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 doublestranded 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-HCI (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 results of by red arrows for PNA 6P008 - strongly suggesting that hybridization results





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 (TMACI, TEACI), 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, TMACI, TEACI, 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,

Α													
CI. A	F21		H14		J20		O21	CI. 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	021	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 M10	0,9454	021	0,9227	1 24	0,3830	L24 C14	0,8972	P 19 11	0,9453	NU7 E14	0,9440	C14	0,9477
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 E13	0,8781	E14	0,3253	F13	0,8476	P06 E13	0,9264	D11 E13	0,9337	P06 H14	0,9322
H14	0.8415	P06	0,8524	P19	0,3084	P06	0,8286	F21	0,9132	H14	0,9058	021	0,8978
O21	0,8198	F21	0,8415	P06	0,3033	F21	0,8198	O21	0,8964	021	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
CI. D	D11		E14		F13		L11	CI. 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
N07	0,9461	1 11	0,9501	P19	0,9440	C14	0,9413	E 14 M10	0,9410	D11	0,9501	1 24	0,9536
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 H14	0,9321	H14	0,9388	H14	0,9185	F21 H14	0,9209	F21	0,9227	H14	0,9204	F13 H14	0,9386
021	0,8705	021	0,8620	021	0,8476	021	0,8572	021	0,8972	021	0,8286	021	0,8468
J20	0,3212	J20	0,3253	J20	0,3328	J20	0,3290	J20	0,3601	J20	0,3033	J20	0,3084
В	F21	1	H14	1	.120	1	021	a c	C14	1	M10	1	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
021	0,9470	P19	0,0973	H14	0,9345	.120	0,9176	F 19 F14	0,9404	021	0,9000	520 F21	0,0002
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	021	0,9190	E14	0,8807	F13	0,8694
L24	0,9034	L24	0,8535	021	0,9176	H14	0,8973	L24	0,9107	P19	0,8801	D11	0,8571
M10 F13	0,8876	E14 D11	0,8501	P06	0,9069	E14 M10	0,8921	M10	0,9000	L24 P06	0,8667	021 M10	0,8413
N07	0.8814	L11	0,8301	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
CI. D	D11		E14		F13		L11	CI. 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
J20	0,8990	J20	0,9310	P19	0,9269	021	0,9391	.120	0,6996	P19	0,9349	⊑14 J20	0,9455
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	0.0260
C14							0.0004	E42	0 8817	120	0.0000		0,3203
	0,8523	N07	0,9132	021	0,9028	J20	0,8901	F I S	0,0011	520	0,9069	L11	0,9209
H14	0,8523 0,8501	N07 D11	0,9132 0,9107	O21 F21	0,9028	J20 E14	0,8901	021	0,8815	L24	0,9089	L11 021	0,9193 0,9178
H14 F21	0,8523 0,8501 0,8379 0,8325	N07 D11 O21 M10	0,9132 0,9107 0,8921 0,8907	O21 F21 C14	0,9028 0,8872 0,8852 0,8817	J20 E14 F13 M10	0,8901 0,8785 0,8564 0,8514	O21 M10 H14	0,8815 0,8667 0,8535	L24 N07 F13	0,9069 0,8843 0,8786 0,8670	L11 O21 N07 D11	0,9193 0,9178 0,9003 0,8990
H14 F21 L24 O21	0,8523 0,8501 0,8379 0,8325 0,8304	N07 D11 O21 M10 L11	0,9132 0,9107 0,8921 0,8807 0,8785	O21 F21 C14 L24 N07	0,9028 0,8872 0,8852 0,8817 0,8694	J20 E14 F13 M10 H14	0,8901 0,8785 0,8564 0,8514 0,8454	C21 M10 H14 E14	0,8815 0,8667 0,8535 0,8515	L24 N07 F13 M10	0,9089 0,8843 0,8786 0,8670 0,8607	L11 O21 N07 D11 L24	0,9193 0,9178 0,9003 0,8990 0,8867
H14 F21 L24 O21 P06	0,8523 0,8501 0,8379 0,8325 0,8304 0,8231	N07 D11 O21 M10 L11 L24	0,9132 0,9107 0,8921 0,8807 0,8785 0,8515	O21 F21 C14 L24 N07 P06	0,9028 0,8872 0,8852 0,8817 0,8694 0,8670	J20 E14 F13 M10 H14 N07	0,8901 0,8785 0,8564 0,8514 0,8454 0,8454	F13 O21 M10 H14 E14 D11	0,8815 0,8667 0,8535 0,8515 0,8325	L24 N07 F13 M10 H14	0,9089 0,8843 0,8786 0,8670 0,8607 0,8392	L11 O21 N07 D11 L24 M10	0,9193 0,9178 0,9003 0,8990 0,8867 0,8801

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

CI. A	C22		F21		G08		J06		021		P22		CI. C	D11		E14		F13		L11		012
C22	0,9928	F21	0,9886	G08	0,9941	J06	0,9776	O21	0,9926	P22	0,9776		D11	0,9882	E14	0,9781	F13	0,9873	L11	0,9882	012	0,9808
P22	0,9638	G08	0,9753	F21	0,9753	G08	0,9719	F21	0,9699	F21	0,9651		L11	0,9561	L11	0,9645	012	0,9271	E14	0,9645	D11	0,9326
021	0,9487	J06	0,9718	J06	0,9719	F21	0,9718	G08	0,9662	C22	0,9638		E14	0,9347	D11	0,9347	D11	0,9230	D11	0,9561	E14	0,9315
J06	0,9455	021	0,9699	A06	0,9688	O21	0,9467	C22	0,9487	G08	0,9634		012	0,9326	012	0,9315	E14	0,9148	A06	0,9137	F13	0,9271
F21	0,9442	P22	0,9651	O21	0,9662	C22	0,9455	J06	0,9467	021	0,9345		F13	0,9230	F13	0,9148	L11	0,9051	012	0,9122	L11	0,9122
G08	0,9420	C22	0,9442	P22	0,9634	P22	0,9293	P22	0,9345	J06	0,9293		E08	0,8827	E08	0,8812	E08	0,8778	F13	0,9051	E08	0,7913
C03	0,9328	A06	0,9195	K04	0,9467	K04	0,8753	A06	0,8741	P19	0,8770		K04	0,8739	K04	0,8667	B03	0,8272	G08	0,8998	K04	0,7701
A06	0,9099	K04	0,9096	C22	0,9420	A06	0,8737	K04	0,8658	P06	0,8762		A06	0,8593	A11	0,8574	A11	0,8060	K04	0,8975	B03	0,7568
L24	0,8986	L24	0,9044	L24	0,9188	K18	0,8646	P19	0,8557	L11	0,7833		A11	0,8431	A06	0,8522	P19	0,7725	E08	0,8908	A11	0,7551
K04	0,8769	003	0,8857	L11	0,8998	C03	0,8598	L24	0,8549	E14	0,7790		GU8	0,8402	GU8	0,8463	K04	0,7666	ATT	0,8765	P19	0,7493
K18	0,8754	P19	0,8787	C03	0,8841	P06	0,8472	C03	0,8434	E08	0,7676		K18	0,8311	P06	0,8337	003	0,7624	K18	0,8732	AUG	0,7374
P19	0,8703	P06	0,8770	E08	0,8806	P19	0,8435	P06	0,8216	011	0,7455		P19	0,8224	K18	0,8284	AU6	0,7540	F21	0,8580	P22	0,7364
P00	0,0002	N IO	0,0752	P 19	0,0772	144	0,0070	K IO	0,0214	512	0,7304		BU3	0,0109	F21	0,0034	L24	0,7352	P00	0,0524	L24	0,7217
EU0 Δ11	0,6495	E08	0,0000	A11	0,6596	E08	0,0271	D11	0,7692	A11	0,7170		124	0,0002	C03	0,7971	P00 K18	0,7311	D10	0,0440	JU0 K18	0,7079
111	0,0122	A11	0,0000	E14	0,0000	E00	0,0000	E08	0,7857	KM	0,6070		C03	0,7018	D10	0,7842	P22	0,7303	106	0,0000	C03	0,0302
D11	0,7508	D11	0,0002	D11	0,0403	A11	0,7037	A11	0,7653	B03	0,6785		POG	0,7886	106	0,7042	C08	0,7170	1.24	0,8058	021	0,0330
E14	0,7346	F14	0.8034	K18	0.8239	D11	0.7697	F14	0 7145	A06	0.6722		021	0 7859	P22	0,7790	F21	0.6836	B03	0 7943	P06	0.6705
012	0.6589	E13	0.6836	B03	0,7192	012	0 7079	012	0.6749	1 24	0.6310		.106	0,7697	1 24	0.7682	.106	0,6668	021	0 7892	C22	0.6589
F13	0.6568	B03	0,6606	F13	0,7001	F13	0.6668	B03	0.6035	C03	0.6021		C22	0,7508	C22	0,7346	C22	0.6568	P22	0 7833	G08	0.6356
B03	0.6097	012	0.5888	012	0.6356	B03	0.6367	F13	0.5979	K18	0 5999		P22	0.7456	021	0.7145	021	0.5979	C22	0 7818	F21	0.5888
	-, 1		-,		-,		-,		-,		-,		1					-,		-,		-,
CI. B	A06		A11		C03		K04		K18		CI. D	L24		P06		P19			singl.	B03	singl.	E08
CI. B A06	A06 0,9935	A11	A11 0,9892	C03	C03 0,9980	K04	K04 0,9920	K18	K18 0,9977		CI. D L24	L24 0,9904	P06	P06 0,9902	P19	P19 0,9905			singl. B03	B03 0,9965	singl. E08	E08 0,9908
<i>Cl. B</i> A06 K18	A06 0,9935 0,9892	A11 K18	A11 0,9892 0,9645	C03 A06	C03 0,9980 0,9790	K04 A06	K04 0,9920 0,9702	K18 A06	K18 0,9977 0,9892		<i>Cl. D</i> L24 P06	L24 0,9904 0,9414	P06 P19	P06 0,9902 0,9738	P19 P06	P19 0,9905 0,9738			singl. B03 E08	B03 0,9965 0,8467	singl. E08 A11	E08 0,9908 0,9458
CI. B A06 K18 C03	A06 0,9935 0,9892 0,9790	A11 K18 A06	A11 0,9892 0,9645 0,9616	C03 A06 K04	C03 0,9980 0,9790 0,9506	K04 A06 K18	K04 0,9920 0,9702 0,9613	K18 A06 A11	K18 0,9977 0,9892 0,9645		<i>CI. D</i> L24 P06 P19	L24 0,9904 0,9414 0,9402	P06 P19 L24	P06 0,9902 0,9738 0,9414	P19 P06 L24	P19 0,9905 0,9738 0,9402			singl. B03 E08 F13	B03 0,9965 0,8467 0,8272	singl. E08 A11 A06	E08 0,9908 0,9458 0,9333
CI. B A06 K18 C03 K04	A06 0,9935 0,9892 0,9790 0,9702	A11 K18 A06 E08	A11 0,9892 0,9645 0,9616 0,9458	C03 A06 K04 K18	C03 0,9980 0,9790 0,9506 0,9362	K04 A06 K18 C03	K04 0,9920 0,9702 0,9613 0,9506	K18 A06 A11 K04	K18 0,9977 0,9892 0,9645 0,9613		<i>Cl. D</i> L24 P06 P19 G08	L24 0,9904 0,9414 0,9402 0,9188	P06 P19 L24 C03	P06 0,9902 0,9738 0,9414 0,9024	P19 P06 L24 A06	P19 0,9905 0,9738 0,9402 0,9174			singl. B03 E08 F13 D11	B03 0,9965 0,8467 0,8272 0,8189	singl. E08 A11 A06 K04	E08 0,9908 0,9458 0,9333 0,8968 0,8968
CI. B A06 K18 C03 K04 G08	A06 0,9935 0,9892 0,9790 0,9702 0,9688 0,9646	A11 K18 A06 E08 K04	A11 0,9892 0,9645 0,9616 0,9458 0,9447 0,9307	C03 A06 K04 K18 C22	C03 0,9980 0,9790 0,9506 0,9362 0,9328 0,9327	K04 A06 K18 C03 G08	K04 0,9920 0,9702 0,9613 0,9506 0,9467	K18 A06 A11 K04 C03	K18 0,9977 0,9892 0,9645 0,9613 0,9362		CI. D L24 P06 P19 G08 A06	L24 0,9904 0,9414 0,9402 0,9188 0,9094	P06 P19 L24 C03 K18	P06 0,9902 0,9738 0,9414 0,9024 0,8992 0,8815	P19 P06 L24 A06 C03	P19 0,9905 0,9738 0,9402 0,9174 0,9040 0,0031			singl. B03 E08 F13 D11 A11 E14	B03 0,9965 0,8467 0,8272 0,8189 0,8106 0,7071	singl. E08 A11 A06 K04 C03	E08 0,9908 0,9458 0,9333 0,8968 0,8934 0,8908
CI. B A06 K18 C03 K04 G08 A11 E08	A06 0,9935 0,9892 0,9790 0,9702 0,9688 0,9616 0,9333	A11 K18 A06 E08 K04 C03	A11 0,9892 0,9645 0,9616 0,9458 0,9447 0,9207 0,8765	C03 A06 K04 K18 C22 A11 P19	C03 0,9980 0,9790 0,9506 0,9362 0,9328 0,9207 0,9040	K04 A06 K18 C03 G08 A11 F21	K04 0,9920 0,9702 0,9613 0,9506 0,9467 0,9447 0,9096	K18 A06 A11 K04 C03 P06	K18 0,9977 0,9892 0,9645 0,9613 0,9362 0,8992 0,8754		CI. D L24 P06 P19 G08 A06 F21	L24 0,9904 0,9414 0,9402 0,9188 0,9094 0,9044 0,8986	P06 P19 L24 C03 K18 A06 E21	P06 0,9902 0,9738 0,9414 0,9024 0,8992 0,8815 0,8770	P19 P06 L24 A06 C03 K04 E21	P19 0,9905 0,9738 0,9402 0,9174 0,9040 0,9031 0,8787			singl. B03 E08 F13 D11 A11 E14 L11	B03 0,9965 0,8467 0,8272 0,8189 0,8106 0,7971 0,7943	singl. E08 A11 A06 K04 C03 L11 D11	E08 0,9908 0,9458 0,9333 0,8968 0,8934 0,8908 0,8827
CI. B A06 K18 C03 K04 G08 A11 E08 E21	A06 0,9935 0,9892 0,9790 0,9702 0,9688 0,9616 0,9333 0,9195	A11 K18 A06 E08 K04 C03 L11	A11 0,9892 0,9645 0,9616 0,9458 0,9447 0,9207 0,8765 0,8621	C03 A06 K04 K18 C22 A11 P19 P06	C03 0,9980 0,9790 0,9506 0,9362 0,9328 0,9207 0,9040 0,9024	K04 A06 K18 C03 G08 A11 F21 P19	K04 0,9920 0,9702 0,9613 0,9506 0,9467 0,9447 0,9096 0,9031	K18 A06 A11 K04 C03 P06 C22 E21	K18 0,9977 0,9892 0,9645 0,9613 0,9362 0,8992 0,8754 0,8752		CI. D L24 P06 P19 G08 A06 F21 C22	L24 0,9904 0,9414 0,9402 0,9188 0,9094 0,9094 0,8986 0,8986	P06 P19 L24 C03 K18 A06 F21 P22	P06 0,9902 0,9738 0,9414 0,9024 0,8992 0,8815 0,8770 0,8762	P19 P06 L24 A06 C03 K04 F21 C08	P19 0,9905 0,9738 0,9402 0,9174 0,9040 0,9031 0,8787 0,8772			singl. B03 E08 F13 D11 A11 E14 L11 K04	B03 0,9965 0,8467 0,8272 0,8189 0,8106 0,7971 0,7943 0,7749	singl. E08 A11 A06 K04 C03 L11 D11 E14	E08 0,9908 0,9458 0,9333 0,8968 0,8934 0,8908 0,8827 0,8812
