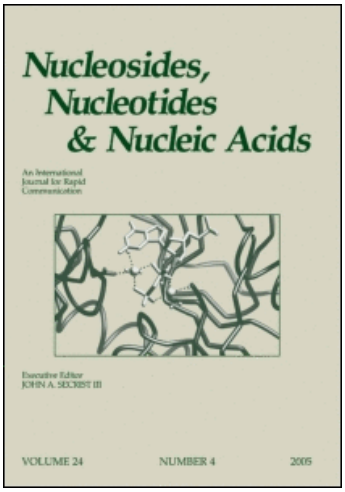

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SEMI-MECHANIZED SIMULTANEOUS SYNTHESIS OF MULTIPLE OLIGONUCLEOTIDE FRAGMENTS

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ABSTRACT: A new strategy for the simultaneous synthesis of multiple oligonucleotides is described, which is based on asynchronous chain growth and use of a maximum number of support segments in each elongation. An approach to the mechanization of this strategy and applications to the preparation of gene fragments and sequentially variant probes are discussed.

INTRODUCTION: Some years ago the concept of simultaneous synthesis of multiple oligonucleotides on segmental solid supports was described by R. Frank et al[1]. For projects demanding a number of oligonucleotides this approach is far more rational than the conventional "consecutive" preparation of each sequence, since the total number of elongations in the synthesis of m sequences of length n can be reduced from n·m to roughly 4·n (Fig.1). All publications on this approach, regardless on whether they use the phosphotriester[1,2] or phosphoramidite chemistry[3], have three things in common: 1. Filter paper disks are used as segmental supports; 2. All chains are growing synchronously, as indicated in Fig.1; 3. All syntheses were done in a manually operated 4-column synthesizer.
This apparative prerequisite somewhat restricts the use of this strategy, since most manual or automatic apparatus for solid-phase oligonucleotide synthesis are of a single-column type. We, therefore, were interested in adapting the general approach of simultaneous solid-phase preparations of multiple sequences to a) the use of deliberate types of

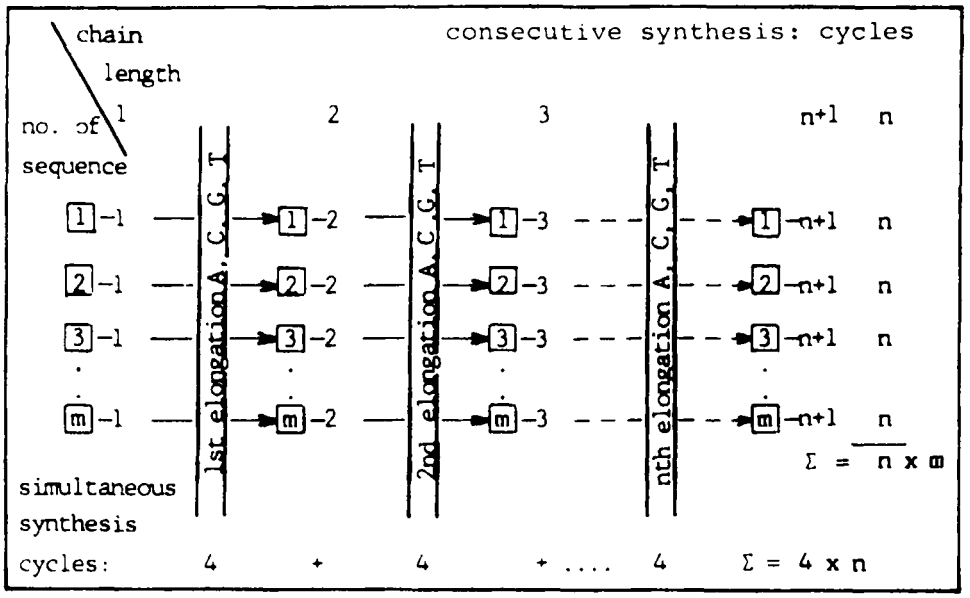

Fig.1: Comparison of synthesis of multiple oligonucleotide sequences, done in a consecutive vs. simultaneous manner, in the latter case using a synchronous chain elongation scheme.

apparatus, and b) the use of deliberate types of polymer supports. Results and applications of this study are reported in this paper[4].

