# 6. Methods

## 6.1 Material preparation

### 6.1.1 Preparation of oligoprobes

7-mer oligoprobes were selected randomly from a full heptamer set, using an algorithm with Shannon entropy as a quality criterion to generate the maximum information content for each hybridisation, and to best partition the human genomic sequences from GenBank based on their theoretical fingerprints (Herwig et al. 2000). Some of the 7-mer sequences were used in previous study in our lab (Bauer et al. 2004). Impractical oligoprobes were removed from the starting set by considering G/C and palindromic and other problematic sequences.

In order to achieve greater stability, most of the oligoprobes were LNA-modified at pyrimidine positions as previously suggested (McTigue et al. 2004). Moreover, building of continuous stretches of LNAs was avoided for most of the oligoprobes, so that particular LNAs were separated by at least one non-modified nucleotide. Due to the difficulties in LNA incorporation, the nucleotides at 3'-end were not subjected to modification. LNA synthesis followed standard amidite chemistry using Expedite 89020 DNA synthesizers and Hyacinth Activator. Cy5 was coupled to the 5'- terminus using amidite chemistry. Deprotection was performed under mild conditions in ammonia. Ammonia was removed by Sephadex gel-filtration disposable columns prior the HPLC-purification. HPLC buffer was removed by gel filtration and the amount of product was quantified based on the absorption at 260 nm. Some DNA oligoprobes of the same sequences but without any LNA modifications were used for comparison.

A series of DNA and DNA–LNA oligoprobes were synthesized by MWG Biotech (Ebersberg, Germany) and TIB MolBiol (Berlin, Germany). The sequences of oligoprobes used in this work, together with some other characteristics are shown in Table 1 for 7-mer LNA-modified oligoprobes, Table 2 for 6-mer LNA-modified oligoprobes, and Table 3 for non-modified DNA oligoprobes. All oligoprobes were 5′-end labelled using Cy5, ROX or Bodipy fluorescent dyes.

The melting temperature (Tm) of DNA and LNA-modified oligoprobes (listed in Table 1-3) was determined using previously described thermodynamic model (Allawi and SantaLucia, 1997; SantaLucia, 1998). The model was modified for prediction of the melting temperatures of DNA/LNA mixmers (more details can be found at http://lna-tm.com). The conditions used for Tm prediction were the same as used for hybridisation (100 mM salt and 0.01 µM oligoprobe).

### 6.1.2 Preparation of synthetic short ssDNAs and PCR products

The templates used in the study were synthetic ssDNAs of 24–40 nt in length or DNA clones of 250–2000 bp in length, with a previously published sequence (Bauer et al. 2004), complementary to the respective oligoprobe sequences. The sequences of the synthetic single stranded DNA targets used in this work are shown in Table 4.

For hybridisation experiments, sixty-six sequence-verified non-identical double-stranded DNA (dsDNA) clones of ~250-2000 bp in length were picked randomly from a human genomic DNA library (Radelof et al. 1998). All the clones were derived from the human chromosome Xq28 cosmid clone (GeneBank accession number: AL034384). The cosmid fragment matching dsDNA sequences are shown in Table 5.

The dsDNA clones were PCR-amplified in 50 µl reactions, using buffer containing Tris-HCl (50 mM, pH 8.3), KCl (50 mM), MgCl2 (1.5 mM), Tween-20 [0.1% (v/v)], dNTPs (200 M each), M13 primer pair (M13-Forward and M13-Reverse, sequences in ‘5.7.1 Primer pair for normal PCR amplification’, 0.4 M each), and Taq DNA polymerase (0.12 U/L). The reaction mixture was incubated for 1 min at 94℃, then thermocycled 30 times on a PTC-100 thermocycler for 20 s at 94℃, 40 s at 60℃ and 100s at 72℃ followed by a final elongation step (5 min at 72℃).

