## 6.3. *In vitro* selection

The main purpose of this work, was the development of *in vitro* selection strategies based on torsionally strained DNA. In chapters 6.1 and 6.2 I have shown that Zα peptide binds to the Z-DNA conformer of DNA. Since the Z-DNA conformation is a high energy form of DNA, some method of stabilization has to be used for *in vitro* experiments involving Z-DNA. Chemical modification of the DNA was deemed undesirable since it possibly could interfere with the interaction of the Zα peptide and DNA. Likewise, high salt conditions were ruled out since the system would not mimic the *in vivo* situation and high salt concentrations most likely inhibit the interaction of the protein with the DNA. Therefore negative supercoiling was chosen as the force used for stabilizing the DNA in the Z-DNA conformation.

I have used two different strategies for *in vitro* selection. In the first, plasmids containing a randomized insert of 16 nucleotides were used, in the second minicircles with a randomized insert of 16 nucleotides were used.

### 6.3.1. *In vitro* selection of plasmids

#### 6.3.1.1. Generation of the plasmid library

A plasmid library containing a 16 bp $(YR)_8$ insert was constructed starting with a

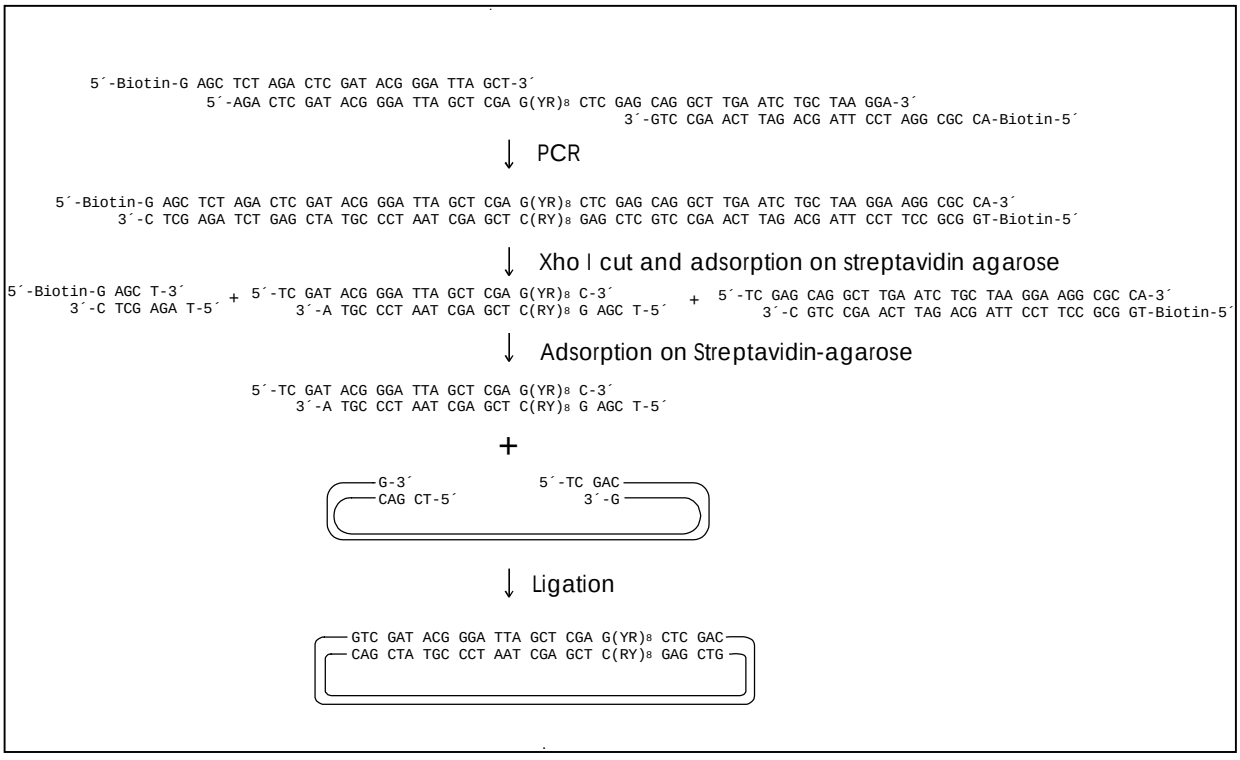


Figure 6.9 Strategy for the generation of the YR8 plamid library

chemically synthesized oligonucleotide containing the randomized region enclosed by Xho I restriction sites and priming sites for two PCR primers. The oligonucleotide was converted into double-stranded DNA by 5 cycles of PCR using primers biotinylated at their 5´ ends. After purification of the PCR products, the DNA was cut with restriction enzymes and incubated with streptavidin-agarose. Unbound DNA was recovered and constituted the insert used for the subsequent ligation. Success of the restriction cut was controlled by electrophoresis of an aliquot on an acrylamide gel and the DNA detected by silver staining (Figure 6.10).

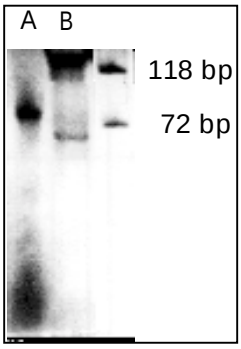


Figure 6.10 Analysis of restriction and streptavidin adsorption steps. An aliquot of the PCR product was loaded in lane A, an aliquot of the purified restriction product was loaded in lane B. Two size marker bands are included. The DNA was visualized by silver staining. In lane B an intensive band of low mobility is visible besides the faster migrating DNA product. This band is caused by streptavidin protein present in the eluate.

The plasmid library was made using the SURE2 bacterial strain in which Z-DNA sequences are supposed to be stably maintained (Greene 1990). Sequences from the unselected library are shown in Figure 6.11 This library was obtained from approximately 200,000 individual colonies. Still, one half of sequences show deletion of dinucleotides within the stretch of randomized alternating pyrimidine and purine bases. In two cases changes outside the randomized sequence were observed. The extent of the sequence modification seems to be dependent on the bacterial strain used.

| | |
|---|---|
| G**YRYRYRYRYRYRYRYR**C | # of YR repeats |
| CCCGGGATCCGTCGAG**TACGCACACACGCG...**TCGACCGATGCCCTT | 7 |
| CCCGGGATCCGTCGAG**CGCATATACACACG..**CTCGACCGATGCCCTT | 7 |
| CCCGGGATCCGTCGAG**TATATACACGTATGCG**CTCGACCGATGCCCTT | 8 |
| CCCGGGATCCGTCGAG**TATATGTGCGCGCATG**CTCGACCGATGCCCTT | 8 |
| CCCGGGATCCGTCGAG**TGTGCATGTGCG....**CTCGACCGATGCCCTT | 6 |
| CCCGGGATCCGTCGAG**TATACATATG......**CTCGACCGATGCCCTT | 5 |
| CCCGGGATCCGTCGAG**TGCATATATGTGCACG**CTCGACCGATGCCCTT | 8 |
| CCCGGGATCCGTCGAG**CGTACACGTGCA....**CTCGACCGATGCCCTT | 6 |
| CCCGGGATCCGTCGAG**TATGCACATGTGCGTG**CTCGACCGATGCCCTT | 8 |
| CCCGGGATCCG.CGAG**TACGTACGTATATACA**CTCGACCGATGCCCTT | 8 |

Figure 6.11 Alignment of the sequences of the unselected YR8 library. The randomized nucleotides are shown in bold letters. The position of the randomized nucleotides are indicated above the alignment. The number of YR repeats in the randomized region are indicated.

