2. Materials and Methods.

2.1 Materials

2.1.1 Laboratory equipment

• Centrifuge	1-13, Sigma Laborzentrifugen GmbH, Osterode am Harz
• CCD camera	PXL CCD video camera, Photometrix, Tucson, Arizona, USA IQLab software, Scientific Analytics, Vienna, Virginia USA
• CCD camera	Fujifilm
• UV trans-illuminator	Herolab GmbH, Wiesloch
• Gel electrophoresis equipment	Hoefer SE 200, Amersham Pharmacia Biotech Europe GmbH,Freiburg
• Expedite 8900 Nucleic Acid Synthesis System	PerSeptive Biosystems, Farmingham, MA, USA
• HPLC system	LKB, Pharmacia
• HPLC SMART system	Pharmacia
• LumiImager LAS 1200	Boehringer Mannheim GmbH
• Multiple gel-caster for SDS-gel electrophoresis	Hoefer SE 215, Amersham Pharmacia Biotech Europe
• Mass spectrometer	Bruker Scout 26 Reflex II, Bruker Franzen Analytik GmbH, Bremen
• Incubator Termomixer	Eppendorf, Koeln
• Pipetts, adjustable,12-channel	Eppendorf, Koeln
• PhosphorImager BAS 5000	Fujifilm
• Power supply	BioRad Laboratories GmbH Munchen
• Robot for DNA spotting	Genetix, Christchurch, Dorset, UK
• Shaker	Rocky, Froebel Labortechnik, Wasserburg
• Spectrophotometer	Shimatzu, Deutschland gmbH, Duisburg
• PCR machine	PTC100 MJ Research, Inc., Watertown, USA
•	
• vacuum blotting manifold	
• Vortex	Vortex Genie 2-Mixer, Bender and Hobein AG, Zurich, Switzerland

2.1.2 Chemicals, enzymes

• Acrylamide, 0.8% Bisacrylamide	Rotiphorese Gel 30, Carl Roth gmbH, Karlsruhe
• Alkaline Phosphatase	Boehringer Mannheim GmbH
• ammonium persulfate	BioRad Laboratories GmbH Munchen
• AttoPhos	JBL Scientific SanLuis Obispo, USA
• m-Cresol	Aldrich
• CDP-Star	Boehringer Mannheim GmbH
• Coomassi Blue	Serva
• dATP, dCTP, dTTP, dGTP, lithium salts	Boehringer Mannheim GmbH

- D-biotin — Sigma, Deisenhofen
- ^{33}P ATP — Amersham Pharmacia Biotech Europe GmbH,Freiburg
- DIPEA, N,N-Diisopropylethylamine — Sigma
- ethydium bromide 1% — Fluka
- glycerol — Merck, Darmshadt
- glycine — Merck, Darmshadt
- Lithium perchlorate — Fluka Chemie AG Buchs
- Streptavidin conjugated Alkaline Phosphatase — Boehringer Mannheim GmbH
- Streptavidin conjugated Phycoerythrin — Molecular Probes Europe BV, Leiden, The Netherlands
- Streptavidin Magnetic Particles — Boehringer Mannheim GmbH
- sodium dodecylsulfate, elecrophoresis grade — BioRad Laboratories GmbH Munchen
- sodium N-lauroyl sarcosinate — Sigma, Deisenhofen

- succinimidyl ester, Biotin-XX — Molecular Probes Europe BV Leiden, The Netherlands
- succinimidyl ester of 5-(and-6)-carboxyfluorescein — Molecular Probes Europe BV Leiden, The Netherlands
- Sybr-DX nucleic acid staining reagent — Molecular Probes Europe BV Leiden, The Netherlands
- SYPRO Orange protein gel staining reagent — Molecular Probes Europe BV Leiden, The Netherlands
- TEMED — BioRad Laboratories GmbH Munchen
- Triton X-100 — Sigma, Deisenhofen
- Tricine — Sigma, Deisenhofen

Inorganic salts, acids and bases, alcohol and solvents were *pro analysi* quality from Merck, Darmstadt. Restriction and DNA modification enzymes were from New England Biolabs GmbH, Schwalbach/Taunus, *Taq* DNA polymerase was a gift from R. Pawlik.

2.1.3 Kits

- Nucleotide Removal Kit — Qiagen GmbH, Hilden
- PCR purification Kit — Qiagen GmbH, Hilden
- Ready gels Precast gels for Polyacrylamide Electrophoresis — BioRad Laboratories GmbH Munchen

2.1.4 Chromatography and separation reagents

- Acetonitrile HPLC grade Uvasol, — Merck, Darmstadt
- Columns,2.5 ml, 10 µm filter — MoBiTec, Goettingen
- DEAE cellulose — Merck, Darmstadt
-
- Glass microfibre filter 2.5 cm — GF/C Whatman GmbH, Goettingen
- HPLC column, DeltaPak 15 µm 7.8x300 µm — Waters Nihon Waters K.K, Japan
- HPLC column µRPC C2/C18, SC 2.1/10 — Pharmacia
-
- TFA, trifluoroacetic acid — Merck, Darmstadt

- Trisacryl GF 05 M, gel-filtration 300Da-2,5 µm — Biosepra GmbH
- Ultrafree- MC PTFE 0.2 µm spin columns — Millipore, NIHON Millipore LTD Yonezawa

