9G DNAChip: Microarray Based on the Multiple Interactions of 9 Consecutive Guanines

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Supporting Information

1. Materials

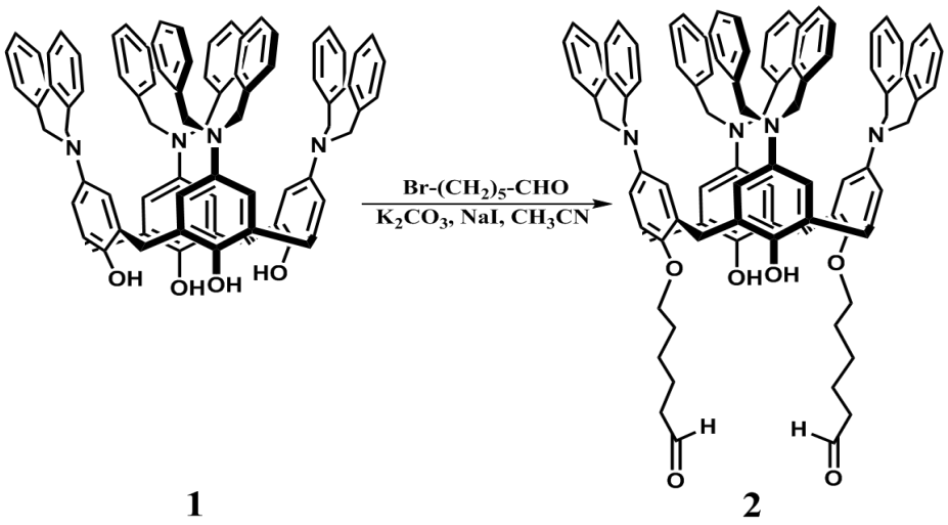
All chemicals were purchased from Sigma-Aldrich Chemicals, Korea. All the oligonucleotides were purchased from Bioneer, Korea. Glass slides (2.5x7.5 cm) were purchased from **Paul Marienfeld GmbH & Co. KG, Germany.** All washing solvents for the substrates are of HPLC grade from SK Chemicals, Korea. Ultrapure water (18 M Ω/cm) was obtained from a Milli-Q purification system (Millipore). ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ using TMS as an internal standard. Chemical shifts (δ) are reported in ppm, and spin-spin coupling constants (*J*) are in Hz.

2. Instruments

Oligonucleotides were spotted using Qarray2 microarrayer (Genetix Technologies, Inc.) Hybridization was done at $25^{0}C$ using the commercial incubator and then the slides were dried using the commercial centrifuge (1000rpm). The fluorescence signal of the microarray was measured on ScanArrayLite (GSI Lumonics), and the images were analyzed by Quant Array software (Packard Bioscience).

3. Host and guests

3a. Scheme and general procedure for synthesis of AMCA-1,3- dialdehyde:



Scheme 1: Synthesis of AMCA-1,3-dialdehyde

The starting material **1** was obtained by the previously reported method.[1] To a solution of **1** (0.5g, 0.41 mmol), K_2CO_3 (0.56g, 4.1 mmol), NaI (0.27g, 1.85 mmol), and acetonitrile (25mL), 6-bromohexanal (0.22g, 1.22 mmol) was added and then the reaction mixture was refluxed with stirring under the argon atmosphere for 12 h. After 12 h, the reaction mixture was then evaporated in vacuo, and the residue was dissolved in 50 mL of dichloromethane and filtered. The filtrate was concentrated and Diisopropyl ether was added. The precipitate was filtered and dried under vacuum to obtain compound **2**. Light brown solid (90%). mp = 291-292.5 ^{0}C.