THE CONCEPT OF SIMULTANEOUS/ASYNCHRONOUS CHAIN ELONGATION:

Our new strategy for the simultaneous synthesis of multiple oligonucleotides is based on the following preliminaries:

1. The different oligonucleotide chains are allowed to grow asynchronously;
2. A maximum number of support segments is introduced into each elongation cycle.

A computer program was written to calculate the optimum elongation scheme for a given set of sequences[5]. The essence of this approach is illustrated in Fig.2 for a random abacus-like arrangement of four types of balls on strings.

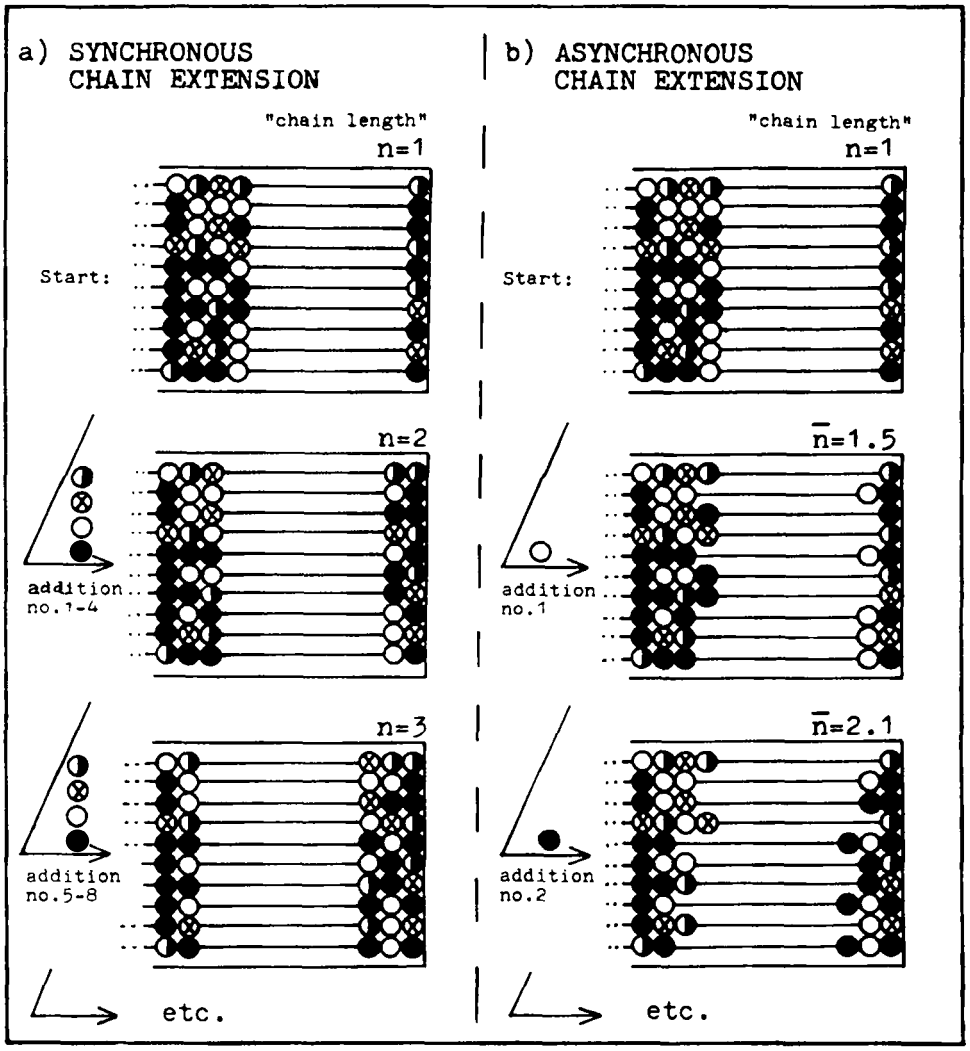

Fig.2: "Abacus game" for illustration of simultaneous/synchronous vs. simultaneous/asynchronous elongation strategies: The game consists of adding 4 types of balls, randomly arranged on the left-hand side of 10 strings and represented by open, half-open, crossed and filled circles, to a given first row by moving them along the strings from left to right. This can be done a) with synchronous chain growth requiring 4 addition steps to lengthen all chains from n=1 to n=2, n=3 etc., or b) with asynchronous chain growth. In this case only one type of balls is added i.e. only one addition step is done in each lengthening. Although the chains are not growing to equal length, the average chain length is $\bar{n}$=1.5 after one addition, $\bar{n}$=2.1 after two additions etc.

