protogene

Department of Energy

Final Technical Report – DOE/ER/62061--T1

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Recipient: Protogene Laboratories, Inc.
Project Director: Thomas M. Brennan

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1. Introduction and Statement of Problem

The subject of this project is to address a pressing need for custom DNA microarrays ("chips") which can be easily and at low cost formatted and revised for research. In this sense, the term "custom" means chips for which there is need for limited quantities (less than hundreds) of any particular chip design which contains a large number of different, user defined sequences.

Of the three principal approaches to fabricate DNA microarrays, the two which have been commercialized (a and b below) are not particularly suited to research purposes because of the significant time and costs required, once a result is obtained, to utilize that result in the design of a new and better chip. :

a. the photodeprotection scheme used by Affymetrix
   Minuses: -high cost of masks and aligners,
   -high cost of synthesis
   -reduced step yield in synthesis means lower quality of oligos and more rigorous analysis of hybridization specificity results
   Plusses: very high density of oligo probes

b. the spotting of pre-synthesized oligos or c-DNA onto surfaces
   Minuses -cost and availability of a large number of oligos or c-DNAs, when it is not clear that many chips with the same raw materials are needed,
   -the tendency, because of high costs to change, of standardizing chip design without verification and validation of optimal designs,
   -the logistics of handling large numbers of samples.
   -the limitations of c-DNAs for detecting SNPs or small mutations
   -the requirement for well-controlled covalent attachment chemistries for smaller DNAs

pg final

DISCLAIMER

Pluses plotter-type arraying machines are relatively simple with low fabrication cost for large quantities of chips.

The methods pursued in this grant work (c below), have resulted in a new, more flexible, less costly approach to create DNA chips e low volume research use.

c. the in-situ synthesis of oligos on a glass surface by a drop-on demand array synthesizer
Minuses: -custom synthesis of one or a few chips at a time is not amenable to very low cost production for very large volumes of a single chip design
Plusses: -Dramatically lower low cost per design,
-Flexibility in design research
-Fast turnaround,
-Higher quality DNA and better quantification of DNA on surface.

2. Protogene Approach to In-situ Synthesis of Oligo Arrays (See Figures 1)

1) Surface tension differences between a non-reactive mask and the individual synthesis locations are used to maintain separation of the liquid contents among the sites. (Figures 2)
2) Drop-on-demand ink jets are used to deliver droplets of reagents to an array of synthesis sites. The principal advantage of in-situ synthesis is the infinite flexibility in chip design. DNA synthesis can be viewed simply as a specialized 4-color printing application. (Figure 3)
3) In order to change the design of the chip, simply change the input file With a 99.5% step yield, it should be possible to synthesize oligos up to 75-mers in good yield
4) To optimize hybridization results, common variants of length and base composition can be easily made..

3. Patterning of Glass Substrates by Photolithography

A positive photoresist is used to define an array of synthesis sites, and then exposed areas are masked with a very hydrophobic, non-reactive perfluoroalkyl silane (Figure 4). After the photoresist is lifted off, a more hydrophylic silyl linker arm is attached to the synthesis sites. (Figure 5)The fluorosilane mask region is sufficiently hydrophobic and lipophobic that it is not wet by polar organic solvents. Droplets of applied reagents tend to bead up and self-register into the more polar synthesis sites.

4. The Linker Arm

The penultimate attachment of the oligo to be synthesized on the glass substrate is through an aminopropyl silane. In order to minimize effects of the glass on hybridization characteristics, the linker arm can be extended with multiple ethylene glycol units. The extended linker is terminated with either a hydroxyl or

amino group, upon which subsequent conventional phosphoramidite oligo synthesis can be initiated.

5. Mechanical Design of the Synthesizer

A square glass chip has been used for synthesis (Figure 6). The same edges of the chip are used for alignment both during photopatterning and then during the actual oligo synthesis. An x-y-z stage is used to transport the chip under the appropriate nozzles during synthesis. The common reagents for capping, oxidation, detritylation and washing are flooded across the surface. The chip holder itself is mounted on a rotary stage so that spent reagents can be removed from the surface simply by spinning the chip.

A bank of 5 piezoelectric impulse jets are used to deliver droplets of the 4 amidites and activator. The jets are operated at a frequency of about 6600 Hz, and eject 55 micron droplets at about 1 m/sec. As the chip is rastered back and forth about 1 mm beneath the nozzles during the coupling cycle, the jets are fired ballistically at the moving surface. The complete coupling pattern for a 1600 oligo chip can be applied in approximately 90 sec using a single bank of nozzles. The spacing or density of synthesis sites which can be achieved is determined by the aiming accuracy of the jets. The densest pattern which can be reliably employed with the current setup is probably about 100 micron targets on 200 micron centers, or 2500 oligos/cm^2.

6. Chemistry

The synthesis reagents employed are those used in conventional solid phase amidite chemistry. A large molar excess of the amidite to linker (about 50,000 X) is used to drive the coupling reaction to completion. Since small drops of acetonitrile tend to evaporate in flight, a higher boiling organic solvent has been substituted.

In order to access the quality of oligos which are synthesized, the inclusion of a cleavable linkage would permit analysis of pooled material by CE. The standard LCAA succinate linker is not sufficiently stable to permit selective side chain deprotection without cleavage of the oligo from the surface. However, a phosphoramidate linkage, which results when the first coupling is to an aminoalkyl rather than hydroxyalkyl residue, is stable to basic sidechain deprotection conditions but readily cleaved by mild acid. Alternatively, the entire assemblage (of silane linker still coupled to the oligo) can be cleaved from the glass by mild fluoride treatment to give the silyl trifluoride derivative. Two different oligos made on alternating sites on a chip are cleaved, and analyzed via capillary electrophoresis (Figure 7).