^{1}H NMR (300 MHz, $CDCl_3$, 298 K) δ (ppm): 9.77 (s, 2H, -CHO), 8.13 (s, 2H, Ar-OH), 7.05-7.24 (m, 40H, Ar-H), 6.26 (d, 8H, *J* = 4.8 Hz, Ar-H), 4.26 (s, 16H, Ar-N-CH2-Ar), 4.16 (d, 4H, *J* = 12.7 Hz, Ar-CH2-Ar), 3.87 (t, 4H, J =11.7, Ar-O-CH2-), 3.00 (d, 4H, *J* = 12.70 Hz, Ar-CH2-Ar), 2.50 (t, 4H, J = 12.7, -OC-CH2-), 2.02 (m, 4H, -O-CH2-CH2-CH2-CH2-CH2-CHO), 1.70 (m, 8H, -O-CH2-CH2-CH2-CH2-CH2-CHO). ^{13}C NMR (300 MHz, $CDCl_3$, 298 K) δ (ppm): 202.5, 146.9, 144.8, 143.9, 139.6, 138.9, 134.6, 129.3, 128.7, 128.6, 127.2, 127.1, 127.0, 126.8, 117.3, 113.2, 113.0, 76.3, 54.8, 54.6, 32.8, 30.0, 25.8, 22.2. HR MALDI-TOF: Cal. for $C_{100}H_{92}N_4O_{20}$ (M+H): m/z = 1401.74026 Found: m/z = 1400.732432 [M^+], 1401.74026 [M^+ H]$^+$.

3b. ^{1}H NMR Spectra of AMCA-1,3-dialdehyde:

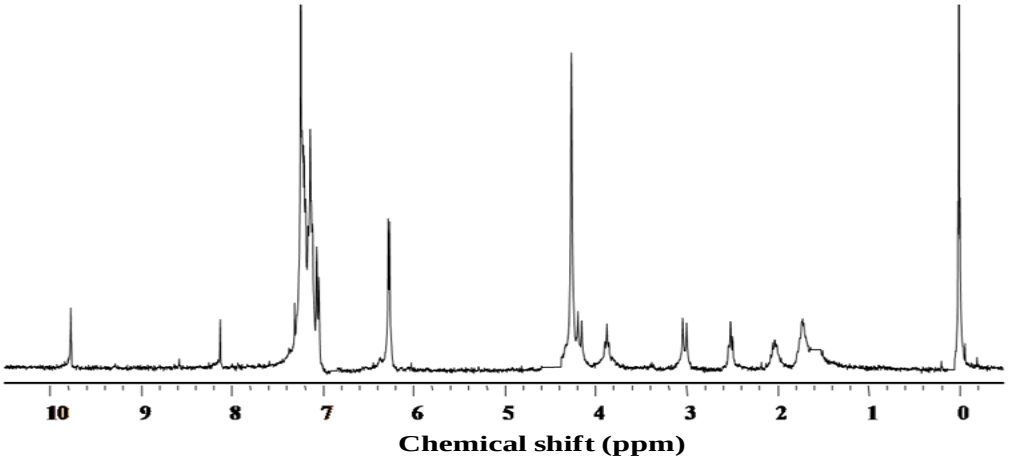
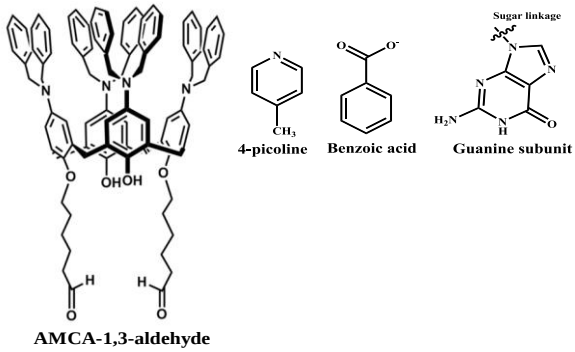


Figure S1: ^{1}H NMR Spectra of AMCA-1,3-dialdehyde

3c. Structures of the AMCA-1,3-aldehyde, 4-picoline, benzoic acid, and guanine subunit



Scheme 2: Structures of the AMCA-1,3-aldehyde, 4-picoline, benzoic acid, and guanine subunit

