

Quantification of nucleic acids

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Abbreviations:

Bisbenzimidide H 33258 (= Hoechst 33258)	– 2-(2-(4-hydroxyphenyl)-6-benzimidazolyl)- 6-(1-methyl-4piperazyl)-benzimidazole, trihydrochloride pentahydrate.
DABA.2HCl	– 3,5-diamino-benzoic acid dihydrochloride;
DAPI	– 4,6-diamidino-2-phenylindole;
EDTA	– sodium ethylene diamine tetraacetate;
PBS	– phosphate buffered saline;
SSC	– standard saline citrate;
Tris	– Tris (hydroxymethyl)-aminomethane

Introduction

Methods for nucleic acids quantification in environmental samples are normally based on spectrofluorometry. When analyzing impure samples containing humic material, autofluorescence has to be measured and subtracted from the nucleic acid specific fluorescence. It is also recommended to use a known amount of nucleic acid as an internal standard, to correct for quenching. There are several methods which are specific for DNA and some which can be used for quantification of both DNA and RNA.

The following methods are more or less specific for DNA and can be used for low concentrations of DNA:

- 1) Reaction with 3,5-diamino-benzoic acid 2HCl (DABA.2HCl) [7]. This reagent is specific for primary aldehydes of the type $R-CH_2-CHO$. With material like soil, great care must be taken in the extraction procedure in order to ascertain that the measured fluorescence stems only from the DNA-DABA.2HCl complex.
- 2) Reaction with mithramycin. This antibiotic reacts with guanine in double stranded DNA, and the complex formed has a fluorescence which can be taken as a measure of the amount of DNA present. This method is absolutely specific

[4], but the binding efficiency is decreased in the presence of nucleoproteins, and it has a somewhat lower sensitivity than the DABA.2HCl-method.

- 3) Reaction with bisbenzimidazole H 33258 (Hoechst 33258). This is a very sensitive and specific reagent for fluorometric determination of DNA as it binds specifically to adenine plus thymidine base pairs [8].

A new fluorochrome which can replace Hoechst 33258 is the fluorochrome 4-[3-methyl-2,3-dihydro-(benzo-1,3-oxazole)-2-methylmethyl-edene]-1-[3'-trimethylammoniumpropyl]-quinolinium diiodide (YO-PROTM-1; Molecular Probes, Inc., Eugene, Oregon, USA). The fluorescence excitation and emission maxima of the DNA-YO-PRO-1 complex are 491 and 509, respectively. The sensitivity is over 400-fold greater than that of Hoechst 33258. The fluorochrome binds to RNA as well as DNA. The RNA and DNA content in a sample may be quantified by measuring the fluorescence before and after treatment with DNase-free RNase.

When applying the mithramycin or Bisbenzimidazole H 33258 method to intact bacteria, the cells first have to be lysed. The most efficient method is sonication, but the sonication conditions are very critical [2]. Sonication energy and length of time must be optimized in order to break as many bacteria as possible without degrading the DNA. Effective cooling during sonication will reduce the DNA degradation.

The first two methods gave similar results, when applied to bacteria extracted from soil, namely 8.4 fg DNA per microscopically counted cell [9]. This is in the upper range of what is normally found in bacteria [1]. Using bisbenzimidazole H 33258, Bakken and Olsen [2] found 2–9 fg per cell in bacterial isolates from soil, and 1.6–2.4 fg in directly extracted bacteria from the same soil. The DNA content of small cells passing through a 0.4 µm membrane filter was close to the average for the total bacterial flora. When the bacterial concentration is 10¹⁰ bacteria per g dry soil, this means that the DNA content is between 20 and 90 µg DNA per g dry weight of soil.