An important criterion for the quality of a library is the presence of sequence biases. Analysis shows that the frequencies for the dinucleotides are roughly equal (Table 6.2).

| Dinucleotide | absolute (relative) frequency | Dinucleotide | absolute (relative) frequency |
|---|---|---|---|
| CA | 18 (0.23) | AC | 17 (0.27) |
| CG | 17 (0.22) | AT | 14 (0.23) |
| TA | 21 (0.28) | GC | 20 (0.32) |
| TG | 20 (0.28) | GT | 11 (0.18) |

Table 6.2 Dinucleotide frequencies of the unselected YR8 plasmid library

Another criterion for the quality of a library is the relative frequency of a nucleotide at a certain position of the sequence. Since the sequences analyzed are of differing lengths the analysis was done with the sequences aligned to either the 5´ end or the 3´ end (Tables 6.3 and 6.4). At most positions the nucleotide frequencies are roughly equal. Near the ends of the randomized insert some differences are observed, with thymidin dominating the most 5´ position and guanosine dominating the most 3´ position. It should be noted, however, that the absolute frequencies are too low for the results to be statistically significant.

| Position | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| A | - | 6 | - | 6 | - | 9 | - | 6 | - | 3 |
| C | 2 | - | 4 | - | 5 | - | 5 | - | 4 | - |
| G | - | 4 | - | 4 | - | 1 | - | 4 | - | 7 |
| T | 8 | - | 6 | - | 5 | - | 5 | - | 6 | - |

Table 6.3 Positional nucleotide frequencies of the YR8 plasmid library.

| Position | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|---|
| A | - | 7 | - | 6 | - | 5 | - | 5 | - | 2 |
| C | 4 | - | 5 | - | 5 | - | 5 | - | 7 | - |
| G | - | 3 | - | 4 | - | 5 | - | 5 | - | 8 |
| T | 6 | - | 5 | - | 5 | - | 5 | - | 3 | - |

Table 6.4 Positional nucleotide frequencies of the YR8 plasmid library

#### 6.3.1.2. *In vitro* selection of plasmids with GST-Zα

Selection of the (YR)8 plasmid library was carried out using a large excess of protein over plasmid DNA. The bound plasmid DNA was electroporated into SURE2 bacteria and the number of colonies obtained was estimated. For comparison, selections were done in parallel with the plasmid pME.A, which is not expected to form Z-DNA. This allowed to

The sequences obtained by *in vitro* selection show some significant changes, when compared to the original sequences. In principal, each sequence can be divided into three parts: A first block of originally randomized alternating purine and pyrimidine bases, a second block of varying length containing a sequence related to a consensus sequence CTCGATCGAG, and a third sequence block consisting of alternating purine and pyrimidine bases :

| | |
|---|---|
| **YR**CTCGATCGAG**YR** | observed consensus sequence |
| GTCGAG**YR** | 5´ boundary of randomized insert |
| **YR**CTCGATC | 3´ boundary of randomized insert |

The consensus sequence of the newly appearing linker is made up of the 5´ boundary and 3´ boundary of the randomized inserts. It is noteworthy that the consensus sequence is palindromic. A comparison of the relative dinucleotide frequencies of the unselected library and after four rounds of selection is shown in Table 6.6.

| Dinucleotide | unselected library | selected library |
|---|---|---|
| CA | 0.23 | 0.18 |
| CG | 0.22 | 0.48 |
| TA | 0.28 | 0.10 |
| TG | 0.28 | 0.23 |

Table 6.6 Dinucleotide frequencies of sequences before and after four rounds of selection

While the selected sequences are enriched in CG dinucleotides and depleted for TA dinucleotides, the frequencies of CA and TG dinucleotides are close to the ones observed in the unselected library.

The selected sequences were analyzed for the presence of common sequence words, using the McSelex computer program. Table 6.7 lists the sequence words and their absolute and relative occurrences.

| Motiv | abs. | rel. |
|---|---|---|
| GCG | 61 | 0,20 |
| CGC | 56 | 0,19 |
| GTG | 28 | 0,094 |
| TGC | 26 | 0,087 |
| CGT | 20 | 0,067 |
| CAC | 18 | 0,060 |
| GCA | 14 | 0,047 |
| ACG | 13 | 0,044 |
| ACA | 11 | 0,037 |
| TGT | 11 | 0,037 |
| GTA | 9 | 0,030 |
| ATG | 8 | 0,027 |
| CAT | 8 | 0,027 |
| TAC | 8 | 0,027 |
| TAT | 5 | 0,017 |
| ATA | 2 | 0,007 |

| Motif | abs. | rel. |
|---|---|---|
| GCGC | 44 | 0,16 |
| CGCG | 33 | 0,12 |
| GTGC | 19 | 0,070 |
| TGCG | 19 | 0,070 |
| GCGT | 17 | 0,063 |
| CGTG | 13 | 0,048 |
| ACGC | 10 | 0,037 |
| TGTG | 10 | 0,037 |
| CACG | 9 | 0,033 |
| CGCA | 9 | 0,033 |
| GCAC | 9 | 0,033 |
| ACAC | 8 | 0,029 |
| GTGT | 8 | 0,029 |
| ATGC | 7 | 0,026 |
| CACA | 6 | 0,022 |
| CGTA | 6 | 0,022 |
| CATG | 5 | 0,018 |
| GCAT | 5 | 0,018 |
| GTAC | 5 | 0,018 |
| GTAT | 4 | 0,015 |
| TACA | 4 | 0,015 |
| ACAT | 3 | 0,011 |
| ACGT | 3 | 0,011 |
| TACG | 3 | 0,011 |
| TATG | 3 | 0,011 |
| ATAC | 1 | 0,004 |
| ATAT | 1 | 0,004 |
| ATGT | 1 | 0,004 |
| CATA | 1 | 0,004 |
| CGCT | 1 | 0,004 |
| CGTC | 1 | 0,004 |
| GTGA | 1 | 0,004 |
| TATA | 1 | 0,004 |
| TGCA | 1 | 0,004 |
| TGTA | 1 | 0,004 |

| Motif | abs. | rel. |
|---|---|---|
| CGCGC | 28 | 0,11 |
| GCGCG | 26 | 0,11 |
| GTGCG | 16 | 0,065 |
| CGTGC | 12 | 0,049 |
| TGCGC | 11 | 0,045 |
| GCGTG | 10 | 0,041 |
| CACGC | 9 | 0,037 |
| TGCGT | 8 | 0,033 |
| GCGCA | 7 | 0,029 |
| GTGTG | 7 | 0,029 |
| GCACG | 6 | 0,024 |
| GCGTA | 6 | 0,024 |
| ACGCG | 5 | 0,020 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| CGCAC | 5 | 0,020 | TATGC | 3 | 0,012 | CGTGT | 1 | 0,004 |
| CGCGT | 5 | 0,020 | ACGCA | 2 | 0,008 | GAGAG | 1 | 0,004 |
| TGTGC | 5 | 0,020 | CACAT | 2 | 0,008 | GAGTA | 1 | 0,004 |
| CACAC | 4 | 0,016 | CGTAT | 2 | 0,008 | GTATA | 1 | 0,004 |
| CATGC | 4 | 0,016 | GCACA | 2 | 0,008 | GTGAG | 1 | 0,004 |
| CGCAT | 4 | 0,016 | GTACG | 2 | 0,008 | GTGCA | 1 | 0,004 |
| CGTAC | 4 | 0,016 | GTATG | 2 | 0,008 | GTGTA | 1 | 0,004 |
| GCATG | 4 | 0,016 | TACGT | 2 | 0,008 | TACGC | 1 | 0,004 |
| TACAC | 4 | 0,016 | ACATA | 1 | 0,004 | TATAC | 1 | 0,004 |
| TGTGT | 4 | 0,016 | ACATG | 1 | 0,004 | TGCAC | 1 | 0,004 |
| ACACA | 3 | 0,012 | ATACA | 1 | 0,004 | TGCCG | 1 | 0,004 |
| ACACG | 3 | 0,012 | ATATG | 1 | 0,004 | TGTAC | 1 | 0,004 |
| ACGTG | 3 | 0,012 | ATGTG | 1 | 0,004 | | | |
| ATGCG | 3 | 0,012 | CATAT | 1 | 0,004 | | | |
| GTACA | 3 | 0,012 | CATGT | 1 | 0,004 | | | |

