7. Results

The objective of this dissertation was to significantly enhance the existing technology of OFP by changing from sequential hybridization and detection to highly multiplexed hybridizations and simultaneous analysis. This was to be achieved by the use of PNA as hybridization probes and MALDI-TOF MS as means of hybridization detection.

Within the scope of this dissertation several pivotal questions were addressed, such as relevant characteristics of PNA hybridizations and detection as well as the impact of different hybridization parameters. In a multiplex OFP pilot study, a number of selected genomic and cDNA clones was analyzed to assess the feasibility of the innovative concept. Furthermore, potential DNA immobilization systems that allow direct hybridization read-out by MALDI-TOF MS were evaluated in terms of their suitability. In the following experimental results addressing these questions are presented.

7.1 Characteristics of PNA hybridizations and MALDI-TOF MS based PNA detection

7.1.1 Significance of probe length

Although the use of DNA oligonucleotide probes as short as six nucleotides has been reported (Drmanac et al., 1990, Chechetkin et al., 2000) hybridizations with octamer or shorter DNA probes fail to yield consistent results of sufficient quality. As a consequence, current OFP technology uses degenerated DNA decamer oligonucleotides as hybridization probes which possess an octamer core as informational entity. However, hybridization frequencies of 8mer probes are much lower in practice than the optimal value of 50%. Assuming such a hybridization probability, about 17 probes would be needed for a successful partitioning of 100,000 clones. Calculations of average hybridization frequencies of randomly chosen oligonucleotide probes to random double-stranded target sequences, that are independent from each other and of equal length of 1000 bp, led to the following results: octamers ~ 3%, heptamers ~ 11%, hexamers ~ 39%, and pentamers ~ 86% (Herwig et al., 2000). Yet, these calculations are only approximations since in real experiments neither target DNA is of equal fixed length nor are hybridization probes randomly selected. In practice, a total number of up to 250 octamer probes has to be employed for the characterization of 50,000-100,000 clones to provide for a meaningful OFP analysis (Herwig et al., 1999 and 2000).

PNA, in contrast to DNA, forms duplex hybrids with complementary DNA, (Egholm et al., 1993. Wittung et al., 1994) that show a greater stability compared to DNA/DNA duplexes. Experiments with fluorescence labeled PNA probes suggested that the hybridization of PNA octamers is feasible (Guerasimova et al., 2001). The authors ascribed that finding to the greater PNA/DNA duplex stability which led to the initial assumption that PNA probes even shorter than 8mers can be reliably hybridized. To prove that assumption right, in the course of this dissertation PNA octamer, heptamer and hexamer probes were tested for their informational as well as experimental applicability.

Due to the respectively higher hybridization frequencies of 7mers and 6mers, the use of these probes compared to 8mers was expected to lead to a higher partitioning and hence better clustering of oligonucleotide-fingerprinted clones (Herwig et al., 2000). This expectation could be confirmed by a clustering simulation where the performance of 8mer, 7mer, and 6mer probes in dependence of the maximum sequence length of target DNA was directly compared (fig. 7.1).

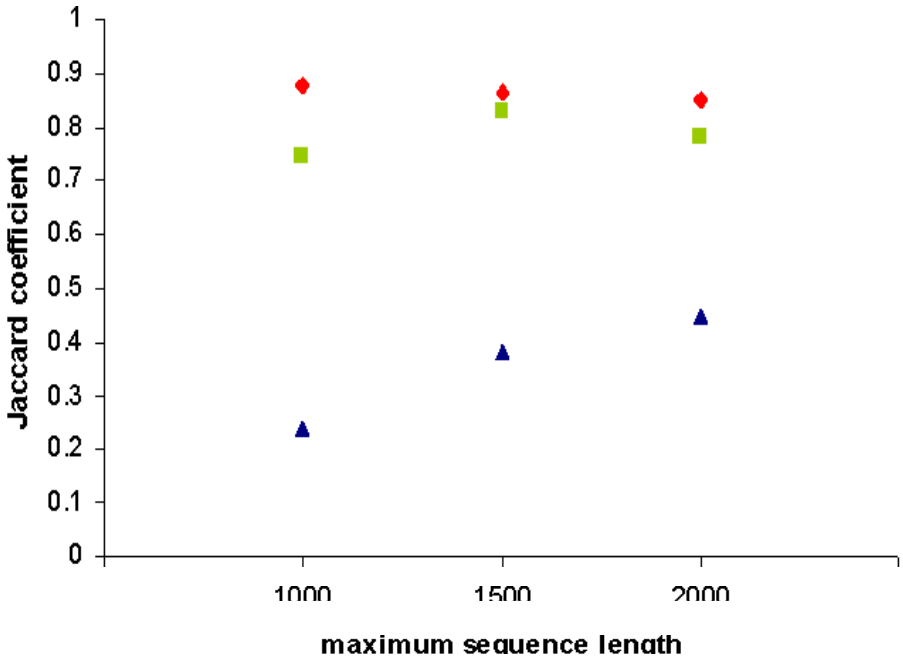

Figure 7.1 Comparison of clustering quality of 8mer, 7mer, and 6mer probes

Clustering quality as measured by Jaccard Coefficient (Herwig et al., 1999) was simulated for 70 8mer, 7mer and 6mer oligonucleotide probes, respectively. A high Jaccard Coefficient reflects a good clustering quality ("1"= perfect clustering). Probes were taken from previously created sets as described in chapter 6.8.1. Target DNA sequences of 300 bp to 1000 bp, 1500 bp, or 2000 bp were generated "in silico" based on Human Brain Unigene set. The experimental noise parameter was set to 20% of false positive/negative hybridization rate.

The comparison of clustering performance clearly demonstrates that, for the given target DNA lengths and oligonucleotide probe number, hexamer and heptamer probes are superior to octamers. Hexamers in turn perform somewhat better than heptamer probes.

To study the impact of varying probe numbers on clustering further simulations were carried out for the better performing 6mers and 7mers (fig. 7.2). From a practical point of view it is impossible to precisely calculate the value of clustering quality due to various

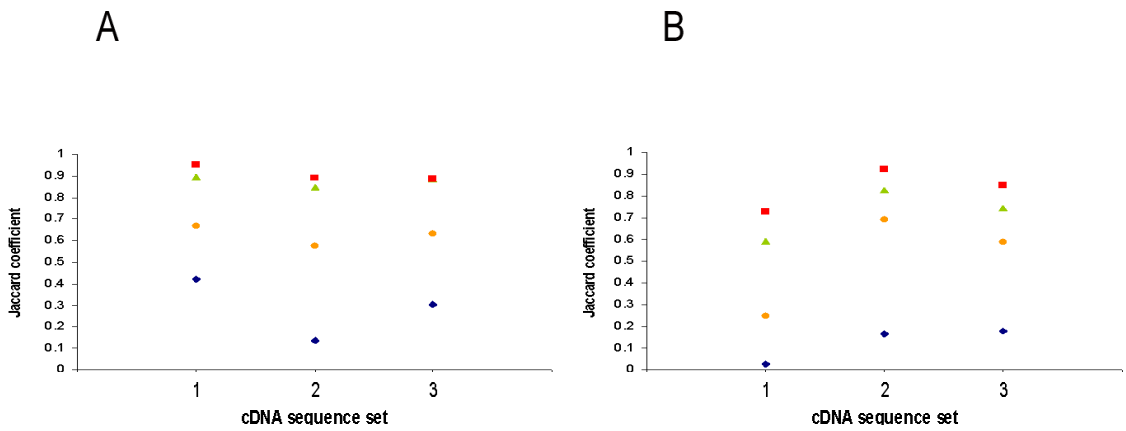

Figure 7.2 Impact of varying probe numbers on clustering quality

Clustering quality as measured by Jaccard Coefficient (Herwig et al., 1999) was simulated for three independent sets of about 7000 known cDNA sequences ("in silico" derived from Human Brain Unigene set) each with a length distribution of 300 bp to 2 kb. Simulations were performed with 30, 50, 70, and 100 hexamer (**A**) or heptamer (**B**) oligonucleotide probes. These were taken from previously created sets as described in chapter 6.8.1. A high Jaccard Coefficient reflects a good clustering quality ("1"= perfect clustering). The experimental noise parameter was set to 20% of false positive/negative hybridization rate.

experimental sources of error. However, recent successfully completed OFP projects suggest that Jaccard coefficients of at least 0.80 have to be achieved for convincing clustering analyses (empirical observations). Therefore, it can be concluded that 70 hexamer probes are sufficient for a meaningful clustering, whereas for heptamers 100 probes are recommended.

Despite the inferior theoretical partitioning of 8mers all three kinds of oligonucleotides were experimentally examined since hybridization properties of PNA oligonucleotides of these short lengths were unknown in practice and are still unpredictable (SantaLucia et al., 1996). PNA oligonucleotides used for hybridizations were produced as described in chapter 6.8.1. As it was demonstrated that the hybridization of PNA octamers is feasible initial experimental efforts focused on the evaluation of 8mers. Subsequently, 7mer and 6mer probes were also tested for their applicability. Results showed that in principle all

three types can be successfully employed. There was no evidence that 8mers hybridize in a more reliable fashion than 7mers and 6mers, respectively. Neither were hexamer nor heptamer probes more specific as thermodynamic considerations would suggest. However, due to their more favorable desorption and ionization properties, 6mers and 7mers are more easily detected than 8mers in the process of MALDI-TOF MS giving rise to higher absolute signal intensities.

7.1.2 Influence of PNA modifications on hybridization and MALDI-TOF MS detection

Different covalent modifications of PNA have been reported that were introduced to either improve PNA hybridization or MALDI properties. Terminal lysine was shown to have a stabilizing role in PNA/DNA duplexes (Ratilainen et al., 1998) presumably because of an increased PNA strand solubility due to the positively charged lysine residue. The use of O-linker 8-amino-3,6-dioxaoctanoic acid offers the possibility to uniquely mass label and detect PNA probes of similar or identical masses (Griffin et al., 1997). Quaternary ammonium fixed charge-tags were shown to increase the detectability of small DNA oligonucleotides about 100-fold compared to unmodified oligonucleotides employing MALDI-TOF MS (Gut et al., 1997).

To test the influence of the above mentioned modifications on PNA hybridization and MALDI properties, varying numbers of O-linker and lysine residues were incorporated at the N- and C-terminus of PNA octamers. As for a few oligonucleotides, either an O-linker was N-terminally attached, or a lysine residue was C-terminally attached; or both O-linker and lysine were N- and C-terminally attached, respectively, or the octamer sequence was left unmodified. Besides, the impact of different numbers of mass tags (one, three, and five N-terminally attached O-linkers) was examined for octamers. In addition, 8mer, 7mer and 6mer PNA probes were charged-tagged as described in chapter 6.8.2.

As for the incorporation of lysine and/or O-linker, experiments revealed no significant improvement neither in hybridization behavior nor in MALDI-TOF MS detection. For some octamer sequences it appeared as if the N-terminal addition of one O-linker resulted in slightly improved detection compared to unmodified sequences. However, this finding was not consistent and could not be observed for hexamers and heptamers. Probes with larger numbers of N-terminally attached O-linkers (three or five) were consistently found to yield weaker signals - with five O-linkers being worse - suggesting that the process of MALDI is increasingly impaired by the presence of many mass-tags. Although it was not found that the detectability of charged-tagged PNA is noticeably increased as in the case of charge-

tagged DNA oligonucleotides it could be observed that charge-tagging of small PNA oligonucleotides renders them significantly more stable and less prone to fragmentation and alkali adduct formation during the MALDI process (fig. 7.3).

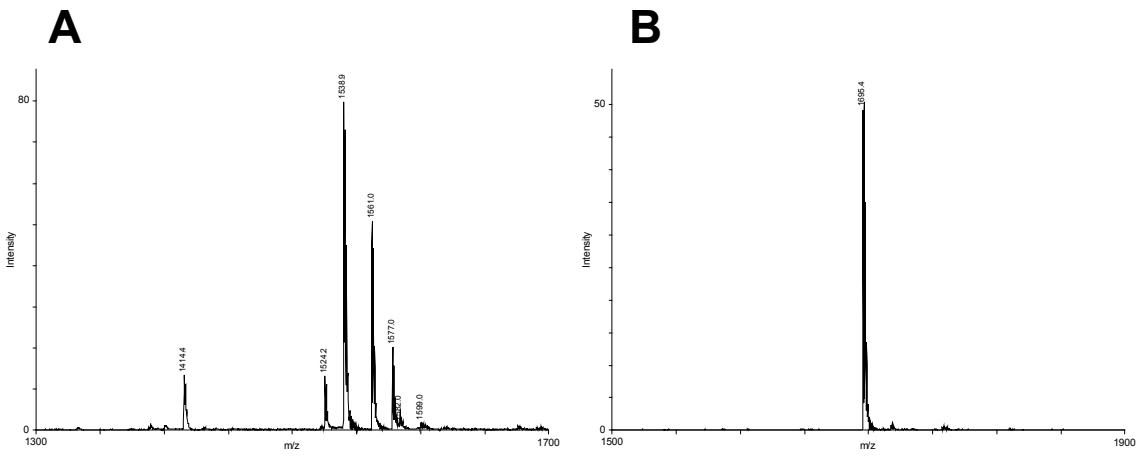

Figure 7.3 Impact of positive charge-tagging on PNA detection

In a direct comparison, a PNA hexamer was left unmodified (**A**) and positively charged-tagged (**B**), before being subjected to analysis by MALDI-TOF MS. In the spectra, relative signal intensity (intensity) as a function of mass-to-charge ratio (m/z) is shown. Charge-tagging of PNA was performed as described in chapter 6.8.2.

7.1.3 Determination of total probe number and creation of PNA sets

The determination of the total number of PNA probes that can be hybridized and detected simultaneously is of utmost importance as it ultimately defines the degree of multiplexing and hence the capacity of the concept of multiplexed OFP.

Little has been reported so far about multiplexed PNA hybridizations. In their MALDI-TOF MS approach to the analysis of genetic variations Griffin et al. (1997) successfully applied duplex PNA hybridizations and detected up to five PNA probes of unique mass in parallel. Furthermore, Ross et al. (1997) demonstrated that it is even feasible to hybridize four PNA probes simultaneously.

To address the issue of multiplexing in PNA hybridizations it was tested how many PNA probes can be resolved in one spectrum in an meaningful fashion. Furthermore, hybridization studies were carried out with different pools of varying probe numbers to evaluate at which degree of multiplexing meaningful results are still to be obtained.

Due to the experimental expertise gained for PNA octamers, the performance of these PNA oligonucleotides was investigated first. Preliminary experiments carried out

with up to 57 different PNA octamer probes, however, revealed their poor hybridization and MALDI properties and showed that, for PNA octamers, the applied degree of multiplexing (57 probes) was too high for sufficient signal resolution and hence analyzable hybridization results. Further experiments were performed with PNA hexamers and heptamers. In contrast to PNA octamers, these showed higher theoretical partitioning and superior desorption and ionization properties. Since for 8mers a total probe number of over fifty resulted in insufficient signal resolution, no additional efforts were made to exceed this figure. As for 6mers and 7mers, global sets were created comprising all respective available PNA that fitted in with respect to their individual mass and mass resolution. In addition, sets consisting of lower numbers of different PNA probes were designed according to their individual MALDI properties (so-called "subsets"), i.e. probes were grouped that, on an equimolar basis, yielded signal intensities of comparative absolute values. The compositions of the respective sets are given in chapter 5.7. To compare resolution and detection sensitivity of highly multiplexed PNA 6mer and 7mer pools, the two global sets of 40 different PNA hexamers and heptamers were directly analyzed by MALDI-TOF MS (fig. 7.4). It appears that hexamers of this number can be

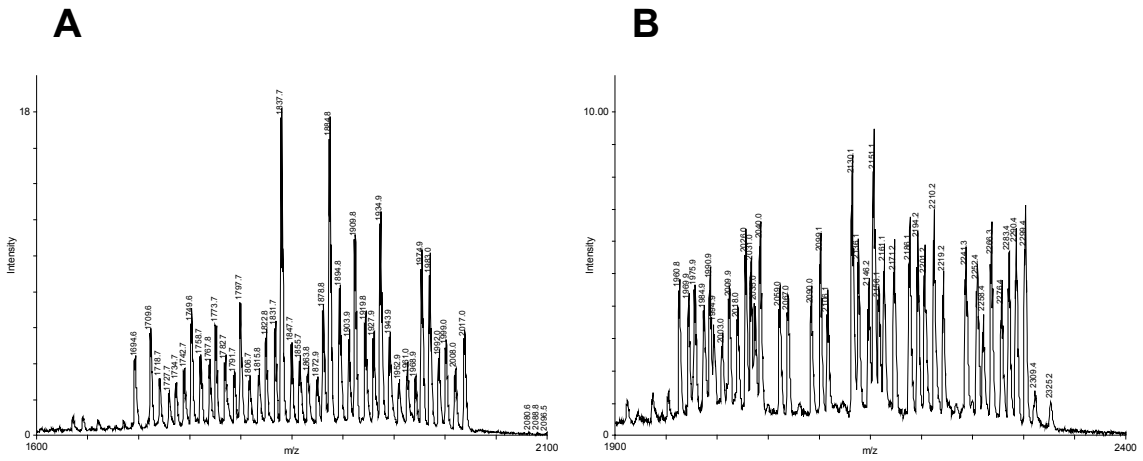

Figure 7.4 Parallel detection of 40 different PNA hexamers and heptamers

40 different PNA hexamers and heptamers (PNA sets "6mer global" and "7mer global") were detected simultaneously by MALDI-TOF MS. Experimental probe masses are annotated. Compositions of the respective sets including PNA names are given in chapter 5.7. Each PNA was applied at a concentration of 667 nM.

slightly better resolved than heptamers. Both overall resolution and signal intensities were found to be significantly higher compared to those obtained for octamers. The better performance is probably due to the superior MALDI properties of 6mers and 7mers. Regarding detection, it can be concluded that for PNA hexamer and heptamer probes an

up to 40-plex approach is feasible. Comprehensive hybridization studies comprising PNA 6mer and 7mer global sets as well as all subsets are presented in chapter 7.3.

