2 dilutions were then assayed as described below in replicates of eight. Aliquots of DNA samples (0.1ml) were pipetted into microplate wells and 0.1 ml of DABA·2HCl (25% in distilled water) was added. The wells were

mixed by several rapid pipettes of the mixture and the microplate was sealed using a plate sealing membrane and incubated for 2 hours at room temperature. Before the fluorescence was read, the reactions were diluted further with 0.1 ml of 0.5 N perchloric acid (PCA). Fluorescence was determined using a BioTek Instruments FL500 fluorescent plate reader with a 410 nm, 10 nm bandwidth, excitation filter and a 508 nm, 20 nm bandwidth, emission filter. The sensitivity setting was at 40 and the data collected using static sampling with a 2 second delay, 100 reads per well. Although in these experiments the plates were read immediately, if they remain sealed the reaction is stable for several days with minimal loss of sensitivity.

Results

The fluorescence intensity was determined for DNA concentrations ranging from 0.0 mg/ml to 250 mg/ml. Over this range the fluorescent intensity increased in a hyperbolic fashion. Using KinetiCalc data reduction software (BioTek Instruments), a 4 parameter non linear equation describing the standard curve can be generated. When the linearity of the reaction is examined, a least means squared straight line can be utilized for DNA concentrations up to 30 mg with very high confidence. The average coefficient of variance for the standards was 7.5% with the greatest variation taking place in the higher DNA concentrations tested (data not shown). In terms of sensitivity the reaction was found to be sensitive to the submicrogram levels, with fluorescent intensity values for 0.24 mg being statistically different from the 0 mg control ($P<0.003$). In practical terms, assuming a DNA content of 4.7 to 6.85 pg/cell (4), DNA can be measured in samples with cell numbers as low as 5 X 104.



Linearity of the Standard assay. Concentration curve from 0.244 mg to 250 mg with 4 parameter regression analysis equation. Insert figure depicts the linearity from 0.244 mg to 31.25 mg with least squares regression analysis. Filled boxes (μ) represent the mean values of eight determinations at each concentration.

In order to achieve the greatest sensitivity a number of measures can be undertaken to reduce background fluorescence. Because impurities in the DABA·2HCl can result in high background levels, dissolving the compound in water and treating it with activated charcoal followed by recrystallization can improve sensitivity (1). Reducing the total hydrolysis volume would also be expected to increase the sensitivity in terms of cell number.

The ability to perform this assay in microplates offers several advantages over the conventional tube-based assay. Like most assays that are performed in microplates, the ability to use multi-channel pipettes greatly reduces the manual labor involved in the assay. The microplate format also lends itself to “off the shelf” automation for laboratories with high volume requirements. The smaller reaction volumes in microplates generally lead to lower overall reagent costs and greater sensitivity with this assay. Finally, if this assay is being run in conjunction with a [3H]-thymidine incorporation study, the use of disposable microplates will generate much less radioactive waste, with its high disposal costs, than the conventional tube-based assay.

References

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Rev. 2-20-01