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Abstract

Non-radioactive hybridization probes were prepared using the M13 phage vector and the universal sequencing primer. The probe sequence to be used was first cloned into the M13 vector, and the minus strand of the template DNA was then synthesized with the Klenow fragment of *E. coli* DNA polymerase I in the presence of the biotinylated nucleotide, biotin-11-dUTP, as a label. Resultant DNA was heavily biotinylated, and made up of the entire minus strand of the template DNA. The long tag sequence derived from the M13 vector may increase the sensitivity of the detection. The biotinylated hybrids were visualized with the streptavidin-alkaline phosphatase conjugate and chromogenic substrates. As shown by Southern hybridization, the probe prepared in this way could be used to detect less than 1 pg of target sequence and a single copy gene sequence in human genomic DNA within several hours of signal development.

KEYWORDS: non-radioactive probe, biotin nucleotide, M13 phage DNA, universal sequencing primer, Southern hybridization

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Non-Radioactive Hybridization Probes Prepared Using M13 Phage Vector and the Universal Sequencing Primer

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Non-radioactive hybridization probes were prepared using the M13 phage vector and the universal sequencing primer. The probe sequence to be used was first cloned into the M13 vector, and the minus strand of the template DNA was then synthesized with the Klenow fragment of *E. coli* DNA polymerase I in the presence of the biotinylated nucleotide, biotin-11-dUTP, as a label. Resultant DNA was heavily biotinylated, and made up of the entire minus strand of the template DNA. The long tag sequence derived from the M13 vector may increase the sensitivity of the detection. The biotinylated hybrids were visualized with the streptavidin-alkaline phosphatase conjugate and chromogenic substrates. As shown by Southern hybridization, the probe prepared in this way could be used to detect less than 1 pg of target sequence and a single copy gene sequence in human genomic DNA within several hours of signal development.

Key words : non-radioactive probe, biotin nucleotide, M13 phage DNA, universal sequencing primer, Southern hybridization

Nucleic acid hybridization is a powerful and extensively used technique for the determination of genetic structure. The application of hybridization technology to the clinical diagnosis of hereditary and infectious diseases and to the discrimination of individuals in forensic medicine is increasing.

Hybridization probes are commonly labeled with the radioisotope ³²P, which is a sensitive marker. However, non-radioactive

probes have recently been developed to avoid the cumbersome procedures and short half-lives associated with radioactive probes (1-6). One of the more successful labeling methods uses biotin as the marker (1, 2, 4). These probes are usually prepared by replacing nucleotides with biotinylated derivatives by nick translation (1, 2). Chemical labeling methods for introducing biotin onto DNA have also been developed (4, 7). After hybridization and washing, the biotin is detected colorimetrically using enzyme-linked avidin or anti-biotin antibody.

Although non-radioactive probes have many

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advantages, they are not as sensitive as radioactive probes. In the present study, we describe a simple method for the preparation of a sensitive biotinylated probe using the M13 phage vector and the universal sequencing primer. The probes provide enough sensitivity for the detection of 1 pg of target DNA and a single copy gene sequence in human genomic DNA by Southern hybridization.

Materials and Methods

Materials. M13mp18 single strand DNA and 17-mer universal M13 sequencing primer (5'-GTA-AAACGACGGCCAGT-3') were purchased from United States Biochemical Co., Cleveland, OH, USA. The Klenow fragment of *E. coli* DNA polymerase I and restriction endonucleases were purchased from Toyobo Co., Ltd., Osaka, Japan and New England Bio Labs, Beverly, MA, USA. Nylon membrane (Hybond-N) was obtained from Amersham Corp., Arlington Heights, IL, USA. Biotin-11-dUTP (0.4 mM solution) and the non-radioactive nucleic acid detection system (BluGENE) were purchased from Bethesda Research Laboratories, Gaithersburg, MD, USA. Single strand DNA (mpRaf) was prepared by subcloning a 1.6 kb human *c-raf-1* cDNA fragment (8) containing exons 14 to 17 between the *EcoRI* and *HindIII* site of the mp18 vector. Most DNA manipulations were carried out as described in standard texts (9, 10).

DNA labeling. Single strand DNA template (5 μ g), 2 pmol of primer, and 10 μ l of 10 \times polymerase reaction buffer (70 mM Tris-HCl, pH 7.5, 70 mM MgCl₂, 200 mM NaCl) were mixed, and the final volume was adjusted to 72.5 μ l with distilled water. The mixture was incubated at 60°C for 20 min, and then cooled slowly at room temperature for more than 30 min. After the annealing, 10 μ l of nucleotide solution (0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP, 0.1 mg/ml bovine serum albumin (BSA) and 20 mM dithiothreitol), 12.5 μ l of 0.4 mM biotin-11-dUTP, and 10 units of Klenow fragment were added to the mixture, and then incubated at 18°C for 16 h. The reaction was stopped by heating at 65°C for 10 min.