EXPERIMENTAL REALIZATION OF SIMULTANEOUS/ASYNCHRONOUS SYNTHESES:

Manual synthesis: A simple and inexpensive system for simultaneous manual preparations of multiple oligonucleotides has been elaborated by stacking filter paper disks in the syringe system published previously[6]. Details of this apparatus and its use for gene synthesis have been described [7].

Semi-mechanized synthesis: A partial mechanization of the simultaneous/asynchronous strategy could be achieved by coupling a commercial SAM I^{R}-nucleotide synthesizer (Biosearch/New Brunswick Sci.) with an Autofix^{R}-system (E. Merck, Darmstadt). This system, originally developed as a pneumatic fitting system for HPLC columns, can replace the reaction column of the synthesizer (Fig.3). Small LiChro-Cart^{R} cartridges[8] originally developed as guard columns, serve as containment for the polymer support. These cartridges can be stacked, as indicated in Fig.3, inside stainless steel tubes. Different tubes were prepared to accomodate up to 10 cartridges. Evidently, segmentation, in our case, is achieved through instrumentation. Thus, we are not confined to the use of the filter paper segments, and can apply any type of support material. During the synthesis the machine is stopped after each cycle. This procedure takes less than one minute. Thus, although the total synthesis time will be generally less for the 4-column synthesizer[1,2,3], the time of personal attention during e.g. a gene synthesis is actually minimized. A further reduction of the time and labor expenditure would be achieved by a fully automated simultaneous synthesis. This is the target of further developments in our laboratory.

Similar realizations for semi-mechanized simultaneous/asynchronous synthesis can be envisaged for most other commercial nucleotide synthesizers, and some of these variants are being studied.

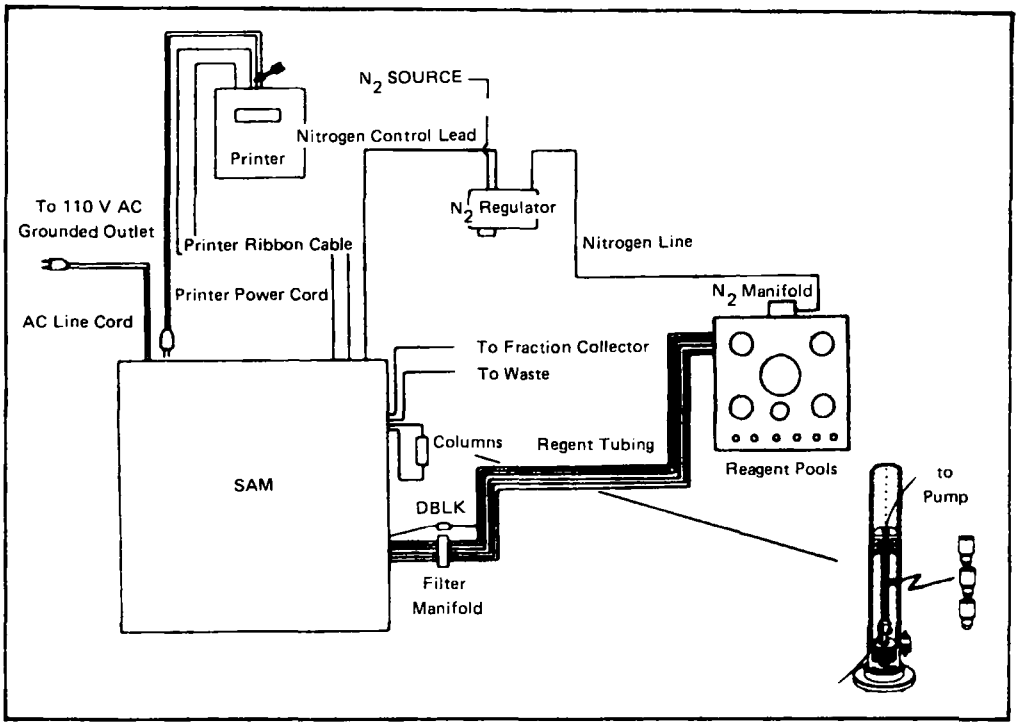

Fig.3: Schematic representation of combined SAM I (Biosearch)/ Autofix (E. Merck) system for simultaneous oligonucleotide synthesis in stacked LiChro-Cart cartridges

