3.1 MATERIALS

3.1.1 FINE CHEMICALS

Fine chemicals and Biochemicals were procured from the following standard commercial sources such as: Sigma-Aldrich Chemicals Co, Bangalore; Bio-Rad Laboratories, California; Bangalore Genei India Pvt. Ltd., Bangalore, India; BIO BASIC INC, Canada; Q-BIO gene molecular biology, USA; Amersham Biosciences, Hong Kong; USB Corporation Cleveland, OH USA; LOBA CHEMIE Pvt. Ltd., Mumbai, India; PIERCE, USA; B D Biosciences clontech, California, USA.

3.1.2 BIOCHEMICALS/GLASSWARES

<table>
<thead>
<tr>
<th>S.no</th>
<th>Items</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Nucleo Trap® Nucleic Acid Purification Kits and accessories</td>
<td>B D Biosciences clontech, California, USA</td>
</tr>
<tr>
<td>2</td>
<td>Acridine orange</td>
<td>HiMedia, India</td>
</tr>
<tr>
<td>3</td>
<td>Bromophenol Blue</td>
<td>HiMedia, India</td>
</tr>
<tr>
<td>4</td>
<td>Bovine Serum Albumin (BSA)</td>
<td>HiMedia, India</td>
</tr>
<tr>
<td>5</td>
<td>Chlorotrimethylsilane</td>
<td>Sigma-Aldrich Chemicals Co, Bangalore</td>
</tr>
<tr>
<td>6</td>
<td>Hydrogen peroxide</td>
<td>Sigma-Aldrich Chemicals Co, Bangalore</td>
</tr>
<tr>
<td></td>
<td>Chemical Name</td>
<td>Supplier</td>
</tr>
<tr>
<td>---</td>
<td>---------------------------------------------------</td>
<td>---------------------------------</td>
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<td>7</td>
<td>Tetrachloroethane</td>
<td>Sigma-Aldrich Chemicals Co, Bangalore</td>
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<td>4', 6- diamidine- 2'- phenylindole, dihydrochloride (DAPI)</td>
<td>Pierce, USA</td>
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<td>9</td>
<td>Dithiothreitol (DTT)</td>
<td>Bio Basic Inc, Canada</td>
</tr>
<tr>
<td>10</td>
<td>Ethylene Diamine Tetra acetic acid (EDTA)</td>
<td>Bio Basic Inc, Canada</td>
</tr>
<tr>
<td>11</td>
<td>Durapore's membrane (0.45μm) using syringe filter technology</td>
<td>Millipore, Ireland</td>
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<td>12</td>
<td>Ethidium bromide (EtBr)</td>
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<td>13</td>
<td>Gold coin (22-Carat)</td>
<td>Tanishq, India</td>
</tr>
<tr>
<td>14</td>
<td>Nucleo Trap PCR extraction kit</td>
<td>New England Biolabs, England</td>
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<tr>
<td>15</td>
<td>Phenol equilibrated with Tris, pH 7.5</td>
<td>Amersham Biosciences, Hong Kong</td>
</tr>
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<td>16</td>
<td>λ-DNA</td>
<td>Amersham Biosciences, Hong Kong</td>
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<td>17</td>
<td>Sodium acetate</td>
<td>LOBA CHEMIE Pvt. Ltd., Mumbai, INDIA</td>
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<tr>
<td>18</td>
<td>Sodium chloride</td>
<td>US Biologicals, USA</td>
</tr>
<tr>
<td>19</td>
<td>Tris-HCl</td>
<td>US Biologicals, USA</td>
</tr>
<tr>
<td>20</td>
<td>All glassware used during present work</td>
<td>Scott glass,</td>
</tr>
<tr>
<td>S.no</td>
<td>Enzymes/DNA ladder</td>
<td>Sources</td>
</tr>
<tr>
<td>------</td>
<td>------------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>T4 DNA ligase</td>
<td>Bangalore Genei India Pvt. Ltd., Bangalore, India</td>
</tr>
<tr>
<td>2</td>
<td>XT-5 Polymerase</td>
<td>Bangalore Genei India Pvt. Ltd., Bangalore, India</td>
</tr>
<tr>
<td>3</td>
<td>Calf Intestine Alkaline Phosphatase (CIP)</td>
<td>Q-BIO gene molecular biology, USA</td>
</tr>
<tr>
<td>4</td>
<td>T4 polynucleotide Kinase</td>
<td>Epicentre, Wisconsin</td>
</tr>
<tr>
<td>5</td>
<td>Ready-to Use DNA Size and Mass Standard is a mix of λ-DNA Hind III digest and Bacteriophage ØX174 DNA Hae III digest</td>
<td>FINNZYMES, Finland</td>
</tr>
<tr>
<td>6</td>
<td>1kb DNA ladder</td>
<td>Bangalore Genei India Pvt. Ltd., Bangalore, India</td>
</tr>
</tbody>
</table>
3.1.4 PRIMERS/OLIGOS

Primers were synthesized and procured from Bio Basic Inc, Canada. These primers were thiol (-SH) modified at 5’ ends. Complementary oligos of the overhang of the Cos-sites of the λ-DNA were synthesized from Microsynth Laboratory, Balgach, Switzerland. These oligos were thiol modified at 3’ ends.

![Figure 3.1: Structure of modification at 5' end of oligonucleotides](image)

Primer-1
1F-5’-SH-ATGCTTGAACCCGCCTATGC-3’ (20)
1R-5’-SH-TCACTTCATGCTTCGGCTTGAC-3’ (22)

Primer-2
2F-5’-SH-TGGGATATTACGTCAGCGAGGAC -3’ (23)
2R-5’-SH-CACTTCATGCTTCGGCTTGAC -3’ (21)

Primer-3
3F-5’-SH-TGACTGCTGCTGCATTGACG-3’ (20)
3R-5’-SH-GCCATGATTACGCCAGTTGTAC-3’ (22)

PCR products amplified by specific primers; Primer-1, Primer-2 and Primer-3 have been denoted as Pr1, Pr2 and Pr3 in further description.
Cos-1 (Oligo-1): 5’ GGG CGG CCT CGA-SH- 3’
Cos-2 (Oligo-2): 5’ AGG TCG CCG CCC –SH- 3’

3.1.5 COMMON REAGENTS AND SOLUTIONS

Solutions and buffers required for purification of DNA samples at different steps, immobilization, PCR and electrophoresis was prepared in deionized (18 MΩ) double distilled ultra pure water obtained from ELGA Purelab ultra system, UK.