7.2 Impact of different parameters on PNA hybridization

Beside the study of impact of overall probe number, multiplexed PNA hybridizations were carried out to examine PNA hybridization properties and optimize hybridization conditions towards better specificity. Experiments were performed in tube format as described in chapter 6.8.3.1. Basic experimental conditions were 20 mM Tris-HCl (pH 8,0) as buffer and 1,5 h as duration of hybridization. Shorter periods (0,5 h) led to weaker results. Longer periods (4 h, 8 h, 16 h) did not yield higher signal intensities nor did they improve specificity. Although first insight on PNA hybridization properties was gained by the use of short synthetic oligonucleotides, the comprehensive examinations presented here were performed with PCR amplified DNA inserts of four different genomic clones. This was because longer PCR products show secondary structure thereby epitomizing target DNA as in “real” OFP projects. As PNA probes, both hexamers and heptamers were tested employing charge-tagged PNA sets “6mer sub1” and “7mer sub2” (refer to chapter 5.7 for composition).

7.2.1 Influence of probe and target DNA concentration

The influence of individual and global probe concentration as well as target DNA concentration on hybridization results was examined for all available 6mer and 7mer PNA sets. As for the study of individual probe concentration, probe sets were created of which all respective concentrations were brought into line with each other, i.e. relative PNA signal intensities were equalized (“equalized set”). In the case of PNA set “6mer global”, for instance, the originally weakest PNA was up to eighteen times more concentrated than the PNA originally yielding strongest signals. In addition, all PNA probes of a respective set were employed in an equimolar fashion (“equimolar set”). Either sets were applied in hybridization experiments by which four different genomic clones were analyzed. Figure 7.5 shows the highly reproducible hybridization results of both an equalized and an equimolar PNA hexamer set with two of these genomic clones. The results clearly demonstrate that altered individual probe concentrations have virtually no impact on PNA presence and signal intensity. PNA probes showing moderate signal intensities in pure PNA mixes may be of highest signal intensities in hybridization spectra and vice versa – as indicated by red arrows for PNA 6P008 - strongly suggesting that hybridization results

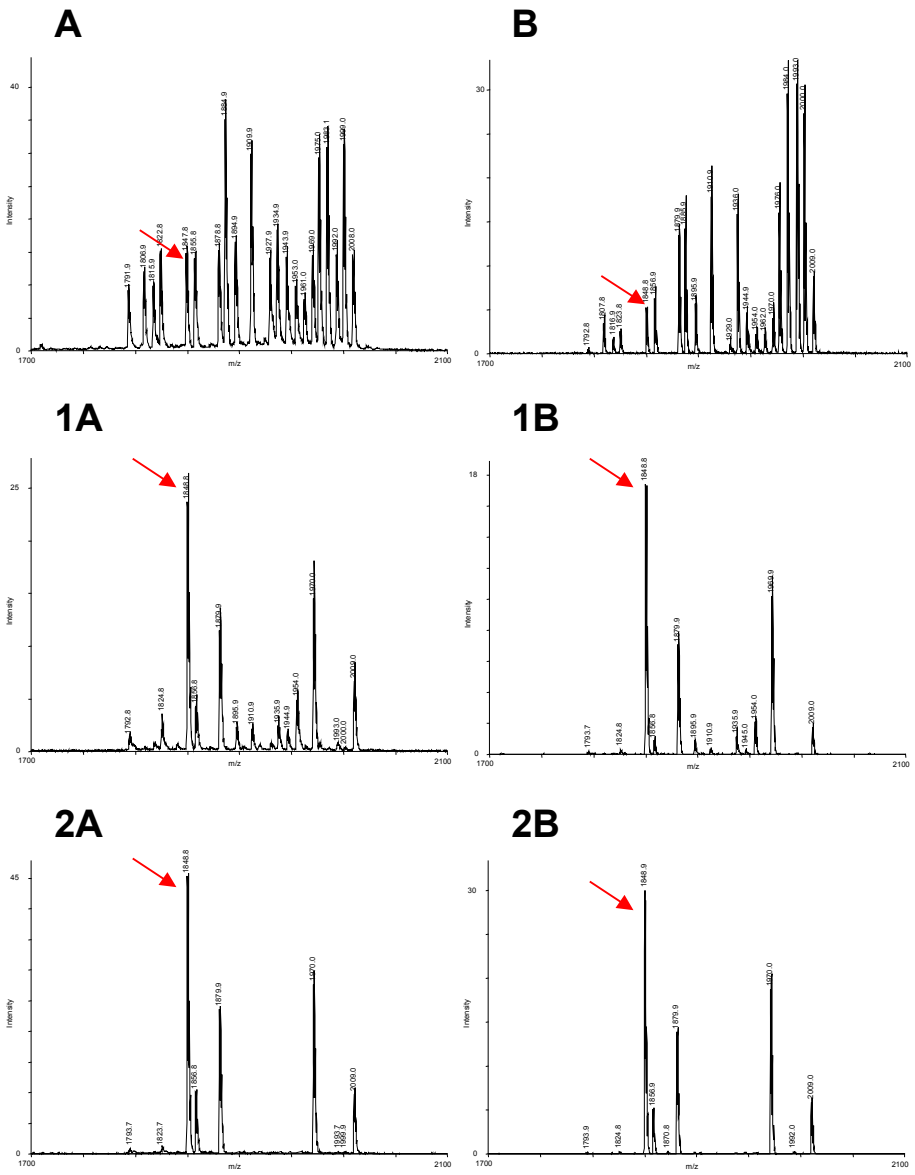

Figure 7.5 Impact of altered individual probe concentration on hybridization

Four genomic clones were hybridized with equalized PNA set “6mer sub1” (**A**) and an equimolar version (**B**) of it. As for the former, the originally weakest PNA was four times more concentrated than the PNA originally yielding strongest signals. Pure PNA mixes are depicted (**A**, **B**) as well as reproducible hybridization results gained with both mixes and two different clones (**1A**, **1B**, **2A**, **2B**). Experimental probe masses are annotated and the relative peak heights of PNA 6P008 is indicated by red arrows. The composition of PNA set “6mer sub1” is given in chapter 5.7.

are target DNA sequence-dependent. Since the alteration of individual probe concentration did neither affect presence nor intensity of PNA signals only equimolar PNA sets were applied for all subsequent hybridization experiments. In a further study the impact of global PNA concentration on hybridization was examined (fig. 7.6). Again, four different genomic clones were analyzed by hybridizing PNA sets of varying equimolar concentration (1333 nM, 667 nM, 333 nM, and 167 nM). The illustration shows that

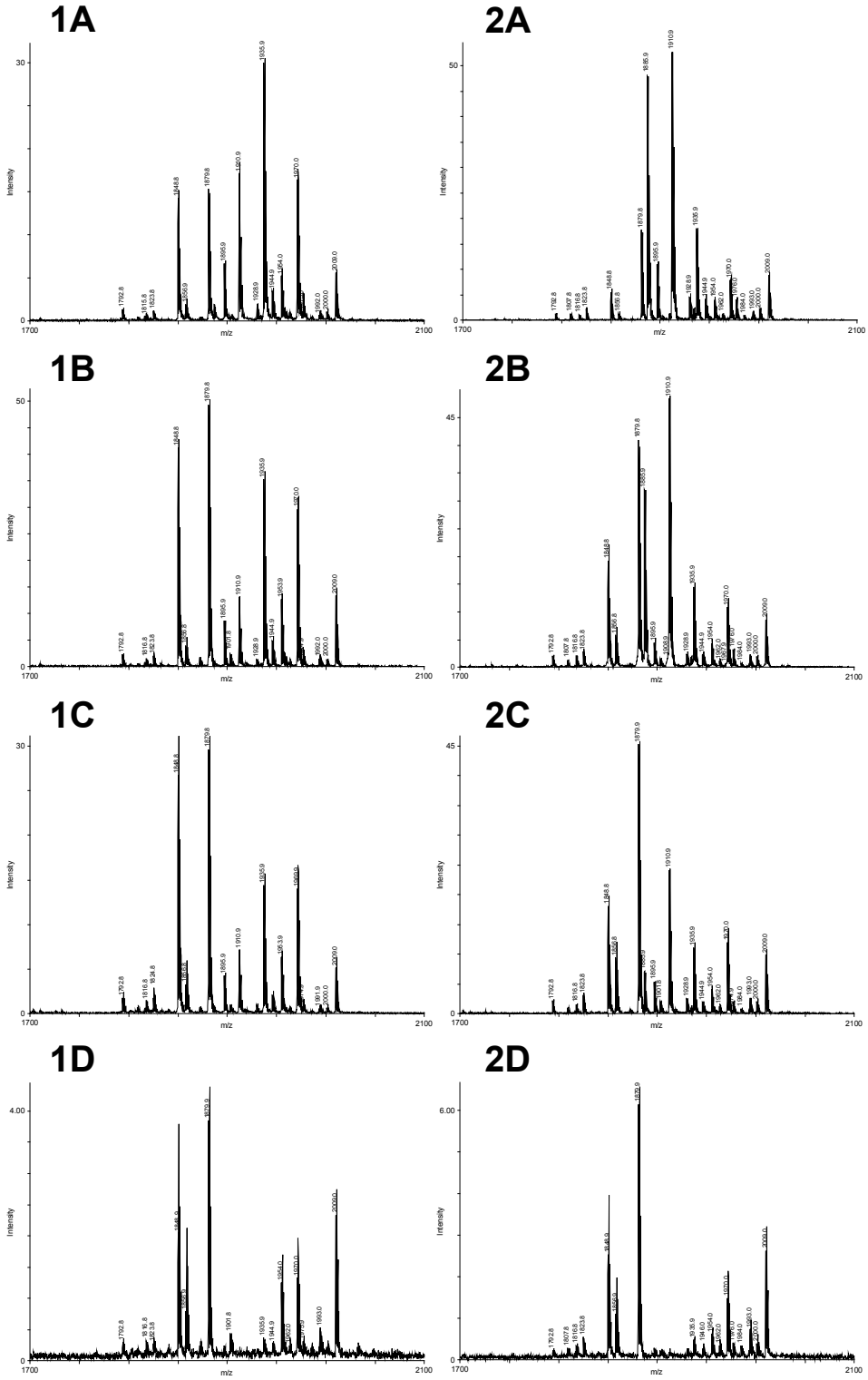

Figure 7.6 Impact of global probe concentration on hybridization

Four genomic clones were hybridized with equimolar PNA set “6mer sub1” of varying global concentrations (**A**: 1333 nM, **B**: 667 nM, **C**: 333 nM, **D**: 167 nM). Reproducible hybridization results gained with all four concentrations and two of the clones are depicted (**1A-D, 2A-D**). Experimental probe masses are annotated. The composition of PNA set “6mer sub1” is given in chapter 5.7. Target DNA was applied at a concentration of 56 nM.

hybridization profiles, i.e. presence and respective signal intensities of probes, are dependent on global probe concentrations. Lower concentrations (fig 7.6: 1D, 2D) gave rise to hybridization spectra of inferior quality that appear to be less distinguishable. Higher concentrations may unexpectedly alter the overall hybridization profile (fig. 7.6: 1A) and are - from an economical point of view - more costly. Due to inconsistent performance no meaningful information with regard to global PNA concentration could be derived for heptamers. As a compromise and to ensure hybridization results of sufficient quality, a global PNA concentration of 667 nM was used as standard for both hexamers and heptamers in subsequent experiments.

Beside individual and global probe concentrations the influence of target DNA concentration on hybridization was also investigated. Figure 7.7 demonstrates that, analogous to the global concentration of probes, hybridization profiles depend very much on overall target DNA concentration. In fact, it appears as if DNA concentration affects hybridization even more critically as is suggested by the prevalent change of hybridization profile through all tested concentrations (fig 7.7: 1A-D, 2A-D). To ensure a sufficient degree of quality, a target DNA concentration of 56 nM was chosen as standard for subsequent hybridization experiments.

7.2.2 Influence of additives and temperature

Elevated temperatures and/or the addition of certain chemicals have been reported to influence the outcome of desoxy- and ribonucleic acid hybridizations towards higher specificity. However, rather little is known for PNA hybridizations.

Within the scope of the dissertation several promising substances have been tested to examine their impact on PNA hybridization and to optimize hybridization conditions towards higher specificity. Furthermore, the impact of temperature on its own as well as in combination with some of these substances was explored. The substances tested comprise sodium chloride, tetramethyl- and tetraethylammonium chloride (TMACl, TEACl), formamide, betaine, sodium N-lauroyl-sarcosine, SDS, and Tween-20.

Sodium chloride has been described to lower T_m in DNA duplexes (Marmur and Doty, 1962) as well as to increase specificity in PNA oligomer array based hybridizations (Weiler et al., 1997). Tetramethyl- and tetralethylammonium salts bind to AT-rich DNA regions thereby abolishing the preferential melting of AT versus GC base pairs (Klump, 1997, Orosz and Wetmur, 1977). Formamide has been known for decades as a DNA melting agent inducing stringency in hybridizations (Bonner et al., 1967, McConaughy et al., 1969) whereas betaine is suggested to reduce the formation of secondary structure caused by

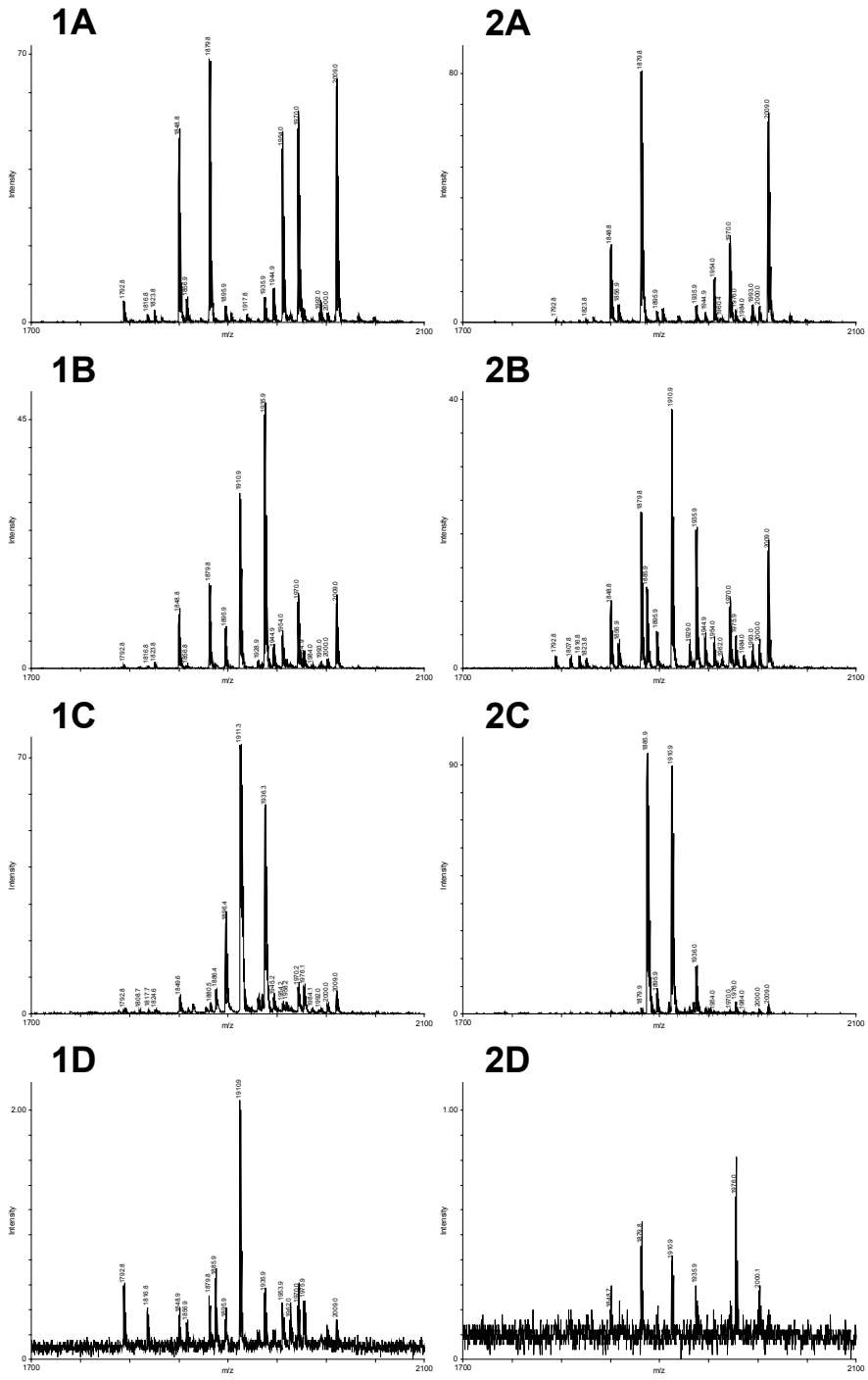

Figure 7.7 Impact of target DNA concentration on hybridization

Four genomic clones were hybridized with equimolar PNA set "6mer sub1" and varying concentrations of target DNA (**A**: 112 nM, **B**: 56 nM, **C**: 28 nM, **D**: 14 nM). Reproducible hybridization results gained with all four concentrations and two of the clones are depicted (**1A-D, 2A-D**). Experimental probe masses are annotated. The composition of PNA set "6mer sub1" is given in chapter 5.7. Each PNA was applied at a concentration of 667 nM.