7. Hybridization Characteristics

A two-color fluorescent laser confocal scanner has recently been assembled based on the design from the labs of Pat Brown and Mike Eisen at Stanford University (available from the lab web site). Software using color coding to indicate location of different sequences on the chip has been developed (Figure 9). Figure 9 shows the specific hybridization of labeled target oligonucleotides. Figure 10 shows a complex pattern of mixed sequences of differeing lengths, with specific hybridization results shown in Figure 11.

These experiments indicate that the quality of oligos which have been synthesized on the surface mirror the results obtained by cleavage and pooled CE analysis.

Acknowledgments:

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Electronics:	Klaus Schaumbeck
Mechanical Design:	Mike Cummer
Hybridization:	Maureen Cronin Mylan Pho

1. Array Design Considerations
2. Surface Tension Array Element
3. Piezoelectric Jet
4. Photo Patterning of Glass
5. Creation of Array Surface
6. Square Chip Designs
7. CE of pooled T-10 + G-10
8. Patterned Synthesis of poly(T) poly(A) poly(TA)
9. Differential Hybridization of Poly_A / Poly_T
10. Patterned multiple mixed sequence
11. Selective hybridization of mixed sequence labeled targets

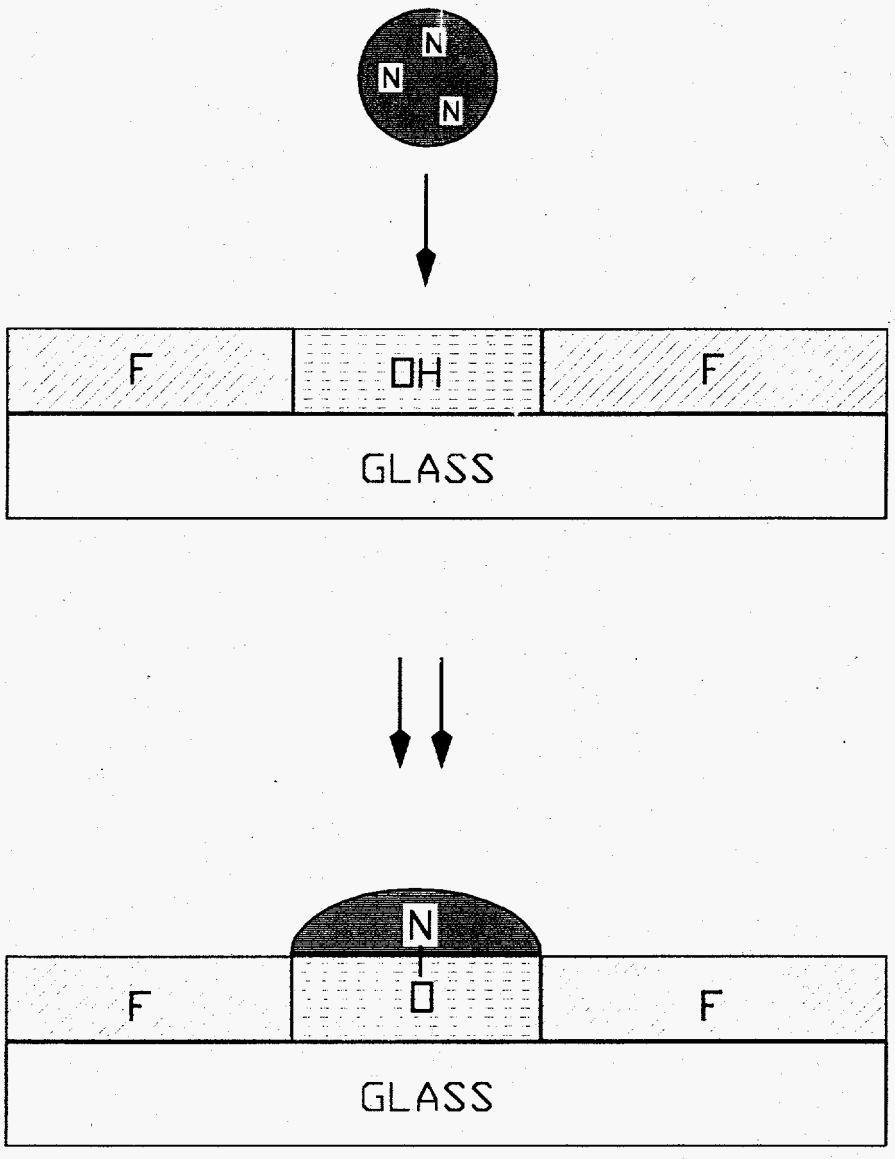
Protogene Approach to Arrays

- Surface tension localizes synthesis; non-wettable hydrophobic matrix separates array elements
- Conventional phosphoramidite chemistry
- Reagent "printing" with piezoelectric jets
- High boiling point solvents and spin-coating for bulk reagent application