According to our previous report, AMCA derivatives can efficiently recognize the structurally flat and hydrophobic molecules in water.[2] We have also reported the molecular recognition of AMCA derivatives with the 4-picoline and benzoic acid. The AMCA derivative showed strong molecular recognition with the 4-picoline (*Ka* = 3.9 x 10^4), while it did not recognize the benzoic acid and other negatively charged guest molecules. Therefore we used the 4-picoline and benzoic acid to probe the molecular recognition of the 9G (9 consecutive guanine subunits) appended probes. The results showed that the 4-picoline moderately prevent the immobilization of the Probe1 on the AMCA slides, while benzoic acid did not show any effect on the immobilization. The 4-picoline competes with the 9G for the cavities of the AMCA molecules and thus prevents the immobilization of the Probe1. This result also proves that guanine subunit being structurally flat can be recognized by the cavity of the AMCA-1,3-aldehide on the AMCA slide.

4. Probes and target oligonucleotides:

Table S1. Sequences and the nomenclature of probes and target oligonucleotides

Capture Probes	**Sequence**
Probe1	5'-GGGGGGGGG CTT TAT CCT ACG ACT TGG GGA GG-3'
Probe1-m1	5'-GGGGGGGGG CTT TAT CCT ACG ACT TGG G**T**A GG-3'
Probe1-m2	5'-GGGGGGGGG CTT TAT CCT ACG ACT T**T**G G**T**A GG-3'
Probe2	5'-GGGGGGGGG CTT TAT TTT AAG GCA AGG TGA AG-3'
Cy5-Probe1	5'-Cy5-GGGGGGGGG CTT TAT CCT ACG ACT TGG GGA GG-3'
Probe3	5-AAAAAAAAA CTT TAT CCT ACG ACT TGG GGA GG-3'
Probe4	5'-TTTTTTTTT CTT TAT CCT ACG ACT TGG GGA GG-3'
Probe5	5'-CCCCCCCCC CTT TAT CCT ACG ACT TGG GGA GG-3'
Probe6	5'- CTT TAT CCT ACG ACT TGG GGA GG-3'
Probe7	5'-GGG CTT TAT CCT ACG ACT TGG GGA GG-3'
Probe8	5'-GGGGGG CTT TAT CCT ACG ACT TGG GGA GG-3'
Probe9	5'-GGGGGGGGGGGG CTT TAT CCT ACG ACT TGG GGA GG-3'
Target DNA	**Sequence**
Cy5-T1	3'-GGA TGC TGA ACC CCT CC TTT TTTTTT TTT-Cy5-5'
Cy5-T2	3'-AAA TTC CGT TCC ACT TC TTT TTTTTT TTT-Cy5-5'

5. Composition of different solutions used:

1. Immobilization solution (pH = 7.4): 15% glycerol, 50mM butyl amine, 600mM NH_4Cl
2. Blocking buffer solution (pH = 7.4): 0.5% milk casein in 4xSSC
3. Hybridization buffer (pH = 7.4): 25% Formamide, 0.1% Triton X-100, 6xSSC
4. Washing buffer solution A (pH = 7.4): 0.1% SDS in 4xSSC
5. Washing buffer solution B (pH = 7.4): 4xSSC

6. Experimental procedures:

6a. Preparation of AMCA slides:

Step 1: Glass microslides were cleaned by immersing them in NaOH (1.0 M) for 2 h at 25℃ followed by rinsing with Milli Q water (2×100 mL). The slides were then subjected to treatment with a solution of the hydrochloric acid (2.0 M) for 30 min, followed by rinsing with Milli Q water (3×100 mL), and finally submerged in 95% ethanol for 10 min. The slides were dried under vacuum. The pre-cleaned and dried slides were treated with water (2 times), and methanol (2 times), and solution of (3-aminopropyl)triethoxysilane (3%, v/v) in methanol for 1h at 25℃ and dried under vacuum to obtain amine modified slides. Finally, these were baked for 1h at 60℃ and stored in an inert atmosphere.[3,4]