GC-rich regions (Henke et al., 1997), a phenomenon exploited primarily in PCR. The addition of detergents, such as sodium N-lauroyl-sarcosine, SDS, and Tween-20, has

been shown to reduce unspecificity in various membrane-based techniques applying any class of probe.

Sodium chloride, TMACl, TEACl, and betaine were tested at concentrations of 20 mM, 50 mM, 200 mM, and 500 mM. The impact of formamide was examined with 0%, 10%, 20%, and 30% (v/v) formamide added whereas 0.2%, 0.5%, 1% and 2% (v/v) of respective detergent were applied. In addition, the influence of formamide, betaine and a combination of both in dependence of temperature was explored. Of the tested substances only formamide showed a favorable impact (fig 7.8). All others did affect results in a rather unfavorable way if there was an impact at all, i.e. overall signal intensities were decreased without a concomitant increase in specificity. The addition of detergents even introduced an extra experimental source of error due to heavy foam formation. As expected beforehand and confirmed by the experiments illustrated in figure 7.8 formamide induces a melting of DNA duplexes rendering target DNA more

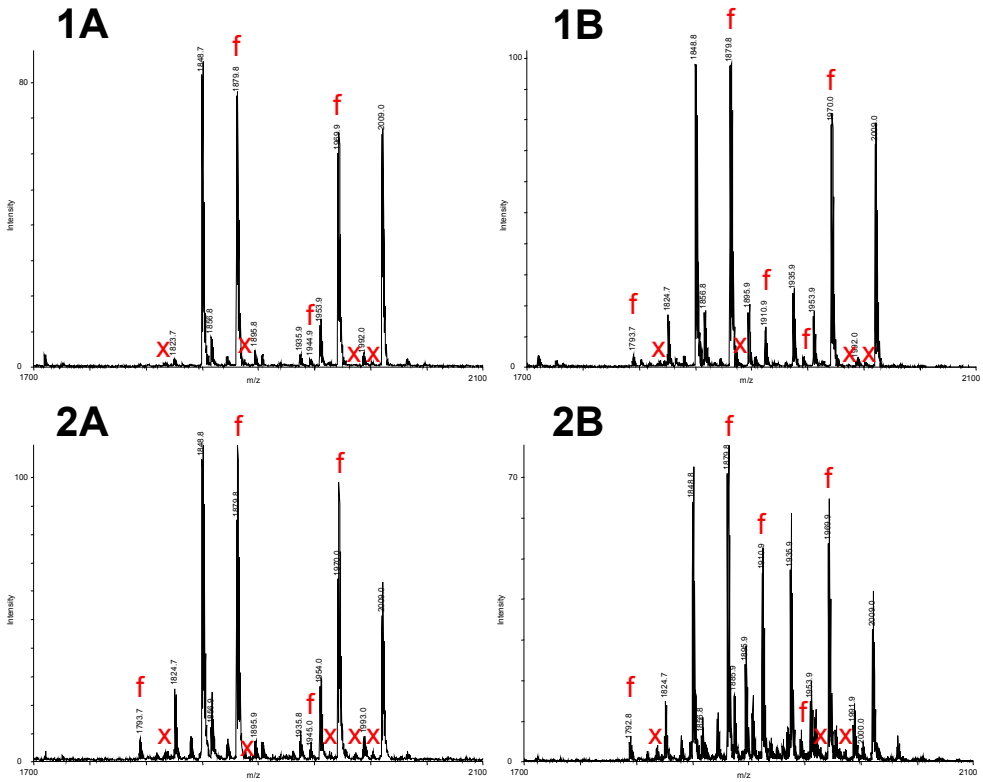

Figure 7.8 Impact of the addition of formamide on hybridization

Four genomic clones were hybridized with equimolar PNA set “6mer sub1” and varying concentrations of formamide [0%, 10%, 20%, 30% (v/v)]. Reproducible hybridization results gained with either no formamide (**A**) or 10% (v/v) formamide (**B**) added and two of the clones are depicted (**1A, 1B, 2A, 2B**). A red “f” stands for a PNA hybridized as false positive, whereas a red “x” represents a missing true positive PNA. Experimental probe masses are annotated. The composition of PNA set “6mer sub1” is given in chapter 5.7. Each PNA was applied at a concentration of 667 nM, target DNA was applied at 56 nM.

accessible to hybridization probes. This is documented by the increase of PNA signal diversity (fig. 7.8: 1B, 2B) compared to hybridization spectra obtained with no additional formamide (fig. 7.8: 1A, 2A). Although it initially appears that extra unspecificity is introduced (slight increase in false positive rate) originally missing true positive PNA probes are also gained. As a consequence, it was decided to include formamide [10% (v/v)] as standard additive for all subsequent hybridizations. Heptamers, however, did not show to be affected to the same extent by the presence of formamide, i.e. the strong melting effect observed for hexamers could not be seen.

In a further study the impact of temperature on hybridization in the presence of 10% (v/v) formamide was examined. In the range from 25° C to 50° C temperature was tested in 5° C steps. Figure 7.9 clearly demonstrates that the elevation of hybridization temperature does not lead to more specificity but to drastically deteriorated hybridization profiles, i.e. presence and respective intensity of PNA signals. At 50° C hybridization temperature, for instance, many true positive PNA probes are missing whereas three prominent false positives still persist (fig. 7.9: 1C, 2C). Similar results were obtained for heptamers which showed a slightly better hybridization reliability at elevated temperatures. Therefore, it was decided to employ 35° C as standard hybridization temperature for 6mers and 40° C as a standard for 7mers.

7.3 Multiplexed OFP analysis of selected clones of known sequence

For a comprehensive evaluation of their hybridization properties PNA hexamers and heptamers were tested in different sets as described in chapter 7.1.3. Within the course of a pilot study, a number of selected genomic as well as cDNA clones of known sequence was analyzed by means of these PNA sets to demonstrate the "proof of principle" of the concept of multiplexed OFP. Hybridizations were carried out for 1,5 h in the presence of 10% (v/v) formamide at 35° C (6mers) and 40° C (7mers). Target DNA was applied at a concentration of 56 nM, each PNA was hybridized at a concentration of 667 nM. Due to persisting PNA unspecificity results were analyzed on the basis of individual hybridization profiles as described in chapter 6.10.

7.3.1 Analysis of genomic DNA clones

31 sequence-confirmed genomic DNA clones, that are void of repeat regions, were analyzed by hybridization to prevent artifacts and hence additional sources of experimental error. These 31 clones fall into five clusters of different size and two cluster-

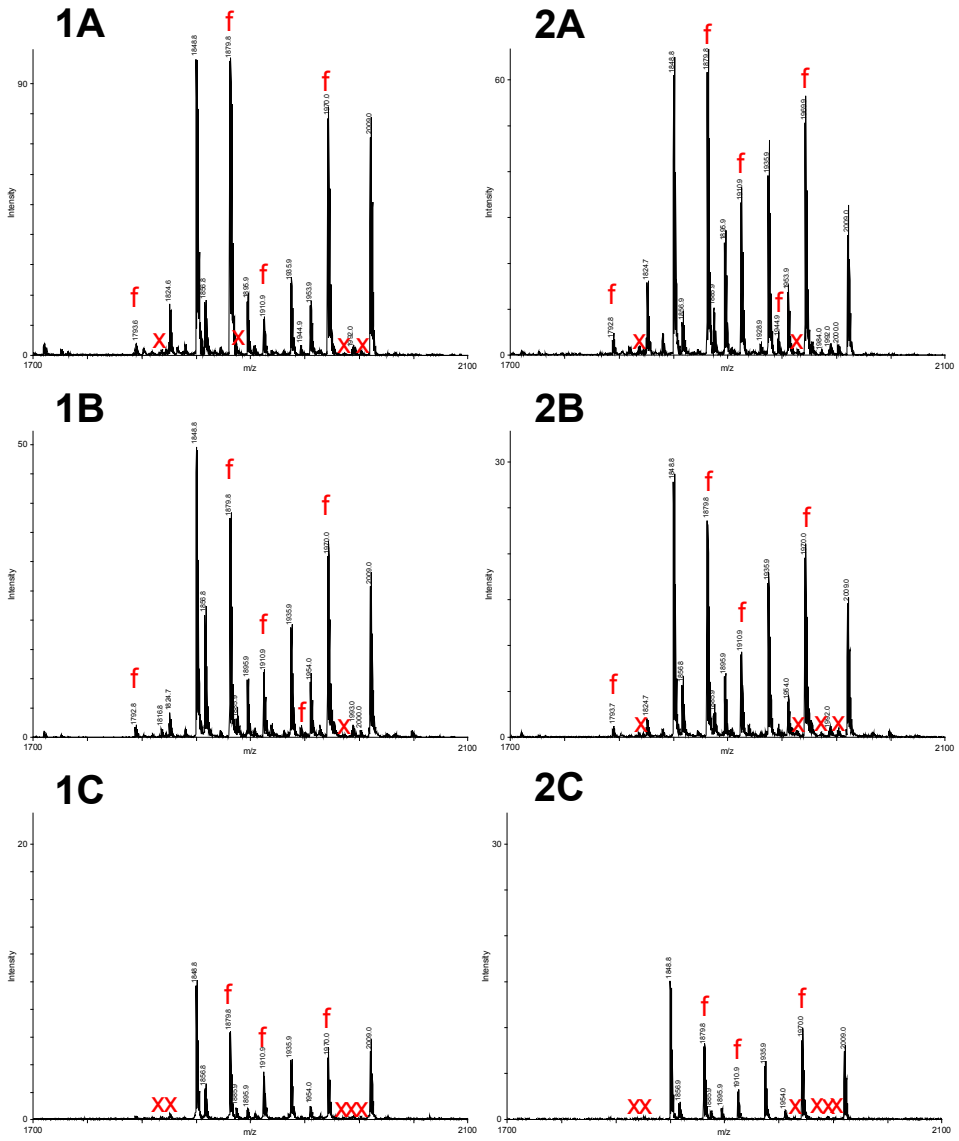

Figure 7.9 Impact of temperature on hybridization in the presence of formamide

Four genomic clones were hybridized with equimolar PNA set "6mer sub1" at varying temperatures (25° C, 30° C, 35° C, 40° C, 45° C, 50° C) in the presence of 10% (v/v) formamide. Reproducible hybridization results gained with two of the clones at 35° C (**A**), 45° C (**B**), and 50° C (**C**) hybridization temperature are depicted (**1A-C, 2A-C**). A red "f" stands for a PNA hybridized as false positive, whereas a red "x" represents a missing true positive PNA. Experimental probe masses are annotated. The composition of PNA set "6mer sub1" is given in chapter 5.7. Each PNA was applied at a concentration of 667 nM, target DNA was applied at 56 nM.

independent singletons. All clones were hybridized with the available PNA 6mer and 7mer subsets. Out of these 31 clones, 14 clones were additionally hybridized with both PNA 6mer and 7mer global set in an approach to determine the practical limit of multiplexing. Figure 7.10 shows the outcome of that approach. With either probe length no convincing data could be gained. It appears that clones belonging to the same cluster are as randomly correlated as are unrelated ones. Figure 7.11 exhibits original mass spectra of,

A

Cl. A	F21		H14		J20		O21	Cl. C	C14		M10		N07
F21	0,9667	H14	0,9623	J20	0,9829	O21	0,9091	C14	0,9696	M10	0,9641	N07	0,9914
P06	0,9632	C14	0,9331	O21	0,4086	H14	0,9088	L24	0,9560	P06	0,9501	L24	0,9733
P19	0,9551	N07	0,9297	H14	0,3890	N07	0,8978	N07	0,9472	P19	0,9493	E14	0,9527
E14	0,9454	L24	0,9227	N07	0,3836	L24	0,8972	P19	0,9453	N07	0,9440	F13	0,9477
M10	0,9344	O21	0,9088	L24	0,3601	C14	0,8964	L11	0,9399	E14	0,9405	C14	0,9472
D11	0,9321	M10	0,9058	C14	0,3483	M10	0,8746	D11	0,9390	L24	0,9381	D11	0,9450
L11	0,9209	L11	0,9042	F13	0,3328	D11	0,8705	E14	0,9388	C14	0,9370	M10	0,9440
F13	0,9196	D11	0,8898	L11	0,3290	E14	0,8620	M10	0,9370	F21	0,9344	L11	0,9423
C14	0,9132	E14	0,8819	M10	0,3267	L11	0,8572	H14	0,9331	L11	0,9339	P19	0,9410
L24	0,9059	P19	0,8781	E14	0,3253	F13	0,8476	P06	0,9264	D11	0,9337	P06	0,9322
N07	0,8955	F13	0,8644	D11	0,3212	P19	0,8468	F13	0,9185	F13	0,9231	H14	0,9297
H14	0,8415	P06	0,8524	P19	0,3084	P06	0,8286	F21	0,9132	H14	0,9058	O21	0,8978
O21	0,8198	F21	0,8415	P06	0,3033	F21	0,8198	O21	0,8964	O21	0,8746	F21	0,8955
J20	0,2787	J20	0,3890	F21	0,2787	J20	0,4086	J20	0,3483	J20	0,3267	J20	0,3836

Cl. D	D11		E14		F13		L11	Cl. E	L24		P06		P19
E14	0,9787	E14	0,9943	F13	0,9814	L11	0,9799	L24	0,9917	P06	0,9866	P19	0,9846
D11	0,9770	F13	0,9807	E14	0,9807	P19	0,9538	N07	0,9733	P19	0,9757	P06	0,9757
F13	0,9656	D11	0,9787	D11	0,9656	E14	0,9529	C14	0,9560	F21	0,9632	E14	0,9601
P19	0,9474	P06	0,9632	N07	0,9477	N07	0,9423	P19	0,9520	E14	0,9632	F21	0,9551
P06	0,9461	P19	0,9601	P06	0,9446	D11	0,9413	E14	0,9410	M10	0,9501	L11	0,9538
N07	0,9450	L11	0,9529	P19	0,9386	C14	0,9399	M10	0,9381	D11	0,9461	L24	0,9520
L11	0,9413	N07	0,9527	L11	0,9321	P06	0,9391	D11	0,9371	F13	0,9446	M10	0,9493
C14	0,9390	F21	0,9454	L24	0,9238	M10	0,9339	P06	0,9357	L11	0,9391	D11	0,9474
L24	0,9371	L24	0,9410	M10	0,9231	F13	0,9321	L11	0,9304	L24	0,9357	C14	0,9453
M10	0,9337	M10	0,9405	F21	0,9196	L24	0,9304	F13	0,9238	N07	0,9322	N07	0,9410
F21	0,9321	C14	0,9388	C14	0,9185	F21	0,9209	H14	0,9227	C14	0,9264	F13	0,9386
H14	0,8898	H14	0,8819	H14	0,8644	H14	0,9042	F21	0,9059	H14	0,8524	H14	0,8781
O21	0,8705	O21	0,8620	O21	0,8476	O21	0,8572	O21	0,8972	O21	0,8286	O21	0,8468
J20	0,3212	J20	0,3253	J20	0,3328	J20	0,3290	J20	0,3601	J20	0,3033	J20	0,3084