| Motif | abs. | rel. |
|---|---|---|
| GCGCGC | 22 | 0,10 |
| CGCGCG | 13 | 0,060 |
| GTGCGC | 10 | 0,046 |
| TGCGCG | 10 | 0,046 |
| CGTGCG | 9 | 0,041 |
| GCGTGC | 9 | 0,041 |
| CGCGCA | 6 | 0,028 |
| GCACGC | 6 | 0,028 |
| GTGCGT | 6 | 0,028 |
| ACGCGC | 5 | 0,023 |
| CACGCG | 5 | 0,023 |
| TGCGTG | 5 | 0,023 |
| TGTGCG | 5 | 0,023 |
| CGCACG | 4 | 0,018 |
| GCATGC | 4 | 0,018 |
| GCGCAT | 4 | 0,018 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GCGCGT | 4 | 0,018 | CGTACG | 2 | 0,009 | CATGTG | 1 | 0,005 |
| GCGTAC | 4 | 0,018 | CGTATG | 2 | 0,009 | CGTGCA | 1 | 0,005 |
| GTGTGC | 4 | 0,018 | GCGTAT | 2 | 0,009 | CGTGTG | 1 | 0,005 |
| TGTGTG | 4 | 0,018 | GTATGC | 2 | 0,009 | GCACAC | 1 | 0,005 |
| ACACGC | 3 | 0,014 | GTGTGT | 2 | 0,009 | GCACAT | 1 | 0,005 |
| ACGTGC | 3 | 0,014 | TACACG | 2 | 0,009 | GCGTGT | 1 | 0,005 |
| CACACA | 3 | 0,014 | TACGTG | 2 | 0,009 | GTACGC | 1 | 0,005 |
| CATGCG | 3 | 0,014 | TGCGTA | 2 | 0,009 | GTACGT | 1 | 0,005 |
| CGCATG | 3 | 0,014 | ACACAT | 1 | 0,005 | GTATAC | 1 | 0,005 |
| CGCGTA | 3 | 0,014 | ACATAT | 1 | 0,005 | GTGCAC | 1 | 0,005 |
| GCGCAC | 3 | 0,014 | ACATGT | 1 | 0,005 | GTGTAC | 1 | 0,005 |
| GTACAC | 3 | 0,014 | ATACAC | 1 | 0,005 | TATACA | 1 | 0,005 |
| ACACAC | 2 | 0,009 | ATATGC | 1 | 0,005 | TGAGAG | 1 | 0,005 |
| ACGCAC | 2 | 0,009 | ATGCGC | 1 | 0,005 | TGCACG | 1 | 0,005 |
| ATGCGT | 2 | 0,009 | ATGTGT | 1 | 0,005 | TGTACA | 1 | 0,005 |
| CACGCA | 2 | 0,009 | CACACG | 1 | 0,005 | TGTGAG | 1 | 0,005 |
| CGCGTG | 2 | 0,009 | CACATA | 1 | 0,005 | | | |
| CGTACA | 2 | 0,009 | CATATG | 1 | 0,005 | | | |

| Motif | abs. | rel. |
|---|---|---|
| CGCGCGC | 13 | 0,066 |
| GCGCGCG | 9 | 0,045 |
| GTGCGCG | 9 | 0,045 |
| TGCGCGC | 8 | 0,040 |
| CGTGCGC | 6 | 0,03 |
| GCGTGCG | 6 | 0,03 |
| CACGCGC | 5 | 0,025 |
| GTGCGTG | 5 | 0,025 |
| TGCGTGC | 5 | 0,025 |
| CGCACGC | 4 | 0,020 |
| GCGCGCA | 4 | 0,020 |
| GTGTGCG | 4 | 0,020 |
| ACACGCG | 3 | 0,015 |
| ACGCGCG | 3 | 0,015 |
| ACGTGCG | 3 | 0,015 |
| CGCATGC | 3 | 0,015 |
| CGCGCAC | 3 | 0,015 |
| CGCGCAT | 3 | 0,015 |
| CGCGTAC | 3 | 0,015 |
| CGTGCGT | 3 | 0,015 |
| GCATGCG | 3 | 0,015 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| GCGCATG | 3 | 0,015 | TGCGCGT | 2 | 0,010 | CGTGCAC | 1 | 0,005 |
| GCGCGTA | 3 | 0,015 | TGTGCGT | 2 | 0,010 | GAGAGTA | 1 | 0,005 |
| TGTGCGC | 3 | 0,015 | ACACACA | 1 | 0,005 | GAGTATA | 1 | 0,005 |
| TGTGTGC | 3 | 0,015 | ACACACG | 1 | 0,005 | GCACACA | 1 | 0,005 |
| ACGCACG | 2 | 0,010 | ACACATA | 1 | 0,005 | GCGCGTG | 1 | 0,005 |
| ACGCGCA | 2 | 0,010 | ACATATG | 1 | 0,005 | GCGTGCA | 1 | 0,005 |
| CACACAC | 2 | 0,010 | ACATGTG | 1 | 0,005 | GCGTGTG | 1 | 0,005 |
| CACGCAC | 2 | 0,010 | ACCGCGC | 1 | 0,005 | GTACGTG | 1 | 0,005 |
| CATGCGT | 2 | 0,010 | AGAGTAT | 1 | 0,005 | GTATACA | 1 | 0,005 |
| CGCGTGC | 2 | 0,010 | AGTATAC | 1 | 0,005 | GTGAGAG | 1 | 0,005 |
| CGTACAC | 2 | 0,010 | ATGCGCG | 1 | 0,005 | GTGCACG | 1 | 0,005 |
| CGTATGC | 2 | 0,010 | ATGCGTA | 1 | 0,005 | GTGCGTA | 1 | 0,005 |
| GCACGCA | 2 | 0,010 | ATGCGTC | 1 | 0,005 | GTGTACA | 1 | 0,005 |
| GCACGCG | 2 | 0,010 | ATGTGTG | 1 | 0,005 | GTGTGAG | 1 | 0,005 |
| GCGCACG | 2 | 0,010 | CACACAT | 1 | 0,005 | TATACAC | 1 | 0,005 |
| GCGTACA | 2 | 0,010 | CACACGC | 1 | 0,005 | TGAGAGT | 1 | 0,005 |
| GCGTACG | 2 | 0,010 | CACATAT | 1 | 0,005 | TGCACGC | 1 | 0,005 |
| GCGTATG | 2 | 0,010 | CATATGC | 1 | 0,005 | TGCGTAC | 1 | 0,005 |
| GTACACG | 2 | 0,010 | CATGCGC | 1 | 0,005 | TGCGTAT | 1 | 0,005 |
| GTGTGTG | 2 | 0,010 | CATGTGT | 1 | 0,005 | TGTACAC | 1 | 0,005 |
| TACACGC | 2 | 0,010 | CGTACGC | 1 | 0,005 | TGTGTGT | 1 | 0,005 |
| TACGTGC | 2 | 0,010 | CGTACGT | 1 | 0,005 | | | |

Table 6.7 The sequence words represented in the selected sequences were extracted using the McSelex program. The words are sorted first according to their frequency and second alphabetically.

