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A COMPARISON OF FOUR FREQUENTLY USED ASSAYS FOR QUANTITATIVE DETERMINATION OF DNA

M. Van Lancker, L.C. Gheyssens

Laboratoria voor Medische Biochemie en voor Klinische Analyse, Harelbekestraat 72, B-9000 Gent, Belgium

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ABSTRACT

Four frequently used DNA determination methods are compared:the diphenylamine colorimetric assay and three fluorescent stain methods, i.e. diaminobenzoic acid diaminophenylindole and bisimidazole (Hoechst 33258). Interference with these assays by thirty-five substances, on different DNA preparations, are examined. The classical diphenylamine method is found to be the most reliable of all, closely followed by diaminobenzoic acid staining. The remaining two methods, although very sensitive, perform rather poorly.

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INTRODUCTION

Numerous procedures for the quantitative determination of DNA have been described since Burton[1] published his classical diphenylamine assay. Most of them are fluorescence-based methods, exhibiting a largely increased sensitivity. Reviewing the recent literature dealing with different aspects of DNA research, three other methods appear well established : the diaminobenzoic acid (DABA) method originally described by Kissane and Roberts[2], the diaminophenylindole (DAPI) method described by Kapuscinski and Skoczylas[3] and finally the bisimidazole (Hoechst 33258) method of Labarco[4]. These assays are used in many situations and in presence of numerous substances, originating from the isolation technique (e.g. buffer components or preservatives coextracted with the DNA, can be expected to interfere with these assays. This can be particulary important when the composition of standards or blanks cannot be exactly matched with those of the samples (e.g., cell homogenates). Nevertheless, although the importance of such interferences is obvious, they are only rarely reported. We examined thirty-five substances, frequently used in extraction methods or occurring in biological matrices, for their possible disturbing activity in these four DNA assays.

EXPERIMENTAL

Stock solutions of DNA were prepared by dissolving highly polymerised calf thymus DNA (Sigma) in double distilled water (48 hours or more at 4°C were needed, eventually with

gentle shaking). Concentrations were measured spectrophotometrically ($E_{260} = 200$). Five different DNA concentrations were used : 0-10-100-500-1000 µg/ml ,except for DAPI and bisimidazole assays. The working ranges of these methods are lower then those of the other two .Moreover, as described[3] , different DAPI concentrations are needed in order to accomodate for extended DNA concentration ranges. We therefore choose a lower and more restricted range 0-10-50-80-100 ug/ml. Aliquots of 100 µl of each were dispensed in glass test tubes, and dried at 40°C in a vacuum evaporator (Vortex Evaporator, Buchler Instruments, Fort Lee, NJ). Prior to use, DNA samples were dissolved again in 100 µl water or 100 µl of a solution containing various (three) concentrations of the test substance. For each test substance, four curves were obtained : a standard curve containing no interfering product and three curves, corresponding to the three different concentrations of the compound tested. This procedure allowed us to measure simultaneously the specificity of the methods and interference of the test substance with the DNA-dye reaction. Moreover, an appreciation of the scatter around the standard curve and eventualy distortions (loss of linearity) could be made. Specificity was derived from the zero DNA concentration intercepts of the curves and expressed as relative color or fluorescence / 100 ug .Interference with the DNA-dye reaction is given as the relative inhibition or enhancement of the slope of the standard curve .

The concentrations of the test substances were: buffer components: 0.1-0.5-1.0 M ; sugars: 0.1-0.5-1.0 mg/ml ; inorganic salts: 0.1-0.5-1.0 M ; albumin: 0.1-0.2-0.4 g/dl ; glycin: 0.1-0.5-1.0 mg/ml ; detergents: 0.1-0.5-1.0 g/dl ; RNA: 0.1-0.5-1.0 mg/ml ; NADP:0.2-1.0-2.0 ug/ml ; ureum: 1.0-2.0-4.0 M ; phenol: 5.0-25.0-50.0 mg/ml ; ethanol: 0.01-0.05-0.1 g/ml ; EDTA: 0.01-0.05-0.1 M ; adriamycin: 10.0-50.0-100.0 ug/ml.