Step 2: The aminated slides were immersed into the 10 mM solution of the AMCA-1,3-dialdehyde in $CHCl_3$ while shaking for 1 h at 25℃. The slides were treated with methanol, water, and methanol in a sequential manner each for 3 min at 25℃. The excess solvents were removed by centrifuge and dried in desiccators for 1 day to obtain the AMCA slide.[5]

6b. Water contact angle of AMCA slide:

The water contact angle of the AMCA slide was measured using a Kruss Easydrop contact angle analyzer (Kruss, Hamburg, Germany) and compared with that of the bare slide glass. The AMCA slide was found to have slightly hydrophobic surface with a contact angle θ = 68.63, whereas the water spread over the surface of a bare slide glass as shown in the **Figure S2.**

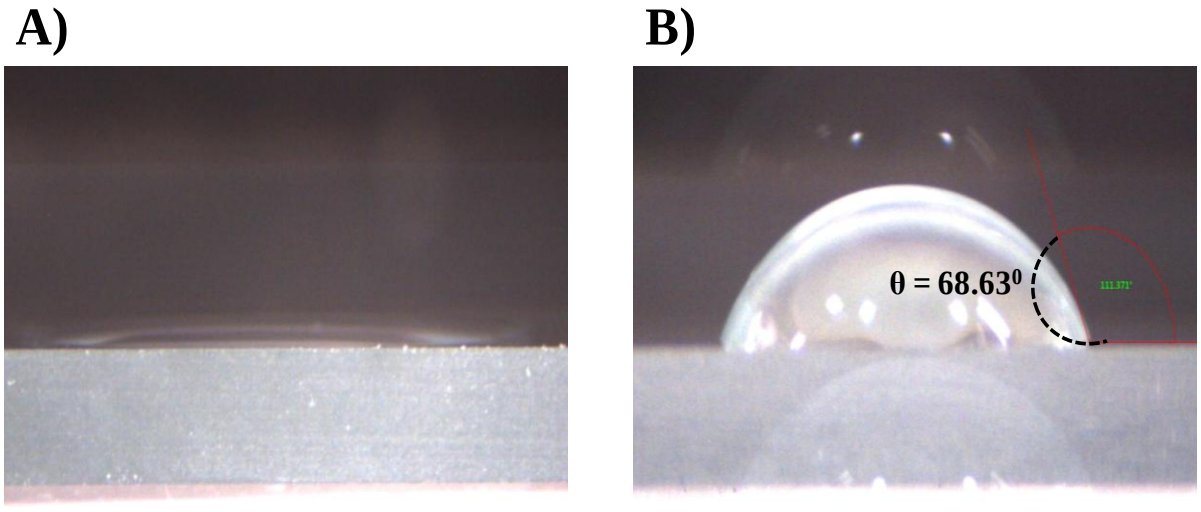


Figure S2: Water contact angle a) Bare slide glass with a 1µl water drop, b) AMCA slide with a 1µl water drop.

6c. Typical method for the preparation of the 9G DNAChip:

9G DNAChip was prepared by spotting the immobilization solution containing oligonucleotide probes with the microarrayer, and the spots were arranged to make 2 by 5 pixels on the AMCA slide. The microarrayed AMCA slide was then kept in the incubator (25^0C, 50% humidity) for 4h to immobilize the oligonucleotides. The slide was then suspended in the blocking buffer solution at 25^0C for 30 min, in order to remove the excess oligonucleotides and to deactivate the non-spotted area. Then the slide was rinsed with washing buffer solutions A and B for 5min each, and then dried with commercial centrifuge to obtain 9G DNAChip. Before hybridization, the 9G DNAChips were covered with Secure-Seal™ hybridization chambers**.**

6d. Preparation of the Cy5-9G DNAChip:

The Cy5-9G DNAchip was obtained by following above method by using the Cy5 dye labeled probe (Cy5-Probe1). The fluorescence intensity on each spot was measured using a Scan Array Lite (GSI Lumonics) and analyzed by Quant Array software (Packard Bioscience).

