US007049073B2

(12) **United States Patent**
Chernov et al.

(10) **Patent No.: US 7,049,073 B2**
(45) **Date of Patent: May 23, 2006**

(54) **DOUBLE STRANDED NUCLEIC ACID BIOCHIPS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 103 days.

(21) Appl. No.: **10/283,670**

(22) Filed: **Oct. 30, 2002**

(65) **Prior Publication Data**

US 2004/0086866 A1 May 6, 2004

(51) **Int. Cl.**
C12Q 1/68 (2006.01)
G01N 33/543 (2006.01)
G01N 33/554 (2006.01)

(52) **U.S. Cl.** **435/6**; 435/DIG. 42; 435/DIG. 40; 435/DIG. 34; 435/DIG. 37; 435/DIG. 2; 435/DIG. 14; 435/DIG. 17; 435/174; 435/180; 436/518; 436/528; 436/529; 536/23.1; 536/25.3; 549/223; 549/227

(58) **Field of Classification Search** ..... 536/23.1, 536/25.3, 25.32; 546/25, 102; 549/223, 549/227; 435/6, DIG. 2, DIG. 34, DIG. 40, 435/DIG. 42; 436/518, 528, 529
See application file for complete search history.

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Assistant Examiner—My-Chau T. Tran
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(57) **ABSTRACT**

This invention describes a new method of constructing double-stranded DNA (dsDNA) microarrays based on the use of pre-synthesized or natural DNA duplexes without a stem-loop structure. The complementary oligonucleotide chains are bonded together by a novel connector that includes a linker for immobilization on a matrix. A non-enzymatic method for synthesizing double-stranded nucleic acids with this novel connector enables the construction of inexpensive and robust dsDNA/dsRNA microarrays. DNA-DNA and DNA-protein interactions are investigated using the microarrays.

21 Claims, 16 Drawing Sheets

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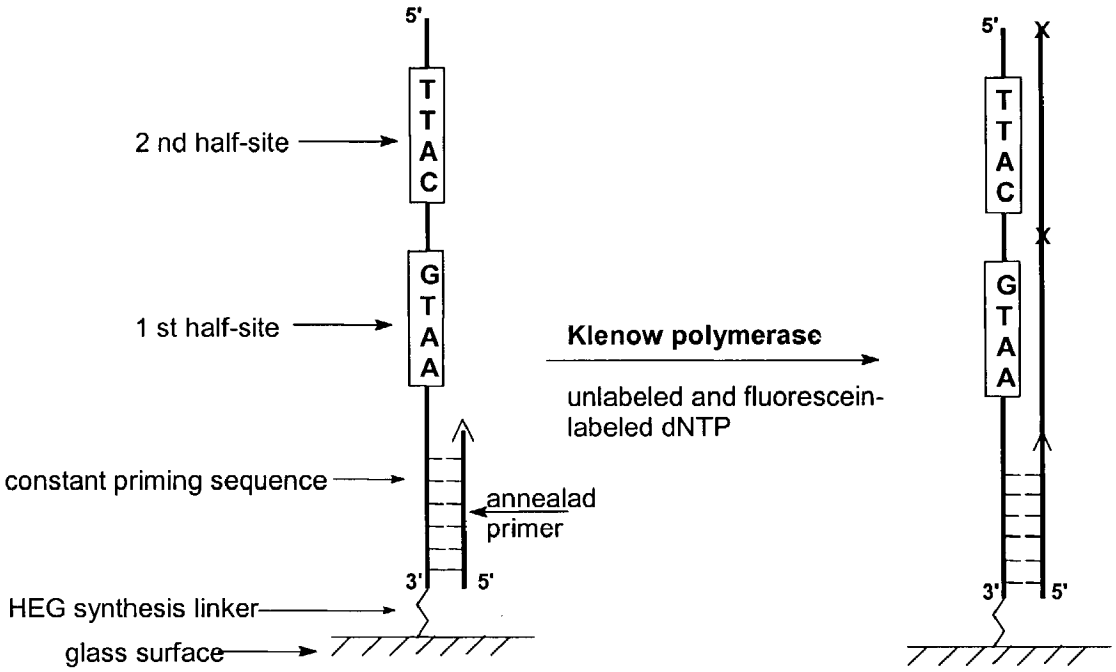

5'
TTAC
2 nd half-site
GTAA
1 st half-site
constant priming sequence
annealad
primer
3'
5'
HEG synthesis linker
glass surface
Klenow polymerase
unlabeled and fluorescein-
labeled dNTP
5'
TTAC
GTAA
3'
5'

FIG. 1

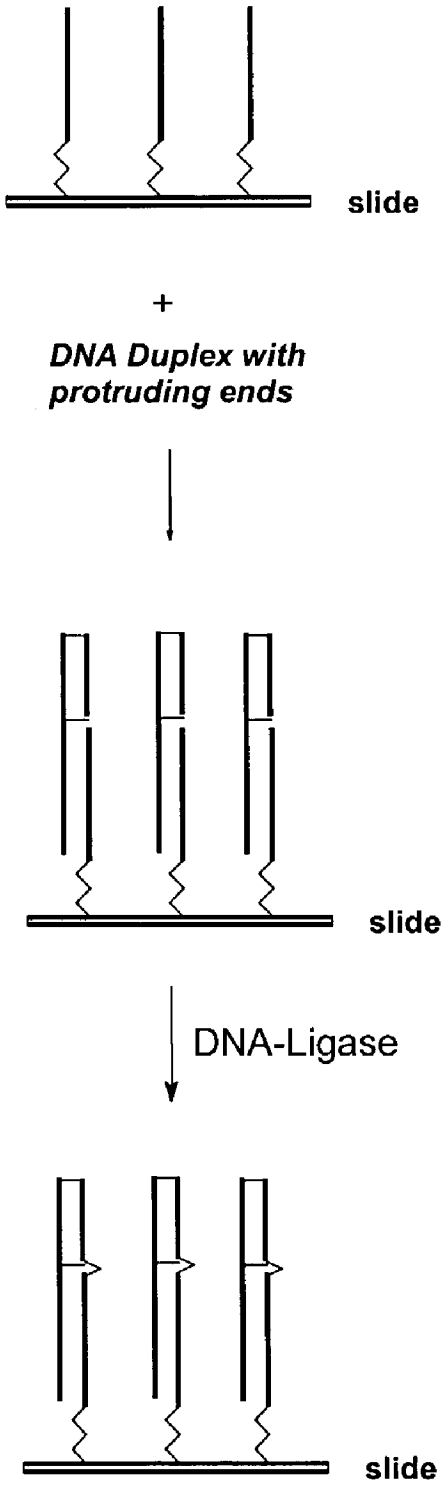

slide
+
DNA Duplex with protruding ends
slide
DNA-Ligase
slide

FIG. 2

$T(CH_2)_6SS(CH_2)_6$-3'-CCC TTT TCC TTA AGC TTT TTT TCC CC-5'--F*
$HO(CH_2)_6SS(CH_2)_6$-5'-GGG AAA AGG AAT TCG AAA AAA AGG GG-3' (SEQ ID NO: 1)

ECoRI

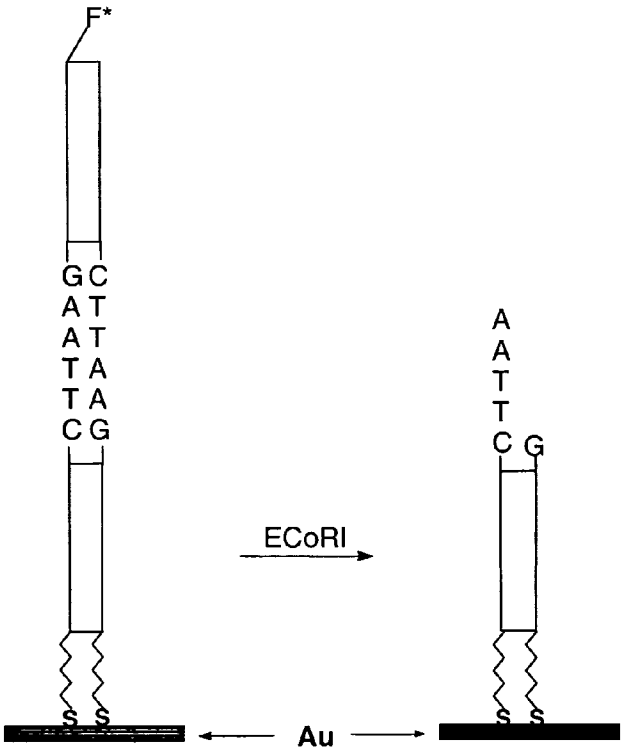
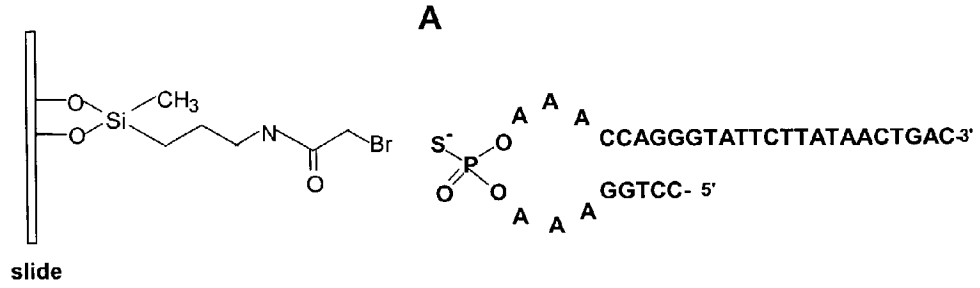


FIG. .3


A
slide
CCAGGGTATTCTTATAACTGAC-3'
GGTCC- 5'