Procedures

Protocol 1

Reaction with DABA (diamino-benzoic acid hydrochloride)[7,9]

The cells are filtered onto a glass fiber filter with a layer of diatomite (diatomaceous earth Hyflo Supercel). The diatomite make it possible to filter a larger sample onto the filter before it get clogged. Some humic matter is removed by washing with sodium pyrophosphate and the cells are pre-extracted with trichloroacetic acid and an ethanol:ether solution to remove lipids, sucrose, etc., which will interfere with the assay. As ether will react with DABA it has to be

completely removed. DABA is allowed to react with deoxyribose at high temperature in the presence of mineral acid. Diatomite, and other particles are then removed by filtering through a membrane filter. It is important to use membrane filters which do not give high blank values. DABA yields a 1000-fold higher fluorescence with DNA than with RNA. The emission light of the DABA-DNA complex is measured in a spectrofluorometer at 500 nm with an excitation light set at 410 nm. Two parallel series are generated; one with an internal standard of calf thymus DNA added. The method is linear for DNA amounts ranging from 1 to 50 µg.

Steps in the procedure

1. Filter 2 ml of a 2% suspension of acid-washed diatomite onto a Whatman glass fiber filter type GF/F (20 mm diameter).
2. Filter a bacterial suspension (i.e. bacteria extracted from soil [3]) containing approximately 10^9 bacteria in 1 ml onto the filter. Take two replicates per sample.
3. Wash the filter with 2×5 ml 0.1 M sodium pyrophosphate, pH 7.0.
4. Extract the material on the filter with 2×5 ml cold 5% trichloroacetic acid, followed by 2×5 ml ethanol:ether solution (3:1).
5. Suck the filter dry, and dry it further in an oven at 60 °C for 1 hour.
6. Transfer the filter to a glass tube.
7. When using DNA as an internal standard, add 5 µg calf thymus DNA in 50 µl PBS to the dried filter of one of the parallel samples.
8. Add 400 µl 20% DABA.2HCl and incubate for 2 hours at 60 °C.
9. Add 4 ml 0.6 M perchloric acid.
10. Pass through a 20 mm Sartorius SM 11106 (0.45 µm pore size) filter to remove particles, diatomite and filter debris.
11. Measure the fluorescence in a spectrofluorometer with a 410 nm excitation light and a 500 nm emission light.
12. Use a sample of 2 ml of a 2% suspension of acid-washed diatomite filtered onto glass fiber filter and treated like the bacterial samples as a blank.
13. Prepare standard series from a stock solution of 1 mg/ml calf thymus DNA in PBS. To standards with 1–50 µg DNA in 50 µl PBS add 400 µl 20% DABA.2 HCl and incubate for 2 hours at 60 °C. Add 4 ml 0.6 M perchloric acid, cool to room temperature and measure the fluorescence.

Note

8. DABA.2HCl is prepared from 3,5 diamino-benzoic acid using the following protocol [5] (should be carried out in a hood):

Dissolve 40 g of 3,5 diamino-benzoic acid in 400 ml boiling 6N HCl. Add 1–2 g charcoal, swirl the suspension briefly to mix well, and filter through a preheated (100 °C) 55 mm diameter Buchner funnel with Whatman glass fiber filter (type GF/C). Cool the filtrate in a refrigerator or cold room for 2–3 hours to crystallize the DABA.2HCl. Collect the crystals by filtering onto a sintered glass funnel, transfer them to a glass vial and dry them at 60 °C for 2 hours. DABA.2HCl can be stored in a closed glass vial in the refrigerator for 2 months. Prepare the DABA.2HCl solution immediately before use.

Equipment

- Ceramic or glass filter holders for 20 mm diameter filters
- Water suction equipment (glass)
- Whatman glass fiber filter, type GF/F, 20 mm diameter
- Sartorius SM 11106 filter, 20 mm diameter (0.45 µm pore size)
- Glass tubes
- Oven with thermostat

For preparation of DABA.2HCl:

- Whatman glass fiber filter, type GF/C, 55 mm diameter.
- Buchner funnel, 55 mm diameter
- Sintered glass funnel
- Oven with thermostat
- Fluorescence spectrophotometer

