6. Methods

6.1 Clone selection, experimental handling and storage

To evaluate the feasibility of the concept of multiplex OFP a number of genomic and cDNA clones has been selected for the optimization of PNA hybridization conditions as well as for performing a multiplex OFP pilot study.

6.1.1 Genomic DNA clones

Fully sequenced genomic DNA clones were taken from human shotgun clone libraries that have already been characterized by oligonucleotide fingerprinting (Radelof et al., 1998), re-arrayed in a 384-well microtiter plate (MTP) containing LB broth, 100 µg/mL ampicillin and 1x HMFM, and incubated at 37° C for 16-18 h. After visual control of growth the clones were shock-frozen on dry ice to avoid contamination by condensation and stored at -80° C.

In principle, clones that are free of repeat regions were chosen according to their sequence similarity or dissimilarity, i.e. groups of clones were created which are dissimilar to each other but of which the respective members are similar or almost identical in sequence. Additionally, autonomous clones were selected that do not belong to any of these groups.

Of the picked clones either plasmid DNA was isolated (chapter 6.2) or the DNA inserts were amplified by PCR and purified (chapters 6.3 and 6.4) for sequence verification (chapter 6.7). Subsequently, large pools of purified PCR product were generated for each sequence-confirmed clone and divided into aliquots to provide sufficient homogeneous target DNA for downstream hybridization experiments.

6.1.2 cDNA clones

Using the GeneNest database (Haas et al., 2000) human cDNA clones were chosen in accordance with the above described procedure of selecting genomic DNA clones. The picked clones were ordered from RZPD Deutsches Ressourcenzentrum für Genomforschung by means of their accession numbers and experimentally handled and stored as described in chapter 6.1.1. Incubation temperatures (30° or 37° C) and antibiotics (12,5 µg/mL chloramphenicol or 100 µg/mL ampicillin) were used as appropriate.

6.2 DNA plasmid isolation

By the alkaline lysis method described by Birnboim and Doly (1979) it is possible to extract plasmid DNA from bacterial cells.

Using the Qiagen "QIAprep™ Spin Miniprep" kit, that employs a modified Birnboim protocol, up to 20 µg of plasmid DNA can be isolated and purified from 1-5 mL of bacterial culture. 1-5 mL of 100 µg/mL Ampicillin containing LB broth were inoculated with a freshly streaked single bacterial colony and incubated at 37° C for 16-18 h. Bacterial cells were pelleted by centrifugation (1000 g, 10 min, 4° C), resuspended in 250 µL Qiagen buffer P1 and lysed (5 min, ambient temperature) adding 250 µL Qiagen buffer P2. By addition of 350 µL Qiagen buffer N3 lysis was halted and chromosomal DNA as well as proteins were preticipated by centrifugation (10,000 g, 10 min, ambient temperature). Plasmid DNA containing supernatant was applied on QIAprep™ spin columns and centrifuged (10,000 g, 1 min, ambient temperature). Columns were washed once with 750 µL Qiagen buffer PE and again centrifuged (10,000 g, 1 min, ambient temperature) to remove remaining buffer PE. Purified plasmid DNA was eluted by applying 50 µL 1x low TE buffer or Millipore water and another centrifugation step (10,000 g, 1 min, ambient temperature). Eluted plasmid DNA was shock-frozen on dry ice and stored at -20° C. The success of DNA plasmid isolation was confirmed by agarose gel electrophoresis (chapter 6.5).

6.3 Polymerase Chain Reaction

The polymerase chain reaction (PCR) proposed by Mullis et al. (1986) is used to amplify segments of DNA that lie between two regions of known sequence. Two oligonucleotides are used as primers for a series of synthetic reactions catalyzed by a DNA polymerase. Typically, these primers of different sequence, are complementary to sequences that lie on opposite strands of the template DNA and flank the segment of DNA that is to be amplified.

PCR was performed in 96-well MTP format either applying plasmid DNA or direct inoculation with bacterial colonies ("colony" PCR) using 1x PCR buffer, 1.5 M betaine, 200 µM of each dATP, dCTP, dGTP, and dTTP, 0.4 µM of each primer, 0.12 U/µL *Taq* DNA polymerase, and 0.008 U/µL *Pfu* DNA polymerase in 50-150 µL reaction volumes. 30 cycles were carried out consisting of a 10 sec 94° C denaturing step, a 30 sec 55° C priming step, and a 3 min 65° C elongation step, preceded by a 1 min 94° C denaturing step and followed by a 5°min 65° C final elongation step. The success of PCR was confirmed by agarose gel electrophoresis (chapter 6.5).