**TE Buffer (200 ml)**

10 mM Tris Cl (pH 8.0)
1mM EDTA (pH 8.0)

The buffer was sterilized by autoclaving at 15 lbs/ in² at 121°C for 15 minutes and stored at 4°C.
10% SDS (100 ml)
10 g of SDS was dissolved in autoclaved deionized double distilled water. Final volume made up to 100 ml and stored at room temperature.

6 M Saturated NaCl solutions
35.064 g - NaCl
Dissolve in deionized double distilled water and final volume made up to 100 ml.

30 mM DTT Solution
Prepared 30 mM working solution in double distilled deionized water by dissolving 9.24 mg in 2 ml. It was sterilized by filtration and kept in vial of volume 2.5 ml at -20°C. Under this condition, DTT is stable to oxidation by air.

NaCl (1M) Solution
5.84 g of NaCl was dissolved in 80 ml. its volume was adjusted to 100 ml with double distilled deionized water. It was sterilized by autoclaving.

Na₂HPO₄ (1M) Solution
26.8 g of Na₂HPO₄ was dissolved in 80 ml of deionized distilled water. Total volume was made up to 100 ml water. The solution was sterilized by autoclaving for 20 minutes at 15 psi (1.05 kg/cm²) on laboratory autoclaving apparatus (METREX). Solution was stored at room temperature.

EDTA (0.5M, pH 8.0) Solution
18.61 g of disodium EDTA.2H₂O was added to 80 ml of double distilled water. It was stirred vigorously on a magnetic stirrer. Its pH was
adjusted to 8.0 with NaOH pellets (20 g each). It was sterilized by autoclaving for a period of 20 minutes at 15 psi (1.05 kg/cm²) on laboratory autoclaving apparatus (METREX). The sodium salt of EDTA will not go into solution until the pH of the solution is adjusted to ~ 8.0 by the addition of NaOH.

**Binding Buffer [KH₂PO₄ (1M)]**
6.80 g of KH₂PO₄ was added in 40 ml deionized double distilled water. It was stirred to dissolve completely. Its pH was adjusted to 7.4 with HCl. Final volume was made up to 50 ml with double distilled water.

### 3.1.6 SOLUTION FOR AGAROSE GEL ELECTROPHORESIS

**Running Buffer: 50X TAE (Stock Solution)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-base</td>
<td></td>
</tr>
<tr>
<td>Glacial Acetic Acid</td>
<td></td>
</tr>
<tr>
<td>0.5 M EDTA, pH 8.0</td>
<td></td>
</tr>
</tbody>
</table>

242 g
57.1 ml
100 ml
Distilled water was added to make the final volume of solution to 1000 ml. 50X TAE were diluted to 1X with distilled water, prior to use.

**DNA Loading dye (5X)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td></td>
</tr>
<tr>
<td>EDTA, pH 8.0</td>
<td></td>
</tr>
<tr>
<td>Tris-HCl, pH 8.0</td>
<td></td>
</tr>
<tr>
<td>Orange G</td>
<td></td>
</tr>
</tbody>
</table>

60%
100 mM
25 mM
0.25%
Appropriate amounts of each were dissolved in a final volume of 100 μl.
**Ethidium Bromide (Stock Solution)**

10 mg/ml stock solution in distilled water was prepared and stored in a screw cap vial wrapped with aluminum foil at 4°C. An aliquot of the stock was added in the agarose solution to get a concentration of 0.5 μg/ml.

### 3.1.7 SOLUTION USED FOR EXTRACTION OF DNA FROM LOW MELTING AGAROSE

DNA eluted form the agarose gel by using the DNA isolation kit (BD Biosciences). All the solutions were supplied by the manufacturer.

- Glass suspension powder
- Sodium iodide solution
- Wash solution
3.2 METHODS
3.2.1 FABRICATION OF AN ARRAY OF ELECTRODES ON GOLD SURFACE

For electrical characterization of λ-DNA, arrays of gold microelectrodes (25 x 35 µm) with pointed ends, were fabricated by lithographic techniques, on a glass wafer and received from GAETEC (Gallium Arsenide Enabling Technology Centre), Hyderabad, India (Fig.3.3). The pointed design was especially created to minimize the number of bridging DNA molecules. The pointed electrodes are used as bonding pads for later on current-voltage measurements, as the pins of the probing station are made to contact the molecule through these protrusions. An interesting possibility will be to gold plate the actual points or to make the electrodes effectively points rather than pointed blocks, which can further limit the number of bridging molecules. Each diced wafer (7 x 7 mm, approximately) had an array of 400 gold microelectrode pairs. Each column has 20 microelectrodes of same spacing and it increases in the row in steps of 1 µm. Thus, the first column has electrodes of 5 µm, the second column of 6 µm and the last (20th column) having electrodes of 25 µm and a total of 400 microelectrodes on each diced wafer (Fig. 3.3). The array format for microelectrodes was especially created since it can play a role in biosensing as a future extension to this work. Such arrays are today’s necessity in nanobiotechnology. In the present study major emphasis was on uniform arrays for DNA immobilization, however, the same methods may be equally useful in the case for individual electrodes or patches of microelectrodes. The glass slides were pretreated with a solution of chlorotrimethylsilane and tetra chloroethane, to avoid spurious binding of DNA to glass before electrode fabrication.
Figure 3.3: A schematic of the ultradense array pattern of gold microelectrodes. Inset shows schematic design of the microelectrodes for DNA immobilization (section view).
Glass substrate was coated with Ti/Pt/Au having thickness around 0.45 μm and ultradense array pattern of gold microelectrodes, using patterned mask was developed. A gold layer was deposited to a thickness of 30 nm. The electrodes have a pointed design and occur in pairs columns separated by one another by a distance of 300 μm. The gap between each electrode pair, in a row, is in increasing order from 5 to 25 μm.