The suitability of each method for the analysis of single-stranded or severely degraded DNA was tested by comparing identical quantities of temperature-degraded calf thymus DNA and Herring sperm DNA type IV (Sigma) with the standards. All experiments were performed in duplicate.

Diphenylamine assay : Richard's modification[5] of the original assay was used, offering higher sensitivity and lower reagent blanks. To 100 µl of sample 1.9 ml of 1.65 M $HClO_4$ was added, followed by 1.2 ml color reagent (diphenylamine 4 % w/v, glacial acetic acid 96 % v/v, paraldehyde 0.01 % v/v). The mixture was kept overnight in the dark and color read at 600 nm.

Diaminobenzoic acid assay (DABA assay) : the Hinegardner method[6] was used. A 100-µl aliquot of DABA solution (300 mg/ml in water) was added to the sample and color developed at 60°C for one hour. After cooling, the mixture was diluted with 2 ml 1N HCl and fluorescence was measured at excitation wavelength 400 nm and an emission wavelength 520 nm.

Before use, impurities in the commercial preparation of DABA were removed by adsorption onto charcoal and repeated precipitation of DABA from concentrated HCl.

DAPI assay. We used a slight modification of the original description of Kapuscinski and Skozylas[3], as the assay was always carried out in the presence of an amount of salt, as this improved considerably the reproducibility.We finally adopted the following procedure : a 100-µl aliquot was dried as above. The dried DNA was redissolved in 100 µl 0.1 M NaCl (instead of pure water) or in 100 µl 0.1 M NaCl plus test substances as described. Two ml DAPI solution (0.3 µg/ml in water) were added and fluorescence read at once (exc. 372 nm, emiss. 454 nm).

Bisimidazole assay was carried out as described by Stout and Becker[7]. This method, according to the authors, has the advantage of being more suited for single-stranded DNA than the original procedure. The sample was mixed with 2 ml of the dye reagent (105 µg/ml in 2.5 mM phosphate buffer, pH 7.0) and fluorescence read immediately (exc. 360 nm, emiss. 450 nm).

RESULTS AND DISCUSSION

A summary of our findings is presented in Table I and Table II. The first Table lists the substances tested, along with their effects on the four assays .Influence of DNA integrity

Table I

		diphenylamine	DABA	DAPI	bisimidazole
Buffer components					
PCA	pH 2,0	(0)	(2) 0,19	(2) 0,00	(2) 0,09
TCA	pH 2,0	(0)	(2) 0,42	(2) 0,00	(2) 0,42
citrate	pH 2,0	(0)	(0)	(2) 0,00	(2) 0,43
ascorbic acid	pH 2,1	(0)	(0)	(2) 0,17	(2) 0,62
acetate	pH 4,3	(0)	(0)	(2) 0,81	(2) 0,54
piperazine	pH 4,3	(0)	(0)	(2) 0,89	(2) 0,62
phosphate	pH 7,0	(0)	(0)	(0)	(0)
HEPES	pH 7,4	(0)	(0)	(0)	(0)
tris	pH 8,0	(0)	(0)	(2) 0,57	(0)
borate	pH 10,0	(2) 0,76	(0)	(2) 0,00	(2) 0,79
NaOH	pH 10,0	(0)	(2) 0,42	(2) 0,61	(2) 0,00
Sugars					
glucose		(0)	(2) 0,94	(0)	(0)
ribose		(0)	(2) 0,89	(0)	(0)
arabinose		(0)	(2) 0,93	(0)	(0)
Inorganic Salts					
KCl		(0)	(0)	(0)	(2) 0,22
NaCl		(0)	(0)	(0)	(2) 0,80
$MgCl_2$		(0)	(0)	(2) 0,09	(2) 0,22
$(NH_4)_2SO_4$		(0)	precipitate formation	(0)	(2) 0,27
NaN_3		(0)	(0)	(0)	(0)
Amino Acids, Protein					
albumin (human)		(0)			
glycin		(0)	(2) 0,76	(0)	(0)
Eagle's MEM		(0)	(0)	(0)	(0)
Detergents					
deoxycholate		(2) 1,42	(0)	erratic curves	(0)
SDS		(0)	(0)	(1) 1,60	precipitate formation
Triton-X-100		(0)	(0)	(1) 0,55	(1) 0,01
Tween 80		(2) 0,84	(1) 0,10	(1) 0,07	(1) 0,16
Varia					
RNA		(0)	(1) 0,11	(1) 0,14	(1) 0,13
NADP		(0)	(0)	(1) 1,29	(0)
urea		(2) 0,09	(2) 0,95	(0)	(0)
phenol		(0)	(0)	(0)	(0)
ethanol		(0)	(0)	(0)	(0)
EDTA		(0)	(0)	(0)	(0)
Filters					
- polyvinyliden fluoride		(0)	(2) 1,04	(0)	(0)
- cellulose esters		(2) 0,46	(2) 1,09	(0)	(0)
adriamycine		(0)	(0)	(0)	(2) 0,51