Figure 1

Figure 2

SURFACE TENSION ARRAY ELEMENT



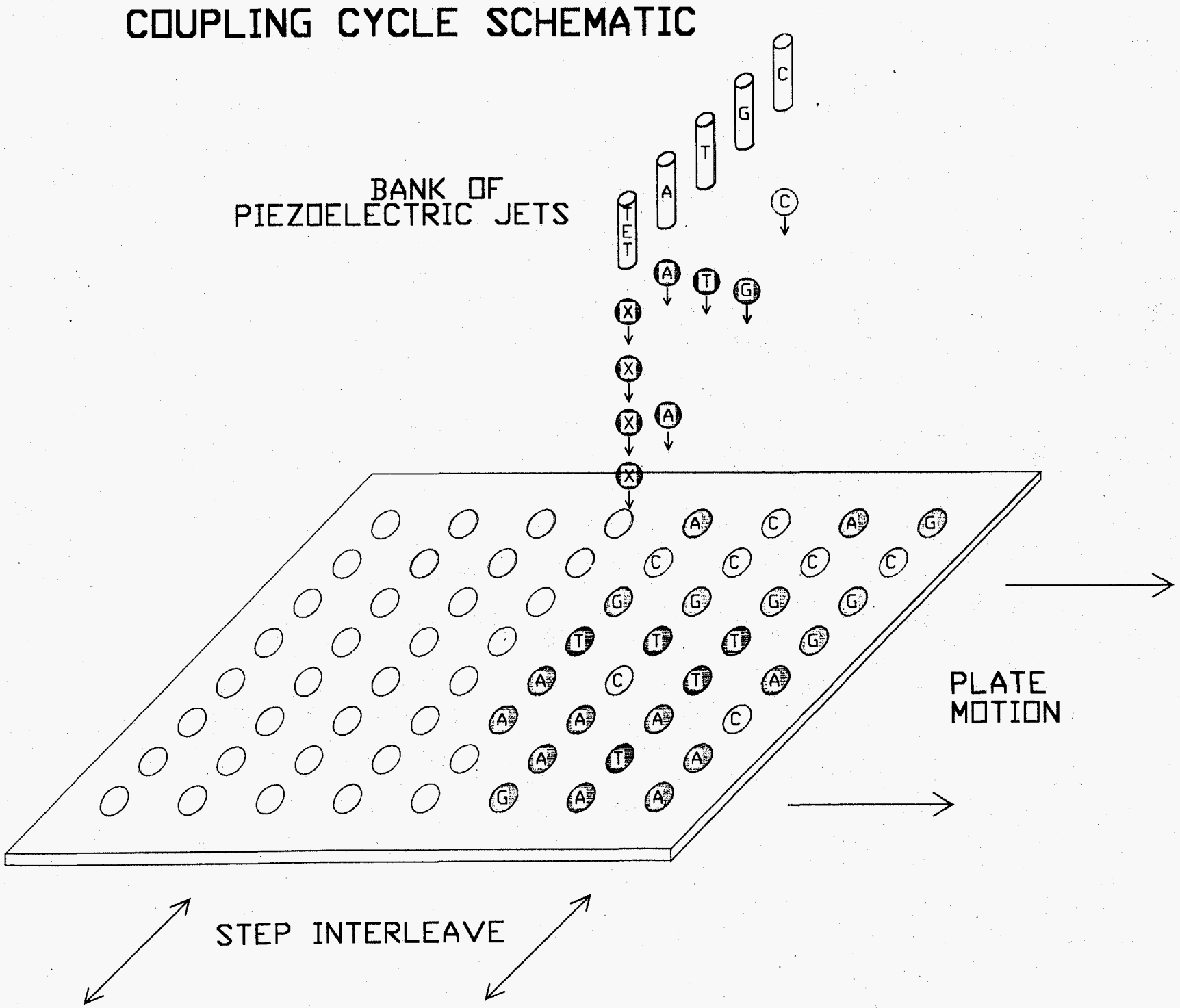
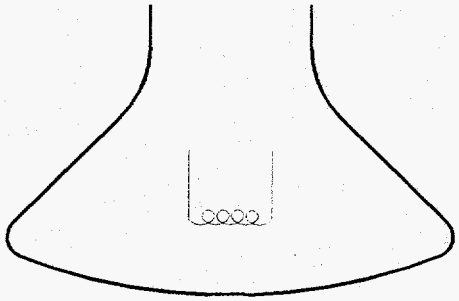