B

Cl. A	F21		H14		J20		O21	Cl. C	C14		M10		N07
F21	0,9903	H14	0,9683	J20	0,9820	O21	0,9616	C14	0,9928	M10	0,9730	E14	0,9132
C14	0,9815	M10	0,9299	M10	0,9503	F21	0,9419	F21	0,9815	J20	0,9503	N07	0,9126
L11	0,9713	J20	0,9291	F13	0,9370	L11	0,9391	P06	0,9650	H14	0,9299	P19	0,9003
P06	0,9682	F13	0,9287	P19	0,9353	C14	0,9190	L11	0,9536	F13	0,9074	C14	0,8859
P19	0,9476	O21	0,8973	C14	0,9345	P19	0,9178	P19	0,9464	C14	0,9000	J20	0,8852
O21	0,9419	P19	0,8732	H14	0,9291	J20	0,9176	E14	0,9420	O21	0,8906	F21	0,8814
E14	0,9316	F21	0,8656	E14	0,9287	P06	0,9147	J20	0,9345	F21	0,8876	P06	0,8786
J20	0,9260	C14	0,8566	F21	0,9260	F13	0,9028	O21	0,9190	E14	0,8807	F13	0,8694
L24	0,9034	L24	0,8535	O21	0,9176	H14	0,8973	L24	0,9107	P19	0,8801	D11	0,8571
M10	0,8876	E14	0,8514	P06	0,9069	E14	0,8921	M10	0,9000	L24	0,8667	O21	0,8413
F13	0,8872	D11	0,8501	D11	0,8961	M10	0,8906	N07	0,8859	P06	0,8607	M10	0,8300
N07	0,8814	L11	0,8454	L11	0,8901	L24	0,8815	F13	0,8852	D11	0,8579	L11	0,8278
H14	0,8656	P06	0,8392	L24	0,8893	N07	0,8413	H14	0,8566	L11	0,8514	H14	0,8272
D11	0,8379	N07	0,8272	N07	0,8852	D11	0,8304	D11	0,8523	N07	0,8300	L24	0,7898

Cl. D	D11		E14		F13		L11	Cl. E	L24		P06		P19
D11	0,9542	E14	0,9710	F13	0,9786	L11	0,9904	L24	0,9617	F21	0,9682	P19	0,9657
F13	0,9366	P19	0,9455	J20	0,9370	F21	0,9713	C14	0,9107	C14	0,9650	F21	0,9476
E14	0,9107	C14	0,9420	D11	0,9366	C14	0,9536	F21	0,9034	P06	0,9650	C14	0,9464
P19	0,8990	F21	0,9316	H14	0,9287	P06	0,9505	L11	0,8998	L11	0,9505	E14	0,9455
J20	0,8961	J20	0,9287	P19	0,9269	O21	0,9391	J20	0,8893	P19	0,9349	J20	0,9353
M10	0,8579	P06	0,9199	E14	0,9158	P19	0,9193	P19	0,8867	E14	0,9199	P06	0,9349
N07	0,8571	F13	0,9158	M10	0,9074	L24	0,8998	P06	0,8843	O21	0,9147	F13	0,9269
C14	0,8523	N07	0,9132	O21	0,9028	J20	0,8901	F13	0,8817	J20	0,9069	L11	0,9193
H14	0,8501	D11	0,9107	F21	0,8872	E14	0,8785	O21	0,8815	L24	0,8843	O21	0,9178
F21	0,8379	O21	0,8921	C14	0,8852	F13	0,8564	M10	0,8667	N07	0,8786	N07	0,9003
L24	0,8325	M10	0,8807	L24	0,8817	M10	0,8514	H14	0,8535	F13	0,8670	D11	0,8990
O21	0,8304	L11	0,8785	N07	0,8694	H14	0,8454	E14	0,8515	M10	0,8607	L24	0,8867
P06	0,8231	L24	0,8515	P06	0,8670	N07	0,8278	D11	0,8325	H14	0,8392	M10	0,8801
L11	0,7834	H14	0,8514	L11	0,8564	D11	0,7834	N07	0,7898	D11	0,8231	H14	0,8732

Figure 7.10 Correlation analysis of genomic DNA clones hybridized with global PNA sets

14 genomic DNA clones were analyzed by hybridization with PNA sets “6mer global” (**A**) and “7mer global” (**B**), respectively. Hybridization data were processed as described in chapter 6.10 using the Bruker-based macro program. For each combinatorial clone pair (clone x versus clone y), Pearson correlations were calculated. Clones are depicted in their respective cluster (Cl. A, C, D, E) and Pearson correlations of each individual clone are sorted by number. Those clones that belong to the same respective cluster are marked by green color. The clone under investigation is made stand out in black bold type. All clones possess internal tracking codes.

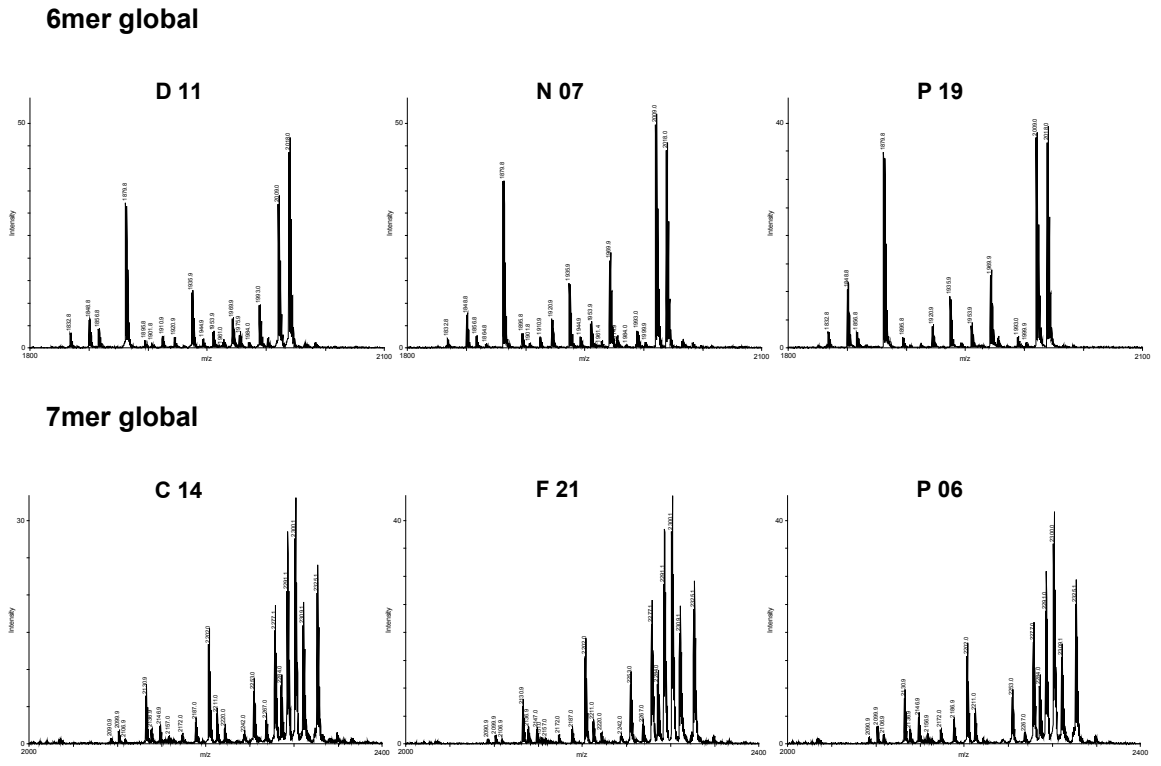

Figure 7.11 Hybridization results of selected genomic DNA clones hybridized with global PNA sets

MALDI-TOF mass spectra of each time three unrelated clones are depicted that were hybridized either with PNA set “6mer global” or PNA set “7mer global”. Experimental probe masses are annotated. Compositions of either PNA sets are given in chapter 5.7.

each time, three unrelated clones that were hybridized with either global PNA sets, respectively. It is evident that out of 40 PNA probes applied only a few distinct PNA probes show up. Furthermore, hybridization profiles are very similar rendering discrimination by profile correlation very difficult. Therefore, it can be concluded that, despite the simultaneous detection of 40 PNA hexamer and heptamer probes, a 40-plex hybridization approach does not deliver meaningful hybridization data and hence is impracticable.

Beside the evaluation of global PNA sets, less complex 6mer and 7mer subsets were tested. These comprise sets “6mer sub1” consisting of 21 PNA probes, “6mer sub2” (17 probes), “6mer sub3” (15 probes), “7mer sub1” (20 probes), and “7mer sub2” (20 probes). In general, hybridization performances of these sets were very heterogeneous, i.e. correlation analyses did not reveal the same quality of results. Figure 7.12 shows the correlation data gained for 21 genomic DNA clones of four different clusters and two independent singletons that were hybridized with PNA set “6mer sub1”. 10 clones, including an entire fifth cluster, that did not yield consistent hybridization data

Cl. A	C22		F21		G08		J06		O21		P22
C22	**0,9928**	**F21**	**0,9886**	**G08**	**0,9941**	**J06**	**0,9776**	**O21**	**0,9926**	**P22**	**0,9776**
P22	**0,9638**	**G08**	**0,9753**	**F21**	**0,9753**	**G08**	**0,9719**	**F21**	**0,9699**	**F21**	**0,9651**
O21	**0,9487**	**J06**	**0,9718**	**J06**	**0,9719**	**F21**	**0,9718**	**G08**	**0,9662**	**C22**	**0,9638**
J06	**0,9455**	**O21**	**0,9699**	A06	0,9688	**O21**	**0,9467**	**C22**	**0,9487**	**G08**	**0,9634**
F21	**0,9442**	**P22**	**0,9651**	**O21**	**0,9662**	**C22**	**0,9455**	**J06**	**0,9467**	**O21**	**0,9345**
G08	**0,9420**	**C22**	**0,9442**	**P22**	**0,9634**	**P22**	**0,9293**	**P22**	**0,9345**	**J06**	**0,9293**
C03	0,9328	A06	0,9195	K04	0,9467	K04	0,8753	A06	0,8741	P19	0,8770
A06	0,9099	K04	0,9096	**C22**	**0,9420**	A06	0,8737	K04	0,8658	P06	0,8762
L24	0,8986	L24	0,9044	L24	0,9188	K18	0,8646	P19	0,8557	L11	0,7833
K04	0,8769	C03	0,8857	L11	0,8998	C03	0,8598	L24	0,8549	E14	0,7790
K18	0,8754	P19	0,8787	C03	0,8841	P06	0,8472	C03	0,8434	E08	0,7676
P19	0,8703	P06	0,8770	E08	0,8806	P19	0,8435	P06	0,8216	D11	0,7456
P06	0,8682	K18	0,8752	P19	0,8772	L24	0,8378	K18	0,8214	O12	0,7364
E08	0,8495	L11	0,8580	P06	0,8598	L11	0,8271	L11	0,7892	F13	0,7170
A11	0,8122	E08	0,8550	A11	0,8509	E08	0,8033	D11	0,7859	A11	0,7015
L11	0,7818	A11	0,8332	E14	0,8463	E14	0,7837	E08	0,7857	K04	0,6970
D11	0,7508	D11	0,8082	D11	0,8402	A11	0,7824	A11	0,7653	B03	0,6785
E14	0,7346	E14	0,8034	K18	0,8239	D11	0,7697	E14	0,7145	A06	0,6722
O12	0,6589	F13	0,6836	B03	0,7192	O12	0,7079	O12	0,6749	L24	0,6310
F13	0,6568	B03	0,6606	F13	0,7001	F13	0,6668	B03	0,6035	C03	0,6021
B03	0,6097	O12	0,5888	O12	0,6356	B03	0,6367	F13	0,5979	K18	0,5999

Cl. C	D11		E14		F13		L11		O12
D11	**0,9882**	**E14**	**0,9781**	**F13**	**0,9873**	**L11**	**0,9882**	**O12**	**0,9808**
L11	**0,9561**	**L11**	**0,9645**	**O12**	**0,9271**	**E14**	**0,9645**	**D11**	**0,9326**
E14	**0,9347**	**D11**	**0,9347**	**D11**	**0,9230**	**D11**	**0,9561**	**E14**	**0,9315**
O12	**0,9326**	**O12**	**0,9315**	**E14**	**0,9148**	A06	0,9137	**F13**	**0,9271**
F13	**0,9230**	**F13**	**0,9148**	**L11**	**0,9051**	**O12**	**0,9122**	**L11**	**0,9122**
E08	0,8827	E08	0,8812	E08	0,8778	**F13**	**0,9051**	E08	0,7913
K04	0,8739	K04	0,8667	B03	0,8272	G08	0,8998	K04	0,7701
A06	0,8593	A11	0,8574	A11	0,8060	K04	0,8975	B03	0,7568
A11	0,8431	A06	0,8522	P19	0,7725	E08	0,8908	A11	0,7551
G08	0,8402	G08	0,8463	K04	0,7666	A11	0,8765	P19	0,7493
K18	0,8311	P06	0,8337	C03	0,7624	K18	0,8732	A06	0,7374
P19	0,8224	K18	0,8284	A06	0,7540	F21	0,8580	P22	0,7364
B03	0,8189	F21	0,8034	L24	0,7352	P06	0,8524	L24	0,7217
F21	0,8082	B03	0,7971	P06	0,7311	C03	0,8448	J06	0,7079
L24	0,7982	C03	0,7844	K18	0,7303	P19	0,8365	K18	0,6982
C03	0,7918	P19	0,7842	P22	0,7170	J06	0,8271	C03	0,6930
P06	0,7886	J06	0,7837	G08	0,7001	L24	0,8058	O21	0,6749
O21	0,7859	P22	0,7790	F21	0,6836	B03	0,7943	P06	0,6705
J06	0,7697	L24	0,7682	J06	0,6668	O21	0,7892	C22	0,6589
C22	0,7508	C22	0,7346	C22	0,6568	P22	0,7833	G08	0,6356
P22	0,7456	O21	0,7145	O21	0,5979	C22	0,7818	F21	0,5888

Cl. B	A06		A11		C03		K04		K18
A06	**0,9935**	**A11**	**0,9892**	**C03**	**0,9980**	**K04**	**0,9920**	**K18**	**0,9977**
K18	**0,9892**	**K18**	**0,9645**	**A06**	**0,9790**	**A06**	**0,9702**	**A06**	**0,9892**
C03	**0,9790**	**A06**	**0,9616**	**K04**	**0,9506**	**K18**	**0,9613**	**A11**	**0,9645**
K04	**0,9702**	E08	0,9458	**K18**	**0,9362**	**C03**	**0,9506**	**K04**	**0,9613**
G08	0,9688	**K04**	**0,9447**	C22	0,9328	G08	0,9467	**C03**	**0,9362**
A11	**0,9616**	**C03**	**0,9207**	**A11**	**0,9207**	**A11**	**0,9447**	P06	0,8992
E08	0,9333	L11	0,8765	P19	0,9040	F21	0,9096	C22	0,8754
F21	0,9195	L24	0,8621	P06	0,9024	P19	0,9031	F21	0,8752
P19	0,9174	E14	0,8574	E08	0,8934	L11	0,8975	L11	0,8732
L11	0,9137	G08	0,8509	F21	0,8857	E08	0,8968	J06	0,8646
C22	0,9099	P19	0,8483	G08	0,8841	L24	0,8964	P19	0,8571
L24	0,9094	D11	0,8431	L24	0,8673	C22	0,8769	L24	0,8496
P06	0,8815	P06	0,8400	J06	0,8598	J06	0,8753	D11	0,8311
O21	0,8741	F21	0,8332	L11	0,8448	D11	0,8739	E14	0,8284
J06	0,8737	C22	0,8122	O21	0,8434	E14	0,8667	G08	0,8239
D11	0,8593	B03	0,8106	D11	0,7918	O21	0,8658	O21	0,8214
E14	0,8522	F13	0,8060	E14	0,7844	P06	0,8496	E08	0,8076
F13	0,7540	J06	0,7824	F13	0,7624	B03	0,7749	F13	0,7303
O12	0,7374	O21	0,7653	O12	0,6930	O12	0,7701	O12	0,6982
B03	0,7303	O12	0,7551	B03	0,6842	F13	0,7666	B03	0,6938
P22	0,6722	P22	0,7015	P22	0,6021	P22	0,6970	P22	0,5999