2.1.5 PNA synthesis reagents

- F-moc PNA monomers and diluent — PerSeptive Biosystems
- Wash solvents A (DMF) and B — PerSeptive Biosystems
- Base solution (2,6-Lutidine and N.N-diisopropylethylamine in DMF — PerSeptive Biosystems
- Activator (HATU) — PerSeptive Biosystems
- Blocking solution — PerSeptive Biosystems
- Expedite AEEA-OH Linker — PerSeptive Biosystems
- Deblocking solution — PerSeptive Biosystems
- Capping solution — PerSeptive Biosystems
- Dicloromethane — Merck, Darmstadt
- Diethyl ether — Aldrich

2.1.6 Other materials

- 3MM blotting paper — Whatman GmbH, Goettingen
- DNA marker, size standart — New England Biolabs GmbH, Schwalbach/Taunus
- Protein size standard low range — Boehringer Mannheim
- Filtration paper — N595, Schleicher &Schuell, Dassel
- Glass microfibre filters — GF/C, Whatman GmbH, Goettingen
- Nylon membrane 222x222 mm^2 — Amersham Pharmacia (Amersham, Arlington Heights, IL)
- Replicators 384-pin — Genetix, Christchurch, Dorset, UK
- Pipett 10µl precision tips, C10 — Gilson, Gilson Medical Electronics S.A.,Villiers-le-Bel, France
- PCR 384 plates — Genetix, Christchurch, Dorset, UK
- PCR plate sealing film Microseal A film — MJ Research Inc., Watertown, USA

2.1.7 Buffers and solutions

Attophos stock solution
2.4 M diethanolamine
5 mM Attophos
0.23 mM $MgCl_2$
pH 9.2
sterilised by filtration through a 0.2 m pore size filter

Coomassi Blue staining
1.25 g Coomassi Brilliant Blue G250 are dissolved in 225ml ethanol. 225 ml distilled water and 50 acetic acid are added. The mixture is stirred for 2 hours and filtered through a folded filter (N595, Schleicher &Schuell, Dassel)

Coomassi De-staining
40% v/v methanol, 10% acetic acid

Denaturing solution
0.5 M NaOH
1.5M NaCl

MALDI matrix solution
1% (w/v) HCCA (α-cyano-4-hydroxycinnamic acid)
50% acetonitrile
0.1% TFA

Neutralising solution
1 M Tris HCl, pH 7.4
1.5 M NaCl

PCR buffer 10-x
1.5 mM m-cresol red (optional)
0.5 M KCl
15 mM $MgCl_2$
0.5 M Tris-HCl, pH 9.0
sterilised by filtration through a 0.2 m pore size filter

10-x PBS
1.37 M NaCl
2.7 mM KCl
100 mM sodium phosphate, pH 7.4

PNA Hybridisation buffer
10 mM Tris-HCl, pH 7.5
5 mM NaCl
7.5% sodium N-lauroyl sarcosinate

PNA Detection buffer
60 mM sodium phosphate, pH 7.5
100 mM NaCl

SDS gel loading buffer 4-x(standard)
0.2 M Tris-HCl, pH 6.8
8% SDS
40 %(w/v)glycerol
0.004% bromophenol blue

SDS gel loading buffer 2-x (modified for PNA)
2x TBE
2%(w/v) SDS
40% glycerol
0.002%(w/v) bromophenol blue

20-x SSC
3 M NaCl
0.3 M sodium citrate, pH 7.5

SSCARC
4-x SSC (pH 7.5)

7.5% sodium N-lauroyl sarcosinate

TAE buffer
40 mM Tris acetate, pH 8.0
1 mM EDTA

TBE buffer
90 mM Tris borate, pH 8.0
1 mM EDTA

TE 10-x
100 mM Tris-HCl, pH 8.0
10 mM EDTA

2.1.8 Oligonucleotides and Primers

4 oligonucleotide targets and 4 probes system

5'-CAGGGATTTCCCAGT
5'-CAGGGCTTTCCCAGT
5'-CAGGGGTTTCCCAGT
5'-CAGGGTTTTCCCAGT

5'-biotinTGGGAAAGCC
5'-biotinTGGGAAAACC
5'-biotinTGGGAAANCC (N=A+G+C+T)

DNA target for PNA-in-gel hybridisation:

5'-TTTGGCTG**C**GGAGGGAGTG
5'-TTTGGCTG**T**GGAGGGAGTG

DNA oligonucleotides for PNA/DNA hybridisation patterns comparison:

5'-NTGGAGCTGN
5'-NTTGTTTTCN
5'- NTGGAGCTGN

DNA target for MALDI multiplex experiment:

5'biotin-TTTGGCGTCGGCACAGTGACAGGAGCA
5'biotin-TTTGGCTGCGGCACAGTGACAGGAGCA

PCR primers:

M13 forward 32-mer 5'-gctattacgccagctggcgaaaggggatgtg

M13 reverse 32-mer 5'-ccccaggctttacactttatgcttccggctcg

2.2 Methods

2.2.1 Oligonucleotide target system preparation

The four target oligonucleotides were transferred as a 3-fold dilution series to Hybond N+ membrane using vacuum dot-blotter. DNA on membrane was denatured by incubation on a denaturing solution for 1min, then 2 min in neutralisation solution, shortly air dried and UV cross-linked at default setting of the Cross-linker and pre-washed in SSARC buffer for 30 min at +65°C before the hybridisation.