3.2.2 FABRICATION OF MICRO/NANO ELECTRODES BY LASER ABLATION USING OPTICAL TWEEZER

A novel methodology was used for fabricating electrodes with spacing as small as 600 nm based on concept of laser ablation of material. For this gold coated glass slides were exposed to focused laser beam of Optical Tweezer cum laser Microdissection system. Ultra violet (nitrogen) laser beam was applied, with a wavelength of 337 nm to the gold coated glass surface. Pulsed laser mode was used that has pulse duration of 4 nsec. and a frequency of 33 Hz. Total maximum energy is 300 μJ and after various losses the energy of UV laser available at objective is 40 μJ. Different energies were applied to gold coated glass surface for optimization.

Maximum energy of UV laser = 300 μJ
Pulse width= 4 nsec
Frequency= 33 Hz
Energy available at Objective after various losses = 40 µJ

Peak power per pulse = Energy/time
= 300 µJ/4 nsec = 75 Kwatt

Peak power per pulse at objective = Energy available at objective/time
= 40 µJ/4 nsec = 10 Kwatt

Average power = P_{peak} x pulse width x frequency = 0.66 mW

The complete data using different percentage of energy with variation of spot size on gold coated glass surface is given in table 3.1

From the data, it was concluded that 60 % energy would give best results and was chosen for subsequent experiments of electrode fabrication (Table 3.1). 60% of the original energy of the Ultra-Violet laser (300 µJ) was used, and after losses during passing in (86.67%) in the microscope, the available energy at the sample was around 24 µJ as shown in the Table 3.1. So, the peak power per pulse is very high (around 75 kilowatt out of which 10 kilowatt is available at objective) and is more than sufficient to remove the gold from the glass surface. The gold surface thickness in the present case used was 30 nm. Gold was coated on the glass surface from a gold coin of 22-carat.
Table 3.1: Variation of spot width as a function of percentage of energy on gold coated glass surface.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>%age Energy</th>
<th>Energy available at objective(μJ)</th>
<th>Peak power per pulse (kilowatt)</th>
<th>Average Power (milliwatt)</th>
<th>Dia. of spot (in μ) on gold slide</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>20</td>
<td>5</td>
<td>0.66</td>
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<tr>
<td>2</td>
<td>53</td>
<td>21.2</td>
<td>5.3</td>
<td>0.69</td>
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<tr>
<td>3</td>
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<td>22</td>
<td>5.5</td>
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<td>1.641</td>
</tr>
<tr>
<td>4</td>
<td>60</td>
<td>24</td>
<td>5.5</td>
<td>0.79</td>
<td>3.359</td>
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<tr>
<td>5</td>
<td>65</td>
<td>26</td>
<td>6.5</td>
<td>0.85</td>
<td>4.698</td>
</tr>
<tr>
<td>6</td>
<td>70</td>
<td>28</td>
<td>7</td>
<td>0.92</td>
<td>5.970</td>
</tr>
<tr>
<td>7</td>
<td>75</td>
<td>30</td>
<td>7.5</td>
<td>0.99</td>
<td>8.047</td>
</tr>
<tr>
<td>8</td>
<td>80</td>
<td>32</td>
<td>8</td>
<td>1.05</td>
<td>8.672</td>
</tr>
<tr>
<td>9</td>
<td>85</td>
<td>34</td>
<td>8.5</td>
<td>1.12</td>
<td>8.359</td>
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<tr>
<td>10</td>
<td>90</td>
<td>36</td>
<td>9</td>
<td>1.18</td>
<td>9.305</td>
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<tr>
<td>11</td>
<td>95</td>
<td>38</td>
<td>9.5</td>
<td>1.25</td>
<td>10.643</td>
</tr>
<tr>
<td>12</td>
<td>100</td>
<td>40</td>
<td>10</td>
<td>1.32</td>
<td>11.719</td>
</tr>
</tbody>
</table>
3.2.3. IMMOBILIZATION OF $\lambda$-DNA

Initially, DNA used for immobilization in the present study was $\lambda$-DNA. The term lambda means that the molecules were extracted from $\lambda$-bacteriophage and does not refer to any structural conformation like B-DNA, or the metallized form of DNA known as M-DNA. $\lambda$-DNA has Guanine-rich regions which serve the purpose of our study on naturally occurring Guanine rich sequence. $\lambda$-DNA was immobilized between electrodes by exploring its Cos-site overhangs for the total length of DNA. Immobilization sample was prepared as describe in schematic of figure 3.4. Calf intestinal phosphatase (CIP) treatment was given to $\lambda$-DNA to remove phosphate group from 5' end and Kinase was used to introduce phosphate at 3' end of the thiolated oligos. Both phosphorylated thiolated Cos oligos and dephosphorylated $\lambda$-DNA was hybridized in hybridization buffer. Ligase was used to join the two strands of DNA. In this was $\lambda$-DNA with thiolated ends was obtained and was used for immobilization in between the gold electrode. The detail protocol followed for the preparation of immobilization sample is mentioned below:
Figure 3.4: A schematic of the immobilization of full length of λ-DNA using thiolated oligonucleotides complementary to Cos site of λ-DNA.
3.2.3.1 CIP TREATMENT

Calf intestine alkaline phosphatase (CIP) hydrolyses 5’-terminal monophosphate groups of DNA and RNA. One unit is the amount of enzyme required for the dephosphorylation of one picomole of 5’ phosphorylated ends of Hind III fragments from λ-DNA (corresponding to approximately 2.5 μg dsDNA) within 1 h at 37°C and pH 9.0. The CIP treatment of λ-DNA was carried out according to the protocol as described below:

- Added 10 μl of 10 X buffer (Tris 50 mM, pH 9.0, MgCl₂ 1mM, ZnCl₂ 0.1 mM)
- Added 25 μl of 15 nM, λ-DNA solution.
- Added 5 U CIP to the solution.
- Made to 100 μl with DI water, incubated the mix at 37°C for 1 h.
- Added EDTA to 5 mM and heated at 70 °C for 10 min, to stop the reaction.
- Extracted with phenol and recovered the DNA by ethanol precipitation.

