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Genome Res. 1992 1: 193-194
Access the most recent version at doi:10.1101/gr.1.3.193

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Avoiding Strand Reassociation in Direct Sequencing of Double-stranded PCR Products with Thermolabile Polymerases

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Subcloning PCR products for plasmid sequencing or performing asymmetric PCR for direct sequencing of single-stranded products is quite time-consuming.[1] Therefore, we have developed a protocol to sequence double-stranded PCR products directly by incorporation-sequencing with thermolabile DNA polymerases.[2] Problems arising from strand reassociation or formation of secondary structures are avoided by the addition of single-strand DNA-binding protein (SSB; see ref. 3) to the DNA immediately after heat denaturation.[4] Using SSB rather than other single-stranded DNA (ssDNA) binding proteins, for example, the gene *32* protein of T4 (gp32), exploits its extremely stable conformation in solution and the observation that the overall binding of SSB to ssDNA is less sensitive to ionic strength and therefore involves fewer ionic interactions than the gp32:ssDNA complex.[3] Likewise, labeling at 10°C further reduces the risk of secondary structure formation that might impede the polymerase and cause it to "pause"

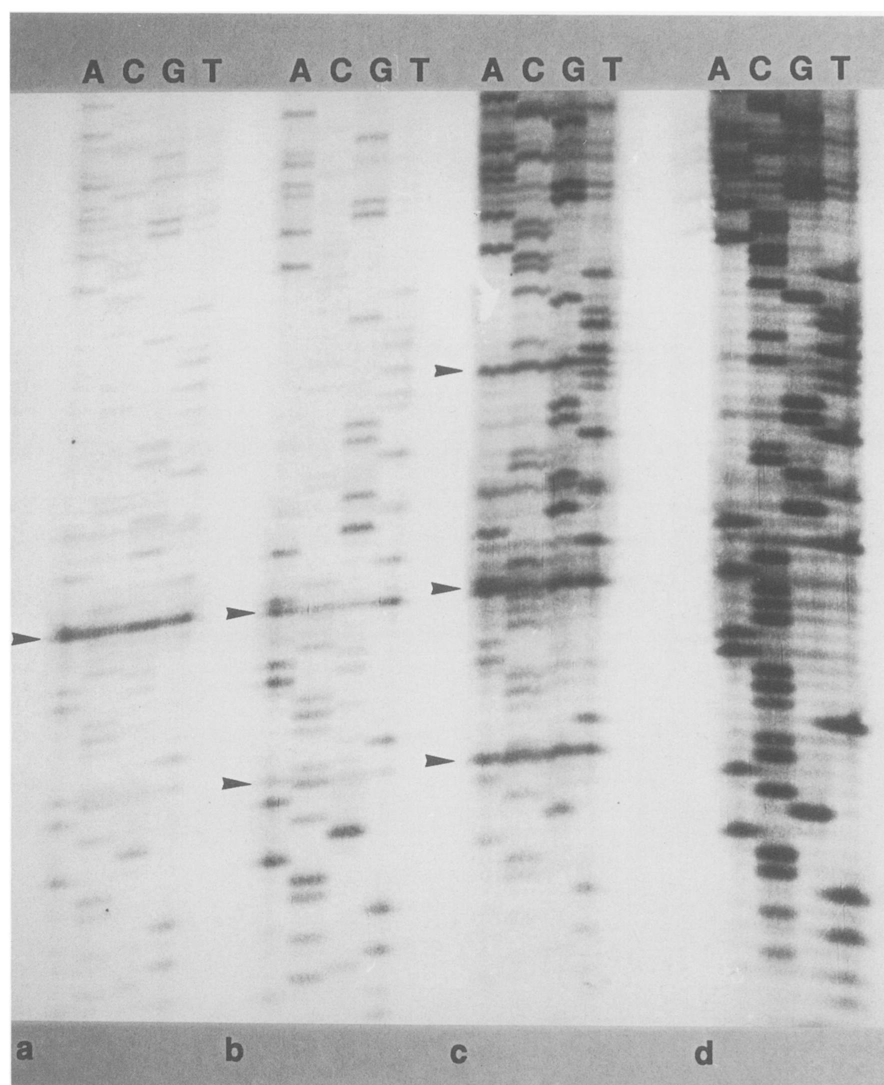

FIGURE 1 Direct sequencing of double-stranded PCR products. DNA sequences generated with the Sequenase 2.0 kit (USB) using the protocol as detailed in the text: (*a*) without the addition of SSB during primer annealing and labeling; (*b*) without SSB but replacing dGTP by 7-deaza-GTP in the labeling and termination reactions; (*c*) omitting SSB during primer annealing; and (*d*) as described with SSB being present throughout primer annealing and labeling. The 593-bp PCR product of the KS gene generated with 22-mer primers located at nucleotides 3–24 and 573–595, respectively,[6] was sequenced using the upstream primer. Arrowheads mark areas of abnormal chain termination.