2.2.2 Radioactive control of blotting and attachment of 15-nt long oligonucleotides

15-mer 15 pmol oligonucleotide was labelled with 50 µCi [γ^{33}P]-ATP and 10 U of T4 polynucleotide kinase and purified on TrisAcryl column as described below. The purity of oligonucleotide in the fractions was confirmed by thin layer chromatography on a DEAE cellulose in $NaHCO_3$ (pH 3.5) buffer. The purification was checked by quantification of amounts of oligonucleotide and label in each fraction. The most pure fraction contained 2/3 of the whole (presuming 100% yield that is 10 pmol or 0.1 pmol/µl) oligonucleotide and 2% from the radioactive label. This fraction was used for control blotting experiments. It is assumed the label has low affinity to nylon membrane.

The 3-fold dilution series of ^{33}P gamma ATP were prepared starting from 0.1 pmol/µl ending 0.03 fmol/µl. 1µL from each was spotted on the membrane. The dilution series in 0.5 M NaCl/ TE buffer were prepared in 40 µl of final volume and blotted using vacuum blot. 1µl from fraction 2 of purified oligonucleotide was spotted manually on N+ membrane as control. The spot diameter was estimated as 3mm, thus the square of the spot ($2\pi r^2$) is 14,14 mm^2.

After blotting the membrane was air dried and co-exposed with dilutions of [γ^{33}P]-ATP and manually spotted DNA to the phosphorscreen for 10-60 sec to estimate the losses from blotting. By comparison of radioactive counts from blotted and spotted oligonucleotide it was revealed that the losses from blotting are non significant.
To estimate the influence of washing steps on retaining of oligonucleotides membrane with blotted 15-mer was treated as described by manufacturer: denaturing and renaturing and UV cross-linked.

2.2.3 Amplification of the clones by PCR

Amplification of the short gun library clones was performed by Polymerase Chain Reaction (PCR). PCR amplifications were carried out in 384-well microtitre plates (Genetix), in 384-termocycling machine (MJ Research, PTC-200). Bacterial colonies were inoculated using disposable plastic 384-pin replicators (Genetix Ltd., Christchurch) into a 30 µl reaction volume containing 50 mM KCl, 10 mM Tris/HCl, pH 8.5, 1.5 mM $MgCl_2$, 200 µM of each dATP, dCTP, dGTP, dTTP, 0.16 µM of each PCR-primer (M13 forward and M13 reverse) and 0.5 units *Thermus aquaticus* (Taq) DNA polymerase. After inoculation, the microtitre plates are sealed using a 0.45 mm thick plastic foil (Biostat or Microseal ^{tm} A Film, MJ Research Inc.). PCR is performed in 30 cycles consisting of 10 sec at 94°C, 10 sec at 65°C and 3 min at 65 °C and 5 min of final extension step at +65 °C.

PCR products were analysed by 1.4% agarose gel electrophoresis. The PCR purification/concentration was performed using Qiagen Kit, the amount of DNA was quantified photometrically.
PCR products were sequenced by dye-terminator cycle sequencing using M13 forward primer and automated ABI sequencer (Perkin Elmer) by service department in the institute.

2.2.4 Arraying of shotgun clones PCR products by spotting

Nylon Hybond N+ membranes (Amersham, UK) membranes carrying 700 PCR products in duplicate were generated using robotic spotting devices developed in house (Maier, 1995). Each PCR product was repeatedly spotted ten times with a 250µm diameter pin, thus transferring approximately 1 µl PCR product (up to 100 ng) ((Maier et al., 1994)).

2.2.5 Direct coupling of oligonucleotide probes to enzyme- Streptavidin conjugate and chemoluminescent detection

For coupling of enzyme 30 pmol of 5´-biotinylated oligonucleotides were incubated with one unit of Str-AP, **Str**eptavidin-**A**lkaline **P**hosphatase conjugate (Cat#1089161), in 10 µl of 20 mM Tris pH 8.0 for 10 min at room temperature. Then 2 µl of 300 µM D-biotin solution were added. The hybridisation was carried out for 5 hours in SSARC buffer at 6 nM probe concentration. All steps were carried out at +4°C. After hybridisation membranes were washed for 30 min in SSARC buffer and rinsed in developing buffer (0.1 M diethanolamine, pH 9.0, 0.5 M NaCl and 1 mM $MgCl_2$). Then membranes were immersed in 0.25 mM solution of CDP-Star in developing buffer, briefly dried on Whatman, wrapped and photographed using Lumi-Imager LAS100 after 15 min of incubation at room temperature in the dark. The signals were evaluated using manufacturer's software. Probe and substrate were stripped off the membrane by incubation in 0.1% SDS/0.5xSSC at +65°C.

2.2.6 Three-step hybridization-detection protocol

DNA oligonucleotides were hybridised as described (Maier et al., 1994)and detected in a three-step protocol with modification. After hybridisation at 6nM probe concentration for 5-16 hours and washing for 30 min in hybridisation buffer (SSARC) the membrane was incubated with the Str-AP conjugate (1U in 5ml) in hybridisation buffer for 15 min at +4°C, followed by two 10 min washings in SSARC buffer and then the membrane was incubated with Alkaline Phosphatase substrate as above prior to imaging.

2.2.7 Radioactive labelling of oligonucleotide and hybridisation

Radioactive probes were prepared by phosphorylation of 30 pmol of non-biotinylated oligonucleotide with 50 µCi [γ^{33}P]-ATP (2500 Ci/ mMol) in 10 µl reaction using 1U of T4 polynucleotide kinase and purified by gel-filtration on Tris Acryl column equilibrated with 2xSSC buffer. The labelled oligonucleotide was eluted in free volume by 2x100µl of 2xSSC buffer by free flow.