Cl. D	L24		P06		P19
L24	**0,9904**	**P06**	**0,9902**	**P19**	**0,9905**
P06	**0,9414**	**P19**	**0,9738**	**P06**	**0,9738**
P19	**0,9402**	**L24**	**0,9414**	**L24**	**0,9402**
G08	0,9188	C03	0,9024	A06	0,9174
A06	0,9094	K18	0,8992	C03	0,9040
F21	0,9044	A06	0,8815	K04	0,9031
C22	0,8986	F21	0,8770	F21	0,8787
K04	0,8964	P22	0,8762	G08	0,8772
E08	0,8695	E08	0,8717	P22	0,8770
C03	0,8673	C22	0,8682	C22	0,8703
A11	0,8621	G08	0,8598	K18	0,8571
O21	0,8549	L11	0,8524	O21	0,8557
K18	0,8496	K04	0,8496	A11	0,8483
J06	0,8378	J06	0,8472	J06	0,8435
L11	0,8058	A11	0,8400	L11	0,8365
D11	0,7982	E14	0,8337	D11	0,8224
E14	0,7682	O21	0,8216	E08	0,8198
F13	0,7352	D11	0,7886	E14	0,7842
B03	0,7315	F13	0,7311	F13	0,7725
O12	0,7217	B03	0,6953	O12	0,7493
P22	0,6310	O12	0,6705	B03	0,7181

singl.	B03	*singl.*	E08
B03	**0,9965**	**E08**	**0,9908**
E08	0,8467	A11	0,9458
F13	0,8272	A06	0,9333
D11	0,8189	K04	0,8968
A11	0,8106	C03	0,8934
E14	0,7971	L11	0,8908
L11	0,7943	D11	0,8827
K04	0,7749	E14	0,8812
O12	0,7568	G08	0,8806
L24	0,7315	F13	0,8778
A06	0,7303	P06	0,8717
G08	0,7192	L24	0,8695
P19	0,7181	F21	0,8550
P06	0,6953	C22	0,8495
K18	0,6938	B03	0,8467
C03	0,6842	P19	0,8198
P22	0,6785	K18	0,8076
F21	0,6606	J06	0,8033
J06	0,6367	O12	0,7913
C22	0,6097	O21	0,7857
O21	0,6035	P22	0,7676

Figure 7.12 Correlation analysis of genomic DNA clones hybridized with PNA set “6mer sub1”

Genomic DNA clones were hybridized with PNA set “6mer sub1”. Hybridization data were processed and Pearson correlations were calculated as described earlier. Clones are depicted in their respective cluster (Cl. A, B, C, D) or as cluster-independent singleton (singl.). Those clones that belong to the same respective cluster are marked by green color. The clone under investigation is made stand out in black bold type. All clones possess internal tracking codes.

were excluded from the analysis. This was done to avoid a subsequent impairment of the overall analysis. The figure demonstrates that - apart from a very few exceptions - related clones of a respective cluster are grouped together and are completely separated from unrelated ones although the numeric difference to those unrelated clones is mostly rather small (< Δ 0,03). For some clones, though, such as O21, P22, or O12, the correlation difference achieved is much higher as is for singletons B03 and E08 (Δ 0,04-0,15). Figure 7.13 exhibits MALDI-TOF mass spectra of six genomic DNA clones that were hybridized with PNA set “6mer sub1”: two clone pairs from two different clusters and two singletons. The figure clearly shows that at least for the clones presented even a visual discrimination would be possible confirming the high performance of PNA set “6mer sub1” in distinguishing clones on the basis of hybridization profiles.

In contrast to PNA set “6mer sub1” hybridization performances of the other two 6mer as well as the 7mer subsets were less satisfactory. Figure 7.14 shows the correlation data gained for 20 genomic DNA clones of four different clusters and two independent

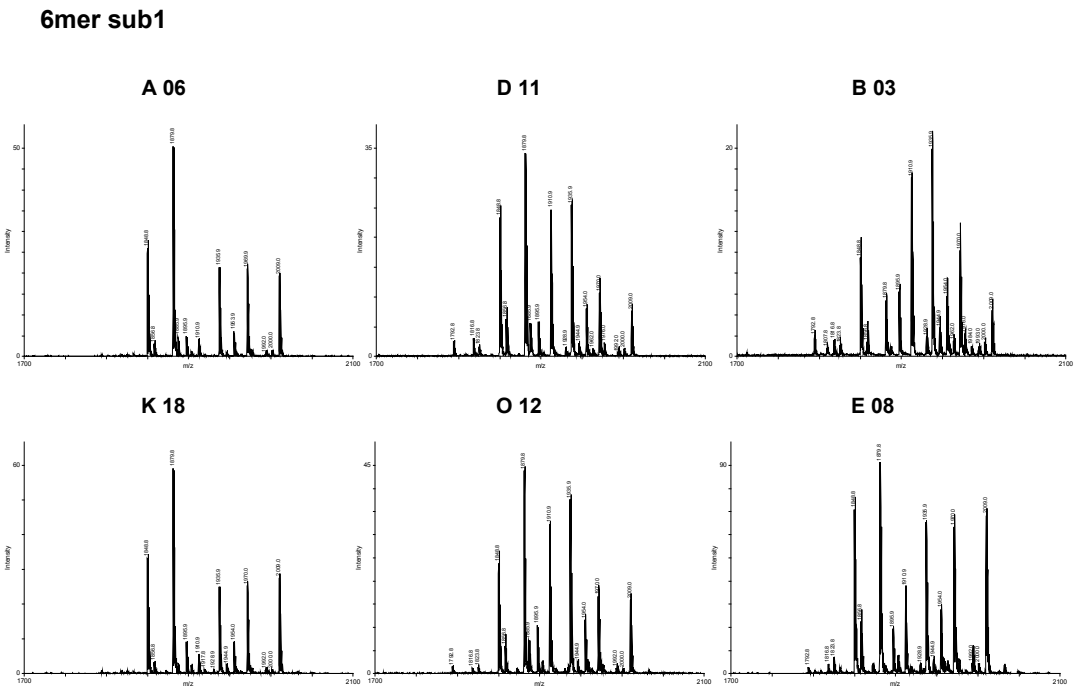

Figure 7.13 Hybridization results of selected genomic DNA clones hybridized with PNA set "6mer sub1"

MALDI-TOF mass spectra of six genomic DNA clones are depicted that were hybridized with PNA set "6mer sub1". A06 and K18 belong to one cluster, D11 and O12 to another one. B03 and E08 are singletons. Experimental probe masses are annotated. The composition of the PNA set is given in chapter 5.7

singletons that were hybridized with PNA set "6mer sub2". To avoid a subsequent impairment of the overall analysis, 11 clones that did not yield consistent hybridization data were again excluded from the analysis The figure clearly shows the inferior performance compared to set "6mer sub1". Related clones of a given cluster are hardly separated from unrelated ones and, if so, numeric correlation differences are negligible. However, for a few clones, such as A11, C03, and singletons B03 and E08, the discrimination achieved is better. Furthermore, a general trend towards correct discrimination is still discernible. As for the remaining PNA sets, set "6mer sub3" and "7mer sub1" yielded correlation data of comparable modest quality whereas PNA set "7mer sub2" failed to generate consistent data at all.

7.3.2 Analysis of cDNA clones

21 sequence-confirmed cDNA clones, that are void of repeat regions, were analyzed by hybridization to prevent artifacts and hence additional sources of experimental error.

Cl. A	C22		F21		H14		J20		O21
C22	**0,9927**	**F21**	**0,9968**	**F21**	**0,9865**	**J20**	**0,9924**	**O21**	**0,9895**
F21	**0,9893**	M10	0,9895	**H14**	**0,9839**	**F21**	**0,9819**	**C22**	**0,9877**
O21	**0,9877**	**C22**	**0,9893**	M10	0,9834	K04	0,9794	**F21**	**0,9837**
M10	0,9817	J19	0,9866	J19	0,9818	**C22**	**0,9726**	**H14**	**0,9748**
H14	**0,9804**	**H14**	**0,9865**	**C22**	**0,9804**	**H14**	**0,9705**	M10	0,9744
J19	0,9763	**O21**	**0,9837**	C14	0,9775	M10	0,9694	**J20**	**0,9667**
C14	0,9731	**J20**	**0,9819**	**O21**	**0,9748**	K18	0,9668	J19	0,9648
J20	**0,9726**	C14	0,9806	**J20**	**0,9705**	J19	0,9667	C14	0,9648
L11	0,9663	H07	0,9714	L11	0,9692	**O21**	**0,9667**	L11	0,9578
H07	0,9598	O12	0,9702	H07	0,9689	C14	0,9622	K04	0,9543
K04	0,9587	L11	0,9679	O12	0,9686	H07	0,9593	H07	0,9482
O12	0,9587	K04	0,9662	K04	0,9535	O12	0,9526	O12	0,9454
N21	0,9428	D11	0,9496	N21	0,9519	A11	0,9464	N21	0,9306
K18	0,9306	N21	0,9489	D11	0,9498	D11	0,9356	K18	0,9237
D11	0,9299	K18	0,9435	P06	0,9321	L11	0,9287	D11	0,9128
P06	0,9143	P06	0,9265	K18	0,9280	C03	0,9104	A11	0,9066
A11	0,9076	A11	0,9159	A11	0,8995	N21	0,9099	P06	0,8997
C03	0,8537	E08	0,8693	E08	0,8625	E08	0,8977	C03	0,8616
E08	0,8337	C03	0,8687	C03	0,8451	P06	0,8769	E08	0,8116
B03	0,8016	B03	0,8101	B03	0,8117	B03	0,8291	B03	0,8048

Cl. B	A11		C03		K04		K18	*singl.*	B03
A11	**0,9910**	**C03**	**0,9702**	**K04**	**0,9946**	**K18**	**0,9938**	**B03**	**0,9350**
K18	**0,9785**	**K04**	**0,9690**	**K18**	**0,9872**	**K04**	**0,9872**	D11	0,8695
K04	**0,9727**	**A11**	**0,9580**	J20	0,9794	**A11**	**0,9785**	E08	0,8434
C03	**0,9580**	**K18**	**0,9488**	**A11**	**0,9727**	J20	0,9668	L11	0,8348
J20	0,9464	J20	0,9104	**C03**	**0,9690**	**C03**	**0,9488**	C14	0,8320
F21	0,9159	F21	0,8687	F21	0,9662	F21	0,9435	H07	0,8291
C22	0,9076	O21	0,8616	C22	0,9587	C22	0,9306	J20	0,8291
O21	0,9066	C22	0,8537	O21	0,9543	M10	0,9289	N21	0,8254
H14	0,8995	H14	0,8451	M10	0,9538	H14	0,9280	P06	0,8223
M10	0,8942	M10	0,8383	H14	0,9535	J19	0,9270	J19	0,8216
J19	0,8870	C14	0,8301	J19	0,9515	O21	0,9237	O12	0,8213
H07	0,8819	H07	0,8214	H07	0,9441	H07	0,9227	M10	0,8170
C14	0,8816	J19	0,8184	C14	0,9416	C14	0,9187	H14	0,8117
O12	0,8709	E08	0,8090	O12	0,9367	O12	0,9126	F21	0,8101
E08	0,8639	O12	0,8010	D11	0,9042	E08	0,9115	O21	0,8048
D11	0,8394	P06	0,7886	L11	0,8960	D11	0,8847	C22	0,8016
L11	0,8123	D11	0,7811	E08	0,8952	L11	0,8558	K18	0,7985
N21	0,7806	N21	0,7716	N21	0,8743	N21	0,8348	K04	0,7938
B03	0,7752	L11	0,7576	P06	0,8347	B03	0,7985	A11	0,7752
P06	0,7351	B03	0,7311	B03	0,7938	P06	0,7954	C03	0,7311

Cl. C	D11		L11		N21		O12
D11	**0,9897**	**L11**	**0,9993**	**N21**	**0,9939**	J19	0,9884
O12	**0,9743**	**N21**	**0,9901**	**L11**	**0,9901**	**O12**	**0,9870**
J19	0,9731	P06	0,9819	P06	0,9869	M10	0,9787
N21	**0,9667**	J19	0,9801	J19	0,9727	H07	0,9783
H07	0,9656	M10	0,9769	**O12**	**0,9669**	C14	0,9778
C14	0,9638	C14	0,9756	**D11**	**0,9667**	**D11**	**0,9743**
M10	0,9592	**O12**	**0,9715**	C14	0,9653	**L11**	**0,9715**
P06	0,9590	H14	0,9692	M10	0,9632	F21	0,9702
L11	**0,9583**	F21	0,9679	H07	0,9567	H14	0,9686
H14	0,9498	C22	0,9663	H14	0,9519	**N21**	**0,9669**
F21	0,9496	H07	0,9636	F21	0,9489	C22	0,9587
J20	0,9356	**D11**	**0,9583**	C22	0,9428	P06	0,9559
C22	0,9299	O21	0,9578	O21	0,9306	J20	0,9526
E08	0,9209	J20	0,9287	J20	0,9099	O21	0,9454
O21	0,9128	K04	0,8960	K04	0,8743	K04	0,9367
K04	0,9042	K18	0,8558	E08	0,8368	K18	0,9126
K18	0,8847	B03	0,8348	K18	0,8348	E08	0,9028
B03	0,8695	E08	0,8187	B03	0,8254	A11	0,8709
A11	0,8394	A11	0,8123	A11	0,7806	B03	0,8213
C03	0,7811	C03	0,7576	C03	0,7716	C03	0,8010

Cl. E	C14		H07		J19		M10	*singl.*	E08
J19	**0,9869**	**J19**	**0,9860**	**J19**	**0,9980**	**M10**	**0,9919**	**E08**	**0,9908**
M10	**0,9835**	**H07**	**0,9820**	**M10**	**0,9915**	**J19**	**0,9915**	D11	0,9209
C14	**0,9829**	O12	0,9783	O12	0,9884	F21	0,9895	K18	0,9115
F21	0,9806	**M10**	**0,9779**	**C14**	**0,9869**	**C14**	**0,9835**	O12	0,9028
O12	0,9778	**C14**	**0,9752**	F21	0,9866	H14	0,9834	H07	0,8982
H14	0,9775	F21	0,9714	**H07**	**0,9860**	C22	0,9817	J20	0,8977
L11	0,9756	H14	0,9689	H14	0,9818	O12	0,9787	J19	0,8957
H07	**0,9752**	D11	0,9656	L11	0,9801	**H07**	**0,9779**	K04	0,8952
C22	0,9731	L11	0,9636	C22	0,9763	L11	0,9769	C14	0,8774
N21	0,9653	C22	0,9598	D11	0,9731	O21	0,9744	M10	0,8720
O21	0,9648	J20	0,9593	N21	0,9727	J20	0,9694	F21	0,8693
D11	0,9638	N21	0,9567	J20	0,9667	N21	0,9632	A11	0,8639
J20	0,9622	O21	0,9482	O21	0,9648	D11	0,9592	H14	0,8625
P06	0,9503	K04	0,9441	P06	0,9582	K04	0,9538	B03	0,8434
K04	0,9416	P06	0,9411	K04	0,9515	P06	0,9455	N21	0,8368
K18	0,9187	K18	0,9227	K18	0,9270	K18	0,9289	C22	0,8337
A11	0,8816	E08	0,8982	E08	0,8957	A11	0,8942	P06	0,8190
E08	0,8774	A11	0,8819	A11	0,8870	E08	0,8720	L11	0,8187
B03	0,8320	B03	0,8291	B03	0,8216	C03	0,8383	O21	0,8116
C03	0,8301	C03	0,8214	C03	0,8184	B03	0,8170	C03	0,8090

Figure 7.14 Correlation analysis of genomic DNA clones hybridized with PNA set "6mer sub2"

Genomic DNA clones were hybridized with PNA set "6mer sub2". Hybridization data were processed and Pearson correlations were calculated as described earlier. Clones are depicted in their respective cluster (Cl. A, B, C, E) or as cluster-independent singleton (singl.). Those clones that belong to the same respective cluster are marked by green color. The clone under investigation is made stand out in black bold type. All clones possess internal tracking codes.

