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Preparation of Sensitive and Specific Oligonucleotide Probes Tailed Using Terminal Transferase and dITP

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ABSTRACT

An oligonucleotide probe tailed with deoxyadenosine-5'-triphosphate or deoxythymine-5'-triphosphate is detectable with high sensitivity, but has a major drawback—the tail co-hybridizes specifically to complementary sequences. This can be a problem when screening cDNA clones that contain poly(dA) sequences. While it is possible to mask the cDNA tail with unlabeled poly(dA) or poly(A) oligonucleotides, false-positive clones are still produced because complete masking of extremely long (dA) tails is difficult. As a result, only cDNA clones that have extremely long poly(dA) sequences are often obtained by hybridization screening using tailed probes. In this report, we describe an oligonucleotide probe tailed with DIG-labeled nucleotide in combination with deoxyinosine-5'-triphosphate that was highly specific and sensitive to cDNAs. Terminal deoxynucleotidyl transferase efficiently adds dI nucleotides to the 3'-end. The dI of the tails did not pair with any nucleotides under stringent hybridization so that the specificity of hybridization assays remained high without affecting the sensitivity of the test. Colony hybridization experiments demonstrated that there were very few (1 of 80 tested) false positives using this technique. Its use may increase the accuracy of cDNA screening.

INTRODUCTION

Labeling of oligonucleotides is important for screening gene libraries using hybridization methods (10). A variety of nonisotopic labeling reactions are used for introducing a labeling molecule to a 3'- or 5'-end (3,4,7), some internal nucleotides (12) or the 3'-tail (5). Specificity to the oligonucleotide sequence is high when using end labeling; however, the sensitivity is low with

nonisotopic labeling. The highest sensitivity is obtained by tailing, using DIG-labeled nucleotide in combination with deoxyadenosine-5'-triphosphate (dATP) as substrates of terminal deoxynucleotidyl transferase (TdT); however, the specificity is often reduced due to the added tail. This is especially true in screening cDNA clones: the homopolymeric dA tail hybridizes to dA/dT regions in the cDNA clones derived from poly(A) sequences of eukaryotic mRNA (2) even if the regions in the cDNA are masked by the addition of unlabeled poly(dA) oligonucleotides. Thus, there has been no nonisotopic method of labeling an oligonucleotide probe for sensitive and specific cDNA screening using hybridization. In this study, we used deoxyinosine-5'-triphosphate (dITP) in combination with DIG-deoxyuridine-5'-triphosphate (DIG-dUTP) as the substrate for TdT tailing. The efficiency of labeling and the specificity of hybridization of the probe were evaluated in comparison with probes tailed with dATP in combination with DIG-dUT

MATERIALS AND METHODS

Materials

dITP was purchased from Sigma (St. Louis, MO, USA). Positively charged nylon membranes were purchased from Roche Molecular Biochemicals (Mannheim, Germany). A synthetic oligonucleotide (5'-CCCTCAAAGAAATGGAGAGCCT-3') to detect the testis-enhanced gene transcript (*TEGT*) gene was designed using an HYBsimulator™ (Advanced Gene Computing Technologies, Irvine, CA, USA). For the colony hybridization experiment, 275 types of synthetic oligonucleotides were designed. Synthetic oligonucleotides were purchased from Life Technologies (Gaithersburg, MD, USA). Plasmid DNA containing cDNA clones was obtained from a cDNA library (11).

Tailing of Oligonucleotide Using TdT

Oligonucleotide probes were tailed with DIG-labeled nucleotide using TdT

from calf thymus (both from Roche Molecular Biochemicals). Each oligonucleotide (100 pmol) was incubated in TdT reaction buffer (0.2 M potassium cacodylate, 25 mM Tris-HCl, pH 6.6, 0.25 mg/mL bovine serum albumin, 5 mM CoCl₂) with 0.05 mM DIG-dUTP and 2.5 U/μL TdT in the presence of 0.1 or 0.5 mM dITP or dATP for 15 min at 37°C. The reaction was stopped

with the addition of 10 μg/mL glycogen and 0.2 mM EDTA. The tailed probe was precipitated by ethanol and dissolved in 100 μL water. It was applied to 15% (wt/vol) polyacrylamide gels with the buffer containing 25 mM Tris and 192 mM glycine (pH 7.9) to determine the tail length. Gels were silver stained using a kit from Daiichi Pure Chemicals (Tokyo, Japan).