6.4 Purification of PCR products

Purification of PCR products is necessary to remove potential contaminants of downstream applications such as enzymes, salts, excess nucleotides, and primers.

The QIAquick™ PCR purification kit is suitable for cleanup of up to 10 µg of either single- or double-stranded DNA in the range of 100 bp to 10 kb. To one volume of PCR reaction five volumes of Qiagen buffer PB were added. The mixture was thoroughly mixed, applied on QIAquick™ spin columns, and centrifuged (10,000 g, 1 min, ambient temperature). Columns were washed once with 750 µL Qiagen buffer PE and again centrifuged (10,000 g, 1 min, ambient temperature) to remove remaining buffer PE. Purified PCR products were eluted by applying 50-100 µL 1x low TE buffer or Millipore water followed by a centrifugation step (10,000 g, 1 min, ambient temperature). Eluted DNA was shock-frozen on dry ice and stored at -20° C. The success of purification of PCR products was confirmed by agarose gel electrophoresis (chapter 6.5).

6.5 Agarose gel electrophoresis

Electrophoresis through agarose gels is the standard method used to separate, identify, and purify DNA fragments of varying size.

Depending on the size of the DNA fragments to separate an agarose concentration of 0,4-1,0% (w/v) in 1x TAE buffer was chosen with ethidium bromide intercalating dye [0,5 µg/mL] added as DNA staining agent. Before separation DNA samples were mixed with gel loading buffer, containing bromophenol blue and xylenecyanol FF dyes, to visualize the progress of electrophoresis. Electrophoresis was carried out routinely with an electric field of 5 V/cm and 1x TAE as buffer. Additionally, a molecular weight marker was carried along to determine the individual size of the DNA fragments. A UV transilluminator (λ= 302 nm) was used to make visible the results of electrophoretic separation by means of ethidium bromide fluorescence.

6.6 Photometric determination of nucleic acid concentration

6.6.1 DNA

The concentration of pure DNA samples, i.e. without significant amounts of contaminants such as proteins, or other nucleic acids, can be simply and accurately

determined by spectrophotometric measurement of the amount of ultraviolet irradiation adsorbed by the nucleobases.

For quantification the amount of isolated DNA adsorption (OD) at 260 nm has to be determined. An OD value of 1,0 at this wavelength corresponds to app. 50 µg/mL double-stranded and 33 µg/mL single-stranded DNA, respectively.

6.6.2 PNA

Analogous to DNA, the concentration of pure PNA samples is determined by measuring adsorption at 260 nm. Routinely, this was done at ambient temperature and 80° C as at the latter temperature PNA is said to be free of base stacking effects manipulating ultraviolet adsorption. PNA concentration was calculated according to $A_{260\ nm} = c * \varepsilon * l$, with "$A_{260\ nm}$" being the OD at 260 nm, "l" the length of the light path within the cuvette (1 cm), and "ε" the extinction coefficient [mL/nmol*cm] at 260 nm. The latter is formed by the sum of the individual nucleobase values (A=13.7, C=6.6, G=11.7, T=8.6) depending on the composition of PNA sequence.

6.7 DNA sequence verification

By means of DNA sequencing unambiguous sequence deciphering is achieved. Nowadays, DNA sequences are almost exclusively determined by refined versions of the enzymatic chain termination method developed by Sanger et al. (1977).

To verify the identity of a clone under investigation it is sufficient to sequence only a few hundred base pairs from either 3'-end or 5'-end. The gained partial clone sequence is then explored by a set of similarity search programs called BLAST[8] (Altschul et al., 1990). One program of that set is "BLAST2sequences" (Tatusova and Madden, 1999), where only two available sequences, already known to be potentially homogeneous, are directly compared. In contrast, the standard BLAST programs are widely used to search for homologous sequences in nucleotide and protein databases.

Circa 2 µg of isolated plasmid DNA dissolved in Millipore water were taken for sequencing which was performed by AGOWA, Germany, with standard sequencing primers according to the Sanger method. Gained sequences were compared with the original sequences of the respective DNA inserts via "BLAST2sequences". Only confirmed cDNA and genomic clones were taken for subsequent hybridization analyses.