6e. Preparation of the 9G DNAChips for competition experiment using 4-picoline and benzoic acid:

9G DNAChips for the competition experiment were prepared by spotting the solutions of Probe1 (20pmol/μl) in immobilization solutions containing 0, 0.1, 1, 10mM of 4-picoline or benzoic acid with the microarrayer, and the spots were arranged to make 2 by 5 pixels on the AMCA slides. The microarrayed AMCA slide was then kept in the incubator (25^0C, 50% humidity) for 4 h to immobilize the oligonucleotides. The slide was then suspended in the blocking buffer solution at 25^0C for 30 min, in order to remove the excess oligonucleotides and to deactivate the non-spotted area. Then the slide was rinsed with washing buffer solutions A and B for 5 min each, and dried with commercial centrifuge to obtain the 9G DNA chip. Before hybridization, the 9G DNAChips were covered with Secure-Seal™ hybridization chambers**.**

6f. Preparation of the Standard slide for the calculation of immobilization density:

Standard slide for calculating immobilization density was prepared by spotting the 1nl of immobilization solution containing 0.23, 0.45, 0.9, 1.8, 3.6pmol/μl of Cy5-Probe1 with the microarrayer, and the spots were arranged to make 2 by 5 pixels on AMCA slides. After drying for 15 min in oven at 60^0C, the fluorescence signal of the microarray was measured on ScanArrayLite, and the images were analyzed by Quant Array software. The average diameter of the dried spot was 180 μm.

6g. Preparation of the 9G imaged slide

9G DNAChip with the 9G image was prepared by spotting the immobilization solution containing oligonucleotide probes with the microarrayer, and the spots were arranged to make 49 by 49 pixels on the AMCA slide. Further the immobilization steps were followed as mentioned earlier.

7. General Hybridization Procedure

Each hybridization chamber of 9G DNAChip was covered with the mixture of 40μl of the hybridization buffer solution and 5μl of target DNA (Cy5-T1 or Cy5-T2) and then incubated at 25^0C for 30 min. Then 9G DNAChip was rinsed with washing buffer solutions A and B successively for 2 min each in order to remove the excess target DNA and dried with commercial centrifuge (1000 rpm). The fluorescence signal of the microarray was measured on ScanArrayLite, and the images were analyzed by Quant Array software.