Legend : (0): no interference. (1) : relative color or fluoresence development of test substances. (2) : relative inhibition or enhancement of DNA-dye reaction.

Table II

	heat denatured DNA	Herring Sperm DNA
diphenylamine	0.00	0.00
DABA	0.00	0.00
DAPI	0.70	0.90
bisimidazole	0.60	0.80

as measured by the relative inhibition of color or fluorescence development is given in the second Table.

In spite of its limited sensitivity (L.D.A., lowest detectable amount: $\pm$ 1 µg DNA), the diphenylamine assay still seems the most reliable method for DNA determination. Its main features are an almost complete independence of pH conditions, ionic strength and the presence of proteins and sugars. Specificity, especially as concerns RNA, is excellent. As some detergents interfere rather strongly with the assay, care must be taken to remove all traces after, e.g., their use in cell lysis prior to determination.The method, however, is equally well suited for severely degraded DNA as it is for intact DNA.

The DABA assay shares many of the former features, but appears nevertheless somewhat less performing. It is not suited for use at extreme pH values, which precludes, e.g., its use for acid-precipitated DNA. Interference is noted from sugars and protein , while an important reaction with RNA exists. Noteworthy is the enhancement of fluorescence as seen for DNA on filter membranes : this effect has been exploited to obtain higher sensitivity and ease of work[8] .

Important differences exist between the two assays discussed above and the DAPI and bisimidazole assays. The former react strongly with the sugar moieties of the DNA , while in the latter, the mechanism is much more complex, as both intercalation and direct binding seem to occur[3,7] .In addition, preference for certain sequences in the DNA molecule has been observed (for instance A/T rich regions for DAPI), and fluorescent yield may depend on the concentration ratio DNA / Dye[3] .

The DAPI assay seems to suffer most from these particularities. Denaturation, degradation and precipitation diminish fluorescence drastically. It can only be used in a very limited pH-range. As already mentioned, there exists a dependence on ionic strenght to obtain good results.

Bisimidazole behaves similarly : limited pH range,susceptibility to the presence of detergents, importance of salt concentration, reaction with RNA.

We conclude that the diphenylamine method still remains the method of choice, although it is somewhat cumbersome and

tedious, and sensitivity is limited. As an alternative, the DABA assay can be used, being more sensitive (L.D.A. : ± 10 ng DNA), but somewhat less generally applicable. Although more sensitive (L.D.A. : ± 5 ng DNA) and very easy to perform, the DAPI and the bisimidazole assays suffer from their numerous interferences . We therefore recommend to use both these assays only under carefully controlled conditions.

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