CI. B A06 K18 C03 K04 G08 A11 E08 F21 P19	A06 0,9935 0,9892 0,9790 0,9702 0,9688 0,9616 0,9333 0,9195 0,9174	A11 K18 A06 E08 K04 C03 L11 L24 F14	A11 0,9892 0,9645 0,9616 0,9458 0,9447 0,9207 0,8765 0,8621 0,8574	C03 A06 K04 K18 C22 A11 P19 P06 E08	C03 0,9980 0,9790 0,9506 0,9362 0,9328 0,9207 0,9040 0,9024 0,9024 0,8934	K04 A06 K18 C03 G08 A11 F21 P19	K04 0,9920 0,9702 0,9613 0,9506 0,9467 0,9447 0,9096 0,9031 0,8975	K18 A06 A11 K04 C03 P06 C22 F21	K18 0,9977 0,9892 0,9645 0,9613 0,9362 0,8752 0,8754 0,8752 0,8752		Cl. D L24 P06 P19 G08 A06 F21 C22 K04 F08	L24 0,9904 0,9414 0,9402 0,9188 0,9094 0,9044 0,8986 0,8964 0,8964 0,8695	P06 P19 L24 C03 K18 A06 F21 P22 E08	P06 0,9902 0,9738 0,9414 0,9024 0,8992 0,8815 0,8770 0,8762 0,8717	P19 P06 L24 A06 C03 K04 F21 G08 P22	P19 0,9905 0,9738 0,9402 0,9174 0,9040 0,9031 0,8787 0,8772 0,8772			singl. B03 E08 F13 D11 A11 E14 L11 K04 012	B03 0,9965 0,8467 0,8272 0,8189 0,8106 0,7971 0,7943 0,7749 0,7568	singl. E08 A11 A06 K04 C03 L11 D11 E14 G08	E08 0,9908 0,9458 0,9333 0,8968 0,8934 0,8908 0,8827 0,8812 0,8812 0,8806
CI. B A06 K18 C03 K04 G08 A11 E08 F21 P19	A06 0,9935 0,9892 0,9790 0,9702 0,9688 0,9616 0,9333 0,9195 0,9174 0,9137	A11 K18 A06 E08 K04 C03 L11 L24 E14 G08	A11 0,9892 0,9645 0,9616 0,9458 0,9447 0,9207 0,8765 0,8621 0,8574 0,8574	C03 A06 K04 K18 C22 A11 P19 P06 E08 F21	C03 0,9980 0,9790 0,9506 0,9362 0,9328 0,9207 0,9040 0,9024 0,8934 0,8934 0,8857	K04 A06 K18 C03 G08 A11 F21 P19 L11 F08	K04 0,9920 0,9702 0,9613 0,9506 0,9467 0,9447 0,9096 0,9031 0,8975 0,8968	K18 A06 A11 K04 C03 P06 C22 F21 L11	K18 0,9977 0,9892 0,9645 0,9613 0,9362 0,8752 0,8754 0,8752 0,8752 0,8732 0,8646		Cl. D L24 P06 P19 G08 A06 F21 C22 K04 E08 C03	L24 0,9904 0,9414 0,9402 0,9188 0,9094 0,9044 0,8986 0,8964 0,8695 0,8673	P06 P19 L24 C03 K18 A06 F21 P22 E08 C22	P06 0,9902 0,9738 0,9414 0,9024 0,8992 0,8815 0,8770 0,8762 0,8717 0,8682	P19 P06 L24 A06 C03 K04 F21 G08 P22 C22	P19 0,9905 0,9738 0,9402 0,9174 0,9040 0,9031 0,8787 0,8772 0,8770 0,8703			singl. B03 E08 F13 D11 A11 E14 L11 K04 O12 L24	B03 0,9965 0,8467 0,8272 0,8189 0,8106 0,7971 0,7943 0,7749 0,7568 0,7315	singl. E08 A11 A06 K04 C03 L11 D11 E14 G08 F13	E08 0,9908 0,9458 0,9333 0,8968 0,8934 0,8908 0,8827 0,8812 0,8806 0,8778
CI. B A06 K18 C03 K04 G08 A11 E08 F21 P19 L11 C22	A06 0,9935 0,9892 0,9790 0,9702 0,9688 0,9616 0,9333 0,9195 0,9137 0,9039	A11 K18 A06 E08 K04 C03 L11 L24 E14 G08 P19	A11 0,9892 0,9645 0,9616 0,9458 0,9447 0,9207 0,8765 0,8621 0,8574 0,8509 0,8483	C03 A06 K04 K18 C22 A11 P19 P06 E08 F21 G08	C03 0,9980 0,9790 0,9506 0,9362 0,9328 0,9207 0,9040 0,9024 0,8934 0,8857 0,8857	K04 A06 K18 C03 G08 A11 F21 P19 L11 E08 L24	K04 0,9920 0,9702 0,9613 0,9506 0,9467 0,9447 0,9096 0,9031 0,8975 0,8968 0,8964	K18 A06 A11 K04 C03 P06 C22 F21 L11 J06 P19	K18 0,9977 0,9892 0,9645 0,9613 0,9362 0,8992 0,8754 0,8752 0,8752 0,8732 0,8646 0,8571		CI. D L24 P06 P19 G08 A06 F21 C22 K04 E08 C03 A11	L24 0,9904 0,9414 0,9402 0,9188 0,9094 0,9044 0,8986 0,8964 0,8695 0,8673 0,8673 0,8621	P06 P19 L24 C03 K18 A06 F21 P22 E08 C22 G08	P06 0,9902 0,9738 0,9414 0,9024 0,8992 0,8815 0,8770 0,8762 0,8717 0,8682 0,8598	P19 P06 L24 A06 C03 K04 F21 G08 P22 C22 K18	P19 0,9905 0,9738 0,9402 0,9174 0,9040 0,9031 0,8787 0,8772 0,8772 0,8770 0,8703 0,8751			singl. B03 E08 F13 D11 A11 E14 L11 K04 O12 L24 A06	B03 0,9965 0,8467 0,8272 0,8189 0,8106 0,7971 0,7943 0,7749 0,7568 0,7315 0,7303	singl. E08 A11 A06 K04 C03 L11 D11 E14 G08 F13 P06	E08 0,9908 0,9458 0,9333 0,8968 0,8934 0,8908 0,8827 0,8812 0,8812 0,8806 0,8778 0,8778 0,8778
CI. B A06 K18 C03 K04 G08 A11 E08 F21 P19 L11 C22 L24	A06 0,9935 0,9892 0,9790 0,9702 0,9688 0,9616 0,9333 0,9195 0,9174 0,9137 0,9099 0,904	A11 K18 A06 E08 K04 C03 L11 L24 E14 G08 P19 D11	A11 0,9892 0,9645 0,9616 0,9458 0,9447 0,9207 0,8765 0,8621 0,8574 0,8574 0,8574 0,8483	C03 A06 K04 K18 C22 A11 P19 P06 E08 F21 G08 L24	C03 0,9980 0,9790 0,9506 0,9362 0,9328 0,9207 0,9040 0,9024 0,8934 0,8857 0,8841 0,8673	K04 A06 K18 C03 G08 A11 F21 P19 L11 E08 L24 C22	K04 0,9920 0,9702 0,9613 0,9506 0,9467 0,9447 0,9096 0,9031 0,8975 0,8968 0,8964 0,8769	K18 A06 A11 K04 C03 P06 C22 F21 L11 J06 P19 L24	K18 0,9977 0,9892 0,9645 0,9613 0,9362 0,8992 0,8754 0,8752 0,8752 0,8754 0,8752 0,8754 0,8752 0,8754 0,87571 0,8496		CI. D L24 P06 P19 G08 A06 F21 C22 K04 E08 C03 A11 O21	L24 0,9904 0,9414 0,9402 0,9188 0,9094 0,8986 0,8964 0,8695 0,8673 0,8621 0,8549	P06 P19 L24 C03 K18 A06 F21 P22 E08 C22 G08 L11	P06 0,9902 0,9738 0,9414 0,9024 0,8815 0,8770 0,8762 0,8770 0,8762 0,8782 0,8598 0,8524	P19 P06 L24 A06 C03 K04 F21 G08 P22 C22 K18 O21	P19 0,9905 0,9738 0,9402 0,9174 0,9040 0,9031 0,8787 0,8772 0,8770 0,8770 0,8770 0,87571 0,8557			singl. B03 E08 F13 D11 A11 E14 L11 K04 O12 L24 A06 G08	B03 0,9965 0,8467 0,8272 0,8189 0,8106 0,7971 0,7943 0,7749 0,7568 0,7315 0,7303 0,7192	singl. E08 A11 A06 K04 C03 L11 D11 E14 G08 F13 P06 L24	E08 0,9908 0,9458 0,8933 0,8968 0,8934 0,8908 0,8827 0,8812 0,8806 0,8778 0,8778 0,8777 0,8695
CI. B A06 K18 C03 K04 G08 A11 E08 F21 P19 L11 C22 L24 P06	A06 0,9935 0,9892 0,9790 0,9702 0,9688 0,9668 0,9333 0,9195 0,9174 0,9137 0,9094 0,8815	A11 K18 A06 E08 K04 C03 L11 L24 E14 G08 P19 D11 P06	A11 0,9892 0,9645 0,9616 0,9458 0,9447 0,9207 0,8765 0,8621 0,8574 0,8509 0,8483 0,8431 0,8400	C03 A06 K04 K18 C22 A11 P19 P06 E08 F21 G08 L24 J06	C03 0,9980 0,9790 0,9506 0,9322 0,9327 0,9040 0,9024 0,8934 0,8857 0,8847 0,8857 0,8673 0,8598	K04 A06 K18 C03 G08 A11 F21 P19 L11 E08 L24 C22 J06	K04 0,9920 0,9702 0,9613 0,9467 0,9447 0,9096 0,9031 0,8975 0,8968 0,8968 0,8753	K18 A06 A11 K04 C03 P06 C22 F21 L11 J06 P19 L24 D11	K18 0,9977 0,9892 0,9645 0,9645 0,8962 0,8754 0,8752 0,8752 0,8752 0,8732 0,8646 0,8571 0,8496 0,8311		Cl. D L24 P06 P19 G08 A06 F21 C22 K04 E08 C03 A11 O21 K18	L24 0,9904 0,9414 0,9402 0,9084 0,9094 0,8086 0,8064 0,8695 0,8673 0,8621 0,8549 0,8496	P06 P19 L24 C03 K18 A06 F21 P22 E08 C22 G08 L11 K04	P06 0,9902 0,9738 0,9414 0,8992 0,8815 0,8770 0,8762 0,8717 0,8682 0,8717 0,8682 0,8524 0,8524	P19 P06 L24 A06 C03 K04 F21 G08 P22 C22 K18 O21 A11	P19 0,9905 0,9738 0,9402 0,9040 0,9040 0,9040 0,9040 0,8787 0,8772 0,8770 0,8703 0,8771 0,8557 0,8483			singl B03 E08 F13 D11 A11 E14 L11 K04 O12 L24 A06 G08 P19	B03 0,9965 0,8467 0,8272 0,8106 0,7971 0,7943 0,7749 0,7568 0,7315 0,7303 0,7192 0,7181	singl. E08 A11 A06 K04 C03 L11 D11 E14 G08 F13 P06 L24 F21	E08 0,9908 0,9458 0,8968 0,8968 0,8934 0,8908 0,8827 0,8812 0,8806 0,8778 0,8778 0,8777 0,8695 0,8550
CI. B A06 K18 C03 K04 G08 A11 E08 F21 P19 L11 C22 L24 P06 O21	A06 0,9935 0,9892 0,9790 0,9688 0,9616 0,9333 0,9195 0,9174 0,9137 0,9099 0,9094 0,8815 0,8741	A11 K18 A06 E08 K04 C03 L11 L24 E14 G08 P19 D11 P06 F21	A11 0,9892 0,9645 0,9458 0,9447 0,9207 0,8765 0,8627 0,8574 0,8574 0,8509 0,8483 0,8431 0,8400 0,8432	C03 A06 K04 K18 C22 A11 P19 P06 E08 F21 G08 L24 J06 L11	C03 0,9980 0,9506 0,9362 0,9328 0,9207 0,9040 0,9024 0,8934 0,8857 0,8841 0,8673 0,8598 0,8448	K04 A06 K18 C03 G08 A11 F21 P19 L11 E08 L24 C22 J06 D11	K04 0,9920 0,9702 0,9613 0,9506 0,9467 0,9447 0,9046 0,9031 0,8975 0,8968 0,8964 0,8759 0,8769 0,8753 0,8739	K18 A06 A11 K04 C03 P06 C22 F21 L11 J06 P19 L24 D11 E14	K18 0,9977 0,9645 0,9643 0,9643 0,9362 0,8754 0,8754 0,8752 0,8732 0,8740 0,8751 0,8466 0,8571 0,8464 0,8571 0,8441		Cl. D L24 P06 P19 G08 A06 F21 C22 K04 E08 C03 A11 O21 K18 J06	L24 0,9904 0,9414 0,9402 0,9188 0,9094 0,9044 0,8964 0,8695 0,8673 0,8621 0,8649 0,8496 0,8499 0,8498	P06 P19 L24 C03 K18 A06 F21 P22 E08 C22 G08 L11 K04 J06	P06 0,9902 0,9734 0,9024 0,8992 0,8815 0,8770 0,8762 0,8777 0,8682 0,8747 0,8682 0,8598 0,8598 0,8598 0,8496 0,8496	P19 P06 L24 A06 C03 K04 F21 G08 P22 C22 K18 O21 A11 J06	P19 0,9905 0,9738 0,9402 0,9174 0,9040 0,9031 0,8787 0,8770 0,8770 0,8703 0,8771 0,8557 0,8483 0,8435			singl. B03 E08 F13 D11 A11 E14 L11 K04 O12 L24 A06 G08 P19 P06	B03 0,9965 0,8467 0,8272 0,8106 0,7971 0,7943 0,7749 0,7568 0,7315 0,7303 0,7192 0,7181 0,7181 0,7182	singl. E08 A11 A06 K04 C03 L11 D11 E14 G08 F13 P06 L24 F21 C22	E08 0,9908 0,9458 0,9333 0,8968 0,8934 0,8908 0,8812 0,8812 0,8806 0,8878 0,8806 0,8778 0,8805 0,8717 0,8655 0,8495
CI. B A06 K18 C03 K04 G08 A11 E08 F21 P19 L11 C22 L24 P06 O21 J06	A06 0,9935 0,9892 0,9702 0,9688 0,9616 0,9333 0,9195 0,9174 0,9137 0,9099 0,9094 0,8731	A11 K18 A06 E08 K04 C03 L11 L24 E14 G08 P19 D11 P06 F21 C22	A11 0,9892 0,9645 0,9645 0,9458 0,9458 0,9458 0,8574 0,8579 0,8574 0,8509 0,8483 0,8431 0,8400 0,8433 0,8431 0,8402	C03 A06 K04 K18 C22 A11 P19 P06 E08 F21 G08 L24 J06 L11 O21	C03 0,9980 0,9506 0,9362 0,9328 0,9207 0,9040 0,9040 0,8934 0,8857 0,8841 0,8867 0,8841 0,8673 0,8848 0,8434	K04 A06 K18 C03 G08 A11 F21 P19 L11 E08 L24 C22 J06 D111 E14	K04 0,9920 0,9702 0,9613 0,9506 0,9447 0,9096 0,9047 0,8975 0,8968 0,8964 0,8769 0,8753 0,8753 0,8753 0,8753	K18 A06 A11 K04 C03 P06 C22 F21 L11 J06 P19 L24 D11 E14 G08	K18 0,9977 0,9645 0,9645 0,9663 0,9362 0,8754 0,8752 0,8752 0,8752 0,8746 0,8571 0,8466 0,8571 0,8466 0,8571 0,8484 0,8284		Cl. D L24 P06 P19 G08 A06 F21 C22 K04 E08 C03 A11 O21 K18 J06 L11	L24 0,9904 0,9414 0,9402 0,9084 0,8094 0,8095 0,8673 0,8673 0,8673 0,8621 0,8549 0,8549 0,8378 0,8358	P06 P19 L24 C03 K18 A06 F21 P22 E08 C22 G08 L11 K04 J06 A11	P06 0,9902 0,9738 0,9414 0,8992 0,8815 0,8770 0,8762 0,8777 0,8682 0,8598 0,8598 0,8524 0,8598 0,8524 0,8400	P19 P06 L24 A06 C03 K04 F21 G08 P22 C22 K18 O21 A11 J06 L11	P19 0,9905 0,9738 0,9402 0,9174 0,9040 0,9031 0,8787 0,8772 0,8770 0,8703 0,8757 0,8757 0,8433 0,8557 0,8433 0,8435			singl. E08 F13 D11 A11 E14 L11 K04 O12 L24 A06 G08 P19 P06 K18	B03 0,9965 0,8467 0,8272 0,8189 0,8106 0,7971 0,7943 0,7749 0,7568 0,7315 0,7315 0,7303 0,7192 0,7181 0,6953	singl. E08 A11 A06 K04 C03 L11 D11 E14 G08 F13 P06 L24 F21 C22 B03	E08 0,9908 0,9458 0,9333 0,8968 0,8934 0,8908 0,8827 0,8806 0,8806 0,8708 0,8777 0,8895 0,8717 0,8695 0,87550 0,8495