When necessary, the PCR products were purified by QIAquick™ PCR Purification Kit and resuspended in water. Purity of the fragments was checked by agarose gel electrophoresis (normally 5 µl sample, 1.2% gel with 5% EB, 80 V). Concentrations of the ethanol precipitated PCR products were measured using a Bio-Photometer and then adjusted to the desired concentration range (e.g., from 0.1 to 0.4 µM). Samples were transferred into 384-well plates. The plates were sealed and stored at –20℃ until further use for spotting.

### 6.1.3 Preparation of RNA Targets

After amplification, DNA clones were purified using a QIAquick™ PCR Purification Kit. The dsDNAs were converted into RNA using a MEGAscript™ Kit according to the manufacturer's instruction. The sequences of the primer pair used for preparation of RNA targets can be found in section 5.7.2.

The 20 µl transcription reaction system containing 2 µl NTP each (75 mM each), 2 µl 10X reaction buffer, 2 µl enzyme mix, about 1 µg linear template DNA was incubated at 37℃ for 5 hours followed by addition with 1 µl DNase I (2 U/µl) and incubation at 37℃ for 15 minutes to remove the template DNA. After the RNA was purified with RNeasy Mini Kit, the subsequent fragmentation was carried out by incubation for 20 min at 94 ℃ in a fragmentation buffer (5X 200 mM Tris– acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc).

RNA quantity and quality were determined by capillary electrophoresis using the Lab-on-a-Chip technique in accordance to the manufacturer's instructions on the RNA 6000 Nano Labchip®. In short, the microchannels were filled by pipetting 9 µl gel-dye mix into the appropriate well and then forcing the mix into the channel network. Two additional buffer wells were filled with 9 µl gel/dye mixture. Sample and ladder wells were filled with 5 µl marker mix before adding 1 µl DNA sizing ladder or sample in the respective wells. The chip was vortexed and placed into the bioanalyzer for analysis. 1 µl of the fragmented product was mixed with 5 µl 25% Sample Buffer from the Agilent RNA 6000 Nano Kit, loaded onto an RNA LabChip (Agilent Technologies) and detected with the Eukaryote Total RNA Nano Assay. All experiments were performed using Agilent biosizing software (Version A.02.12).

### 6.1.4 Preparation of fragmented single-stranded PCR fragments

Several methods have been described to generate ssDNA from double-stranded (ds) PCR products. Asymmetric PCR generates single-stranded (ss-) PCR products only linearly with variable results (Ulf et al. 1988). The second method for single-stranded DNA preparation using streptavidin-coupled beads (www.invitrogen.com/dynal; Green et al. 1990) involves too many steps and therefore is laborious. Another method utilized 5' phosphorylated PCR primer and exploits the preference of the bacteriophage λ

exonuclease for phosphorylated over nonphosphorylated 5' ends of dsDNA (Higuchi and Ochman, 1989). A major operational disadvantage of this method is the need for an additional buffer exchange step after the PCR amplification, because the λ exonuclease has a relatively narrow activity opitimum at a pH of ~9.4. Nonphosphorylated 5' ends are also attacked by λ exonuclease, although at a slower rate, and thus are not completely protected from degradation.

Regarding the disadvantages of the methods mentioned above, we exploited another approach for convenient ssDNA production. The incorporation of one partially phosphorothioated primer at the 5'-end into the double-stranded PCR product allowed the selective enzymatic hydrolysis of the opposite strand by T7 gene 6 exonuclease (Nikiforov et al. 1994). To protect one of the strands of the double-stranded PCR product from T7 Gene 6 exonuclease hydrolysis, four phosphorothioate bonds (e.g., the first five bases were phosphorothioated) were introduced during synthesis at the 5' end of one of PCR primers.

In our experiments, for generation of single-stranded PCR products, following the PCR amplification with one normal M13 primer (M13-Reverse or M13-Forward) and one phosphorothioated opposite M13 primer (M13-Forward-S or M13-Reverse-S), T7 gene 6 exonuclease was added directly into the reaction without any purification to a final concentration of 2 U/µl of PCR reaction mix. Incubation was carried out for 20 minutes at 37℃ followed by an inactivation at 80℃ for 10 min .