3.2.3.2 T4 POLYNUCLEOTIDE KINASE

T4 polynucleotid e kinase (T4 PNK) catalyzes the transfer of γ-phosphate of ATP to the 5’-terminus of single and double stranded DNA or RNA molecules that have a 5’-OH. T4PNK was used for phosphorylate the 5’ ends of oligodeoxynucleotides prior to ligation. One unit catalyzes the transfer of one nanomole of phosphate from ATP to the 5’ hydroxyl ends of micrococal nuclease-treated calf thymus DNA in 30 min at 37°C. The reaction was set as mentioned below:
• Added 0.1 nmole of oligonucleotides to appropriate amount of 10X reaction buffer (330 mM tris-acetate (pH 7.8), 660 mM potassium acetate, 100 mM magnesium acetate and 5 mM DTT).
• Added ATP to a final concentration of 1 mM.
• Added 3 U of T4 PNK before adjusting the final concentration of buffer to 1x by adding DI water.
• Incubated the mixture at 37°C for 30 min.
• Inactivated the T4 PNK by incubating for 5 min at 70°C.

3.2.3.3. T4 DNA LIGASE

T4 DNA ligase was used to covalently join DNA fragments with cohesive ends or blunt ends. One unit is defined as the amount of enzyme required to give approximately 50% ligation of 2 µg λ-DNA, Hind III digest in 30 min at 16°C in a 20 µl reaction mixture. Thiolated oligonucleotides were covalently joined to their complementary sites in λ-DNA by setting the ligase reaction as mentioned below:

• Added 10 µl of 10X buffer, 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 20 mM DTT, 50 µg/ml nuclease free BSA and 1 mM ATP to a clean microfuge tube.
• Added DI water, pre-calculated, to make 100 µl final reaction mixtures.
• Added 25 µl, 15 nM λ-DNA.
• Added 25 U (Weiss units) of the enzyme.
• Incubated the mixture at 16°C overnight.
• Inactivated the enzyme by heating at 70°C for 10 min.
3.2.3.4 PHENOL CHLOROFORM PURIFICATION

Phenol-chloroform purification method underlies the principal of like dissolves like. This purification separates component of a mixture in two different liquid phases i.e. aqueous and organic. DNA being a hydrophilic molecule will be obtained in aqueous phase as a supernatant. Proteineous impurities will be obtained at the interface of organic and aqueous phase. Organic contaminants remains dissolve on lower organic phase.

- An equal volume of buffer-saturated phenol: chloroform (1:1) was added to the DNA solution.
- The mixture was gently mixed using micropipettes to prevent the shearing of the long strands of DNA.
- It was spun at 12000 g for a period of 2 minutes.
- The aqueous layer was carefully removed to a new vial; care was taken to avoid the interface. (Steps 1-4 can be repeated until an interface is no longer visible)
- An equal volume of chloroform was added to the aqueous layer to remove traces of the phenol.
- It was spun at 12000 g for a period of 2 minutes.
- Aqueous layer was carefully transferred to a new vial.
- Finally DNA was ethanol precipitated.
- 30 μl of the prepared λ-DNA was pipetted out on the activated gold electrode surface.
- This glass wafer containing gold electrodes were put in a plastic Petridish and incubated for a period of 16 h.
- Thereafter, it was gently rinsed in deionized double distilled water and was taken for staining and visualization.
3.2.3.5 BEAD PURIFICATION

Using Nucleo Trap® Nucleic Acid Purification Kits and accessories from BD Biosciences clontech, California, USA. We were able to purify the thiol modified λ-DNA reaction mixture. The Nucleo Trap matrix binds DNA fragments larger than 120 bp. Therefore, unligated or excess oligos stay in solution during the extraction procedure and can be separated from the ligated thiol modified DNA. The DNA can be eluted in TE buffer (pH 8.0) or another low-salt buffer (e.g. H2O). The manufacturer’s instructions were followed to purify the PCR product as described below. All the solutions were provided in the manufacturer’s kit.

- The reaction mixture containing thiolated λ-DNA was transferred to a vial of 500 or 1000 µl capacity.
- The volume of the reaction mixture was adjusted to 100 µl with TE buffer (pH 8.0).
- Nucleo trap PCR suspension was vortexed thoroughly until the beads were completely resuspended.
- 400 µl of buffer NT2 and 10 µl of Nucleo Trap PCR suspension were added to the reaction mixture. (Note: if the volume of the original PCR reaction was > 100 µl, increase the volume of buffers NT2 and the Nucleo Trap PCR suspension proportionally. For example, a 150 µl PCR reaction needs 600 µl of buffer NT2 and 15 µl of Nucleo Trap PCR suspension.)
- Sample was incubated at room temperature for 10 min. It was vortexed briefly every 2-3 min during the incubation period.
- The sample was centrifuged at 10,000 X g for 30 sec at room temperature. Thereafter, the supernatant was discarded.

62
• 400 μl of buffer NT2 was added to the pellet. It was vortexed briefly. Again the centrifugation was performed at 10,000 X g for 30 sec at room temperature. Supernatant was removed completely.

• 400 μl of buffer NT3 was added to the sample. It was vortexed briefly. It was centrifuged at 10,000 X g for 30 sec at room temperature. Supernatant was removed completely. (Note: Increase the volume of buffer NT3 proportionally if the original volume of the reaction mixture was > 100 μl).

• Last step was repeated.