While Table 6.7 lists the occurrences of sequence words summed over all selected sequences, it is of interest, whether certain sequence words are common to all selected sequences. The only sequence word of 5 nucleotides length present in all selected sequences is GCGCG. No other sequence words of 5 nucleotide length or longer are

present in all sequences. The only sequence words of 4 nucleotides length present in all selected plasmids are : (CGCG/GCGC); CGCA/TGCG); (ACGC/GCGT) and (CACG/CGTG); (ACGC/GCGT).

### 6.3.2. *In vitro* selection of DNA minicircles

#### 6.3.2.1. Generation of a minicircle library containing a $N_{16}$ insert

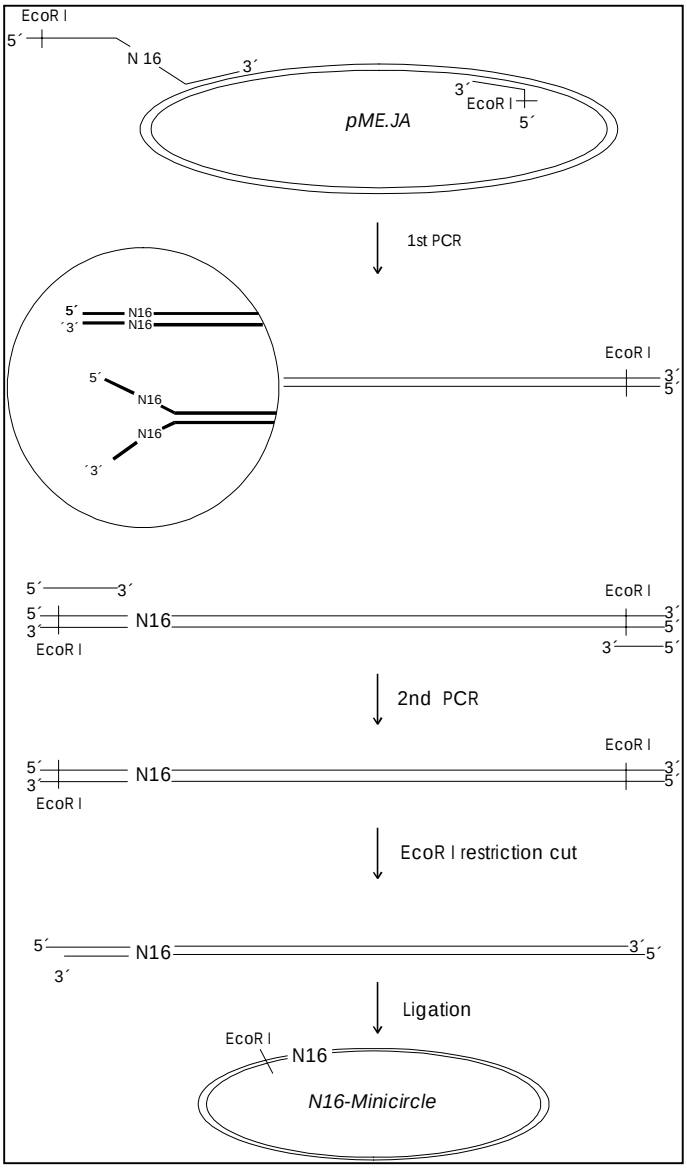


Figure 6.13 Strategy for the generation of the minicircle library

A minicircle library was generated of DNA molecules of 580 nt length containing a randomized insert of 16 nucleotides. A cartoon depicting the strategy for the generation of the minicircle library is shown in Figure 6.13 . A 560 base pair long stretch of the plasmid pME.JA was amplified using primers that had recognition sites for the EcoR I restriction endonuclease included at their 5´ ends. Furthermore, one of the primers contained a stretch of 16 randomized nucleotides and a stretch of nucleotides used for priming the second PCR. The first PCR reaction led to linear DNA molecules of 580 base pairs length, which at one of their ends, contained the randomized stretch of nucleotides. This stretch of nucleotides could be present either base paired or mismatched (enlarged inset). Hence, a second PCR reaction was performed using one of the primers from the first PCR reaction and a new one, which hybridized to the new priming site introduced during the first PCR. The product of the second PCR was treated with the EcoR I restriction endonuclease in order to obtain staggered ends, and ligated into minicircles.

For DNA molecules of this length, agarose gel electrophoresis does not allow circular DNA to be distinguished from linear DNA. Therefore, the DNA molecules were labeled radioactively and resolved on 4 % non denaturing polyacrylamide gels. The mobility of circular DNA molecules in polyacrylamide gels is different than in agarose gels. Whereas, in agarose gels circular DNA tends to have a higher electrophoretic mobility than linear DNA, in polyacrylamide gels circular DNA migrates slower than linear DNA. Since in polyacrylamide gels individual topoisomers can be resolved, it is of interest to look at their relative mobilities. Whereas nicked circular DNA migrates slower than relaxed circular DNA, negatively supercoiled DNA molecules migrate faster. However, once the Z-DNA forms, no simple rules can be given (Nordheim and Meese 1988).

First it had to be shown that ligation of the linear DNA does indeed lead to the generation of closed circular DNA. Figure 6.14 shows a gel in which linear DNA of 580 bp length generated from the plasmid pME.A was ligated in the absence or presence of 10 µM ethidium bromide. The results show that upon addition of ligase two new bands of lower electrophoretic mobility appear and the intensity of the band corresponding to the 580 bp linear DNA diminishes. The mobility of the new bands is slower than would be expected for linear dimers or trimers, suggesting, that indeed circular DNA was formed. When the DNA is ligated in the presence of ethidium bromide one of the new bands disappears almost completely and another slightly faster migrating band is detected. This implies that intercalation of ethidium bromide led to the formation of a different topoisomer of circular DNA, an effect only possible if the circular DNA is not nicked, but covalently closed. The absence of a band of 1200 base pairs size, indicates that essentially no dimers were formed during the ligation reaction.

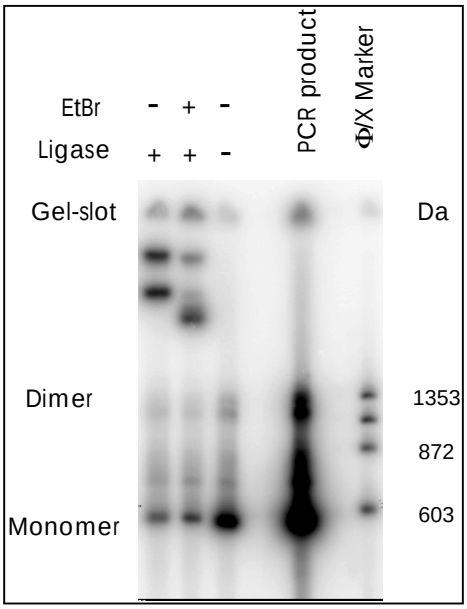


Figure 6.14 Generation of minicircles. Radioactively end labeled PCR product was incubated with or without T4 DNA Ligase in the presence or absence of ethidium bromide as indicated above each lane. The DNA was resolved on a 4 % (w/v) polyacrylamide gel. The PCR product and a size marker are shown for comparison. The positions of monomer and dimer are indicated, as are the lengths of some of the size marker bands.

