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INVESTIGATION ON THE NON-RADIOACTIVE LABELING WITH DIGOXIGENIN-11-2'-DEOXYURIDINE-5'-TRIPHOSPHATE FOR SOUTHERN BLOT ANALYSIS

(Southern blot analysis/non-radioactive labeling/digoxigenin-11-2'-deoxyuridine-5'-triphosphate)

Atsushi AKANE, Kazuo MATSUBARA, Hiroshi SHIONO, Hiroaki NAKAMURA, Masanori HASEGAWA, Masato KAGAWA and Setsunori TAKAHASHI

Department of Legal Medicine, Shimane Medical University, Izumo 693, Japan

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Southern blot analysis using probes labeled with digoxigenin-11-2'-deoxyuridine-5'-triphosphate (digoxigenin-11-dUTP) instead of ^{32}P-labeled deoxyribonucleotide was investigated. A molecular weight standard marker λ-HindIII should have been labeled with pure digoxigenin-11-dUTP instead of the DIG DNA labeling mixture containing both digoxigenin-11-dUTP and 2'-deoxythymidine-5'-triphosphate. For the labeling of probes, nick translation using pure digoxigenin-11-dUTP was more suitable than random primer extension using the labeling mixture. Furthermore, some modifications on stringency were discussed to identify hybridization signals of the target genes more clearly.

Southern blotting is a commonly applied technique for DNA analysis, by which the target genes are detected as hybridization signals using labeled DNA probes specific to them (1). The probes are usually labeled with a radioisotope ^{32}P, and the signals are

Abbreviations: digoxigenin-11-dUTP, digoxigenin-11-2'-deoxyuridine-5'-triphosphate; dATP, 2'-deoxyadenosine-5'-triphosphate; dCTP, 2'-deoxycytidine-5'-triphosphate; dGTP, 2'-deoxyguanosine-5'-triphosphate; dTTP, 2'-deoxythymidine-5'-triphosphate.

detected on X-ray films by autoradiography (2). Recently, several methods for non-radioactive labeling of probes are established (3-5). By these new techniques, hybridization signals are detected either on sample filters directly by color reaction between alkaline phosphatase and nitroblue tetrazolium (NBT) (3, 4) or on X-ray films by chemiluminescence reaction (5). These non-radioactive methods are less dangerous than radioactive ones. However, they are also less sensitive. Then, the authors investigated a DIG-ELISA method for sufficiently sensitive non-radioactive Southern blot analysis. By this method, the probes were labeled with digoxigenin-11-dUTP and the signals are detected using ELISA and the color reaction.

MATERIALS AND METHODS

1. Sample preparation

DNA samples were prepared from peripheral blood leukocyte as described previously (6). These samples were digested with appropriate restriction enzymes, electrophoresed in 0.7% agarose gel, and then transferred to nylon membrane filter (Sartorius, Göttingen, Germany) by the Southern blotting method.

2. Labeling of a molecular weight standard marker

λ-HindIII digests [bacteriophage λ cI 857 Sam7 DNA (Takara, Kyoto, Japan) digested with a restriction endonuclease HindIII (Toyobo, Osaka, Japan)] are frequently used as a molecular weight standard marker for Southern blot analysis. Its fragments were end-labeled by Klenow enzyme (Toyobo) (2) either with DIG DNA labeling mixture (Boehringer Mannheim, Mannheim, Germany) containing 2 nmol each of dATP, dCTP, dGTP and digoxigenin-11-dUTP/dTTP (35:100) or with 2 nmol of dNTP solution (dATP, dCTP and dGTP) and 2 nmol of pure digoxigenin-11-dUTP (Boehringer Mannheim). Thus labeled standard marker was electrophoresed and transferred in the same way as digested DNA samples.

3. Detection of target DNA according to the supplier's instruction

Labeling of probes: 1 μg probe was labeled by random primer extension (2) with DIG DNA labeling mixture. After purification by rapid gel filtration, labeled probe was boiled and placed into hybridization buffer.