• The pellet was centrifuged at 10,000 X g for 30 sec at room temperature. Pellet was air dried for 15 min at room temperature (or at 37°C to speed up evaporation). (Note: Do not use a speed vacuums to dry the pellets. Speed vacuums tend to overdry the beads as it leads to lower recovery rates.)

• 20-50 μl of TE buffer (pH 8.0) or another appropriate low-salt buffer was added to the pellet. Pellet was resuspended by vortexing it. [Note: Expected recovery rates range from 60% (eluting with 20 μl of TE) to 80% (eluting with 50 μl of TE)].

• The DNA was eluted by incubating the sample at 50°C for 5 min or at room temperature for 10 min. Vortexed the mixture 2-3 times during the incubation step.

• The sample was centrifuged at 10,000 X g for 30 sec at room temperature. Supernatant containing the pure DNA fragment was transferred to a clean 1.5 ml microcentrifuge tube. (Repeating last three steps can increase yields approximately 10-15%)

• 30 μl of bead purified DNA (thiol modified λ-DNA) was pipetted out on the array of gold electrodes.

• It was incubated for 24 hours.
Eventually, it was washed and taken for visualization after staining with a fluorescent DAPI Dye.

3.2.3.6 ACTIVATION OF ELECTRODES

Electrodes were fabricated on gold coated (30 nm) glass wafers. The adhesion of gold to glass substrate is very poor. To promote adhesion a thin layer (10 nm) of Ti was pre-deposited. The glass wafer was of 75 mm diameter, 0.5 mm thick and polished to a surface finish to a surface finish of 1.5 nm on both sides. The cleaned glass slide was dried thoroughly and then pretreated for 12 h with a 1:5 v/v solution of chlorotrimethylsilane and tetrachloroethane. This treatment is necessary to avoid spurious binding of DNA on glass. Gold microelectrodes on glass wafer were washed with trichloroethylene to remove the resist on the diced wafers. Resist can also be removed from the microelectrode surface by incubating them in boiling acetone for a time period of half an hour. The resist was deposited, to avoid scraping of glass during the process of ‘dicing’. After drying, wafers were stored at room temperature and treated with Piranha solution before DNA immobilization. Piranha solution is used to clean the gold surface. A wet chemical process removes organic contamination, which invariably absorbed on the surface during prolonged exposure. The gold microelectrodes were soaked in Piranha solution 1:3 v/v (30% H₂O₂, conc H₂SO₄) for 2 min at room temperature. Following the cleaning regime, copious rinsing with pyrogen free, DI water, was carried out. It should be noted that latex rubber gloves tend to leave behind small residues of latex particles or talc. So gloves made from polyethylene were worn during substrate electrode array processing. Gold electrodes were prepared in large batches and stored over long periods of time until were required to use for DNA immobilization.
3.2.3.7 FLUORESCENCE VISUALIZATION OF DNA

Immobilized DNA between/on microelectrodes was stained with DAPI fluorescent dye. The DAPI stock solution was prepared by dissolving 2 mg of DAPI in 10 ml of DAPI buffer solution (10 mM EDTA, 0.1 M NaCl, 10 mM Tris) and dispensed in 1 ml aliquots and stored at -20°C for six months (Schweizer et al., 1978). The working solution of the dye was prepared by dissolving 0.1 ml of stock solution in buffer to make 100 ml. The process of staining the sample was carried out, by flooding the diced wafer with a working solution of the dye. The incubation was done in the dark at room temperature for 15 min. During incubation period DAPI intercalates in DNA and gives fluorescence when excited. The sample was washed three times in a stream of buffer solution (without DAPI) to remove excess of dye and any unbound material that may retain dye and may result in background imaging. After draining buffer from the electrodes surface, DNA bridges were visualized under an Axiovert 25CFL, inverted fluorescence microscope, equipped with mercury vapor short arc lamp HBO 50 (Carl Zeiss, Germany). The correct filter sets for DAPI (excitation 358 nm and emission 461 nm) were used. For magnification higher than 200X Axiovert 200, inverted fluorescence microscope, (Carl Zeiss, Germany) was used. It was equipped with a motorized stage and a mercury vapor short arc lamp, HBO 100. The position of electrode pairs with DNA bridges were noted in a computer generated work sheet having 400 cells with each cell representing an electrode on the chip. On visualization of a DNA bridge the representative cell on the worksheet is marked with red pencil. This worksheet is helpful for later on analysis and current-voltage measurements. Soon after fluorescence microscopy the samples were kept in desiccators at -20°C for further studies.
3.2.3.8 COLUMN CHROMATOGRAPHY PURIFICATION OF DTT TREATED OLIGOS.

Clear visualization of DNA on surface was not observed following the above mentioned protocols for DNA immobilization. Dendrite like structure and non-specific binding of DNA was observed. It was difficult to visualize DNA on microelectrodes. Such result was due to salts present in the reaction mixture along with the small fragments of DNA which acquired dendrite shapes once it was dried as reported earlier (Ostuka et al., 2002). Aggregates of DNA were also observed. It was assumed that aggregation probably was due to sulfur bond formation among the thiolated oligos. Due to this reason, oligos were first treated with DTT and then it was immobilized on gold substrate. Hence, protocol was modified to get rid of such effects. Efforts were made to minimize impurities and salts in the prepared sample. Common impurities includes enzymatic remains, inorganic remains i.e. ions, salts and organic components i.e. dithiothreitol etc. Proteinous impurities were already tried to remove using purification methods mentioned above i.e. phenol-chloroform and bead purification. For removing dithiothreitol column chromatography was adopted (Fig. 3.5). For this purpose G-25 column (Lab Mate PD-10 Buffer Reservoir, was purchased from Amersham Biosciences, USA; rest column detail below). Synthesized thiolated Cos-I and Cos-II were treated with dithiothreitol (30 mM) to reduce the disulfide bond and to maintain the sulfhydryl group (-SH). The mixture was purified by column chromatography. Gel exclusion chromatography facilitates the separation of molecules based on their size. Sephadex beads were being used in the column of different pore size. Molecules of large size were unable to pass through the bead pores hence they pass through the column quickly.
Column detail:
Matrix: Sephadex G-25 Medium
Bed Volume: 8.3 ml
Particle size range: 85-260 micrometer
Bed height: 5 cm
Exclusion limit: Mr 5000

Figure 3.5: Schematics of Gel Exclusion Chromatograph for oligos purification from DTT.
3.2.3.8.1 COLUMN SEPARATION PROTOCOL OF Cos I AND Cos II OLIGOS

Molecules of size less than the bead pore size enters the bead adopts zig-zag path and hence they take comparatively longer duration to pass through the column than large molecular size. Cos oligos being large in size will not be entering into the bead pores and will come out quickly.