Hybridisation was held at 6nM probe concentration in SSARC buffer at +4°C for 5 hours. The filters were washed for 30 min at +4°C. Membranes were exposed to Fuji imaging plate for 12 hours and scanned on a FUJI BAS 5000 system at the highest resolution. Data analysis was performed using AIDA 2.0 software.

2.2.8 Data capture

Radioactive hybridisation were exposed to the screens various times, the screens were scanned at a resolution of 25-50 µm and the images captured in 16bit TIFF format with a phosphor imager (Fuji).
The fluorescence hybridisation detected by chemiluminescence images were generated using LumiImager (Boehringer Mannheim).
The detection of phycoethrin-coupled probe hybridisation was performed using built in house laser scanner (Nyarsik, in preparation) using 40 mW laser power and 70-100% laser intensity. The size of image with reduced to 8bit depth is 30 MB in size at 40 µm resolution. The laser scanner consists of galvanometer-based optical scanner, lasers, photomultipiers and optical filtering. The excitation is made by argon-ion laser at 488 nm and emission filter of 570 nm. The detection is performed using Photomultiplier with analogue signal.

2.2.9 Data analysis

The hybridisation data analysis was performed using commercial software TINA2.0 (Raytest). The output median count within specified area is recorded and corrected by subtracting its local background. Local background is determined as darkness of pixels around the signal area. is the list of grey values correspondent to clone coordinate.

The reproducibility of hybridisation is verified within the same experiment by quantifying the duplicates and drawing a logarithmic diagonal plot of corresponding intensities.

2.2.9.1 Raw data normalisation by ranking of intensities

Signals from clones obtained upon hybridisation with one probe are ranked by comparison. The highest signal is assigned value of 1, the lowest- 0. All the rest clones in between are assigned values at regular intervals in between, e.g. at intervals of 1/N (N-number of clones).

2.2.9.2 Reproducibility plots generation

The reproducibility of hybridisation is calculated as the sum of positive clones from same region divided by total number of clones in this overlap. The value of each positive clone (the clone which intensity was above the threshold of 0.9) is set to 1, value of negative (if ranked value was below the threshold) to 0 and their sum divided by number of clones in the region. The calculation was performed for each nucleotide position of the contig using routines written by C. Wierling. The value for each nucleotide value is then plotted. Alternatively, raw intensities were integrated for each nucleotide in order to avoid bias of an artificial binary system. The results of plotting using raw values showed similar profiles (not shown).

2.2.10 PNA methods

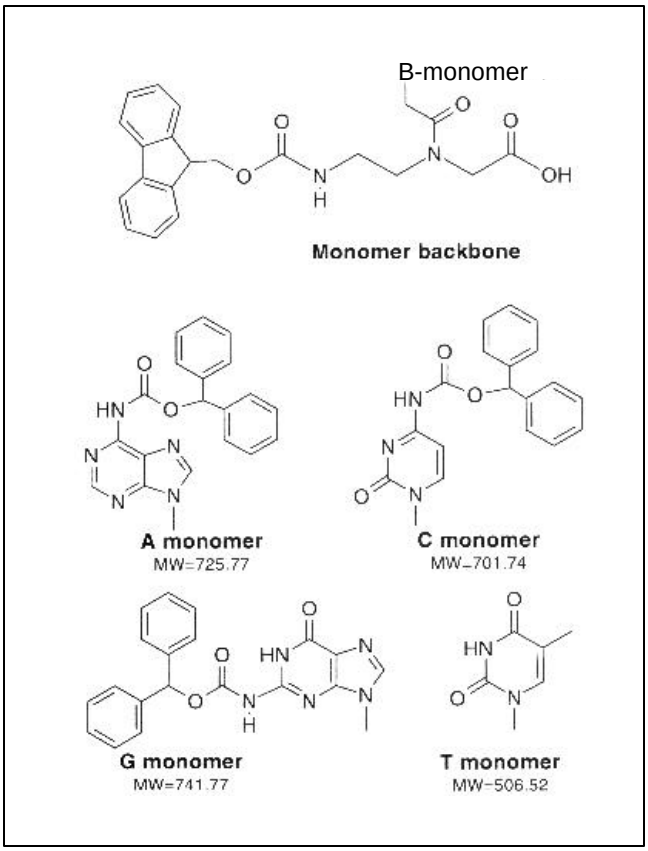
2.2.10.1 PNA synthesis

The PNAs were synthesised at a 2 µmol scale on Expedite PNA columns using an Expedite 8900 Nucleic Acid Synthesis System (PerSeptive Biosystems, Framingham, MA, USA). The monomers and activator (HATU) were diluted freshly with PNA diluent and DMF respectively.

When stated PNA were coupled with a linker (L), hydrophilic Fmoc-AEEA-OH spacer (8-amino-3,6-dioxaoctonoic acid) (GEN 063032), in additional cycle.