Hybridization: The sample filter (14×12 cm^2) was incubated with 25 ml prehybridization buffer containing 5×SSC (20×SSC is 3 M NaCl, 0.3 M Na-citrate, pH 7.0), 0.1% (w/v) N-lauroyl-sarcosine, 0.02% (w/v) sodium dodecyl sulfate (SDS) and 1% (w/v) blocking reagent (Boehringer Mannheim) at 65°C for 2-3 h. Then, the hybridization was performed in 10 ml hybridization buffer containing 5×SSC, 50% (v/v) deionized formamide, 0.1% N-lauroyl-sarcosine, 0.02% SDS and 5% blocking reagent at 42°C overnight.

Wash: The filter was washed twice with 100 ml Wash II solution (2×SSC, 0.1% SDS) at room temperature for 5 min and twice with 1 l Wash III solution (0.1×SSC, 0.1% SDS) at 65°C for 15 min.

Detection (performed at room temperature): Detection of the hybridization signals were based on ELISA and the color reaction (Fig. 1). After washing with 100 ml buffer-1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 min, the filter was incubated with 100 ml buffer-2 (0.5% blocking reagent in buffer-1) for 30 min. After washing with buffer-1 for 1 min again, the filter was incubated with 10 ml buffer-1 containing 1.5 U anti-digoxigenin-alkaline phosphatase conjugate for 30 min. After washing twice with buffer-1 for 15 min, the filter was equilibrated with 100 ml buffer-3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 2 min. Then, hybridization signals were stained in 10 ml buffer-3 containing 3.375 mg NBT and 1.75 mg 5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt for 1-16 h. Then color reaction was terminated by incubation of the filter with buffer-4 (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

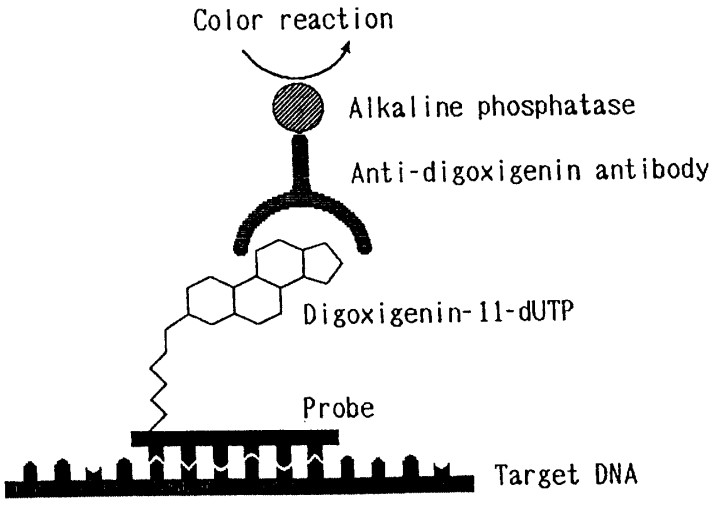

Fig. 1 Non-radioactive detection based on the digoxigenin-anti digoxigenin ELISA.

4. Modified procedures for detection of hybridization signals

Labeling of probes: 1 µg probe was labeled by nick translation (2) in the reaction mixture containing 2 nmol of dNTP solution (dATP, dCTP and dGTP), 2 nmol of pure digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany) and 5 µl enzyme solution (1 unit/µl DNA polymerase I and 40 pg/µl DNase I, Biotin-21-dUTP Nick Translation Labeling Kit, Clontech, Palo Alto, CA, USA).

Wash: The filter was washed with Wash I (2×SSC, 0.5% SDS) and Wash II for 10 min at room temperature, and then twice with Wash III at 50°C for 1 h.

Hybridization and detection procedures were the same as described above.

RESULTS

1. Labeling of a molecular weight standard marker

When λ-HindIII digests were labeled with the labeling mixture containing both digoxigenin-11-dUTP and dTTP, signals for 9.4, 6.6, 2.3 and 2.0 kb fragments were less or not visible whereas only 23 and 4.4 kb bands were detectable (Fig. 2). On the contrary, all fragments were clearly identified when the marker was labeled with pure digoxigenin-11-dUTP.