To fragment the single-stranded PCR products into short segments for sequential hybridisation, single-stranded PCR products were mixed with equal volume of DNase I solution (0.04 U/µl) and then incubated at 37℃ for 20 minutes, followed by inactivation at 95℃ for 15 minutes.

## 6.2 Liquid-based hybridisation assays with real-time detection of iFRET signals

### 6.2.1 Assays in cuvette and droplet formats

Hybridisation experiments were carried out in buffer-2 containing Sybr Green I at 1:1000 dilution. Real-time evaluation of hybridisations was performed in a volume of 100 µl buffer-2 in quartz cuvettes (Qs 10 mm) by thermo-controllable spectrophotometer and processed using Avasoft 5.1 software. The final amounts in the 100 µl hybridisation mixture were 100 pmol for oligoprobes and 30 pmol for the DNA/RNA targets.

In-droplet (Fig. 5) hybridisation was performed in 2 µl buffer-2. In this case, soak the pre-cleaned ready-to-use microscope slides in silane by gently shaking for about 30 minutes, wash fully with di-water and then 100% ethanol, dry water. 1 µl oligoprobe and 1 µl target solutions were dispensed onto the air-dried glass slide surface. The slides were then covered with a second glass slide, spaced with an adhesive tape providing a distance of 255 µm between the two slides to prevent evaporation. The slides were placed onto a thermal cycler that was set to a heating increment of 3℃/min. The final concentrations in the hybridisation mixture were 0.5–1.5 µM for oligoprobes and 0.15–0.5 µM for the DNA/RNA targets, for both the micro-drop and nano-well formats.

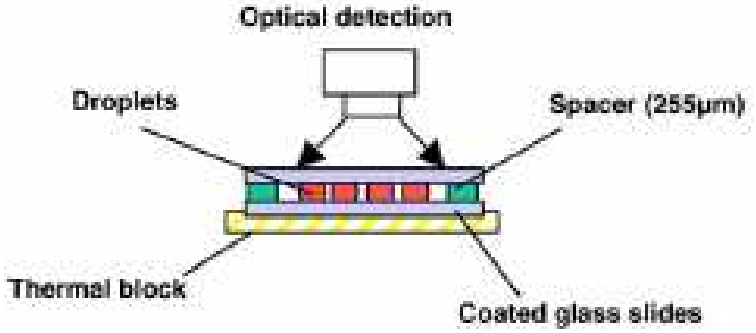



**Figure 5**. Scheme of the ‘sandwich’ set-up in the droplet experiment.

### 6.2.2 Assays in nano-well plate format

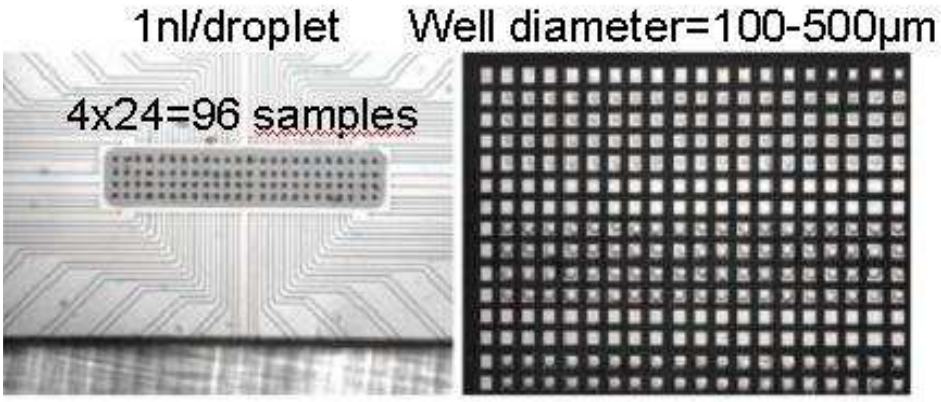
For nano-dispensing, the TopSpot/E Micro-Arrayer module (Fig. 6, modified from *Sauer* et al. 2005) was used. In contrast to other microarray spotting techniques the highly integrated TopSpot technology delivers the spotted oligoprobes at a very small pitch of 500 µm. This significantly reduces the effort for automation. The TopSpot