[8] basic local alignment search tool (http://www.ncbi.nlm.nih.gov)

6.8 Experimental handling of PNA

6.8.1 Generation of PNA oligonucleotide sets for OFP

The choice of probes is an important element of hybridization experiments. Such probes can either be selected randomly, according to their hybridization frequency, or with respect to the entropy optimization of a probe set as a whole. The latter method of probe selection based on Shannon entropy as a quality criterion is superior to the former ones as demonstrated by Herwig et al. (2000).

Three independent oligonucleotide probe sets that consist of 300 octamers, 289 heptamers, and 300 hexamers, respectively, were generated according to Herwig et al. (2000) using 6000 known human brain cDNA sequences as a training set. Since the hybridization quality in practice is still not predictable from the sequence level (SantaLucia et al., 1996) some probe sequences were added that yielded good practical hybridization results. Out of the created probe sets 67 octamer, 40 heptamer and 56 hexamer oligonucleotides were chosen. To avoid mass clashes of oligonucleotides with different sequences but similar or identical masses O-linker mass tags (8-amino-3,6-dioxaoctanoic acid)[9] were used for an unambiguous discrimination (Griffin et al. 1997). Gaps between oligonucleotides with contiguous masses were restricted to a minimal size of 5 Da to ensure sufficient resolution in MALDI-TOF MS. Respective PNA probes were synthesized by TibMolBiol, Germany, and Applied Biosystems, USA.. Lyophilized PNA probes were dissolved in 1% (v/v) TFA, incubated at 65° C for 30 min and their concentration was determined (chapter 6.6.2). By means of MALDI-TOF MS (chapter 6.9) PNA sequence and integrity was verified. PNA stock solutions were stored at 4° C.

6.8.2 Charge-tagging of PNA

The introduction of quaternary ammonium fixed charge-tags was shown to increase the detectability of small DNA oligonucleotides about 100-fold compared to unmodified oligonucleotides employing MALDI-TOF MS (Bartlet-Jones et al., 1994, and Gut et al., 1997). Although it has not yet been demonstrated that the detection limit of charged-tagged PNA is significantly lower than the detection limit of unmodified counterpart it could be empirically observed that charge-tagging of small PNA oligonucleotides renders them significantly more stable and less prone to fragmentation during MALDI.

[9] 8-amino-3,6-dioxaoctanoic acid is used as so-called "O-linker" in PNA synthesis, for instance, to covalently attach biotin moieties to the N-terminus of PNA molecules.

A solution of 100 µM PNA (100 µL) was added to 2M TEA/CO_2 buffer (10 µL) and Millipore water (100 µL) and cooled on ice. Freshly prepared ice-cold 1.5% (w/v) 6-trimethylammoniumhexyryl-N-hydroxy-succinimidylester in Millipore water (200 µL) was added and mixed. The reaction proceeded for 30 min on ice. Thereafter, the volatile buffer was removed *in vacuo* at ambient temperature. Lyophilized charge-tagged PNA was resuspended in 1% (v/v) TFA (100 µL) and stored as stock solution at 4° C. The success of charge-tagging was verified by MALDI-TOF MS (chapter 6.9).

6.8.3 Hybridization of PNA

In order to optimize PNA hybridization conditions and to evaluate the concept of multiplex OFP, PNA probes have been hybridized in varying numbers and different pools and with different additives applying varying temperatures.

6.8.3.1 Tube format

Selected numbers of different PNA probes (667 nM each) were hybridized with target DNA (56 nM) in standard PCR tubes containing 18 µL 20 mM Tris-HCl (pH 8.0) and 10% (v/v) formamide. Target DNA was denatured before hybridization by incubating it at 95° C for 4 min and on ice for another 5 min. Hybridization was carried out at 15° C to 50° C for 1.5 h. DNA and hybridized PNA were isolated using the Bruker genopure magnetic bead purification kit according to a modified protocol of the manufacturer. 5 µL magnetic beads and 50 µL binding buffer BP (diluted 1:1.786 with Millipore water) were added to the hybridization mixture, thoroughly mixed and kept at ambient temperature for 2 min. Magnetic beads were collected in a magnetic separation device for 15 min and the supernatant was removed. Subsequently, beads were washed 5-times with 100 µL washing buffer WP1 and twice with 150 µL washing buffer WP2 using the magnetic separation device for thorough mixing and separation. Before resuspension in 5 µL Millipore water the beads were allowed to dry for ca. 30 min at ambient temperature to remove residual ethanol by evaporation. Elution was carried out at 37° C for 30 min. The eluate was transferred into a fresh tube and acidified with 0.5 µL 10% (v/v) TFA.