In order to confirm that ligation resulted in the formation closed circular negatively supercoiled minicircles, the ligation product was treated with different enzymes and resolved on a polyacrylamide gel (Figure 6.15). The leftmost lane contains the PCR product used for generation of the minicircles. Due to overloading a

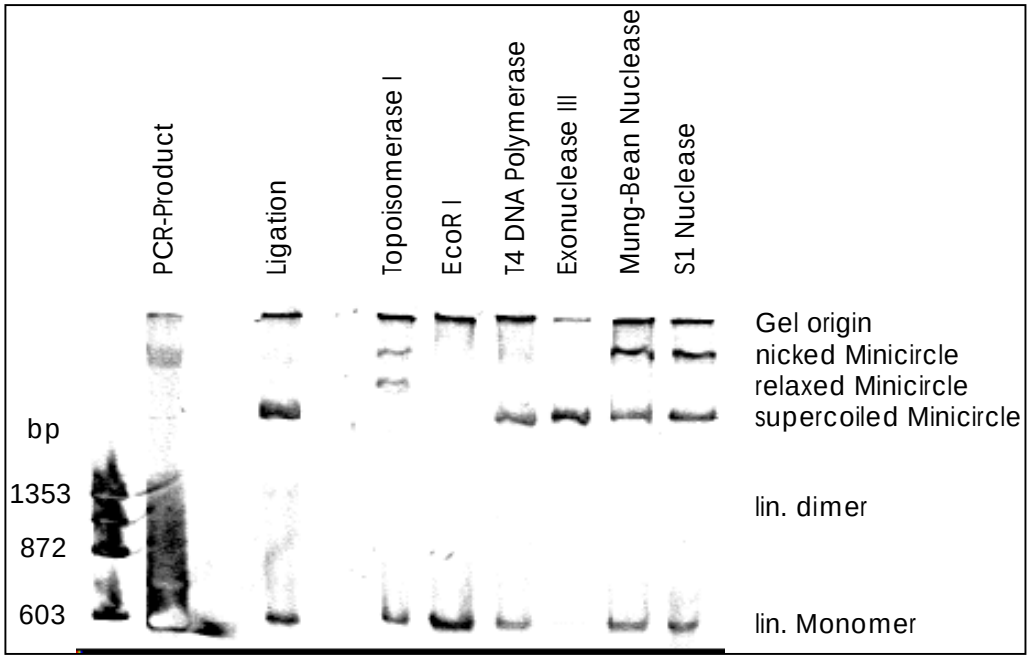


Figure 6.15 Treatment of minicircles with different enzymes. Radioactively end labeled PCR product was ligated with T4 DNA Ligase and incubated with different enzymes. The samples were resolved on a 4 % (w/v) polyacrylamide gel and detected by autoradiography. The lanes containing the PCR product is indicated, as is the lane containing the ligation product. The enzymes used to treat the ligation product are indicated aboveeach lane. A size marker has been included and the lengths of some of the size marker bands are indicated on the left. The assignments of the observed bands to different ligation products and topoisomers is indicated on the right.

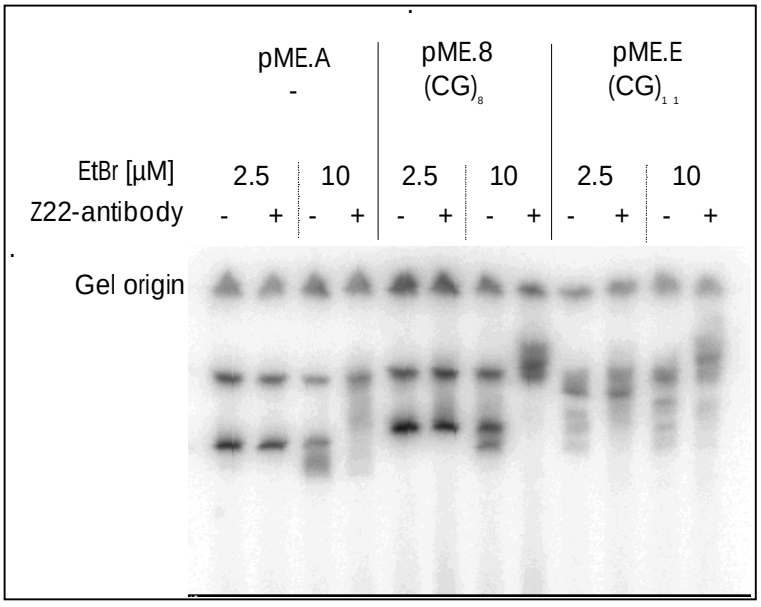


Figure 6.16 Radioactively labeled minicircles were generated using the plasmids pME.A, pME.8 and pME.E as templates and ligated in the presence of either 2.5 µM or 10 µM ethidium bromide. Equal amounts of radioactivity were resolved on a 4 % (w/v) polyacrylamide gel in the presence or absence of 0.5 µg Z22 antibody. The type of plasmid DNA, amount of ethidium bromide and presence or absence of Z22 antibody is indicated above each lane.

smear is present but the main product of 580 bp length is clearly visible. Some high molecular weight material is visible too, probably caused by the plasmid DNA used as a template for the PCR reaction. Following ligation a new band of low electrophoretic mobility appears. Upon addition of EcoR I restriction endonuclease the low mobility band disappeared, as would be expected for DNA produced by ligation. Likewise, treatment with Topoisomerase I lead to

the loss of the ligation product band. However, two new bands of even lower electrophoretic mobility appeared. Therefore the new band created by the ligation reaction was not a linear dimer of the PCR product, but circularized monomer. Treatment with T4 DNA Polymerase had no effect. Under the conditions used, the strong 3´-5´ exonuclease activity of T4 DNA Polymerase would have digested any DNA if blunt ends or nicks had been present. The fact that the ligation product was not affected, proved that the DNA was present as covalently closed circles. However the linear monomer still present in the ligation product mixture was only partially digested, indicating that the T4 DNA Polymerase was not sufficiently active to fully digest the DNA. The enzyme Exonuclease III is an 3´-5´ exonuclease specific for double-stranded DNA that has a blunt end, a 5´ ovy t.55(e-)6.3(NA]TJT eAotlyaatment othe lc( t.55ti)5.25on prch t.55(Ben )N5.5(un-0.5(rl)5.5(ease )anew)15.455(s)5.5(