The biotinylated DNA was separated from unincorporated biotin nucleotides by electrophoresis

with 0.6% low-melting-temperature agarose (Nippon Gene Co., Ltd., Toyama, Japan) in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA) (9). The gel slice containing the labeled DNA was soaked in cold deionized water 3 times for 1 h to remove the electrophoresis buffer. The gel was then melted at 65°C, diluted 5-fold with TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA), and stored at -20°C until needed. Just before the hybridization, the probe DNA was denatured by boiling for 5 min and cooling quickly.

DNA blot and hybridization. DNA samples digested with restriction endonuclease were subjected to agarose gel electrophoresis, and blotted on nylon membranes in alkaline solution according to the method of Reed and Mann (11).

The filters were incubated at 42°C for 4 h in hybridization buffer consisting of 50% deionized formamide, 4 \times SSC (1 \times SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 10 \times Denhardt's solution (0.2% BSA, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone 360), 0.5% sodium dodecyl sulfate (SDS), and 0.3 mg/ml denatured salmon sperm DNA. Hybridization was carried out overnight at 42°C in the hybridization buffer containing

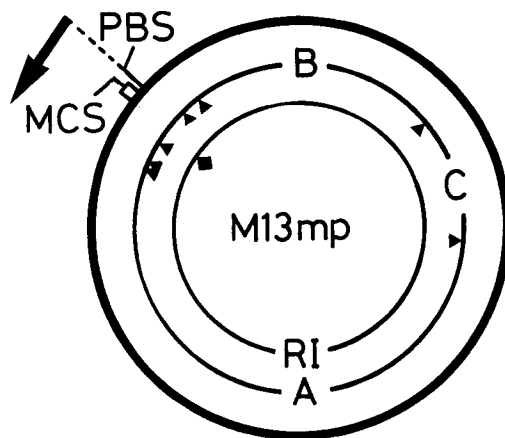


Fig. 1 Restriction map of M13 mp series. The cleavage sites of the enzymes, *EcoRI* (◆) and *BstNI* (▼), in M13 replication form DNA are shown in the inner circles (12). RI, A, B, and C indicate the fragments generated by the digestion of M13 replication form DNA with the enzymes. The probe sequences can be cloned into the MCS (multiple cloning sites). PBS (primer binding site) is a sequence complementary to the universal sequencing primer. The arrow indicates the direction of DNA synthesis.

50 to 100 $\mu\text{g}/\text{ml}$ of denatured probe. Following the hybridization, the filters were washed twice for 10 min in $2\times\text{SSC}$, 0.1% SDS, and six times for 15 min in $0.1\times\text{SSC}$, 0.1% SDS. All wash steps were performed at 50°C . The filters were then blocked with 3% BSA in 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl at 65°C for 1 h.

Detection of biotinylated DNA. Colorimetric DNA detection system was performed according to the BRL kit instructions, except that buffer containing 0.1 M Tris-HCl (pH 7.5), 1 M NaCl, 2 mM MgCl_2 , and 0.05% Triton X-100 was used for the dilution of streptavidin-alkaline phosphatase (SA-AP) conjugate and the washing of excess conjugate. Briefly, the blocked filters were incubated with SA-AP conjugate, washed, and visualized

with a dye solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Color development was allowed to proceed for 1 to 3 h, and stopped by washing in TE.

Results and Discussion

Our strategy to improve the sensitivity of non-radioactive hybridization was to prepare heavily biotinylated probe DNA with a long non-specific tag sequence. The DNA which is to be used as the probe is first cloned into the M13 vector mp18 (Fig. 1). The 17-base sequence of the universal prim-