COUPLING CYCLE SCHEMATIC
BANK OF
PIEZOELECTRIC JETS
TET
A
T
G
C
X
PLATE
MOTION
STEP INTERLEAVE

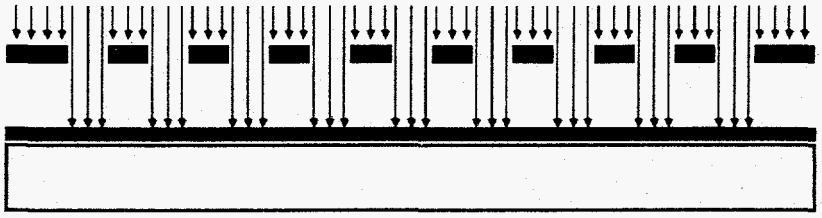
Figure 3

Figure 4

PHOTO PATTERNING OF GLASS



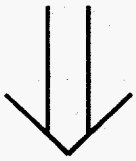
UV LAMP



MASK

PHOTORESIST

GLASS

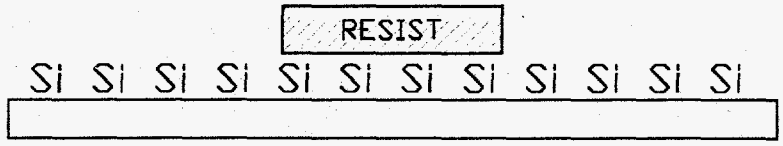


DEVELOP

GLASS WITH
PATTERNED
PHOTORESIST

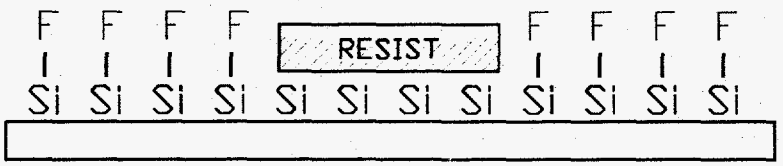
Figure 5

CREATION OF ARRAY SURFACE



PATTERN GLASS CHIP

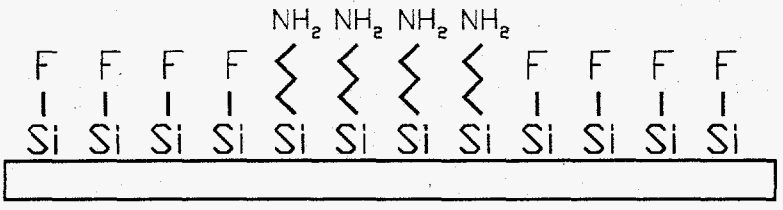
