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A SUPERSENSITIVE DOT-HYBRIDIZATION METHOD: RAPID AND QUANTITATIVE DETECTION OF HOST-DERIVED DNA IN RECOMBINANT PRODUCTS AT THE ONE PICOGRAM LEVEL

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SUMMARY: We have developed a highly sensitive method of DNA dot-blotting hybridization to detect host-derived DNA (nuclear DNA and plasmid DNA) in a recombinant product. This method has two distinctive features compared to the conventional hybridization method: firstly, a highly specific radioactive probe is prepared by using ultrasonicated DNA, instead of untreated DNA, as a template for the oligo-labeling reaction; secondly, the signal to noise ratio is increased by the use of lambda phage DNA as non-homologous DNA. This method enabled us to detect host-derived DNA at the one picogram level without using a radioisotope of high specific activity and long exposure times.

Recently some products made by genetic engineering techniques are being marketed world-wide. The content of host-derived DNA (mainly nuclear DNA) in the product is an important factor in the manufacturing guidelines. There is a possibility that host-derived DNA contains unfavorable DNA fragments, such as viral DNA, provirus DNA and oncogenes. Therefore, the development of a supersensitive method for detecting host-derived DNA is urgently needed (1).

Filterbound nucleic acid hybridization has been widely used to analyze trace amounts of nucleic acids quantitatively (2,3,4). This method can be utilized to detect a specific sequence of DNA at the one picogram level (5,6,7). However, in the case of detection of nuclear DNA (heterogeneous DNA), a multicopy gene such as a rRNA gene has usually been used as a probe, and this does not allow detection of nuclear DNA with the same sensitivity (8,9). In addition, it is difficult to find a suitable multicopy gene for nuclear DNA from various origins.

In this report, we describe that ultrasonicated DNA is a good template for oligo-labeling (10), and that using this probe we have developed a quantitative dot-blot method to rapidly detect host-derived

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DNA at the one picogram level. This method does not require the use of a radioisotope of high specific radioactivity or long exposure times.

MATERIALS AND METHODS

Host-derived DNA and plasmid: Host-derived DNA was prepared from Saccharomyces cerevisiae AH22R⁻/pGLDP31-RcT (11,12) using the method of Cryer et al. (13). The plasmid pBD4, containing the ribosomal RNA genes of S.cerevisiae (14), was a gift from Dr.Valenzuela of Chiron Research Laboratories.

Fragmentation of nuclear DNA: The host-derived DNA (50µg) in 500µl of TE buffer (10mM Tris,pH8.0 and 1mM EDTA) was ultrasonicated with a Kaijo Denki ultrasonic generator 4280 (300W, 19.5kHz) at the minimum output level for 60 sec. The resultant DNA fragments were electrophoresed on 1.1% agarose gel. The fraction between 200bp and 1000bp was recovered using an electroelution method (4) and purified with RDP Mini-Columns (Bio-Rad). Digestion with Sau3AI (Takara Biochemicals) was performed by the method of Davis et al. (15). The resulting DNA fragments (200-1000bp) were recovered and purified as described for ultrasonicated DNA.

Radiolabeling of probes: The DNA fragments were ^{32}P-labeled by nick translation or oligo-labeling. Nick translation was performed with Nick translation kits (Amersham) using 1µg of the DNA fragment and 100µCi of [α-^{32}P]dCTP (>400Ci/mmol). For oligo-labeling, the Multiprime DNA labeling system (Amersham) was used with 25ng of the DNA fragment and 50µCi of [α-^{32}P]dCTP (>400Ci/mmol). The labeling procedures were performed according to the supplier's protocol.

DNA extraction from highly purified HBsAg preparation: HBsAg carrying pre-S2 region was purified from S.cerevisiae AH22R⁻/pGLDP31-RcT as described elsewhere (16). The 500µg of HBsAg samples were extracted with phenol and chloroform (17), precipitated with 10µg of glycogen (Boehringer) and ethanol (18,19) and then resuspended in 10µl of TE buffer.

DNA dot blotting: A known quantity of untreated host-derived DNA (<1ng) and proteinized extracts of HBsAg, both volumes were under 2µl, were incubated in 2µl of 50mM NaOH for 1 min at room temperature; an equal volume of a mixture of 1.5M NaCl, 1mM EDTA, and 0.5M Tris (pH7.2) was added; and the mixture was spotted directly onto a nitrocellulose filter (S&S BA85) pre-immersed in 20X SSC (1X SSC=0.15M NaCl, 15mM Na_3citrate). The filter was baked for 2 hrs at 80°C to fix the DNA.