8. Immobilization kinetics of the oligonucleotide probes on the 9G glass slide:

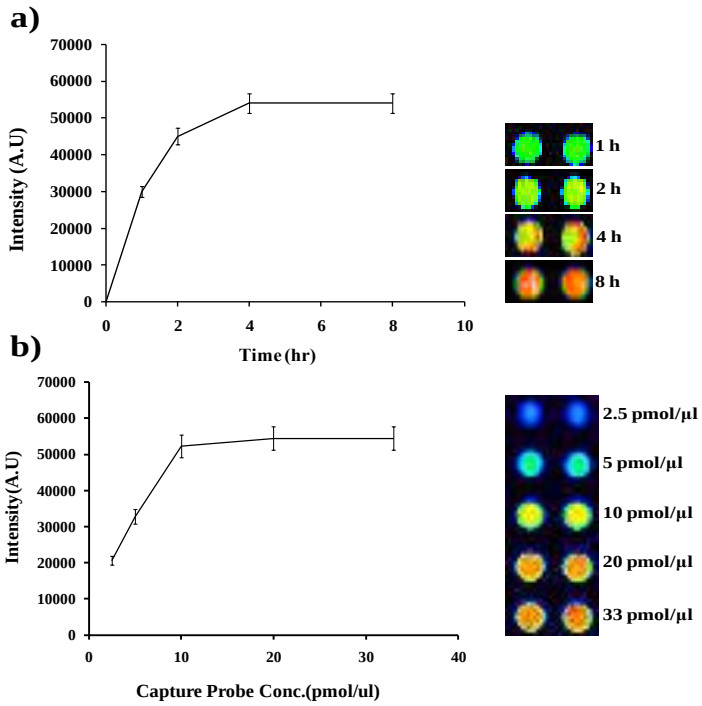


Figure S3: Optimum time and optimum concentration required for immobilization of probes on the AMCA slides a) Immobilization of Probe1 with 20pmol/µl concentration for 2, 4, 6, and 8 h and followed hybridization with Cy5-T1, respective fluorescence images (left), PMT gain = 48, b) Immobilization of Probe1 with 2.5, 5, 10, 20, and 33pmol/µl concentrations for 4, followed hybridization with Cy5-T1, respective fluorescence image (left) PMT gain = 48.

8a. Optimum time required for the immobilization:

In order to determine the optimum time required for immobilization of Probe1, the immobilization solution of Probe1 (20pmol/µl) was spotted onto the AMCA slide using microarrayer. The microarrayed AMCA slide was then kept in the incubator (25^0C, 50% humidity) for 2, 4, 6, and 8 h and subjected to capping, washing, and hybridization with Cy5-T1 as described in the experimental procedure for 9G DNAChip. After drying in a commercial centrifuge (1000 rpm) for 2 min, the spots were visualized under a laser scanner and quantified. As shown in the **Figure S3a**, the immobilization reaches to 80% in 2 h and maximum in 4 h at 25^0C, 50% humidity. The optimum time required for the immobilization is 4 h and is used for the rest of the experiments.

8b. Optimum concentration required for the immobilization:

The optimum concentration of Probe1 required for the immobilization on the surface of the AMCA slide in order to achieve the highest density is determined by spotting Probe1 at various concentrations ranging from 2.5 to 33pmol/µl. The obtained microarray was incubated for 4 h at 25^0C, 50% humidity. After capping, washing, and hybridization with Cy5-T1, the dried spots were visualized under a laser

scanner and quantified. As shown in the **Figure S3b**, the fluorescence intensity for immobilized probes reaches a maximum at the Probe1 concentration of 10pmol/µl and stays at the maximum at the concentration of 20, and 33pmol/µl. Therefore, the optimum concentration for the immobilization has been chosen as 20pmol/µl and used for the rest of the experiments.

8c. Immobilization density:

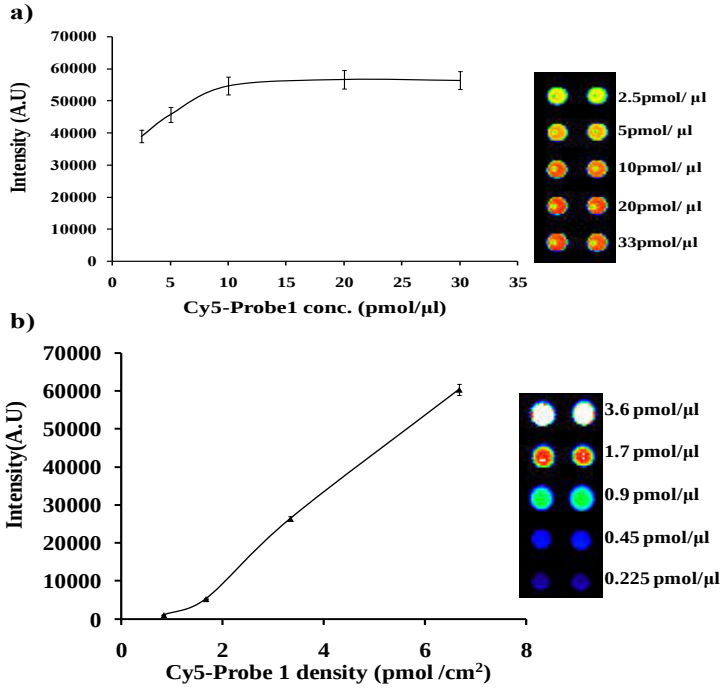


Figure S4. Immobilization density a) Immobilization of Cy5-Probe1 with 2.5, 5, 10, 20, and 33pmol/µl concentrations for 4 h followed by capping and washing to obtain Cy5-9G DNAChip, respective fluorescence image (left), PMT gain = 48, b) Standard slide prepared by the spotting of Cy5-Probe1 with 0.23, 0.45, 0.9, 1.8, 3.6pmol/µl concentrations followed by drying, respective fluorescence image (the fluorescence intensity for the spot with 3.6pmol/µl conc. is oversaturated and hence did not shown in the graph) (left), PMT gain = 48.