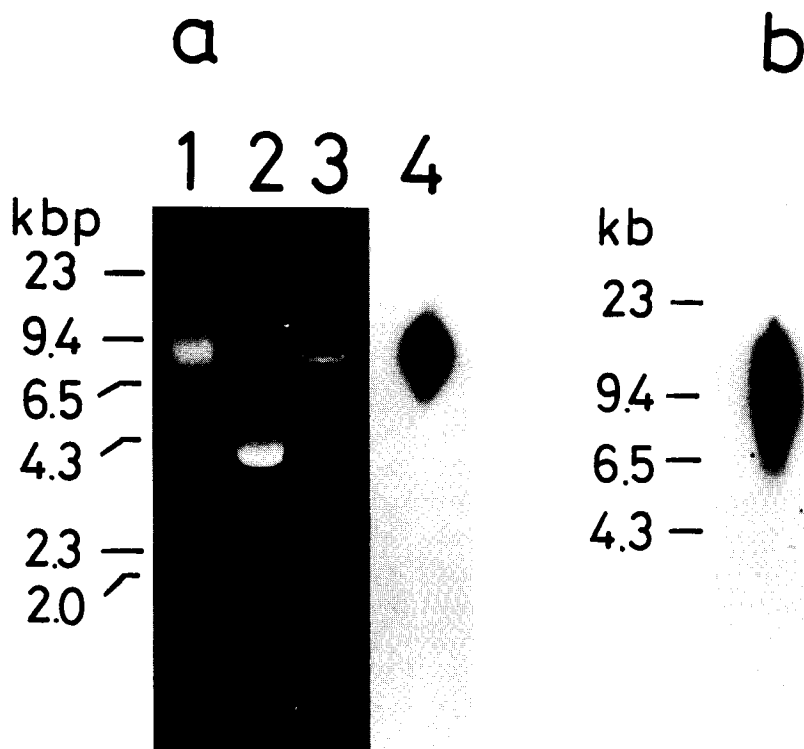


Fig. 2 Preparation of biotinylated probes. The biotinylated probe was synthesized with the template DNA of mpRaf as described in Materials and Methods. Five microcuries of $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ (3000 Ci/mmol, ICN Radiochemicals, Irvine, CA, USA) was added to the reaction mixture to detect newly synthesized DNA by autoradiography. (a) After stopping the reaction, $5\ \mu\text{l}$ of the sample was loaded on 0.7% agarose gel prepared with TBE buffer (lane 1). Single strand mpRaf DNA (lane 2) and mpRaf RF DNA digested with *EcoRI* (lane 3) was also loaded on the same gel. After electrophoresis, the DNAs were stained with ethidium bromide, and the photograph was taken (lanes 1 to 3). The gel was then dried, and exposed on Fuji RX film with intensifying screens. Lane 4 shows the autoradiogram of lane 1. (b) A $5\text{-}\mu\text{l}$ aliquot of the reaction mixture was subjected to alkaline agarose gel (0.7%) electrophoresis (8). An autoradiogram of the gel was taken as in (a).

er is complementary to the 3' side of the multiple cloning sites of the M13 vector (13) and is used to initiate synthesis of the minus strand from the plus strand template by the Klenow fragment. The newly synthesized DNA, which has the probe sequence at its 5' region and the tag sequence derived from the M13 vector at the 3' region, is labeled by incorporation of the biotinylated nucleotide. The long tag sequence may increase the sensitivity of the detection of the target sequence. The conditions for making probe DNA by this method were those of standard techniques for DNA sequencing (10), although the nucleotides were suitably modified for the long elongation polymerase reaction. No dTTP was included in the reaction mixture.

We prepared the biotinylated probe using a test template DNA, mpRaf, which contains the anti-sense strand of human *c-raf-1* cDNA (Fig. 2). The resultant DNA migrated slower than the template DNA in neutral agarose gel, and its mobility was almost the same as that of linear mpRaf replication form (RF) DNA. The length of the synthesized strand estimated by alkaline agarose gel electrophoresis was about 9 kilobases on the average. These results indicate that the probe was made up of the entire minus strand of the template. To evaluate the labeling efficiency, serially diluted probe DNA was blotted and detected with the color development reaction (Fig. 3). One picogram of labeled DNA was visible within 1 h of development. This high level of labeling efficiency was not obtained with probes made by the usual nick translation reaction (data not shown).

The sensitivity of the probe was tested using mp18 RF DNA as the target sequence for Southern hybridization (Fig. 4). mp18 RF DNA was digested with *Eco*RI or *Bst*NI, serially diluted, and blotted on nylon membrane. The probe was made from mp18 template DNA without using an insert sequence.

1 2 3 4 5 6

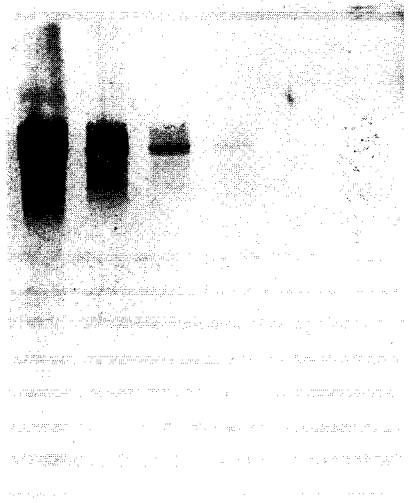


Fig. 3 Labeling efficiency of biotinylated probes. The probe DNA prepared as shown in Fig. 1 was serially diluted, electrophoresed on 0.7% agarose gel in TBE, and blotted on nylon membrane in $10\times$ SSC. The filter was baked at 80°C for 30 min and blocked with 3% BSA. Detection of biotinylated probes was carried out as described in Materials and Methods. The amount of biotinylated DNA loaded per lane was as follows: lane 1, 1 ng; lane 2, 100 pg; lane 3, 10 pg; lane 4, 1 pg; lane 5, 0.1 pg; lane 6, 0 pg.