Chemicals and solutions

- 2% suspension of acid-washed diatomaceous earth (Hyflo Supercel, Johns-Manville Products, New York) in distilled water
- 0.1 M sodium pyrophosphate, pH 7.0
- 5% trichloroacetic acid
- Ethanol:ether solution (3:1)
- Calf thymus DNA
- 0.6 M perchloric acid
- Phosphate buffered saline (PBS); 0.8 g sodium chloride, 0.2 g potassium chloride, 1.44 g disodium hydrogen phosphate, 0.24 g potassium dihydrogen phosphate, 1000 ml sterile distilled water, pH 7.5
- 20% solution of DABA.2HCl in distilled water

For preparation of DABA.2HCl:

- 3,5 diamino-benzoic acid
- 6 N hydrochloride
- Charcoal, Norit A

Protocol 2

Reaction with mithramycin [9]

The DNA is measured in the extracts of cells after sonication. Two parallel series are generated; one with mithramycin added and one with buffer in order to measure the autofluorescence. In addition, fluorescence is measured in a standard series of calf thymus DNA. The fluorescence of the DNA-mithramycin complex is measured in a spectrofluorometer with an excitation light set at 410 nm, and the emission light measured at 515 nm. The method is linear with DNA amounts ranging from 5 to 50 µg.

Steps in the procedure

1. Take two series of aliquots from a suspension of soil bacteria containing approximately $2-5 \times 10^9$ bacteria, and pellet the bacteria by centrifugation at $10,000 \times g$ for 15 minutes.
2. Wash the bacteria twice in 0.1 M sodium pyrophosphate and once in PBS by resuspending the cells and then pelleting them by centrifugation as above.
3. Add 3 ml PBS with 15 mM $MgCl_2$, pH 7.5 to the pellets of one of the series, and 3 ml mithramycin reagent to the other.
4. Place the samples in a cold bath (ethanol-dry ice, or $-70^\circ C$ methanol) and sonicate them with a sonifier (Branson B-12 sonifier with a 1.25 cm titanium probe) at maximum output (100 W) for 5 minutes with 120 seconds pulses and 20 seconds pauses.
5. Centrifuge the samples for 10 minutes at $20,000 \times g$ and transfer the supernatant to new glass tubes.
6. Measure the fluorescence of DNA-mithramycin complex in a fluorescence spectrophotometer at 515 nm with an excitation light set at 410 nm.
7. Add 20 µg calf thymus DNA in 50 µl PBS to the series with mithramycin, and measure the fluorescence again after 5 minutes to determine the quenching.

8. Measure the autofluorescence of the samples in the series with only PBS and MgCl_2 added.
9. Measure the fluorescence of the mithramycin reagent separately.
10. Prepare standard series from a stock solution of 1 mg/ml calf thymus DNA in PBS. To a series of standards with 1–50 μg DNA in 50 μl PBS add 3 ml mithramycin reagent and measure the fluorescence.

Equipment

- High-speed centrifuge with a rotor for 15–30 ml tubes (Sorvall SS-34 rotor)
- Branson B-12 sonifier (Branson Sonic Power SA, D-6055 Heusenstam) with a 1.25 cm titanium probe
- Fluorescence spectrophotometer

Chemicals and solutions

- Mithramycin solution (Mithracin; Pfizer Ltd); 200 $\mu\text{g}/\text{ml}$ in 300 mM MgCl_2 , stored frozen and diluted 1:20 with PBS, pH 7.5 before use
- 0.1 M sodium pyrophosphate, pH 7.0
- Phosphate buffered saline (PBS) with 15 mM MgCl_2 ; 0.8 g sodium chloride, 0.2 g potassium chloride, 1.44 g disodium hydrogen phosphate, 0.24 g potassium dihydrogen phosphate, 3.05 g magnesium chloride hexahydrate, 1000 ml sterile distilled water, pH 7.5
- Calf thymus DNA

Protocol 3

Reaction with bisbenzimidazole H 33258 [1, 8]

This method has become widely used for quantification of DNA. The DNA concentration is determined in crude cell extracts which are obtained by sonication in the presence of SSC and a detergent. DNA can be measured down to the nanogram range. The fluorescence is highly specific for DNA, fluorescence enhancement of RNA relative to DNA is below 1%. To correct for quenching by humic material an internal standard should be added to the samples. By using two different dye concentrations (0.15 μM and 1.5 μM), a linear response in the range from 0.05 to 10 μg DNA can be obtained.