Hybridization: The nitrocellulose filter was prehybridized in a SSC system for 1 hr at 65°C (6X SSC, 5X Denhardt's, 0.1% SDS, 20µg/ml ultrasonicated non-homologous DNA). Hybridization was performed in a SSC system containing a 1X 10^7cpm/ml probe labeled by oligo-labeling or a 2X 10^6cpm/ml probe labeled by nick translation for 16 hrs at 65°C. Two 30 min posthybridization washes were then done in 2X SSC containing 0.1%SDS at 40°C and then in 0.2X SSC containing 0.1%SDS at 50°C.

Autoradiography: The filter was exposed to Kodak XAR-5 film for 4 hrs at room temperature with a Cronex intensifying screen. Radioautographs were analyzed with a Shimadzu chromatoscanner CS-910 operated in the transmittance mode.

RESULTS

Conditions for the fragmentation of host-derived DNA and the labeling system: Among the probes labeled by nick translation, the specific activity (1.2X 10^6cpm/µgDNA) of the ultrasonicated host-derived DNA probe was unexpectedly one-fifth of that (5.7X 10^7cpm/µgDNA) of the Sau3AI

Table 1. Specific radioactivity and sensitivity of probes

Labeling system	Host-derived DNA(cpm/μgDNA)		
	Untreated (>20kbp)	Ultrasonicated (200-1000bp)	Sau3AI digested (200-1000bp)
Nick translation	$4.4X\ 10^7$ (500pg)[a]	$1.2X\ 10^6$ (<1ng)	$5.7X\ 10^7$ (100pg)
Oligo-labeling	$8.2X\ 10^7$ (100pg)	$8.0X\ 10^8$ (1pg)	$1.5X\ 10^8$ (50pg)

a, Numbers in parentheses indicate the sensitivity of the probe under the conditions described in MATERIALS AND METHODS.

digested host-derived DNA probe. However, the probes being prepared by oligo-labeling system, the specific activity of the ultrasonicated host-derived DNA probe ($8.0X\ 10^8$cpm/μgDNA) was more significantly elevated than that of the others (Table 1). These results suggest that ultrasonic treatment generates a suitable template for the oligo-labeling system.

Carriers used for ethanol precipitation: Carrier is necessary for ethanol precipitation of a trace of host-derived DNA extracted from highly purified recombinant products. Conventional nucleic acid carriers, such as ribosomal RNA, transfer RNA and heterogeneous DNA, were unfavorable for the detection of host-derived DNA, because such carriers hybridized with the host-derived DNA probe. Then, we tested glycogen (18,19) as a non-nucleic acid carrier. It was found that glycogen completely reduced non-specific hybridization (Fig.1).

Non-homologous DNA used in hybridization: As shown in Fig.2, labeled host-derived DNA slightly hybridized with the conventional non-homologous

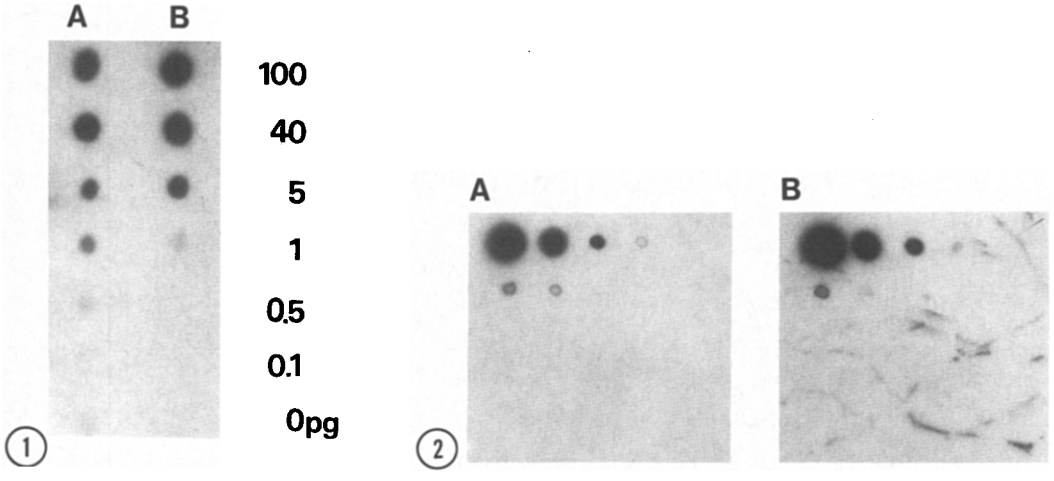

Fig.1. Effect of carriers used in ethanol precipitation. Various amounts of host-derived DNA were precipitated with 10μg of E.coli rRNA (A) and glycogen (B). The hybridization was performed with ultrasonicated host-derived DNA labeled by oligo-labeling ($8.0X\ 10^8$cpm/ μgDNA).

Fig.2. Effect of non-homologous DNA on hybridization. Mock hybridization was carried out with lambda DNA (A) and calf thymus DNA (B). The conditions of hybridization were the same as in Fig.1.