APPLICATIONS:

Gene synthesis: Two examples of gene synthesis were done, to date, by simultaneous/asynchronous preparation of blocks. The first example, the preparation of a gene for an LHRH precursor peptide, has been described in previous publications[4,7]. Recently, a second example was studied, namely the simultaneous preparation of the blocks for assemblage of two genes for γ_1- and γ_3-MSH. The melanocyte stimulating hormones are encoded in the human proopiomelanocortin gene[9], γ_1- arising from γ_3-MSH by processing at the two basic amino acid residues no.64 and 65 of the POMC translate. The sequence of the two peptides is listed in Fig.4.

The figure also shows the two derived genes, which were tailored to be cloned into the Pst I/Eco RI-sites of pBR 322

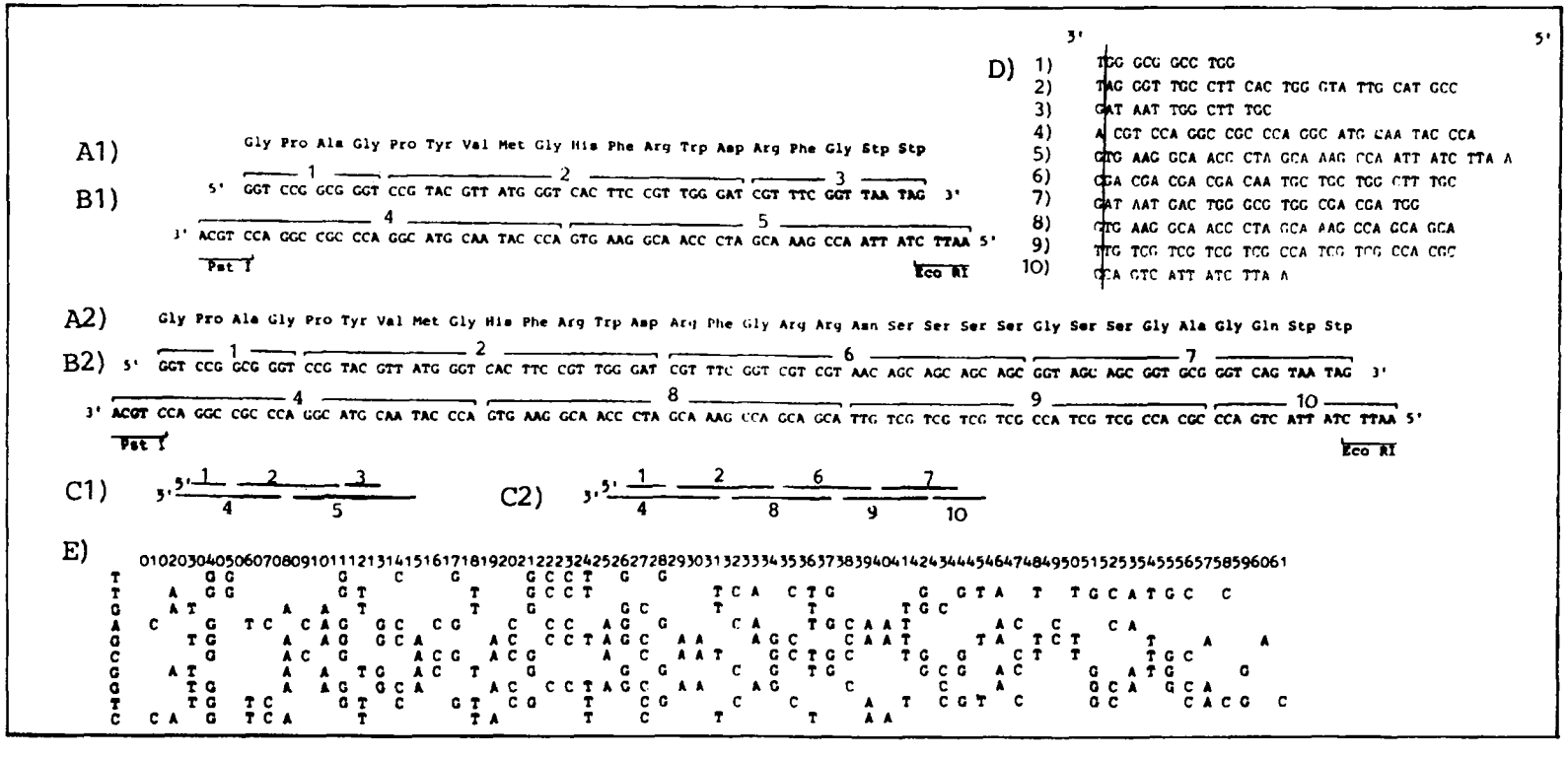

Fig.4: Plan for the synthesis of genes for γ_1- and γ_3-MSH.
A=Peptide sequences of γ_1-MSH (1) and γ_3-MSH (2); B=respective gene sequences (blocks underlined); C=ligation schemes for genes (1) and (2); D=list of blocks (from 3'-end); E= computer outprint of optimized strategy for simultaneous/asynchronous elongation.

Table 1: Simultaneous synthesis of fragments for the preparation of genes for γ_1- + γ_3-MSH