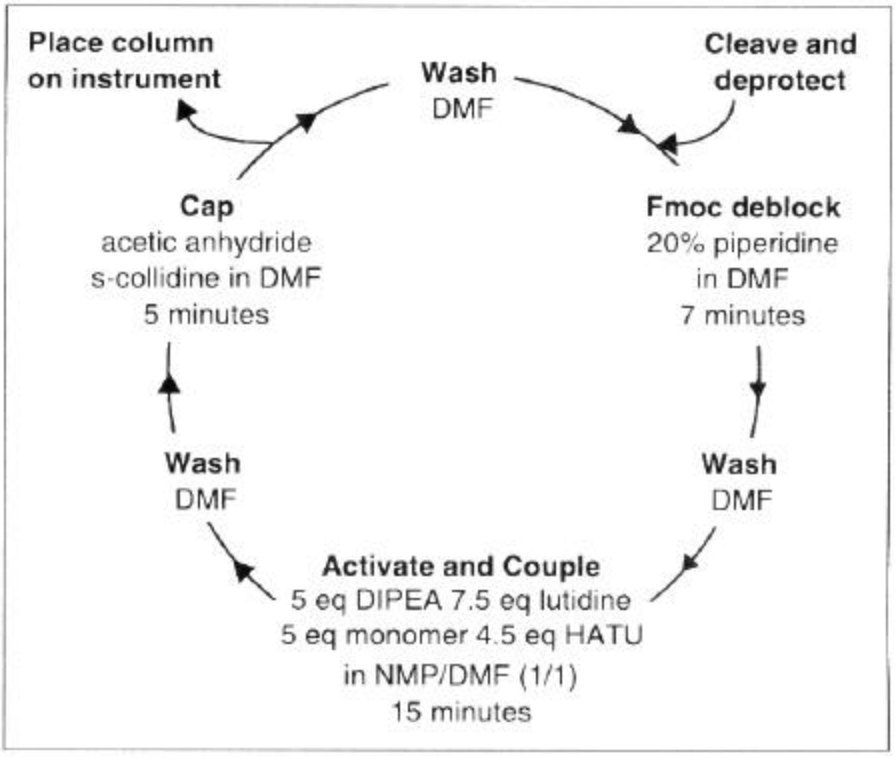
The monomers are protected with Fmoc (9-fluorenylmethoxycarbonyl), which is base labile and removed by 20%piperidine in DMF, and exocyclic aminogroups of A,C,G are protected with Bhoc (benzhydryloxycarbonyl) group, which is removed in the end by TFA treatment.

Figure 1. F-moc/Bhoc PNA monomers (from PerSeptive Biosystems User's Guide).



Fmoc monomer activation and coupling (acetylation) should be efficient so that PNA product is homogeneous. Expedite chemistry of PNA synthesis uses as activator (HATU,(o-(7-azabenzo-triazol-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate in presence of tertiary amine (DIPEA). Activation and coupling steps are carried in so called base solution (2,6-Lutidine and N.N-diisopropylethylamine in DMF). Usually HATU is used to enhance reactivity of amino acids, suited for difficult couplings. After coupling step the uncoupled PNA are blocked with capping solution (acetic anhydride s-collidine in DMF) to prevent generation of wrong sequences in next cycles.

Figure 2. Expedite PNA synthesis cycle (from PerSeptive Biosystems User's Guide)



2.2.10.2 PNA deprotection and precipitation

The columns were removed from the Synthesiser and flushed with 5 ml of dichloromethane (DCM) and subsequently dried by blowing nitrogen to remove DCM. Then they were dried in vacuum for 2hours. When not immediately used dried columns were stored at -20°C.

PNA on resin were transferred to Millipore Ultrafree- MC PTFE (SE3P230J3) and cleaved from the support with TFA: m-cresol mixture (4:1, v/v) for 1,5 hour at room temperature. The resign was centrifuged at 2,000 rpm for 5min. To precipitate PNA from the resulting solution 800 µl of diethyl ether was added, mixed by vortexing and centrifuged at 2,000 rpm 5min. Supernatant was removed, the precipitant mixed with the same volume of diethyl ether and precipitation procedure was repeated two more times. The residual ether was removed by heating at 55°C for 5min. The PNAs were dissolved in 200 µl of aqueous 0.1% TFA by vortexing.

The list of synthesised PNA is in Table 1. As with peptides, the PNA sequences are written from the N to the C-terminus. The PNA with double linker were purchased from Metabion (Martinsried).

Table 1. The sequences of synthesised or purchased PNAs.

+ a single modification group (linker or biotin),

++ a double modification group(linker),

+/- PNA exists as modified and non-modified with biotin)

sequence	linker	biotin	sequence	linker	biotin
agggagt	++	+	ctggagca	++	+
agggagt			gaagcaga	++	+
ctggaag	++	+	gacgaggt		+
gacgagg	++	+	gcagcagc		+
aatgagga	+	+/-	gctgctgc		+/-
aggagtaa	+	+	ggagcagc		+
aggagtaa	++	+	ggagcagc	+	+
agggagtg		+/-	tcactgtg		+/-
agtggctg			tcctcctg		+
cacagtga		+/-	tcctcctg	+	+
cactccct		+/-	tgctcctg		+/-
cagccact		+/-	tgctcctg	++	+
ccacagcc	+	+	tgctggtg	+	+/-
ccaggagg	++	+	tgctggtg	+	+
ccccagcc	+	+/-	tggagcag	+	+/-
ccacagcc	+	+/-	tggagctg		+
ccgcagcc	+	+/-	tggagctg	++	+
cctcagcc	+	+/-	tggctctg	++	+
cctcctgc		+	tgttattt		+/-
cctcctgc	++	+	ttctcctg		+/-
cctgctgc	+	+/-	ttgccttt	+	
cctgggca	++	+	ttgttttc	+	+/-
ctcaccat		+/-	NcactccctN		
ctccggcc	+		NtgctcctgN		
ctggagca	++	+			