Figure 1. Hybridization of cDNA clones to the probe tailed with dITP and DIG-dUTP. Plasmids bound to a nylon membrane were hybridized with probes tailed with dITP and DIG-dUTP or dATP and DIG-dUTP. Plasmids containing cDNAs encoding *TEGT* (rows 1, 11), human cysteine protease CMH-1 (row 2), human chaperonin containing the t-complex polypeptide 1 eta subunit (row 3), the human ATP synthase alpha subunit (row 4), human alpha enolase (row 5), expressed sequence tag (EST) clone of GenBank® Accession No. AA888820 (row 6), EST clone of Accession No. AA062589 (row 7), translocated in liposarcoma (row 8), EST clone of Accession No. N54208 (row 9), and human initiation factor 4B (row 10) with various lengths of poly(dA) polynucleotide sequences were spotted on a membrane. Panels (a) and (b): hybridization with probe that was tailed in a reaction solution containing a 2/1 ratio of dITP/DIG-dUTP. Panels (c) and (d): hybridization with probe that was tailed in a reaction solution containing a 10/1 ratio of dITP/DIG-dUTP. Membranes were hybridized with a probe specific for the *TEGT* gene (panels a and c) or a vector plasmid probe (panels b and d).

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Table 1. Colony Hybridization Specificity of Oligodeoxynucleotides Tailed with dITP and DIG-dUTP

	Total Clone Number	Sequencing Success Rate	Identity of Sequenced Clone to the Target Gene of Probe by BLASTn		Probe Sequence Within the Identical Clone	
			Identical	Not Identical	Including	Not Including
Positive clones	10	9/10	8	1	8	0
Negative clones	70	58/70	3	55	0	3

Hybridization

Plasmids were denatured for 5 min at 96°C, spotted onto nylon membranes and cross-linked using a Stratalinker® UV cross-linker (Stratagene, La Jolla, CA, USA). Membranes were prehybridized in hybridization solution [6× standard saline citrate (SSC), 1% (wt/vol) blocking reagent (Roche Molecular Biochemicals), 0.1% (wt/vol) *N*-lauroylsarkosine, 0.02% (wt/vol) SDS] with or without 0.1 mg/mL poly(A) and 5 mg/mL poly(dA) solution for 3 h at 68°C, followed by hybridization with 5

pmol/mL tailed probe in hybridization solution for 12 h at 60°C. The membranes were washed 4× with 6× SSC, 0.1% (wt/vol) SDS for 15 min at 60°C. Hybridized probes were detected using a DIG luminescence detection system.

Preparation of High-Density Colony Filters

High-density filters (HDF) were prepared using a Biomek 2000® robotics workstation with a 384-pin tool (Beckman Coulter, Fullerton, CA, USA). Transformants cultured in nine 384-well plates were spotted onto a nylon membrane filter (11 × 7.5 cm) on LB medium in a 3 × 3 array and incubated at 37°C for 24 h. HDF were then treated as described (8) for subsequent hybridization with probes tailed with dITP and DIG-dUTP.

Sequencing

Plasmids appearing in the first row, first column of each array were prepared from the original transformants stock. The 5' sequences of these clones were determined using Big-Dye™ Terminator Cycle Sequencing (PE Biosystems, Foster City, CA, USA). The sequences were compared with nucleotide sequences in the GenBank® database using WU-BLAST 2.0© (1) to identify the cDNA clones.