6.8.3.2 Slide format

Similar to tube format hybridizations, selected numbers of different PNA probes (varying concentrations) were hybridized in 20 mM Tris-HCl (pH 8.0) to DNA immobilized

on various slide substrates. Hybridizations were carried out at 4° C or ambient temperature for 2 h to 16 h using coverslips - underneath of which the hybridization mixture was pipetted - and self-made hybridization chambers. Slides were washed twice or 3-times for 5-10 min in 20 mM Tris-HCl (pH 8.0) and Millipore water and centrifuged (2,500 g, 5 min, ambient temperature) to remove residual liquid.

6.9 Analysis of pure PNA and PNA hybridizations by MALDI-TOF MS

By MALDI-TOF MS PNA sequence and integrity are verified and results of PNA hybridization experiments are detected. This is achieved by comparing the experimentally determined molecular mass of a given PNA with its calculated mass.

6.9.1 Sample preparation

Samples and MALDI matrix are generally applied on stainless steel MALDI targets and subjected to the process of MALDI. Two common matrix preparation methods exist: thin-layer and dried-droplet preparation. As for the former method, matrix is spread over a MALDI target in a volatile solvent, such as acetone. The solvent evaporates immediately leaving behind a homogeneous thin layer of small matrix crystals onto which the analyte is dispensed. Analyte molecules co-crystallize homogeneously into the matrix surface resulting in low spot-to-spot variations, high mass accuracy and resolution. In contrast, dried-droplet preparations, where matrix solution is mixed with analyte prior to MALDI target application, give rise to “sweet spots”. These are certain regions within a preparation that produce better results in terms of signal presence and sensitivity, rendering this kind of preparation rather unsuitable for use in automated processes.

Bruker AnchorChip™ 400/384 targets were neatly cleaned by sonication for 10 min in 40% (v/v) isopropanol and 10% (v/v) acetic acid before being thoroughly rinsed with Millipore water and dried in a nitrogen stream. MALDI matrix solution (I or II) was applied according to the thin-layer preparation method and 0.5 µL to 1.0 µL analyte was dispensed on top of the dried thin matrix layer. Analyte incubation was allowed to continue for 2-3 min before excess liquid was removed by means of a water beam pump. Subsequently, all sample positions where washed once with 5 µL 0.2% (v/v) TFA. Again, excess liquid was removed via a water beam pump.

As for hybridized microarray slides, MALDI matrix solution (III) was directly spotted onto the respective sample positions and let dry completely. No subsequent wash step was performed.

6.9.2 Signal acquisition

Mass spectra were recorded on either a Bruker Biflex III or Autoflex time-of-flight mass spectrometer equipped with a Scout 384™ ion source and delayed extraction using positive ion and reflector mode. The chosen acceleration potential was 19 kV. Measurements were performed manually or in an automatic run averaging 200-300 shots. Microarray slides were first mounted onto an in-house designed adapter before being inserted into the mass spectrometers.

6.10 Data analysis

The ultimate goal of MALDI-TOF MS based PNA hybridization analysis is to distinguish between genomic or cDNA clones of similar or even identical sequence and those that are dissimilar. Consequently, generated hybridization data have to be highly reproducible and meaningful, i.e. hybridization artifacts and data of poor quality have to be excluded. In a second step raw data have to be processed before being subjected to the original OFP analysis. This is an essential prerequisite for successfully performing clustering analyses and eventually OFP projects.

6.10.1 Visualization and quality verification

General hybridization quality and reproducibility were visually verified using Bruker XMASS 5.1 and Proteometrics m/z software. Ambiguous results were checked by re-measurement. Only reproducible data were taken for further analysis.