2.2.10.3 Reversed-phase purification of PNA

PNA were purified on C18 DeltaPak column 15 µm, 300 A 7.8 x300 mm (Waters, #011803) on LKB HPLC (Pharmacia) apparatus with 2ml/min flow rate. The separation was performed using 0.1% TFA in water as Eluent A and 70% ACN/0.1% TFA as Eluent B. The gradient used was 0%B in 0 to 5 min, 0 to 40% B in 5 to 25 min, 40% B in 25-30min, 40 to 0% B in 30-40 min. 50-100 µl of PNA were injected into a 200 µl loop. The 8mer unmodified PNA eluted typically at 30% range of solvent B(20% of acetonitrile). The separation was monitored at 260 nm, the fractions were

collected manually. Spectra was recorded on LKB recorder. Manually collected fractions were vacuum dried (without heating) and sequences confirmed by MALDI TOF.

2.2.10.4 Determination of PNA concentration

The concentrations was determined according to (Sambrook et al., 1989):

$A_{260} = C * \varepsilon * l$.

A_{260} is the optical density at 260 nm, L-length of the light path in the cuvette (1 cm) and ε -extinction coefficient.

Extinction coefficients ε were determined as sum of dNTP, using following values for nucleobases

C= 6.6, T=8.6, A=13.7, G=11.7.

The absorption was taken at the maximum, since for the short oligonucleotides the maximum of absorption is shifted from 260nm.

2.2.10.5 Small scale PNA HPLC purification

When PNA were labelled in analytical quantities they were purified on SMART (Pharmacia) system using reverse phase (µRPC C2/C18, SC 2.1/10 column) and 100 Ml injection loop. 50 µl was injected. PNA were separated using linear gradient of 70%ACN in 0.1% TFA and monitored at 260 nm. PNA eluted at 15-17 % of solvent B. PNA after reaction were vacuum dried.

2.2.10.6 PNA labelling and modification

2.2.10.6.1 Biotin labelling of PNA

To couple PNAs with biotin the resin-bound PNA (approximately 1/3 from that recovered after drying) were mixed with 50 µL DMF, 5µL DIPEA and 150 µL Biotin-XX succinimide ester dissolved in DMF at 60µg/µL. The reaction was held overnight at ambient temperature. The resin was washed twice with DMF on spin-columns Ultrafree-MC PTFE (Millipore, SE3P230J3), deprotected and precipitated as above. Modified PNA are not going in spontaneous degradation in the amino-end and can be stored in water solutions.

2.2.10.6.2 Fluorescein labelling of PNA

For the labelling 5-10-fold excess of succinimide ester of Fluorescein is used. 230 µg of Fluorescein:(MW 457g/Mol) were dissolved in DMSO 40µl, diluted 1:1000 in 20 mM Tris-HCl buffer pH 8.0 to estimate concentration. To measure concentration of fluorescein the absorption spectra was taken from 600 to 400nm and the value at 495 nm maximum was used for calculation. 1Unit corresponds to 13.5nmol/µl, (extinction coefficient is 74 e^{-3})

For labelling of 4 nmol of PNA with fluorescein 40 nmoles of succinimide ester of Fluorescein (Haugland, 1996) have been used which corresponds to at least a 10-fold excess of the modification reagent. The 3-5 nmoles of purified PNA in 2µl of water were mixed with 4µL DMF, 4µl of 5 mM 5(6)-FAM in DMSO and 0.5µL DIIPE. The mixture was incubated at 60°C for 30 min. During reaction Fluorescein changes colour from red to orange.

After first incubation 4µl of 5 mM succinimide ester of Fluorescein in DMSO were added once more and incubation was proceeded for another 30 min. The reaction was diluted with water to 50µl and excess of fluorescein was removed by adding 5µL of 2M acetic acid followed by extractions with water saturated n-butanol. This procedure was repeated at least four times. The labelled PNAs were precipitated by adding 10 volumes of acetone followed by incubation for one hour at –20°C and centrifugation for 5 min at 13,000 rpm. The precipitant was air dried and dissolved in water.

2.2.10.7 Analysis of PNA by SDS PAGE

SDS polyacrylamide gels (12%-20%T, 2.6%C) and running buffers were prepared according to(10) using standard Mini-gel chamber (Amersham Pharmacia) and run at a constant current setting in most cases.
%T and %C were calculated as follows:

$$\%T = \frac{\text{acrylamide + bis (g)}}{100\ \text{ml}} \times 100\%$$

$$\%C = \frac{\text{bis (g)}}{\text{acrylamide + bis}} \times 100\%$$

Thus a stock solution prepared as follows: 30%acrylamide and 0.8% has %T=30.8%, %C =2.6%

2.2.10.7.1 Preparation of polyacrylamide gels

2.2.10.7.1.*1* SDS- PAGE.

SDS- polyacrylamide gels were prepared according to Laemmly (Sambrook et al., 1989).

Separation and stacking gels were prepared as follows: separation gel (20%T, 2.6%C): 0.1%SDS, 0.38 M Tris-HCl, 0.1% APS, polymerisation is started by adding 0.03% TEMED; stacking gel: 0.1% SDS 0.125 M Tris-HCl, pH 6.8, 0.1% APS, polymerisation is started by adding 0.1% TEMED.

Probes at approximately 1 µg/µl in water were mixed with the Loading buffer (10% glycerol, 0.125M Tris-HCl pH 6.8, 2%(w/v) SDS, 0.001%(w/v) bromophenol blue final concentration) and heated at 95°C for 3 min.