nanoliter dispenser system enables non-contact printing of different media like oligonucleotides, DNA or protein solutions. It provides contact-free distribution of nanolitre droplets on nanowell plates and microfluidic chips without disturbing or changing the surface properties. TopSpot printheads with 96 channels can be loaded with reagent volumes up to 6 µl per channel. The highly parallel and integrated non-contact approach of this new technology accelerates the deposition of oligoprobes onto a substrate. It enables high-throughput printing of low and medium density microarrays in cost-effective way. One of the major advantages of the TopSpot technology over other nanoliter dispensing systems (*e.g.* an inkjet printer) is the fact that every nozzle can be supplied with another printing medium. Thus a contact-free high-throughput production allows to overcome the problems arising from the adhesion force acting between oligoprobes, nozzles and the substrate surface.

For the model experiment only one head was used to prevent evaporation. Prior to oligoprobe dispensing the nano-well plate was pre-filled with 30nl of buffer-2 containing Sybr Green I dye at 1:1000 dilution. Oligoprobes and DNA/RNA targets were dispensed into wells at volumes of 4 nl in two directions on the nano-well plate (Fig. 7). Finally, the plate was covered with mineral oil to prevent evaporation. Final concentrations of the reagents were the same as in the micro-drop experiments.

### 6.2.3 Image acquisition

For imaging, a CCD camera of type CH350 (shown in Fig. 8) cooled to −40 ℃ was used, which was integrated with a thermal cycler and operated by controlling software developed in-house. The camera had a chip size of 512 × 512 pixels so that object field of 400 $mm^2$ can be detected. The excitation light was generated in a halogen light source with an interference filter. The emission filter was placed directly to the front side of the camera objective. For excitation, 480 and 620 nm filters were used and for emission, 540 and 670 nm filters. The images were evaluated using an UTHSCASA Image Tool v3.00.




**A** **B**

**Figure 6**. The multi-channel print head TopSpot (**A**) and the nanowell plate (**B**). Ninety-six droplets are arrayed in a 4 × 24 format. The print head (left picture) with microchannels for liquid transport from reservoirs to the nozzles, is placed at a 500µm distance above a glass substrate in the dispensing regime. The print head is connected to a positioning table (400 × 400 mm²) in order to allow serial dispensing in requested positions. It can be adjusted in all directions and angles. The dispensing procedure is monitored with a camera from the bottom of a transparent plate. In high-density nano-well plate (B), the distance between neighbouring nano-wells is 500 µm in order to achieve an exact overlapping with the nozzle grid of the TopSpot print head. Dispensing volumes of about 1–100 nl can be adjusted for particular samples thus leading to reduction of evaporation. The fluorescence was detected using a CCD-camera.