Less than 1 pg of *Eco*RI-digested DNA was detected after only 1 h of signal development. The *Bst*NI B fragment, which is farthest from the priming site of the template in the 3' direction (Fig. 1), was also visible, indicating that the full length of the complementary strand to the template DNA was synthesized. A probe concentration of 50 to 100 $\mu\text{g}/\text{ml}$ in hybridization buffer gave maximal sensitivity with a low background.

We used the non-radioactive probe to detect a single copy gene sequence in human genomic DNA. *c-raf-1* is a human oncogene which has serine/threonine kinase activity (8). The hybridization results obtained with the biotinylated *c-raf-1* probe are shown in Fig. 5. A 5.9 kb *Pst*I fragment and a 3.0

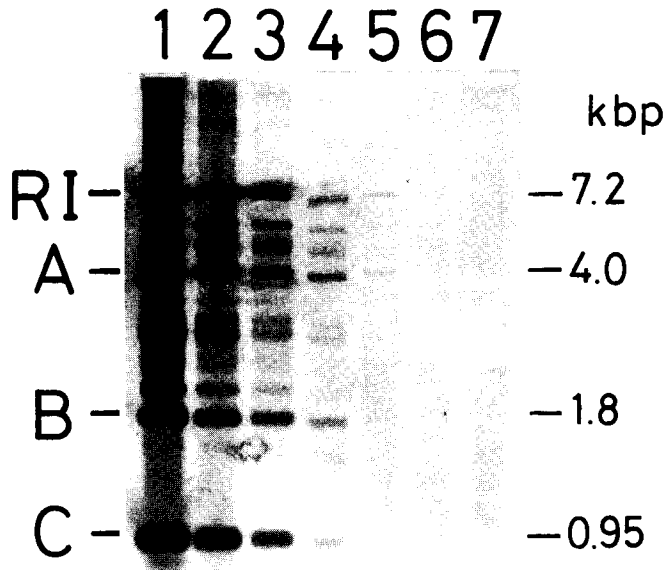


Fig. 4 The sensitivity of the biotinylated probes. mp18 RF DNA was digested with *Eco*RI or *Bst*NI, and the digestion products were mixed at a 1:1.8 ratio so that the mixture contained the same amount of *Eco*RI fragment and *Bst*NI A fragment. The mixture was serially diluted with TE buffer containing *Eco*RI-digested salmon sperm DNA, and subjected to 1.0% agarose gel electrophoresis. The DNAs blotted on nylon membrane were hybridized with the probe prepared using mp18 single strand DNA. Lanes 1 to 7 contain 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 0.1 pg, and 0 pg of the *Eco*RI fragment of mp18 RF DNA, respectively. RI, A, B, and C indicate *Eco*RI-digested mp18 RF DNA, A, B, and C fragments of the *Bst*NI-digested DNA, respectively, as shown in Fig. 1.

kb *Eco*RI fragment were detected as expected from the physical map of the human *c-raf-1* locus (8). The *c-raf-1* fragment could also be detected in genomic blots of only 1 μ g. This result shows that the sensitivity of the detection of target DNA with our probe in Southern blots was equivalent to that obtained with 32 P-labeled DNA probes. Recently it was discovered that the protein III gene in M13 can recognize a family of hypervariable minisatellites in vertebrates (14). However, these repetitive sequences were not detected under the conditions of our hybridization, *i. e.*, high stringency and high concentration of carrier DNA. The present method for the preparation of non-radioactive probes might be applicable to the detection of other single copy gene sequences in mammalian DNA.

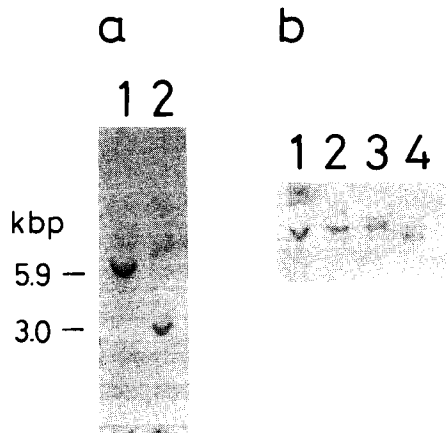


Fig. 5 Detection of the *c-raf-1* sequence in human genomic blots. (a) Normal human placental DNA (10 μ g) was digested with *Pst*II (lane 1) and *Eco*RI (lane 2), subjected to 0.7% agarose gel electrophoresis, and blotted on nylon membrane. The *c-raf-1* sequence was detected with the probe prepared using mpRaf template DNA. (b) Lanes 1 to 4 contain 10 μ g, 5 μ g, 2 μ g, and 1 μ g, respectively, of *Eco*RI-digested human placental DNA. The *c-raf-1* sequence was detected as in (a).

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