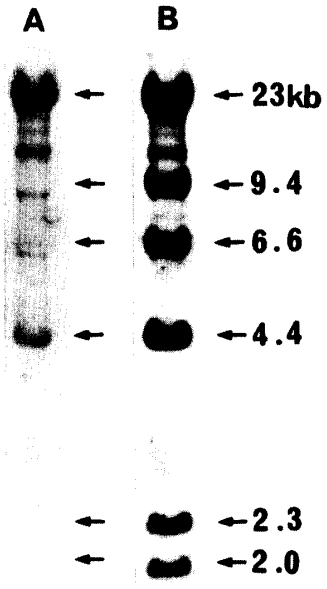

Fig. 2 Detected fragments of λ-HindIII digests labeled with DIG DNA labeling mixture (A) and pure digoxigenin-11-dUTP (B). Numbers indicate the fragment length (kb).

2. Labeling of probes

When any probe was labeled by random primer extension using digoxigenin-11-dUTP as described above, the hybridization signals were sometimes too unclear to identify the target gene under the same experimental conditions. Even by nick translation, any probe was insufficiently labeled using DIG DNA labeling mixture. Labeling by nick translation using pure digoxigenin-11-dUTP could make the signals sufficiently clear. Figure 3 shows an example of restriction fragment length polymorphism (RFLP) analyses in two cases of parentage testing. The probe used was pYNH24 for a variable number of tandem repeat locus (6), which was established by Nakamura et al. (7), and was supplied from Japanese Cancer Research Resources Bank. DNA samples were digested with MspI. As shown in this example, the non-radioactive signals (Fig. 3, right) are, usually, less intense than radioactive ones (Fig. 3, left), but were proven to be sufficiently clear for the testing. However, shorter fragments seemed to be less clear by non-radioactive detection.

3. Stringency

When sample filters were washed with Wash III at 65°C for 15

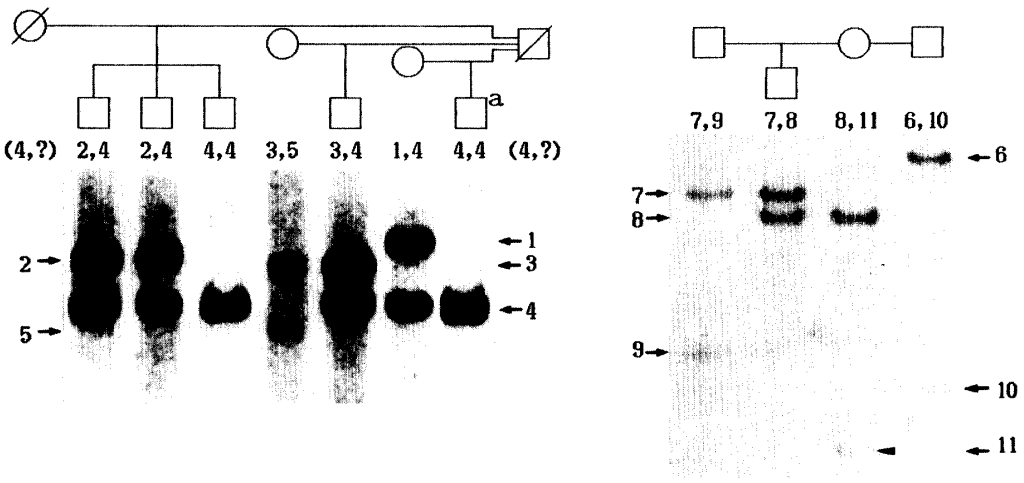

Fig. 3 Comparison between radioactive (left) and non-radioactive labeling (right). Cases of parentage testing are shown (probe: pYNH24, restriction enzyme: MspI, radioactivity of the probe: 2,000,000 cpm, autoradiography: 2 days, see Ref. 1). Alleles are arbitrary numbered in this paper.

min, hybridization signals of some probes such as cDNA clones could not be identified. When washed at 50°C for 15 min, signals became clearer but the background was also strengthened. When filters were washed with more stringent Wash I for 10 min and Wash II for 10 min at room temperature, and subsequently twice with Wash III at 50°C for 1 h, signals were enhanced with the diminishment of background, as shown in Fig. 3 (right).

