Quantification of picogram levels of specific DNA immobilized in microtiter wells

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A non-radioisotopic and sensitive method for quantification of specific DNA immobilized in microtiter wells has been developed. This method is based upon the immobilization of DNA in microtiter wells and hybridization with biotinylated DNA probe which is followed by complexing with avidin-β-galactosidase. By measuring fluorescence emitted from the hydrolyzed product by β-galactosidase of 4-methylumbelliferyl-β-D-galactoside, it has become possible to quantify a few picograms of specific DNA in DNA samples immobilized in plastic microtiter wells.

Nonradioisotopic DNA probe *Avidin-biotinylated β-galactosidase complex* *Immobilized DNA* *Microtiter well* *DNA probe quantitation*

1. INTRODUCTION

In general, the detection of specific nucleotide sequences in DNA preparations is carried out by DNA-DNA (or DNA-RNA) hybridization with DNA (or RNA) probes labeled with radioisotopes. Recently, biotin-labeled [1] or chemically modified [2] polynucleotides were reported as non-radioisotopic probes for detection of specific nucleotide sequences. In the former case, the biotin-labeled probe hybridized with specific DNA then becomes associated with avidin which is coupled with horseradish peroxidase [3] or alkaline phosphatase [4] and the associated enzyme activity is assayed.

Here, we report the development of a method using β-galactosidase (EC 3.2.1.23)–avidin complex as a new detector for biotin-labeled DNA probe and 4-methylumbelliferyl-β-D-galactoside as a substrate. To extend this methodology to quantification of specific viral or other DNAs in clinical tissue specimens, optimum conditions for assay of specific DNA were explored with DNA immobilized in microtiter plates. By this method, we were able to detect a few picograms of DNA with specific nucleotide sequences in a well of microtiter plates.

2. MATERIALS AND METHODS

2.1. *DNA probe synthesis*

Purified λ DNA was labeled with biotinylated dUTP (Enzo, purchased through Wako) by nick-translation [5]. The extent of incorporation of biotinylated dUTP into DNA in each sample was estimated from the radioactivities incorporated into DNA from [^{3}H]dATP (Amersham) which was present in the reaction mixture. Approx. 50% of the total thymine residues was replaced by biotinylated uracil. Radioisotope-labeled probes were prepared in a similar way by labeling the DNA with [^{3}H]dATP or [α-^{32}P]dCTP (Amersham).

2.2. *Immobilization of DNA in microtiter wells*

Heat-denatured DNA (100 μl) dissolved in phosphate-buffered saline (PBS; 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2, 137 mM NaCl, 2.7 mM

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KCl) containing 0.1 M $MgCl_2$ (PBSM) was incubated overnight at room temperature in a microtiter well (MicroFluor, Dynatech). The solution was then removed by aspiration and the plate irradiated under UV lamp (Toshiba GL15) with a total intensity of 1.6 kJ/m^2 for securing the immobilization of DNA.

2.3. *Hybridization*

To microtiter wells in which DNA was immobilized, 200 μl prehybridization solution was added and the plate incubated at 65°C for 2 h. Hybridization was carried out overnight at 65°C in 200 μl hybridization solution (4 vols each of 10 × SSC, 8 × Denhardt's solution, and 2 vols of 50% dextran sulfate, containing 50 μg/ml of sonicated salmon sperm DNA and 20 ng/ml of biotin-labeled λ DNA). After hybridization each well was washed with 2 × SSC at 65°C for 30 min. Since as little as 5 pg of specific DNA could be quantified for 1–50 ng/ml of biotinylated DNA probe, we used 20 ng/ml of the probe in the following experiments.

2.4. *Preparation of avidin-β-galactosidase complex*

Biotinylated β-galactosidase and avidin (avidin DN) were products of Vector Laboratories. They were dissolved in PBS containing 1% bovine serum albumin (BSA, Sigma) at a concentration of 100 μg/ml. Fifty μl each of the solutions were mixed and after incubation for 1 h at 4°C, avidin-biotinylated β-galactosidase (ABG complex) was separated from free avidin by column chromatography on Sephadex G-200 (1 × 35 cm). Complete removal of free avidin from ABG complex is essential in quantifying picogram levels of specific DNA.

