Fluorometric Determination of DNA in Epidermis and Cultured Fibroblasts, Using 4′-6-Diamidino-2-Phenyindole (DAPI)

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Summary. 4′-6-diamidino-phenylindole (DAPI) which is used for the detection of mycoplasmic infections of cell cultures or cytofluorometric investigations proved to be a sensitive agent for the fluorometric assay of alkali extracted DNA in cultured cells and human epidermis. Various experiments to optimize assay conditions led to the following procedure: 1 part of freeze dried tissue (1 mg/ml) and 1 part of 0.2 mol/l NaOH (w/v) were heated at 95° for 40 min. After cooling for 1 h to room temperature, 0.1 ml alkaline extract was mixed with 2.9 ml DAPI-solution (0.2 gg/ml in McIlvaine buffer containing 1 mmol/l MgCl₂). After 10 min in the dark the emitted fluorescence was estimated at 453 nm with an excitation at 360 nm. This assay procedure allowed a detection limit of 25 ng DNA.

Key words: Fluorometry - DNA - 4′-6-Diamidino-2-phenylindole - Fibroblasts - Epidermis

Introduction

4′-6-diamidino-phenylindole (DAPI), which was first synthesized by Dann et al. [3] as trypanocide, has become well known as a specific fluorescent stain for DNA. Looking for a simple and specific DNA determination method in skin and fibroblasts we remembered our mycoplasma detection technique for cell cultures, which requires a simple fluorescent staining of cytoplasmic DNA by DAPI [12]. The same staining technique has also been applied to cytofluorometric investigations [2, 9, 10]. 3,5-diaminobenzoic acid, mithramycin, ethidiumbromide, Hoechst H 33258 and DAPI have been used for the fluorometric determination of DNA, with different preparation techniques [1, 4 – 8, 13].

Using the alkali extraction method described by Halprin et al. [5] DAPI proved to be a very sensitive fluorescent dye for DNA. The details and properties of this method are described below.

Materials and Methods

Reagents

McIlvaine Buffer: 0.2 mol/l Na₂HPO₄ was adjusted to pH 7.0 with 0.1 mol/l citric acid. DAPI (Serva No. 18860, Heidelberg, FRG): A stock solution contained 1 mg DAPI in 50 ml distilled water. This stock solution remained stable for at least 3 months at 4°C. For routine use 0.4 ml 0.25 mol/l MgCl₂ was added to 1 ml DAPI stock solution and adjusted to 100 ml with McIlvaine buffer to give a final concentration of 0.2 µg DAPI/ml. This solution was always immediately prepared before use. According to Halprin et al. [5] ethidium bromide (EtBr) (Fluka No. 46065, Buchs, Switzerland) was used in a concentration of 10 µg/ml in 0.1 mol/l Tris-HCL pH 7.4 in 0.1 mol/l NaCl. Stock solutions of highly polymerized DNA (Calbiochem No. 62011, La Jolla, CA, USA), salmon sperm or thymonucleic acid (calf thymus) (Fluka No. 89370) were, like tissues or cells, identically treated by the alkali extraction procedure and then diluted by NaOH to the appropriate concentrations for calibration. Ribonuclease was obtained from Fluka (No. 83822).

Cell Cultures and Epidermis

Human skin fibroblasts were grown from skin biopsies in DMEM + 10% FCS as described elsewhere [11]. After propagating in roller flasks (Falcon Plastics, Cockeysville, MD, USA), cells were scraped off by a rubber policeman, washed with PBS without calcium and magnesium and then freeze dried. 3T6 and 3T3 cells (Flow Labs, Irvine, Scotland) were treated in the same manner. Epidermis from human skin biopsies was separated from dermis by heat (3 min at 56°C) and then freeze dried.

Extraction Procedure

1 mg dry weight (dw) of epidermis or cultured cells were extracted in NaOH as described in Results and Discussion.

Quantitation of DNA

To 0.1 ml filtrated extract 2.9 ml DAPI solution (0.2 µg/ml) was added. After standing for 10 min in the dark, the fluorescence...
intensity was estimated at 453 nm with an excitation wavelength of 360 nm, using a Kontron spectrofluorometer SFM 23 (Kontron Analytik, Zürich, Switzerland) and a standard 3 ml fluorescence cuvette.

**Results and Discussion**

Stocks of freeze dried 3T6 cell cultures were used to evaluate optimal conditions. In preliminary experiments freeze dried cells were extracted 30 min at 95°C in 0.5 mol/l NaOH as recommended by Halprin et al. [5]. The addition of RNase did not reduce the fluorescence intensity confirming the fact that this alkaline extraction completely destroys RNA [5], which on the other hand only minimally enhances DAPI fluorescence. Before adding the DAPI solution the hot alkaline DNA extract was allowed to cool to room temperature for at least 1 h to obtain maximal fluorescence. In the following three experiments DNA from samples of 1 mg dry weight of cells were extracted under different conditions and then, after the addition of DAPI solution, the relative fluorescence intensity estimated as described in Material and Methods.

**Duration of Extraction**

Treating 3T6 cells various times with 0.5 mol/l NaOH at 95°C, maximal fluorescence was obtained after 40 min of extraction (Fig. 1). No significant change in fluorescence intensity was observed, when reference DNA was subjected to identical conditions between 30–60 min. We therefore concluded that the maximal fluorescence at 40 min was due to optimal DNA extraction. Because RNA only minimally contributes to the enhancement of DAPI fluorescence [1, 7], the optimal DNA extraction can be considered as the main result of the alkali treatment.

**Extraction Temperature**

Extraction of 3T6 cells with 0.5 mol/l NaOH at different temperatures yielded maximal fluorescence at 95°C, whereas the treatment of reference DNA with hot alkali at various temperatures between 30 and 95°C did not alter its fluorescence intensity (Fig. 2). Again the higher fluorescence at 95°C reflects a more efficient DNA extraction.

**Alkali Concentration**

In contrast to the first two experiments, similar curves were obtained for 3T6 cell extracts and reference DNA by treatment with different concentrations of NaOH, yielding a maximum at 0.2 mol/l NaOH (Fig. 3). The decrease of fluorescence observed with 1 mol/l NaOH corresponds to the total loss of fluorescence with ethidium bromide after 4 h in 1 mol/l NaOH at 95°C [5]. On the other hand the decreased fluorescence might also be partly explained by the increased pH (pH 9) in the fluorescing solution.