2.1 Source of Chemicals

3-Aminopropyltriethoxysilane (APTS), 3-chloropropyltriethoxysilane (CPTS), 3-glycidyloxypropyltrimethoxysilane (GOPTS), 3-isocyanatopropyltriethoxysilane (IPTS), 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride), N-methyl-2-aminoethanol, N,N-diisopropylethylamine, long chain alkylamine-controlled pore glass (LCAA-CPG), controlled pore glass (CPG) (500Å), dithiothreitol (DTT), N,N’-diisopropylcarbodiimide (DIPCI), N,N’-dicyclohexylcarbodiimide (DCC), 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDAC), N-methylimidazole (NMI), diethylene glycol (DEG), 4-dimethylaminopyridine (DMAP), amino-functionalized magnetic particles (particle size 1 micron) and glass microslides were procured from Sigma-Aldrich Chemical Co., St Louis, MO, US. TET-phosphoramidite was purchased from Applied Biosystems Inc., Foster City, CA, US. Fractosil 500Å and triphenylphosphine (TPP) were obtained from Fluka Chemie Gmbh, Buchs, Switzerland. Pre-coated silica gel TLC plates were purchased from Merck, India. Other chemicals and reagents were procured from local vendors.

2.2 Instrumentation

2.2.1 Microarray Scanner

Microslides were scanned using laser scanners ScanArray Lite™, Micro Array Analysis System, GSI Lumonics, CA, USA, and ScanArray Gx Plus™, Micro Array Analysis System, Perkin Elmer, Massachusetts, USA, fitted with a Cy3 and Cy5 optical filter with 30 μm resolutions.

2.2.2 Infrared Spectroscopy (IR)

FTIR spectra were recorded on a single beam Perkin Elmer (Spectrum BX Series) and Beckmann Acculab-10 systems, USA with the following scan parameters: scan range 4400-400 cm⁻¹; number of scans 16; resolution 4.0 cm⁻¹; interval 1.0 cm⁻¹; unit %T. The samples were prepared in KBr pellets.

2.2.3 Ultraviolet Spectroscopy (UV)

Ultraviolet spectra of organic compounds and oligonucleotides were recorded on a Lambda Bio 20 UV-VIS spectrophotometer (Perkin Elmer). The
cuvette has an optical path length of 1.0 cm and $\lambda_{\text{max}}$ values are expressed in nanometers (nm).

### 2.2.4 NMR Spectroscopy

$^1$H NMR spectra were recorded on a Brüker Avance 300 system operating at 300 MHz. Chemical shifts are shown in parts per million (ppm) in the spectra and tetramethylsilane (TMS) was used as an internal standard. The notations used are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad; R, aliphatic; and Ar, aromatic.

### 2.2.5 Mass Spectroscopy

Mass spectra of the organic compounds were recorded on Waters KC455 TOF-MS and oligonucleotides on a SELDI-TOF (Surface enhanced laser desorption ionization-time of flight) PBS-II, Ciphergen Biosystems, Freemont, CA using an 8-well gold plated chip. 3-Hydroxypicolinic acid mixed with picolinic acid and diammonium citrate was used as matrix for oligomer analysis.

### 2.2.6 Zeta Potential Measurements

The zeta potential measurement of the particles was carried out on a Nano ZS Zetasizer (Malvern instruments, UK) equipped with a 5 mW He-Ne laser, operating at 633 nm. The analyses were performed either in automatic or manual mode. Measured size range was presented as the average value of 20 runs.

### 2.3 Purification of Solvents

Analytical grade solvents, viz., acetonitrile (ACN), pyridine (Py), dichloromethane (DCM), $N,N$-dimethylformamide (DMF), ethylenedichloride (EDC), tetrahydrofuran (THF), triethylamine (TEA) and methanol, were purified and dried according to reported protocols.¹

### 2.4 Chromatography

#### 2.4.1 Thin Layer Chromatography (TLC)

All compounds synthesized in the present work were analyzed on TLC plates ($F_{254}$, Merck, India). Following solvent systems were used in present investigation:
• EDC
• EDC : Methanol (9 : 1 v/v)
• EDC : Methanol (8 : 2 v/v)

The spots on the TLC plates were detected under single wavelength UV irradiator and/or developed by using the following reagents: (i) 0.5% Ninhydrine in methanol, (ii) Perchloric Acid: 50 ml perchloric acid in 75 ml of water.