2.5. *Reaction of biotin-labeled DNA probe with ABG complex*

The microtiter well was washed with PBS containing 2% BSA and 0.1% Triton X-100 (Packard) at room temperature for 10 min. ABG complex (0.5 units β-galactosidase equivalent) was added and incubated at 37°C for 30 min. After incubation, the well was washed 3 times with the same solution and once with PBS.

2.6. *Assay of β-galactosidase activity*

To the well, 50 μl of buffer A (10 mM Na-phosphate, pH 7.0, 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% BSA, 0.1% NaN_3) containing 3×10^{-4} M 4-methylumbelliferyl-β-D-galactoside (4-MUF-β-Gal, Sigma) was added and the plates incubated at 30°C for 120 min. The reaction was terminated by adding 200 μl of 0.1 M glycine-NaOH, pH 10.3. The fluorescence intensity of the 4-MUF released was measured with a MicroFluor Reader (Dynatech) at a wavelength of 360 nm for excitation and 450 nm for emission. One unit of enzyme activity was defined as that which hydrolyzes 1 μmol 4-MUF-β-Gal per min under the conditions described.

3. RESULTS AND DISCUSSION

The procedure for quantification of specific DNA by the non-radioisotopic method described in this study includes 4 steps:

Step 1: Immobilization of DNA to microtiter wells.

Step 2: Hybridization of the DNA with biotinylated DNA probe.

Step 3: Reaction of the probe with ABG complex.

Step 4: Assay of β-galactosidase activity.

3.1. *Immobilization of DNA*

While almost no detectable amount of DNA was associated with the wells after incubation with DNA in PBS, addition of $MgCl_2$ to PBS enhanced the association of DNA to the wells dramatically, reaching a plateau at 0.1 M $MgCl_2$ (fig.1A). Although Ca^{2+} was found to be as effective as Mg^{2+}, phosphate or NaCl in PBS has little effect on the association. The effect of incubation time on DNA binding to the well is shown in fig.1B, requiring approx. 16 h to reach a plateau. Under these conditions (16 h incubation in PBSM), the amount of immobilized DNA was proportional to the DNA added up to at least 1 ng per well and approx. 80% of the DNA added was retained in the wells (fig.1C).

3.2. *Sensitivity of the procedure*

As shown in fig.2, using β-galactosidase as a detector for biotinylated DNA probe, we could quantify specific sequences in a DNA preparation

