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GENERATION OF HIGH EFFICIENCY ssDNA HYBRIDIZATION PROBES BY LINEAR POLYMERASE CHAIN REACTION (LPCR)

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Abstract

The polymerase chain reaction (PCR) methodology can be employed to produce DNA hybridization probes. The major advantages of this paradigm over other techniques include superior specific activity of the probes, the versatility of sequence selection, the ability to produce short probes, and the simplicity of the procedure. We have further improved the efficiency of PCR probes by generating single stranded (ssDNA) probes that do not reanneal with themselves in solution, and hence, their availability for the interaction with the complementary sequences of the target is profoundly increased. Protocols for ^{32}P-dCTP labeled and digoxigenin-dUTP labeled probes have been elaborated to maximize the incorporation rate of the label as well as to provide for the production of full-length probes. The ssDNA probes may be particularly suitable for nucleic acid detection in tissues by *in situ* hybridization.

Key Words: ssDNA hybridization probes, linear polymerase chain recation (PCR), digoxigenin-labeled probes, radiolabeled probes.

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Introduction

The quality of hybridization probes is a decisive factor in the sequence-specific detection and/or quantitation of nucleic acids. The polymerase chain reaction (PCR) (Saiki *et al.*, 1985, 1988) provides a convenient technique to generate hybridization probes that in many aspects are superior to those prepared by other techniques (Konat *et al.*, 1994). Both internal labeling, i.e., the incorporation of labeled dNTPs into the extending strand, and end labeling, i.e., the extension of labeled primer can be performed. Protocols for both isotopic (Jansen and Ledly, 1989; Schowalter and Sommer, 1989; Bednarczuk *et al.*, 1991; Konat *et al.*, 1991; Blakeley and Carman, 1991) and nonradioactive variety (Day *et al.*, 1990; Lo *et al.*, 1988; Konat *et al.*, 1991; Liesack *et al.*, 1990; Lion and Haas, 1990; Lanzillo, 1990; Emanuel, 1991; Tabibzadeh *et al.*, 1991; Uchimura *et al.*, 1991) are available. The detection sensitivity of such probes is comparable to riboprobes (Schowalter and Sommer, 1989). Other major advantages of PCR include the versatility of sequence selection, the ability to produce discrete short probes, the requirement for only small amounts of even unpurified DNA template, and the simplicity of the procedure.

The efficiency of PCR probes can further be improved by generating single stranded (ssDNA) probes (Bednarczuk *et al.*, 1991; Konat *et al.*, 1991) that do not reanneal with themselves in solution, and hence, their availability for the interaction with the complementary sequences of the target is profoundly increased. For example, the use of ssDNA probes results in an approximately 8-fold increase in the signal intensity as compared to double stranded (dsDNA) probes as assessed by Northern blot analysis (Bednarczuk *et al.*, 1991; Fig. 1). The reaction conditions for the generation of ^{32}P-dCTP-labeled and digoxigenin-dUTP labeled probes have been elaborated to maximize the incorporation rate of the label as well as to provide for the production of full-length probes (Konat *et al.*, 1991). The ssDNA PCR technology that yields discrete, high efficiency probes may be particularly suitable for nucleic acid detection in tissues by *in situ* hybridization.

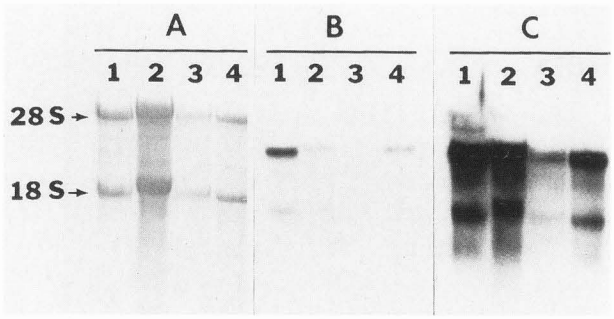

Figure 1: Hybridization efficiency of ssDNA versus dsDNA PCR probes. Total rat brain RNA was isolated by the acidic guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987), size fractionated by electrophoresis on 1% agarose gel, vacuum transferred onto a Nytran membrane (Schleicher and Schuell, Keen, NH), and UV cross-linked. The membranes were stained in 0.02% methylene blue to visualize the RNA (panel A). The membranes were subsequently hybridized with radiolabeled probes, and washed at high stringency (0.2XSSC, 0.1% SDS, 60°C, 60 min). Both, the dsDNA probe (panel B) and the ssDNA probe (panel C) were generated using 250 μCi of α^{32}P-dCTP (Du Pont NEN, Boston, MA). The autoradiograms show Northern blots following 4 h exposure with an intensifying screen. The positions of 28S and 18S ribosomal RNA are the same in all three panels. The lanes 1 and 4 contained 8 and 3 μg, respectively, of total RNA from 3-week-old rat brain, while the lanes 2 and 3 contained 24 and 2 μg, respectively, of total RNA from 3-month-old rat brain. Densitometric quantitation revealed that ssDNA hybridization probes resulted in approximately 8 times higher signal intensities as compared to dsDNA probes.