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at these regions in the template. Addition of SSB apparently also does not require primers that hybridize to the end of the template; rather, the primer may be located anywhere along the DNA strand to be sequenced, thus allowing the use of nested primers (our observation).

PROTOCOL

Adjust 1.5 pmoles of gel-purified PCR product (e.g., 500 ng of a 500-bp fragment) to a volume of 7 μl in H_2O and heat at 95°C for 5 min. Quickly chill on ice and add 2 μl of sequencing buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), 1.5 pmoles of sequencing primer (for example, one of the PCR primers), and 1 μg SSB (from U.S. Biochemical) for a volume of 10 μl. Anneal the primer at 37°C for 15 min and chill on ice again.

To the annealed mixture add 1 μl of 100 mM DTT, 2 μl of a solution of dGTP, dTTP, and dCTP (1.5 μM each), and 5 μCi [^{35}S]dATPαS (1000 Ci/mmoles). Heat the tube to 10°C (we use the thermal cycler) and add 2 μl Sequenase 2.0 or T7 DNA polymerase [1.8 U/μl] (diluted in 10 mM Tris-HCl, pH 7.5, 5 mM DTT, 0.5 mg/ml bovine serum albumin). Label for 4 min.

Transfer 3.5 μl of the labeling reaction to each of the termination mixtures (2.5 μl of dATP, dTTP, dGTP, and dCTP 80 μM each, 50 mM NaCl, and 8 μM of the respective ddNTP; prewarmed to 37°C). Stop the reaction by adding 4 μl of 95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and xylene cyanol. Add 1 μl proteinase K (0.1 mg/ml) and incubate for at least 20 min at 65°C.

Using a 40-cm-long 6% polyacrylamide gel containing 50% (wt/vol) urea run in a discontinuous buffer system,[5] at least 250 bp can be read. Thus, the sequence of a 500-bp PCR product can be determined on a single gel by sequencing with both PCR primers.

Figure 1 shows part (nucleotides 5-65 on the gel) of the 5′upstream nontranslated sequence of the human K5 keratin gene (see ref. 6; nucleotides 34-94). Please note the elimination of regions with banding in all four lanes when SSB is present both during primer annealing and labeling (Fig. 1d) as well as the concurrent increase of band intensities in all tracks.

ACKNOWLEDGMENTS

Funding from the Deutsche Forschungsgemeinschaft (Mi 210/6-1) is gratefully acknowledged. We thank Dr. Michael Hummel (Institut fuer Pathologie, Universitaetsklinikum Steglitz, FU Berlin) for synthesizing primers and Dr. A. Ziegler (this insitute) for emphatic encouragement.

REFERENCES

1. Gyllensten, U. 1989. Direct sequencing of in vitro amplified DNA. In *PCR technology* (ed. H.A. Erlich), pp. 45–60. Stockton Press, New York.
2. Sanger, F., S. Niklen, and A.R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Nat. Acad. Sci.* **74:** 5463–5467.
3. Chase, J.W. and K.R. Williams. 1986. Single-stranded DNA binding protein required for DNA replication. *Annu. Rev. Biochem.* **55:** 103–136.
4. Kaspar, P., S. Zadrazil, and M. Fabry. 1989. An improved double stranded DNA sequencing method using gene 32 protein. *Nucleic Acids Res.* **17:** 3616.
5. Sheen, J. and B. Seed. 1988. Electrolyte gradient gels for DNA sequencing. *BioTechniques* **6:** 942–944.
6. Eckert, R.L. and E.A. Rorke. 1988. The sequence of the human epidermal 58-kD (#5) type II keratin reveals an absence of 5′upstream sequence conservation between coexpressed epidermal keratins. *DNA* **7:** 337–345.

Received September 24, 1991, accepted in revised form November 19, 1991.