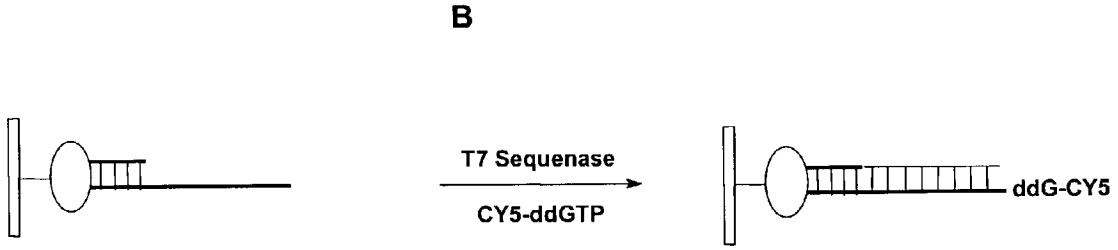

B
T7 Sequenase
CY5-ddGTP
ddG-CY5

FIG. 4

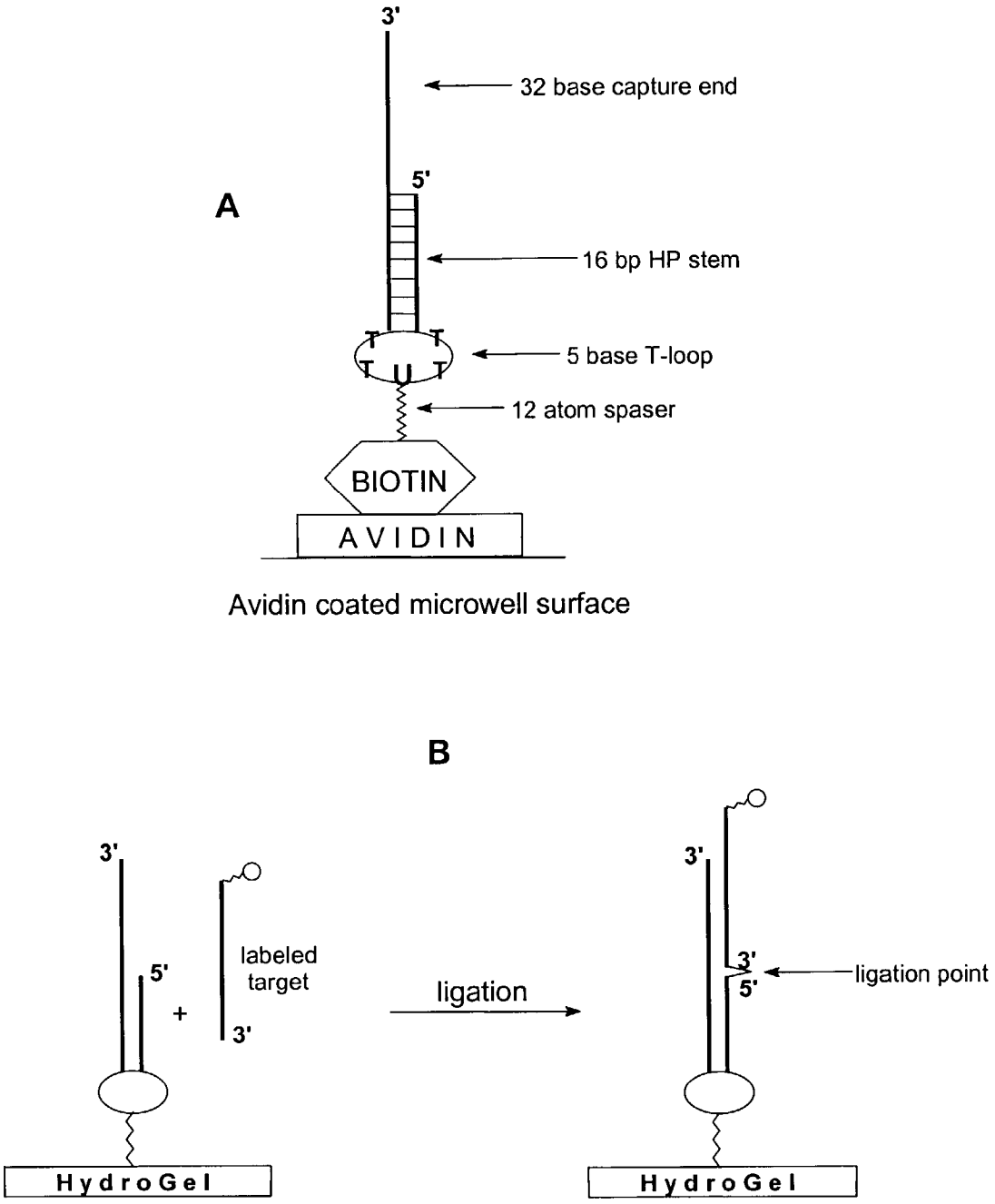
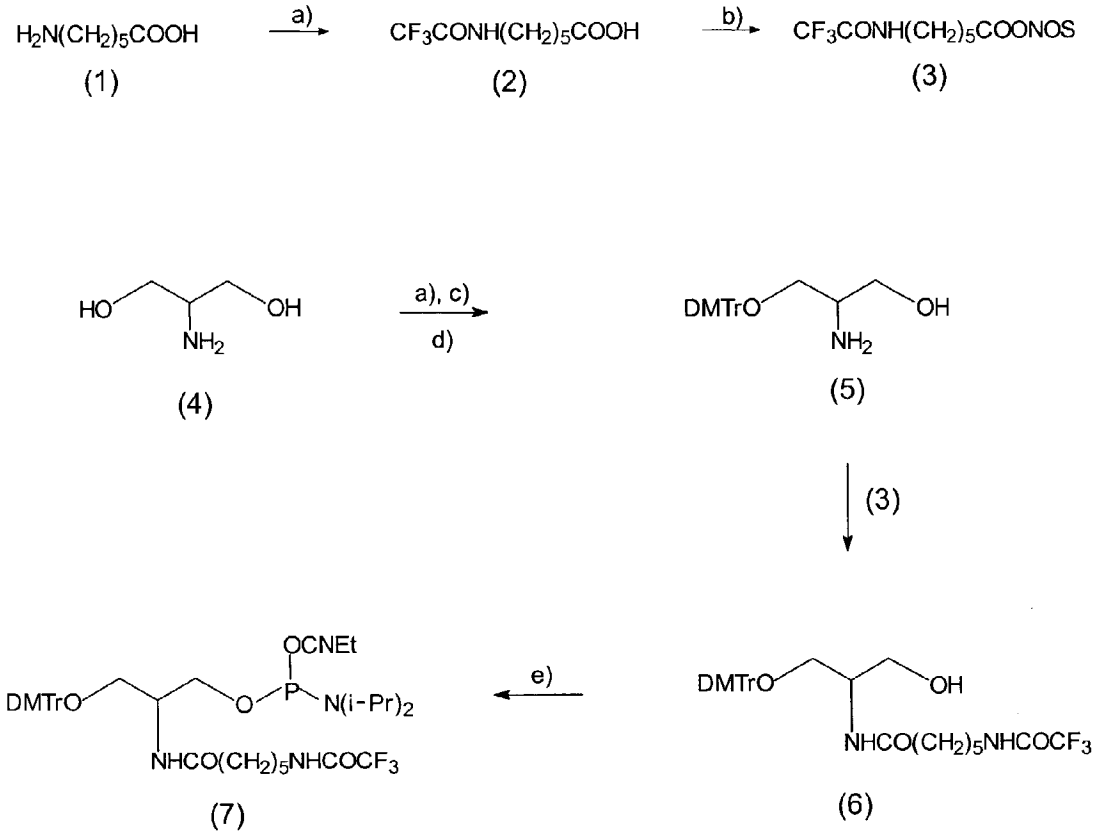

A
3'
32 base capture end
5'
16 bp HP stem
T T T T U
5 base T-loop
12 atom spaser
BIOTIN
AVIDIN
Avidin coated microwell surface
B
3'
5'
+
labeled target
3'
ligation
HydroGel
3'
3'
5'
ligation point
HydroGel

FIG. 5

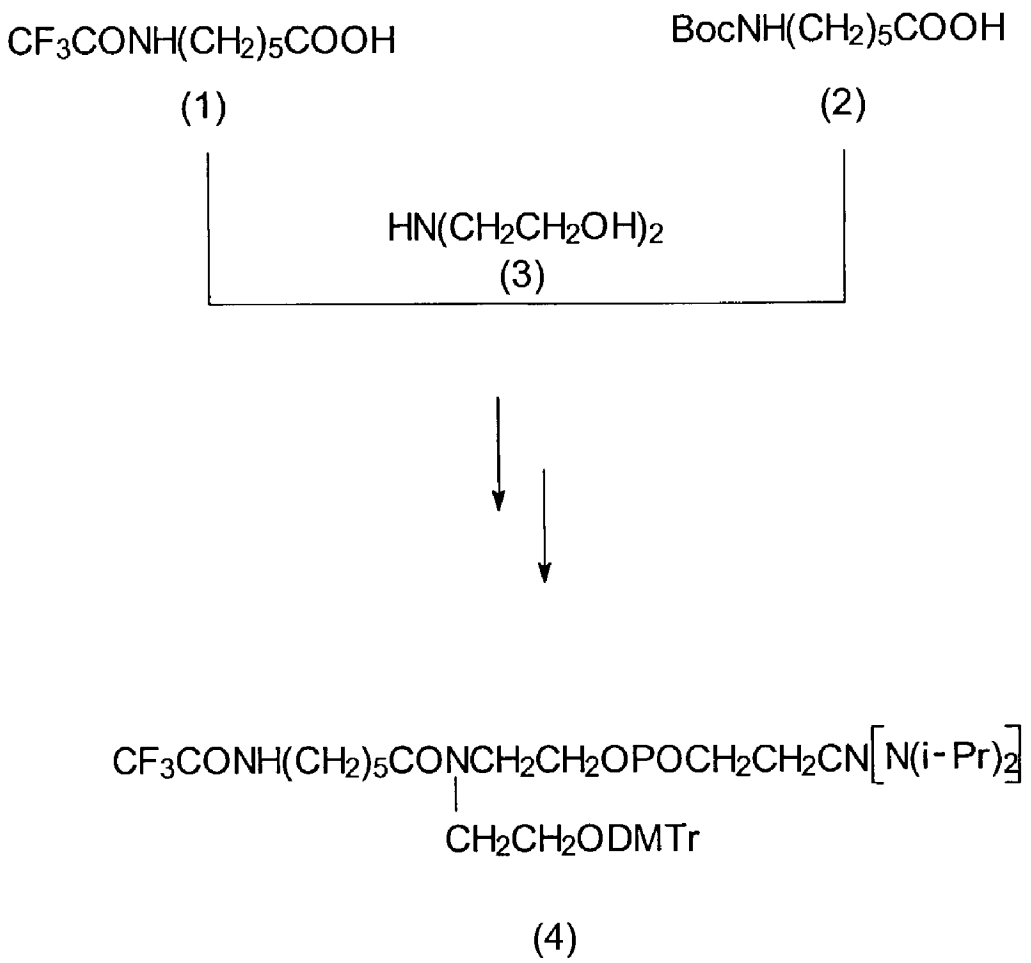


Scheme1

The strategy of synthesis of connector phosphoramidite (7)

Reagents: a) Ethyl trifluoroacetate
b) N,N-Dicyclohexylcarbodiimide/N-Hydroxysuccinimide
c) 4,4'-Dimethoxytrityl chloride
d) Methylamine
e) 2-Cyanoethyl diisopropylchlorophosphoramidite/ N,N-Diisopropylethylamine

FIG. 6



Scheme 2

Preparation of connector phosphoramidite (4)

FIG. 6 cont.

1. L
```
      CfoI        HaeIII
TT GCGCTCGAGGCC
TT CGCGAGCTCCGG
          XhoI
```
(SEQ ID NO: 3)

L - Connector

2. L
```
      CfoI        HaeIII
TTTT GCGCTCGAGGCC
TTTT CGCGAGCTCCGG
          XhoI
```
(SEQ ID NO: 4)

3. L
```
      CfoI        HaeIII
TTTT GCGCTCGAGGCC
TTTT CGCGAGCTCCGG-NH-FITC
          XhoI
```
(SEQ ID NO: 5)

FITC - fluorescent label

4. L
```
      HindIII    EcoRI
TT(A)nAAGCTTCGAATTCGA GG
TT(T)nTTCGAAGCTTAAGCT CC
          Csp      TagI
```
(SEQ ID NO: 6)

n=4

5. L
```
      HindIII    EcoRI
TT(A)nAAGCTTCGAATTCGA GG
TT(T)nTTCGAAGCTTAAGCT CC-NH-FITC
          Csp      TagI
```
(SEQ ID NO: 7)

n=4

FITC - fluorescent label

Scheme 3

FIG. 6 cont.

1. L T T C TATCACCGCCAGAGGTA GC 3'
T T G ATAGTGGCGGTCTCCAT CG 5' (SEQ ID NO: 8) **OR** (var.1)

L - Connector

2. L T T A_nTATCACCGCCAGAGGTA GC 3'
T T T_nATAGTGGCGGTCTCCAT CG 5' (SEQ ID NO: 9) **OR** (var.2)

n=4

3. L T T C TTCCGAACGCAAGCTCA GC 3'
T T G AAGGCTTGCGTTCGAGT CG 5' (SEQ ID NO: 10) **Antisense I**

4. L T T A_nTTCCGAACGCAAGCTCA GC 3'
T T T_nAAGGCTTGCGTTCGAGT CG 5' (SEQ ID NO: 11) **Antisense II**

n=4

Scheme 4

FIG. 6 cont.

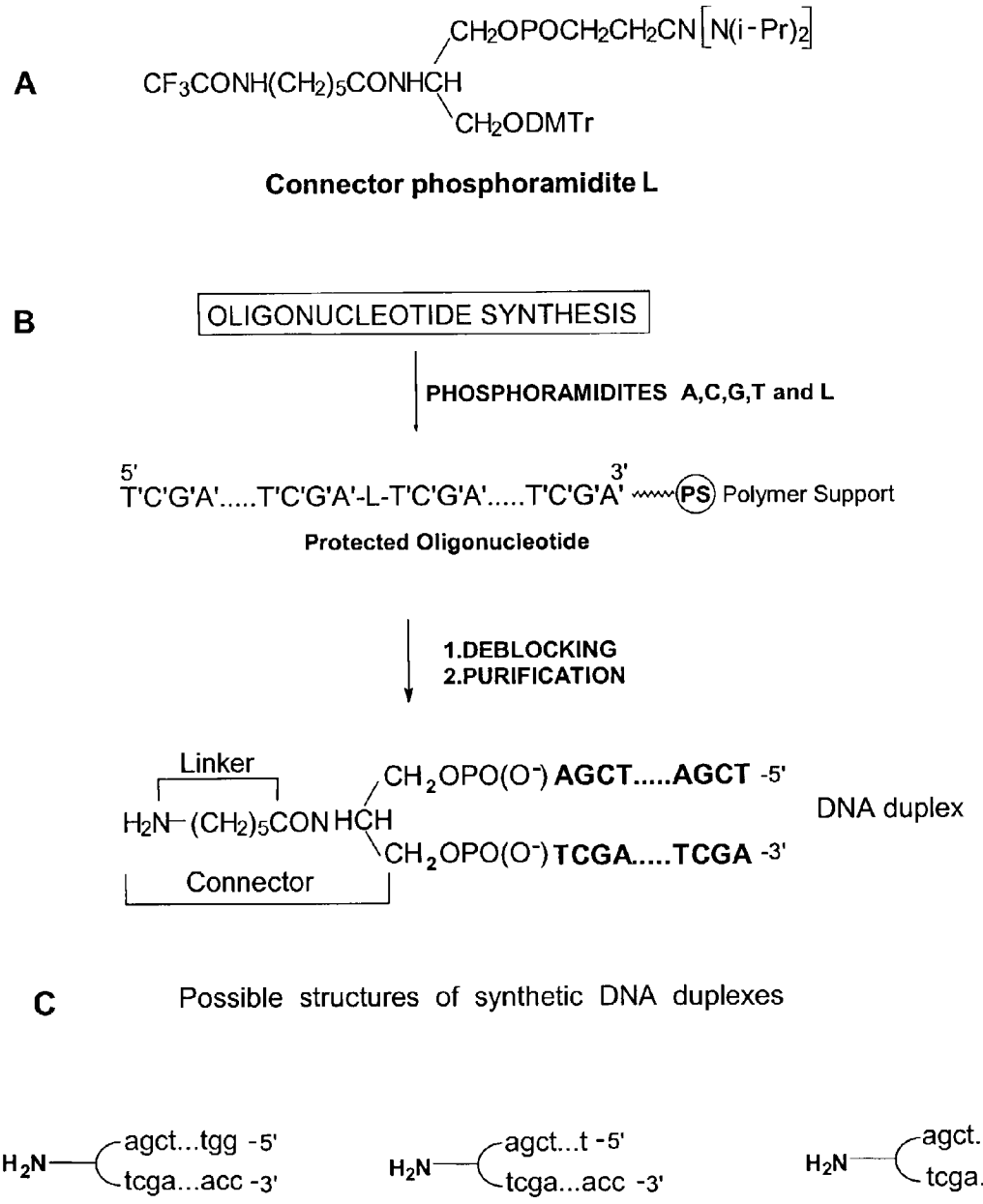
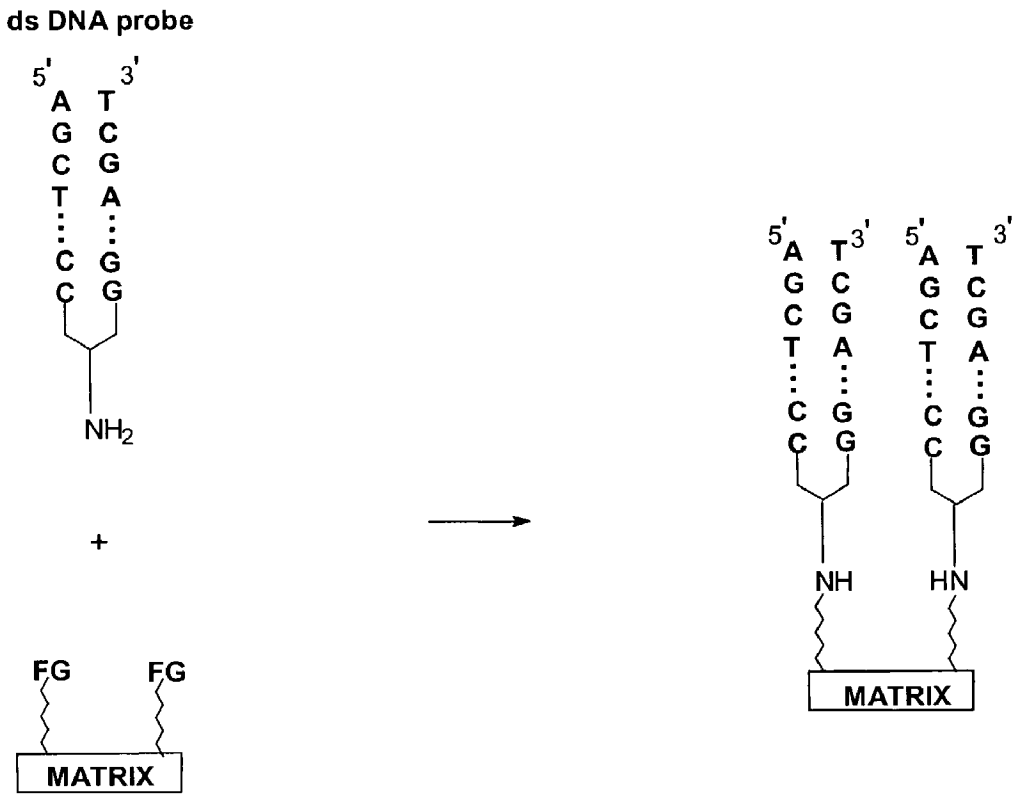