DISCUSSION

According to the supplier's instruction, the labeling efficiency by random primer extension is the highest when DIG DNA labeling mixture containing digoxigenin-11-dUTP/dTTP (35:100) is used. However, fragments of λ-HindIII digests were not labeled by primer extension with the mixture (Fig. 2). In a 5'-stalk end (3'-TCGA-5') of a DNA fragment produced by HindIII, one molecules each of dATP, dGTP, dCTP and dTTP (dUTP) are taken by Klenow enzyme: One digoxigenin-11-dUTP molecule can be incorporated at an end. Because the labeling mixture contained more dTTP than digoxigenin-11-dUTP, dTTP might be taken in the end more easily than digoxigenin-11-dUTP, so that 9.4, 6.6, 2.3 and 2.0 kb fragments were undetectable. On the other hand, 23 and 4.4 kb fragments were labeled with the labeling mixture (Fig. 2). These fragments include left- and right-hand ends of the original λDNA, respectively. Thus, each of these fragments has a 5'-stalk end and a single-stranded terminus 12 nucleotides in length (cohesive terminus) (8). It might be possible to take digoxigenin-11-dUTP in this cohesive terminus from the labeling mixture.

Random primer extension is known to have higher labeling efficiency than nick translation (2). By the former, digoxigenin-11-dUTP is taken by Klenow enzyme in denatured template DNA, following the annealing of hexanucleotide primers [$dp(N)_6$]. However, random primer extension seems unsuitable for labeling of some types of probes. For example, a pHY10 probe for DYZ1 locus can not be labeled by random primer extension, since the locus consists of a tandem array of pentanucleotides (9), which is hardly annealed with $dp(N)_6$. Furthermore, even when the same probe DNA was used, linear fragment DNA seemed to be less labeled than circular plasmid DNA by the reaction in our experience.

Nick translation is suitable for labeling of various types of

probes. By this reaction, pre-existing nucleotides in probe DNA nicked by DNase I are replaced with labeled molecules by DNA polymerase I (2). However, it is difficult to regulate DNase I activity by dilution before the use. If the activity was too high, the probe DNA would be digested to short and less specific fragments. Less specific probes would hybridize not only to the target genes but also to the other sequences of sample DNA, and the hybridization signals would be detected as enhanced background. In our laboratory, the mixture of DNA polymerase I and DNase I in Nick Translation Labeling Kit is thus used, which needs no further preparation before the use. Although nick translation is usually performed at 15-18°C (2), this enzyme mixture can label probes at room temperature.

When the filter was washed with Wash III at 65°C, not only background but also weakly-hybridized probe were faded away. For example, the cDNA probe may hybridize weakly to the target gene; cDNA generally consists of short sequences of the target gene's exons and does not include relatively longer sequences of the introns. That is to say, the cDNA probe is hybridizing based on the weak complementarity of short and separated sequences of the exons. To maintain weak hybridization of such probes, the temperature of filter washing should have been decreased to 50°C. To avoid background hybridization, however, sample filters were washed for much longer time (1 h). Furthermore, instead of washing twice with Wash II for 5 min, filters were washed with Wash I for 10 min, and subsequently with Wash II for 10 min. Wash I is more stringent than Wash II, eliminating the background. With these modifications, hybridization signals of target genes could be clearly identified.

Detection of radioisotopes is highly sensitive so as to be applied to many and various analytical techniques. However, radiation is also known to damage live tissues. If sufficiently sensitive, non-radioactive analyses are better than radioactive ones. Non-radioactive labeling of DNA probes was first established based on the biotin-avidin coupling reaction. Following hybridization, biotinylated DNA probe was identified with streptavidin-alkaline phosphatase conjugate, and the enzyme catalyzed <u>in situ</u> the color reaction of NBT. However, the sensitivity of this method appeared to be very low. Although repetitive genes such as pHY10 and Alu sequences were reported to be identified (3, 4), no single copy gene is detectable using biotinylated probes. On the other hand, detection of

hybridization signals by digoxigenin-11-dUTP labeling method based on ELISA (Fig. 1) was sufficiently sensitive for any single copy gene, although some modifications were required as described in this paper. To increase the sensitivity, chemiluminescence reaction instead of color reaction should be applied to the detection (5).

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