F = Perfluoro Octyl Silane



COUPLE HYDROPHOBIC MASK

REMOVE RESIST

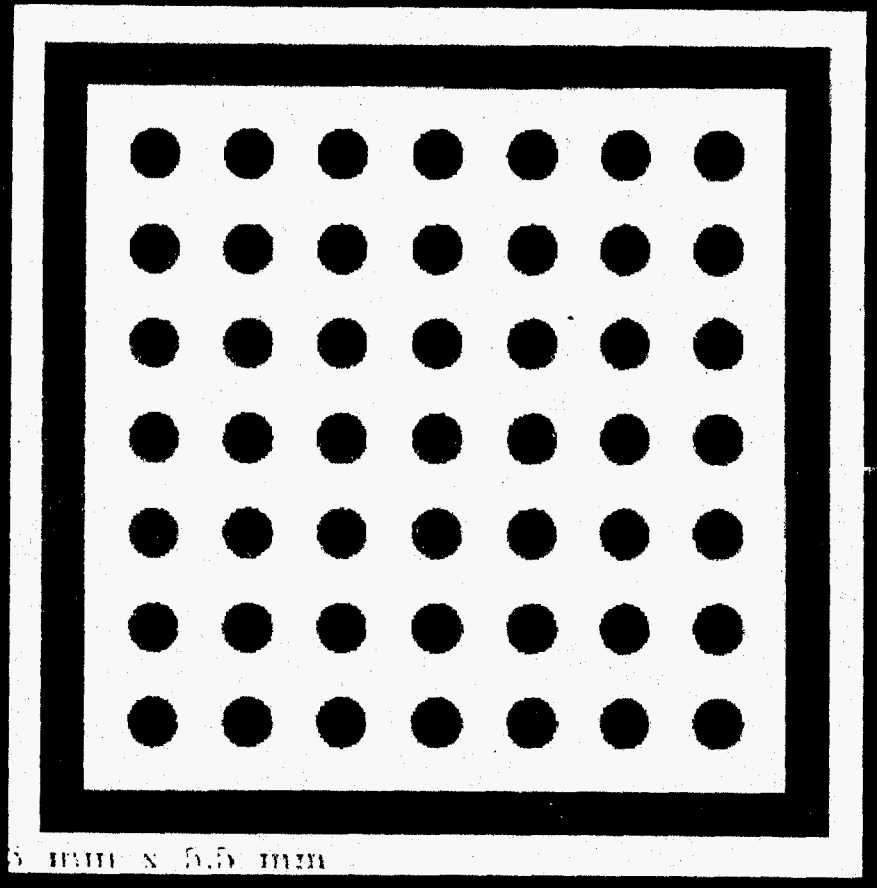
NH_2 = Aminopropyl Silane



ADD LINKER

CHIP READY FOR OLIGO SYNTHESIS

Lithography Patterning Masks



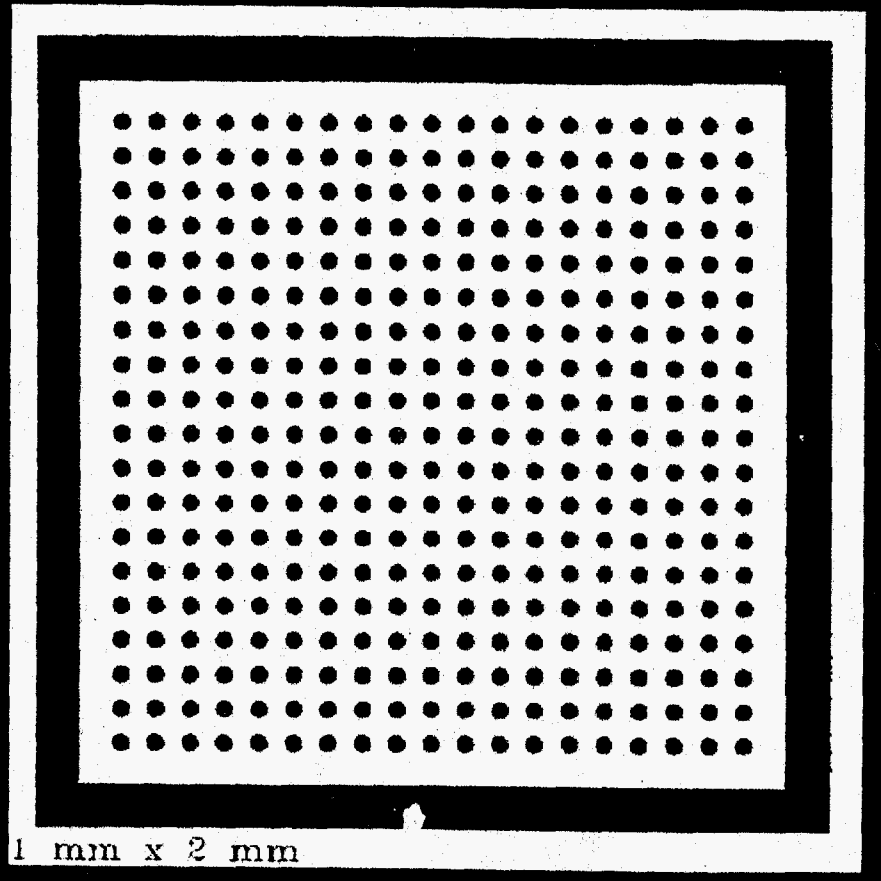


Figure 6

Figure 7

CAPILLARY ELECTROPHEROGRAM
UV DETECTOR
OF POOLED SYNTHESIS
T-10 + G-10
USING PAC-AMIDITES

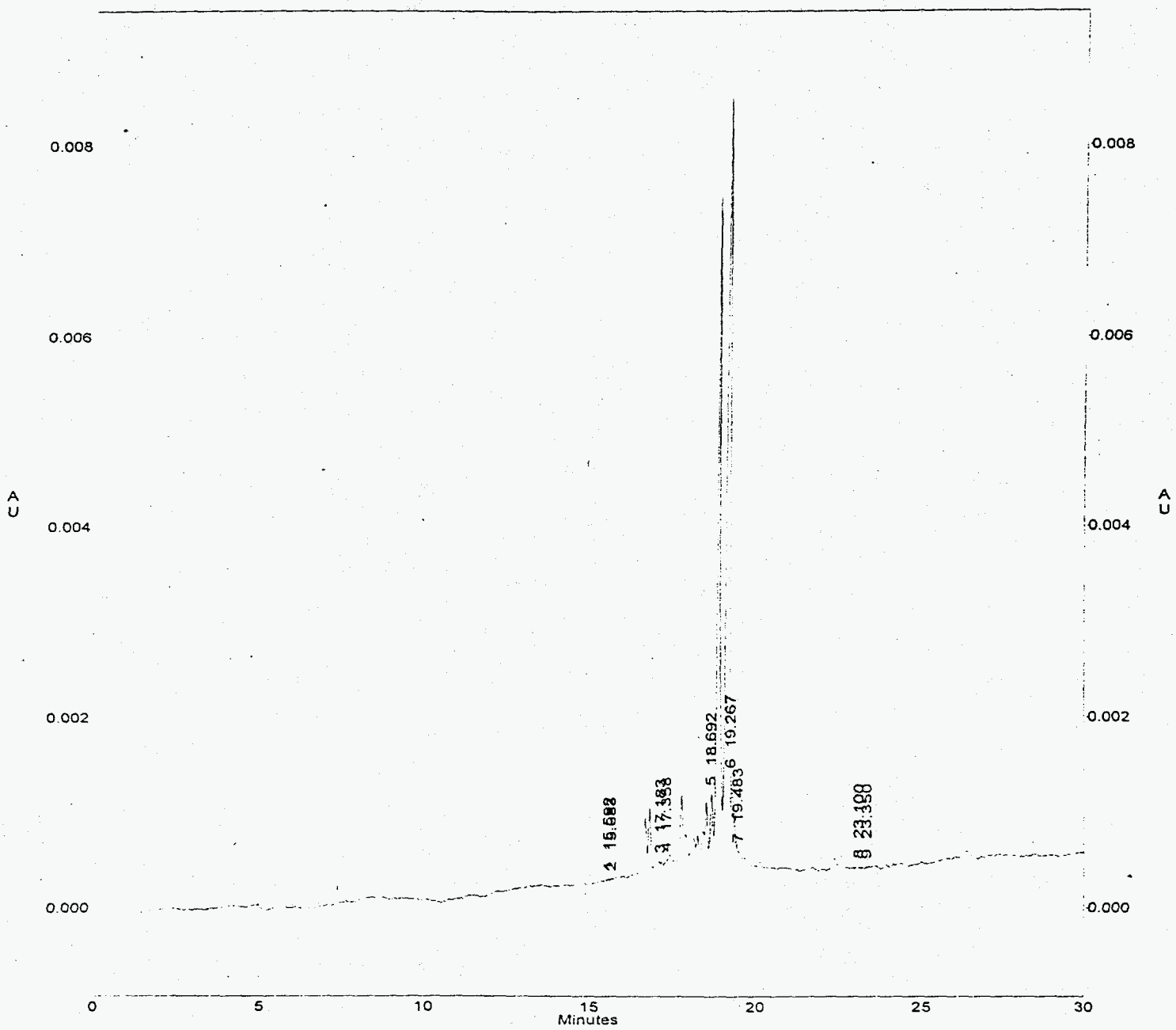


Figure 8

PATTERNED SYNTHESIS OF poly(T) + poly(A) + poly(TA)