A
CF3CONH(CH2)5CONHCH
CH2OPOCH2CH2CN[N(i-Pr)2]
CH2ODMTr
Connector phosphoramidite L
B
OLIGONUCLEOTIDE SYNTHESIS
PHOSPHORAMIDITES A,C,G,T and L
5'
T'C'G'A'.....T'C'G'A'-L-T'C'G'A'.....T'C'G'A'
3'
PS
Polymer Support
Protected Oligonucleotide
1.DEBLOCKING
2.PURIFICATION
Linker
H2N-(CH2)5CONHCH
CH2OPO(O-) AGCT.....AGCT -5'
CH2OPO(O-) TCGA.....TCGA -3'
DNA duplex
Connector
C
Possible structures of synthetic DNA duplexes
H2N
agct...tgg -5'
tcga...acc -3'
H2N
agct...t -5'
tcga...acc -3'
H2N
agct...tgg -5'
tcga...a-3'

FIG. 7



MATRIX:

1. Polyacryamide gel pads
2. Hydro-gels
3. Glass surface (slides, beads)
4. Silicon surface
5. Gold surface (slides, beads)
6. Magnetic beads covered by latex containing carboxylic groups (COOH)

FIG. 8

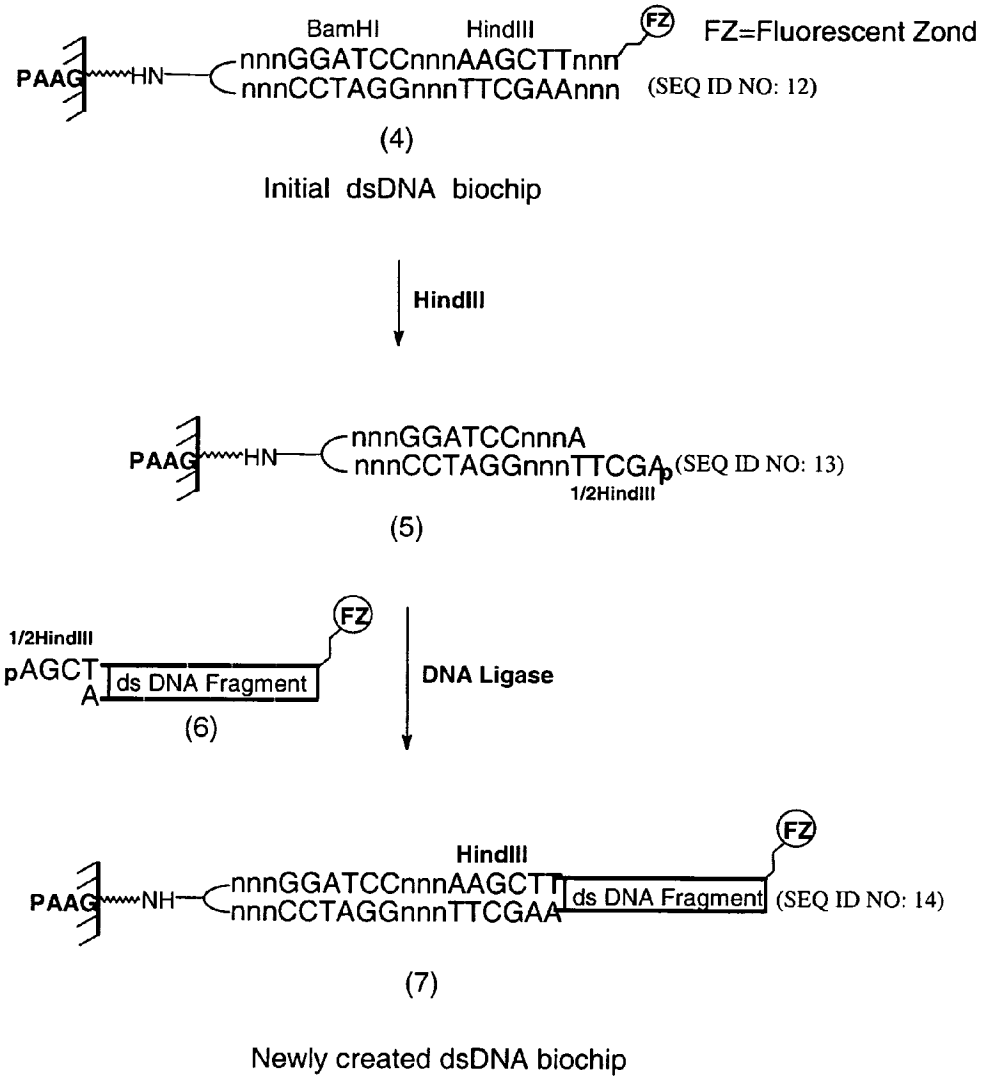

BamHI
HindIII
FZ
FZ=Fluorescent Zond
PAAG
HN
nnnGGATCCnnnAAGCTTnnn
nnnCCTAGGnnnTTCGAAnnn
(SEQ ID NO: 12)
(4)
Initial dsDNA biochip
HindIII
PAAG
HN
nnnGGATCCnnnA
nnnCCTAGGnnnTTCGAp
(SEQ ID NO: 13)
1/2HindIII
(5)
1/2HindIII
pAGCT
A
ds DNA Fragment
FZ
(6)
DNA Ligase
HindIII
FZ
PAAG
NH
nnnGGATCCnnnAAGCTT
nnnCCTAGGnnnTTCGAA
ds DNA Fragment
(SEQ ID NO: 14)
(7)
Newly created dsDNA biochip

FIG. 9

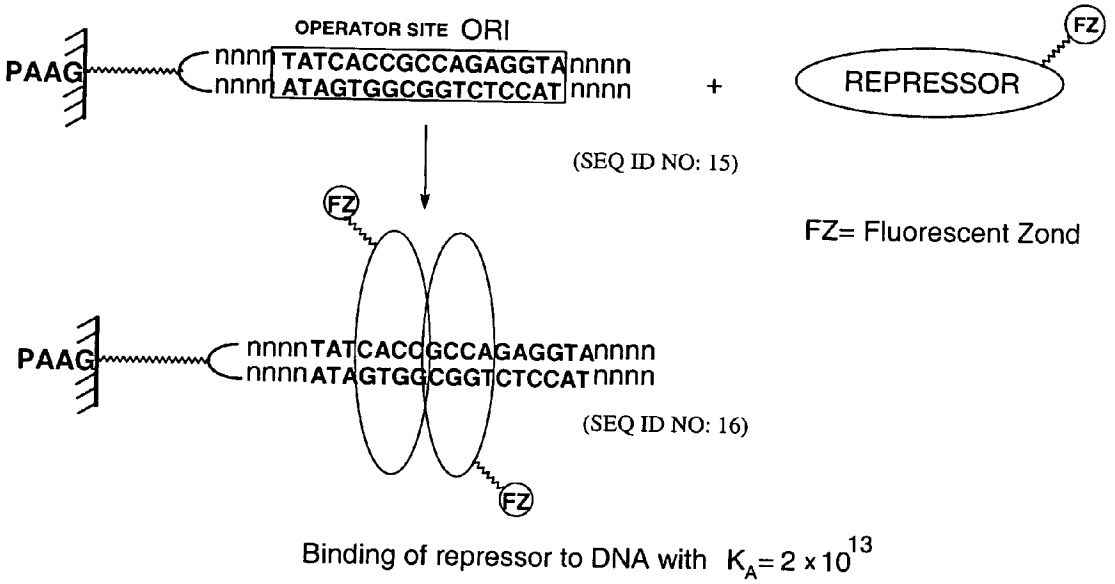
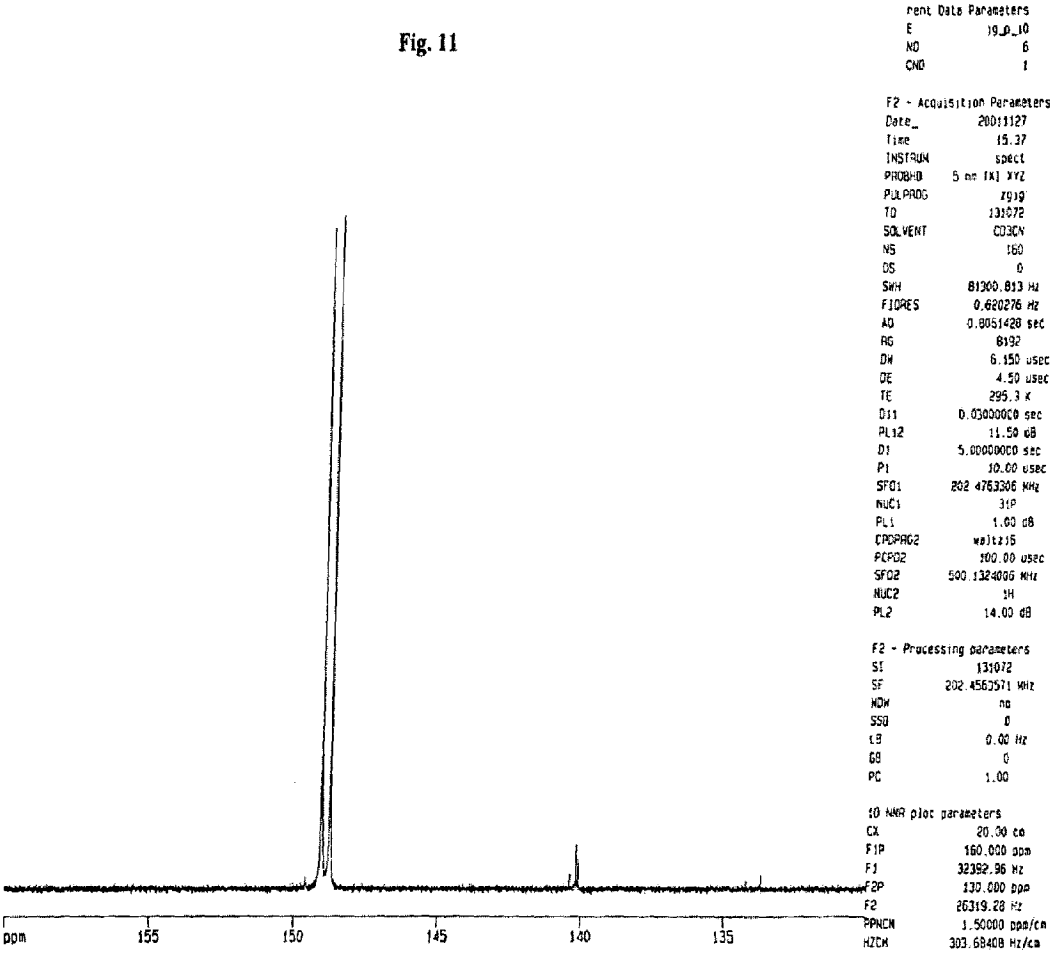

OPERATOR SITE ORI
PAAG
nnnn TATCACCGCCAGAGGTA nnnn
nnnn ATAGTGGCGGTCTCCAT nnnn
+
REPRESSOR
FZ
(SEQ ID NO: 15)
FZ= Fluorescent Zond
PAAG
nnnn TATCACCGCCAGAGGTA nnnn
nnnn ATAGTGGCGGTCTCCAT nnnn
(SEQ ID NO: 16)
Binding of repressor to DNA with $K_A = 2 \times 10^{13}$