CI. B A06 K18 C03 K04 G08 A11 E08 F21 P19 L11 C22 L24 P06 O21 J06 D11	A06 0,9935 0,9790 0,9790 0,9702 0,9688 0,9164 0,9137 0,9174 0,9099 0,9094 0,8815 0,8741 0,8737	A11 K18 A06 E08 K04 C03 L11 L24 E14 G08 P19 D11 P06 F21 C22 B03	A11 0,9892 0,9645 0,9458 0,9447 0,8765 0,8621 0,8574 0,8574 0,8509 0,8483 0,8431 0,8400 0,8432 0,8132 0,8106	C03 A06 K04 K18 C22 A11 P19 P06 E08 F21 G08 L24 J06 L11 O21 D11	C03 0,9980 0,9790 0,9506 0,9362 0,924 0,9024 0,8934 0,8857 0,8841 0,8673 0,8598 0,8448 0,8598	K04 A06 K18 C03 G08 A11 F21 P19 L11 E08 L24 C22 J06 D111 E14 O21	K04 0,9920 0,9613 0,966 0,9467 0,9096 0,9096 0,9096 0,9096 0,9096 0,9096 0,9096 0,9096 0,9096 0,9096 0,8753 0,8753 0,8753 0,8753 0,8668	K18 A06 A11 K04 C03 P06 C22 F21 L11 J06 P19 L24 D11 E14 G08 O21	K18 0,9877 0,9892 0,9645 0,9613 0,9362 0,8752 0,8752 0,8752 0,8752 0,8752 0,8752 0,8752 0,8752 0,8752 0,8751 0,8496 0,8311 0,8244 0,8239 0,8214		Cl. D L24 P06 P19 G08 A06 F21 C22 K04 E08 C03 A11 O21 K18 J06 L11 D11	L24 0,9904 0,9412 0,9188 0,9094 0,8986 0,8964 0,8695 0,8673 0,8627 0,8627 0,8649 0,8496 0,8496 0,8496 0,8378 0,8058	P06 P19 L24 C03 K18 A06 F21 P22 E08 C22 G08 L11 K04 J06 A11 E14	P06 0,9902 0,9414 0,9024 0,8992 0,8750 0,8762 0,8777 0,8682 0,8598 0,8524 0,8598 0,8524 0,8496 0,8472 0,8400 0,8337	P19 P06 L24 A06 C03 K04 F21 G08 P22 C22 K18 O21 A11 J06 L11 D11	P19 0,9905 0,9738 0,9402 0,9174 0,9040 0,9040 0,8787 0,8772 0,8770 0,8770 0,8773 0,8577 0,8557 0,8557 0,8483 0,8557 0,8483 0,8485 0,8365			singl. B03 E08 F13 D11 A11 E14 L11 K04 O12 L24 A06 G08 P19 P06 K18 C03	B03 0,9965 0,8467 0,8129 0,8189 0,8189 0,7943 0,7749 0,7545 0,7315 0,7303 0,7315 0,7303 0,7192 0,7181 0,6933 0,6893	singl. E08 A11 A06 K04 C03 L11 D11 E14 G08 F13 P06 L24 F21 C22 B03 P19	E08 0,9908 0,9458 0,8968 0,8933 0,8968 0,8934 0,8827 0,8812 0,8806 0,8717 0,8695 0,8550 0,8495 0,8495 0,8495 0,8496
CI. B A06 K18 C03 K04 G08 A11 E08 F21 P19 L11 C22 L24 P06 O21 J06 D11 E14	A06 0,9935 0,9790 0,9702 0,9688 0,9688 0,9616 0,9133 0,9195 0,9174 0,9137 0,9094 0,904 0,8615 0,8741 0,8737 0,8593 0,8522	A11 K18 A06 E08 K04 C03 L11 L24 E14 G08 P19 D11 P06 F21 C22 B03 F13	A11 0,9892 0,9645 0,9616 0,9458 0,9447 0,8765 0,8621 0,8764 0,8509 0,8483 0,8400 0,8332 0,8400 0,8332 0,8122 0,8106	C03 A06 K04 K18 C22 A11 P19 P06 E08 F21 G08 L24 J06 L11 O21 D11 E14	C03 0,9980 0,9506 0,9362 0,9328 0,9207 0,9040 0,9024 0,8934 0,8857 0,8843 0,8673 0,8598 0,8448 0,8434 0,7918	K04 A06 K18 C03 G08 A11 F21 P19 L11 E08 L24 C22 J06 D11 E14 O21 P06	K04 0,9702 0,9613 0,9506 0,9467 0,9046 0,9096 0,9096 0,9096 0,9096 0,9096 0,9096 0,9096 0,8753 0,8759 0,8753 0,8759 0,8753 0,8759 0,8667 0,8658	K18 A06 A11 K04 C03 P06 C22 F21 L11 J06 P19 L24 D11 E14 G08 O21 E08	K18 0,9977 0,9892 0,9645 0,9613 0,9362 0,8752 0,8752 0,8752 0,8752 0,8732 0,8646 0,8571 0,8446 0,8311 0,8284 0,8214 0,8284 0,8216		Cl. D L24 P06 P19 G08 A06 F21 C22 K04 E08 C03 A11 O21 K18 J06 L11 D11 E14	L24 0,9904 0,9414 0,9402 0,9188 0,9094 0,8086 0,8695 0,8673 0,8621 0,8649 0,8496 0,8378 0,8496 0,8378 0,8378	P06 P19 L24 C03 K18 A06 F21 P22 E08 C22 G08 L11 K04 J06 A11 E14 O21	P06 0,9902 0,9738 0,9414 0,8024 0,8815 0,8762 0,8762 0,8762 0,8762 0,8762 0,8784 0,8472 0,8496 0,8472 0,8496 0,8437 0,8216	P19 P06 L24 A06 C03 K04 F21 G08 P22 C22 K18 O21 A11 J06 L11 D11 E08	P19 0,9905 0,9738 0,9402 0,9174 0,9040 0,9040 0,8787 0,8772 0,8770 0,8773 0,8773 0,8773 0,8773 0,8757 0,8483 0,8435 0,8483 0,8435 0,8435			singl. B03 E08 F13 D11 A11 E14 L11 K04 O12 L24 A06 G08 P19 P06 K18 C03 P22	B03 0,8965 0,8272 0,8189 0,8106 0,7941 0,7943 0,7749 0,7568 0,7315 0,7303 0,7152 0,7152 0,7181 0,6953 0,6882 0,6882	singl. E08 A11 A06 K04 C03 L11 D11 E14 G08 F13 P06 L24 F21 C22 B03 P19 K18	E08 0,9908 0,9458 0,9333 0,8968 0,8934 0,8827 0,8812 0,8806 0,8778 0,8717 0,8695 0,8717 0,8550 0,8550 0,8467 0,8495 0,8467
CI. B A06 K18 C03 K04 G08 A11 E08 F21 P19 L11 C22 L24 P06 O21 J06 D11 E14 F13	A06 0,9355 0,9590 0,9702 0,9688 0,9195 0,9195 0,9137 0,9094 0,8815 0,8741 0,8737 0,8593 0,8524 0,7540	A11 K18 A06 E08 K04 C03 L11 L24 E14 G08 P19 D11 P06 F21 C22 B03 F13 J06	A11 0,9645 0,9616 0,9458 0,9477 0,9207 0,8765 0,8621 0,8579 0,8483 0,8431 0,8403 0,8433 0,8431 0,8403 0,8332 0,8122 0,8166 0,8060 0,7824	C03 A06 K04 K18 C22 A11 P19 P06 E08 F21 G08 L24 J06 L11 O21 D11 E14 F13	C03 0,9980 0,9506 0,9362 0,9362 0,9024 0,9024 0,8934 0,8857 0,8841 0,8673 0,8867 0,8841 0,8673 0,8448 0,8448 0,8444 0,7918 0,7918	K04 A06 K18 C03 G08 A11 F21 P19 L11 E08 L24 C22 J06 D11 E14 O21 P06 B03	K04 0,9920 0,9613 0,9506 0,9031 0,9031 0,8958 0,8964 0,8769 0,8753 0,8769 0,8769 0,8769 0,8753 0,8739 0,8667 0,8668 0,8749	K18 A06 A11 K04 C03 P06 C22 F21 L11 J06 P19 L24 D11 E14 G08 O21 E08 F13	K18 0,9977 0,9892 0,9645 0,9643 0,8962 0,8754 0,8752 0,8732 0,8746 0,8752 0,8732 0,8646 0,8571 0,8496 0,8571 0,8496 0,8239 0,8214 0,8239 0,8214		Cl. D L24 P06 P19 G08 A06 F21 C22 K04 E08 C03 A11 O21 K18 J06 L11 D11 E14 F13	L24 0,9904 0,9412 0,9188 0,9044 0,8986 0,8964 0,8695 0,8673 0,8621 0,8549 0,8649 0,8549 0,8549 0,8378 0,8378 0,8378 0,7382 0,7382	P06 P19 L24 C03 K18 A06 F21 P22 E08 C22 G08 L11 K04 J06 A11 E14 O21 D11	P06 0,9902 0,9414 0,9024 0,8902 0,8815 0,8700 0,8762 0,8717 0,8682 0,8598 0,8598 0,8598 0,8598 0,8496 0,8490 0,8442 0,8400 0,8337 0,8216	P19 P06 L24 A06 C03 K04 F21 G08 P22 C22 K18 O21 A11 J06 L11 D11 E08 E14	P19 0,9905 0,9402 0,9174 0,9001 0,8787 0,8772 0,8770 0,8773 0,8773 0,8557 0,84835 0,8485 0,8485 0,8485 0,8485 0,8485 0,8485 0,8485			singl. B03 E08 F13 D11 A11 E14 L11 K04 O12 L24 A06 G08 P19 P06 K18 C03 P22 F21	B03 0,9965 0,8467 0,8272 0,8189 0,7971 0,7943 0,7749 0,7568 0,7375 0,7303 0,7192 0,7182 0,6938 0,6938 0,6696	singl. E08 A11 A06 K04 C03 L11 D11 E14 G08 F13 P06 L24 F21 C22 B03 P19 K18 J06	E08 0,9908 0,9458 0,8933 0,8968 0,8934 0,8827 0,8812 0,8806 0,8717 0,8550 0,8778 0,8757 0,8550 0,8467 0,8467 0,8198 0,8033
CI. B A06 K18 C03 K04 G08 F21 P19 L11 C22 L24 P06 O21 D11 E14 F13 O12	A06 0,935 0,9790 0,9790 0,9702 0,9688 0,9174 0,9137 0,9174 0,9174 0,9099 0,9094 0,8091 0,8741 0,8737 0,8593 0,8593 0,8593 0,7540	A11 K18 A06 E08 K04 C03 L11 L24 E14 G08 P19 D11 P06 F21 C22 B03 F13 J06 O21	A11 0,9892 0,9645 0,9616 0,9458 0,9447 0,8207 0,8274 0,8509 0,8433 0,8431 0,8400 0,8332 0,8122 0,8122 0,8126 0,8126 0,8126 0,8126 0,8126 0,8126 0,8126 0,8126 0,8126 0,8126 0,8126 0,8126 0,8126 0,8126 0,8126 0,9616 0,9645 0,9616 0,9645 0,9616 0,9458 0,9457 0,8207 0,8207 0,8207 0,8207 0,8207 0,8458 0,8458 0,8458 0,9447 0,8207 0,8207 0,8574 0,8574 0,8458 0,8458 0,8458 0,8457 0,8458 0,8458 0,8457 0,8574 0,8458 0,8458 0,8458 0,8458 0,8458 0,8457 0,8458 0,8458 0,8459 0,8458 0,8459 0,8458 0,8459000000000000000000000000000000000000	C03 A06 K04 K18 C22 A11 P19 P06 E08 F21 G08 F21 G08 F21 G08 L24 J06 L11 O21 D11 E14 F13 O12	C03 0,9980 0,9790 0,9506 0,9328 0,9207 0,9040 0,8934 0,8857 0,8843 0,8673 0,8673 0,8598 0,8448 0,7918 0,7844 0,7918	K04 A06 K18 C03 G08 A11 F21 P19 L11 E08 L24 C22 J06 D11 E14 O21 P06 B03 O12	K04 0,9920 0,9702 0,9467 0,9467 0,9447 0,906 0,9031 0,8975 0,8968 0,8753 0,8769 0,8753 0,8769 0,8753 0,8739 0,8668 0,8769 0,8658 0,8668 0,8749 0,8658	K18 A06 A11 K04 C03 P06 C22 F21 L11 J06 P19 L24 D11 E14 G08 O21 E08 F13 O12	K18 0,9977 0,9882 0,9645 0,9645 0,8962 0,8754 0,8752 0,8732 0,8746 0,8754 0,8754 0,8754 0,8754 0,8764 0,8239 0,8214 0,8224 0,8224 0,8224 0,8224 0,8224 0,8224 0,8224 0,8224 0,8224 0,8224 0,8224 0,8224 0,8224 0,8224 0,8224 0,8245 0,8245 0,8245 0,8252 0,825		Cl. D L24 P06 P19 G08 F21 C22 K04 E08 C03 A11 O21 K18 J06 L11 D11 E14 F13 B03	L24 0,9904 0,9442 0,9084 0,9094 0,8095 0,8673 0,8621 0,8549 0,8549 0,8549 0,8549 0,8549 0,8549 0,8549 0,8549 0,8549 0,8549 0,8549 0,8558 0,7682 0,7682 0,7315	P06 P19 L24 C03 K18 A06 F21 P22 E08 C22 G08 L11 K04 J06 A11 E14 O21 D11 F13	P06 0,9902 0,9738 0,9414 0,8092 0,8815 0,8770 0,8762 0,8747 0,8682 0,8747 0,8682 0,8496 0,8420 0,8490 0,8437 0,8216 0,7831	P19 P06 L24 A06 C03 K04 F21 G08 P22 C22 K18 O21 A11 J06 L11 D11 D11 E08 E14 F13	P19 0,9905 0,9738 0,9402 0,9040 0,9040 0,9041 0,8770 0,8770 0,8770 0,8770 0,8773 0,8773 0,8773 0,8757 0,8483 0,8483 0,8485 0,8224 0,8188 0,7825			singl. B03 E08 F13 D11 A11 E14 L11 K04 O12 L24 A06 G08 P19 P06 K18 C03 P22 F21 J06	B03 0,8467 0,8272 0,8189 0,8168 0,7971 0,7943 0,7749 0,7568 0,7303 0,7303 0,7303 0,7303 0,7303 0,7192 0,7181 0,6953 0,6958 0,6842 0,6785 0,6606	singl. E08 A11 A06 K04 C03 L11 D11 E14 G08 F13 P06 L24 F21 C22 B03 P19 K18 J06 O12	E08 0,9908 0,9458 0,9333 0,8968 0,8908 0,8812 0,8812 0,8816 0,8816 0,8778 0,8895 0,8550 0,8495 0,8495 0,8495 0,8495 0,8495 0,8495 0,8495
Cl. B A06 K18 C03 K04 G08 A11 E08 F21 P19 L11 C22 L24 P06 O21 J06 D11 E14 F13 O12 B03	A06 0,9935 0,9790 0,9702 0,9688 0,9174 0,9137 0,9174 0,9137 0,9099 0,815 0,8741 0,8741 0,8743 0,8593 0,8522 0,7374 0,7333	A11 K18 A06 E08 K04 C03 L11 L24 E14 E14 G08 P19 D11 P06 F21 C22 B03 F13 J06 O21 O12	A11 0,9892 0,9645 0,9616 0,9488 0,9447 0,8765 0,8627 0,8574 0,8509 0,8483 0,8430 0,8483 0,8400 0,8332 0,8106 0,8060 0,7653	C03 A06 K04 C22 A11 P19 P06 E08 F21 G08 L24 J06 L11 O21 D11 E14 F13 O12 B03	C03 0,9980 0,9790 0,9506 0,9322 0,9328 0,9207 0,9040 0,9024 0,8934 0,8857 0,8841 0,8658 0,8598 0,8598 0,8598 0,8448 0,7918 0,7948 0,7948 0,7948	K04 A06 K18 C03 G08 A11 F21 P19 L11 E08 L24 C22 J06 D11 E14 O21 P06 B03 O12 F13	K04 0,9920 0,9702 0,9467 0,9467 0,9467 0,9096 0,9037 0,8968 0,8968 0,8753 0,8753 0,8753 0,8753 0,8753 0,8753 0,8753 0,8753 0,8753 0,8753 0,8753 0,8753 0,8753 0,8753 0,8753 0,8753 0,8753 0,8753 0,8668	K18 A06 A11 K04 C03 P06 C22 F21 L11 J06 P19 L24 D11 E14 G08 O21 E08 F13 O12 B03	K18 0,9977 0,9892 0,9645 0,9645 0,8962 0,8754 0,8752 0,8742 0,8752 0,8742 0,8752 0,8466 0,8311 0,8284 0,8311 0,8284 0,8214 0,8076 0,7303 0,6982		Cl. D L24 P06 P19 G08 A06 F21 C22 K04 E08 C03 A11 O21 K18 J06 L11 D11 E14 F13 B03 O12	L24 0,9904 0,9414 0,9402 0,908 0,8964 0,8964 0,8665 0,8673 0,8621 0,8549 0,8648 0,8678 0,8678 0,8678 0,8378 0,8378 0,8378 0,8378 0,7082 0,7352 0,7315	P06 P19 L24 C03 K18 A06 F21 P22 E08 C22 G08 L11 K04 J06 A11 E14 O21 D11 F13 B03	P06 0,9902 0,9738 0,9414 0,8992 0,8815 0,8770 0,8682 0,8717 0,8682 0,8747 0,8682 0,8472 0,8496 0,8472 0,8496 0,8472 0,8496 0,8437 0,8216 0,7311 0,6953	P19 P06 L24 A06 C03 K04 F21 G08 F22 C22 K18 O21 A11 J06 L11 D11 E08 E14 F13 O12	P19 0,9905 0,9738 0,9402 0,9040 0,9040 0,9040 0,8703 0,8772 0,8770 0,8703 0,8703 0,8557 0,8483 0,8483 0,8483 0,8485 0,8483 0,8493 0,8493 0,8493 0,8493 0,8793 0,779			singl. B03 E08 F13 D11 A11 E14 L11 K04 O12 L24 A06 G08 P19 P06 K18 C03 P22 F21 J06 C22	B03 0,9965 0,8467 0,8272 0,8106 0,7971 0,7943 0,7749 0,7588 0,7315 0,7303 0,7192 0,7181 0,6953 0,6983 0,6842 0,6785 0,6606 0,6367	singl. E08 A11 A06 K04 C03 L11 D11 E14 G08 F13 P06 L24 F21 C22 B03 P19 K18 J06 O12 O21	E08 0,9458 0,9458 0,9333 0,8868 0,8827 0,8827 0,8827 0,8842 0,8878 0,8778 0,8778 0,8778 0,8778 0,8749 0,8749 0,8749 0,8467 0,8495 0,8467 0,8495 0,8467 0,8495 0,8467 0,8198

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.