Bisbenzimidazole H 33258 can be replaced by equimolar amounts of 4',6-diamidino-2-phenylindole (DAPI) which is also an

adenine-thymine specific dye [6]. DAPI gives higher background fluorescence than bisbenzimidazole H 33258.

Steps in the procedure

1. Pellet the bacteria in samples containing 2×10^8 – 10^9 cells by centrifugation at $10,000 \times g$ for 15 minutes.
2. Resuspend the bacteria in 6 ml $1 \times$ SSC and add 10 μ l 5% Triton X-100.
3. Sonicate the bacteria in a cold bath (ethanol-dry ice, or -70°C methanol) with an ultrasound sonifier (Branson B-12 sonifier with a 1.25 cm titanium probe) at maximum output (100 W) for 5 minutes with 120 seconds pulses and 20 seconds pauses.
4. Centrifuge the homogenate at $10,000 \times g$ for 10 minutes at 5°C .
5. Take 2 ml aliquots in duplicate from the cell extract. Mix one of the parallels with 1 ml $0.15 \mu\text{M}$ bisbenzimidazole solution (for samples containing 50 ng–1 μg DNA) or $1.5 \mu\text{M}$ bisbenzimidazole solution (for samples containing 1–10 μg DNA). Protect the samples with bisbenzimidazole from light.
6. Measure the fluorescence at 450 nm (emission) with an excitation light of 350 nm.
7. To determine the quenching add 0.1 or 1 μg calf thymus DNA in 50 μ l $1 \times$ SSC to the series with bisbenzimidazole, mix well and measure the fluorescence again.
8. Measure the autofluorescence of the samples without bisbenzimidazole.
9. Measure the fluorescence of the bisbenzimidazole reagent (diluted 1:2 with $1 \times$ SSC) separately.
10. From a stock solution of 1 mg/ml calf thymus DNA in $1 \times$ SSC prepare working stock solutions by diluting to 10 $\mu\text{g}/\text{ml}$ (low range standards) and 100 $\mu\text{g}/\text{ml}$ (high range standards) with $1 \times$ SSC. Prepare series of low range standards containing 50 ng–1 μg DNA and high range standards containing 1–10 μg DNA, in 2 ml $1 \times$ SSC. Add 1 ml $0.15 \mu\text{M}$ or $1.5 \mu\text{M}$ bisbenzimidazole reagent to the low and high range standards respectively.

Equipment

- Branson B-12 sonifier (Branson Sonic Power SA, D-6055 Heusenstam) with a 1.25 cm titanium probe

- High-speed centrifuge with rotor for 10–15 ml tubes
- Fluorescence spectrophotometer

Chemicals and solutions

- Bisbenzimidazole H 33258 solution (Hoechst 33258, Calbiochem or Boehringer Mannheim). A stock solution is prepared by dissolving 1 mg/ml of bisbenzimidazole H 33258 in ultrapure distilled water. Can be stored at 4 °C in the dark for a week.
- Working solution of bisbenzimidazole; dilute 10 µl of the stock solution in 100 ml TEN buffer (10 mM Tris, 1 mM sodium ethylene diamine tetraacetate (EDTA), 0.1 M sodium chloride, pH 7.4).
- Standard Saline Citrate (SSC); 1 × SSC is 0.15 M sodium chloride, 0.15 M trisodium citrate in distilled water, pH adjusted to 7.0. Prepare as a 10 × SSC stock solution and dilute as needed.
- 5% Triton X-100
- Calf thymus DNA

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