2.4.2 Column Chromatography
Silica gel (60-120 mesh size, SRL, India) was used for purification of compounds.

2.5 Oligonucleotide Synthesis
In the present study, oligonucleotide synthesis was performed on a Pharmacia LKB Gene Assembler Plus according to the standard protocol provided by the manufacturer. Various reagents and chemicals required in a synthetic cycle are detailed below:

• Dry acetonitrile
• Dry dichloroethane
• Detritylation solution : TCA (1.0 g) dissolved in EDC (100 ml)

The following solutions were freshly prepared prior to commencement of oligonucleotide synthesis:

• **Capping solutions**
  Capping solution A: 6% 4-Dimethylaminopyridine (DMAP) dissolved in dry acetonitrile.
  Capping solution B: 20% Acetic anhydride in dry acetonitrile.
• **Oxidation solution**: Iodine (0.02 M) dissolved in H$_2$O: Pyridine: MeCN (1: 10: 40) solvent system.
• **Deoxyribonucleoside phosphoramidites**: Solution of each phosphoramidite (0.1 M) was prepared in acetonitrile and kept for at least 2 h over molecular sieves (3Å) prior to the commencement of synthesis.
• **1H-Tetrazole (activator)**: A solution of sublimed tetrazole (0.45 M) was prepared in dry acetonitrile and kept for 2h over molecular sieves (3Å) before use.
2.6 Deprotection and Desalting

The support bound fully protected oligonucleotides were subjected to deprotection with aqueous ammonium hydroxide (30%) at 60°C for 16 h in a sealed deprotection vial to effect the cleavage of oligonucleotide chains from the support and removal of protecting groups from the exocyclic amino functionalities of the nuclear bases as well as from internucleotidic phosphates. After stipulated time, the vial was removed from the oven and kept in a refrigerator for 1 h. After evaporation of ammoniacal solution, the residue was suspended in distilled water and subjected to desalting on C-18 reverse phase silica gel column. The oligomers were eluted with 30% aqueous acetonitrile solution and concentrated in a speed vac (Heto) prior to purification step.

2.7 Synthesis of 5’- and 3’-Modified Oligonucleotides

2.7.1 Preparation of 5’-Modified Oligonucleotides

Synthesis of 5’-modified oligonucleotides is relatively simple. A reactive functional group (aminoalkyl / mercaptoalkyl / phosphoryl / thiophosphoryl) can be easily introduced in the protected form with the help of a suitable linker phosphoramidite in the last coupling step. The desired oligonucleotide with a free nucleophilic group is obtained after deprotection.

(a) Synthesis of 5’-Aminoalkylated Oligonucleotides

Oligonucleotides were assembled at 0.2 µmol scale on a Pharmacia LKB Gene Assembler Plus following manufacturer’s recommendations. The last coupling step was performed with the phosphoramidite reagent, \( N-(4,4’\text{-dimethoxytrityl})-5\text{-aminohexyl}-2\text{-cyanoethyl}-N,N\text{-diisopropylphosphoramidite} \),\(^2\) (Figure 1) in a similar manner the normal nucleoside phosphoramidites were coupled except with an extended coupling time (5 min) and with DMTr off option.\(^3\) The 5’-aminoalkyl-oligonucleotides were fully deprotected by the treatment with 30% aqueous ammonium hydroxide at 60°C for 16 h. The ammoniacal solution was concentrated in a speed vac and subjected to desalting, as described above.

![Figure 1: \( N-(4,4’\text{-Dimethoxytrityl})-5\text{-aminohexyl}-2\text{-cyanoethyl}-N,N\text{-diisopropylphosphoramidite} \)](image_url)
(b) Synthesis of 5’-Mercaptoalkylated Oligonucleotides

Oligonucleotides synthesis was carried out as described above. The last coupling step was performed with one of the phosphoramidite reagents, S-acetyl-6-mercaptohexyl-2-cyanoethyl-N,N-diisopropylphosphoramidite (A) or S-benzoyl-6-mercaptohexyl-2-cyanoethyl-N,N-diisopropylphosphoramidite (B) (Figure 2) in an analogous manner to the normal nucleoside phosphoramidites.4 The mercaptoalkylated oligonucleotides were deprotected from the support on treatment with 30% aqueous ammonium hydroxide containing 50 mM DTT at 60°C for 16 h. The ammoniacal solution was concentrated, desalted and stored under inert atmosphere (Ar or N2).

![Figure 2: Reagents for 5'-mercaptoalkylation of oligonucleotides](image_url)

(c) Synthesis of 5’-Phosphorylated Oligonucleotides

Synthesis and deprotection of 5’-phosphorylated oligonucleotides was carried out, as described earlier. The last coupling step of synthesis was performed using the following phosphoramidite (Figure 3).5

![Figure 3: 2-\{(4,4’-Dimethoxytrityloxy)\}-ethanesulphonylethoxy-(2-cyanoethyl-N,N-diisopropylphosphoramidite](image_url)

2.7.2 Preparation of 3’-Modified Oligonucleotides

Unlike 5’-end modifications, 3’-modifications are not that straightforward. Since 3’- hydroxyl group of the leader nucleoside is not available for manipulation during solid phase synthesis, as it is attached to the support via its hydroxyl group, engineered supports are required to incorporate modifications at the 3’ terminus of oligonucleotides.6-11
(a) Synthesis of 3'-Aminoalkylated Oligonucleotides

3'-Aminoalkylated oligonucleotides were synthesized on the polymer support prepared according to the published protocol. A modified controlled pore glass support (Figure 4) was synthesized in order to incorporate aminoalkyl groups at the 3'-end of oligonucleotides. Oligonucleotides were synthesized in a Gene Assembler Plus using the functionalized support. After assembly of the desired sequence, the support was subjected to deprotection with aq. NH₄OH (30%) at 60°C for 16 h.