CI. A	C22		F21		H14		J20		O21	CI. B	A11		C03		K04		K18	singl.	B03
C22	0,9927	F21	0,9968	F21	0,9865	J20	0,9924	021	0,9895	A11	0,9910	C03	0,9702	K04	0,9946	K18	0,9938	B03	0,9350
F21	0,9893	M10	0,9895	H14	0,9839	F21	0,9819	C22	0,9877	K18	0,9785	K04	0,9690	K18	0,9872	K04	0,9872	D11	0,8695
O21	0,9877	C22	0,9893	M10	0,9834	K04	0,9794	F21	0,9837	K04	0,9727	A11	0,9580	J20	0,9794	A11	0,9785	E08	0,8434
M10	0,9817	J19	0,9866	J19	0,9818	C22	0,9726	H14	0,9748	C03	0,9580	K18	0,9488	A11	0,9727	J20	0,9668	L11	0,8348
H14	0,9804	H14	0,9865	C22	0,9804	H14	0,9705	M10	0,9744	J20	0,9464	J20	0,9104	C03	0,9690	C03	0,9488	C14	0,8320
J19	0,9763	021	0,9837	C14	0,9775	M10	0,9694	J20	0,9667	F21	0,9159	F21	0,8687	F21	0,9662	F21	0,9435	H07	0,8291
C14	0,9731	J20	0,9819	O21	0,9748	K18	0,9668	J19	0,9648	C22	0,9076	O21	0,8616	C22	0,9587	C22	0,9306	J20	0,8291
J20	0,9726	C14	0,9806	J20	0,9705	J19	0,9667	C14	0,9648	O21	0,9066	C22	0,8537	O21	0,9543	M10	0,9289	N21	0,8254
L11	0,9663	H07	0,9714	L11	0,9692	021	0,9667	L11	0,9578	H14	0,8995	H14	0,8451	M10	0,9538	H14	0,9280	P06	0,8223
H07	0,9598	012	0,9702	H07	0,9689	C14	0,9622	K04	0,9543	M10	0,8942	M10	0,8383	H14	0,9535	J19	0,9270	J19	0,8216
K04	0,9587	L11	0,9679	012	0,9686	H07	0,9593	H07	0,9482	J19	0,8870	C14	0,8301	J19	0,9515	021	0,9237	012	0,8213
012	0,9587	K04	0,9662	K04	0,9535	012	0,9526	012	0,9454	H07	0,8819	H07	0,8214	H07	0,9441	H07	0,9227	M10	0,8170
N21	0,9428	D11	0,9496	N21	0,9519	A11	0,9464	N21	0,9306	C14	0,8816	J19	0,8184	C14	0,9416	C14	0,9187	H14	0,8117
K18	0,9306	N21	0,9489	D11	0,9498	D11	0,9356	K18	0,9237	012	0,8709	E08	0,8090	012	0,9367	012	0,9126	F21	0,8101
D11	0,9299	K18	0,9435	P06	0,9321	L11	0,9287	D11	0,9128	E08	0,8639	012	0,8010	D11	0,9042	E08	0,9115	021	0,8048
P06	0,9143	P06	0,9265	K18	0,9280	C03	0,9104	A11	0,9066	D11	0,8394	P06	0,7886	L11	0,8960	D11	0,8847	C22	0,8016
A11	0,9076	A11	0,9159	A11	0,8995	N21	0,9099	P06	0,8997	L11	0,8123	D11	0,7811	E08	0,8952	L11	0,8558	K18	0,7985
C03	0,8537	E08	0,8693	E08	0,8625	E08	0,8977	C03	0,8616	N21	0,7806	N21	0,7716	N21	0,8743	N21	0,8348	K04	0,7938
E08	0,8337	C03	0,8687	C03	0,8451	P06	0,8769	E08	0,8116	B03	0,7752	L11	0,7576	P06	0,8347	B03	0,7985	A11	0,7752
B03	0,8016	B03	0,8101	B03	0,8117	B03	0,8291	B03	0,8048	P06	0,7351	B03	0,7311	B03	0,7938	P06	0,7954	C03	0,7311
cı. c	D11		L11		N21		012			CI. E	C14		H07		J19		M10	sinal.	E08
D11	0.9897	L11	0.9993	N21	0.9939	J19	0.9884			J19	0.9869	J19	0.9860	J19	0.9980	M10	0.9919	E08	0.9908
012	0,9743	N21	0,9901	L11	0,9901	012	0,9870			M10	0,9835	H07	0,9820	M10	0,9915	J19	0,9915	D11	0,9209
J19	0,9731	P06	0,9819	P06	0,9869	M10	0,9787			C14	0,9829	012	0,9783	012	0,9884	F21	0,9895	K18	0,9115
N21	0,9667	J19	0,9801	J19	0,9727	H07	0,9783			F21	0,9806	M10	0,9779	C14	0,9869	C14	0,9835	012	0,9028
H07	0,9656	M10	0,9769	012	0,9669	C14	0,9778			012	0,9778	C14	0,9752	F21	0,9866	H14	0,9834	H07	0,8982
C14	0,9638	C14	0,9756	D11	0,9667	D11	0,9743			H14	0,9775	F21	0,9714	H07	0,9860	C22	0,9817	J20	0,8977
M10	0,9592	012	0,9715	C14	0,9653	L11	0,9715			L11	0,9756	H14	0,9689	H14	0,9818	012	0,9787	J19	0,8957
P06	0,9590	H14	0,9692	M10	0,9632	F21	0,9702			H07	0,9752	D11	0,9656	L11	0,9801	H07	0,9779	K04	0,8952
L11	0,9583	F21	0,9679	H07	0,9567	H14	0,9686			C22	0,9731	L11	0,9636	C22	0,9763	L11	0,9769	C14	0,8774
H14	0,9498	C22	0,9663	H14	0,9519	N21	0,9669			N21	0,9653	C22	0,9598	D11	0,9731	O21	0,9744	M10	0,8720
F21	0,9496	H07	0,9636	F21	0,9489	C22	0,9587			021	0,9648	J20	0,9593	N21	0,9727	J20	0,9694	F21	0,8693
J20	0,9356	D11	0,9583	C22	0,9428	P06	0,9559			D11	0,9638	N21	0,9567	J20	0,9667	N21	0,9632	A11	0,8639
C22	0,9299	O21	0,9578	O21	0,9306	J20	0,9526			J20	0,9622	O21	0,9482	O21	0,9648	D11	0,9592	H14	0,8625
E08	0,9209	J20	0,9287	J20	0,9099	021	0,9454			P06	0,9503	K04	0,9441	P06	0,9582	K04	0,9538	B03	0,8434
021	0,9128	K04	0,8960	K04	0,8743	K04	0,9367			K04	0,9416	P06	0,9411	K04	0,9515	P06	0,9455	N21	0,8368
K04	0,9042	K18	0,8558	E08	0,8368	K18	0,9126			K18	0,9187	K18	0,9227	K18	0,9270	K18	0,9289	C22	0,8337
K18	0,8847	B03	0,8348	K18	0,8348	E08	0,9028			A11	0,8816	E08	0,8982	E08	0,8957	A11	0,8942	P06	0,8190
B03	0.8695	F08	0.8187	B03	0.9254		0.0700				0.0774		0.0010	A11	0.9970		0.9700	1.4.4	0.8187
	-,		0,0101	000	0,0234	ATT	0,8709			E08	0,8774	A11	0,0019	A 11	0,0070	EUO	0,0720	LUU	-,
A11	0,8394	A11	0,8123	A11	0,7806	B03	0,8709 0,8213			E08 B03	0,8774 0,8320	A11 B03	0,8819	B03	0,8216	C03	0,8383	021	0,8116

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.

CI. 1	H109		H182		H182a	1	H182b		1151		P156		CI. 5	B112		C017		F231		N158		P091			
H109	0,9918	H182b	0,9765	H182a	0,9953	H182b	0,9927	1151	0,9713	P156	0,9811		B112	0,9863	C017	0,9971	F231	0,9955	N158	0,9958	P091	0,9854			
P156	0,9732	H182	0,9727	H182b	0,9716	H182	0,9765	P156	0,9489	H109	0,9732		F231	0,9788	F231	0,9914	C017	0,9914	P091	0,9727	F231	0,9727			
1151	0,9430	H182a	0,9562	H182	0,9562	H182a	0,9716	H109	0,9430	1151	0,9489		C017	0,9748	B112	0,9748	B112	0,9788	F231	0,9723	N158	0,9727			
H182	0,9274	P156	0,9299	P156	0,9241	P156	0,9295	H182	0,9291	H182	0,9299		N158	0,9685	N158	0,9732	P091	0,9727	B112	0,9685	B112	0,9675			
H182a	0,9222	1151	0,9291	H109	0,9222	H109	0,9213	H182b	0,9005	H182b	0,9295		P091	0,9675	P091	0,9665	N158	0,9723	C017	0,9642	C017	0,9665			
H182b	0.9213	H109	0.9274	A071a	0.9187	A071a	0.9101	H182a	0.8992	H182a	0.9241		K022b	0.9451	N178	0.9642	H035	0.9466	1051	0.9341	P156	0.9117			
A071	0.8848	A071b	0.8061	K022a	0.9006	1051	0.9021	N158	0.8094	P091	0.9117		1051	0.9379	H035	0.9454	K022b	0.9419	K022a	0.9171	K022b	0.8982			
F231	0,8722	A071a	0,8015	1051	0,8994	K022b	0,9008	A071a	0,7880	N158	0,9090		A071a	0,9315	A071a	0,9453	A071a	0,9393	K022b	0,9151	1051	0,8935			
N158	0.8602	N178	0.7851	1151	0,8992	1151	0,9005	P091	0.7718	F231	0.9032		H035	0.9232	1051	0.9248	N178	0.9388	A071a	0.9115	A071a	0.8890			
A071b	0,8588	H035	0,7664	K022b	0,8909	K022a	0,9002	M098	0,7638	C017	0,8901		K022a	0,9219	K022b	0,9214	D178	0,9201	P156	0,9090	D178	0,8877			
H035	0,8576	A071	0,7445	B112	0,8878	H035	0,8885	1051	0,7635	B112	0,8897		D178	0,9146	A071b	0,9177	1051	0,9190	H035	0,9079	N178	0,8872			
C017	0,8540	M098	0,7274	N158	0,8838	B112	0,8883	N178	0,7570	H035	0,8860		M098	0,9102	M098	0,9157	A071b	0,9134	N063	0,9077	H035	0,8834			
B112	0,8450	1051	0,7259	N063	0,8831	F231	0,8832	H035	0,7502	A071a	0,8807		N178	0,9096	D178	0,9147	A071	0,9122	H182a	0,8838	K022a	0,8788			
P091	0,8425	N158	0,7229	H035	0,8771	N063	0,8780	K022a	0,7433	K022b	0,8806		N063	0,8967	A071	0,9123	K022a	0,9103	D178	0,8809	N063	0,8771			
A071a	0,8362	K022	0,7079	F231	0,8748	N158	0,8775	K022	0,7424	D178	0,8766		A071	0,8944	K022a	0,9039	M098	0,9058	H182b	0,8775	M098	0,8702			
M098	0,8336	P091	0,7053	C017	0,8726	N178	0,8743	B112	0,7313	K022a	0,8764		P156	0,8897	N063	0,8957	P156	0,9032	N178	0,8708	H182b	0,8550			
K022b	0,8332	B112	0,7006	M098	0,8701	C017	0,8692	A071b	0,7211	1051	0,8729		H182b	0,8883	P156	0,8901	N063	0,8922	M098	0,8703	A0/1	0,8501			
D178	0,8216	KUZZA	0,6771	D178	0,8671	K022	0,8650	F231	0,6824	M098	0,8648		H1828	0,8878	H1828	0,8726	H182D	0,8832	H109	0,8602	H1828	0,8429			
N179	0,0104	C017	0,0070	N1/0	0,0091	D179	0,0570	K022h	0,6765	NU03	0,0040		AU7 ID	0,0010	L102D	0,0092	H102a	0,0740	A0710	0,0570	A071b	0,0425			
N063	0,0149	E231	0,0031	K022	0,8520	P001	0,8550	A071	0,0755	N178	0,8533		K022	0,8430	K022	0,0340	K022	0,0722	K022	0,0420	K022	0,0300			
1051	0 7740	N063	0.6519	P091	0.8429	A071b	0.8318	D178	0.6469	A071b	0.8383		1151	0 7313	1151	0.6761	1151	0.6824	1151	0.8094	1151	0 7718			
K022	0.7245	K022b	0.6393	A071	0.8418	A071	0.8075	N063	0.6304	K022	0 7781		H182	0,7006	H182	0.6651	H182	0.6574	H182	0,7229	H182	0,7053			
CI 2	A071		Δ071a	I	A071b	1	M098		CI 3	K022		K022a		K022h		N178		CI 4	D178		H035		1051		N063
<i>Cl. 2</i>	A071	A071a	A071a	A071b	A071b	M098	M098		CI. 3 K022	K022	K022a	K022a	K022b	K022b	N178	N178		<i>Cl. 4</i>	D178	H035	H035	1051	1051	N063	N063
<i>Cl. 2</i> A071 A071b	A071 0,9903 0.9785	A071a M098	A071a 0,9926 0.9717	A071b A071	A071b 0,9903 0.9785	M098 A071a	M098 0,9796 0.9717		<i>Cl.</i> 3 K022 K022a	K022 0,9777 0.9424	K022a K022b	K022a 0,9810 0.9523	K022b K022a	K022b 0,9890 0.9523	N178 C017	N178 0,9963 0.9732		<i>Cl. 4</i> D178 H035	D178 0,9953 0.9672	H035 D178	H035 0,9914 0.9672	1051 N063	1051 0,9929 0.9794	N063	N063 0,9929 0.9794
<i>Cl. 2</i> A071 A071b A071a	A071 0,9903 0,9785 0,9526	A071a M098 A071	A071a 0,9926 0,9717 0,9526	A071b A071 A071a	A071b 0,9903 0,9785 0,9512	M098 A071a A071	M098 0,9796 0,9717 0,9481		<i>Cl.</i> 3 K022 K022a K022b	K022 0,9777 0,9424 0.9352	K022a K022b K022	K022a 0,9810 0,9523 0.9424	K022b K022a B112	K022b 0,9890 0,9523 0,9451	N178 C017 K022b	N178 0,9963 0,9732 0,9426		Cl. 4 D178 H035 N063	D178 0,9953 0,9672 0,9641	H035 D178 N063	H035 0,9914 0,9672 0.9618	1051 N063 D178	1051 0,9929 0,9794 0,9602	N063 1051 D178	N063 0,9929 0,9794 0.9641
<i>Cl. 2</i> A071 A071b A071a M098	A071 0,9903 0,9785 0,9526 0,9481	A071a M098 A071 A071b	A071a 0,9926 0,9717 0,9526 0,9512	A071b A071 A071a M098	A071b 0,9903 0,9785 0,9512 0,9480	M098 A071a A071 A071b	M098 0,9796 0,9717 0,9481 0,9480		<i>Cl.</i> 3 K022 K022a K022b N178	K022 0,9777 0,9424 0,9352 0,9277	K022a K022b K022 N178	K022a 0,9810 0,9523 0,9424 0.9397	K022b K022a B112 N178	K022b 0,9890 0,9523 0,9451 0,9426	N178 C017 K022b K022a	N178 0,9963 0,9732 0,9426 0,9397		<i>Cl. 4</i> D178 H035 N063 I051	D178 0,9953 0,9672 0,9641 0,9602	H035 D178 N063 1051	H035 0,9914 0,9672 0,9618 0,9597	1051 N063 D178 H035	1051 0,9929 0,9794 0,9602 0,9597	N063 1051 D178 H035	N063 0,9929 0,9794 0,9641 0,9618
<i>CI. 2</i> A071 A071b A071a M098 C017	A071 0,9903 0,9785 0,9526 0,9481 0,9123	A071a M098 A071 A071b	A071a 0,9926 0,9717 0,9526 0,9512 0,9457	A071b A071 A071a M098 C017	A071b 0,9903 0,9785 0,9512 0,9480 0,9177	M098 A071a A071 A071b C017	M098 0,9796 0,9717 0,9481 0,9480 0,9157		CI. 3 K022 K022a K022b N178 H182b	K022 0,9777 0,9424 0,9352 0,9277 0,8650	K022a K022b K022 N178	K022a 0,9810 0,9523 0,9424 0,9397 0,9338	K022b K022a B112 N178 F231	K022b 0,9890 0,9523 0,9451 0,9426 0,9419	N178 C017 K022b K022a F231	N178 0,9963 0,9732 0,9426 0,9397 0,9388		Cl. 4 D178 H035 N063 I051 F231	D178 0,9953 0,9672 0,9641 0,9602 0,9201	H035 D178 N063 I051 F231	H035 0,9914 0,9672 0,9618 0,9597 0,9466	1051 N063 D178 H035 A071a	1051 0,9929 0,9794 0,9602 0,9597 0,9457	N063 1051 D178 H035 K022a	N063 0,9929 0,9794 0,9641 0,9618 0,9295