Reproduced from Bednarczuk *et al.* (1991) by permission of Eaton Publishing Co., 154 E. Central St., Natick, MA 01760-3644 USA.

Materials and Methods

The strategy for the production of ssDNA probes is schematized in Fig. 2, and detailed protocols for the generation of ^{32}P-dCTP-labeled and digoxigenin-dUTP labeled probes (Konat *et al.*, 1991) are provided below.

Materials and equipment

GeneAmpT DNA Amplification Reagent Kit is obtained from Perkin Elmer Cetus (Norwalk, CT). ^{32}P-α-dCTP (>3,000 Ci/mmol) is from ICN Biochemicals (Irvine, CA). Digoxigenin-11-dUTP (DIG-dUTP) and Quick Spin G-50 columns are purchased from

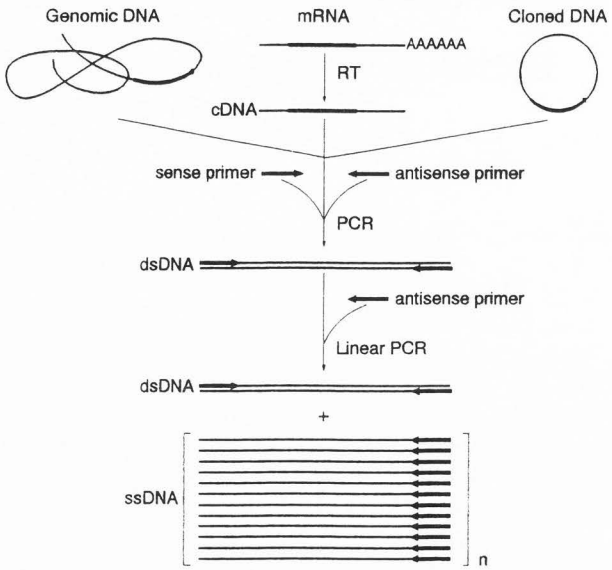

Figure 2: Flow diagram for generation of hybridization probes by linear PCR. In the initial step, the DNA fragment of interest is amplified either from reversely transcribed (RT) mRNA, from cloned DNA, or from genomic DNA by conventional PCR using a pair of specific primers. This double stranded (dsDNA) fragment is subsequently used as a template for the generation of single stranded DNA molecules (ssDNA) by linear PCR with only one, e.g., antisense primer. Because the ssDNA is arithmetically amplified, the number of ssDNA strands (n) generated on one molecule of dsDNA template corresponds to the number of PCR cycles. The labeling of ssDNA probes is attained by the incorporation of labeled nucleotides into the linear PCR reaction.

Boehringer Mannheim (Indianapolis, IN), and Elutip-d from Schleicher and Schuell (Keen, NH). Oligonucleotide primers (20-mers) are custom synthesized and HPLC-purified by Operon Technologies (Alameda, CA). All PCR amplifications are performed in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT) in standard 0.5 ml eppendorf tubes. The reagents and equipment of comparable quality obtained from other commercial suppliers may also be used.

Preparation of dsDNA template

A DNA fragment is amplified by the conventional two-primer PCR from an appropriate DNA, e.g., reversely transcribed RNA, genomic digest, or cloned inserts (Fig. 2). The amplicon is subsequently isolated by 1% agarose gel electrophoresis and purified by Elutip-d elution using the manufacturer's protocol. The dsDNA template is quantitated spectrophotometrically at 260 nm.

Generation of radiolabeled ssDNA probes

The PCR amplification reaction contains in a total volume of 25 μl: 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, pH 8.3), 20 μM of each dATP, dTTP and dGTP, approximately 25 ng of dsDNA template, 1 μM primer (antisense), 2 μM ^{32}P-dCTP (150 μCi), and 0.62 units of Taq polymerase. The mix is overlaid with approximately 25 μl of mineral oil, and processed through 30 cycles at the following profile: (1) denaturation for 2 min at 94°C, (2) annealing for 2 min at appropriate temperature (determined for a particular template/primer set), (3) extension for 5 min at 72°C.