- 25 μl of thiolated oligos (Cos I) mix in 30 mM DTT solution to reduce the thiol bonds.
- It was incubated for a period of 30 minutes.
- PD-10 desalting column was calibrated with the immobilization buffer (KH₂PO₄, 1M, pH 3.8)
- Reduced thiolated oligos was poured in the column.
- The bed volume 8.3 ml was collected in microfuge tube of 1.5 ml volume and consecutively sample was eluted.
- O.D. of the eluents was taken immediately on eppendorf spectrophotometer then immobilization was performed (Table 3.2).
Table 3.2: Removal of DTT from oligos using Sephadex G-25 column.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Conc. of primer 1 (μg/μl)</th>
<th>Conc. of primer 2 (μg/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.0029</td>
<td>0.0024</td>
</tr>
<tr>
<td>2.</td>
<td>0.0014</td>
<td>0.0023</td>
</tr>
<tr>
<td>3.</td>
<td>0.0043</td>
<td>0.0041</td>
</tr>
<tr>
<td>4.</td>
<td>0.0061</td>
<td>0.0050</td>
</tr>
<tr>
<td>5.</td>
<td>0.0038</td>
<td>0.0032</td>
</tr>
<tr>
<td>6.</td>
<td>0.0012</td>
<td>0.0015</td>
</tr>
<tr>
<td>7.</td>
<td>0.0008</td>
<td>0.008</td>
</tr>
<tr>
<td>8.</td>
<td>0.0000</td>
<td>0.00</td>
</tr>
</tbody>
</table>
3.2.3.9 IMMOBILIZATION OF DTT TREATED AND PURIFIED OLIGOS BY DROP APPROACH.

Figure 3.6 represents molecular interaction in Drop approach of immobilization process. A drop of sample containing different kind of molecules has been shown. Drop approach represents the aggregation of molecules in receding meniscus and hence, its adsorption.

![Diagram showing molecular interaction](image)

Figure 3.6: Schematics of immobilization by Drop approach showing receding meniscus leading to aggregation of molecules.

Principal underlying such aggregation has been described in discussion. Detail protocol followed for the immobilization of DNA by Drop approach is mentioned below:-

3.2.3.9.1 IMMOBILIZATION OF THIOLATED Cos I OLIGOS.

Cos oligos were labeled with sulphydryl group (thiol) as mentioned in section 3.1.6. When eluted oligos were applied over the electrode
surface, they got covalently attached to the gold surface as mention in section 2.3.1. This forms a stable bond between gold and thiol.

- Gold electrodes were activated using piranha solution (70% \( \text{H}_2\text{SO}_4 \): 30% \( \text{H}_2\text{O}_2 \); 3:1) as mentioned in step 3.2.3.6.
- Activated surface was dried under nitrogen gas stream.
- 30 µl of the eluted oligos was pipetted out over the surface of the gold electrodes.
- The electrodes were put in a petridish (TARSON Petridish, Disposable) and it was sealed with paraffin film.
- Sealed petridish was fixed in a floater.
- Floater was kept in covered water tank so that sample should not be allowed to dry.
- Sample was incubated for 24 hours.
- Finally, Gold electrodes were gently rinsed with autoclaved double distilled water.

### 3.2.3.9.2 HYBRIDIZATION OF IMMobilIZED OLIGOS WITH \( \lambda \)-DNA

Hybridization is a self recognition property in DNA. When two complementary strands of DNA come close to each other of complementary bases, this results in hydrogen bond formation between nucleobases. This process is called hybridization. As mentioned below in protocol, when \( \lambda \)-DNA was applied to the surface with already immobilized Cos oligos, hybridization will take place due to complementary regions between them. Hybridization was confirmed by staining with DAPI dye. DAPI dye doesn’t stains to single strands of DNA. Only after hybridization DNA will remain on substrate or it will be washed in the washing buffer.
20 µl of λ-DNA (0.5 µg/µl) was mixed with 480 µl of hybridization buffer (150 mM NaCl, 20 mM Na₂HPO₄, 0.1 mM EDTA, pH 7.4).

- Heat shock at 74°C was given for a period of 2 minutes.
- 30 µl of the DNA in hybridization buffer was applied on electrode surface with immobilized complementary oligos.
- Gold electrode surface was put in a plastic petridish (TARSON Petridish, Disposable)
- Plastic petridish was sealed with paraffin film. It was fixed in a floater.
- This set up was floated in covered water tank so that sample should not allow to be dried.
- It was incubated for a period of half an hour.
- The electrodes were gently rinsed with autoclaved double distilled water.

3.2.3.9.3 HYBRIDIZATION AND BINDING OF Cos I AND Cos II

This step again repeats the process of hybridization for Cos-II as was performed for Cos-I in section 3.2.3.9.2. In this Cos-II will be hybridized to the complementary end of the X-DNA available on the gold surface.

- 30 µl of eluted reduced Cos II oligos was dispensed over the electrodes surface having immobilized Cos I and hybridized λ-DNA.
- Immobilization buffer (20 µl) was applied over its surface and it was incubated for 24 hours.
- The reaction mixture was incubated in covered water tank for a period of half an hour.
• It was rinsed gently with double distilled water. Rinsing was done in such a way that it leads to stretching of the DNA strands.
• Surface was gently washed with double distilled water and then proceeded for ligation.