<i>Cl. 2</i> A071 A071b A071a M098 C017 F231	A071 0,9903 0,9785 0,9526 0,9481 0,9123 0,9122	A071a M098 A071 A071b I051 C017	A071a 0,9926 0,9717 0,9526 0,9512 0,9457 0,9453	A071b A071 A071a M098 C017 F231	A071b 0,9903 0,9785 0,9512 0,9480 0,9177 0,9134	M098 A071a A071 A071b C017 B112	M098 0,9796 0,9717 0,9481 0,9480 0,9157 0,9102		Cl. 3 K022 K022a K022b N178 H182b H182a	K022 0,9777 0,9424 0,9352 0,9277 0,8650 0,8529	K022a K022b K022 N178 I051 N063	K022a 0,9810 0,9523 0,9424 0,9397 0,9338 0,9295	K022b K022a B112 N178 F231 K022	K022b 0,9890 0,9523 0,9451 0,9426 0,9419 0,9352	N178 C017 K022b K022a F231 A071a	N178 0,9963 0,9732 0,9426 0,9397 0,9388 0,9317		Cl. 4 D178 H035 N063 I051 F231 C017	D178 0,9953 0,9672 0,9641 0,9602 0,9201 0,9147	H035 D178 N063 I051 F231 C017	H035 0,9914 0,9672 0,9618 0,9597 0,9466 0,9454	1051 N063 D178 H035 A071a B112	1051 0,9929 0,9794 0,9602 0,9597 0,9457 0,9379	N063 1051 D178 H035 K022a K022b	N063 0,9929 0,9794 0,9641 0,9618 0,9295 0,9120
CI. 2 A071 A071b A071a M098 C017 F231 H035	A071 0,9903 0,9785 0,9526 0,9481 0,9123 0,9122 0,8981	A071a M098 A071 A071b I051 C017 E231	A071a 0,9926 0,9717 0,9526 0,9512 0,9457 0,9453 0,9393	A071b A071 A071a M098 C017 F231 H035	A071b 0,9903 0,9785 0,9512 0,9480 0,9177 0,9134 0,8957	M098 A071a A071 A071b C017 B112 F231	M098 0,9796 0,9717 0,9481 0,9480 0,9157 0,9102 0,9058		CI. 3 K022 K022a K022b N178 H182b H182a A071a	K022 0,9777 0,9424 0,9352 0,9277 0,8650 0,8529 0,8480	K022a K022b K022 N178 I051 N063 A071a	K022a 0,9810 0,9523 0,9424 0,9397 0,9338 0,9295 0,9242	K022b K022a B112 N178 F231 K022 C017	K022b 0,9890 0,9523 0,9451 0,9426 0,9419 0,9352 0,9214	N178 C017 K022b K022a F231 A071a K022	N178 0,9963 0,9732 0,9426 0,9397 0,9388 0,9317 0,9277		Cl. 4 D178 H035 N063 I051 F231 C017 B112	D178 0,9953 0,9672 0,9641 0,9602 0,9201 0,9147 0,9146	H035 D178 N063 I051 F231 C017 K022a	H035 0,9914 0,9672 0,9618 0,9597 0,9466 0,9454 0,9235	1051 N063 D178 H035 A071a B112 N158	1051 0,9929 0,9794 0,9602 0,9597 0,9457 0,9379 0,9341	N063 1051 D178 H035 K022a K022b N158	N063 0,9929 0,9794 0,9641 0,9618 0,9295 0,9120 0,9077
CI. 2 A071 A071b A071a M098 C017 F231 H035 B112	A071 0,9903 0,9785 0,9526 0,9481 0,9123 0,9122 0,8981 0,8944	A071a M098 A071 A071b I051 C017 F231 N178	A071a 0,9926 0,9717 0,9526 0,9512 0,9457 0,9453 0,9393 0,9317	A071b A071 A071a M098 C017 F231 H035 N178	A071b 0,9903 0,9785 0,9512 0,9480 0,9177 0,9134 0,8957 0,8826	M098 A071a A071 C017 B112 F231 I051	M098 0,9796 0,9717 0,9481 0,9480 0,9157 0,9102 0,9058 0,8811		CI. 3 K022 K022a K022b N178 H182b H182a A071a I051	K022 0,9777 0,9424 0,9352 0,9277 0,8650 0,8529 0,8480 0,8388	K022a K022b K022 N178 I051 N063 A071a H035	K022a 0,9810 0,9523 0,9424 0,9397 0,9338 0,9295 0,9242 0,9235	K022b K022a B112 N178 F231 K022 C017 N158	K022b 0,9890 0,9523 0,9451 0,9426 0,9419 0,9352 0,9214 0,9151	N178 C017 K022b K022a F231 A071a K022 B112	N178 0,9963 0,9732 0,9426 0,9397 0,9388 0,9317 0,9277 0,9096		Cl. 4 D178 H035 N063 I051 F231 C017 B112 K022a	D178 0,9953 0,9672 0,9641 0,9602 0,9201 0,9147 0,9146 0,9133	H035 D178 N063 I051 F231 C017 K022a B112	H035 0,9914 0,9672 0,9618 0,9597 0,9466 0,9454 0,9235 0,9232	1051 N063 D178 H035 A071a B112 N158 K022a	1051 0,9929 0,9794 0,9602 0,9597 0,9457 0,9379 0,9341 0,9338	N063 1051 D178 H035 K022a K022b N158 B112	N063 0,9929 0,9794 0,9641 0,9618 0,9295 0,9120 0,9077 0,8967
CI. 2 A071 A071b A071a M098 C017 F231 H035 B112 H109	A071 0,9903 0,9785 0,9526 0,9481 0,9123 0,9122 0,8981 0,8944 0,8848	A071a M098 A071 A071b I051 C017 F231 N178 B112	A071a 0,9926 0,9717 0,9526 0,9512 0,9457 0,9453 0,9393 0,9317 0,9315	A071b A071 A071a M098 C017 F231 H035 N178 B112	A071b 0,9903 0,9785 0,9512 0,9480 0,9177 0,9134 0,8957 0,8826 0,8815	M098 A071a A071 C017 B112 F231 I051 N178	M098 0,9796 0,9717 0,9481 0,9480 0,9157 0,9102 0,9058 0,8811 0,8783		Cl. 3 K022 K022a K022b N178 H182b H182a A071a I051 N158	K022 0,9777 0,9424 0,9352 0,9277 0,8650 0,8529 0,8480 0,8388 0,8252	K022a K022b K022 N178 I051 N063 A071a H035 B112	K022a 0,9810 0,9523 0,9424 0,9397 0,9338 0,9295 0,9242 0,9235 0,9219	K022b K022a B112 N178 F231 K022 C017 N158 N063	K022b 0,9890 0,9523 0,9451 0,9426 0,9419 0,9352 0,9214 0,9151 0,9120	N178 C017 K022b K022a F231 A071a K022 B112 H035	N178 0,9963 0,9732 0,9426 0,9397 0,9388 0,9317 0,9277 0,9096 0,8982		Cl. 4 D178 H035 N063 I051 F231 C017 B112 K022a K022b	D178 0,9953 0,9672 0,9641 0,9602 0,9201 0,9147 0,9146 0,9133 0,9120	H035 D178 N063 I051 F231 C017 K022a B112 A071a	H035 0,9914 0,9672 0,9618 0,9597 0,9466 0,9454 0,9235 0,9232 0,9166	1051 N063 D178 H035 A071a B112 N158 K022a C017	1051 0,9929 0,9794 0,9602 0,9597 0,9457 0,9379 0,9341 0,9338 0,9248	N063 1051 D178 H035 K022a K022b N158 B112 C017	N063 0,9929 0,9794 0,9641 0,9618 0,9295 0,9120 0,9077 0,8967 0,8957
CI. 2 A071 A071b A071a M098 C017 F231 H035 B112 H109 N178	A071 0,9903 0,9785 0,9526 0,9481 0,9123 0,9122 0,8981 0,8944 0,8848 0,8828	A071a M098 A071 A071b I051 C017 F231 N178 B112 K022a	A071a 0,9926 0,9717 0,9526 0,9512 0,9457 0,9453 0,9393 0,9317 0,9315 0,9242	A071b A071 A071a M098 C017 F231 H035 N178 B112 K022b	A071b 0,9903 0,9785 0,9512 0,9480 0,9177 0,9134 0,8957 0,8826 0,8815 0,8804	M098 A071a A071 C017 B112 F231 I051 N178 K022b	M098 0,9796 0,9717 0,9481 0,9480 0,9157 0,9102 0,9058 0,8811 0,8783 0,8771		Cl. 3 K022 K022a K022b N178 H182b H182a A071a I051 N158 H035	K022 0,9777 0,9424 0,9352 0,9277 0,8650 0,8529 0,8480 0,8388 0,8252 0,8105	K022a K022b K022 N178 I051 N063 A071a H035 B112 N158	K022a 0,9810 0,9523 0,9424 0,9397 0,9338 0,9295 0,9242 0,9235 0,9219 0,9219 0,9171	K022b K022a B112 N178 F231 K022 C017 N158 N063 D178	K022b 0,9890 0,9523 0,9451 0,9426 0,9419 0,9352 0,9214 0,9151 0,9120 0,9120	N178 C017 K022b K022a F231 A071a K022 B112 H035 I051	N178 0,9963 0,9732 0,9426 0,9397 0,9388 0,9317 0,9277 0,9096 0,8982 0,8925		Cl. 4 D178 H035 N063 I051 F231 C017 B112 K022a K022b P091	D178 0,9953 0,9672 0,9641 0,9602 0,9201 0,9147 0,9146 0,9133 0,9120 0,8877	H035 D178 N063 I051 F231 C017 K022a B112 A071a N158	H035 0,9914 0,9672 0,9618 0,9597 0,9466 0,9454 0,9235 0,9232 0,9166 0,9079	1051 N063 D178 H035 A071a B112 N158 K022a C017 F231	1051 0,9929 0,9794 0,9602 0,9597 0,9457 0,9379 0,9341 0,9338 0,9248 0,9190	N063 1051 D178 H035 K022a K022b N158 B112 C017 F231	N063 0,9929 0,9794 0,9641 0,9618 0,9295 0,9120 0,9077 0,8967 0,8957 0,8922
CI. 2 A071 A071b A071a M098 C017 F231 H035 B112 H109 N178 K022b	A071 0,9903 0,9785 0,9526 0,9481 0,9123 0,9122 0,8981 0,8984 0,8848 0,8828 0,8794	A071a M098 A071 I051 C017 F231 N178 B112 K022a H182a	A071a 0,9926 0,9717 0,9526 0,9512 0,9453 0,9393 0,9315 0,9315 0,9242 0,9187	A071b A071 A071a M098 C017 F231 H035 N178 B112 K022b D178	A071b 0,9903 0,9785 0,9512 0,9480 0,9177 0,9134 0,8957 0,8826 0,8815 0,8804 0,8729	M098 A071a A071 C017 B112 F231 I051 N178 K022b N158	M098 0,9796 0,9717 0,9481 0,9480 0,9157 0,9102 0,9058 0,8811 0,8783 0,8771 0,8703		C/. 3 K022 K022a K022b N178 H182b H182a A071a I051 N158 H035 P091	K022 0,9777 0,9424 0,9352 0,9277 0,8650 0,8529 0,8480 0,8388 0,8252 0,8105 0,7963	K022a K022b K022 N178 I051 N063 A071a H035 B112 N158 D178	K022a 0,9810 0,9523 0,9424 0,9397 0,9338 0,9295 0,9242 0,9235 0,9242 0,9219 0,9219 0,9171 0,9133	K022b K022a B112 N178 F231 K022 C017 N158 N063 D178 A071a	K022b 0,9890 0,9523 0,9421 0,9426 0,9429 0,9352 0,9214 0,9151 0,9120 0,9120 0,9109	N178 C017 K022b K022a F231 A071a K022 B112 H035 I051 P091	N178 0,9963 0,9732 0,9426 0,9397 0,9388 0,9317 0,9277 0,9096 0,8982 0,8982 0,8925 0,8872		Cl. 4 D178 H035 N063 I051 F231 C017 B112 K022a K022b P091 N158	D178 0,9953 0,9672 0,9641 0,9602 0,9201 0,9147 0,9146 0,9133 0,9120 0,8877 0,8809	H035 D178 N063 I051 F231 C017 K022a B112 A071a N158 K022b	H035 0,9914 0,9672 0,9618 0,9597 0,9466 0,9454 0,9235 0,9232 0,9166 0,9079 0,9033	1051 N063 D178 H035 A071a B112 N158 K022a C017 F231 K022b	1051 0,9929 0,9794 0,9602 0,9597 0,9457 0,9379 0,9341 0,9338 0,9248 0,9190 0,9097	N063 1051 D178 H035 K022a K022b N158 B112 C017 F231 H182a	N063 0,9929 0,9794 0,9641 0,9295 0,9120 0,9077 0,8967 0,8957 0,8922 0,8831
CI. 2 A071 A071b A071a M098 C017 F231 H035 B112 H109 N178 K022b D178	A071 0,9903 0,9785 0,9526 0,9481 0,9123 0,9122 0,8981 0,8984 0,8848 0,8828 0,8794 0,8692	A071a M098 A071 I051 C017 F231 N178 B112 K022a H182a H035	A071a 0,9926 0,9717 0,9526 0,9457 0,9453 0,9393 0,9315 0,9242 0,9187 0,9166	A071b A071 A071a M098 C017 F231 H035 N178 B112 K022b D178 K022a	A071b 0,9903 0,9785 0,9512 0,9480 0,9177 0,9134 0,8957 0,8826 0,8815 0,8804 0,8729 0,8675	M098 A071a A071 C017 B112 F231 I051 N178 K022b N158 P091	M098 0,9796 0,9717 0,9481 0,9157 0,9102 0,9058 0,8811 0,8783 0,8771 0,8703 0,8702		C/. 3 K022 K022a K022b N178 H182b H182a A071a I051 N158 H035 P091 M098	K022 0,9777 0,9424 0,9352 0,9277 0,8650 0,8529 0,8480 0,8252 0,8105 0,7963 0,7914	K022a K022b K022 N178 I051 N063 A071a H035 B112 N158 D178 F231	K022a 0,9810 0,9523 0,9424 0,9337 0,9235 0,9242 0,9235 0,9242 0,9239 0,9219 0,9219 0,9171 0,9133 0,9103	K022b K022a B112 N178 F231 K022 C017 N158 N063 D178 A071a I051	K022b 0,9890 0,9523 0,9451 0,9426 0,9352 0,9352 0,9214 0,9151 0,9120 0,9120 0,9109 0,9097	N178 C017 K022b K022a F231 A071a K022 B112 H035 I051 P091 A071	N178 0,9963 0,9732 0,9426 0,9397 0,9388 0,9317 0,9277 0,9096 0,8982 0,8982 0,8925 0,8872 0,8828		Cl. 4 D178 H035 N063 I051 F231 C017 B112 K022a K022b P091 N158 A071a	D178 0,9953 0,9672 0,9641 0,9201 0,9147 0,9146 0,9130 0,9120 0,8877 0,8809 0,8767	H035 D178 N063 I051 F231 C017 K022a B112 A071a N158 K022b N178	H035 0,9914 0,9672 0,9618 0,9466 0,9454 0,9235 0,9235 0,9235 0,9166 0,9079 0,9033 0,8982	1051 N063 D178 H035 A071a B112 N158 K022a C017 F231 K022b H182b	1051 0,9929 0,9794 0,9602 0,9597 0,9457 0,9379 0,9341 0,9338 0,9248 0,9190 0,9097 0,9021	N063 1051 D178 H035 K022a K022b N158 B112 C017 F231 H182a A071a	N063 0,9929 0,9794 0,9641 0,9618 0,9295 0,9120 0,9077 0,8957 0,8957 0,8952 0,8818