Electrophoresis was conducted at 20 mA for 30 min. Running buffer was 0.1% SDS, 25 mM Tris base, 250 mM glycine (pH 8.3).

If pre-cast Tris/Tricine peptide gels (16.5%T, 3.3%C, Bio-Rad) were used the running conditions were 50 mA for 1 hour with Tris/Tricine buffer (0.1 M Tricine, 1%(w/v) SDS, 0.1 M Tris base pH 8.2) in both chambers.

2.2.10.7.1.*2* TBE SDS-PAGE

TBE (90 mM Tris-borate pH 8.3, 2 mM EDTA) gels were cast at 7.5-15%T.10 ml of corresponding amount of 30% (29:1) acrylamide to bis-acrylamide solution in 1X TBE (without SDS) were polymerised by adding 100 µl of 10% ammonium persulfate and 4µl TEMED. 1mm thick gels were cast in the multiple gel caster Hoefer SE 215(Amersham Pharmacia). Probes were loaded in 2-4 µl volume of the Loading buffer B (20% glycerol, 1X TBE, 1%(w/v) SDS, 0.001%(w/v) bromophenol blue in the final concentration). Electrophoresis was conducted at 20mA for 30-40 min with 1X TBE/0.1%SDS buffer in both chambers.

2.2.10.7.2 Detection of PNA in gels

The PNAs were visualised either by UV shadowing or by staining with Coomassie or SYPRO. For staining with Coomassie gels were immersed in 100 ml Coomassi Staining solution (0.3mM Coomassie, 40%v/v methanol, 10% acetic acid) and gently shaken for 30 min and then destained with several changes of destaining solution (40% v/v methanol, 10% acetic acid). Alternatively, gels were stained for 30 min with SYPRO dye by diluting 5000X concentrated stock in 7.5% (v/v) acetic acid, followed by 30 min destaining in 7.5% acetic acid without dye. PNA bands were visualised using a CCD camera (Herolab GmbH, Germany) with the supplied by-pass filter.

2.2.10.7.3 Detection of PNA hybridisation by gel electroforesis

100 pmol of PNA-A or PNA-G were mixed with 200 pmol of the corresponding target DNA oligonucleotides (5'-TTTGGCTG**C**GGAGGGAGTG or 5'-TTGGCTG**T**GGAGGGAGTG) in a total volume of 2 µl in 5 mM Tris HCl, pH 8.0. The hybridisation was carried for 15 min at 20°C. The reactions and a control (100 pmol of PNA in 2 µl water) were mixed with 2 µl of the loading buffer B and analysed by 15% TBE PAGE using the same settings as above. Detection was done by UV shadowing and SYPRO staining.

2.2.10.7.4 Calculation of enthalpy for PNA duplex

The stability of PNA duplexes was estimated based on the sum of nearest neighbour bases interactions which is temperature dependent (Breslauer et al., 1986) The prediction was made using the following 10 values out of 16 possible due to symmetry:

pair	AA/TT	AT/TA	TA/AT	CA/GT	GT/CA	CT/GA	GA/CT	CG/GC	GC/CG	GG/CC

ΔH value	9.1	8.6	6.0	5.8	6.5	7.8	5.6	11.9	11.1	11.0

The prediction for PNA was made via the simplified estimation of the ΔH, using the factor of nearest neighbour bases interaction as the main component to estimate ΔH (transition enthalpy) which is calculated as

$\Delta H = \Delta h_i + \Sigma_x \Delta h_x$

where the helix initiation enthalpy (Δh_i) equals zero (Breslauer et al., 1986).

According to the pairwise interactions, ΔH for PNA CCTCCTGC is calculated as sum of 7 pairs: (CC-TC-CT-GC and CT-CC-TG):

CA*1+ CT*2 +GA*1 +GC*1 +GG*2 = 5.8 + 7.8 *2 +5.6 + 11.1 +11.2 *2 = 60.1 (kcal/mol)

Similarly, ΔH for AGGAGTAA is calculated as 53.8.

Melting curves were obtained using UV spectrophotometry from Exiqon (Denmark). These Tm measurements were performed in 100 mM NaCl, 0,1 mM EDTA, 10 mM sodium phosphate buffer (160 mM Na^+) at a 1 µM concentration of the PNAs and their complementary oligonucleotides in a volume of 500 µl.

2.2.10.8 Hybridisation of fluorescent PNAs to the DNA filters

2.2.10.8.1 Labelling with Phycoerythrin

The labelling was performed essentially the same as with the other streptavidin conjugate (Guerasimova et al., 1999). 30-50 pmol of biotinylated PNA were incubated in 10 µl of Tris-HCl pH 8.0 with 1.5 of Str-Phycoerythrin conjugate (1µg/µl) for 20 min. Then 1µl of 300µM D-biotin was added and incubated for 5 min at RT.

2.2.10.8.2 Hybridisation

Prior to hybridisation DNA membranes were pre-hybridised at +65°C for 20 min in hybridisation buffer. Probes were hybridised in PNA hybridisation buffer (10 mM Tris-HCl pH 7.5, 5 mM NaCl and 7.5% sarcosinate) at 6-10 nM concentration in volume of 3 ml of hybridisation buffer. The standard time was set as 3 hours of hybridisation. The filters were inserted in hybridisation glass tubes and incubated while rotated. The washing was performed on a Rocky shaker for 30 min at the same temperature as was temperature of hybridisation in a plastic box in volume of 100 ml of hybridisation buffer. Usually the same buffer which was used for pre-hybridisation was used for washing.