3.2.3.9.4 LIGATION OF THE HYBRIDIZED DNA ON THE GOLD ELECTRODE SURFACE

This is the step allowing joining the nicks between the Cos oligos and complementary end of the λ-DNA. T4 DNA ligase is used to covalently join DNA fragments. This reaction mixture was incubated O/N at 4°C. Thiolated oligonucleotides were covalently joined to their complementary sites in λ-DNA by setting the ligase reaction as mentioned below:

• 2 μl of 10X ligation buffer, 2 μl of ATP (10 mM), 14 μl of double distilled water and 2 μl of T4 DNA ligase enzyme was added in a vial of 50 μl.
• Mixed 20 μl of ligation reaction mixture was applied on the immobilized DNA surface and was incubated for 5-6 hours at 16⁰C.
• Reaction was terminated by gently washing the surface.
• Sample was taken for staining with DAPI Dye and eventually, for fluorescence visualization.

3.2.3.10. IMMOBILIZATION OF λ-DNA BY DIP METHOD

Dip approach represents reduced probability of islanding as the non-thiolated DNA and undesired molecules remain suspended in solution due to hydrophilic interaction as shown in the figure 3.7. Its
detail mechanism is mentioned in discussion. In this approach, results were far better than Drop approach (Conventional method).

Hydrophilic suspension of non-thiolated DNA

Figure 3.7: Schematics of immobilization by dip approach showing no-aggregation of suspending molecules.

Only the thiolated DNA which is having more affinity for the gold surface would overcome the hydrophilic force and would be immobilized on the gold surface. In this way most of the impurities will be left behind in the buffer.

3.2.3.10.1 IMMOBILIZATION OF THIOLATED Cos-I OLIGOS.

This section is same as describe in 3.2.3.9.1 Cos oligos were labeled with sulphonyl group (thiol) as mentioned in section 3.1.4. When eluted oligos were applied over the electrode surface, they got covalently attached to the gold surface following a chemical process as mentioned in section 2.3.1. This results in stable bond formation between gold and thiol that cannot be simply removed in washing processes. Detail protocol is described below.
• Gold electrodes were activated as mentioned in step 3.2.3.6.
• Activated surface was dried with nitrogen stream.
• 30 µl of the eluted oligos was dispensed over the surface of the gold electrodes.
• Electrodes were put in a petridish (TARSON Petridish, Disposable) fitted in a floater.
• Floater was kept in water tank so that sample should not be allowed to dry.
• Sample was incubated for a period of 24 hours.
• Finally, Gold electrode surface was gently rinsed with autoclaved double distilled water.

3.2.3.10.2 IMMOBILIZATION OF HYBRIDIZED λ-DNA AND Cos II OLIGO

Protocol mentioned below describes the hybridization of λ-DNA with Cos-II oligo and its immobilization on gold surface. Cos-II oligos and λ-DNA was hybridized in a vial. Above to this reaction mixture, gold electrodes with already immobilized Cos-I was put for the hybridization of λ-DNA with Cos-I.

• 20 µl of λ-DNA (0.5 µg/µl) was mixed with 480 µl of hybridization buffer (150 mM NaCl, 20 mM Na₂HPO₄, 0.1 mM EDTA, pH 7.4) containing Cos-II oligo.
• Mixture was incubated for a period of half an hour.
• Back side of the surface containing gold electrodes was made to stick to the double sided adhesive and then to a thread.
• This arrangement was floated (up side down) on the top surface of the reaction mixture in the vial.
• Set up was left for a period of 24 hours.
• Gold electrode surface was taken out of the set up.
• It was rinsed with double distilled water, ligated and eventually, stained with DAPI.
• Stained sample was taken for visualization as mentioned in section 3.2.3.7.

3.2.4. CURRENT VOLTAGE MEASUREMENT

I-V characterization of DNA makes us familiar with the electronic properties that it carries. In-depth understanding of molecular electronic properties enables us to use them for practical aspects. This also narrates the nature of the molecule whether it is conductor, semiconductor or insulator. I-V characterization of the bio-molecules at the molecular level demands sophisticated instrumentation facility. I-V measurements were made at ambient atmosphere in class-100 clean laboratory conditions using probing station from Signatone Corporation, USA. The probe station was located in a chamber, to provide dark environment, for making light sensitive measurements. It was having a wafer stage with coarse and fine stage movement using manual control. The probing station was fitted with a microscope having 10X resolutions. The sample on the stage was probed using probe pins, which were connected to vacuum chucks. The probe pins of 10 μm tip diameter were used to make good Ohmic contact with the gold microelectrodes. The Hewlett-Packard HP4155A, Semiconductor Parameter Analyzer having an internal resistance of $\geq 10^{13}$ Ω and current resolution of 10 fA was used to measure current voltage characteristics of the DNA immobilized between the electrodes. This facility is available at Semi Conductor Laboratories (SCL), Mohali, Panjab, India. The current-voltage measurements were made on the microelectrodes.
having DNA bridge between them. Electrode pairs without DNA bridge but same spacing were used as control during electrical measurements.

### 3.2.5. AFM SURFACE ROUGHNESS STUDY

Topography of chemically treated gold surface was analyzed by AFM (Nanoscope II, Digital instruments, USA) for its topography. The AFM records contours of force resulting from the repulsion generated by the overlap of the electron cloud at the tip with the electron clouds of the sample. The sample is mounted on a stub using double sided carbon tape, placed on xyz-piezo-translator and scanned by using a sharp diamond tip mounted on a gold coated 200 μm triangular Si₃N₄ microfabricated cantilever (force constant = 0.6 N/m). Deflection sensors detect the angular displacement of a laser beam reflected off the back of cantilever and monitor by the photodiode and pass to the Digital Signal Processor (DSP) to convert into topographic image. The forces between tip and sample ranges from 10⁻⁷ to 10⁻⁹ N. Images consisted of 400 scans of 400 pixels each. Typical image acquisition time was 150-200 sec/scan.