CI. 2 A071 A071b A071a M098 C017 F231 H035 B112 H109 N178 K022b D178 K022a	A071 0,9903 0,9785 0,9526 0,9481 0,9123 0,9122 0,8981 0,8944 0,8848 0,8794 0,8622 0,8653	A071a M098 A071 I051 C017 F231 N178 B112 K022a H182a H182a H182a SN158	A071a 0,9926 0,9717 0,9526 0,9512 0,9453 0,9393 0,9315 0,9242 0,9187 0,9166 0,9115	A071b A071 A071a M098 C017 F231 H035 N178 B112 K022b D178 K022a I051	A071b 0,9903 0,9785 0,9512 0,9480 0,9177 0,9134 0,8957 0,8826 0,8815 0,8804 0,8729 0,8675 0,8633	M098 A071a A071 C017 B112 F231 I051 N178 K022b N158 P091 H182a	M098 0,9796 0,9717 0,9481 0,9480 0,9157 0,9102 0,9058 0,8811 0,8783 0,8771 0,8703 0,8702 0,8701		CI. 3 K022 K022a K022b N178 H182b H182a A071a I051 N158 H035 P091 M098 P156	K022 0,9777 0,9424 0,9352 0,9277 0,8650 0,8529 0,8480 0,8388 0,8252 0,8105 0,7963 0,7914 0,7781	K022a K022b K022 N178 I051 N063 A071a H035 B112 N158 D178 F231 C017	K022a 0,9810 0,9523 0,9424 0,9397 0,9338 0,9295 0,9242 0,9235 0,9219 0,9171 0,9133 0,9103 0,9039	K022b K022a B112 N178 F231 K022 C017 N158 N063 D178 A071a I051 H035	K022b 0,9890 0,9523 0,9451 0,9426 0,9419 0,9352 0,9214 0,9151 0,9120 0,9120 0,9120 0,9109 0,9097 0,9033	N178 C017 K022b K022a F231 A071a K022 B112 H035 I051 P091 A071 A071b	N178 0,9963 0,9732 0,9426 0,9397 0,9388 0,9317 0,9277 0,9096 0,8982 0,8925 0,8872 0,8828 0,8826		Cl. 4 D178 H035 N063 I051 F231 C017 B112 K022a K022b P091 N158 A071a P156	D178 0,9953 0,9672 0,9641 0,9201 0,9147 0,9146 0,9133 0,9120 0,9120 0,8877 0,8809 0,8767	H035 D178 N063 I051 F231 C017 K022a B112 A071a N158 K022b N178 A071	H035 0,9914 0,9672 0,9618 0,9597 0,9466 0,9454 0,9235 0,9232 0,9166 0,9079 0,9033 0,8982 0,8981	1051 N063 D178 H035 A071a B112 N158 K022a C017 F231 K022b H182b H182b	1051 0,9929 0,9794 0,9602 0,9597 0,9457 0,9379 0,9341 0,9338 0,9248 0,9190 0,9097 0,9021 0,9094	N063 1051 D178 H035 K022a K022b N158 B112 C017 F231 H182a A071a H182b	N063 0,9929 0,9794 0,9641 0,9295 0,9295 0,9220 0,9077 0,8967 0,8957 0,8957 0,8922 0,8831 0,8818 0,8818
CI. 2 A071 A071b A071a M098 C017 F231 H035 B112 H109 N178 K022b D178 K022a P156	A071 0,9903 0,9785 0,9526 0,9481 0,9123 0,9122 0,8981 0,8944 0,8848 0,8794 0,8692 0,8653 0,8537	A071a M098 A071 I051 C017 F231 N178 B112 K022a H182a H035 N158 K022b	A071a 0,9926 0,9717 0,9526 0,9457 0,9453 0,9393 0,9315 0,9242 0,9187 0,9166 0,9115 0,9109	A071b A071 A071a M098 C017 F231 H035 N178 B112 K022b D178 K022a I051 H109	A071b 0,9903 0,9785 0,9512 0,9480 0,9177 0,9134 0,8957 0,8826 0,8815 0,8804 0,8729 0,8675 0,8633 0,8588	M098 A071a A071 D017 B112 F231 I051 N178 K022b N158 P091 H182a H035	M098 0,9796 0,9717 0,9481 0,9480 0,9157 0,9102 0,9058 0,8811 0,8783 0,8771 0,8703 0,87703 0,8701 0,8702		CI. 3 K022 K022a K022b N178 H182b H182a A071a I051 N158 H035 P091 M098 P156 B112	K022 0,9777 0,9424 0,9352 0,9277 0,8650 0,8529 0,8480 0,8388 0,8252 0,8105 0,7963 0,7914 0,7781 0,7734	K022a K022b K022 N178 I051 N063 A071a H035 B112 N158 D178 F231 C017 H182a	K022a 0,9810 0,9523 0,9424 0,9397 0,9338 0,9295 0,9242 0,9235 0,9219 0,9171 0,9133 0,9103 0,9103 0,9006	K022b K022a B112 N178 F231 K022 C017 N158 N063 D178 A071a I051 H035 H182b	K022b 0,9890 0,9523 0,9451 0,9426 0,9352 0,9352 0,9151 0,9120 0,9120 0,9109 0,9033 0,9008	N178 C017 K022b K022a F231 A071a K022 B112 H035 I051 P091 A071 A071b M098	N178 0,9963 0,9732 0,9426 0,9397 0,9388 0,9317 0,9277 0,9096 0,8982 0,8925 0,8872 0,8828 0,8828 0,8826 0,8783		Cl. 4 D178 H035 N063 I051 F231 C017 B112 K022a K022b P091 N158 A071a P156 A071b	D178 0,9953 0,9672 0,9641 0,9602 0,9201 0,9147 0,9146 0,9133 0,9120 0,8877 0,8809 0,8767 0,8766 0,8729	H035 D178 N063 I051 F231 C017 K022a B112 A071a N158 K022b N178 A071 A071b	H035 0,9914 0,9672 0,9618 0,9597 0,9466 0,9454 0,9235 0,9232 0,9166 0,9079 0,9033 0,8982 0,8982 0,8981 0,8957	1051 N063 D178 H035 A071a B112 N158 K022a C017 F231 K022b H182b H182a P091	1051 0,9929 0,9794 0,9602 0,9597 0,9457 0,9379 0,9341 0,9338 0,9248 0,9190 0,9097 0,9021 0,8994 0,8935	N063 1051 D178 H035 K022a K022b N158 B112 C017 F231 H182a A071a H182b P091	N063 0,9929 0,9794 0,9641 0,9641 0,9295 0,9120 0,9077 0,8967 0,8957 0,8957 0,8957 0,8818 0,8818 0,8818 0,8780 0,8771
CI. 2 A071 A071b A071a M098 C017 F231 H035 B112 H109 N178 K022b D178 K022a P156 P091	A071 0,9903 0,9785 0,9526 0,9481 0,9123 0,8981 0,8981 0,8984 0,8848 0,8794 0,8692 0,8653 0,85501	A071a M098 A071 I051 C017 F231 N178 B112 K022a H182a H035 N158 K022b H182b	A071a 0,9926 0,9717 0,9526 0,9512 0,9457 0,9453 0,9315 0,9315 0,9315 0,9242 0,9187 0,9166 0,9115 0,9109 0,9101	A071b A071 A071a M098 C017 F231 H035 N178 B112 K022b D178 K022a I051 H109 N158	A071b 0,9903 0,9785 0,9512 0,9480 0,9177 0,9134 0,8856 0,8826 0,8815 0,8804 0,8729 0,8675 0,8675 0,8635 0,8635 0,8635	M098 A071a A071 C017 B112 F231 I051 N178 K022b N158 P091 H182a H035 P156	M098 0,9796 0,9717 0,9481 0,9480 0,9157 0,9102 0,9058 0,8811 0,8783 0,8771 0,8703 0,8702 0,8701 0,8702 0,8702		Cl. 3 K022 K022a K022b N178 H182b H182a A071a I051 N158 H035 P091 M098 P156 B112 F231	K022 0,9777 0,9424 0,9352 0,9277 0,8650 0,8388 0,8388 0,8388 0,8388 0,8388 0,8388 0,8388 0,8388 0,8388 0,8388 0,8388 0,7914 0,7784 0,7734	K022a K022b K022 N178 I051 N063 A071a H035 B112 N158 D178 F231 C017 H182a H182b	K022a 0,9810 0,9523 0,9424 0,9397 0,9235 0,9245 0,9245 0,9249 0,9171 0,9133 0,9039 0,9006 0,9002	K022b K022a B112 N178 F231 K022 C017 N158 N063 D178 A071a I051 H035 H182b P091	K022b 0,9890 0,9523 0,9451 0,9426 0,9132 0,9352 0,9151 0,9120 0,9120 0,9109 0,9093 0,9008 0,9008 0,9008	N178 C017 K022b K022a F231 A071a K022 B112 H035 I051 P091 A071 A071b M098 H182b	N178 0,9963 0,9732 0,9426 0,9397 0,9377 0,9377 0,9377 0,9096 0,8982 0,8925 0,8872 0,8828 0,8828 0,8878 0,88743		Cl. 4 D178 H035 N063 I051 F231 C017 B112 K022a K022b P091 N158 A071a P156 A071b A071	D178 0,9953 0,9672 0,9641 0,9201 0,9147 0,9146 0,9133 0,9120 0,8877 0,8809 0,8767 0,8766 0,8729 0,8729	H035 D178 N063 I051 F231 K022a B112 A071a N158 K022b N178 A071b H182b	H035 0,9914 0,9672 0,9618 0,9597 0,9466 0,9232 0,9232 0,926 0,9079 0,9033 0,8982 0,8981 0,89857 0,8855	I051 N063 D178 H035 A071a B112 N158 K022a C017 F231 K022b H182b H182b H182a P091 N178	1051 0,9929 0,9794 0,9602 0,9457 0,9379 0,9341 0,9338 0,9248 0,9190 0,9097 0,9021 0,8994 0,8935 0,8925	N063 1051 D178 H035 K022a K022b N158 B112 C017 F231 H182a A071a H182b P091 N178	N063 0,9929 0,9794 0,9641 0,9648 0,9295 0,9120 0,9077 0,8957 0,8757 0,8957 0,8757 0,8957 0,8957 0,8771 0,8771 0,8771 0,8771 0,8771 0,8771 0,8771 0,8771 0,8771 0,8771 0,8771 0,8771 0,8771 0,8771 0,8771 0,8771 0,8771 0,8757 0,8757 0,87571 0,87571 0,87571 0,87571 0,87571 0,87571 0,87571 0,87571 0,87571 0,87571 0,87571 0,87571 0,87571 0,87711 0,87571 0,87571 0,87571 0,87571 0,87571 0,87571 0,87571 0,87711 0,97711 0,97711 0,97711 0,97711 0,97711 0,977110 0,977110 0,977110 0,977110 0,97710000000000000000000000000000000000
CI. 2 A071 A071b A071a M098 C017 F231 H035 B112 H109 N178 K022b D178 K022a P156 P091 I051	A071 0,9903 0,9785 0,9526 0,9481 0,9122 0,8981 0,8944 0,8848 0,8794 0,8653 0,8537 0,8501 0,8531 0,8531	A071a M098 A071 A071b I051 C017 F231 N178 B112 K022a H182a H182a H182b H182b P091	A071a 0,9926 0,9717 0,9526 0,9457 0,9453 0,9313 0,9315 0,9242 0,9187 0,9166 0,9109 0,9109 0,9101 0,9109	A071b A071 A071a M098 C017 F231 H035 N178 B112 K022b D178 K022a I051 H109 N158 H182a H182a	A071b 0,9903 0,9785 0,9512 0,9140 0,9177 0,8826 0,8815 0,8826 0,8815 0,8867 0,8675 0,8633 0,8570 0,8557 0,8567	M098 A071a A071 C017 B112 F231 I051 N178 K022b N158 P091 H182a H035 P156 K022a	M098 0,9796 0,9717 0,9481 0,9162 0,9102 0,9102 0,9102 0,9102 0,9058 0,8811 0,8783 0,87701 0,8702 0,8701 0,8702 0,8701 0,8692 0,8648 0,8631 0,9796		Cl. 3 K022 K022a K022b N178 H182b H182a A071a I051 N158 H035 P091 M098 P156 B112 F231 A071b	K022 0,9777 0,9424 0,9352 0,9277 0,8650 0,8388 0,8388 0,8388 0,8252 0,8105 0,7963 0,7963 0,7784 0,7784 0,7763 0,7763	K022a K022b K022 N178 I051 N063 A071a H035 B112 N158 D178 F231 C017 H182a H182b P091	K022a 0,9810 0,9523 0,9424 0,9397 0,9295 0,9245 0,9245 0,9249 0,9171 0,9133 0,9103 0,9008 0,9006 0,9002 0,8788 0,9788	K022b K022a B112 N178 F231 K022 C017 N158 N063 D178 A071a I051 H0355 H182b P091 H182a P091	K022b 0,9890 0,9523 0,9426 0,9426 0,9419 0,9352 0,9214 0,9151 0,9120 0,9120 0,9120 0,9109 0,9097 0,9033 0,9008 0,8982 0,8909	N178 C017 K022b K022a F231 A071a K022 B112 H035 I051 P091 A071b A071b M098 H182b N158	N178 0,9963 0,9732 0,9326 0,9388 0,9317 0,9388 0,8982 0,8982 0,8982 0,8828 0,8828 0,8828 0,8828 0,8828 0,8783 0,8743 0,8743		Cl. 4 D178 H035 N063 I051 F231 C017 B112 K022a K022b P091 N158 A071a P156 A071b A071 H182a	D178 0,9953 0,9672 0,9641 0,9602 0,9201 0,9147 0,9146 0,9133 0,9120 0,8877 0,8809 0,8767 0,8766 0,8729 0,8671 0,8692	H035 D178 N063 I051 F231 C017 K022a B112 A071a N158 K022b N178 A071b A071b H182b P156	H035 0,9914 0,9672 0,9618 0,9597 0,9466 0,9232 0,9232 0,9166 0,9079 0,9033 0,8982 0,8982 0,89857 0,8885 0,8860 0,9079	1051 N063 D178 H035 A071a B112 N158 K022a C017 F231 K022b H182b H1	1051 0,9929 0,9794 0,9602 0,9457 0,9345 0,9345 0,9348 0,9348 0,9190 0,9097 0,9021 0,8994 0,8935 0,8935 0,8925 0,8811	N063 1051 D178 H035 K022a K022b N158 B112 C017 F231 H182a A071a H182b P091 N178 P156	N063 0,9929 0,9794 0,9641 0,9295 0,9120 0,9077 0,8967 0,8957 0,8922 0,8831 0,8818 0,8780 0,8771 0,8632 0,8740
CI. 2 A071 A071b A071a M098 C017 F231 H035 B112 H109 N178 K022b D178 K022a P156 P091 I051 N063	A071 0,9903 0,9785 0,9526 0,9481 0,9122 0,8981 0,8944 0,8848 0,8794 0,8653 0,8537 0,8537 0,8537 0,8537 0,8548 0,8434	A071a M098 A071 I051 C017 F231 N178 B112 K022a H182a H182a H182a H182a H182b H182b H182b H182b H182b H182b H182b	A071a 0,9926 0,9717 0,9526 0,9453 0,9453 0,9313 0,9315 0,9242 0,9187 0,9166 0,9115 0,9109 0,9101 0,9109 0,9109 0,9101 0,9109 0,9109	A071b A071a A071a M098 C017 F231 H035 N178 B112 K022b D178 K022a I051 H109 N158 H182a N063 D456	A071b 0,9903 0,9785 0,9512 0,9134 0,8957 0,8826 0,8815 0,8804 0,8729 0,8633 0,8567 0,8633 0,8588 0,8570 0,8567 0,8567	M098 A071a A071 C017 B112 F231 I051 N178 K022b N158 P091 H182a H035 P156 K022a H182b	M098 0,9796 0,9717 0,9481 0,9102 0,9102 0,9058 0,8811 0,8783 0,87703 0,8703 0,87703 0,8702 0,8704 0,8704 0,8648 0,8641 0,8656 0,8656		Cl. 3 K022 K022a K022b N178 H182b H182a A071a I051 N158 H035 P091 M098 P156 B112 F231 A071b I151	K022 0,9777 0,9424 0,9352 0,8650 0,8529 0,8480 0,8388 0,8252 0,8105 0,7963 0,7963 0,7914 0,7781 0,7784 0,7648 0,7638 0,7638	K022a K022b K022 N178 I051 N063 A071a H035 B112 N158 F231 C017 H182a H182b P091 P156	K022a 0,9810 0,9523 0,9424 0,9338 0,9295 0,9242 0,9235 0,9219 0,9171 0,9133 0,9103 0,9039 0,9002 0,9002 0,8788 0,8764	K022b K022a B112 N178 F231 K022 C017 N158 N063 D178 A071a I051 H035 H182b P091 H182a P156	K022b 0,9890 0,9523 0,9451 0,9426 0,9419 0,9352 0,9214 0,9151 0,9120 0,9120 0,9109 0,9109 0,9097 0,9033 0,9003 0,8982 0,8982 0,8909 0,8806	N178 C017 K022b K022a F231 A071a B112 B112 B112 B112 B112 H035 I051 P091 A071b A071b A071b M098 H182b N158 N063 D178	N178 0,9963 0,9732 0,9426 0,9397 0,9388 0,9317 0,9277 0,9096 0,8982 0,8822 0,8822 0,8828 0,8826 0,8783 0,8743 0,8748 0,8748		Cl. 4 D178 H035 N063 I051 E231 C017 B112 K022a K022b P091 N158 A071a P156 A071b A0711 H182a N178	D178 0,9953 0,9672 0,9641 0,9101 0,9147 0,9146 0,9133 0,9120 0,8877 0,8809 0,8767 0,8766 0,8729 0,8762 0,8671 0,8692 0,8671	H035 D178 N063 I051 C017 K022a B112 A071a N158 K022b N178 A0711 A07118 H182b P156 P091	H035 0,9914 0,9672 0,9618 0,9466 0,9454 0,9235 0,9232 0,9166 0,9079 0,9033 0,8982 0,8085 0,8885 0,8885 0,8885 0,88860 0,8884	1051 N063 D178 H035 A071a B112 N158 K022a C017 F231 K022b H182b H182a P091 N178 M098 P156	1051 0,9929 0,9794 0,9602 0,9457 0,9341 0,9338 0,9248 0,9190 0,9097 0,9021 0,8094 0,8935 0,8925 0,8811 0,8729	N063 1051 D178 H0355 K022a K022b N158 B112 C017 F231 H182a A071a H182b P091 N178 P156 M098	N063 0,929 0,9794 0,9641 0,9641 0,9295 0,9120 0,8957 0,8957 0,8957 0,8957 0,8831 0,8831 0,8818 0,8780 0,8780 0,8711 0,8632 0,8540 0,8540 0,8510 0,8540