FIG. 10

P^{31}NMR Spectrum of Connector Phosphoramidite (7)

Fig. 11



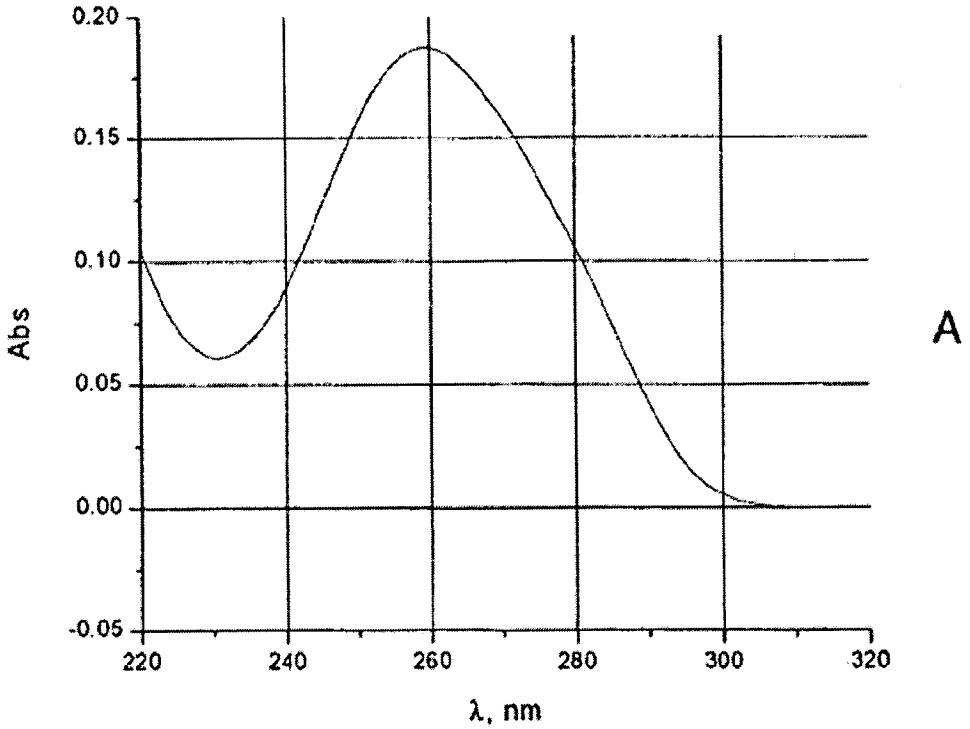
rent Data Parameters
E 19_p_10
ND 6
CND 1

F2 - Acquisition Parameters
Date_ 20011127
Time 15.37
INSTRUM spect
PROBHD 5 mm [X] XYZ
PULPROG zgig
TD 131072
SOLVENT CD3CN
NS 160
DS 0
SWH 81300.813 Hz
FIDRES 0.620276 Hz
AQ 0.8051428 sec
RG 8192
DW 6.150 usec
DE 4.50 usec
TE 295.3 K
D11 0.03000000 sec
PL12 11.50 dB
D1 5.00000000 sec
P1 10.00 usec
SFO1 202.4763306 MHz
NUC1 31P
PL1 1.00 dB
CPDPRG2 waltz16
PCPD2 100.00 usec
SFO2 500.1324006 MHz
NUC2 1H
PL2 14.00 dB

F2 - Processing parameters
SI 131072
SF 202.4563571 MHz
WDW no
SSB 0
LB 0.00 Hz
GB 0
PC 1.00

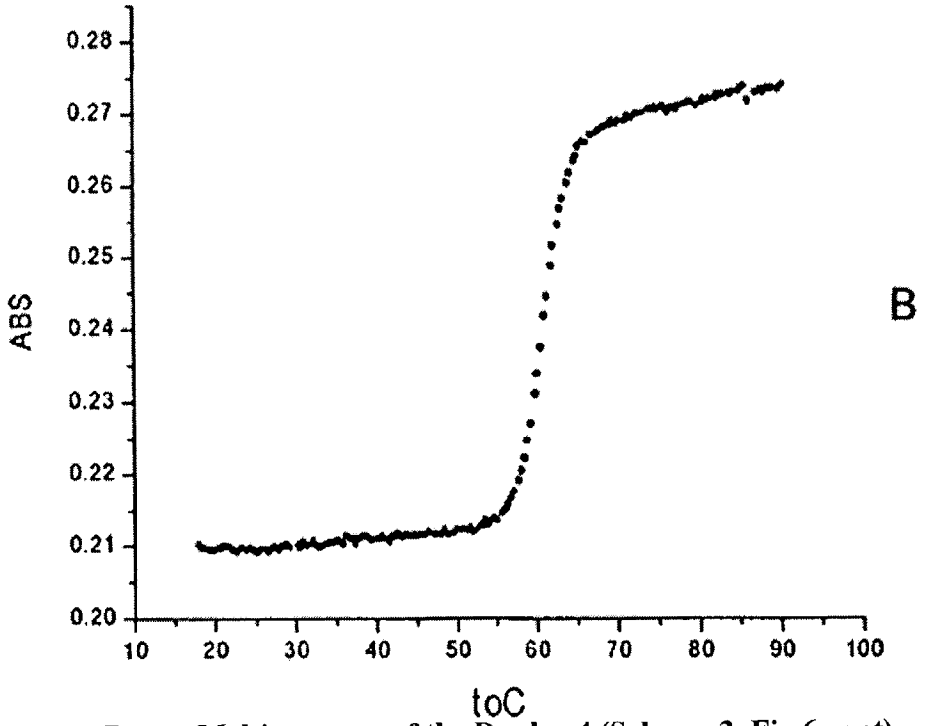
1D NMR plot parameters
CX 20.00 cm
F1P 160.000 ppm
F1 32392.96 Hz
F2P 130.000 ppm
F2 26319.28 Hz
PPMCM 1.50000 ppm/cm
HZCM 303.6840B Hz/cm

FIG. 11



A. UV-Spectrum of the Duplex 4 (Scheme 3, Fig.6 cont),

Abs – absorbtion, λ – wave length



B. Melting curve of the Duplex 4 (Scheme 3, Fig.6 cont)

ABS- absorbtion, t oC – temperature in Celsius

FIG. 12

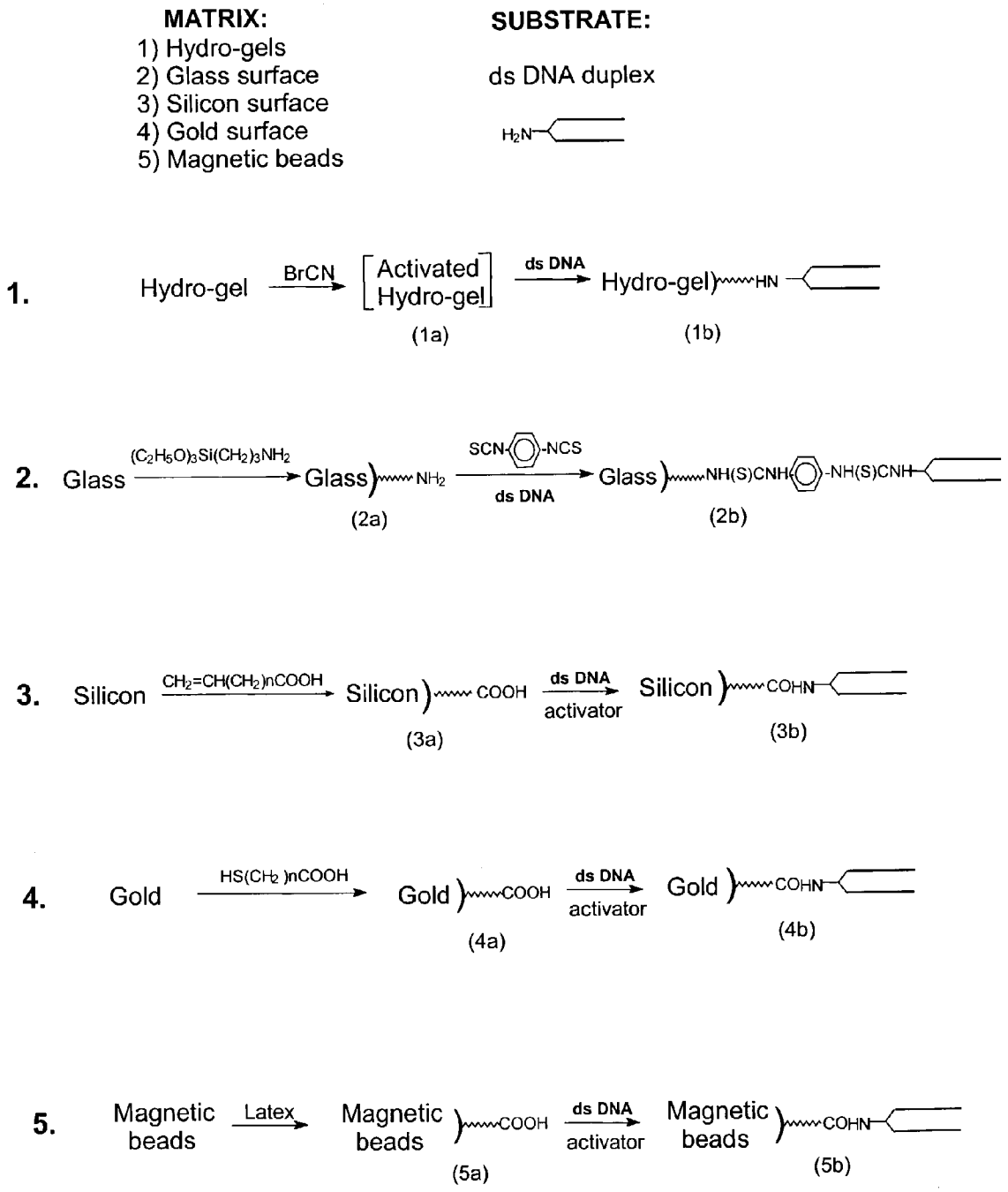

MATRIX:
1) Hydro-gels
2) Glass surface
3) Silicon surface
4) Gold surface
5) Magnetic beads
SUBSTRATE:
ds DNA duplex
H2N
1.
Hydro-gel
BrCN
Activated Hydro-gel
(1a)
ds DNA
Hydro-gel
HN
(1b)
2.
Glass
(C2H5O)3Si(CH2)3NH2
Glass
NH2
(2a)
SCN
NCS
ds DNA
Glass
NH(S)CNH
NH(S)CNH
(2b)
3.
Silicon
CH2=CH(CH2)nCOOH
Silicon
COOH
(3a)
ds DNA
activator
Silicon
COHN
(3b)
4.
Gold
HS(CH2)nCOOH
Gold
COOH
(4a)
ds DNA
activator
Gold
COHN
(4b)
5.
Magnetic beads
Latex
Magnetic beads
COOH
(5a)
ds DNA
activator
Magnetic beads
COHN
(5b)

FIG. 13

DOUBLE STRANDED NUCLEIC ACID BIOCHIPS

The United States Government has rights in this invention under Contract No. W-31-109-ENG-38 between the U.S. Department of Energy (DOE) and the University of Chicago representing Argonne National Laboratory.

BACKGROUND

Methods and compositions for preparation of double stranded nucleic acid molecules, e.g. dsDNA, by novel connectors that include linkers that attach the molecules to a matrix, are useful for the investigation of protein-nucleic acid interactions and nucleic acid structure.

Single-stranded DNA (ssDNA) microarrays have been extensively reported and used to screen genetic polymorphisms, mutations and gene expression to answer various questions. However, analysis of genetic transcription, replication, and restriction enzyme engineering requires dsDNA microarrays.

During the past several years many developments were reported in DNA microarray (biochip)-based automated technology that allowed parallel analysis of multiple DNA samples. Most of these biochips are based on application of single stranded DNA (ssDNA) microarrays, which are used in hybridization experiments with different kinds of DNA samples. Single-stranded DNA biochips are reported for gene expression profiling, gene polymorphism analysis, and mutation screening.

However, many important investigations in molecular biology cannot be served by this format of DNA microarrays. For example, systematic investigation of DNA-protein interactions which includes investigation of recombination, transcription, replication, and also discovery and engineering of new restriction enzymes, requires double stranded DNA microarrays.

There are many different approaches to the creation of double stranded DNA (dsDNA) microarrays. Different supports, linkers, base pair lengths, and types of DNA are reported for various applications.

A brief review of the major work and achievements in dsDNA biochip manufacturing is as follows:

There were two main approaches to construct dsDNA arrays. In the first, a single oligonucleotide strand was attached to a matrix, and then duplexes were formed by hybridization of the attached strand with complementary chains. The second method is based on manufacturing dsDNA arrays by using hairpin stem-loop DNA molecules attached to the matrix across the loop-part and hybridized to form only a partial duplex structure. The full duplex can be obtained by using enzyme polymerase if needed.

A method used for fabrication of dsDNA microarrays on a glass surface is illustrated in FIG. **1** (Bulyk, 1999). In the first step of creation of dsDNA arrays, single stranded DNA probes are synthesized on a glass surface by light-directed methods of oligonucleotide synthesis. Unfortunately, the efficiency of this method of synthesis is not high—92–94% per one step of oligonucleotide synthesis, thus for a 40-mer oligonucleotide, only from 5 to 20% of the synthesized oligonucleotides on the glass surface will be of the desired length and sequence (Carlson, 1999). Short synthetic primers are then annealed to the single stranded oliogonucleotide probes on the glass surface. Finally, the full duplex is obtained by Klenow polymerase reaction. The labeling of dsDNA probes thus created is carried out by fluorescein labeled dNTPs during a polymerization reaction, or by use of terminal transferase addition of fluorescein-labeled ddNTPs. However, this method is very time consuming and it is impossible to guarantee the high precision of answers using these microarrays.