These 21 clones fall into five clusters of different size. In addition, 8 shorter PCR fragments of four of these clones were generated to yield a total of 29 clones to be analyzed. Analogous to genomic DNA clones, all clones were hybridized with the available PNA 6mer and 7mer subsets which, in turn, revealed very heterogeneous hybridization performances. Figure 7.15 shows the correlation data gained for 23 cDNA clones of five different clusters that were hybridized with PNA set "6mer sub1". 6 clones that did not yield consistent hybridization results were excluded from the analysis to avoid a subsequent impairment of the overall analysis. The figure clearly shows that –as for genomic DNA clones and apart from a very few exceptions - related clones of a respective cluster are grouped together and are completely separated from unrelated ones. Numeric differences to unrelated clones are mostly rather small though (< Δ 0,03). For some clones, however, such as H182, I151, or K022, the correlation difference achieved is much higher (Δ 0,06-0,12). As in the case of genomic DNA clones, the high performance of PNA set "6mer sub1" in distinguishing clones was also confirmed by original spectra.

Cl. 1	H109		H182		H182a		H182b		I151		P156
H109	**0,9918**	H182b	0,9765	H182a	0,9953	H182b	0,9927	I151	0,9713	P156	0,9811
P156	**0,9732**	**H182**	**0,9727**	H182b	0,9716	H182	0,9765	P156	0,9489	H109	0,9732
I151	**0,9430**	H182a	0,9562	H182	0,9562	H182a	0,9716	H109	0,9430	I151	0,9489
H182	**0,9274**	P156	0,9299	P156	0,9241	P156	0,9295	H182	0,9291	H182	0,9299
H182a	**0,9222**	I151	0,9291	H109	0,9222	H109	0,9213	H182b	0,9005	H182b	0,9295
H182b	**0,9213**	H109	0,9274	A071a	0,9187	A071a	0,9101	H182a	0,8992	H182a	0,9241
A071	0,8848	A071b	0,8061	K022a	0,9006	I051	0,9021	N158	0,8094	P091	0,9117
F231	0,8722	A071a	0,8015	I051	0,8994	K022b	0,9008	A071a	0,7880	N158	0,9090
N158	0,8602	N178	0,7851	I151	0,8992	I151	0,9005	P091	0,7718	F231	0,9032
A071b	0,8588	H035	0,7664	K022b	0,8909	K022a	0,9002	M098	0,7638	C017	0,8901
H035	0,8576	A071	0,7445	B112	0,8878	H035	0,8885	I051	0,7635	B112	0,8897
C017	0,8540	M098	0,7274	N158	0,8838	B112	0,8883	N178	0,7570	H035	0,8860
B112	0,8450	I051	0,7259	N063	0,8831	F231	0,8832	H035	0,7502	A071a	0,8807
P091	0,8425	N158	0,7229	H035	0,8771	N063	0,8780	K022a	0,7433	K022b	0,8806
A071a	0,8362	K022	0,7079	F231	0,8748	N158	0,8775	K022	0,7424	D178	0,8766
M098	0,8336	P091	0,7053	C017	0,8726	N178	0,8743	B112	0,7313	K022a	0,8764
K022b	0,8332	B112	0,7006	M098	0,8701	C017	0,8692	A071b	0,7211	I051	0,8729
D178	0,8216	K022a	0,6771	D178	0,8671	K022	0,8650	F231	0,6824	M098	0,8648
K022a	0,8184	D178	0,6670	N178	0,8591	M098	0,8576	C017	0,6761	N063	0,8540
N178	0,8149	C017	0,6651	A071b	0,8567	D178	0,8572	K022b	0,6755	A071	0,8537
N063	0,7799	F231	0,6574	K022	0,8529	P091	0,8550	A071	0,6563	N178	0,8533
I051	0,7740	N063	0,6519	P091	0,8429	A071b	0,8318	D178	0,6469	A071b	0,8383
K022	0,7245	K022b	0,6393	A071	0,8418	A071	0,8075	N063	0,6304	K022	0,7781

Cl. 5	B112		C017		F231		N158		P091
B112	**0,9863**	**C017**	**0,9971**	**F231**	**0,9955**	**N158**	**0,9958**	**P091**	**0,9854**
F231	**0,9788**	**F231**	**0,9914**	**C017**	**0,9914**	**P091**	**0,9727**	**F231**	**0,9727**
C017	**0,9748**	**B112**	**0,9748**	**B112**	**0,9788**	**F231**	**0,9723**	**N158**	**0,9727**
N158	**0,9685**	**N158**	**0,9732**	**P091**	**0,9727**	**B112**	**0,9685**	**B112**	**0,9675**
P091	**0,9675**	**P091**	**0,9665**	**N158**	**0,9723**	**C017**	**0,9642**	**C017**	**0,9665**
K022b	0,9451	N178	0,9642	H035	0,9466	I051	0,9341	P156	0,9117
I051	0,9379	H035	0,9454	K022b	0,9419	K022a	0,9171	K022b	0,8982
A071a	0,9315	A071a	0,9453	A071a	0,9393	K022b	0,9151	I051	0,8935
H035	0,9232	I051	0,9248	N178	0,9388	A071a	0,9115	A071a	0,8890
K022a	0,9219	K022b	0,9214	D178	0,9201	P156	0,9090	D178	0,8877
D178	0,9146	A071b	0,9177	I051	0,9190	H035	0,9079	N178	0,8872
M098	0,9102	M098	0,9157	A071b	0,9134	N063	0,9077	H035	0,8834
N178	0,9096	D178	0,9147	A071	0,9122	H182a	0,8838	K022a	0,8788
N063	0,8967	A071	0,9123	K022a	0,9103	D178	0,8809	N063	0,8771
A071	0,8944	K022a	0,9039	M098	0,9058	H182b	0,8775	M098	0,8702
P156	0,8897	N063	0,8957	P156	0,9032	N178	0,8708	H182b	0,8550
H182b	0,8883	P156	0,8901	N063	0,8922	M098	0,8703	A071	0,8501
H182a	0,8878	H182a	0,8726	H182b	0,8832	H109	0,8602	H182a	0,8429
A071b	0,8815	H182b	0,8692	H182a	0,8748	A071b	0,8570	H109	0,8425
H109	0,8450	H109	0,8540	H109	0,8722	A071	0,8428	A071b	0,8308
K022	0,7734	K022	0,7204	K022	0,7649	K022	0,8252	K022	0,7963
I151	0,7313	I151	0,6761	I151	0,6824	I151	0,8094	I151	0,7718
H182	0,7006	H182	0,6651	H182	0,6574	H182	0,7229	H182	0,7053

Cl. 2	A071		A071a		A071b		M098
A071	**0,9903**	A071a	0,9926	A071b	0,9903	M098	0,9796
A071b	**0,9785**	M098	0,9717	A071	0,9785	A071a	0,9717
A071a	**0,9526**	A071	0,9526	A071a	0,9512	A071	0,9481
M098	**0,9481**	A071b	0,9512	M098	0,9480	A071b	0,9480
C017	0,9123	I051	0,9457	C017	0,9177	C017	0,9157
F231	0,9122	C017	0,9453	F231	0,9134	B112	0,9102
H035	0,8981	F231	0,9393	H035	0,8957	F231	0,9058
B112	0,8944	N178	0,9317	N178	0,8826	I051	0,8811
H109	0,8848	B112	0,9315	B112	0,8815	N178	0,8783
N178	0,8828	K022a	0,9242	K022b	0,8804	K022b	0,8771
K022b	0,8794	H182a	0,9187	D178	0,8729	N158	0,8703
D178	0,8692	H035	0,9166	K022a	0,8675	P091	0,8702
K022a	0,8653	N158	0,9115	I051	0,8633	H182a	0,8701
P156	0,8537	K022b	0,9109	H109	0,8588	H035	0,8692
P091	0,8501	H182b	0,9101	N158	0,8570	P156	0,8648
I051	0,8488	P091	0,8890	H182a	0,8567	K022a	0,8631
N063	0,8434	N063	0,8818	N063	0,8486	H182b	0,8576
N158	0,8428	P156	0,8807	P156	0,8383	N063	0,8510
H182a	0,8418	D178	0,8767	H182b	0,8318	D178	0,8416
H182b	0,8075	K022	0,8480	P091	0,8308	H109	0,8336
H182	0,7445	H109	0,8362	H182	0,8061	K022	0,7914
K022	0,7303	H182	0,8015	K022	0,7638	I151	0,7638
I151	0,6563	I151	0,7880	I151	0,7211	H182	0,7274

Cl. 3	K022		K022a		K022b		N178
K022	**0,9777**	K022a	0,9810	K022b	0,9890	N178	0,9963
K022a	**0,9424**	K022b	0,9523	K022a	0,9523	C017	0,9732
K022b	**0,9352**	K022	0,9424	B112	0,9451	K022b	0,9426
N178	**0,9277**	N178	0,9397	N178	0,9426	K022a	0,9397
H182b	0,8650	I051	0,9338	F231	0,9419	F231	0,9388
H182a	0,8529	N063	0,9295	K022	0,9352	A071a	0,9317
A071a	0,8480	A071a	0,9242	C017	0,9214	K022	0,9277
I051	0,8388	H035	0,9235	N158	0,9151	B112	0,9096
N158	0,8252	B112	0,9219	N063	0,9120	H035	0,8982
H035	0,8105	N158	0,9171	D178	0,9120	I051	0,8925
P091	0,7963	D178	0,9133	A071a	0,9109	P091	0,8872
M098	0,7914	F231	0,9103	I051	0,9097	A071	0,8828
P156	0,7781	C017	0,9039	H035	0,9033	A071b	0,8826
B112	0,7734	H182a	0,9006	H182b	0,9008	M098	0,8783
F231	0,7649	H182b	0,9002	P091	0,8982	H182b	0,8743
A071b	0,7638	P091	0,8788	H182a	0,8909	N158	0,8708
I151	0,7424	P156	0,8764	P156	0,8806	N063	0,8632
N063	0,7331	A071b	0,8675	A071b	0,8804	D178	0,8596
A071	0,7303	A071	0,8653	A071	0,8794	H182a	0,8591
H109	0,7245	M098	0,8631	M098	0,8771	P156	0,8533
C017	0,7204	H109	0,8184	H109	0,8332	H109	0,8149
D178	0,7203	I151	0,7433	I151	0,6755	H182	0,7851
H182	0,7079	H182	0,6771	H182	0,6393	I151	0,7570

Cl. 4	D178		H035		I051		N063
D178	**0,9953**	**H035**	**0,9914**	**I051**	**0,9929**	**N063**	**0,9929**
H035	**0,9672**	**D178**	**0,9672**	**N063**	**0,9794**	**I051**	**0,9794**
N063	**0,9641**	**N063**	**0,9618**	**D178**	**0,9602**	**D178**	**0,9641**
I051	**0,9602**	**I051**	**0,9597**	**H035**	**0,9597**	**H035**	**0,9618**
F231	0,9201	F231	0,9466	A071a	0,9457	K022a	0,9295
C017	0,9147	C017	0,9454	B112	0,9379	K022b	0,9120
B112	0,9146	K022a	0,9235	N158	0,9341	N158	0,9077
K022a	0,9133	B112	0,9232	K022a	0,9338	B112	0,8967
K022b	0,9120	A071a	0,9166	C017	0,9248	C017	0,8957
P091	0,8877	N158	0,9079	F231	0,9190	F231	0,8922
N158	0,8809	K022b	0,9033	K022b	0,9097	H182a	0,8831
A071a	0,8767	N178	0,8982	H182b	0,9021	A071a	0,8818
P156	0,8766	A071	0,8981	H182a	0,8994	H182b	0,8780
A071b	0,8729	A071b	0,8957	P091	0,8935	P091	0,8771
A071	0,8692	H182b	0,8885	N178	0,8925	N178	0,8632
H182a	0,8671	P156	0,8860	M098	0,8811	P156	0,8540
N178	0,8596	P091	0,8834	P156	0,8729	M098	0,8510
H182b	0,8572	H182a	0,8771	A071b	0,8633	A071b	0,8486
M098	0,8416	M098	0,8692	A071	0,8488	A071	0,8434
H109	0,8216	H109	0,8576	K022	0,8388	H109	0,7799
K022	0,7203	K022	0,8105	H109	0,7740	K022	0,7331
H182	0,6670	H182	0,7664	I151	0,7635	H182	0,6519
I151	0,6469	I151	0,7502	H182	0,7259	I151	0,6304

Figure 7.15 Correlation analysis of cDNA clones hybridized with PNA set "6mer sub1"

cDNA clones were hybridized with PNA set "6mer sub1". Hybridization data were processed and Pearson correlations were calculated as described earlier. Clones are depicted in their respective cluster (Cl. 1, 2, 3, 4, 5). Those clones that belong to the same respective cluster are marked by green color. The clone under investigation is made stand out in black bold type. All clones possess internal tracking codes. Shorter PCR fragments of original clones are indicated by codes with lower case letters "a" and "b".

Figure 7.16 exhibits MALDI-TOF mass spectra of six cDNA clones that were hybridized with PNA set "6mer sub1": three clone pairs from three different clusters. The figure visually supports the successful clone discrimination.

Similar to genomic DNA clones, the other two 6mer as well as the 7mer subsets performed worse. Figure 7.17 shows the correlation data gained for 17 cDNA clones of four different clusters that were hybridized with PNA set "6mer sub2". To avoid a subsequent impairment of the overall analysis, 12 clones, including an entire fifth cluster, that did not yield consistent hybridization results were excluded from the analysis The figure clearly demonstrates the inferior performance compared to set "6mer sub1". Related clones of a given cluster are again hardly separated from unrelated ones and numeric correlation differences are negligible. Although a general trend towards correct discrimination is still somewhat discernible, for the cDNA clones tested the discrimination performance of PNA set "6mer sub2" was even worse compared to genomic DNA clones.

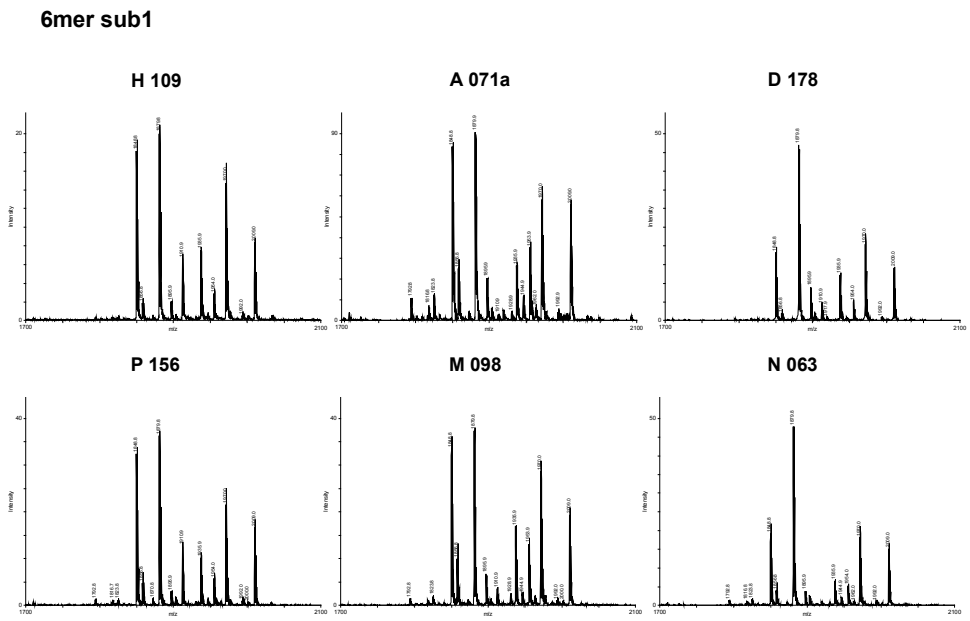

Figure 7.16 Hybridization results of selected cDNA clones hybridized with PNA set "6mer sub1"

MALDI-TOF mass spectra of six cDNA clones are depicted that were hybridized with PNA set "6mer sub1". H109 and P156 belong to one cluster, A071a and M098 to another one, D178 and N063 to a third cluster. Experimental probe masses are annotated. The composition of the PNA set is given in chapter 5.7.