Chemical treatment was given to the gold surface before proceeding for the immobilization of DNA. First of all acetone was used to remove the photoresist from the gold surface. This treatment was given at 60°C for about two hours. Thereafter it was washed in deionized distilled water and its topographic analysis was done by AFM. Secondly, the same surface was treated with piranha solution for its activation.

### 3.2.6. DESIGNING OF PRIMERS

Primers were designed in order to amplify the Guanine rich regions of λ-DNA. First of all, the nucleotide sequence of λ-DNA
bacteriophage was retrieved from the database website http://www.ncbi.nlm.nih.gov with the accession number NC_001416 (Source: Enterobacteria phage lambda) and was crossed checked from the literature. The forward and reverse primers were designed online as well as with the help of the downloaded bioinformatics programs like DNATOOLS 6. The primers so designed were checked for hairpin loops, nucleotide repeats, internal loops, primer-dimers, GC percentage, Tm etc. with the help of Gene Runner Program. The primers were further checked for homology search either in FASTA program of www.ebi.ac.uk or blasted with the database at http://www.ncbi.nlm.nih.gov/BLAST/. Further the primers were rechecked for primer-dimer, nucleotide repeats, GC content, hairpin loop formation etc. at Dr. B. R. Ambedker Centre for Biomedical Research, Delhi University, Delhi.

3.2.7. STANDARDIZATION AND AMPLIFICATION OF PCR CONDITIONS

In order to amplify Guanine rich sequences, λ-bacteriophage DNA purchased from Amersham Biosciences, Hong Kong was used as template. Different sets of conditions were tried, so that the maximum amplification can be obtained. The PCR product was visualized by EtBr staining on agarose gel. The intensity of band on agarose gel was used as an approximate measure of product amplification. The various conditions tried for standardization were as follows:

- Concentration of MgCl₂ in amplification buffer: Taken as 1.25 mM, 1.5 mM, 1.75 mM, 2.0 mM and 3.0 mM.
- Concentration of DNA taken as template: 10 ng, 20 ng, 50 ng, 80 ng and 100 ng.
Annealing temperature: Various temperatures tried were 53°C, 54°C, 55°C, 56°C and 57°C.

3.2.8. AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was carried out in a horizontal matrix of agarose with 1X TAE buffer as described by Sambrook et al. (1989). Required amount of agarose (depending upon the percentage) was added to flask (2 to 4 times the volume of the solution) containing distilled water. The agarose was melted by microwave (2 mins at maximum). The molten agarose was kept in 50°C water bath for 10 mins and 50X TAE were added to get a final concentration of 1X TAE. An aliquot of EtBr was added to get a final concentration of 0.5 µg/ml. The agarose solution was then poured on to gel template sealed with tape and fitted with comb. The agarose was allowed to polymerize for 30 mins. The comb was then removed and the gel was immersed in 1X TAE buffer in horizontal electrophoresis tank. The DNA was mixed with 1/6 volume of 6X loading buffer and electrophoresed at 5 V/cm. DNA ladder was run in parallel as size standard. The bands were visualized using short wave (300 nm) transilluminator and photographed using a gel documentation system fitted with a camera. The characterization of the PCR products was done by comparing the electrophoretic mobility of the PCR products with standard DNA ladder marker.

3.2.9. PCR PRODUCT PURIFICATION

PCR amplified DNA was purified to remove the Proteinaceous impurities. These impurities interfere during immobilization of DNA. Using Nucleo Trap® Nucleic Acid Purification Kits from BD
Biosciences Clontech, California, USA PCR amplified products were purified. All the solutions were provided in the manufacturer’s kit.

- After the PCR was completed, tube was removed from the thermal cycler. The aqueous phase was transferred to a new micro-centrifuge tube.
- The volume of the reaction mixture was adjusted to 100 µl with TE buffer (pH 8.0).
- Nucleo trap PCR suspension was vortexed thoroughly until the beads were completely resuspended.
- 400 µl of buffer NT2 and 10 µl of Nucleo Trap PCR suspension were added to the reaction mixture.
- Sample was incubated at room temperature for 10 min. It was vortex briefly every 2-3 min during the incubation period.
- The sample was centrifuge at 10,000 X g for 30 sec at room temperature. Thereafter, the supernatant was discarded.
- 400 µl of buffer NT2 was added to the pellet. It was vortexed briefly. Again the centrifugation was performed at 10,000 X g for 30 sec at room temperature. Supernatant was removed completely.
- 400 µl of buffer NT3 was added to the sample. It was vortexed briefly. It was centrifuged at 10,000X g for 30 sec at room temperature. Supernatant was removed completely.
- Last step was repeated.
- The pellet was centrifuge at 10,000 X g for 30 sec at room temperature. Pellet was air dried for 15 min at room temperature (or at 37°C to speed up evaporation).
- 20-50 µl of TE buffer (pH 8.0) or another appropriate low-salt buffer was added to the pellet. Pellet was resuspended by vortexing it.
• The DNA was eluted by incubating the sample at 50°C for 5 min or at room temperature for 10 min. Vortexed the mixture 2-3 times during the incubation step.

• The sample was centrifuged at 10,000X g for 30 sec at room temperature. Supernatant containing the pure DNA fragment was transferred to a clean 1.5 ml microcentrifuge tube. (Repeating last three steps can increase yields approximately 10-15%).

• PCR purified products were pipetted out over the fabricated electrodes incubated for a period of 16h and then washed thoroughly.

• DNA was stained with DAPI and was visualized under fluorescence microscope as mentioned in section 3.2.3.7.

### 3.2.10 AFM CHARACTERIZATION OF IMMOBILIZED PCR PRODUCT

Immobilized DNA was analyzed by AFM (Nanoscope II, Digital instruments, USA) for its topography. The sample was mounted on a stub using double sided carbon tape, placed on xyz-piezo-translator and scanned by using a sharp diamond tip mounted on a gold coated 200 µm triangular Si₃N₄ microfabricated cantilever (force constant = 0.6 N/m). Detail of AFM scanning is mentioned in section 3.2.5.