Another approach to manufacturing dsDNA arrays is shown in FIG. **2**. In this method (Braun, 1998) the first step uses the same light-directed oligonucleotide synthesis as in FIG. **1** on a glass or gold surface e.g. a microscope "slide." Then, double stranded DNA fragments with protruding ends (complementary to probes attached to the chip) are hybridized to the immobilized oligonucleotides. The treatment of the resulting complex with DNA ligase creates a surface coupled dsDNA microarray. However, all disadvantages of the previously described method are present here too.

Another way for obtaining dsDNA arrays was demonstrated by O'Brien and coworkers (see FIG. **3**). The authors made dsDNA microarrays for protein—dsDNA screening and investigation of antigen-antibody binding using Atomic Force Microscopy. First, the set of complementary oligonucleotide pairs was synthesized by means of a standard solid phase approach using phosphoramidite chemistry (Sinha, 1983). Then, self-assembled DNA duplexes with recognition sites for the EcoRI restriction endonuclease and fluorescein moieties above the recognition sequences were obtained by annealing complementary chains in solutions before the creation of microarrays. Each of the complementary oligonucleotides contained a disulfide moiety, which provided further bonding of the duplexes to the surface of slide. However, the authors emphasized that there were serious problems with the standardization of capacity parameters of microarrays in that method. Also the high level of nonspecific adsorption of DNA probes can dramatically affect further experiments with this chip and interpretation of obtained results.

Other methods created DNA microarrays with hairpin structured probes (see FIG. **4**A, Zhao et al., 2001). One method was based on the covalent attachment of the hairpin stem-loop structure to a matrix. In this work oligonucleotides with five phosphorothioate residues in the loop were synthesized. The presence of these multiple phosphorothioate functions in the hairpin structure was used to anchor the oligonucleotide to a glass slide surface. A problem was that covalent attachment to the slide may occur statistically in every position of the loop, so geometric parameters of the hairpin can vary and as a result, can influence the formation of correct duplexes, especially for short complements (e.g. 5 base pairs). Then, complete labeled duplexes were obtained by extension of hairpin structures on a glass slide by use of T7 sequenase in the presence of cyanine dye labeled ddGTP (see FIG. **4**B)

The paper of Riccelli and coworkers (see FIG. **5**A) reported the use of hairpin probes made from a partial duplex (16 base pairs and a 32-base long single strand end) and a loop with biotinylated uracil as a linker in the middle. By this linker the structure was coupled to avidin coated microtiter wells. It was reported that such hairpin DNA probes attached to the chip displayed higher rates of hybridization and larger equilibrium amounts of captured targets in comparison with linear probes. Also, hairpin DNA—target complexes were thermodynamically more stable.

DNA microarrays containing stem-loop DNA probes with short single-stranded overhangs were immobilized on 3-dimensional Packard HydroGel chips by Broude et al. (2001). Microarrays were fabricated by immobilizing pre-synthesized, self-complementary single-stranded oligonucleotides which adopt partially duplex dsDNA was then biotinylated at single stranded regions. The biotinylated dsDNA was then

used as a ligand at a gold electrode covered by avidin. The obtained biosensors were said to be useful to determine small molecular weight organics, that is, a dsDNA based sensor, and for monitoring DNA-analyte interactions.

Although much effort has been expended in this research area, improvements are needed so that dsDNA microchips can be manufactured efficiently and used effectively.

SUMMARY OF THE INVENTION

This invention describes novel methods and compositions to create double-stranded nucleic acid, e.g. DNA (dsDNA) microarrays. Nucleic acid duplexes are synthesized to include connectors and linkers, and methods for immobilization of the dsDNA to biochips is by novel connectors that include linkers.

The resulting DNA duplexes do not have the cumbersome loops at the point of connection of complementary chains characteristic of some DNA duplexes synthesized in the art and their construction does not require enzymes. DNA-DNA, RNA-RNA, DNA-RNA, RNA-protein and DNA-protein interactions are investigated using the biochips.

A method for creating dsDNA biochips (microarrays) includes the steps of:

1. synthesizing at least one DNA duplex in which complementary oligonucleotude chains are covalently joined together by a novel connector;
2. attaching the synthesized DNA duplexes to a matrix to form a biochip (microarray) by free amino-groups of linkers incorporated into the connector.

The connector is a bifunctional molecule that can be placed between the two complementary nucleotide chains, which causes the respective strands on each side of the connector to hybridize without a loop formation. The smaller size of the attachment region facilitates the incorporation of more DNA duplexes to the same area of the matrix than would be possible using a stem loop structure. Additionally, this synthesis method does not involve enzymatic reactions, which tend to be more stem-loop structures upon denaturing and re-annealing. Stem-loop DNA probes were covalently attached to the matrix by modified nucleosides incorporated in the middle of the loop. The structure obtained was used in hybridization experiments followed by ligase reaction, and the results in searching and analysis of single nucleotide polymorphisms in the p53 gene were reported as good (FIG. **5**B).

Another approach for making dDNA microarrays was to use thiol-derivatized 15 base pair duplexes tethered through single 3' and 5' linkages to a gold [Au(III)] surface. The long-range film structure was measured by scanning probe microscopy. These microarrays are designed for use in nanotechnology or as biosensors. One conclusion of this study was that placement and composition of linkers will affect the film structure of DNA microarrays e.g. 3' duplex linkage results in a flat surface, also linker length and composition may induce different chain-duplex interactions and possibly duplex self-assembly (Sam et al., 2001). Therefore, different tethering methods likely result in different dsDNA microarrays.

Brockman et al. (1999) related a chemical modification procedure to create DNA arrays on gold surfaces for the study of protein-DNA interactions. Surface plasma resonance (SPR) imaging was used. The authors noted that "DNA arrays on glass supports from commercially available sources such as Affymetrix[13] are not a viable option" for SPR imaging investigations of protein-DNA binding interactions (p. 8045, col. 1), because this method needs to use DNA arrays on the metal surface. The authors investigated the binding of proteins that are specifically bonded with single-stranded DNA. Both single and double stranded DNA were spotted in a microarray. It was shown, that these proteins bound to single stranded DNA, but only very little bound to dsDNA.

Some goals of microarray DNA molecules, single or double stranded, immobilized on solid supports, are to use DNA chips as films for, e.g. electric charge transport. Jackson and Hill (2001) studied a charge transport through DNA double helices. The presynthesized duplexes were self-assembled onto gold surface, and the resulting films have been characterized by electrochemistry.

For the development of biosensors dsDNA was immobilized on self-assembled avidin monolayer onto a metal surface (Yan and Sadik, 2001). Circular plasmid dsDNA was linearized using the restriction endonuclease BamHI. The expensive and less efficient than the present methods. Additionally, dsDNA molecules without a loop structure are preferred for interaction with target substances.

A method of investigating dsDNA-protein interactions includes the following steps:

1. synthesizing at least one dsDNA probe with a specific nucleotide sequence and a connector that forms a DNA duplex;
2. attaching the synthesized DNA duplex to a matrix to form a biochip;
3. providing conditions for individual peptide targets to interact with specific dsDNA probes on the biochip; and
4. measuring parameters from which the dsDNA-protein interactions are inferred.

A method for designing a dsDNA biochip by using dsDNA specific enzymes such as restriction endonucleases and ligases, includes the following steps:

1. synthesizing at least one dsDNA probe with a specific nucleotide sequence and containing at least one site for restriction endonuclease recognition, and a connector to form a duplex;
2. attaching the synthesized DNA duplex to a matrix to form a biochip;
3. digesting the DNA duplexes on the biochip with site specific restriction enzymes to create dsDNA probes with protruding ends;
4. annealing the dsDNA probes on the biochip with dsDNA fragments containing complementary protruding ends; and
5. ligating annealed dsDNA fragments at the restricted point.

A method of chemical synthesis of specific connector phosphoramidite L (7) of the present invention is shown in FIG. **6**, Scheme 1.

An aspect of the invention is a microchip with dsDNA oligonucleotides each anchored to gel pads on the surface of the microchip by the linkers of the present invention.

An aspect of the invention is a connector characterized as a bifunctional phosphoramidite L (FIG. **7**A) wherein the connector contains a linker with free amino-groups for immobilization on a biochip matrix (FIG. **7**B).

A composition of the present invention is a connector constructed by Scheme 1 of FIG. **6**. An NH_2-group of aminoacid 1 was blocked by reaction of ethyltrifluoroacetate in methanol (1→2). Then a carboxylic group in 2 was converted in activated ester 3 by reaction with N-hydroxysuccinimide and N,N-dicyclohexylcarbodiimide. To obtain compound 5 a one-flask method of synthesis was used. It included sequential reactions of serinol 4 with ethyl trifluo-

roacetate 4a and 4,4'-dimethoxytritylchloride followed by treatment of methylamine 4d. Condensation of substances 3 and 4 leads to formation of synthone 6, which gives connector phosphoramidite 7 after phosphorylation by using 2-cyanoethyl diisopropylchlorophosphoramidite. An embodiment of a connector is shown in FIG. **6**.

dsDNA probes for biochip manufacturing (FIG. **7**C) are synthesized by using a connector phosphoramidite and standard phosphoramidites, and may be a full duplex. Also alternatives to a full duplex can be synthesized, which include a partial duplex with a 3'-protruding end, and a partial duplex with a 5'-protruding end.

Connector phosphoramidite (7) [FIG. **6**, Scheme 1] also can be used in standard procedures for oligoribonucleotide synthesis to obtain RNA or RNA-DNA duplexes. The connector phosphoramidite on FIG. **6**, Scheme 1=connector phosphoramidite L on FIG. **7**A

An aspect of the invention is a method of attachment of dsDNA probes including a connector to a biochip (microarray) matrix, which contains active functional groups able to react with amino-groups in connector construction. Novel in this procedure is using dsDNA probes, with the special structure.

Definitions and Abbreviations

Matrix=support (glass slide, gold slide, gel pad, others) on which an array (microarray) of molecules is formed; contains functional groups.

Array, microarray=molecules connected to the matrix in a specific arrangement relative to each other.

Biochip (DNA chip, DNA microarray, DNA array, peptide chip, peptide array and so on)=array of biological molecules e.g. bioprobes (DNA fragments, peptides, others) connected to the matrix in a specific arrangement to each other and designed to answer a specific question.

Bioprobe=molecule which can be used to identify or characterize another molecule e.g. by hybridizing.

Connector=a novel molecule linking together complementary oligonucleotide chains of a DNA/DNA, DNA/RNA or RNA/RNA duplex.

DMTr=dimethoxytrityl.

Linker=a structural element of a connector providing connection of a DNA-duplex to functional groups of a matrix.

MALDI=MS=matrix assisted laser desorption ionization mass spectroscopy.

HEG=hexaethylene glycol.

dNTP=deoxynucleoside triphosphate.

ddNTP=dideoxynucleoside triphosphate.

CY5-ddGTP=cyanine-labeled dideoxeriboguanosinetriphosphate.

FG=functional group.

FZ=Fluorescent Label

PAAG=polyacrylamide gel.

DNA=deoxyribonucleic acid.

RNA=ribonucleic acid.

UV=ultraviolet.

NMR=Nuclear Magnetic Resonance.

Phosphoramidite=building blocks for chemical synthesis of oligodeoxyribonucleotides by phosphoramidite method.

Methods and compositions described herein that relate to dsDNA also include DNA/RNA and RNA/RNA duplexes.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** shows creation of dsDNA array by annealing and further enzymatic extension of the complementary primer (Bulyk et al., 1999).

FIG. **2** shows manufacturing steps for surface-coupled dsDNA by hybridization of dsDNA containing a protruding end with a single stranded complement attached to the glass slide surface followed by treatment with DNA-ligase (Braun et al., 1998); protruding ends in DNA duplex means, that one oligonucleotide chain is longer then the other. > shows the ligation point, in which two oligonucleotide chains are connected together by a ligase reaction.

FIG. **3** shows creation of a dsDNA microchip (SEQ ID NO: 1) by connection of self-assembling dsDNA to a gold slide. Disulfide-modified dsDNA 26-mer containing the recognition sequence specific for cleavage by EcoRI restriction endonuclease were synthesized using phosphoramidite chemistry and annealed in solution. Oligonucleotides were labeled with fluorescein (F*) at the **5'**-end for use in an optical verification of sequence specific cleavage by EcoRI restriction endonuclease (O'Brien et al., 2000).

FIG. **4**(A) shows a hairpin structure primer with five phosphorothioate groups in the loop comprising six adenosines. The surface of the glass slide is coated with bromoacetamidopropylsilane; (B) shows an arrayed extension of hairpin primer on a glass slide by T7 Sequenase in the presence of dye-labeled ddGTP to obtain full length labeled duplex (Zhao et al., 2001).

FIG. **5**(A) shows creation of a dsDNA chip with a partial duplex structure on an avidin coated microwell surface (Riccelli et al., 2001). Hairpin DNA probes contain biotinylated uracil in the middle of a loop to be captured by avidin; (B) shows an outline of the experiments with stem-loop DNA probes immobilized on a HydroGel (Broude et al., 2001), which were used for searching single-nucleotide mutations by hybridization of labeled targets with hairpin DNA probes followed ligase reaction.