Cl. 1	B219		H109		H182		I151		P156
P156	0,9879	H109	0,9948	H182	0,9960	I151	0,9951	P156	0,9991
H109	0,9854	B219	0,9854	H109	0,9853	P156	0,9892	H035	0,9900
B219	0,9843	H182	0,9853	K022a	0,9814	P091	0,9853	I151	0,9892
H182	0,9787	K022a	0,9813	B219	0,9787	H035	0,9836	B219	0,9879
I151	0,9770	I151	0,9738	P156	0,9719	D178	0,9822	N158	0,9871
K022a	0,9759	K022	0,9718	E033	0,9675	N063	0,9772	L028	0,9856
G173	0,9672	G173	0,9680	I151	0,9658	B219	0,9770	P091	0,9847
E033	0,9651	P156	0,9673	P091	0,9632	N158	0,9748	N063	0,9796
K022	0,9596	E033	0,9672	K022	0,9607	L028	0,9743	I051	0,9747
P091	0,9500	P091	0,9631	B112	0,9545	B112	0,9739	D178	0,9744
B112	0,9440	B112	0,9567	G173	0,9510	H109	0,9738	K022	0,9721
D178	0,9337	D178	0,9501	H035	0,9439	H182	0,9658	H182	0,9719
H035	0,9259	H035	0,9430	N158	0,9339	I051	0,9594	B112	0,9710
N158	0,9093	N158	0,9284	D178	0,9324	K022a	0,9579	H109	0,9673
N063	0,9089	N063	0,9270	L028	0,9253	K022	0,9547	K022a	0,9543
L028	0,9014	L028	0,9220	N063	0,9229	E033	0,9253	E033	0,9238
I051	0,8760	I051	0,8977	I051	0,9064	G173	0,9228	G173	0,9045

Cl. 3	E033		G173		K022		K022a
K022a	0,9754	G173	0,9961	K022	0,9932	K022a	0,9955
E033	0,9740	K022	0,9791	G173	0,9791	H182	0,9814
H182	0,9675	K022a	0,9776	K022a	0,9755	H109	0,9813
H109	0,9672	H109	0,9680	P156	0,9721	G173	0,9776
G173	0,9664	B219	0,9672	H109	0,9718	B219	0,9759
B219	0,9651	E033	0,9664	D178	0,9647	K022	0,9755
K022	0,9422	H182	0,9510	H182	0,9607	E033	0,9754
I151	0,9253	I151	0,9228	H035	0,9600	I151	0,9579
P156	0,9238	P156	0,9045	B219	0,9596	P156	0,9543
P091	0,9076	P091	0,8999	P091	0,9571	P091	0,9379
B112	0,9001	B112	0,8938	I151	0,9547	B112	0,9273
D178	0,8875	D178	0,8936	N063	0,9546	H035	0,9238
H035	0,8852	H035	0,8719	L028	0,9465	D178	0,9230
N158	0,8686	N063	0,8563	B112	0,9449	N158	0,9071
N063	0,8630	N158	0,8443	N158	0,9442	N063	0,9071
L028	0,8597	L028	0,8393	E033	0,9422	L028	0,9023
I051	0,8318	I051	0,8036	I051	0,9238	I051	0,8769

Cl. 4	D178		H035		I051		L028		N063
D178	0,9937	H035	0,9979	I051	0,9971	L028	0,9994	N063	0,9981
N063	0,9859	L028	0,9949	L028	0,9953	N158	0,9965	H035	0,9944
H035	0,9831	N063	0,9944	N158	0,9928	I051	0,9953	L028	0,9935
I151	0,9822	N158	0,9943	H035	0,9875	H035	0,9949	N158	0,9900
P091	0,9759	P156	0,9900	N063	0,9857	N063	0,9935	D178	0,9859
L028	0,9756	I051	0,9875	P156	0,9747	P156	0,9856	I051	0,9857
P156	0,9744	P091	0,9858	P091	0,9645	D178	0,9756	P156	0,9796
N158	0,9733	I151	0,9836	D178	0,9597	P091	0,9744	I151	0,9772
B112	0,9697	D178	0,9831	I151	0,9594	I151	0,9743	P091	0,9767
K022	0,9647	B112	0,9732	B112	0,9479	B112	0,9602	B112	0,9659
I051	0,9597	K022	0,9600	K022	0,9238	K022	0,9465	K022	0,9546
H109	0,9501	H182	0,9439	H182	0,9064	H182	0,9253	H109	0,9270
B219	0,9337	H109	0,9430	H109	0,8977	H109	0,9220	H182	0,9229
H182	0,9324	B219	0,9259	K022a	0,8769	K022a	0,9023	B219	0,9089
K022a	0,9230	K022a	0,9238	B219	0,8760	B219	0,9014	K022a	0,9071
G173	0,8936	E033	0,8852	E033	0,8318	E033	0,8597	E033	0,8630
E033	0,8875	G173	0,8719	G173	0,8036	G173	0,8393	G173	0,8563

Cl. 5	B112		N158		P091
N158	0,9895	N158	0,9969	P091	0,9979
P091	0,9869	L028	0,9965	B112	0,9869
B112	0,9865	H035	0,9943	H035	0,9858
I151	0,9739	I051	0,9928	N158	0,9854
H035	0,9732	N063	0,9900	I151	0,9853
P156	0,9710	B112	0,9895	P156	0,9847
D178	0,9697	P156	0,9871	N063	0,9767
N063	0,9659	P091	0,9854	D178	0,9759
L028	0,9602	I151	0,9748	L028	0,9744
H109	0,9567	D178	0,9733	I051	0,9645
H182	0,9545	K022	0,9442	H182	0,9632
I051	0,9479	H182	0,9339	H109	0,9631
K022	0,9449	H109	0,9284	K022	0,9571
B219	0,9440	B219	0,9093	B219	0,9500
K022a	0,9273	K022a	0,9071	K022a	0,9379
E033	0,9001	E033	0,8686	E033	0,9076
G173	0,8938	G173	0,8443	G173	0,8999

Figure 7.17 Correlation analysis of cDNA clones hybridized with PNA set "6mer sub2"

cDNA clones were hybridized with PNA set "6mer sub2". Hybridization data were processed and Pearson correlations were calculated as described earlier. Clones are depicted in their respective cluster (Cl. 1, 3, 4, 5). Those clones that belong to the same respective cluster are marked by green color. The clone under investigation is made stand out in black bold type. All clones possess internal tracking codes. Shorter PCR fragments of original clones are indicated by codes with lower case letters "a" and "b".

Correlation data yielded with the remaining sets were as random as they were for global PNA sets (“6mer sub3” and “7mer sub1”) or failed to generate consistent data at all (“7mer sub2”).

7.4 Evaluation of potential DNA immobilization systems for direct hybridization read-out by MALDI-TOF MS

For full automation and acceleration of the OFP process, a platform would be essential that allows on-site DNA immobilization, probe hybridization and read-out of hybridization events by MALDI-TOF MS. At the beginning of this thesis such a system did not exist - neither in the academic scientific community nor commercially.

Available detection platforms (so-called MALDI targets) normally consist of conductive surfaces (metal, silicon etc.) onto which MALDI matrix and samples are transferred. Optionally, these surfaces can be preloaded with optimized MALDI matrix formulations as in the case of Sequenom´s SpectroCHIP™[12]. In either case, neither immobilization nor hybridization is performed on the surface rendering such a platform unsuitable for the concept of multiplexed OFP. As a consequence, in the scope of the present dissertation it was aimed at the development of a DNA microarray that is compatible with MALDI-TOF MS and suitable for multiplexed OFP.

The majority of conventional DNA microarrays are based on glass slides of 75 mm x 25 mm x 1 mm dimension. This format is used as a universal standard for almost all academically and commercially available microarray chips. To boost the development of a MALDI-TOF MS compatible DNA microarray and to enhance industrial cooperations, a prototype adapter was designed and fabricated that functions as an interface between Bruker MALDI-TOF mass spectrometers and potential DNA chips of universal glass slide format. The adapter was subsequently optimized and possesses the advantage of a flexible design that allows to mount potential chips of roughly the above mentioned dimensions. This flexibility is particularly advantageous for the evaluation of multiple DNA immobilization systems since an individual re-adjustment step for either microarray under investigation or adapter would be practically and economically unfeasible.

[12] http://www.sequenom.com

7.4.1 Promising surfaces and attachment chemistries

A MALDI-TOF MS compatible DNA immobilization system should feature a high DNA immobilization capacity, a stable attachment chemistry on a solvent resistant conductive surface and a high accessibility of immobilized DNA to hybridization probes and laser desorption. Therefore, in the scope of this dissertation various surfaces and attachment chemistries were evaluated that were likely to fulfill the above mentioned requirements.

7.4.1.1 Acrylamide-based immobilization system

The immobilization of DNA via a polymeric three-dimensional matrix is supposed to offer a higher immobilization capacity compared to two-dimensional systems. Additionally, no hybridization probe and/or DNA interferences which impair hybridization and its specificity are expected to occur.

Polyacrylamide embodies such a three-dimensional matrix. Two main strategies of immobilizing DNA within such a gel matrix have been proposed. The first is based on an activation of the gel matrix by a reducing agent, mostly hydrazide. Subsequently, modified DNA can be covalently bound to the gel matrix (Khrapko et al., 1991, Yershov et al., 1996). The second strategy focuses on the immobilization of acrylamide-modified oligonucleotides or PCR products by co-polymerization (Rehman et al., 1999). DNA bearing 5'-terminal acrylamide modifications was shown to efficiently co-polymerize with acrylamide monomers to form thermally stable DNA-containing polyacrylamide co-polymers.

Since the activation of gel matrices by strong reducing agents is cumbersome, time-consuming and hazardous, it was decided to concentrate on the co-polymerization strategy. Besides, acrylamide modified PCR products needed for co-polymerization can be readily generated via PCR, employing 5´-acrylamide modified primers which are commercially available. Preliminary experiments on an acrylic silane functionalized MALDI target covered with DNA-containing acrylamide co-polymers showed that - in principle - it is possible to hybridize and detect PNA probes on such a surface. However, several reasons led to the cessation of that approach. First, the co-polymerized gel matrix needs to be completely dried prior to vacuum applied during MALDI-TOF MS measurements. Unfortunately, dried gel matrix on functionalized metal is very unstable and disintegrates. Second, acrylic silane needed for stable functionalization of MALDI targets or other metal surfaces is commercially not available, a custom synthesis would be astronomically expensive. Third, an industrial cooperation giving access to either acrylic silane coated

metal slides or completely functional acrylamide DNA microarrays could not be established. Last, the polymerization mix is very toxic and the time point of polymerization difficult to control. Although polymerization by photo-initiation instead of radical initiation has been successfully employed (Lyubimova et al., 1993), avoiding some of the above mentioned problems, still the required instrumental setup is very costly.

7.4.1.2 Streptavidin-based immobilization system

Another promising three-dimensional matrix is a streptavidin-based immobilization system based on self-assembling monolayers (SAM) of long-chain thiol alkanes adsorbed onto a gold layer. Biotin is coupled covalently to the surface and saturated with streptavidin to form an interface for the binding of biotinylated DNA. This system overcomes the length limitation of thiolated DNAs directly coupled to a pure gold layer (Steel et al., 2000) since it combines the robustness of a gold-based SAM immobilization scheme with the high accessibility of streptavidin bound biotinylated DNA to hybridization probes.

Regarding such a streptavidin-based system the XNA on Gold™[13] affinity biochip developed by Thermohybaid, Germany, was tested. It employs the above described chemistry for the immobilization of biotinylated DNA. Preliminary results obtained with a linear mode MALDI-TOF mass spectrometer under non-optimized detection conditions suggested that specific and reproducible results can be obtained. Figure 7.18 shows a 6-plex hybridization result of a PCR amplified insert of a genomic DNA clone of known sequence that was recorded by means of a high-resolution reflector-mode MALDI-TOF mass spectrometer. However, despite conditions and instruments settings optimized for PNA detection, hybridization results could not be consistently reproduced, i.e. sometimes no signals were detected at all. In addition, as suggested by control experiments without immobilized DNA, unspecific binding of PNA probes, presumably to streptavidin, frequently occurred even in the presence of detergents, such as Tween-20. Hence, there was no guarantee that obtained results were sequence-dependent. The persisting problem of unspecificity of the XNA on Gold™ biochip was too crucial to follow up with this approach.

[13] http://www.thermohybaid.com

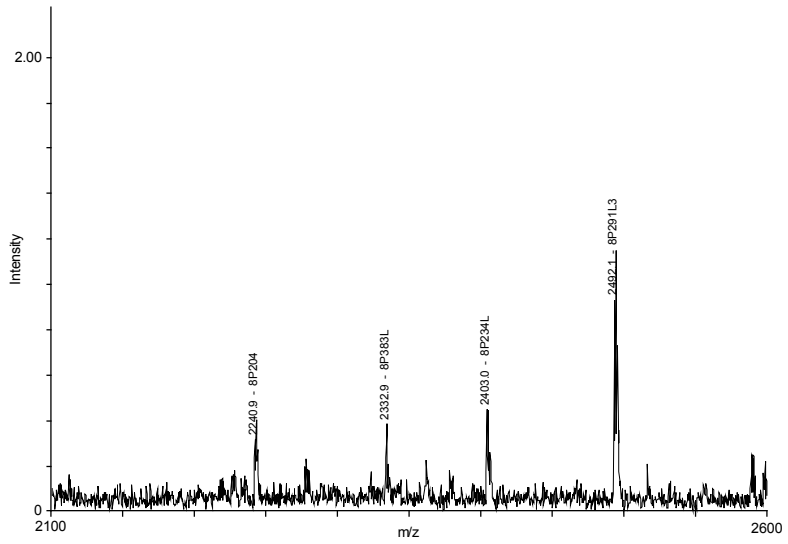

Figure 7.18 6-plex PNA hybridization with a PCR product immobilized on a XNA on Gold™ biochip

The MALDI-TOF mass spectrum shows the reproducible and specific hybridization result of four out of six different PNA octamers with a PCR product of known sequence.

7.4.1.3 Nylon-based immobilization system

Unprecedented experience in conventional nylon membrane hybridizations and blotting gathered over the last three decades made it very reasonable to pursue the development of a nylon-based immobilization system. Furthermore, many studies have shown that for mass spectrometric analyses of proteins and protein digest different types of membranes can be applied for direct MALDI-TOF MS measurements (McComb et al., 1997, Worrall et al., 1998, Binz et al., 1999, Hung et al., 1999) supporting a nylon-based approach.

MALDI-TOF MS compatibility of such a nylon-based DNA immobilization system was demonstrated by the immobilization of DNA on small pieces of nylon membrane and subsequent multiplexed PNA hybridization. Following hybridization the membrane pieces were incubated with MALDI matrix solution III and mounted onto a modified MALDI target using double adhesive conductive tape (figure 7.19 A). For independent experiments under initial conditions reproducible results could be obtained suggesting that nylon membranes are suitable. To follow up with that approach, the development of a nylon-based DNA microarray was intensively pursued on the basis of industrial collaborations. A cooperation with Schleicher & Schuell Bioscience, USA, was initiated aiming at the further development of their nylon-based CAST™ slides towards MALDI-TOF-MS compatibility.

The CAST™ membrane was deposited on metal slides (Al or $TiAlV_6$ alloy) which proved to be a stable and adequate solution (figure 7.19 B). Such prepared slides were used for DNA immobilization and multiplexed PNA hybridization. Experiments with the metallic

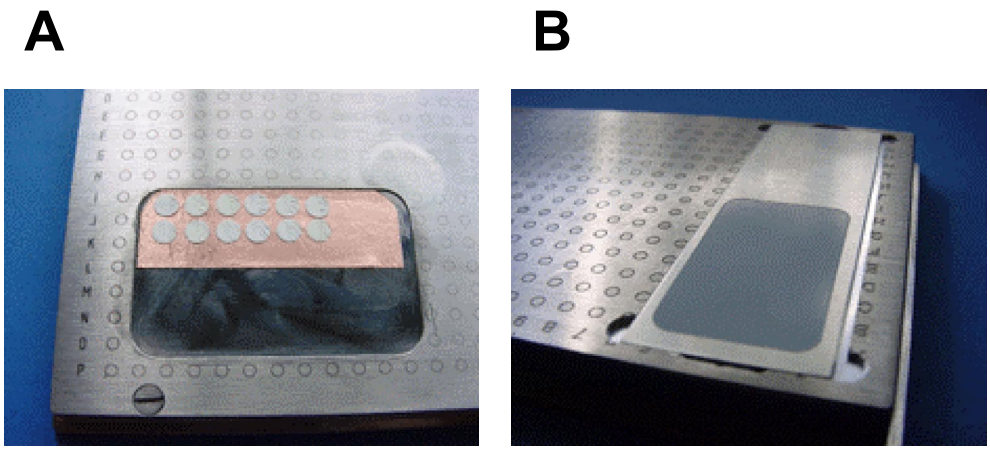

Figure 7.19 Development of nylon-based immobilization system

A: Milled conventional MALDI target with small pieces of nylon membranes (0,45 µm pore size) of about 10 mm^2 size and that have been mounted on double adhesive conductive tape.
B: CAST™ membrane (0,45µm thick) on aluminum slide that has been mounted in the self-designed adapter.

