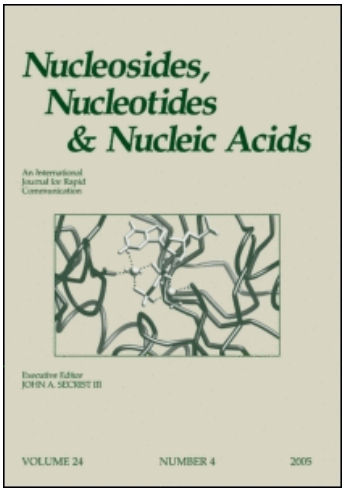

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New Methods for the Solid-Phase Sequence Analysis of Nucleic Acid Fragments Using the Sanger Dideoxy Procedure

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NEW METHODS FOR THE SOLID-PHASE SEQUENCE ANALYSIS OF NUCLEIC ACID FRAGMENTS USING THE SANGER DIDEOXY PROCEDURE

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Abstract. 3'-Terminal tailing of a given DNA fragment with 5-bromodeoxyuridine-5'-triphosphate or biotinylated deoxyuridine-5'-triphosphate and deoxynucleotidyl terminal transferase allows its immobilization to an anti-bromo-deoxyuridine-antibody column or to a streptavidin column. The immobilized DNA could be subjected to enzymatic sequencing following the usual protocol. The dideoxy-nucleotide-terminated fragments were eluted with buffer containing formamide / dye mixture and directly applied to gel electrophoresis, allowing reading of ca. 600 bases. Several "sequencing cycles" could be performed with the same DNA column. Semi-mechanization of the process is described.

The enzymatic sequence analysis of nucleic acids as described by F. Sanger and coworkers (1) has been one of the methodological bases for a dramatic increase in knowledge on nucleic acids structures yielding a total volume of sequenced nucleotides of several millions within roughly ten years. Most of this amazing success has been achieved using a solution phase sequencing technique in combination with gel electrophoresis. A possibility of simplifying the rather tedious manual operations has first come up with the use of robots (2). More recently, the part of the procedure including gel electrophoresis and gel reading has been mechanized and respective machines have been made commercially available. Large scale sequencing projects culminating in an interest to elucidate the physical structure of the complete

human genome require a further boost in the development of sequencing methods and a significantly higher degree of automation. Astonishingly, solid phase techniques which are the basis for highly efficient methods of polynucleotide synthesis have received little attention in enzymatic sequence analysis. In fact, the first paper on this topic has been published only last year by M. Uhlen and coworkers (3) who immobilized the target DNA via biotinylation through a specific cloning procedure. We had, at this time, initiated studies directed at developing a very simple labelling and immobilization procedure through tailing with modified nucleoside triphosphates + terminal deoxynucleotidyl transferase, which can be done without preliminary cloning for any single- or double-stranded DNA (4). A brief description and progress report of this work will be given here.

Results. 1. Immobilization Methods

Non-covalent immobilization was achieved by tailing of the DNA to be sequenced ("target" DNA; 0.2 - 10 pmol) with up to ca. 70 5-bromodeoxyuridylate units using a solution containing 400 µM 5-bromodeoxyuridine-5'-triphosphate, 1 mM ß-mercaptoethanol, 100 mM potassium cacodylate, 1 mM $CoCl_2$ and 100 µg/ml bovine serum albumin (5). The incubation was done with 16 u terminal deoxynucleotidyl transferase for 1 h at 37°, resulting in the attachment of approximately 70 units of bromodeoxyuridylate. For the preparation of the column material 500 mg protein A - Sepharose, swollen in 5 ml phosphate buffered saline (0.9 % NaCl, 0.05 % NaN_3) were treated with 25 mg mouse anti-BrdU antibody in 500 ml water (6). 40 µl of swollen antibody column were then treated with 10 pmol bromodeoxyuridylate-tailed DNA resulting in the immobilization of up to 0.2 pmol DNA per µl antibody resin.

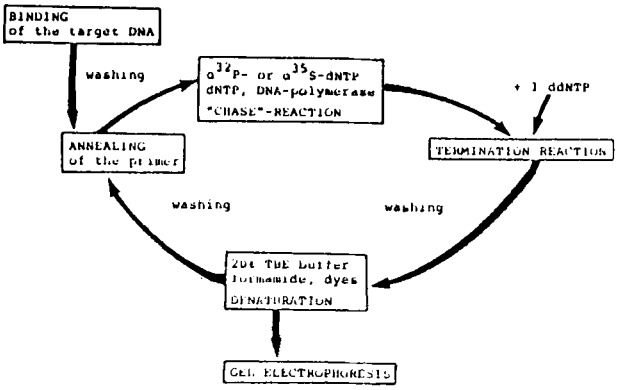


FIG. 1: Cycle for solid phase sequence analysis using the Sanger dideoxy method

Alternatively, tailing with biotinylated dUTP was done as described in a recent paper (7). In this case, commercial streptavidin Sepharose was used as a support for sequence analysis, and a loading capacity similar to the previously described experiment was obtained.