FIG. **6**, Scheme 1 shows the synthesis strategy for a connector phosphoramidite (7) of the present invention. Scheme 2 shows the synthetic way for preparation of connector phosphoramidite (4), an unsuccessful embodiment proposed for a connector structure. Scheme 3 demonstrates the structures of synthesized duplexes (SEQ ID NOS 3–7, respectively, in order of appearance) to be used for the formation of new duplex formats on 3D dsDNA biochips by use of restriction digestion followed by ligase reaction. Scheme 4 shows the structures of DNA duplexes (SEQ ID NOS 8–11, respectively, in order of appearance) containing Lambda Phage operator site ORI and antisense-duplexes useful for investigation of dsDNA-protein interaction on 3D biochips.

FIG. **7**(A) shows a structure of connector phosphoramidite L; oligonucleotides as a sequence of letters "A,C, G and T" which correspond to the nucleotides (adenosine, cytosine, guanosine and thymidine). The letter "L" shows the place where the connector is in the synthesized oligonucleotide chain. This is 7 from Scheme 1, FIG. **6**.

(B) shows a scheme for chemical synthesis of a DNA duplex by using commercial phosphoramidites and connector phosphoramidite L; (C) shows possible structures of synthetic DNA duplexes.

FIG. **8** is a schematic illustration of biochip formation by immobilization of a DNA duplex on a matrix. Six types of matrices are exemplified.

FIG. **9** shows a scheme for creation of a new format for dsDNA-biochips (SEQ ID NOS 12–14, respectively, in order of appearance) by use of enzymatic reactions (restriction endonuclease HindIII and DNA ligase). ½ HindIII is an abbreviation that means half-site of HindIII with protruding ends which appears after digestion of the recognition site with HindIII restriction endonuclease.

FIG. **10** shows the binding or Lamda Cro-repressor with operator site ORI as a model for investigation of dsDNA-protein interactions by use of a dsDNA-biochip (SEQ ID NOS 15 & 16, respectively, in order of appearance).

FIG. **11** shows the P_{31}NMR Spectrum of connector phosphoramidite 7.

FIG. **12** shows (A) a UV spectrum and (B) melting curve of synthesized DNA duplex 4 (see FIG. **6** cont., Scheme 3).

FIG. **13** demonstrates approaches to immobilization of dsDNA probes on different kinds of matrices.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to novel methods and compositions to create and use double stranded DNA microarrays (biochips). dsRNA or RNA/DNA duplexes can be made similarly. Methods are based on use of synthesized DNA duplexes in which complementary chains are covalently bonded by a novel connector including a linker for attachment to a support (matrix). A range of suitable oligonucleotide lengths attached to the connector includes:

about 10–40 base pairs—for synthesized dsDNA probes; and

up to about 400 base pairs—for natural dsDNA fragments.

Natural DNA fragments can be obtained from commercial sources or isolated by methods known to those of skill in the art. These can be longer than DNA made synthetically because of technical restrictions on the latter.

The present invention is a new and promising tool for many molecular biological investigations based on DNA-protein interactions, and can also be used for creation of new formats of microarrays including the biochips bearing the fragments of natural DNA (e.g. genomic, bacterial, phage, and the like).

dsDNA microarrays of dsDNA will significantly enhance the performance of nucleic acid assays for known applications such as gene expression profiling, analysis of single nucleotide polymorphism or high-throughput diagnostic and also investigations of DNA-protein interactions. The latter application includes studying fundamental cell processes, such as recombination, transcription, replication in which dsDNA chains are involved. Aspects of experiments based on use of dsDNA-protein interactions include restriction enzyme discovery and engineering. dsDNA arrays also can be useful in design and engineering of new peptide structures to control gene expression in biotechnology applications ranging from functional genomics to gene therapy.

In an embodiment, 3-D Acrylamide Gel Chips created in Argonne National Laboratory are used as matrices for manufacturing 3-dimensional (3D) dsDNA biochips. Other kinds of matrices (such as glass slides or beads, gold slides) are also suitable. A preferred matrix used for manufacturing of dsDNA-biochip contains active functional groups, which are able to react with free amino-functional groups of the connector (FIG. **8**).

The fields of application for the double-stranded DNA biochips include:

High-throughput diagnostics;

Single-nucleotide polymorphism genotyping;

Gene expression profiling;

Investigation of DNA-protein interactions such as: recombination, transcription, replication;

Discovery and engineering of restriction enzymes;

Design and engineering of new peptide structures to control gene expression in biotechnology applications.

Design and Chemical Synthesis of a Connector of the Present Invention

General characteristics of a connector include:

Geometrical parameters and conformational mobility of the connector allows the correct duplex formation.

Hydrophilic properties are close to properties of the nucleic acids. The molecules of the present invention are soluble in water at the same concentrations as molecules without a connector.

A connector of the present invention includes a bifunctional phosphoramidite [FIG. **7**(A)]. This structure is suitable for use in automated oligonucleotide synthesis by procedures known to those of skill in the art [FIG. **7**(B)]. A connector phosphoramidite can be introduced in any position of a synthesized oligonucleotide chain to obtain different structures of DNA duplexes, such as: full duplex or partial duplexes with protruding 3'- or 5'-ends [FIG. **7**(C.)]. The linker with a free amino-group incorporated into the structure of the connector provides the covalent immobilization of dsDNA probe on matrices supplied with active functional groups (FG) able to react with amino-groups (FIG. **8**).

Aspects of a suitable connector include:

1. Can be synthesized very simply by using inexpensive commercial chemicals. A major cost savings is that enzymes are not needed.
2. Can be used as a phosphoramidite in the protocol for automated oligonucleotide synthesis known to those of skill in the art.
3. Is chemically stable during oligonucleotide synthesis and post synthesis procedures.
4. The bifunctional structure of the connector phosphoramidite allows it to be incorporated in any position of a synthesized oligonucleotide chain to receive variants of DNA duplexes such as:
   Full size duplex,
   Duplex with 3'-protruding end,
   Duplex with 5'-protruding end.
5. The structure of the connector improves the thermodynamic parameters of DNA duplex formation in comparison with duplexes obtained by hybridization from two separate oligonucleotide chains, and also with hairpin duplex structures (Tchurikov, 1998; Riccelli, 2001). The connector group also guarantees the stability of a duplex because the entropy factor is decreased during duplex formation (Schyolkina, 2000).

The structure of a connector of the present invention is a bifunctional phosphoramidite and is suitable for use in automatic oligonucleotide synthesis known to those of skill in the art. The connector includes a linker group for immobilization of dsDNA probe on a matrix. Factors to be considered for construction of a connector molecule include:

1. Incorporating chemical groups with different functions from each other (for example, one amino group and two hydroxyl groups) to be able to manipulate the molecule selectively during chemical synthesis of the connector; for example, the synthesis strategy described in Scheme 1, FIG. **6** involved selective protection of the primary-amino group in serinol 4 by a trifluoroacetic group and subsequent protection of one of the hydroxylic functions by the DMTr-group. Then trifluoroacetic group was removed selectively and deblocked amino function was reacted with activated ester of aminocaproic acid 3. Phosphitilation of obtained synthon resulted in the desired connector phosphoramidite 7.
2. Choosing the length of the linker, which can affect the efficiency of interaction of dsDNA probe on the biochip

with target molecule (Carmon, 2002). Derivatives of aminocaroic acid 1 and 2 (see: Scheme 2 on FIG. **6**) and diethanolamine 3 were used as base components for obtaining the compound 4. The connector phosphoramidite 4 was used in the procedure of solid phase oligonuceotide synthesis by a phosphoramidite method known to those of skill in the art (Sinha, 1983). Limitations in structure and properties of this connector included phosphoramidite 4 demonstrating low yield of coupling reaction (90% instead of 99%), which is needed for successful results in the synthesis of prolonged oligonucleotides. Insufficient yields were encountered in all different variants of synthesis conditions and programs. Next, the oligonucleotides that contained connector 4 in their composition demonstrated abnormal low stability during the deprotection step, in which ammonium hydroxide is used for removing of N-acylic protective group from heterocycle bases. Therefore, a new connector for dsDNA probe was constructed.

Subsequently, a preferred embodiment was developed as shown in Scheme 1 (FIG. **6**), 7. A preferred structure for a connector was obtained from serinol and 6-aminocaproic acid 1 as base substances.

For construction of the new connector (FIG. **6**, Scheme 1, 7) serinol 4 and activated ester of N-trifluoroacetyl-aminocaproic acid 1 were used. Efficiency of the elongation step with participation of phosphoramidite 7 in oligonucleotide synthesis reaches more than 98%, estimated by spectorophotometer monitoring of DMTr-cation UV-absorbance, which is correlated with the efficiency of each coupling step in oligonucleotide synthesis. Oligonucleotides containing this connector do not change their structure during ammonium treatment during a deprotection step.

By use of newly synthesized connector 7 DNA duplexes for manufacturing of 3D biochips were synthesized (see Scheme 3 and Scheme 4, FIG. **6**). There are structures containing recognition sites for several restriction endonucleases (1–5, Scheme 3, FIG. **6**) and also Phage λ operator sites ORI (1 and 2, Scheme 4, FIG. **6**).

Chemical synthesis of compound 7 was carried out in the manner shown in Scheme 1 (FIG. **6**): The NH_2-group of amino acid 1 was blocked by reaction of ethyltrifluoroacetate in methanol (1→2). Then carboxylic group in 2 was converted in activated ester 3 by reaction with N-hydroxysuccinimide and N,N-dicyclohexyicarbodiimide. To obtain compound 5 a one-flask method of synthesis was used. It included sequential reactions of serinol with ethyl trifluoroacetate 4a and 4,4'-dimethoxytritylchloride followed by treatment of methylamine 4d. Resulting O-dimethoxytritylserinol is isolated by column chromatography on silica gel with the yield 64%. Condensation of substances 3 and 4 leads to formation of synthone 6, which gives connector phosphoramide 7 after phosphorylation by use of 2-cyanoethyl diisopropylchlorophosphoramidite. FIG. **11** demonstrates the P^{31}NMR spectrum of phosphoramidite 7, which contains a representative signal of P(III) atom in the prospective range (around 150 ppm).

Then, the linear DNA fragment was synthesized by automated solid phase oligonucleotide synthesis carried out from commercial phosphoramidite and connector phosphoramidite [FIG. **7**(B)]. After deblocking and purification procedures, a structure was obtained, in which two complementary oligonucleotide chains are joined together by a connector via their 3'- and 5'-ends and can form a duplex. The formation of a duplex structure from synthesized linear fragments was confirmed by melting curves (FIG. **12**).

As it was shown in background—nobody before used this chemical structure in the synthesis of oligonucleotides. The connector phosphoramidite L is a new substance in oligonucleotide synthesis and its use is a new method for creating improved nucleic acid duplexes.

Double-Stranded DNA Fragments Synthesized to Have Restriction Sites

The sequences of synthetic DNA duplexes are designed for specific purposes (Scheme 3, FIG. **6**), for example, to create different new formats of dsDNA biochips using restriction endonuclease and ligases. The structures 1, 2 and 3 (Scheme 3, FIG. **6**) have identical combinations of restriction sites, but they differ in the length and structure of the loops, connecting complementary oligonucleotide chains. These structures are useful to investigate the role and effect of the loop structure on duplex formation and its interaction with the enzymes. Structure 3 also has a fluorescence label (fluorescein isothiocyanate—FITC) to control the restriction reaction on a biochip.

Enzymatic cleavage of duplexes results in protruding ends that are available for further reactions with other dsDNA fragments or oligonucleotides.

Combinations of restriction sites presented herein were not reported previously for use in dsDNA-biochips. However, they are available and used for other purposes.

dsDNA duplexes may be synthesized that contain different restriction sites. The combination of two sequential enzymatic reactions (restriction cleavage and ligation) allows the microarrays to have selected functional parts of genomic DNAs or their combinations.

The sequence of a synthetic DNA duplex (FIG. **9**) containing recognition sites for restriction enzymes BamHI and HindIII is an example demonstrating how dsDNA-biochips can be used for creation of new dsDNA biochip format. Enzymatic cleavage of such duplexes 3 by BamHI and HindIII leads to formation of new DNA duplexes 4 and 5 with protruding ends. This step can be controlled by MALDI-MS or by monitoring the loss of fluorescent label incorporated at the cleavage site. Further fluorescent labeled dsDNA fragments 6 and 7 with protruding half-sites BamHI and HindIII can be covalently bonded by DNA-ligase to the duplexes 4 and 5 respectively. Thus, new dsDNA biochips are formed. The appearance of fluorescent signals during this step should indicate that the appropriate reactions proceeded.

Double-Stranded DNA Fragments Synthesized to Have CRO-Repressor Recognition Sites

For investigation of specific DNA-protein interactions, several DNA duplexes, containing Lambda phage operator site ORI for specific interaction with CRO-repressor, were designed and synthesized (Scheme 4, FIG. **6**).

dsDNA duplexes were obtained in two variants with different structures and different lengths of the loop part of the duplexes to investigate their influence on the binding of CRO-repressor to synthesized duplexes. To control the specificity of dsDNA-protein interactions, two antisense duplexes (Antisense 1 and 2 on Scheme 4, FIG. **6**) were synthesized. The antisense duplexes do not contain a specific site for CRO-repressor binding and should be used as a control for non-specific binding of CRO-repressor with double stranded DNA fragments if such binding exists.

The Lambda phage repressor-operator system is used as an example (FIG. **10**) for investigations of DNA-protein interaction, which can be carried out with a dsDNA-biochip. For this purpose a dsDNA-biochip can be fabricated with duplexes containing Lambda ORI operator sites and used as

a target for fluorescent labeled CRO-repressor to demonstrate efficient interactions of dsDNA probes with the peptide. Then, specificity of protein-DNA interactions in this system is estimated by using a mixture of CRO-repressor with other proteins and further analysis of the effect of administration on the intensity of the fluorescent signal, which appears after target interaction with dsDNA probe. dsDNA biochips with antisense duplexes are used as negative controls in these experiments.