| | Sequence | Z-Score | A | C | G | T |
|---|---|---|---|---|---|---|
| G0-1 | CGCTC..**GGTACCTACGGTGTGTC**GAAGGG | 8 | 2 | 4 | 6 | 5 |
| G0-2 | CGCTCA.**CCGGAGCCCTTGTTC....**AGGG | - | 1 | 6 | 4 | 4 |
| G0-3 | CGCTCAG**CAAAGTTGGTACGCCTG**GAAGGG | 6 | 4 | 4 | 5 | 4 |
| G0-5 | CGCTCAG**GACAAACGGTTCTGTC.**GAAGGG | - | 4 | 4 | 4 | 4 |
| G0-6 | CGCTCAG**CCTGCCTAGGTGGTGT**.GAAGGG | 4 | 1 | 4 | 6 | 5 |
| G0-7 | CGCTCAG**GACGGGCATCGCACGG**.GAAGGG | 8 | 3 | 5 | 7 | 1 |
| G0-8 | CGCTCAG**CGTCCACCAAGACGTT.**GAAGGG | - | 4 | 6 | 3 | 3 |
| G0-10 | CGCTCAG**GCAGCTTATGGCCCCAC**GAAGGG | - | 3 | 7 | 4 | 3 |
| G0-12 | CGCTCAG**CGCCCTTGACGCCGCG.**GAAGGG | - | 1 | 8 | 5 | 2 |
| G0-13 | CGCTCAG**TGGGCCGAGACGTGAG**.GAAGGG | 5 | 3 | 3 | 8 | 2 |
| G0-14 | CGCTCAG**GTAGGGGGACTGTATCT**GAAGGG | 2 | 3 | 2 | 7 | 5 |
| G0-15 | CGCTCAG**TCTCTCGACGTGCGGC**.GAAGGG | 12 | 1 | 6 | 5 | 4 |
| G0-18 | CGCTCAG**CGGGGAATGTAGGTC..**GAAGGG | 2 | 3 | 2 | 7 | 3 |
| G0-19 | CGCTCAG**TGCGGCGGGTTCGGGA**.GAAGGG | 9 | 1 | 3 | 9 | 3 |
| G0-23 | CGCTCAG**CAAACCGTTGTCTTGC**.GAAGGG | - | 3 | 5 | 3 | 5 |
| G0-24 | CGCTCAG**ATGTCGTGGAGGCT...**GAAGGG | - | 2 | 2 | 6 | 4 |
| G0-25 | CGCTCAG**GGGGGTATCACCGCTGT**GAAGGG | - | 2 | 4 | 7 | 4 |
| G0-26 | CGCTCAG**ATCGGCAAGGAGCCTGC**GAAGGG | - | 4 | 5 | 6 | 2 |
| G0-27 | CGCTCCA**GGCACCAGCACCAGTCC**GAAGGA | - | 4 | 8 | 4 | 1 |
| G0-28 | CGCTCAG**ACGTATCGCAGAACAC**.GAAGGG | 8/5 | 6 | 5 | 3 | 2 |
| G0-30 | CGCTCAG**CCTTGAAACCCTCTCC**.GAAGGA | - | 3 | 7 | 1 | 4 |
| G0-31 | CGCTCAG**GTGTCAGTTGGCTGC**..GAAGGG | - | 1 | 3 | 6 | 5 |
| G0-32 | CGCTCAG**GCGGCGTCCCGCGGAA**.GAAGGG | - | 2 | 6 | 7 | 1 |
| G0-33 | CGCTCAG**AGGGCACAAGGCACGC**.GAAGGG | 5/10 | 5 | 5 | 6 | 0 |
| G0-34 | CGCTCAG**GCACCAGCACCAGTCC**.GAAGGA | - | 4 | 8 | 3 | 1 |
| G0-35 | CGCTCAG**CGGACGGTTTTTGACCT**GAAGGG | - | 2 | 4 | 6 | 5 |
| G0-36 | CGCTCAG**GCCCCCGAGTAAAGCA**.GAAGGG | - | 5 | 6 | 4 | 1 |
| | Absolute occurrences | | 77 | 134 | 142 | 83 |
| | Average occurrences | | 2.9 | 5.0 | 5.3 | 3.1 |
| | Relative occurrences | | 0.18 | 0.31 | 0.33 | 0.19 |

AAA AAAGGG (plasmid sequence)

Figure 6.17 Randomized sequences of the un-selected minicircle library. The sequences are listed together with their Z-scores and the absolut frequencies of the four bases. The sums of the absolute occurrences of the bases and the average and relative occurences of the bases are shown at the bottom. The original sequence of the plasmid sequence is indicated to show that no sequence bias was introduced by the plasmid.

shift was visible in the case of the minicircles generated in the presence of 2.5 µM ethidium bromide. In the presence of 10 µM ethidium bromide the lower band was seemingly split into two bands and upon addition of Z22 antibody both bands were shifted into a smear. In the case of pME.8 as template for the generation of minicircles, the results in the case of 2.5 µM ethidium bromide were the same as for pME.A. The same was true in the case of 10 µM ethidium bromide, except that upon addition of Z22 antibody a clearly defined shifted band appeared that migrated even slower than the smear seen

with pME.A . In the case of pME.E more than 2 bands were visible in all cases. This is indicative of the presence of different topoisomers. Apparently, the sequences were able to adopt the Z-DNA conformation even in the absence of Z22 antibody, leading to another set of bands. At both concentrations of ethidium bromide, addition of the Z22 antibody caused the formation of a shifted smear. Summarizing, it can be said that at 10 µM ethidium bromide, the negative supercoiling introduced in the minicircles is great enough to cause the formation of Z-DNA even in a DNA sequence that is normally deemed not to adopt the Z-DNA formation. However, in the case of 2.5 µM ethidium bromide long stretches of alternating CG bases are necessary for Z-DNA formation. The minicircle containing a $(CG)_8$ sequence, representing the theoretically best Z-DNA forming sequence in the minicircle library does not adopt the Z-DNA conformation under these conditions. In conclusion, an ethidium bromide concentration intermediate between 2.5 µM and 10 µM seems best to on the one hand avoid the formation of Z-DNA in the non-randomized part of the minicircles and on the other hand supply sufficient negative supercoiling to stabilize the Z-DNA conformation, if a suitable sequence is present in the randomized area. Accordingly, a concentration of 5 µM ethidium bromide was used in the selection experiments.

The minicircle library was constructed according to a strategy in which a part of the plasmid pME.JA of the desired length was amplified by PCR using primers containing a unique restriction site. Additionally, one of the primers contained the randomized nucleotides and a novel primer binding site. The stretch of 16 randomized nucleotides was synthesized by mixing the 4 phosphoamidites in the ratio A:C:G:T = 20:30:30:20. The DNA resulting from the first PCR was purified and used as template for the second PCR done in a large volume for just 5 cycles. An aliquot of the unselected library was cloned into the pCR2.1-TA vector and sequenced (Table 6.17)

The sequences show that cytosine-, guanosine- and thymidine bases are present roughly according to their intended frequency. Adenosine bases are somewhat underrepresented. One possible problem that might have occurred during construction of the library was that a bias could have been introduced during the first PCR in which those sequences of the randomized pool would have been favored that matched those of the template DNA. Analysis of the sequences showed that no such bias is present. It is interesting that, again, the length of the randomized insert is somewhat variable. However, in most case only one nucleotide is missing or added. It may be speculated, that these changes resulted from inefficiencies in the chemical synthesis of the PCR primer.

#### 6.3.2.2. Development of the selection protocol

Initial attempts of *in vitro* selection using minicircles were not successful. Therefore, a number of modifications of the protocol were introduced, which in the end led to success.

##### 6.3.2.2.1. Development of the 2 PCR protocol

Initially, only the generation of the minicircle library was done using two consecutive PCR reactions. However, it was found necessary to use a similar approach during the amplification reactions following each selection cycle. During a PCR reaction the two complementary strands of DNA are melted at high temperature to allow annealing of a primer. In a usual PCR reaction, if no primer anneals, two complementary strands will rejoin and hybridize, yielding fully base paired double stranded DNA molecule. However, in the case of a DNA molecules containing a randomized region, any two different DNA molecules will most likely not contain the same randomized region and therefore lead to a mismatch. Since a mismatched region cannot adopt the Z-DNA conformation, these molecules no longer participate effectively in the selection reaction. Therefore it proved crucial for the success of *in vitro* selection, that the PCR reaction had to be very efficient. Ideally, every melted DNA molecule had to be bound by a primer and converted into double stranded DNA in each PCR cycle. This was best achieved if primer and polymerase were present during the reaction in excess of template DNA. Therefore, a two step amplification protocol was adopted, in which the DNA was amplified in a first small scale PCR reaction, followed by a second PCR reaction performed in a large volume and for just 4 to 6 PCR cycles.