After hybridisation the DNA membrane were washed in PNA Detection buffer (100 mM NaCl, 60 mM sodium phosphate (pH 7.5)) for 15 min, placed on plastic support with DNA side up, covered with Saran wrap and scanned on laser scanner. PNA could be stored wrapped for 3-5 hours with no decay of signal.

2.2.10.8.3 Comparison of sensitivity of Phycoerythrin and Fluorescein

DNA was transferred onto membrane using highly precise system of liquid micro-delivery Micro-dispenser ((Eickhoff, 1998)). The system allows to prepare precise series of 8 two-fold dilutions for generating wide range of

DNA to be detected. Pooled PCR reaction from 20 clones were used as source of DNA. Fluorescein label was introduced via amino-terminus of a free PNA as described in Methods. Phycoerythrin protein label was coupled via Streptavidin-biotin link to the biotinylated PNA of the same sequence. Hybridisation was performed using the same conditions apart the final wash to equilibrate pH. The optimal pH for detection of Fluorescein is higher, thus the membrane was washed in Tris-HCl pH 8.0 buffer.
Dependence between amount of DNA and fluorescent signal was detected on laser scanner (Nyarsik, in preparation) with 20μm resolution using 570 nm by-pass filter for Phycoerythrin and 520 nm for Fluorescein.

2.2.10.8.4 Stripping of probes

The probes were stripped from filter by incubation in heated to +70°C in 0.1x SSARC buffer twice for 30 min. After all treatments the membranes could be used reliably for 10 -15 times.

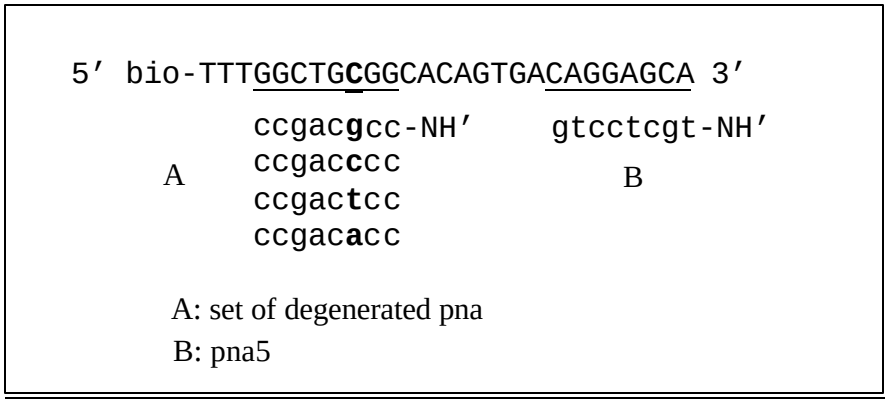
2.2.10.9 Analysis of PNA by MALDI

Several matrices were examined for detection of the PNAs including sinapic acid (SA), CHCA (α-cyano-4-hydroxycinnamic acid), and DHB. Best results were obtained with CHCA dissolved in 50% ACN/0.1% TFA. For sample preparation, 0.5 μl of PNA solution were mixed with 0.5 μl matrix solution on the stainless steel MALDI sample support. After solvent evaporation at ambient temperature, the crystalline samples were analysed in a Bruker Scout 26 Reflex II MALDI mass spectrometer, which is equipped with 377nm laser, reflector and upgraded with delayed extraction. The spectra recorded in positive ion mode using linear calibration. Acceleration voltage was +20kV and data collected with 30-100 individual laser shots. External mass calibration was performed with a number of peptide standards using the same matrix.

2.2.10.10 Hybridisation with mixture of PNAs

As target DNA two biotinylated 27 nt-long synthetic oligonucleotide was used. The oligonucleotides conferred also a complementary site for PNA5. This oligonucleotide is a model for the DNA target which contains closely situated hybridisation sites for several PNAs, so that discrimination properties could have been estimated as well (Figure 3).

Figure 3. System of DNA oligonucleotide targets (DNA-C which is complementary to PNA-G) and PNA probes.



Both targets were hybridised to a mixture of PNA-A, PNA-G, PNA-C and PNA-T. PNA5 was included to mixture to normalise the signal.

To bind oligonucleotides to the beads the were incubated in excess (60pmol)with 100 µg of the beads equilibrated in binding buffer for 2 hours at +37°C with shaking. 100 µg of Dynobeads should bind ca 20pmol of oligonucleotide (Dynal manual). The binding was monitored on UV by taking spectra from the supernatant against binding buffer and confirmed binding of ca 70%. After binding the beads were washed twice with 20 mM Tris-HCl, pH 7.4 and resuspended in 10 µl.

Hybridisation of DNA bound to the beads with PNA mixture (each PNA at 2pmol/µl in water) was held at +4°C for 15 min in volume of 40 µl. As control for non specific PNA adsorption the beads with no DNA bound were incubated with PNA mixture in parallel.

Unhybridised probe was removed by double washing with 0.2% SDS/20 mM Tris-HCl (pH7.4) buffer at RT, then washed with Tris-HCl buffer to remove SDS and 100 mM ammonium acetate to suppress formation of Na/K adducts with PNA by substitution of Na^+ and K^+ ions. Since it is a volatile compound it is also easily removed PNA extraction was performed by incubation with 0.1% TFA at +90°C for 1min. PNA were mixed with HCCA matrix and analysed as above.