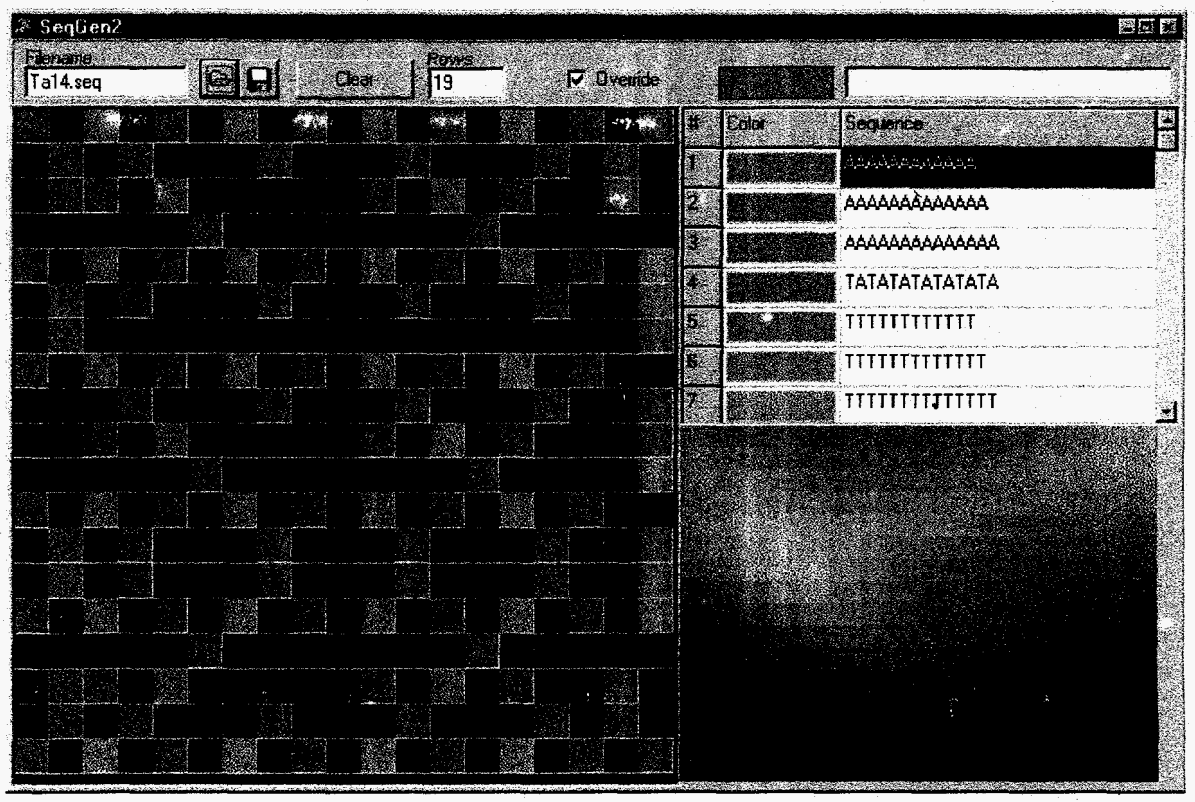
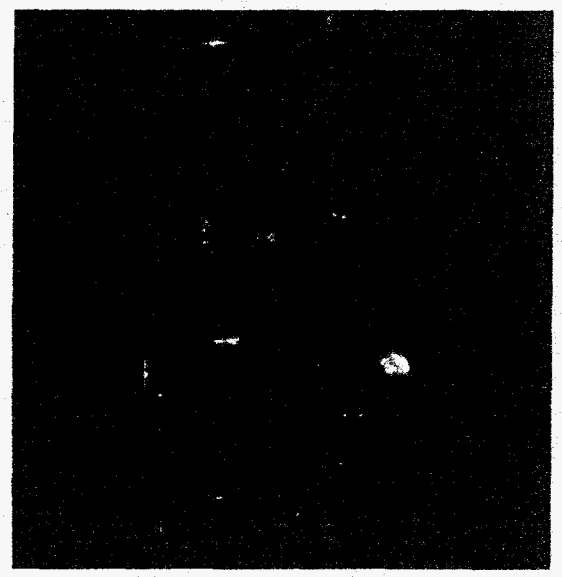
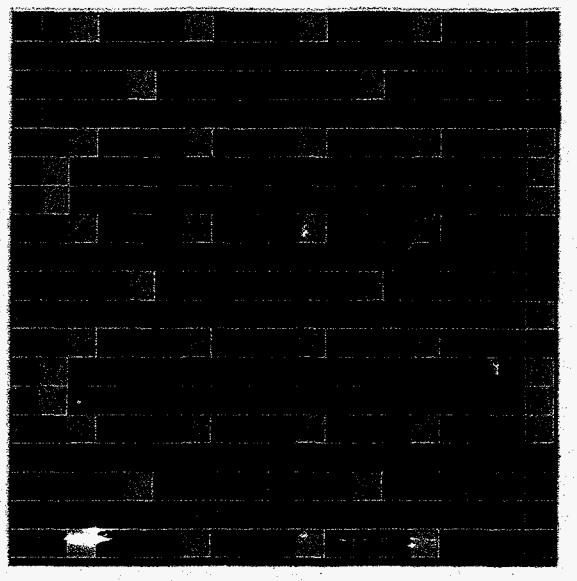