Subsequently, the reaction mix is diluted with 25 μl water, and the probe is purified from unincorporated nucleotides by passing the mix through a Quick Spin G50 column. Although the best results are obtained with freshly prepared probes, the probe can be stored for several days at -20°C.

Generation of digoxigenin-labeled ssDNA probes

The PCR amplification reaction contains in a total volume of 100 μl: 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, pH 8.3), 100 μM of each dATP, dCTP and dGTP, 90 μM dTTP, 10 μM DIG-dUTP, approximately 400 ng of dsDNA template, 3 μM primer (antisense), and 7.5 units of Taq polymerase. The mix is overlaid with approximately 25 μl of mineral oil, and processed through 45 cycles at the following profile: (1) denaturation for 1.5 min at 94°C, (2) annealing for 1.5 min at appropriate temperature (determined for a particular template/primer set), (3) extension for 2 min at 72°C.

Subsequently, the aqueous phase is mixed with 0.1 vol of 4 M LiCl and 3 vols of ethanol, and placed at -20°C for 30 min. The DNA is spun down (12,000 g, 20 min), and the pellet is washed with 100 μl 70% ethanol, air-dried, dissolved in TE buffer, and stored at -20°C.

Commentary

The identity of templates prepared from complex nucleic acid mixtures, i.e., genomic digest or total RNA should be confirmed by restriction and sequence analysis. The removal of unincorporated dNTPs and primers from dsDNA template is another critical step required for successful generation of high quality ssDNA probes. Although the procedure described in Materials and Methods yields highly purified templates, alternative methods can also be employed.

The ssDNA protocols employ a linear (arithmetic) amplification paradigm in which the production of probes is proportional to the number of thermal cycles, and to the amount of dsDNA template. Thus, relatively high concentrations of the template have to be used to maximize the generation of the probes. Excessive amounts of template, however, are undesirable as they result in higher proportion of unlabeled DNA, and consequently may reduce the intensity of hybridization signal through annealing with the labeled probe.

The experimental conditions for individual template/primer sets may require adjustment to optimize the quality (average length) and the quantity (yield) of the probes (Konat *et al.*, 1991). This pertains chiefly to the annealing temperature and magnesium concentration. Furthermore, some DNAs may require DMSO or other agents of choice as well as hot start to attain efficient amplification/labeling. In general, the optimal conditions found for the dsDNA template amplification should also be optimal for the generation of ssDNA probes.

In the radiolabeled protocol the reaction conditions are suboptimal in regard to dNTP, and especially, dCTP concentration. Consequently, the radiolabeling reaction requires a 5 min extension time to ensure high proportion of full-length probe. Under these conditions, approximately 50% yield of ^{32}P-dCTP incorporation is reached after 30 cycles of amplification (Konat *et al.*, 1991), which is comparable to the efficiency of incorporation observed for dsDNA probes (Jansen and Ledley, 1989). For some applications (e.g., *in situ* hybridization) ^{35}S or ^{3}H labeled nucleotides can be incorporated using the same protocol.

In the nonradioactive probe paradigm the template concentration is increased four fold as compared to the radiolabeling protocol to maximize the production of the ssDNA probe at high dNTP concentration. The optimal concentration of both Taq polymerase and the primer is three times higher, whereas the concentration of dNTPs is 50% lower than the standard concentrations for exponential (two primer) PCR (Saiki, 1989). Under these conditions, approximately 8 μg of full-length digoxigenin-labeled ssDNA probe (60% incorporation) can be generated in a 100 μl reaction using 45 cycles (Konat *et al.*, 1991). The specific labeling of these probes can be further augmented by increasing the ratio of DIG-dUTP to dTTP up to 1:2 (Emanuel, 1991). The DIG-labeled probes preserve their activity for at least one year when stored at -20°C (Lion and Haas, 1990).

The versatility of ssDNA technique combined with high efficiency of the probes will undoubtedly be valuable for the detection of nucleic acids in tissues. For example, short discrete probes specific to any segment of a message or a gene can be easily produced by selecting appropriate primers. Such discrete probes will be highly desirable for detection of differentially spliced messages, or genetic alleles. The simplicity of sequence selection will also help to overcome possible problems of disadvantageous secondary structure of certain regions

of nucleic acids that may impede hybridization. Furthermore, if required, both sense and antisense probes can be conveniently generated from the same dsDNA template by extending one of the two primers.