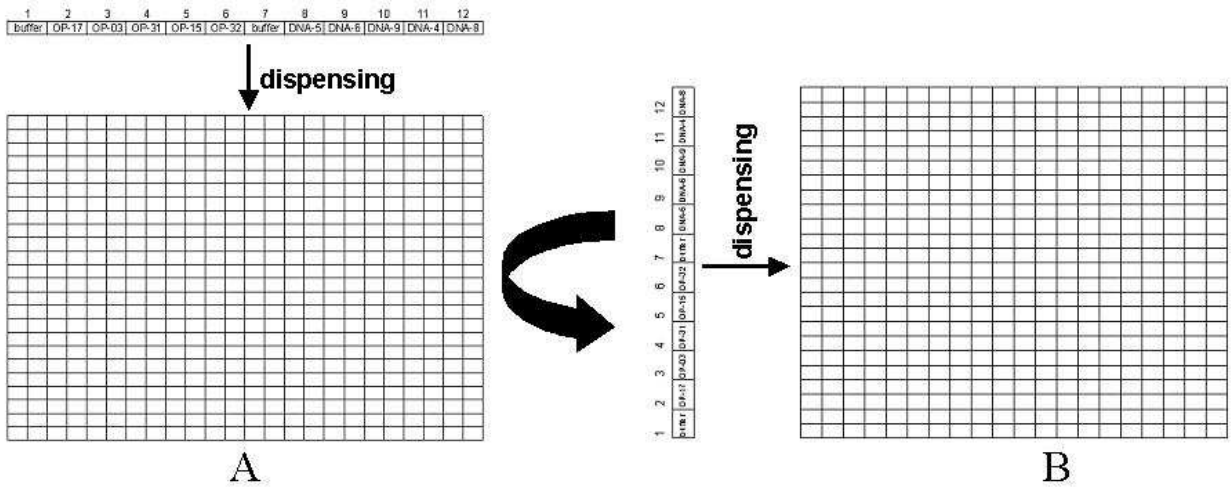



**Figure 7**. The two steps of nano-dispensing using the 24X24 nano-well plate. The multichannel print head TopSpot was filled with buffer, oligoprobes (in this case, non-modified 7-mer DNA oligoprobes such as OP-17, OP-03, OP-31, OP-15, OP-32) and targets (in this case, short synthetic ssDNAs such as, DNA-5, DNA-6, DNA-9, DNA-4, DNA-8), each in duplicates. After dispensing in one direction (**A**), the print head TopSpot was turned 90 degree and the dispensing was continued (**B**).

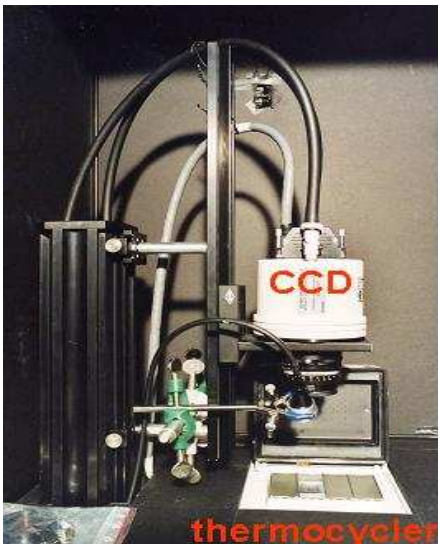



**Figure 8**. CCD camera with high sensitivity for fluorescence detection.

### 6.3 Microarray-based hybridisation assays with LNA-modified oligoprobes

PCR products were spotted onto nanoporous membranes by SciFlexArrayer. DNA was then fixed by exposure to UV and prehybridised in a blocking buffer to prevent nonspecific hybridisation signals. After hybridisation with Cy5 labeled DNA oligoprobes or with LNA modifications, the membranes were washed to remove non-specifically bound oligoprobes and then exposed to a CCD-camera for image acquisition. Image analysis and quantification was carried out with AlphaEaseFC$^{TM}$ software.

#### 6.3.1 Microarray dispensing

Protocols for microarray preparation were modified from the 'instruction for use', available at http://www.pall.com/pdf/1193_87485B.pdf. Target DNA samples were dissolved in water and spotted onto the Vivid Pall nanoporous membrane slides with a printable area of 60 mm × 20 mm using a piezoelectric spotting robot (SciFlexArrayer, Fig. 9). A single nozzle was used to avoid differences between pins. The system was programmed to release about 4-40 nl DNA solution for each spot. Spots were arrayed in a 10X10 arrangement (100 spots in a 1.2 × 1.2 cm area), or totally 1,152 spots were arrayed in a printable area of 60 mm × 20 mm, with a center-to-center distance between spots of 500–1000 µm. The DNA samples were printed in quadruplicate or in duplicate. Six copies were prepared for parallel hybridisation experiments. After deposition, slides were left at room temperature to dry the water and then heat-treated on a metal block at 95℃ for 5 min.