Figure 9

DIFFERENTIAL HYBRIDIZATION

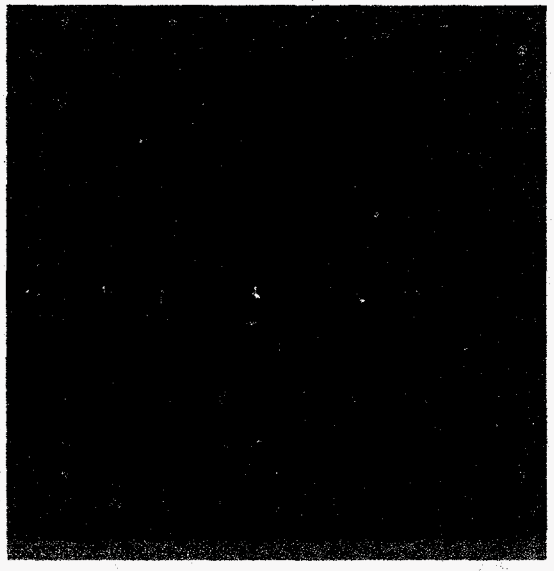


A20-Cy5 50% EtOH / 3M TMACl Hybridization

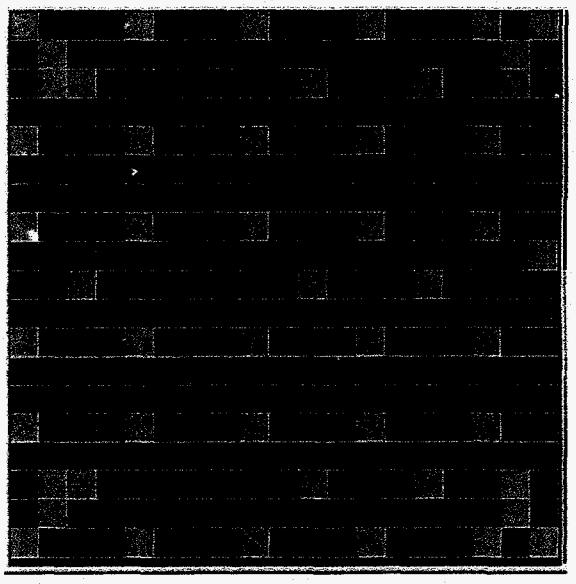


T14 Synthesis Pattern

Strip and Reprobe



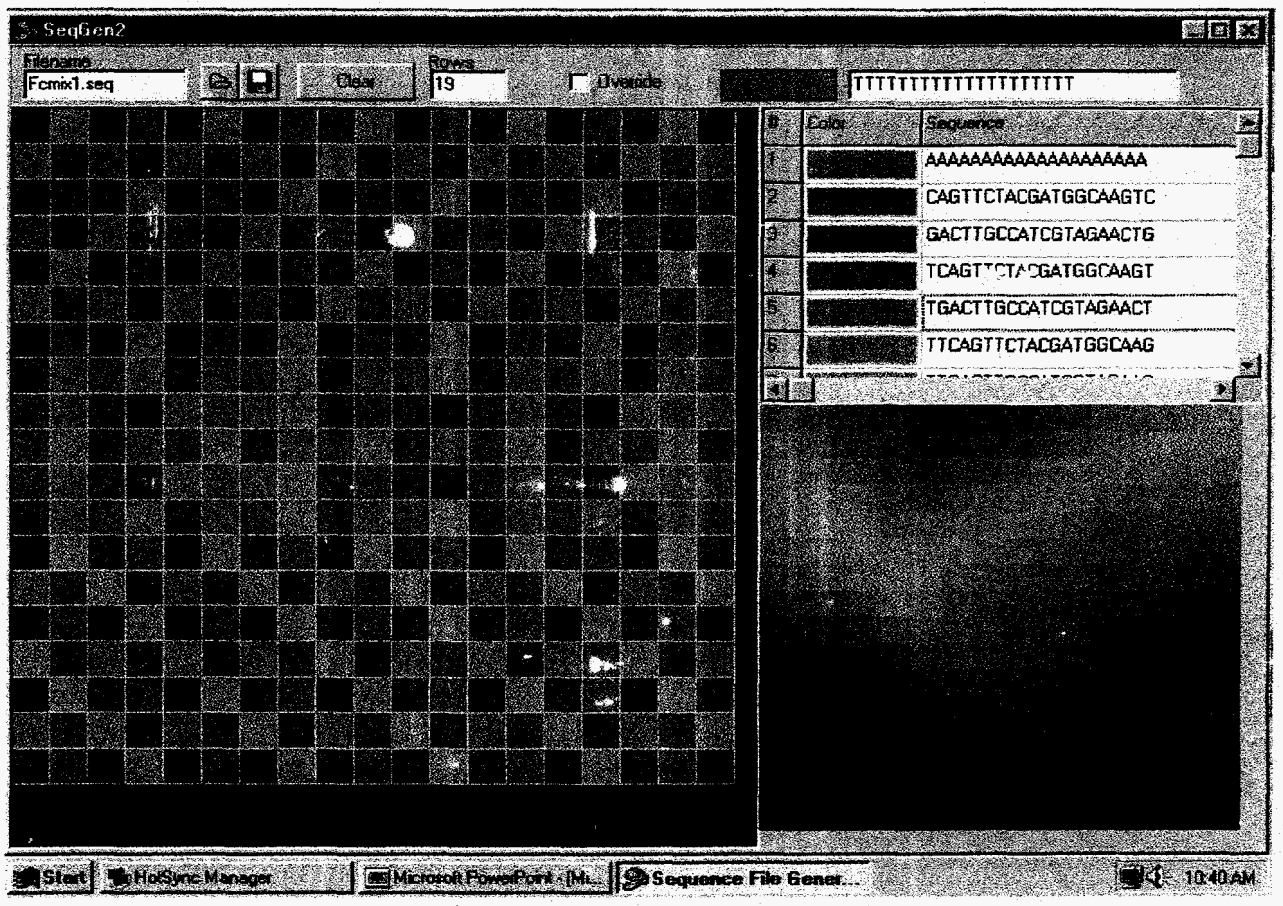
T20-Cy5 50% EtOH / 3M TMACl Hybridization