2. Sequence Analysis and Electrophoretic Separation

A "sequencing cycle" shown in Fig. 1 (6) consists of a) primer hybridization (0.5 - 2 pmol primer, 15 min at 37°), b) the "chase reaction" applying to 10 µl DNA resin 1 µl 100 mM DTT, 2 µl of a solution containing 1.5 µM dCTP, dGTP and dTTP, 0.5 µl ^{32}P- or ^{35}S-labelled dATP and incubating with 0.2 u DNA polymerase at room temperature for 10 min., c) the "stop reaction" by addition of 2.5 µl each of a solution containg 80 µM dATP, dCTP, dGTP, dTTP and dideoxy-NTP (N = one of four nucleotides) and further incubation for 30 min at 37°. After washing with aqueous buffers to remove excess enzyme and monomers the dideoxy-terminated fragments were finally eluted from the column with buffer containing formamide and dye markers and directly applied on top of a sequencing gel. The gel electrophoresis (0.2 mm denaturing PAGE) was run under the usual conditions (8), applying per well ca. 40 000 cpm. Reading of up to ca. 600 bases per sequencing cycle was possible in examples including both single stranded and double stranded DNA (Fig. 2).

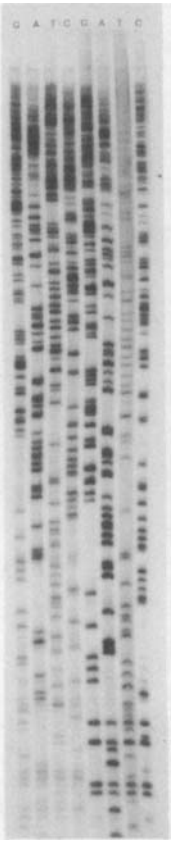
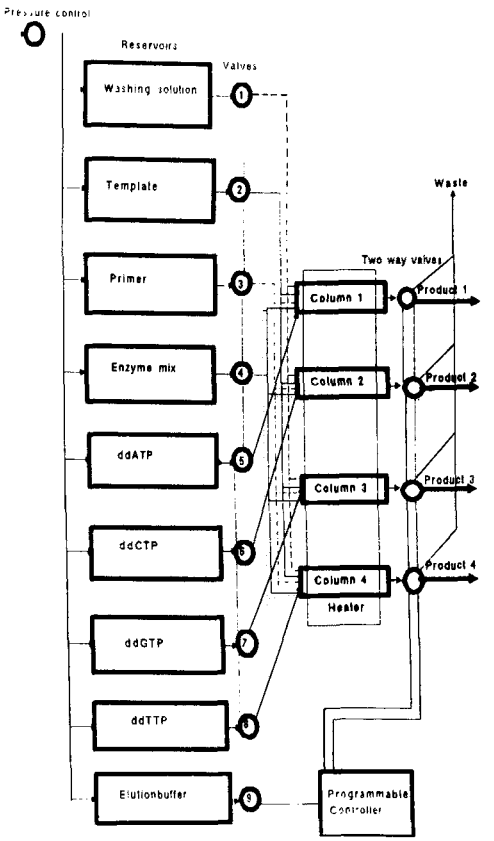


FIG. 2: Autoradiography of the polyacrylamide gel separation of the fragments released from the support, showing the result of the sequencing reactions with pUC 19 (left), a double stranded vector, and with M13mp9 (right), a single stranded vector.

3. Release of the Immobilized Target DNA

Release of the target DNA from the support could be effected in the case of bromodeoxyuridiylate tailing by denaturation using acid or high salt conditions (e.g. glycine buffer, pH 3). Biotinylated DNA could not be recovered from the streptavidin resin. At pH around 7, i.e. under sequencing conditions, release of DNA is quite slow in both cases. Thus, the sequencing cycle could be re-run 5 times still giving a clearly readable sequencing gel. Experiments directed at effecting a covalent linkage of the target DNA to a carrier are under way.

4. Approach to Mechanization of the Enzymatic Solid-Phase Sequencing



A manually operated apparatus was assembled, which allowed to perform the sequencing cycles in ca. 25 min. A scheme of this apparatus is shown in Fig. 3.

FIG. 3: Scheme of apparatus for solid phase sequencing

Discussion : The new route we describe here for enzymatic dideoxy sequencing is based on a simple enzymatic tailing procedure and thus, with respect to the previously published method (3), has the advantage of facile sample preparation and applicability to both single and double stranded DNA. In the latter case, a denaturation and washing procedure is recommended after the support loading to remove the non-immobilized second strands. Not more than 1 pmol DNA is required per sequence analysis with the option of "proof-reading" or "chromosome walking" by re-running the sequencing cycles with the same primer or with a series of con-

secutive primers. The stability of binding of the target DNA to the columns, which was already satisfactory in the non-covalent linking procedure described here, should be further enhanced by covalent attachment methods, which are the target of studies currently done in our laboratory. With the possibility of mechanization and direct coupling of an automatic sequencing unit to existing gel reading apparatus (9) the solid-phase technique can be expected to be most useful for routine and large-scale DNA sequencing projects.

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