Lambda phage repressor-operator systems are useful as a model for investigation of protein-dsDNA interactions. For example, Lambda operator ORI (dsDNA) can be immobilized on 3D chips. The process of interaction of labeled repressor with dsDNA target is monitored by appearance of fluorescent signal. The specificity of protein-DNA interaction is estimated by measuring change in the intensity of a fluorescent signal during interaction of the repressor and some other proteins with the operator.

3-Dimensional Acrylamide Gel Biochips

dsDNA-biochips are constructed using many different kinds of matrices [i.e. supports]. They can be fabricated in 2- or 3-dimensional formats. General requirements for the supports to be used as matrices for fabrication of biochips bearing the dsDNA-probes of the present invention, include the presence of active functional groups that are able to react with free amino-functional groups of the connectors.

Possible variants of covalent immobilization of dsDNA probes on different matrices are demonstrated in FIG. **13**. Hydro-gels formed from polysaccharides (for example, dextrane, agarose and others) may form covalent bonds with amino-groups of dsDNA after standard activation by bromocyane 1. The glass surface matrix is modified by 3-aminopropylethoxysilane, and then, sequential treatment of amino-glass surface 2a with phenylendiisothiocyanate and dsDNA probes leads to formation of a microarray 2b. The strategy for immobilization of dsDNA fragments on matrixes 3–5 assumes preliminary modifications of surfaces with carboxylic functions. Silicon 3 is modified by unsaturated acids to obtain 3a, gold 4—by mercaptoacids with formation 4a, magnetic beads is covered by special latex 5a. Formation of covalent (in these cases—peptide bonds) occurs as a consequence of interaction of carboxy-modified matrixes 3a–5a with dsDNA probes in the presence of water-soluble carbodiimide as an activator.

Porous 3-dimensional (3D) substrates, such as polyacrylamide gel pads, posses very good properties to be used as a matrix for DNA chips. These chips have higher capacity and as a consequence—higher sensitivity (U.S. Pat. No. 5,552,270). For these reasons 3D polyacryamide gel pads are suitable as a matrix for manufacturing 3D dsDNA-biochips. Fabrication of 3D dsDNA-biochips was carried out using standard conditions for 3D DNA-biochips (see: Materials and Methods).

Materials and Methods

A. Chemical Synthesis of Connector Phosphoramidite (7) FIG. **6**, Scheme 1

1. N-(Trifluoroacetyl)-6-aminocaproic acid (2)

6-Aminocaproic acid (1, 6.55 g, 50 mmol) was suspended in methanol (30 ml) and treated with triethylamine (6.9 ml, 100 mmol) followed by ethyl trifluoroacetate (7.2 ml, 60 mmol). The mixture was stirred at room temperature (r.t.) during 40 hours. The unreacted solid precipitate was filtered, the filtrate was evaporated in vacuo and crystallized from benzene-hexane mixture. Trifluoroacetyl derivative (2) had an 84% yield (9.5 g).

Mp 92–93° C.

2. N-hydroxysuccinimide ester of N-(Trifluoroacetyl)-6-aminocaproic acid (3)

N,N-Dicyclohexylcarbodiimide (7.8 g, 34.4 mmol) was added to a solution of compound 2 (7.1 g, 31.3 mmol) in 100 ml of acetone or tetrahydrofurane (TGF). The reaction mixture was stirred at room temperature overnight. The formed precipitate was filtered and washed with acetone or TGF. The filtate was concentrated in vacuo.

A. The resulting oil was purified by chromatography on a silica gel column using chloroform-acetone (8:1) as a mobile phase. The collected fractions were evaporated to dryness to obtain compound 3 at a yield of 89.7% (9.1 g). C12H15F3N2O5, Mass Spectra (MS): calc. 324.04. found 324.9.

B. Alternatively, the resulting oil was dissolved in ethyl acetate (100 ml), washed with 5% aqueous NaHCO3 (3×50 ml). Organic layer was dried over Na2SO4, filtrated and concentrated in vacuo. The residue was crystallized from isopropanol to give compound 3 with yield 79.8% (8.1 g).

3. 1-O-(4,4'-Dimethoxytrityl)-2-amino-1,3-propanediole (5)

The suspension of serinol (4, 1.82 g, 20 mmol) in 50 ml of methanol, triethylamine (2.77 ml, 20 mmol) and ethyltrifluoroacetate (3.0 ml, 25 mmol) were added. The mixture was stirred at room temperature for 40 hours and then evaporated. The residue was dried by coevaporation with anhydrous pyridine, dissolved in 40 ml of dry pyridine, and 4,4'-dimethoxytrityl chloride (7.4 g, 22mmol) was added and the mixture was stirred overnight at r.t. Then the solution was evaporated in vacuo and taken up in ethyl acetate (100 ml). The solution was sequentially washed with saturated aqueous solution of NaHCO3 (2×50 ml), water, dried over Na2SO4 and evaporated in vacuo. The residue was dissolved in 40 ml of methanol, then treated with aqueous methylamine (1 h, r.t.) and evaporated. The product was purified by silica gel column chromatography and eluted with chloroform-methanol (8:1). Collected fraction was evaporated to give compound 5 (5.0 g, 64% yield). C24H27NO4, MS: calc. 393.2. found 394.5.

4. N-(Trifluoroacetyl)-6-aminocaproylic derivative of 1-O-(4,4'Dimethoxytrityl)-2-amino-1,3-propanediole (6)

O-Dimethoxytrityl serinol 6 (3.93 g, 10 mmol) and N-hydroxysuccinimide ester 3 (3.56 g, 11 mmol) were dissolved in tetrahydrofurane (25 ml) and the mixture was stirred for 1.5 hours at room temperature. Then the mixture was evaporated in vacuo, diluted with chloroform (50 ml) and washed with aqueous 5% solution of NaHCO3. The organic layer was dried over Na2SO4, filtered and concentrated to dryness. The residue was applied to a silica gel column. Chromatography was performed with chloroform, applying gradient of methanol (1–5%), to give compound 6 (4.5 g, 75%). C32H37F3N2O6, MS: calc. 602.23. found 603.1.

5. Connector Phosphoramidite (7)

To compound 6 (424 mg, 0.7 mmol) in dry dichloroethane N,N-diisopropylethylamine (2.82 ml, 2.8 mmol) was added. The mixture was cooled and 2-cyanoethyl diisopropylchlorophosphoramidite (250 mg, 1.05 mmol) was added with vigorous stirring. The reaction was allowed to proceed at room temperature for one hour. Dry methanol (0.2 ml) was added, the mixture was diluted with dichloroethane (20 ml), and washed with 5% solution of Na2CO3 (2×10 ml) and

brine (2×10 ml), dried over Na2SO4, and evaporated in vacuo. The residue was purified on a silica gel column. Chromatography was performed with dichloroethane-ethyl acetate-triethylamine (60:35:5) to give compound 7 (480 mg, 85%).

6. Standardized Chemicals and Equipment for Chemical Synthesis of Connector Phosphoramidite (7)

1. Ethyl trifluororacetate, 99%	Aldrich, Cat# E5,000-0
2. 6-Aminocaproic acid, 98%	Aldrich, Cat# A4,460-6
3. 4,4'-Dimethoxytrityl chloride, 95%	Aldrich, Cat# 10,001-3
4. Triethylamine, 99.5%	Aldrich, Cat# 47,128-3
5. Methyl alcohol, 99.9%	Aldrich, Cat# 15,490-3
6. Tetrahydrofuran, HPLC grade	Fisher, Cat# T425-4
7. Pyridine, 99.8% anhydrous	Aldrich, Cat# 27,097-0
8. N-Hydroxysuccinimide, 97%	Aldrich, Cat# 13,067-2
9. 1,3-Dicyclohexylcarbodiimide, 98%	Aldrich, Cat# D8,000-2
10. Cyanoethyldiisopropylchloro phosphoroamidite	Aldrich, Cat# 30,230-9
11. 1,2-Dichloroethane, 99.8%, anhydrous	Aldrich, Cat# 28,450-5
12. Acetonitrile, 99.8%, anhydrous	Aldrich, Cat# 27,100-4
13. Serinol, 98%	Aldrich, Cat# 35,789-8
14. N,N-Diisopropylethylamine, 99.5%	Aldrich, Cat# 49,621-9
Cromatographic Silica Gel, 100–200 MESH	Fisher, Cat# S734-1
16. Ethyl acetate, 99.5%	Aldrich, Cat# 15,485-7
17. Ammonium hydroxide, 28.0–30.0%	Aldrich, Cat# 32,014-5
18. Chloroform, A.C.S.	Fisher, Cat# C 298-4

B. Chemical Synthesis of Oligonucleotides by Using Liquid Chemical Dispensing Robot

Standard Protocol

1. Loading oligonucleotide sequences information for 96-wells plate in computer controlled Liquid Chemical Dispensing Robot (LCDR).
2. Loading information about the parameters of reaction cycles in the LCDR Computer:
   Injection times for reagent valves;
   Wait times for each step of synthesis;
   Wash cycles after each step;
   Valve assignment for each reagent.
3. Loading chemicals into the reagent bottles:
   0.075 M solutions of phosphoramidites (dA, dC, DG and T) in acetonitrile;
   0.45 M solution of tetrazole in acetonitrile;
   Capping mixture A (acetic anhydride-lutidine-tetrahydrofurane, v/v 10:10:80);
   Capping mixture B (N-methylimidazole-tetrahydrofurane, v/v 10:90);
   Deblocking solution (3% dicloroacetic acid in dichloromethane);
   Oxidizing solution (0.1 M iodine in tetrahydrofurane-pyridinewater, v/v 60:20:10);
   Acetonitrile.
4. Filling of 96-wells plate with Control Pore Glass (CPG) Support.
5. Setting up the machine for a run and automated chemical synthesis of oligonucleotides.
6. Post processing treatment:
   cleaving synthesized oligonucleotides from CPG support with simultaneous elution by using a 30% ammonium hydroxide solution (1 h under 25° C.);
   deprotection stage in 30% ammonium hydroxide solution (10 h under 25° C. or 5 min in mW-assisted procedure);
   evaporation on SpeedVac centrifuge.
7. HPLC purification of synthesized oligonucleotides:
   dissolving evaporated crude reaction mixtures in 1.0 ml of 0.05M triethylammonium acetate buffer, pH 7.0;
   filtration
   chromatography on Rainin HPLC System, column C18 (4.6–9.0×250 mm), gradient of acetonitrile in 0.05 triethylammonium acetate buffer, pH 7.0, 25–50%, 8 min;
   evaporation of HPLC elutes on SpeedVac centrifuge.
8. Final removing of 5'-protecting group by treatment of evaporated oligonucleotides with 80% acetic acid solution during 5 min under 25° C. followed by evaporation on SpeedVac centrifuge and precipitation of oligos by iso-propanol.
9. Preparation of probes in 96-wells format for immobilization on the acrylamide micro-matrixes:
   dissolving of oligonucleotides in 500 ml of MQ-water (Stock solutions) and preparation of diluted (×100) solutions for UV spectroscopy;
   UV spectroscopy of diluted solutions of oligonucleotides;
   evaporation of stock solutions of oligonucleotides;
   calculation of amount of oligonucleotides based on information from UV spectrums;
   final preparation of oligonucleotide solutions in MQ-water in concentrations appropriate for immobilization on the acrylamide micro-matrixes.

TABLE 1

Materials and Equipment

Chemicals/Equipment	Manufacturer	Catalog #
Phosphoramidite		
-dA	Glen Research	10-1000-10
-dG		10-1020-10
-dC		10-1015-10
-dT		10-1030-10
-5'-Amino		10-1936-02
CPG Support	Glen Research	
-dA		20-2000-10
-dG		20-2020-10
-dC		20-2010-10
-dT		20-2030-10
-3'-Amino		20-2957-10
Activator	Glen Research	30-3100-52
		30-3100-57
Acetonitrile	Aldrich	43,913-4
Acetic amhydride		32,010-2
Acetic Acid		10,908-8
Ammonium hydroxide		38,053-9
Dichloroacetic acid		D5,470-2
Iodine		37,655-8
2,6-Lutidine		33,610-6
1-Methylimidazole		33,609-2
2-Propanol		10,982-7
Pyridine		27,097-0
Dichloromethane		15,479-9
Tetrahydrofuran		18,656-2
		40,175-7
Triethylamine		47,128-3
Liquid Chemical Dispensing Robot	Avantech	P98363
HPLC system	Rainin	9920-113
HPLC-column C18, 4.6 × 250 mm	Supelco	58355-U
	Varian	CP29519
9.4 × 250 mm	Zorbax	880975.202
CentriVap	Labconco	U28652-00
Centrifuge	Eppendorf	5415C
96 well plate	BioLogical Brand	p9605
Eppendorf microcentrifuge tubes, 1.5 ml	Fisher	05-402-24B
Syringe filter, 0.2 um pore size	Aldrich	Z25994-2

C. Preparation of Acrylamide Micro-Matrices by Photo-polymerization

1. Preparation of Glass Slides

(a) Immerse 10 glass slides in 10 M sodium hydroxide in a Wheaton glass-slide container (volume 150 ml) for 30 minutes.

(b) Rinse with five changes of double-distilled water in a container.

(c) Immerse 10 slides in concentrated sulfuric acid in container for 30 minutes

(d) Rinse five times in double-distilled water and allow to stand in double distilled water for 5 minutes then rinse again.

(e) Remove water drops with nitrogen stream. Dry for 1 h at 60° C.