(b) Synthesis of 3'-Mercaptoalkylated Oligonucleotides

The polymer support for the synthesis of 3'-mercaptoalkylated oligonucleotides was prepared according to the published protocol. The functionalized support (figure 4) was dried under vacuum and loading was determined following standard protocol.

In order to incorporate mercaptoalkyl groups at the 3'-end of oligonucleotides, synthesis was carried out on the above support in a Gene Assembler Plus. After assembly of the desired sequence, the support was subjected to deprotection with aq. NH₄OH (30%) containing 0.5 M DTT at 60°C for 16 h.

(c) Synthesis of 3'-Phosphorylated Oligonucleotides

3'-Phosphorylated oligonucleotides were synthesized on an aminopropylated CPG support depicted in figure 6. After assembly of the desired sequence, the support was subjected to deprotection with aq. NH₄OH (30%) at 60°C for 16 h, followed by treatment with 80% acetic acid for 4 h.
2.7 Purification and Characterization of Oligonucleotides

The crude product, obtained after deprotection and desalting steps, consists of the desired oligonucleotide chains along with a mixture of truncated shorter oligonucleotides. Therefore, a rapid and efficient purification or separation technique is required. Among the various purification techniques reported, the RP-HPLC technique was extensively used and is described below in brief:

**Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)**

Reverse-phase high performance liquid chromatography (RP-HPLC) is an important technique for the purification of oligonucleotides. Traditional adsorption chromatography is performed on a polar stationary phase such as silica gel, and uses a non-polar mobile phase. In this ‘normal phase’ chromatography, polar solutes are tightly bound to the polar stationary phase and are eluted later than non-polar solutes. Polar compounds are eluted from the stationary phase by increasing the polarity of the mobile phase. In reversed phase chromatography, as implied, the conditions are reversed. The stationary phase is non-polar and the mobile phase is polar. It is the hydrophobic interactions, which determine the velocity of the migration along the stationary phase. Polar solutes are eluted relatively early from the column. Elution of a particular compound, in this case, is effected by reducing the polarity of the aqueous mobile phase usually by the addition of an organic solvent. The technique may be used in three modes depending on the size of the column, viz., analytical, semi-preparative and preparative chromatography. Separation of oligonucleotide chains is decided by the hydrophobicity imparted by heterocyclic bases present in the chain. Therefore, a slight variation in the composition does not alter the overall chromatographic behavior of the chromatogram, which may sometimes pose problems in identification of the desired product. This problem is normally circumvented by introducing a hydrophobic group, 4,4’-dimethoxytrityl, at the 5’-position of the synthetic oligomers. The group provides an affinity handle and increases the retention of the desired chain on the matrix in the column. During the synthesis, if the capping takes place perfectly, then only the desired oligonucleotide chains carry the 4,4’-dimethoxytrityl (DMTr) group, not the truncated or failure sequences. During
the deprotection step, all the protecting groups are removed except 5'-O-DMTr group. The truncated sequences are eluted first and the desired material elutes at a higher concentration of non-polar solvent, acetonitrile. Because of the large difference in hydrophobicity, the desired sequence is well resolved from the shorter ones. After removal of DMTr group, the desired material can be reanalyzed on the same column.

In the present work, RP-HPLC was performed on a Agilent 1100 series attached with a variable wavelength PDA (Photodiode array) detector operating at 260 nm from Agilent Technologies, USA. Ammonium acetate (0.1 M, pH 7.1) was used as a general purpose buffer with acetonitrile as an eluent.

2.8 Cleaning of Glass Microslides

Unmodified glass microslides were cleaned by immersing them in 1M NaOH (100 ml) for 2 h, followed by washings under running water. Slides were then submerged in a solution of 1 M HCl (100 ml) for 2 h, followed by washings under running water, and finally immersed in 95% ethanol for 1 h at room temperature. The glass slides were dried under vacuum and stored under inert atmosphere.

2.9 Epoxylation of Glass Microslides

The silanization was accomplished using GOPTS (2%, v/v) in toluene. Briefly, washed glass microslides were dipped in a 2% solution of GOPTS and kept for 4 h at 50°C. After the reaction, the slides were washed with dry toluene (3 x 30 ml), dried and stored under Ar atmosphere.11

2.10 Scanning Parameters

Microarrays were scanned on a microarray fitted with a Cy3 optical filter at 20 μm resolution, PMT and Laser power were set to 55% and 80%, respectively. For quantification of immobilized oligonucleotides, a TET-labeled oligonucleotide sequence was diluted from 1 to 0.05 μM concentrations and spotted on a virgin micro slide. After drying, the slide was scanned and spots were quantified. A standard calibration curve was plotted between fluorescence
intensity (A.U) and concentration (μM). For each experimental condition tested on the microarrays, the experiment was repeated 2-3 times. The immobilization and hybridization data presented are the average of these repetitions, and the error bars represent the percentage error (±2-4%) on this average.
2.11 References