A14 Synthesis Pattern

Figure 10

Patterned synthesis of mixed sequences



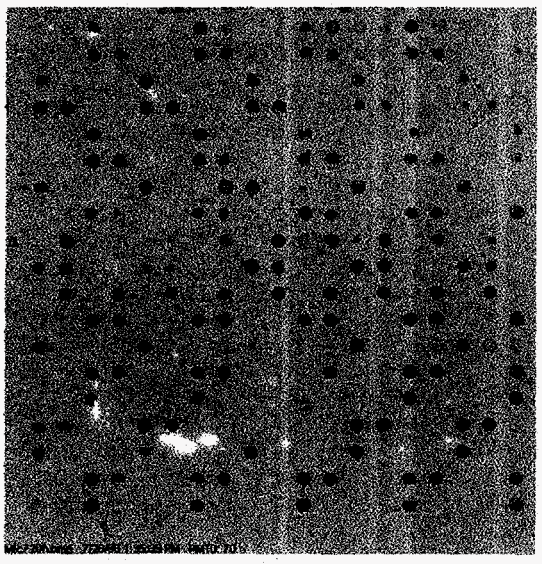
Sequences

CAGTTCTACGATGGCAAGTC
TCAGTTCTACGATGGCAAGT
TTCAGTTCTACGATGGCAAG
TTTCAGTTCTACGATGGCAA
TTTTCAGTTCTACGATGGCA
TTTTTCAGTTCTACGATGGC
TTTTTTTTTTTTTTTTTTT

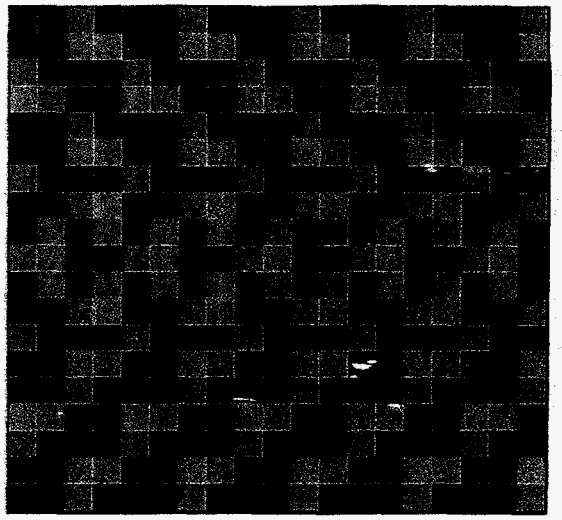
GACTTGCCATCGTAGAACTG
TGACTTGCCATCGTAGAACT
TTGACTTGCCATCGTAGAAC
TTTGACTTGCCATCGTAGAA
TTTTGACTTGCCATCGTAGA
TTTTTGACTTGCCATCGTAG
AAAAAAAAAAAAAAAAAAA

Figure 11

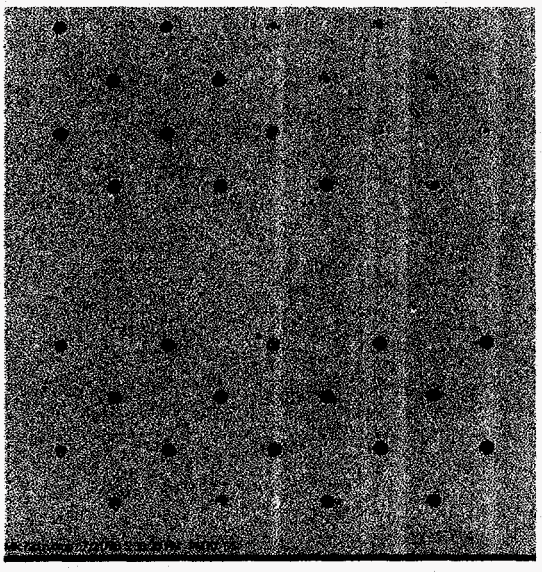
Differential Hybridization



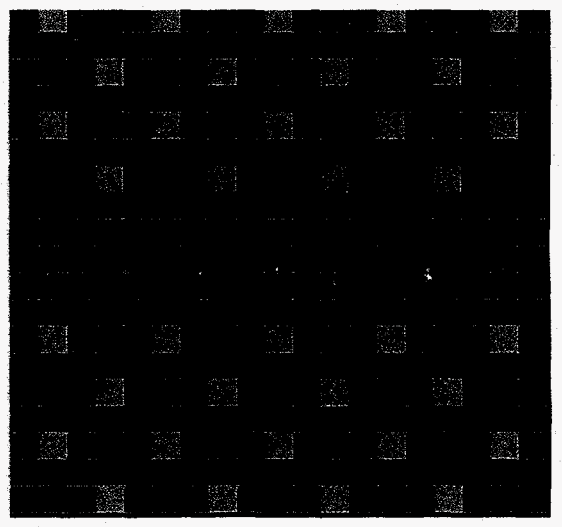
Hybridization
Target : d(CAGTTCTACGATGGCAAGTC)



Synthesis Pattern



Hybridization
Target : A20-Cy3



T20 Synthesis Pattern