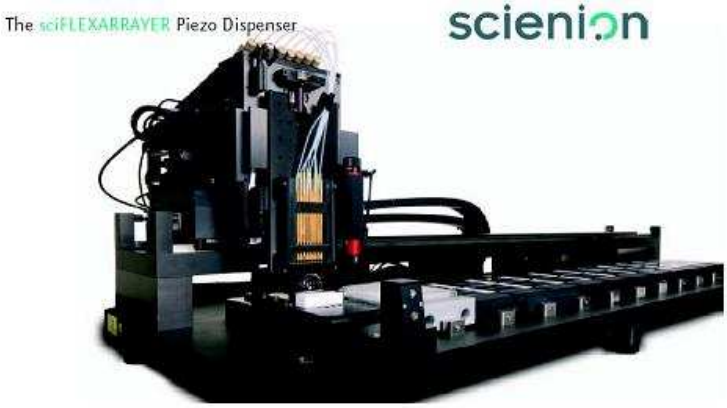



**Figure 9**. sciFLEXARRAYER. The sciFLEXARRAYER is the automated ultra low volume liquid handling tool with the dispense volume of 100-500 pl per drop.

### 6.3.2 Crosslinking and blocking

After DNA samples were air-dried after being arrayed onto nanoporous membrane slides. The samples were then cross-linked by ultraviolet irradiation using a Stratalinker$^{TM}$ oven, that was set at 1200 mJ, by placing the slides array side up, to form covalent bonds between the thymidine residues in the DNA and the positively charged amine groups on the membrane slides (Church and Gilbert, 1983; Vollrath and Davis, 1987). This step lead to increase in the amount of hybridisable DNA stably attached at each spot, especially when the DNA concentration in the spotting solution was low. Under− and over−irradiation caused excessive loss of DNA by insufficient binding and over−nicking of the DNA samples, respectively.

The most critical step in post-processing of dispensing is the blocking step. If the nanoporous membranes are not blocked, labelled oligoprobes will bind indiscriminately and non-specifically to the surface with excessively high background. According to the recommendation of the Pall membrane manufacture, blocking of the non-reacted surface can be achieved by incubating the slides at 4℃ for at least 30 min in a solution of 3% (w/v) casein-hammerstein, 150 mM NaCl, 10 mM Tris pH 8.0 adjusted using HCl, and 1% (w/v) SDS (sodium dodecyl sulfate), followed by washing in buffer-2 (50 mM NaCl, 20 mM Tris pH 7.0 adjusted using HCl and 0.2% (v/v) Tween 20) by gentle shaking at 4℃ for about 10 min. The slides were th en air-dried and stored at 4℃ until hybridisation.

### 6.3.3 Nanoporous slide hybridisation and stripping

Before hybridisation, the arrays were pre-hybridised for 30 min at room temperature with hybridisation buffer (100 mM NaCl, 40 mM Tris pH 7.0 adjusted using HCl and 0.2% (v/v) Tween 20). A total of 200 pmol of Cy5-labeled oligoprobe was added to 20ml of hybridisation buffer in a glass box and then denatured at 98℃ for 5 min. The membrane slides were incubated in hybridisation solution at 98℃ for 5 min with gentle shaking and then cooled down on ice, followed by washing with hybridisation buffer at 4℃ for about 5 min. As presented in Fig. 10, the s lides were successively subjected to hybridisation with all of the oligoprobes.