sequence no.	Yield after Completion of Cycles (O.D.$_{260}$)	Average Yield per Condensation (%)	Yield of Purified Sequence (O.D.$_{260}$)
55	98	91,7	10,5
56	127	94,8	3,1
57	148	96,1	14,9
58	205	95,2	3,2
59	210	96,5	3,1
60	215	95,1	4,3
61	175	96,1	5,2
62	154	95,0	5,3
63	150	95,5	4,1
64	110	95,7	14,5

and to contain an expression linker sequence coding for a collagenase recognition site[10]. The two genes can be ligased from 10 sequences of 12-34 b, which are listed in Fig.4 together with the computer-optimized elongation scheme. A calculation of the number of condensations, done as outlined in Fig.1, counts 245 vs. 106 condensations for the consecutive vs. simultaneous/synchronous[1-3] synthesis. The computer outprint for our simultaneous/asynchronous strategy lists only 61 condensations. This roughly corresponds to a ration of 97 vs. 40 vs. 25 condensations previously calculated for the LHRH precursor gene[7]. Thus, by partial mechanization and minimization of condensation steps, our strategy will also substantially reduce the risk of error, that arises through sorting of support segments and other repeated manual operations.

The γ_1- and γ_3-MSH gene fragments were prepared by standard phosphoramidite chemistry using a Fractosil 500 support[11].