CI. 2 A071 A071b A071a M098 C017 F231 H035 B112 H109 N178 K022b D178 K022b D178 K022a P156 P091 I051 N063 N158 N158	A071 0,9903 0,9785 0,9526 0,9123 0,9122 0,8981 0,8944 0,8848 0,8894 0,8894 0,8693 0,8653 0,8553 0,8551 0,8501 0,8488 0,8434 0,8434 0,8428 0,8419	A071a M098 A071 I051 C017 F231 N178 B112 K022a H182a H035 N158 K022b H182b P091 N063 P156 D176	A071a 0,9926 0,9717 0,9526 0,9453 0,9453 0,9313 0,9315 0,9242 0,9186 0,9115 0,9109 0,9109 0,9109 0,8818 0,8807 0,872	A071b A071 A071a M098 C017 F231 H035 N178 B112 K022b D178 K022a I051 H109 N158 H182a N063 P156	A071b 0,9903 0,9785 0,9512 0,9480 0,9177 0,9134 0,8826 0,8815 0,8845 0,8845 0,88729 0,8675 0,8633 0,8588 0,8570 0,8633 0,8577 0,8486 0,8577 0,8486 0,8333 0,85490,8549 0,8549 0,8549 0,85490,8549 0,8549 0,85490,8549 0,8549 0,85490,8549 0,8549 0,85490,8549 0,8549 0,85490,8549 0,8549 0,85490,8549 0,8549 0,85490,8549 0,8549 0,85490,8549 0,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,85490,8549 0,85490,85500 0,85490,8550000000000000000000000000000000000	M098 A071a A071 C017 B112 F231 I051 N178 K022b N158 P091 H182a H035 P156 K022a H182b N063 D178	M098 0,9796 0,9717 0,9481 0,9182 0,9157 0,9102 0,9058 0,8713 0,8783 0,8771 0,8703 0,8703 0,8701 0,8703 0,8701 0,8692 0,8648 0,8576 0,8510 0,8510 0,8416		Cl. 3 K022 K022a K022b H18	K022 0,9777 0,9424 0,9352 0,8529 0,8480 0,8529 0,8480 0,8388 0,8252 0,8105 0,7963 0,7963 0,7914 0,7781 0,7734 0,7648 0,7648 0,7638 0,7424 0,7331 0,7331	K022a K022b K022 N178 I051 N063 A071a H035 B112 N158 F231 C017 H182a H182b P091 P156 A071b	K022a 0,9810 0,9523 0,9424 0,9338 0,9295 0,9242 0,9235 0,9219 0,9171 0,9133 0,9103 0,9039 0,9006 0,9002 0,8788 0,8764 0,8675 0,8675	K022b K022a B112 N178 F231 K022 C017 N158 N063 D178 A071a 1051 H035 H182b P091 H182a P156 A071b A071b	K022b 0,9880 0,9451 0,9451 0,9426 0,9419 0,9352 0,9140 0,9120 0,9120 0,9120 0,9190 0,9093 0,9008 0,8082 0,8082 0,8806 0,8804 0,8804	N178 C017 K022b F231 A071a K022 B112 H035 I051 A071b A071 A071b M098 H182b N158 N063 D178 N063	N178 0,9963 0,9732 0,9426 0,9337 0,9388 0,9317 0,9096 0,8982 0,8982 0,8982 0,8828 0,8828 0,8783 0,8743 0,8743 0,8743 0,8763 0,8763 0,8763 0,8763 0,8763 0,8763 0,8763 0,8763 0,8763 0,8763 0,8763 0,8763 0,8763 0,8763 0,8763 0,8763 0,8763 0,8773 0,9963 0,9963 0,9963 0,9963 0,9963 0,9963 0,9963 0,9963 0,9963 0,9963 0,9963 0,9963 0,9972 0,9986 0,9972 0,9986 0,9972 0,9987 0,9987 0,9987 0,9987 0,9987 0,9987 0,9987 0,9987 0,9987 0,9987 0,9987 0,9987 0,9987 0,9987 0,9987 0,9977 0,9988 0,8987 0,9987 0,9987 0,9977 0,9988 0,8987 0,9977 0,9988 0,8987 0,9977 0,9988 0,8987 0,9977 0,9988 0,8987 0,8987 0,8987 0,8987 0,8987 0,8987 0,8987 0,8987 0,8987 0,8987 0,8987 0,8987 0,8987 0,8987 0,8987 0,8987 0,8985 0,8987 0,8985 0,8987 0,8985 0,8987 0,8985 0,8995 0,8995 0,8995 0,8995 0,8995 0,8995 0,8995 0,8995 0,99		Cl. 4 D178 H035 N063 I051 F231 C017 B112 K022a P091 N158 A071a P156 A071b A071b H182a N178 H182a N178	D178 0,9953 0,9672 0,9641 0,9102 0,9147 0,9146 0,9133 0,9120 0,8877 0,8809 0,8767 0,8766 0,8729 0,8692 0,8671 0,8596 0,8572 0,8416	H035 D178 N063 I051 F231 C017 K022a B112 A071a N158 K022b N178 A071 A071b H182b P156 P091 H182a M09P	H035 0,9914 0,9672 0,9618 0,9454 0,9235 0,9232 0,9166 0,9079 0,9033 0,8982 0,8981 0,8957 0,8885 0,8860 0,8834 0,8834 0,8834	1051 N063 D178 H035 A071a B112 N158 K022a C017 F231 K022b H182b H182a P091 N178 M098 P156 A071b	1051 0,9929 0,9794 0,9602 0,9597 0,9341 0,9338 0,9248 0,9190 0,9097 0,9021 0,8094 0,8095 0,8825 0,8811 0,8729 0,8633 0,8483	N063 1051 D178 H035 K022a K022b N158 B112 C017 F231 H182a A071a H182b P091 N178 P091 N178 P156 M098 A071b	N063 0,9929 0,9794 0,9641 0,9618 0,9295 0,9120 0,8967 0,8957 0,8957 0,8952 0,8831 0,8838 0,8780 0,8771 0,8632 0,8540 0,8541 0,8540 0,8541 0,8541 0,8541 0,8557 0,8550 0,8570 0,8550 0,8570 0,8550 0,8570 0,8550 0,8570 0,8570 0,8570 0,8550 0,8570 0,8700 0,8700 0,8700 0,8570 0,8570 0,87000 0,87000 0,870000000000
Cl. 2 A071 A071b A072b A072b A072b A072b A072b A072b A072b A072b A072b A072b A075b A072b A075b A07	A071 0,9903 0,9785 0,9526 0,9123 0,9122 0,8981 0,8944 0,8848 0,8848 0,8874 0,8652 0,8537 0,8551 0,8551 0,8488 0,8448 0,8448 0,8448 0,8448 0,8448	A071a M098 A071 A071b I051 C017 F231 N178 B112 K022a H182a H182a H182a H182b F091 N158 K022b H182b F093 P156 D178 K022	A071a 0,9926 0,9717 0,9526 0,9457 0,9453 0,9393 0,9317 0,9165 0,9115 0,9105 0,9101 0,8188 0,8807 0,8818 0,8807 0,8767 0,8480	A071b A071 A071a M098 C017 F231 H035 N178 B112 K022b D178 K022a I051 H109 N158 H182a N063 P156 H182b P091	A071b 0,9903 0,9785 0,9512 0,9480 0,9177 0,9134 0,8857 0,8826 0,8875 0,8865 0,8875 0,8675 0,8588 0,8570 0,8588 0,8570 0,85867 0,8486 0,8383 0,8388 0,8388	M098 A071a A071 C017 B112 F231 I051 N178 K022b N158 F091 H182b K022a H1035 F156 K022a H182b N063 D178 H109	M098 0,9796 0,9717 0,9481 0,9157 0,9102 0,9058 0,8811 0,8703 0,8703 0,87703 0,87703 0,8702 0,8648 0,8631 0,8576 0,8510 0,8510 0,8416 0,8331		CI. 3 K022 K022b N178 H182b H182b H182a A071a I051 N158 H035 P091 M098 P156 B112 F231 A071b I151 N063 A071 H109	K022 0,9777 0,9424 0,9352 0,8650 0,8529 0,8480 0,8522 0,8105 0,7963 0,7963 0,7781 0,7784 0,77649 0,7649 0,7649 0,7648 0,7444 0,7638	K022a K022b K022 N178 I051 N063 A071a H035 B112 N158 D178 F231 C017 H182a H182b P091 P156 A071b A071b A071b	K022a 0,9810 0,9523 0,9424 0,9237 0,9235 0,9242 0,9235 0,9219 0,9171 0,9133 0,9103 0,9002 0,9002 0,8788 0,8764 0,8653 0,8653	K022b K022a B112 N178 F231 K022 C017 N158 N063 D178 A071a I051 H0355 H182b P091 H182a P1566 A071b A071b A071b	K022b 0,9830 0,9451 0,9451 0,9452 0,9352 0,9151 0,9120 0,9120 0,9120 0,9109 0,9097 0,9033 0,9098 0,8080 0,8804 0,8804 0,8804 0,8874 0,8774	N178 C017 K022ba F231 A071a K022 B112 H035 I051 P091 A071b A071b M098 H182b N158 N063 D178 H182a P156	N178 0,9963 0,9732 0,9426 0,9387 0,9387 0,9277 0,9096 0,8982 0,8982 0,8982 0,8982 0,8826 0,8783 0,8743 0,8743 0,8748 0,8743 0,8748 0,8743 0,8749 0,8596 0,8591 0,8591 0,8591		Cl. 4 D178 H035 N063 I051 F231 C017 B112 K022a K022b P091 N158 A071a P156 A071b A071 H182a N178 H182b M098 H109	D178 0,9953 0,9672 0,9641 0,9142 0,9146 0,9133 0,9120 0,8877 0,8766 0,8766 0,8766 0,8766 0,8766 0,8766 0,8766 0,8766 0,8766 0,8766 0,8766 0,8729 0,8692 0,8692 0,8692 0,8692 0,8572 0,8416 0,8596 0,8572 0,8416 0,8596 0,8572 0,8416 0,8572 0,8416 0,8572 0,8416 0,8572 0,8572 0,8572 0,8572 0,872 0,872 0,9147 0,9146 0,9147 0,9146 0,9147 0,9146 0,9147 0,9146 0,9147 0,9146 0,9147 0,9146 0,9147 0,9146 0,9147 0,9146 0,9147 0,9146 0,9147 0,9146 0,9147 0,9146 0,9147 0,9146 0,9147 0,9146 0,9146 0,9146 0,9147 0,9146 0,9146 0,9146 0,9146 0,9146 0,9146 0,9146 0,9146 0,9146 0,9146 0,9146 0,9146 0,9146 0,9146 0,9146 0,9146 0,9146 0,9146 0,8776 0,8766 0,8772 0,8766 0,8772 0,8767 0,8766 0,8772 0,8767 0,8767 0,8767 0,8776 0,8772 0,8772 0,8776 0,8776 0,8776 0,8772 0,8776 0,9776 0,9776 0,9776 0,9776 0,9766 0,9766 0,9766 0,976	H035 D178 N063 I051 F231 C017 K022a B112 A071a N158 K022b N178 A071b H182b P156 P091 H182a M098 H109	H035 0,9914 0,9672 0,9618 0,9597 0,9466 0,9235 0,9166 0,9166 0,9079 0,9033 0,8982 0,8981 0,8985 0,8885 0,8885 0,8884 0,88771 0,8882 0,8874	1051 N063 D178 H035 A071a B112 N158 K022a C017 F231 K022b H182a P091 N178 M098 P156 A071b A071 K022	1051 0,9929 0,9794 0,9602 0,9597 0,9379 0,9341 0,9338 0,9248 0,9190 0,9097 0,9021 0,8994 0,8935 0,8944 0,8729 0,8633 0,8729 0,8633 0,8729 0,8633	N063 1051 D178 K022a K022b N158 B112 C017 F231 H182a A071a H182b P091 N178 P156 M098 A071b A071 H109	N063 0,9929 0,9794 0,9641 0,9641 0,9618 0,9295 0,9120 0,9077 0,8967 0,8927 0,8927 0,8828 0,8831 0,8831 0,8838 0,8780 0,8771 0,8632 0,8510 0,8486 0,8486 0,8434 0,7799
CI. 2 A071 A071b A071b A071a M098 C017 F231 H035 B112 H109 N178 K022b D178 K022b D178 K022b D178 K022a P156 P091 N053 N158 H182a H182b	A071 0,9903 0,9785 0,9526 0,9481 0,9123 0,9122 0,8981 0,8984 0,8848 0,8848 0,8848 0,8653 0,8537 0,8501 0,8488 0,85301 0,8434 0,8434 0,8434 0,8434 0,8434 0,8434 0,8435 0,8435 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,8436 0,844 0,844 0,845 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8543 0,8544 0,8448 0,8448 0,8448 0,8448 0,8448 0,8448 0,8457 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,8537 0,9745 0,77450,7745 0,77450,7745 0,77450,7745 0,77450,7745 0,77450,7745 0,77450,7745 0,77450,7745 0,77450,7745 0,77450,7745 0,77450,7745 0,77450,77450,7745 0,77450,77450,7745 0,77450,77450,7745 0,77450,775 0,7750,775 0,7750,775 0,7750,775 0,775 0,7750,775 0,7750,775 0,7750,775 0,7750,775 0,7750,775 0,775 0,7750,775 0,775 0,7750,775 0,775 0,775 0,7750,775 0,775 0,7750,775 0,7750,775 0,775 0,7750,775 0,775 0,775 0,775 0,7750,775 0,775	A071a M098 A071 I051 C017 F231 N178 B112 K022a H182a H182a H182b P091 N063 P156 D178 K022 H109	A071a 0,9926 0,9717 0,9526 0,9457 0,9453 0,9317 0,9315 0,9242 0,9186 0,9115 0,9101 0,8890 0,9101 0,8818 0,8807 0,8818 0,8807 0,88480 0,8807 0,8767	A071b A071 A071a M098 C017 F231 H035 N178 B112 K022b D178 K022a I051 H109 N158 H182a N063 P156 H182b P0912	A071b 0,9903 0,9785 0,9512 0,9480 0,9177 0,9134 0,8957 0,8826 0,8815 0,8804 0,8729 0,8633 0,8578 0,8570 0,8567 0,8587 0,8587 0,8570 0,85870 0,85700 0,85700 0,85700 0,85700000000000000000000000000000000000	M098 A071a A071 C017 B112 F231 I051 N178 K022b N158 P091 H182a H035 P156 K022a H182b N063 D178 H109 K022	M098 0,9796 0,9717 0,9481 0,9167 0,9102 0,9058 0,8711 0,8783 0,8771 0,8703 0,8702 0,8701 0,8703 0,8702 0,8648 0,8631 0,8576 0,8510 0,8416 0,8536 0,8510 0,8416 0,8336 0,97914		Cl. 3 K022a K022b N178 H182b H182a A071a I051 N158 H035 P091 M098 P156 B112 F231 A071b I151 N063 A071 H109 C017	K022 0,9777 0,9424 0,9352 0,9277 0,8650 0,8388 0,8252 0,8105 0,7963 0,7914 0,7781 0,7781 0,7638 0,7649 0,7638 0,7638 0,7638 0,7245 0,7204	K022a K022b N178 I051 N063 A071a H035 B112 N158 D178 F231 C017 H182b P091 P156 A071b A071 M098 H109	K022a 0,9810 0,9523 0,9424 0,9397 0,9338 0,9295 0,9245 0,9249 0,9173 0,9103 0,9103 0,9002 0,80788 0,8675 0,8653 0,8653 