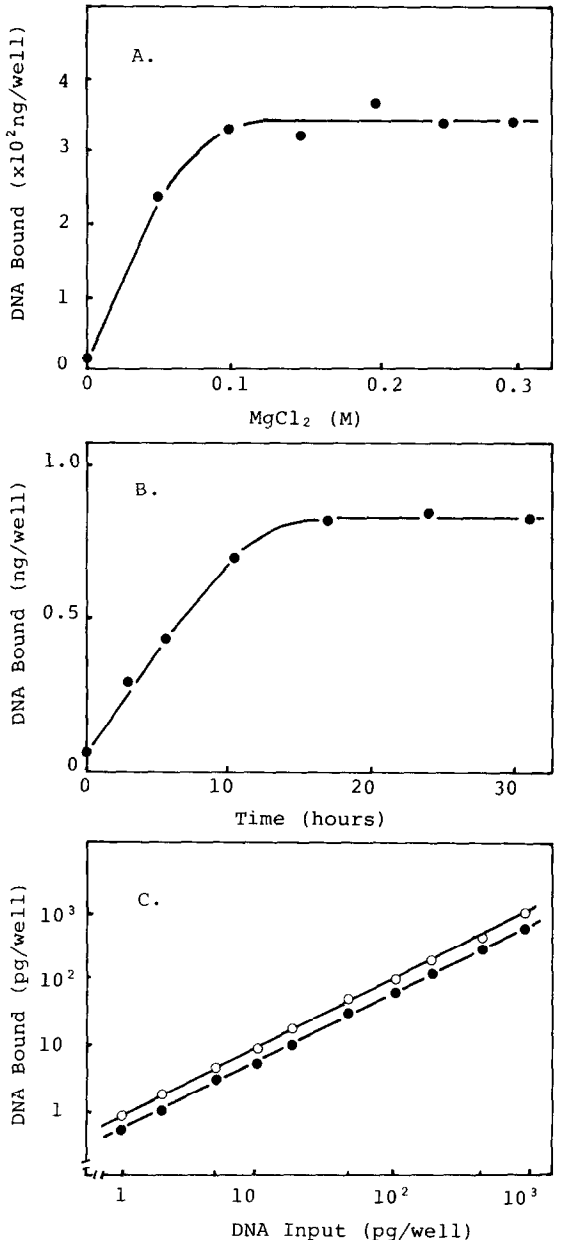

Fig.1. Immobilization of DNA in microtiter wells. (A) Five μg [^{3}H]DNA (salmon sperm, 10^3 cpm/μg) in PBS was incubated with various concentrations of $MgCl_2$ for 16 h at 20°C. The wells were washed with PBSM. Bound DNA was extracted with 0.2 N NaOH and assayed by counting in a scintillation counter (Beckman LS 7500). (B) One ng ^{32}P-labeled DNA (pBR322, 10^5 cpm/ng) in PBSM was incubated at 20°C for various periods of time. (C) Various amounts of ^{32}P-labeled DNA (pBR322, 10^5 cpm/ng) in PBSM were incubated for 16 h at 20°C. In (B) and (C) the wells were washed, cut into pieces and counted (●). In (C), (○) refers to added DNA, i.e., no wash control.

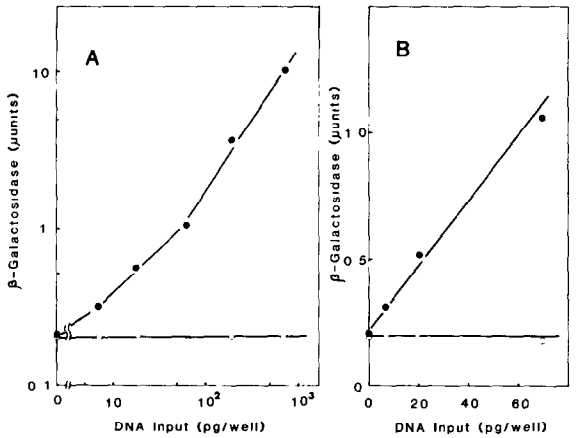

Fig.2. Quantification of λ DNA immobilized in microtiter wells. Various amounts of DNA [λ (●) or pBR322 (○)] in PBSM were incubated for 24 h at 20°C. The wells were processed for hybridization with biotinylated λ DNA (20 ng/ml), reaction with ABG complex and assay of β-galactosidase as described in the text. (A) 0–1 ng on a log scale. (B) 0–70 pg on a linear scale.

from as little as a few picograms to nanograms (fig.2A). The assay was specific, since no β-galactosidase activity was detected when pBR322 DNA was employed in place of λ DNA. The few picograms of specific DNA, which can be quantified by this procedure (fig.2B), are roughly equivalent to the amount of DNA of an average-sized gene present in 10^6 mammalian cells.

In general when either ^{32}P-labeled DNA probes or biotin-labeled probes coupled with horseradish peroxidase or alkaline phosphatase avidin complex are used [3,4], detection and quantification of specific DNA are carried out by DNA-DNA hybridization with DNA immobilized on nitrocellulose filters. In either case, since detection and quantification were achieved by visualization of the signals originating from the probes on the filters, these methods are considered to be semi-quantitative. Utilization of DNA-immobilized plastic wells and β-galactosidase as a detector in our procedure has made it possible to quantitate specific DNA and to handle a great number of samples at the same time, thus leading to an automated assay procedure for specific DNA. This may be of great importance for the future application of this method to determination of pathogens in infectious diseases or diagnosis of genetically disordered patients.

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