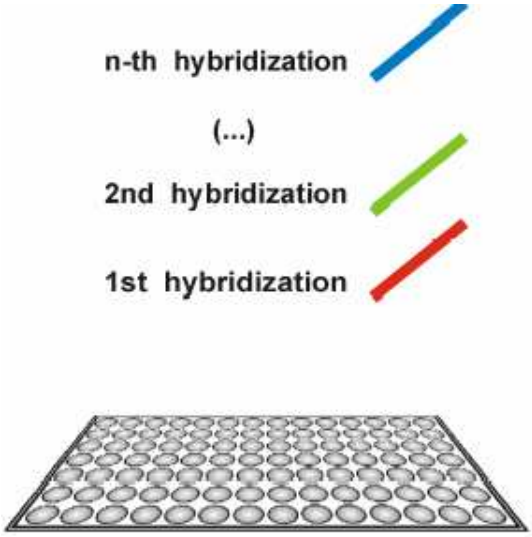



**Figure 10**. Cy5-labelled short oligoprobes were hybridised sequentially to arrays of immobilised clones (PCR products) with stripping intervals.

The oligoprobe was removed by incubation of the nanoporous membrane in stripping buffer (100 mM NaCl, 40 mM Tris pH 7.4 adjusted using HCl, 0.2%(v/v) Tween-20, 0.4× SSC pH 7.5 (adjusted using tri-sodium citrate dihydrate and sodium chloride), 0.2%(w/v) SDS) at 80℃ for 30 min in case the LNA-m odified oligoprobes were used. In cases in which non-modified oligoprobes were used, an extra period of 10 min incubation in stripping buffer was applied. Subsequently, the slides submerged in hybridisation buffer were cooled down to room temperature. The membranes were verified using a charge-coupled-device (CCD) camera to ensure that all Cy5 fluorescence had been removed. Once the fluorescent intensity had been eliminated, dried membranes were stored at room temperature.

### 6.3.4 Microarray image acquisition and data analysis

For hybridisation signal imaging, we used the 16-bit CCD-camera type CH350, with the 512 × 512 pixel chip size cooled down to –40℃. The camera allowed the measurement of pixel values in the range from 0 to 65535. The acquired pixel values were corrected for background and presented as arbitrary units of fluorescence intensity. The CCD-camera was operated by in-house developed software. An object field of 20 × 20 $mm^2$ could be detected using this approach. The excitation light was generated by a

halogen light source with an interference filter. The emission filter was placed directly on the front side of the camera objective. A 620 nm filter and a 670-nm filter were used for excitation and emission, respectively.

For signal quantification, the images were evaluated using AlphaEaseFC™ Software version 4.0.0. The intensity for each spot was calculated by integrating the gray scale values within the spot and subtracting the local background. Deviating spots (e.g. high background, dust, irregularities, etc.) were manually removed from the analysis. The mean background-corrected spot intensity of each sample was used in subsequent data analysis, as described previously (Guerasimova et al, 2006).

### 6.3.5 Data normalisation and cluster analysis

The reproducibility of each hybridisation signal was assessed through spotting each dsDNA sample in 4 replicas. The raw fingerprint data were normalised in order to eliminate experimental error. For each oligoprobe (this corresponds to a data-column in the cluster matrix), the median of all values was calculated and each value of the oligoprobe was divided by this median. To test the performance of the hybridisation, we computed receiver operating characteristic (ROC) curves. A ROC curve is a graphical plot of sensitivity vs. specificity. It can also be represented by plotting the fraction of true positives (TP) vs. the fraction of false positives (FP). Sensitivity/specificity was judged with the theoretical sequence matches between the oligoprobes, genomic DNA sequences, and a continuous discrimination threshold across the signal range.

For clustering the hybridisation fingerprints of the 66 clones across the set of oligoprobes, we applied a hierarchical clustering method with Pearson correlation as similarity measure and average-linkage as an update rule using J-Express Pro V 2.7 (http://www.molmine.com). Pairwise similarity was calculated based on median-row-centered hybridisation profiles with 26 LNA-modified oligoprobes. The fingerprint of every clone was compared to every other clone. Subsequently clones with similarity scores were merged into clusters if their member lists overlapped by 60% or more, as described previously by Meier-Ewert et al. (1998).