Equation 1.

$$\text{Immobilization density} = \frac{\text{amt. of immobilized probe (pmol)}}{\text{area of the spot } (cm^2)}$$

Where, diameter of the spot = 180µm , Spotting volume = 1nl

Table S2. The concentration (Conc., pmol/µl) of Cy5-Probe1 used for immobilization and the immobilization density (ID, pmol/cm^2):

Conc., pmol/µl	2.5	5.0	10	20
ID, pmol/cm^2	4.6	5.3	6.1	6.3

Quantification of immobilized oligonucleotides was carried out by using a standard calibration curve plotted between fluorescence intensity (a. u.) and the immobilization density (pmol/cm^2) obtained for the standard slide following **Equation 1**.[6] Here, the Cy5-Probe1 was spotted on the AMCA slide with varying concentrations ranging from 0.23 to 3.6pmol/µl using the microarrayer and dried without further treatment as mentioned in the preparation of the standard slide. The dried spots on the standard slide were scanned and quantified. As shown in the **Figure S4b (**standard calibration curve**)**, the fluorescence intensity of the spots was plotted against immobilization density calculated for respective concentrations (0.23 to 3.6pmol/µl). A linear correlation was observed between the concentration of the spotted oligonucleotide and its fluorescence intensity. The graph was then used to quantify the Cy5-9G DNAChip which was prepared by immobilization of Cy5-Probe1 with 2.5, 5, 10, 20, and 33pmol/µl concentrations for 4 h followed by capping and washing to obtain Cy5-9G DNAChip on the AMCA slide as mentioned in the experimental procedure for Cy5-9G DNAChip**.** The data obtained after scanning the dried slide is presented in the **Figure S4a**. The immobilization density for Cy5-Probe1 on Cy5-9G DNAChip is calculated for each immobilization concentration by extrapolating the obtained fluorescence intensity to its counterpart on the X-axis of standard calibration curve shown in **Figure S4b**. As shown in the **Table 2**, the immobilization density for Cy5-Probe1 after immobilizing 20pmol/µl was found to be 6.3pmol/cm^2.

9. Hybridization kinetics (for the target probes on the AMCA slide) and hybridization efficiency:

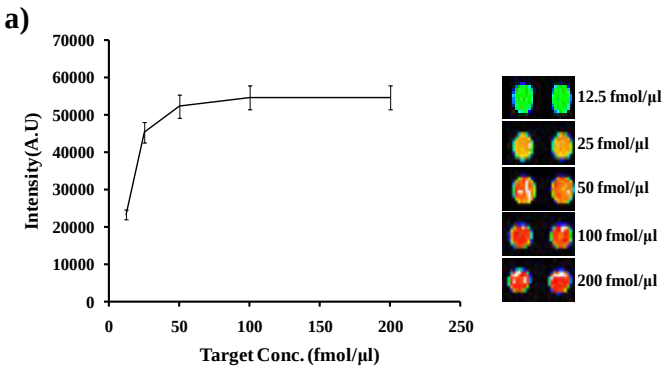


Figure S5. Optimum concentration of target probe required for the hybridization, and the hybridization efficiency a) Hybridization of Probe1 (20pmol/µl) immobilized on the AMCA slide with Cy5-T1 at 12.5, 25, 50, 100, and 200fmol/µl concentrations (fluorescence map on the left), PMT gain = 48.