##### 6.3.2.2.2. Primer dimers

Primer dimers are a common problem in PCR reactions. Even when the primer sequences are chosen such that they cannot anneal, it appears that during a PCR reaction such hybridization occasionally does occur. Once formed, such a primer dimer product, due to its small size, is amplified much more efficiently than the intended PCR product and, over the course of a PCR reaction, the primer dimer can become the dominant product. Using well designed primers the formation of primer dimers is not a significant problem since the

incidence of annealing is low enough so that it is unlikely to happen within the usually used numbers of PCR cycles. However, in the *in vitro* selection experiments described here it proved that primer dimers can become a significant problem. During the selection certain amount of unspecifically bound DNA is recoverd. Primer dimers once formed can seed the PCR reactions during later amplification rounds and then will become the major product. It proved that normal methods used to eliminate primer dimers were not

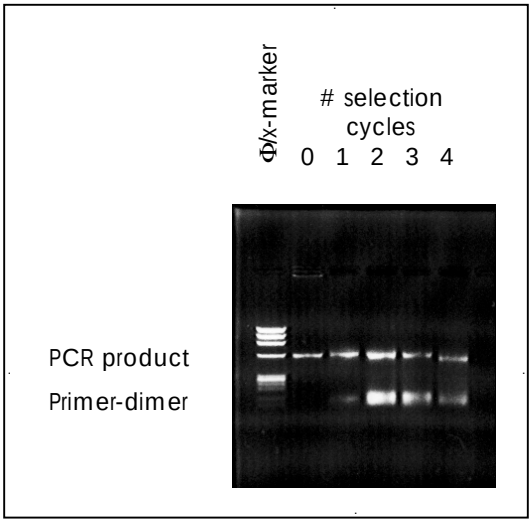


Figure 6.18 PCR amplification of the minicircle libraries obtained during successive rounds of in vitro selection. Four rounds of in vitro selection using the randomized library and GST-Zα peptide were performed and the libraries regenerated after each round of selection without PEG precipitation step. Equal amounts of DNA from the unselected library and from the regenerated libraries (200 ng) were resolved on a 2 % (w/v) agarose gel and stained with ethidium bromide. The positions of the minicircle PCR product and the primer dimer are indicated on the left.

sufficient. The DNA was generally purified following each PCR or EcoR I digestion by adsorption to a silica matrix at high salt conditions, washed and eluted at low salt (Qiagen Qiaquick PCR purification kit). Such a procedure is generally deemed useful to eliminate primer dimers since short DNA molecule bind only inefficiently to the silica matrix. Nevertheless, it proved that residual primer dimers were sufficient to cause problems in later selection cycles (Figure 6.18).

Therefore it was decided to include an PEG precipitation step in order to eliminate short DNA molecules. By varying the concentration of the PEG the efficiency of precipitation of small DNA molecules can be influenced. In the present case 10 % PEG-4000 and 10 mM $MgCl_2$ proved optimal, resulting in almost complete recovery of DNA longer than 250 bp, while eliminating shorter DNA molecules (Figure 6.19).

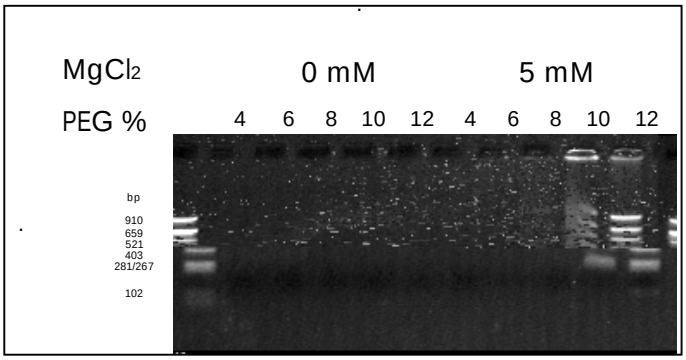


Figure 6.19 Effect of PEG and $MgCl_2$ concentration on the precipitation of small nucleic acids. Equal amounts (0.5 µg) of pBR322 / Alu I digest were precipitated with different concentrations of $MgCl_2$ and PEG (MW 4,000). A control sample that had not been treated was used for comparison. The percentages of $MgCl_2$ and PEG are indicated above each lane. The lengths of the size marker are indicated on the left.

### 6.3.2.3. *In vitro* selection of minicircles with GST-Zα

Using the *in vitro* selection protocol described above, five rounds of selection of the minicircle library with the GST-Zα peptide were carried out. For these selections 10 µg of peptide and minicircles from a 1 µg ligation reaction were used. After each round of selection a small part of the eluates was amplified by PCR. During the PCR reaction aliquots were removed after every second cycle and resolved on an agarose gel (Figure 6.20). The relative amount of DNA contained in the eluates was determined by comparing the number of PCR cycles needed to generate a visible DNA band. The different amounts