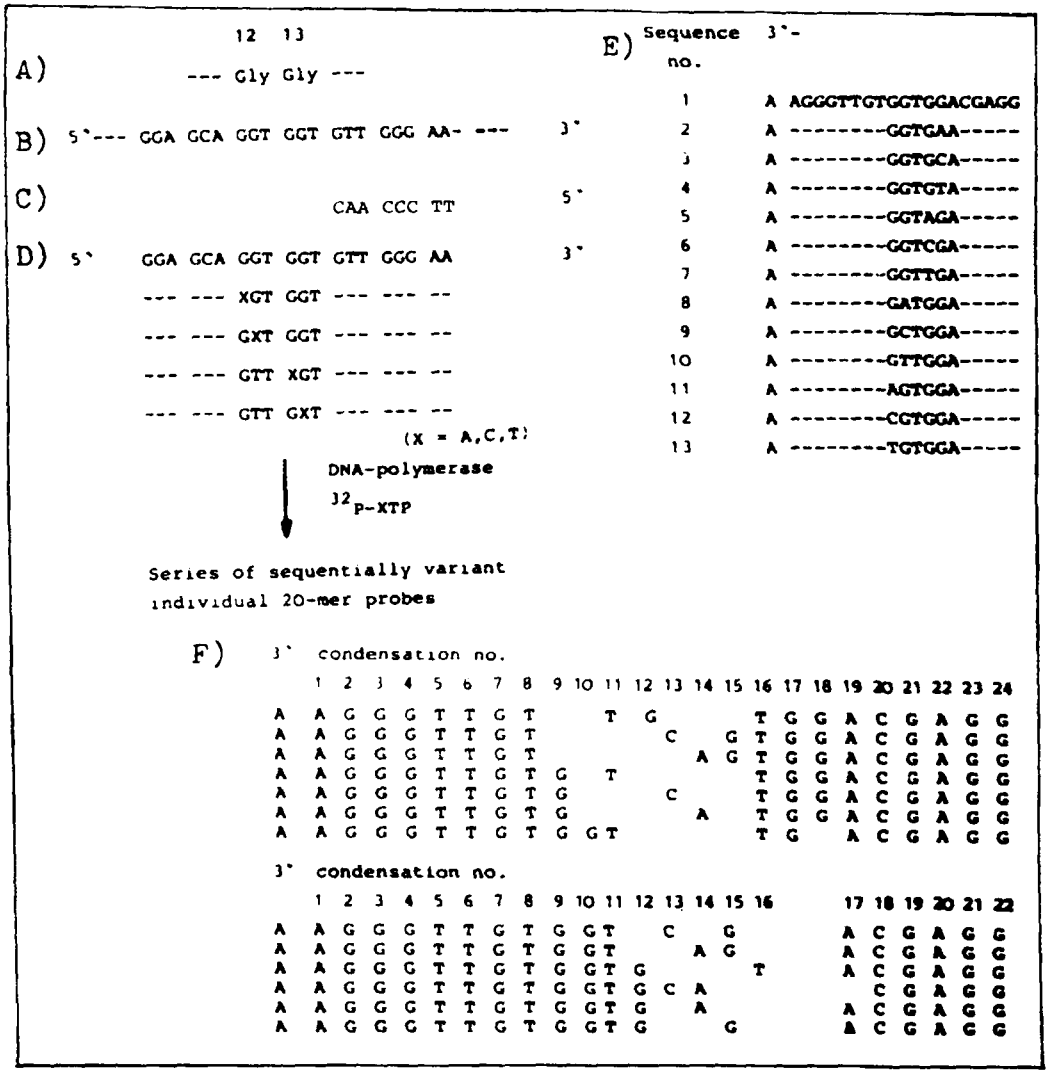

Fig.5: Plan for the simultaneous synthesis of variant fragments of a human N-ras gene as templates for the preparation of related probes. A,B= relevant segments of N-ras protein and gene; C,D=sequences of general replication primer and variant templates; E=list of sequentially variant templates (from 3'-end); F=computerized elongation scheme.

Table 2: Yields of oligonucleotide sequences used as templates for the preparation of probes for detection of N-ras gene mutations (sequence numbers correspond to Fig.5)

no. of sequence	Yield of purified probes (O.D. 260)
1	1.06
2	1.84
3	0.70
4	2.89
5	2.20
6	1.00
7	2.80
8	1.65
9	0.45
10	0.96
11	0.53
12	0.10
13	1.76

The oligonucleotides were purified by standard workup[11] and their correct sequence established by the "wandering spot" resp. Maxam-Gilbert technique. The yields are given in Table 1.

Hybridization Probes: In the preparation of primers and probes for hybridization to biological nucleic acids on the basis of a known peptide sequence the degeneracy of the genetic code often requires the screening of a large number of sequentially variant fragments in order to establish a perfect match. The possibility of wobble pairing and the insertion of mixed bases at critical positions has been the only way out of this dilemma. In such cases the strategy of simultaneous synthesis allows to rapidly generate a series of individual probes of variant sequence. This was examplified for the preparation of a set of sequences required to test for mutant N-ras genes. N-ras is among a group of genes, which have been identified as onco-genes in the human genome[12], and mutations of aminoacids 12, 13, 59, 61 or 63 have been found in malignantly transformed cells.[13]. In order to test such mutations by standard hybridization techniques eicosanucleotides of different sequence have been prepared as mixed probes[14]. Although these mixed probes permitted to

detect the occurence of a mutation, they did not in all cases allow to identify the nature of the mutated amino a first study we now targeted possible mutations at amino acids 12 and 13, and, in order to account for all relevant variations, prepared a set of 13 individual eicosamer sequences according to the scheme shown in Figure 5. These sequences serve as templates, from which, with the help of a general primer, probes for mutations can be prepared deliberately by the use of DNA-polymerase and deoxynucleoside-triphosphates. The synthesis of these template sequences was done according to the elongation scheme given in Fig.5 following the usual phosphoramidite protocol[11]. The oligonucleotides were purified and characterized as previously[11], and their yields are given in Table 2.

Other series of probes have been prepared similary, and their synthesis together with the hybridization studies will be published elsewhere[15].

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