In order to determine the optimum concentration of the target probe (Cy5-T1) required for the hybridization of the oligonucleotide probes on the AMCA slide, the kinetics of the hybridization of probes at various concentrations was investigated. The immobilization of the probe was carried out by spotting Probe1 (20pmol/µl), following the method to obtain the 9G DNAChip. The hybridization is performed by using Cy5-T1 at various concentrations ranging from 12.5 to 200fmol/µl for 30min. After washing with washing buffers A and B, dried spots on the 9G DNAChip were visualized under a laser scanner and quantified. As shown in the **Figure S5**, the hybridization signal increases with the increase in concentration and reaches a steady state between 100-200fmol/µl. The 100fmol/µl of the target probe concentration is considered to be optimum and used for further experiments.

The hybridization efficiency of the probes on the AMCA slide was obtained by comparing the result of Cy5-9G DNAChip (**Figure S4a**) which was prepared by immobilizing Cy5-Probe1 and the result of 9G DNAChip (**Figure S3b**) which was obtained by immobilizing Probe1. In both cases, concentrations of the immobilized probe were 2.5, 5, 10, 20, 33pmol/µl. The data for Cy5-9G DNAChip was obtained by experimental procedure as mentioned earlier. The microarray on 9G DNAChip after the immobilization of Probe1 was hybridized with Cy5-T1 and final data obtained by subsequent washing, drying and scanning is depicted in the manuscript **Figure 4** (Manuscript). The hybridization efficiency of more than 95% achieved on 9G DNAChip with the concentration 20pmol/µl and 33pmol/µl of the probe immobilized on the AMCA slide indicates that the immobilized probes provide good accessibility for the incoming target probe during the hybridization.

10. Sensitivity and the Specificity of the 9G DNAChip

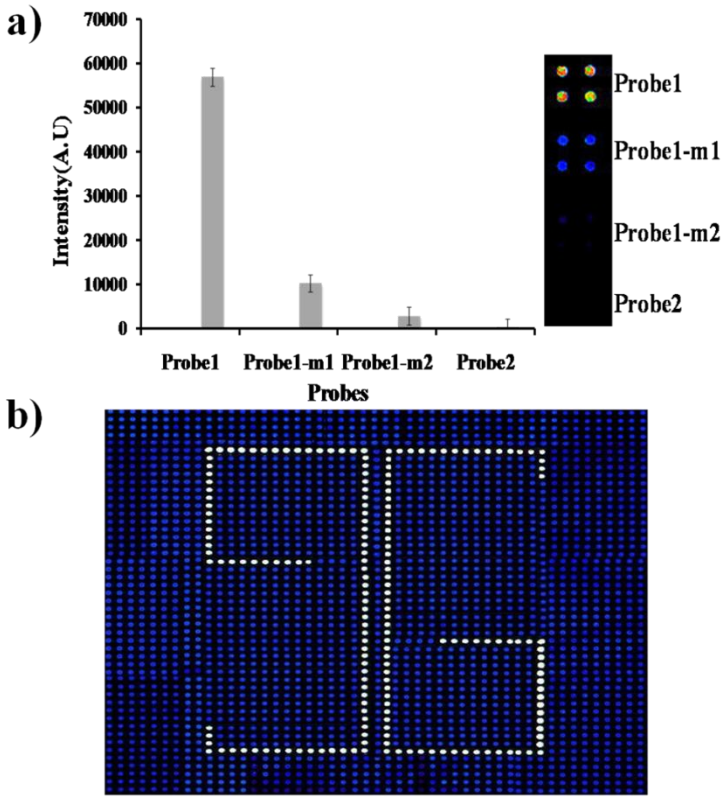


Figure S6. Specificity of 9G DNAChips a) Fluorescence intensities after hybridization of immobilized Probe1, Probe1-m1, Probe1-m2, and Probe2 with Cy5-T1, respective fluorescence image (left), PMT gain = 48. b) Fluorescence map obtained by immobilization of Probe1 (9G character, white spots) and Probe2 (surrounding the 9G character, blue spots) after hybridization with a 6:1 mixture of Cy5-T1 and Cy5-T2, PMT gain = 48.

11. References:

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