2. Treatment of Cleaned Slide with Bind Silane.

Immerse slides in 3-(Trimethoxysilyl)propyl methacrylate and incubate for 40 h min at 37° C.

Rinse thoroughly with ethanol and then double-distilled water and dry under a nitrogen stream.

3. Preparation of Solutions for Aldehyde Matrices

(a) Composition of 5% polyacrylamide solution 0.5 ml 40% Acrylamide/Bis solution (19:1) 1.82 ml 0.2M sodium phosphate buffer (consists of equal volumes of 0.2M sodium phosphate monobasic monohydrate and 0.2M sodium phosphate dibasic anhydrous pH=6.8, store at 4° C.). 1.6 ml glycerol 0.08 ml monomer I solution (N-(5,6-di-O-isopropylidene)hexyl acrylamide). For monomer I solution add 1 ml MilliQ water to aliquot of monomer I stock (25 mg) located in −8° C. freezer. Aliquot and store these at −2° C. for approximately 1 month.

(b) filter.

(c) Prepare solution weekly and store at 4° C. Allow solution to reach room temperature before use.

4. Assembly of Gel-Casting Cassette.

(a) Clean mask surface with ethanol.

(b) Rinse thoroughly with distilled water stream rubbing briskly with lint-free tissue.

(c) Dry under a nitrogen stream.

(d) Place spacers (audio tape film) on chrome side of mask; two spacers from both sides and one in the center.

(e) Place slide over mask and spacers with treated surface facing mask.

(f) Clamp in place.

5. Photo-Polymerization (Optimized for 4-Cluster Mask).

Prepare mixture: 100 μl of above polyacrylamide solution
0.4 μl Methylene blue (0.04%)
1.2 μl TEMED
Vortex 3 seconds
Degas solution 3 min

(a) Pipette mixture between the slide and the mask allowing the solution to move between the space by capillary action. Take care that air does not enter the pipette or space. Pipette off excess solution.

(b) Turn cassette over so that glass slide is underneath the mask. Place in Oriel chamber.

(c) Irradiate for 300 sec.

(d) Carefully disassemble the cassette under water using the point of a scalpel to separate the slide and mask (the slide floats free without pressure being placed on the gel elements.) Take care not to scratch mask.

(e) Rinse 30 seconds under running distilled water and soak in distilled water for 15 minutes

(f) Air dry in a laminar-flow hood

(g) Keep in dust -free slide box at room temperature. Matrices can be kept for at least 1 year.

6. Procedure for Activation (Deprotection) of Aldehyde Matrices

(a) Place matrix in 2% trifluoro-acetic acid for 10 minutes at room temperature (prepare fresh solution after every 10 microchips).

(b) Rinse well (5 or 6 times) with filtered distilled water for 1 min

(c) Wash in distilled water ×3 times then leave 3–5 mins in last rinse and dry 20 min in air.

(d) Put slide into Repel Silane™ (use fresh solution for each treatment) for one minute.

(e) Wash with acetone or dichloromethane (15 sec) and then thoroughly with tap-distilled water (15 sec under stream).

(f) Load oligonucleotides.

(g) Put microchip into freshly prepared solution of pyridine-borane complex in chloroform (0.1M)(80 ml chloroform/1 ml pyridine borane) and cover chloroform layer with water; (approx ¼ inch) hold 12 hours at room temperature (O. N.)

(h) Wash microchip with distilled water.

(i) Place microchip into 0.1M sodium borohydride on microchip for 20 min.

(j) Wash with distilled water 1 min.

(k) Heat microchip in 0.1×SSPE with 0.1% SDS at 60° C. for 1 h (50 ml).

(l) Wash biochip in Hybridization Station for 15 min on a stirrer.

(m) Wash with distilled water 1 min.

(n) Dry microchip in a dust-free environment in the air for 20 min.

(o) The chip is now ready for hybridization. The chip could be kept at room temperature.

7. Standardized Sources of Chemicals and Equipment

(a) DEPC-Treated Water (Ambion, cat#9920)

(b) 0.5M EDTA, pH 8.0 (Ambion, cat#9260G)

(c) Eppendorf Centrifuge 5417C (Fisher, cat#05-406-11)

(d) Eppendorf microcentrifuge tubes, 1.5 ml (Fisher, cat#05-402-24B)

(e) Acetone (Sigma, cat#A4206)

(f) Guanidine Thiocyanate (Fisher, cat#BP221-1)

(g) 1M HEPES (Sigma, cat#H4034)

(h) Hybridization chamber: Probe-Clip Press-Seal Incubation Chamber (Sigma, cat#Z36,157-7)

(i) Kimwipes (Fisher, cat#06-666A)

(j) 20×SSPE (Sigma, cat#S2015)

(k) Tween 20 (Fisher, cat#BP337-100)

(l) Imaging Chamber (Sigma, cat#Z36,585-8)

(m) Ultrafree-MC 0.45 μm filter unit (Millipore, cat#UFC30HVNB)

(n) Triton X-100 (Sigma, cat#T9284)

(o) Ethyl Alcohol, absolute 200 proof, 99.5%, A.C.S. reagent (Aldrich, cat#45,984-4)

(p) QIAquick PCR Purification Kit (50) (Qiagen, cat#28104)

(q) Taq DNA Polymerase (includes 10×PCR reaction buffer) (Amersham Pharmacia Biotech, cat#T0303Z)

(r) PCR Nucleotide Mix: PCR nucleotide mix (10 mM each dATP, dCTP, dGTP, dTTP) (Amersham Pharmacia Biotech, cat# US77212)

(s) Sonicated Salmon Sperm DNA, Phenol Extracted (Amersham Pharmacia Biotech, cat#27-4565-01)

(t) Albumin from bovine serum, 20 mg/ml in water (Sigma, cat#B8667)

(u) Luer Lok syringe, 60 cc/2 oz, B-D (Fisher cat#14-823-2D)

(v) Millex-GN 0.20 filter units (Millipore, cat#SLGN025NS)

TABLE 2

Preparation of Complex Buffers

Buffer	Chemical/ Solvent/ Elementary buffer	Amount	Final Concentration	Comments
Wash Buffer	20 × SSPE buffer	15 ml	3xSSPE	Filter by using Millex GN 0.20 Filter and Luer Lok Syringe, B-D, 60 cc/2oz Note: Discard first 5 ml of Wash Buffer when you start filtration Keep at room temperature
	Tween 20	500 μl	1% (v/v)	
	MQ H2O	34.5 ml	—	
3xHybridization Buffer	6 M GuSCN	50 ml	3 M	Store at room temperature
	1 M HEPES, pH 7.5	15 ml	0.15 M	
	0.2 M EDTA, pH 8.0	7.5 ml	15 mM	
	MQ H2O	27.5 ml	—	
Stripping buffer	Guanidinium thiocyanate	300 g	4.9 M	Store solution at room temperature in a bottle with dark glass. Use for 20 stripping procedures (see below) then prepare a new portion.
	1 M HEPES, pH 7.5	13.2 ml	25 mM	
	10% (w/v) Triton X-100	5.2 ml	0.1%	
	Distilled water	250 ml		
Blocking solution	BSA, 20 mg/ml	200 μl	13.33 mg/ml	Store solution at −20° C.
	Salmon sperm DNA, ~20 A260 U/ml (to prepare, add 5 ml of 10 mM HEPES to a bottle with the DNA)	100 μl	6,67 A260 U/ml	

NOTE:
KEEP ALL BUFFERS IN BOTTLES WITH PLASTIC CAPS

TABLE 3

Materials and Equipment

Chemicals/Equipment	Manufacturer	Catalog #	Lot#
Acetone	Fisher	A18-4	11685
Acrylamide/Bis (19/1) solution 40%	BioRad	161-0144	66767
3-(Trimethoxysilyl)propyl methacrylate	Aldrich	Z-6030	03915TI
Pyridine-borane complex	Aldrich	17,975-2	13905MU
Glycerol	Sigma	G-7893	118H0280
Methylene blue	Merck	73881	51076
Ethyl Alcohol (absolute, 200 proof	Aapec Alcohol and Chemical Co	N/A	099I15UA
Chloroform	Aldrich	31,998-8	CO 09980AO
Repel Silane	Amersham-Pharmacia-Biotech	39422	17-332-01
Sodium borohydride	Aldrich	21,346-2	DU 00220MS
Sodium Hydroxide Solid	Sigma	S-5881	11K0116
Sodium Periodate (meta)	Aldrich	S-1878	11K3644
Sodium Phosphate, dibasic. anhydrous	Sigma	S-9763	119H0196
Sodium Phosphate, monobasic monohydrate	Fisher	S-369	792237
Sulfuric Acid	Fisher	A300-500	994173
Sodium Dodecyl Sulfate	Sigma	L3771	83H08411
SSPE, 20X	Sigma	S-2015	107H8508
(N-(5,6-di-O-isopropylidene)hexyl acrylamide)	Argonne, custom made		
TEMED	Sigma	T-7024	67H0136
Trifluoro-acetic Acid	Aldrich	T6,220-0	8K3483
Filter (0.45 um filter unit); Millex-HV 0.4	Millipore	SLHV 025 LS	
Glass slides, 3□ × 1□ Plain;	Corning	2947	
Mask	Nanofilm, California		
Audio tape film	Radioshak XR 60; Type I		

TABLE 3-continued

Materials and Equipment			
Chemicals/Equipment	Manufacturer	Catalog #	Lot#
Clamp. Medium Binger clips	Masterbrand	BTM00252	
Oriel Light Source	Oriel Instruments	92532-1000	S/N 139

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U.S. Pat. No. 5,552,270

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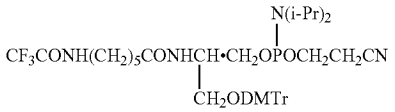
We claim:

1. A nucleotide-matrix connector comprising a bifunctional phosphoramidite and a nitrogen linker atom adapted for connecting to the matrix, the connector having the formula:



wherein DMTr is dimethoxytrityl and the connector, as part of a nucleic acid strand enables the formation of a synthetic duplex structure and immobilizes the synthetic duplex on the matrix.

2. A nucleic acid comprising strands linked by the connector of claim **1**.

3. The nucleic acid of claim **2** is DNA.

4. The nucleic acid of claim **2** is RNA.

5. The nucleic acid of claim **2** is a DNA-RNA hybrid.

6. The nucleic acid of claim **2** is a full duplex.

7. The nucleic acid of claim **2** is a partial duplex with a 3'-protruding end.

8. The nucleic acid of claim **2** is a partial duplex with a 5'-protruding end.

9. The nucleic acid of claim **2** is synthetic.

10. A method for synthesizing a connector designated phosphoramidite of claim **1**, said method comprising:

(a) synthesizing an activated ester of aminocaproic acid by

(i) protecting the NH_2 group of 6-aminocaproic acid with trifluoraccetic protective group; and

(ii) reacting with N-hydroxysuccinimide and N,N-dicylohexylcarbodiimide;

(b) obtaining a serinol derivative through

(i) reacting sequentially with ethyl trifluroacetate and 4,4'-dimethoxytritylchloride and

(ii) treating with methylamine;

(c) condensing the serinol derivative with an activated ester of N-trifluoroacetyl-6-aminocaproic acid; and

(d) phosphitylating the obtained compound with 2-cyanoethyl diisopropylchlorophosphoramidite.

11. A method for constructing double-stranded nucleic acid biochips (microarrays), said method comprising:

(a) synthesizing at least one double-stranded nucleic acid in which complementary oligonucleotide chains are attached together by the connector of claim **1**; and

(b) attaching the synthesized double-stranded nucleic acid to a matrix through a free-amino group of the linker.

12. A method for constructing a double-stranded nucleic acid microarray, said method comprising:

(a) synthesizing at least one double-stranded nucleic acid with the connector of claim **1** with at least one specific sequence for restriction endonuclease recognition;

(b) attaching the synthesized double-stranded nucleic acid to a matrix;

(c) digesting the attached double-stranded nucleic acid on the matrix using appropriate restriction endonucleases; and

(d) annealing the digested double-stranded nucleic acid on the matrix with double-stranded nucleic acid fragments having complementary protruding ends; and

(e) ligating the annealed result of (d).

13. The method of claim **12**, wherein the double-stranded nucleic acid is DNA.

14. The method of claim **12**, wherein the double-stranded nucleic acid is RNA.

15. The method of claim **12**, wherein the double-stranded nucleic acid is a DNA-RNA hybrid.

16. A method for investigating double-stranded nucleic acid-protein interactions, said method comprising:

(a) synthesizing a double-stranded nucleic acid probe of a specific sequence having a connector as in claim **1**;

(b) attaching the synthesized double-stranded nucleic acid probe to a matrix;

(c) providing conditions necessary for the double-stranded nucleic acid-protein interaction; and

(d) determining results of the interaction.

17. The method of claim **16**, wherein the double-stranded nucleic acid is DNA.

18. A method for non-enzymatically synthesizing a double-stranded nucleic acid without forming a stem-loop structure, said method comprising:

synthesizing a linear chain of complementary nucleic acids with the connector of claim **1** at a specific position within the chain; and

providing conditions necessary for double-strand structure formation from the complementary chains.

19. A method to construct double-stranded DNA microarrays, said method comprising:

(a) synthesizing complementary oligonucleotide chains bonded together by a novel connector to form a DNA duplex;

(b) incorporating a linker to the connector, said linker capable of anchoring the DNA duplex to a support; and

(c) contacting the chemically synthesized duplex-connector-linker molecule to a support.

20. A microchip with dsDNA oligonucleotides each anchored to gel pads by the connector of claim **1**.

21. A dsDNA microarray constructed using the method of claim **12**.

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