CAST™ slides were reproducible and confirmed the preliminary results obtained with small pieces of nylon membrane (figure 7.20). However, signals were unspecific, resolution of signals was rather poor and hybridization results were not always detectable presumably due to membrane thickness and insufficient MALDI matrix crystallization. Less volatile matrix formulations, such as MALDI matrix solution III, on the other hand, improved the outcome. Experiments with CAST™ nylon membranes of smaller pore size (0,2 µm) and smaller membrane pieces did not improve results. Empirical observations revealed that signals were detected at the edges of a membrane piece rather than in the center. The evaluation of the FAST™ nitrocellulose membrane failed due to the chemical instability of nitrocellulose to organic solvents.

Despite the above mentioned problems, a nylon membrane based approach still remains promising once those drawbacks are overcome.

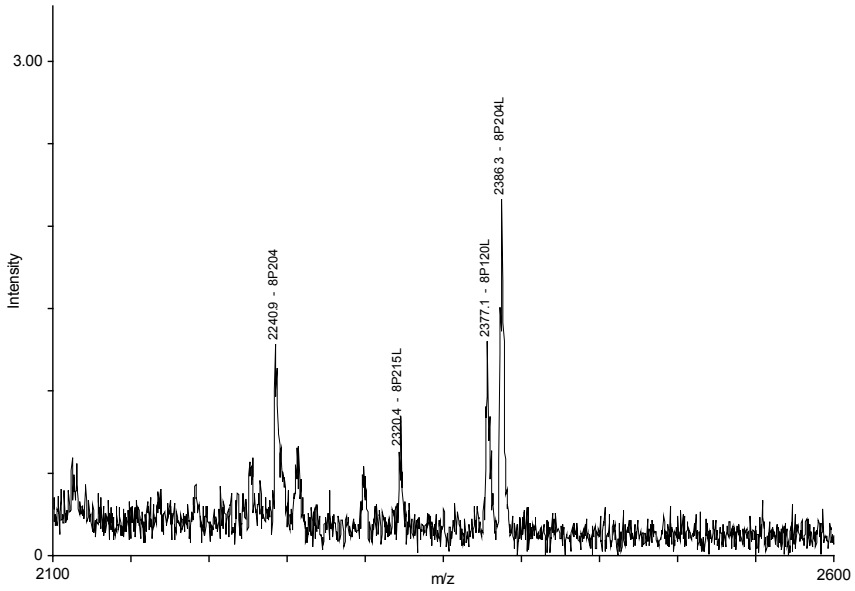

Figure 7.20 6-plex PNA hybridization with a PCR product immobilized on a metallic CAST™ slide

The MALDI-TOF mass spectrum shows the reproducible but unspecific hybridization result of four out of six different PNA octamers with a PCR product of known sequence.

7.4.1.4 Dendritic immobilization system

Polyamidoamine (PAMAM) starburst dendrimers belong to polyfunctional dendritic linker systems that have been initially developed by Tomalia et al. (1980). Their suitability as pre-fabricated dendrimers for efficient DNA immobilization has been described by Benters et al. (2001, 2002) who demonstrated a 10-20 times higher DNA immobilization capacity compared to planar glass slides.

To exploit this feature for the concept of multiplexed OFP a collaboration with Chimera Biotec, Germany, who commercializes the PAMAM technology, was initialized. As a standard, PAMAM starburst dendrimers are applied on conventional glass slides. However, initial experiments with PAMAM functionalized silicon performed at Chimera suggested that a conductive silicon surface would be at least equally suitable. Therefore, it was decided to evaluate PAMAM functionalized silicon in comparison to conventional PAMAM glass slides. The latter were directly obtained from Chimera Biotec. In contrast, for the generation of PAMAM functionalized silicon doped silicon wafers were first polished, covered with a defined layer of thermally applied oxide (~ 1000 nm) and then cut into pieces of uniform glass slide format. Subsequently, PAMAM starburst dendrimers were applied as described (Benters et al., 2002). Two batches of silicon-based PAMAM

slides were produced of which the second one was methodically optimized. Figure 7.21 shows the reproducible hybridization result of a 6-plexed PNA hybridization with DNA immobilized on a conventional PAMAM glass slide (A) and PAMAM functionalized conductive silicon (B). Signal intensities yielded from silicon slides were consistently

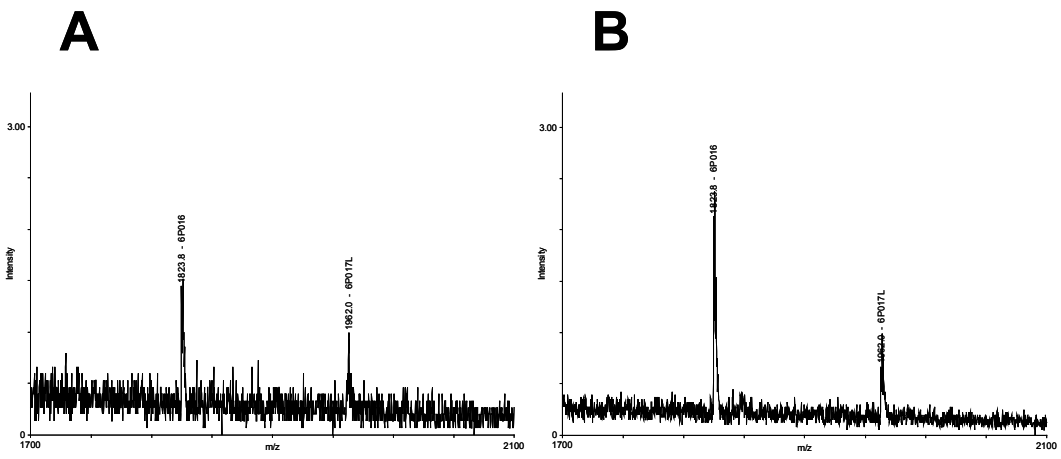

Figure 7.21 6-plex PNA hybridization with a PCR product immobilized on PAMAM functionalized surfaces

The MALDI-TOF mass spectra show the reproducible hybridization results of two out of four expected different PNA hexamers with a PCR product of known sequence immobilized on a conventional PAMAM glass slide (**A**) and PAMAM functionalized conductive silicon (**B**, second batch).

higher and showed a better resolution suggesting a superior performance compared to conventional non-conductive PAMAM glass slides. However, due to a lack of pre-structuring of the solid support an intricate on-slide sample localization occurred. Besides MALDI matrix crystallization appeared to be inconsistent resulting in impaired signal acquisition. Nevertheless, a PAMAM based dendritic immobilization system represents a powerful alternative once those challenges are overcome.

7.4.2 Comparison of DNA immobilization systems

From the obtained results it became apparent that the success and quality of MALDI-TOF mass spectrometric measurements is highly dependent on MALDI matrix and sample crystallization. As for the evaluation of two immobilization systems, it is hence almost impossible to directly compare two microarray slides of different nature exclusively on the basis of hybridization signal intensities and resolution yielded by MALDI-TOF MS. Even

an excellent hybridization performance could be superimposed by an insufficient matrix crystallization resulting in very bad or no signals at all.

Consequently, to assess and compare their individual binding capacity the DNA immobilization systems tested in the course of this dissertation were evaluated with regard to their performance in fluorescent DNA hybridizations. Fluorescence intensities and signal-to-noise ratios simultaneously give information about DNA immobilization capacity as well as accessibility to hybridization probes. However, in contrast to direct labeling assays, where radioactively or fluorescence labeled DNA is used, it is not possible to determine the individual impact of one parameter separately. Nevertheless, the straightforwardness of the experimental setup and the rapidness of data acquisition compensate for this drawback. Furthermore, direct labeling assays do only provide information about one parameter at the same time. A simultaneous and separate determination of the influence of DNA immobilization capacity and accessibility is also unfeasible.

In the course of fluorescence DNA hybridization experiments the following immobilization systems were evaluated: streptavidin-based XNA on Gold™ biochip, metallic as well as conventional glass-based CAST™ nylon membrane slides, PAMAM functionalized conductive silicon (initial and refined batch) as well as conventional PAMAM glass slides, and Quantifoil QMT™ aldehyde glass. Figure 7.22 shows the highly reproducible hybridization results of a Cy-3 fluorescence labeled DNA oligonucleotide complementary to a PCR priming region that was hybridized to PCR amplified DNA of different size. Results of the XNA on Gold™ biochip and the conventional glass-basedmembrane slide are not illustrated. The latter yielded unsatisfactory results as did the metallic CAST™ slide (fig. 7.22 A) whereas the former failed to deliver any meaningful data. Both findings can be explained as follows: Gold which forms the basis of the streptavidin-based XNA chip is known to quench fluorescence thereby eliminating any fluorescent signal. In fact, only weak signals at high DNA concentrations could be detected. Nylon membranes, as already mentioned before, act as a sponge resulting in a spread of DNA applied which caused smeared fluorescence signals.

Figure 7.23 shows the numeric analysis of fluorescence hybridization results of the DNA microarrays that could be analyzed using the GenePix Pro 4.1 software package. From the figure it is apparent that increasing DNA concentrations lead to higher overall signal intensities and slightly improved signal-to-noise ratios. Besides, within the range of the applied dilution series of spotted DNA concentrations no plateau seems to be reached. As expected, DNA of decreasing length yields increasing hybridization signal intensities which are due to higher numbers of molecules arrayed per given concentration unit. All spotted slides showed an increase in fluorescent signal intensities as a function of

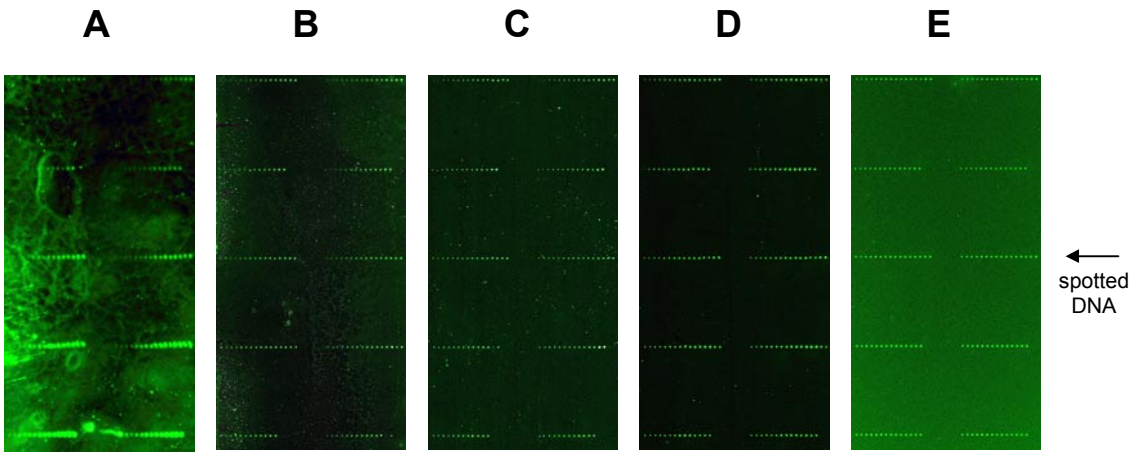

Figure 7.22 Comparison of DNA microarrays hybridized with a fluorescent DNA probe

DNA microarrays were spotted, processed, subsequently hybridized with a Cy-3 fluorescence labeled DNA 18mer oligonucleotide and scanned as described in chapter 6.11. Microarrays on the following solid supports are depicted: metallic CAST™ nylon membrane slide (**A**), PAMAM functionalized conductive silicon initial (**B**) and refined batch (**C**), conventional PAMAM glass slide (**D**), and Quantifoil QMT™ aldehyde glass slide (**E**). PCR amplified DNA of five different lengths (450 bp, 650 bp, 1060 bp, 1350 bp, 1520 bp) was spotted as a dilution series (25, 50, 100, 200, 400, 600, 800, 1000 ng/µL). Each row represents a specific PCR product that was arrayed with increasing concentrations (from left to right) in adjacent duplicates and duplicate subarrays (horizontally neighboring rows). DNA length decreases from top rows (1520 bp) to bottom rows (450 bp). Fluorescence at λ=532 nm was scanned with a PMT voltage of 550.

the applied dilution series, except Quantifoil aldehyde slides, which behaved conversely. Considering the different immobilization systems, it appears that PAMAM functionalized silicon is by far superior to glass based attachment chemistries. Noticeably, slides of the methodically refined silicon batch perform considerably better than slides of the initial batch. There is, however, only little difference in performance between PAMAM glass and Quantifoil aldehyde glass slides.

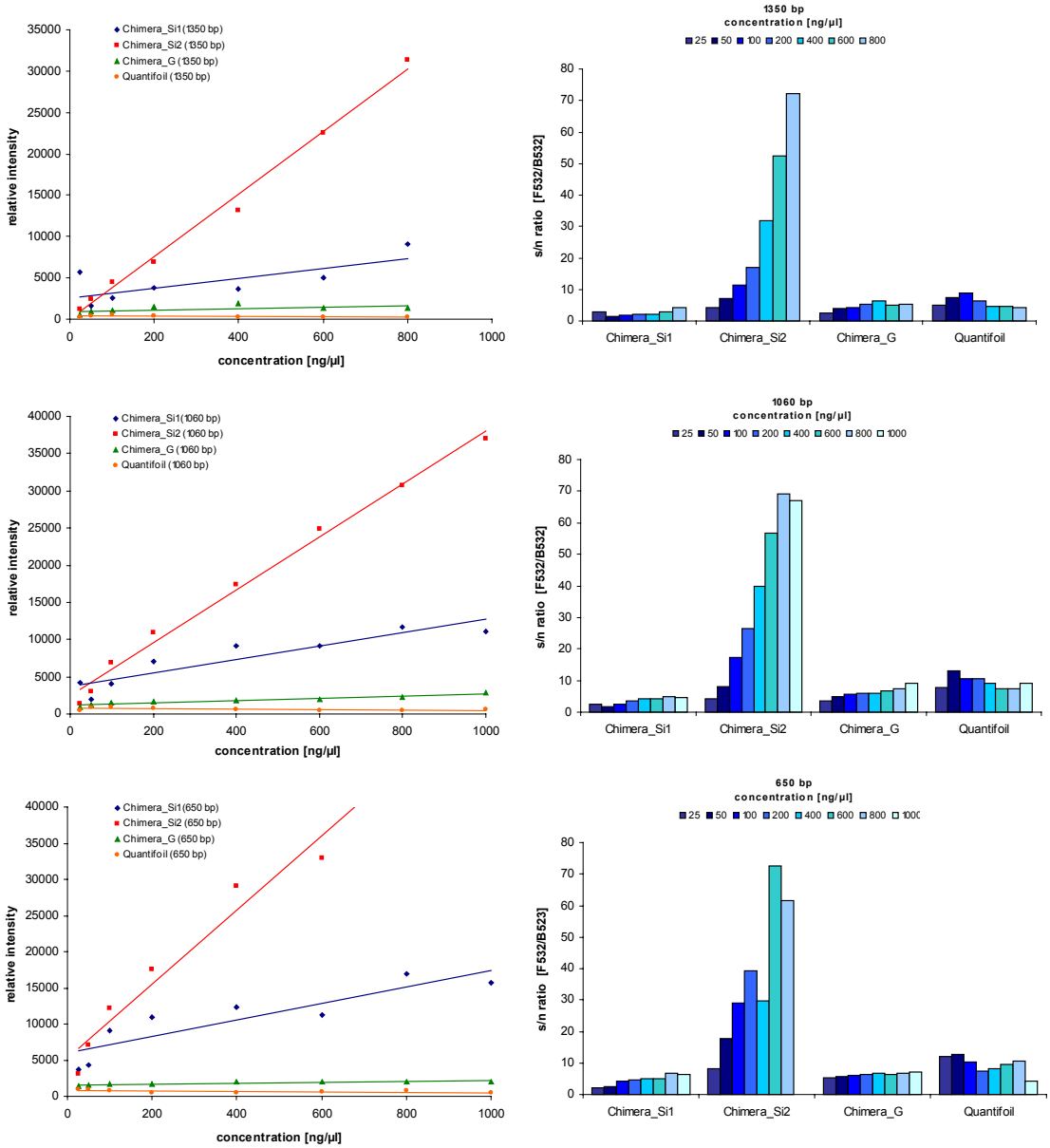

Figure 7.23 Numeric analysis of fluorescence DNA hybridization results

The results of numeric analysis of fluorescence DNA hybridizations of three different DNA lengths (1350 bp, 1060 bp, 650 bp) gained on PAMAM functionalized silicon initial batch (Chimera_Si1) and refined batch (Chimera_Si2) as well as PAMAM functionalized glass slides (Chimera_G) and Quantifoil QMT™ aldehyde glass slides (Quantifoil) are depicted. On the left, normalized relative fluorescence signal intensities in dependence of spotted DNA concentration are shown for the respective microarrays. On the right, signal-to-noise ratios for each slide under investigation are given, in turn, in dependence of spotted DNA concentration.