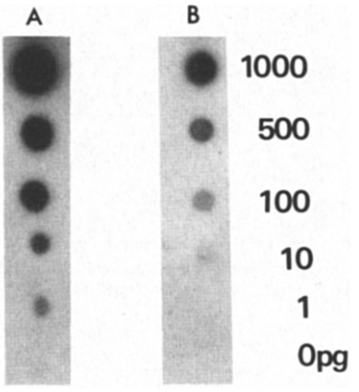

Fig.3. Comparison between the ultrasonicated host-derived DNA (A) and the conventional probe pBD4 (B). The specific radioactivities of the ultrasonicated host-derived DNA and EcoRI digested pBD4 were $8.0X\ 10^8$cpm/μgDNA and $2.3X\ 10^8$ cpm/μgDNA, respectively.

DNA, such as ultrasonicated calf thymus DNA in this system. We used other non-homologous DNAs in place of calf thymus DNA. Lambda DNA was found to reduce the background efficiently at the same concentration of calf thymus DNA.

Comparison between host-derived DNA and pBD4: Using the oligo-labeling method with the modifications as described above, we compared the sensitivity of ultrasonicated host-derived DNA probe with that of EcoRI digested plasmid pBD4, which has been usually used as a probe. As shown in Fig.3, the sensitivity of the ultrasonicated host-derived DNA probe was 100-fold greater than that of pBD4. Thus, it was possible to detect one picogram level of the host-derived DNA within less than 5 hrs.

Quantitative detection of host-derived DNA in HBsAg: A series of known quantity of host-derived DNA (standard) and the DNA extracted from the 500μg of the highly purified HBsAg with or without premixing the 50pg of host-derived DNA (referred to as positive control or samples) were spotted onto filters, hybridized with oligo-labeled ultrasonicated host-derived DNA probe and then autoradiographed for 4 hrs at room temperature. The relation between the amount of DNA spotted and the autoradiographic intensity was observed (Fig.4). Fig.5 showed that the 50pg of added host-derived DNA was quantitatively extracted from the 500μg of HBsAg (P1 and P2). However, no signal was found at the spots of all the sample (A2,B2,C2,D2 and E2) and the sensitivity of this assay was 5-10pg (standard). This meant that the content of host-derived DNA in all the HBsAg samples was less than 10pg per 100μg of total HBsAg protein.

DISCUSSION

Preliminary results showed that ultrasonicated host-derived DNA was degraded faster than Sau3AI digested host-derived DNA by S1 nuclease

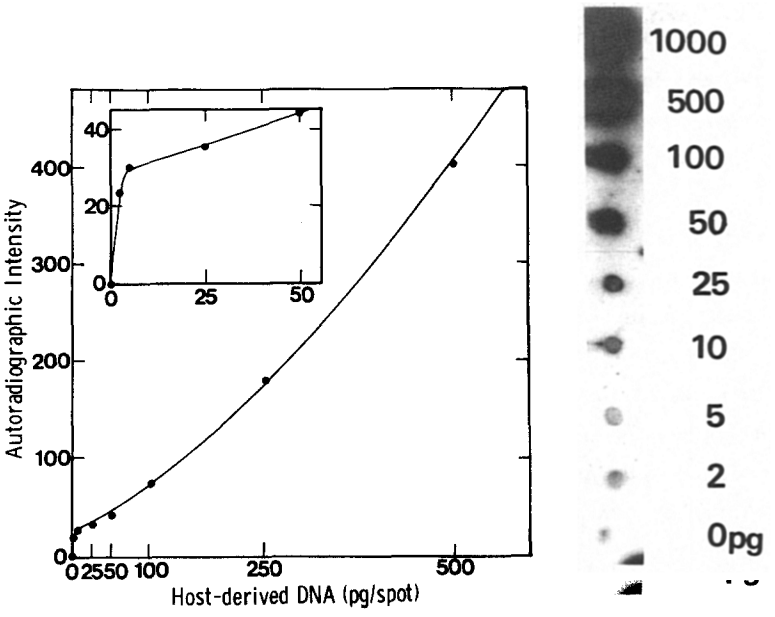

Fig.4. Relation between autoradiographic intensity and the amount of host-derived DNA spotted. The specific radioactivity of the probe was $8.0X\ 10^8$cpm/μgDNA.