6.10.2 Processing

Two different programs for data processing were used: an in-house developed peak finding program and a macro program based on Bruker XMASS 5.1. As for the latter program, spectra were first calibrated with two known PNA masses. Subsequently, all present PNA peaks were labeled with their corresponding molecular mass on the basis of a pre-defined custom peak list. An XML output file was automatically created that contained molecular masses of all identified and hence labeled PNA signals, their absolute peak intensities as well as other parameters describing resolution and quality of a given signal. The in-house developed program also makes use of a pre-defined peak

list. With a tolerance of $\Delta \pm 0.3$ Da potential PNA signals were identified. Per PNA signal at least three isotopic peaks were expected. The intensity of the (first) monoisotopic peak was compared with the local background intensity[10]. If the signal-to-background ratio was lower than five the entire PNA signal was set to zero, i.e. declared as not present. Otherwise, the average intensity of all three isotopic peaks was taken as output data for a given PNA signal.

6.10.3 Pearson correlation

A means of interpreting hybridization data in the form of mass spectra is to compare their profiles. For a given spectrum, all individual PNA signals and their respective intensities are correlated with each other. Pearson correlations[11] are calculated for each two spectra compared that have been generated with the same set of hybridization probes. Clones of similar or identical sequence as well as hybridization repetitions of a given clone are expected to show a high correlation because of their similar MS profiles (i.e. same PNA signals present with similar absolute peak intensities). In contrast, clones with little or no similarity are supposed to show a low correlation. As input data complete unprocessed spectra and processed data were applied. The latter comprise PNA signals and corresponding absolute peak intensities generated either by the macro program based on Bruker XMASS 5.1 or the in-house developed peak finding software.

6.11 Fabrication and use of DNA microarrays

A DNA immobilization system that allows direct read-out of hybridization events by MALDI-TOF MS would be a valuable asset towards a complete and efficient automation of the OFP process. Therefore, promising substrates (i.e. surface materials and DNA attachment chemistries) were evaluated in terms of their suitability.

6.11.1 Spotting and processing

DNA microarrays were either fabricated manually by pipetting DNA on distinct positions or by means of an in-house designed or a Virtek Vision SDDC-3 microarrayer. Prior to spotting target DNA was dissolved in an appropriate spotting buffer, such as

[10] molecular mass of monoisotopic peak minus 1 Da
[11] see: Krengel (1991), p54 et sqq.

3x SSC, depending on the demands of the respective functionalization of the surface under investigation. After spotting microarrays were allowed to incubate at ambient temperature for 12-16 h for proper immobilization, before they were processed according to the publicly available manufacturers´ protocols. Processing steps generally comprise snap-drying and rehydration to guarantee a uniform spot morphology, backing and/or UV crosslinking to complete DNA on-chip immobilization, blocking to inactivate free functional groups on the respective surface, and intense washing and centrifugation to remove excess DNA and all kinds of salts or solutions used during processing. Processed DNA microarrays were used for PNA hybridizations followed by MALDI-TOF MS detection or fluorescent DNA hybridizations (chapter 6.11.2) or stored dessicated at ambient temperature.

6.11.2 Fluorescent DNA hybridization and detection

By means of comparative fluorescent oligonucleotide hybridizations it is possible to simply evaluate the capacity of a DNA immobilization system. This is achieved by immobilizing target DNA on different slides under investigation and a subsequent hybridization of the same fluorescent DNA oligonucleotide. Although no absolute data, such as the total amount of DNA immobilized or DNA accessibility towards hybridization probes, are gained, the capacity of a respective system can be deduced from a relative comparison of normalized hybridization signal intensities and signal-to-noise ratios.

Prior to hybridization a fluorescence-labeled oligonucleotide was denatured at 95° C for 5 min and immediately put on ice. An appropriate amount of oligonucleotide was diluted in 1x SSARC buffer to yield a concentration of 6 µM. Per microarray slide 60 µL of that hybridization solution were pipetted on top of a cleaned cover slip onto which the respective slide was placed upside-down. Hybridization was carried out in self-designed hybridization chambers at ambient temperature for 16 h in the dark. After hybridization, microarray slides were washed twice in 1x SSARC buffer at ambient temperature for 15 min, once in 0.2x SSC/0.1% (w/v) SDS at ambient temperature for 5 min, once in 0.1x SSC at ambient temperature for 5 min, and once in Millipore water for 10 sec. By centrifugation (2.500 g, 3 min, ambient temperature) residual liquid was removed. Fluorescence hybridized DNA microarrays were analyzed using a GenePix 4000B fluorescence scanner and the GenePix Pro 4.1 software package and stored desiccated at ambient temperature in the dark.