RESULTS AND DISCUSSION

Tailing with dITP and DIG-dUTP Using TdT

The oligonucleotide for the *TEGT* gene was labeled to evaluate the length

Figure 2. Colony Hybridization with Probes Tailed with dITP and DIG-dUTP. A total of 3456 colonies on an HDF were hybridized with probes tailed with dITP and DIG-dUTP.

and the sensitivity of probes tailed with dITP and DIG-dUTP, in comparison with those of typical probes tailed with dATP and DIG-dUTP. The tail length was increased by the elevation of the dITP/DIG-dUTP ratio in the reaction mixture. The probe labeled with dITP and DIG-dUTP was slightly longer than that labeled with dATP and DIG-dUTP, indicating efficient incorporation of dITP in the tail of the probe (data not shown). The signal intensity increased 10× by increasing the ratio of dITP/DIG-dUTP from 2/1 to 10/1 (data not shown). These results indicated that the highly sensitive tailed probe was prepared using a mixture of dITP and DIG-dUTP as substrates of TdT.

Hybridization of cDNA clones with a Probe Tailed with dITP and DIG-dUTP

To examine the hybridization characteristics of the tailed probes, nine types of cDNA clones containing poly(dA) regions from 23–140 bases long, but not containing sequences complementary to the designed sequence, were used as negative controls. The melting temperature of the original nucleotide of the probe was estimated to be 65°C in the hybridization solution according to a previously described method (9). Hybridization was carried out at 60°C, 5°C below the melting temperature. Regardless of the ratio of dITP/DIG-dUTP in the reaction mixture, specific hybridization of the probe tailed with dITP and DIG-dUTP was observed without the addition of poly(A) and poly(dA) mixed nucleotides (Figure 1, a and c).

In contrast, the probe tailed with dATP and DIG-dUTP did not specifically hybridize to *TEGT* cDNA without the addition of poly(A) and poly(dA) mixed nucleotides (Figure 1, a and c). Background hybridization was reduced by either decreasing the ratio of dATP/DIG-dUTP in the reaction mixture or by adding poly(A) and poly(dA) mixed nucleotides (Figure 1a), however, background hybridization was not eliminated completely. These results suggest that the dI tails do not hybridize to any sequence of the cDNA clones, while the homopolymeric dA tails specifically hybridize to poly(dT) regions of the cDNA

clones under stringent conditions. It is possible that the poly(dI) tail have a lower melting temperature compared with the poly(dA) tail since dI:dG, dI:dT, dI:dA and dI:dT pairs are less stable than dA:dT pairs (6). The character of the tail is thought to result in the high specificity of hybridization.

Colony Hybridization with Probes Tailed with dITP and DIG-dUTP

Probes tailed with dITP and DIG-dUTP were applied to colony hybridization screening. Oligonucleotides were designed for highly redundant genes in a library; 169 genes were selected, consisting of approximately 30% of the clones in the library. Oligonucleotides for 106 types of ribosomal protein cDNAs were also designed. Figure 2 shows the results of colony hybridization with the tailed oligonucleotides. Spots were labeled in an all-or-none

manner, indicating high specificity. The probes did not hybridize to the genomic sequence of *Escherichia coli*. To confirm the results of the hybridization, 80 clones were selected in order and a single-pass sequence from the 5'-end was determined for each (Table 1). Among 58 clones with negative signals, three clones lacked the region to which the oligonucleotides were designed, indicating that the tailed probes did not hybridize with these cDNA clones. Among nine clones with positive signals, there was only one false positive. The clone may contain a sequence similar to the probe within the unsequenced region. Thus, it is shown that sensitive and specific hybridization can be carried out using the tailed probe in colony hybridization screening.

The oligonucleotide labeling described in this report did not require any specialized methods except the use of dITP. We used the labeled

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nucleotide DIG-dUTP as a model in the present study. It may be possible to use other labeled nucleotides, such as fluorescein-labeled nucleotides or biotin-labeled nucleotides. Oligonucleotide probes tailed with dITP and labeled nucleotide may facilitate cDNA screening by hybridization.

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