(data not shown). This suggests that ultrasonic treatment generates a number of single strand regions in the host-derived DNA. These regions are likely to promote the efficiency of annealing between hexanucleotide primers and template DNA in the first step of the oligo-labeling reaction. As a result, we could obtain a probe with high specific radioactivity (Table 1). In the case of nick translation, this single

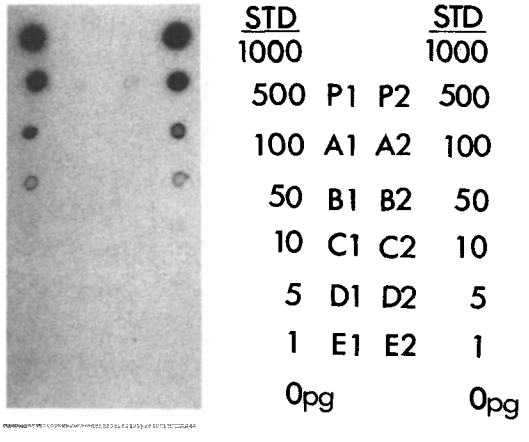

Fig.5. Quantitative detection of host-derived DNA in HBsAg. A1,B1,C1,D1 and E1 indicated the DNA spots from the 50μg of samples (lot 21,22,23,24 and 25, respectively). A2,B2,C2,D2 and E2 indicated that from the 100μg of samples (lot 21,22,23,24 and 25, respectively). P1 and P2 indicated that from 50μg and 100μg of positive controls, respectively. The specific activity of the probe was $8.0X\ 10^8$cpm/μgDNA.

strand region might reduce the efficiency of the reaction seriously, because double strand region necessary for nicking by DNase I (20) was greatly damaged by ultrasonication.

Radioisotopes having high specific activity (ex.>3000Ci/mmol) and long-term exposure (ex.3-7 days) are usually required to gain high sensitivity in dot-hybridization. However, it is not easy to satisfy with these two conditions. Therefore, we tried to develop a supersensitive method of dot-hybridization. It was found that small host-derived DNA (200-1000bp) generated by ultrasonication could be used as a good template for oligo-labeling. The resultant probe had high specific radioactivity and this probe enabled us to detect the host-derived DNA at the one picogram level using an exposure time of less than 5 hrs. This method could be applied to detect the DNA derived from other host cells, such as bacteria, fungi, streptomycetes and animal cells.

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REFERENCES

1. WHO.(1987) WHO Technical Report Series, No.760, p.87. WHO, Geneva.
2. Southern,E.(1980) In Methods in Enzymology(Wu,R.,ed.), Vol.68, pp.152-176. Academic Press, New York.
3. Meinkoth,J., and Wahl,G.(1984) Anal.Biochem.138,267-284.
4. Maniatis,T., Fritsch,E.F., and Sambrook,J.(1982) Molecular Cloning: A Laboratory Manual,p.382. Cold Spring Harbor, New York.
5. Scotto,J., Hadchouel,M., Hery,C., Yvart,J., Tiollais,P., and Brechot,C.(1983) Hepatology 3,279-284.
6. Guntaka,R.V., Rao,A.S., Green,T.J., and Collins,W.E.(1986) Biochem.Biophys.Res.Commun.138,363-368.
7. Lieberman,H.M., LaBrecque,D.R., Kew,M.C., Hadziyannis,S.J., and Shafritz,D.A.(1983) Hepatology 3,285-291.
8. Thomas,P.S.(1980) Proc.Natl.Acad.Sci.USA 77,5201-5205.
9. Dasgupta,S., Adhya,S., and Majumder,H.K.(1986) Anal.Biochem.158,189-194.
10. Feinberg,A.P. and Vogelstein,B.(1983) Anal.Biochem.132,6-13.
11. Kuroda,S., Itoh,Y., and Fujisawa,Y. in preparation.
12. Itoh,Y. and Fujisawa,Y.(1986) Biochem.Biophys.Res.Commun. 141,942-948.
13. Cryer,D.R., Eccleshall,R., and Marmur,J.(1975) In Methods in Cell Biology (Prescott,D.M.,ed.), Vol.12, pp.39-44.
14. Bell,G.I., DeGennaro,L.J., Gelfand,D.H., Bishop,R.J., Valenzuela,P.,and Rutter,W.J.(1977) J.Biol.Chem.252,8118-8125.
15. Davis,L.G., Dibner,M.D., and Battey,J.F.(1986) Basic Methods in Molecular Biology, p.171.Elsevier,New York.
16. Kobayashi,M., Asano,T., Utsunomiya,M., Itoh,Y., Fujisawa,Y., Nishimura,O., Kato,K., and Kakimuma,A.(1988) J.Biol.Chem. in press.
17. Berninger,M., Hammer,M., Hoyer,B. and Gerin,J.L.(1982) J.Med.Virol.9,57-68.
18. Tracy,S.(1981) Prep.Biochem.11,251-268.
19. Helms,C., Graham,M.Y., Dutchik,J.E., and Olson,M.V.(1985) DNA 4,39-49.
20. Rigby,P.W.J., Dieckmann,M., Rhodes,C., and Berg,P.(1977) J.Mol.Biol.113,237-251.