0,8651 0,8184	K022b K022a B112 N178 F231 K022 C017 N158 N063 D178 A071a I051 H0355 H182b P091 H182a P156 A071b A071 M098 H109	K022b 0,9890 0,9523 0,9451 0,9426 0,9419 0,9151 0,9120 0,9120 0,9109 0,9097 0,9033 0,9093 0,8082 0,8909 0,8806 0,8804 0,8809 0,8806 0,8804 0,8794 0,8771 0,8332	N178 C017 K022ba F231 A071a K022 B112 H035 I051 P091 A071b A071b M098 H182b N158 N063 D178 H182a P156 H109	N178 0,9963 0,9732 0,9426 0,9397 0,9388 0,9397 0,9096 0,8992 0,8925 0,8822 0,8828 0,8783 0,8743 0,8743 0,8743 0,8768 0,8591 0,8533 0,8149		CL. 4 D178 H035 I051 F231 C017 B112 K022a K022b P091 N158 A071a P156 A071b H182a N178 H182b M098 H182b M098 H182b	D178 0,9953 0,9672 0,9641 0,9147 0,9146 0,9133 0,9120 0,8877 0,8809 0,8767 0,8766 0,8729 0,8692 0,8672 0,8692 0,8672 0,8672 0,8692 0,8572 0,8671 0,8592 0,8572 0,8416 0,8572 0,8416 0,8572 0,8416 0,8572 0,8416 0,8572 0,8572 0,8572 0,8572 0,8572 0,8572 0,8572 0,8572 0,8572 0,8572 0,8572 0,8572 0,8572 0,8572 0,9541 0,9545 0,9541 0,9545 0,95550 0,95550 0,95550 0,95550000000000	H035 D178 N063 I051 F231 C017 K022a B112 A071a N158 K022b N178 A071b H182b P156 P091 H182a M098 H109 K022	H035 0,9914 0,9672 0,9618 0,9597 0,9466 0,9232 0,9166 0,9023 0,9079 0,9033 0,8982 0,8981 0,8957 0,8885 0,8860 0,8834 0,8871 0,8875 0,8876 0,8105	1051 N063 D178 H035 A071a B112 N158 K022a C017 F231 K022b H182b H182b H182a P091 N178 M098 P156 A071b K022 H109	1051 0,9929 0,9794 0,9597 0,9457 0,93457 0,9348 0,9348 0,9348 0,9190 0,9097 0,9021 0,8935 0,8944 0,8935 0,8944 0,8935 0,88411 0,8729 0,8633 0,86488 0,8388 0,8388 0,7740	N063 1051 D178 H035 K022a K022b N158 B112 C017 F231 H182a A071a H182b P091 N178 P156 M098 A071 H1097 A071 H1092	N063 0,9929 0,9794 0,9641 0,9641 0,9295 0,9295 0,8927 0,8957 0,8922 0,8831 0,8818 0,8818 0,8818 0,88780 0,8540 0,8540 0,8540 0,8540 0,8540 0,8540 0,8540 0,8540 0,8484 0,7331
CI. 2 A071 A071b A071a M098 C017 F231 H035 B112 H109 H109 D178 K022a D178 K022a P091 I051 N063 N158 H182a H182a H182b K022	A071 0,9903 0,9526 0,9526 0,9481 0,9123 0,9123 0,8924 0,8924 0,8848 0,8794 0,8652 0,86537 0,8501 0,8458 0,8434 0,8428 0,8448 0,8448 0,8448 0,8448 0,8448 0,8448 0,8475 0,7445 0,7303	A071a M098 A071 I051 C017 F231 N178 B1122 K022a H182a H182a H182b P091 N063 P156 D178 K022 H109 H182	A071a 0,9926 0,9717 0,9526 0,9457 0,9453 0,9315 0,9315 0,9315 0,9166 0,9116 0,9109 0,9110 0,9109 0,9109 0,9101 0,8890 0,8818 0,8807 0,8767 0,8767 0,8480 0,88162 0,8767	A071b A071a A071a M098 C017 F231 H035 N178 B112 K022b D178 B112 K022a I051 H109 N158 H182a N063 P156 H182b P091 H182 K022	A071b 0,9903 0,9785 0,9512 0,9480 0,9177 0,8826 0,8815 0,8804 0,8729 0,8675 0,8888 0,8675 0,8888 0,8675 0,8588 0,8577 0,8486 0,8588 0,8570 0,8588 0,8570 0,8588 0,8570 0,8588 0,8570 0,8588 0,8570 0,8588 0,8570 0,8588 0,8570 0,8588 0,8570 0,8570 0,8588 0,8570 0,8800 0,8750 0,8800 0,8750 0,8800 0,8750 0,8800 0,8750 0,8800 0,8750 0,8800 0,8750 0,8800 0,8577 0,8860 0,8800 0,8570 0,8850 0,8800 0,8800 0,8800 0,8570 0,8800 0,8800 0,8570 0,8800 0,8570 0,8800 0,8570 0,8800 0,8570 0,8800 0,8570 0,8800 0,8570 0,8580 0,8570 0,8500 0,80000 0,80000 0,80000 0,800000000	M098 A071a A071 D017 B112 F231 I051 N178 K022b N158 K022b H182a H035 P156 K022a H182b N063 D178 H109 K022 I151	M098 0,9796 0,9481 0,9480 0,9157 0,9102 0,9058 0,8811 0,8783 0,8771 0,8703 0,8771 0,8702 0,8648 0,8576 0,8510 0,8546 0,8510 0,8416 0,8336 0,8416 0,8336 0,7914 0,7638		Cl. 3 K022 K022b N178 H182b H182b H182b H182b H182b H182b H182b H071 N158 H035 P091 N098 P156 B112 F231 A071b H151 N098 H151 N071b H151 N071 H109 C017 D178	K022 0,9777 0,9424 0,9352 0,8650 0,8529 0,8480 0,8388 0,8252 0,8105 0,7963 0,7963 0,7914 0,7781 0,7781 0,7784 0,7648 0,7638 0,7638 0,7638 0,7245 0,7204 0,7204	K022a K022b K022 N178 I051 N063 A071a H035 B112 N158 D178 D178 D178 D178 D178 D178 D171 H182a H182b P091 P156 A0711 M098 H109 I151	K022a 0,9810 0,9523 0,9424 0,9397 0,9338 0,9295 0,9245 0,9245 0,9245 0,9249 0,9133 0,9039 0,9006 0,9002 0,8788 0,80764 0,8675 0,8653 0,8653 0,8631 0,8184 0,7433	K022b K022a B112 N178 F231 K022 C017 N158 N063 D178 A071a H035 H182b P091 H182a P156 A071b M098 H109 H151	K022b 0,9830 0,9451 0,9451 0,9426 0,9352 0,9352 0,9151 0,9120 0,9120 0,9120 0,91920 0,9093 0,9098 0,8082 0,8080 0,8804 0,8804 0,8771 0,8332 0,6755	N178 C017 K022b K022a F231 A071a K022 B112 H035 I051 P091 A071b A071b A071b M098 H182b N158 N063 D178 H182a P156 H182	N178 0,9963 0,9732 0,9342 0,9397 0,9397 0,9377 0,9096 0,8982 0,8982 0,8872 0,8872 0,8872 0,8878 0,8783 0,8743 0,8753 0,8743 0,8753 0,8743 0,8753 0,8743 0,87530 0,87530 0,87530 0,8753000000000000000000000000000000000000		CL 4 D178 H035 N063 I051 F231 C017 B112 K022a K022b P091 N158 A071a P156 A071b A071b A071b H182a N178 H182b M098 H109 K022 H182	D178 0,9953 0,9672 0,9641 0,9147 0,9146 0,9133 0,9120 0,8877 0,8766 0,8762 0,8762 0,8762 0,8671 0,8596 0,8572 0,8671 0,8596 0,8572 0,8416 0,8572 0,8416 0,8216 0,8216 0,8216 0,8216 0,8216	H035 D178 N063 I051 F231 C017 K022a B112 A071a N158 K022b N178 K022b N178 A071 A071b H182a M098 H109 K022 H182	H035 0,9914 0,9672 0,9618 0,9597 0,9466 0,9232 0,9166 0,9033 0,8982 0,8981 0,8957 0,8885 0,8885 0,8885 0,8886 0,8884 0,8876 0,8876 0,8876 0,8692 0,8576 0,8105 0,7664	1051 N063 D178 H035 A071a B112 N158 K022a C017 F231 K022b H182b H182b H182a P091 N178 M098 P156 A071b K022 H109 H151	1051 0,9929 0,9794 0,9597 0,9457 0,93457 0,9348 0,9348 0,9248 0,9097 0,9021 0,8094 0,8025 0,8811 0,8729 0,8633 0,8488 0,8488 0,8488 0,8488 0,8488 0,7740 0,7635	N063 1051 H035 K022a K022b N158 B112 C017 F231 H182a A071a H182b P091 N178 P156 M098 A071b H109 K022 H182	N063 0,9929 0,9794 0,9641 0,9295 0,9120 0,9077 0,8957 0,8957 0,8957 0,8922 0,8831 0,8818 0,8780 0,8771 0,8540 0,8770 0,8770 0,8957 0,8770 0,8770 0,8770 0,8770 0,8771 0,8540 0,8771 0,8540 0,8570 0,8570 0,8540 0,8570 0,8540 0,8570 0,8540 0,8570 0,8570 0,8570 0,8570 0,8570 0,8570 0,8771 0,8773 0,8770 0,8771 0,8773 0,8770 0,8771 0,8773 0,8770 0,8771 0,8770 0,8771 0,8770 0,8771 0,8770 0,8771 0,8770 0,8771 0,8770 0,8771 0,8770 0,8771 0,8770 0,8771 0,8770 0,8771 0,8770 0,8770 0,8771 0,87700 0,87700 0,87700 0,8770000000000

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.

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Cl. 1	B219		H109		H182		1151		P156	C	. 3	E033		G173		K022		K022
P156	0,9879	H109	0,9948	H182	0,9960	1151	0,9951	P156	0,9991	K0	22a	0,9754	G173	0,9961	K022	0,9932	K022a	0,995
H109	0,9854	B219	0,9854	H109	0,9853	P156	0,9892	H035	0,9900	E)33	0,9740	K022	0,9791	G173	0,9791	H182	0,9814
B219	0,9843	H182	0,9853	K022a	0,9814	P091	0,9853	1151	0,9892	H	182	0,9675	K022a	0,9776	K022a	0,9755	H109	0,9813
H182	0,9787	K022a	0,9813	B219	0,9787	H035	0,9836	B219	0,9879	H	109	0,9672	H109	0,9680	P156	0,9721	G173	0,977
1151	0,9770	1151	0,9738	P156	0,9719	D178	0,9822	N158	0,9871	G	173	0,9664	B219	0,9672	H109	0,9718	B219	0,9759
K022a	0,9759	K022	0,9718	E033	0,9675	N063	0,9772	L028	0,9856	B	219	0,9651	E033	0,9664	D178	0,9647	K022	0,975
G173	0,9672	G173	0,9680	1151	0,9658	B219	0,9770	P091	0,9847	K)22	0,9422	H182	0,9510	H182	0,9607	E033	0,975
E033	0,9651	P156	0,9673	P091	0,9632	N158	0,9748	N063	0,9796	- 11	51	0,9253	1151	0,9228	H035	0,9600	1151	0,9579
K022	0,9596	E033	0,9672	K022	0,9607	L028	0,9743	1051	0,9747	P	156	0,9238	P156	0,9045	B219	0,9596	P156	0,9543
P091	0,9500	P091	0,9631	B112	0,9545	B112	0,9739	D178	0,9744	P	91	0,9076	P091	0,8999	P091	0,9571	P091	0,9379
B112	0,9440	B112	0,9567	G173	0,9510	H109	0,9738	K022	0,9721	B	12	0,9001	B112	0,8938	1151	0,9547	B112	0,9273
D178	0,9337	D178	0,9501	H035	0,9439	H182	0,9658	H182	0,9719	D	178	0,8875	D178	0,8936	N063	0,9546	H035	0,9238
H035	0,9259	H035	0,9430	N158	0,9339	1051	0,9594	B112	0,9710	H)35	0,8852	H035	0,8719	L028	0,9465	D178	0,9230
N158	0,9093	N158	0,9284	D178	0,9324	K022a	0,9579	H109	0,9673	N	158	0,8686	N063	0,8563	B112	0,9449	N158	0,907
N063	0,9089	N063	0,9270	L028	0,9253	K022	0,9547	K022a	0,9543	N)63	0,8630	N158	0,8443	N158	0,9442	N063	0,907
L028	0,9014	L028	0,9220	N063	0,9229	E033	0,9253	E033	0,9238	LC	28	0,8597	L028	0,8393	E033	0,9422	L028	0,9023
1051	0,8760	1051	0,8977	1051	0,9064	G173	0,9228	G173	0,9045	10	51	0,8318	1051	0,8036	1051	0,9238	1051	0,8769
CI. 4	D178		H035		1051		L028		N063	C	. 5	B112		N158		P091		
D178	0,9937	H035	0,9979	1051	0,9971	L028	0,9994	N063	0,9981	N	58	0,9895	N158	0,9969	P091	0,9979		
N063	0,9859	L028	0,9949	L028	0,9953	N158	0,9965	H035	0,9944	P	91	0,9869	L028	0,9965	B112	0,9869		
H035	0,9831	N063	0,9944	N158	0,9928	1051	0,9953	L028	0,9935	B	12	0,9865	H035	0,9943	H035	0,9858		
1151	0,9822	N158	0,9943	H035	0,9875	H035	0,9949	N158	0,9900	- 11	51	0,9739	1051	0,9928	N158	0,9854		
P091	0,9759	P156	0,9900	N063	0,9857	N063	0,9935	D178	0,9859	H)35	0,9732	N063	0,9900	1151	0,9853		
L028	0,9756	1051	0,9875	P156	0,9747	P156	0,9856	1051	0,9857	P	156	0,9710	B112	0,9895	P156	0,9847		
P156	0,9744	P091	0,9858	P091	0,9645	D178	0,9756	P156	0,9796	D	178	0,9697	P156	0,9871	N063	0,9767		
N158	0,9733	1151	0,9836	D178	0.9597	P091	0,9744	1151	0,9772	N	063	0,9659	P091	0.9854	D178	0,9759		
B112	0,9697	D178	0.9831	1151	0,9594	1151	0,9743	P091	0,9767	LC	28	0,9602	1151	0,9748	L028	0,9744		
K022	0,9647	B112	0,9732	B112	0,9479	B112	0,9602	B112	0,9659	H	109	0,9567	D178	0,9733	1051	0,9645		
1051	0.9597	K022	0,9600	K022	0,9238	K022	0,9465	K022	0,9546	H	182	0,9545	K022	0,9442	H182	0.9632		
H109	0,9501	H182	0,9439	H182	0,9064	H182	0,9253	H109	0,9270	10	51	0,9479	H182	0,9339	H109	0,9631		
B219	0,9337	H109	0,9430	H109	0,8977	H109	0,9220	H182	0,9229	K)22	0,9449	H109	0,9284	K022	0,9571		
H182	0,9324	B219	0,9259	K022a	0,8769	K022a	0,9023	B219	0,9089	B	219	0,9440	B219	0,9093	B219	0,9500		
K022a	0,9230	K022a	0,9238	B219	0,8760	B219	0,9014	K022a	0,9071	KO	22a	0,9273	K022a	0,9071	K022a	0,9379		
G173	0,8936	E033	0,8852	E033	0,8318	E033	0,8597	E033	0,8630	E)33	0,9001	E033	0,8686	E033	0,9076		
F033	0.8875	G173	0.8719	G173	0.8036	G173	0.8393	G173	0.8563	G	73	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^{TM12}. 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, timeconsuming 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 CASTTM membrane was deposited on metal slides (Al or TiAlV₆ 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 mm² 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 prestructuring 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 CASTTM nylon membrane slide (**A**), PAMAM functionalized conductive silicon initial (**B**) and refined batch (**C**), conventional PAMAM glass slide (**D**), and Quantifoil QMTTM 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.