References

Bednarczuk TA, Wiggins RC, Konat G (1991) Generation of high efficiency, single-stranded DNA hybridization probes by PCR. BioTechn **10**: 478.

Blakeley MS, Carman MD (1991) Generation of an S1 probe using arithmetic polymerase chain reaction. BioTechn **10**: 52-51.

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acidic guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem **162**: 156-159.

Day PJR, Bevan IS, Gurney SJ, Young LS, Walker MR (1990) Synthesis *in vitro* and application of biotinylated DNA probes for human papilloma virus type 16 by utilizing the polymerase chain reaction. Biochem J **267**: 119-123.

Emanuel JR (1991) Simple and efficient system for synthesis of non-radioactive nucleic acid hybridization probes using PCR. Nucleic Acid Res **19**: 2790.

Jansen R, Ledley FD (1989) Production of discrete high specific activity DNA probes using the polymerase chain reaction. Gene Anal Techn **6**: 79-83

Konat G, Laszkiewicz I, Bednarczuk TA, Kanoh M, Wiggins RC (1991) Generation of radioactive and nonradioactive ssDNA hybridization probes by polymerase chain reaction. Technique **3**: 64-68.

Konat GW, Laszkiewicz I, Grubinska B, Wiggins RC (1994) Generation of labeled DNA probes by PCR. In: PCR Technology: Current Innovations. Griffin HG, Griffin AM (eds) CRC Press Inc, pp. 37-42.

Lanzillo JJ (1990) Preparation of digoxigenin-labeled probes by the polymerase chain reaction. BioTechn **8**: 621-622.

Liesack W, Menke MAOH, Stackebrandt E (1990) Rapid generation of vector-free digoxigenin-dUTP labeled probes for nonradioactive hybridization using the polymerase chain reaction (PCR) method. System Appl Microbiol **13**: 255-256.

Lion T, Haas OA (1990) Nonradioactive labeling of probe with digoxigenin by polymerase chain reaction. Anal Biochem **188**: 335-337.

Lo YMD, Mehal WZ, Fleming KA (1988) Rapid production of vector-free biotinylated probes using the polymerase chain reaction. Nucleic Acid Res **16**: 8719.

Saiki RK (1989) The design and optimization of the PCR. In: PCR Technology: Principles and Applications for DNA Amplification. Erlich HA (ed) Stockton Press, New York, pp 7-16.

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of β-globin genomic sequences and restriction site analysis for the diagnosis of sickle-cell anemia. Science **230**: 1350-1354.

Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**: 487-491.

Schowalter DB, Sommer SS (1989) The generation of radiolabeled DNA and RNA probes with polymerase chain reaction. Anal Biochem **177**: 90-94.

Tabibzadeh S, Bhat UG, Sun X (1991) Generation of nonradioactive bromodeoxyuridine labeled DNA probes by polymerase chain reaction. Nucleic Acid Res **19**: 2783.

Uchimura Y, Ishida H, Asada K, Mukai H, Kato I (1991) Nonradioactive labeling with chemically modified cytosine tails by polymerase chain reaction. Gene **108**: 103-108.

Discussion with Reviewers

M. Malecki: In your work you reported 8-fold increase in signal intensity generated with ssDNA as compared to the dsDNA. Have you noticed variability in signal intensity dependent on the reporter molecules used?
Author: The increased hybridization efficiency of ssDNA probes as compared to dsDNA probes is mostly attributed to their inability to reanneal in solution. The sequence of a probe itself should not affect the ratio of hybridization efficiencies, however, we only compared probes generated on the PLP-specific template.

M. Malecki: *Taq* polymerase is recommended in your protocol. Would you anticipate a higher fidelity through proof reading using, e.g., *Pfu*?
Author: A few misincorporated bases introduced *in vitro* by DNA polymerases would in most cases have a minimal effect on the stringency of hybridization. However, during the generation of radiolabeled probes when the concentration of dNTPs (and especially dCTP) is greatly reduced the infidelity of polymerases may increase significantly, and consequently reduce the stringency. *Pfu* polymerase could reduce the mutation rate by approximately 10-fold. However, it should also be considered that the integral 3'-(editing)-exonuclease activity of *Pfu* may increase at low dNTP concentrations, and hence, profoundly reduce the amount of ssDNA molecules, or their length.