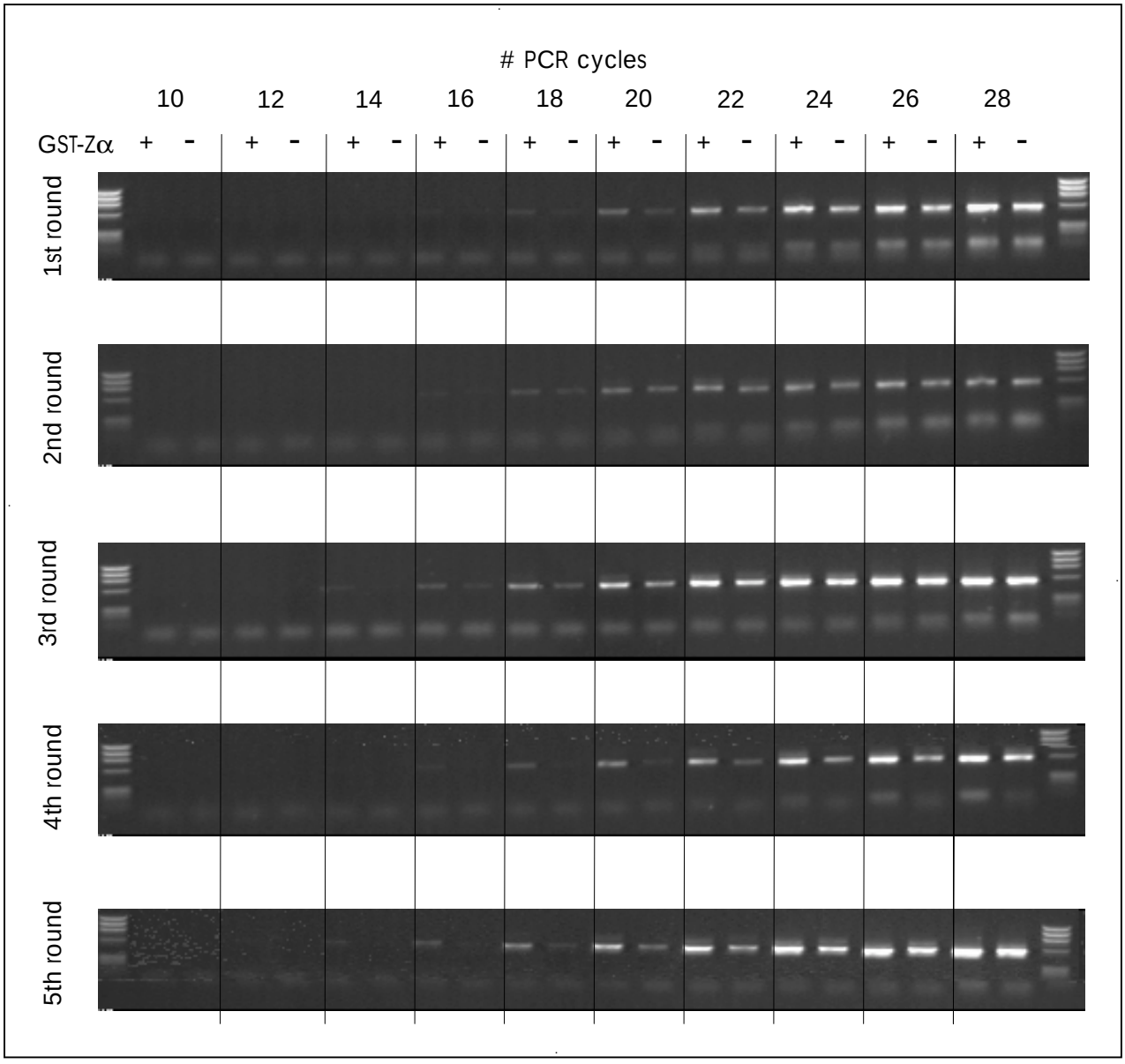


Figure 6.19 PCR amplification of the eluates from the minicircle selection with GST-Zα peptide. The eluates of both the sample incubated with and without GST-Zα peptide were used as templates in a PCR reaction. Aliquots were removed after every other PCR cycle and resolved on a 2 % (w/v) agarose gel. The results are shown for the first five rounds of selection. The number of PCR cycles and the presence or absence of GST-Zα peptide during the selection step is indicated.

of eluted DNA of the sample incubated with protein (+ GST-Zα) and the control sample incubated without protein (- GST-Zα) served as a measure for the extent to which binding of the minicircle population to the GST-Zα peptide exceeded the unspecific binding. The results of the first round of selection shows that right from the beginning the minicircle library showed some specific binding to the protein. In later selection rounds the differences increased. After the fifth round of selection at least 4 more PCR cycles were needed to generate a visible PCR product from the mock selected DNA than from DNA that was selected for binding to GST-Zα. Due to varying efficiency of the steps used to generate the minicircles, the absolute number of PCR cycles cannot be compared between different selection rounds.

After five rounds of *in vitro* selection, an aliquot of the eluted DNA was subcloned into the pCR2.1-TA vector and individual plasmids sequenced. An alignment of the sequences is shown in Table 6.8 together with their Z-scores. The sequences were further analyzed for the frequency of sequence words (Appendix).

| | Sequence | Z-score |
|---|---|---|
| G51-2 | CGCTCAG**GCAGATCGCGCGCCT**....GAAGGG | 12 |
| G51-3 | CGCTCAG**GAACGCGTACACGTGT**...GAAGGG | 17 |
| G51-4 | CGCTCAG**AAATGCCGGCGCTGCA**...GAAGGG | - |
| G51-5 | CGCTCAG**TACGTGCATGCGCACG**...GAAGGG | 20 |
| G51-6 | CGCTCAG**TGCATACGCGAGCGTG**...GAAGGG | 12/6 |
| G51-7 | CGCTCAG**GCGCGCGCGACCGTGCAA**.GAAGGG | 16/7 |
| G51-8 | CGCTCAG**GCGAGTTTGCGCGCAC**...GAAGGG | 15 |
| G51-9 | CGCTCAA**GGAATGGCGGAACGAG**...GAAGGG | - |
| G51-10 | CGCTCAG**GCGCGCGCGGGCTCCT**...GAAGGG | 16 |
| G51-11 | CGCTCAG**CGCGCCCGTGTGTGCAG**..GAAGGG | 10/11 |
| G51-13 | CGCTCAG**TCGCGCGTGCGGGGCC**...GAAGGA | 16 |
| G51-14 | CGCTCAG**GCCCGCCTGCGCGTGC**...GAAGGG | 15 |
| G51-15 | CGCTCAG**ACCTGACACGTCTGC**....GAAGGG | 6 |
| G51-17 | CGCTCAG**CCGGATTGGCAGTCTA**...GAAGGG | - |
| G51-18 | CGCTCAG**TCGGGTGTGCGTGCAC**...GAAGGG | 16 |
| G51-19 | CGCTCAA**CACGTCCCAGTGGCGC****....**AAGGG | 6/7 |
| G51-20 | CGCTCAG**GCCCCCGCGCACGCTC**...GAAGGG | 14 |
| G51-21 | CGCTCAG**CGGGCCCGTAAACGCC**...GAAGGA | - |
| G51-22 | CGCTCAG**GCGCGCGCGTCGCGAG**...GAAGGG | 22 |
| G51-23 | CGCTCAA**TATAACAAGGTTTGT**.....AAGGG | 1 |
| G51-27 | CGCTCAG**GCGGCGCGGGGGCACGT**..GAAGGG | 8/8 |
| G51-28 | CGCTCAG**GCACGCGGACGTCTGGGTG**AGAAGG | 10 |
| G51-53 | CGCTCAG**TGAGGCCAGAGGTCTG**...GAAGGG | - |
| G51-54 | CGCTCAG**CATGCTGGCACGCGCG**...GAAGGG | 6/14 |
| G51-55 | CGCTCAG**CCGGATTGGCAGTCTA**...GAAGGG | - |
| G51-56 | CGCTCAG**GGAGCCGAGGTCTGTG**...GAAGGG | - |
| G51-57 | CGCTCAG**ATGCGGGTGAGCACGC**...GAAGGG | 5/10 |
| G51-58 | CGCTCAG**CGCGCGCGATCGTGTG**...GGAGGA | 15/6 |
| G51-60 | CGCTCAG**GCGTGGTGCGGGCGT****....**GAAGGG | 11/6 |
| G51-61 | CGCTCAG**GGCTGTCGGTACGCAGG**..GAAGGG | 7 |
| G51-63 | CGCTCAG**GCTCGGGCGTGCGGGG**...GAAGGA | 10 |
| G51-65 | CGCTCAG**TAGCGCGCGCCGGGG**....GAAGGG | 15 |
| G51-66 | CGCTCAG**CGCGCGCGATCGTGTG**...GAAGGG | 13 |
| G51-67 | CGCTCAG**CCGCGCGCGTCCGC**......AAGGG | 15 |
| G51-68 | CGCTCAG**GAGAGCGGGCGTGTGC**...GAAGGG | 12 |
| G51-69 | CGCTCAG**CCGCCGGCATCCGTTA**...GAAGGG | - |
| G51-72 | CGCTCAG**TGTGCTGGTTACCACC**...GAAGGA | 5 |
| G51-73 | CGCTCAA**CTCTCGATGCCCTCTC****....**AAGGA | - |
| G51-74 | CGCTCAG**GCGTGGCAGCGCGCGAT**..GAAGGG | 8/11 |

Table 6.8 Alignment of the sequences obtained after five rounds of selection. Stretches of five or more alternating purines and pyrimidines are underlined. Nucleotides originating from the randomized sequence are shown in bold characters. The Z-scores were determined using the Interpret program of the MacMolly program package.

Following the fifth round of selection, 3 further selections cycles were done using less GST-Zα peptide ( 6th round : 7 µg; 7th round 3.5 µg; 8th round 1.7 µg). As before, the success of the selection was checked by PCR analysis (Figure 6.21). The selections showed increasing differences between protein specific binding and non specific binding.

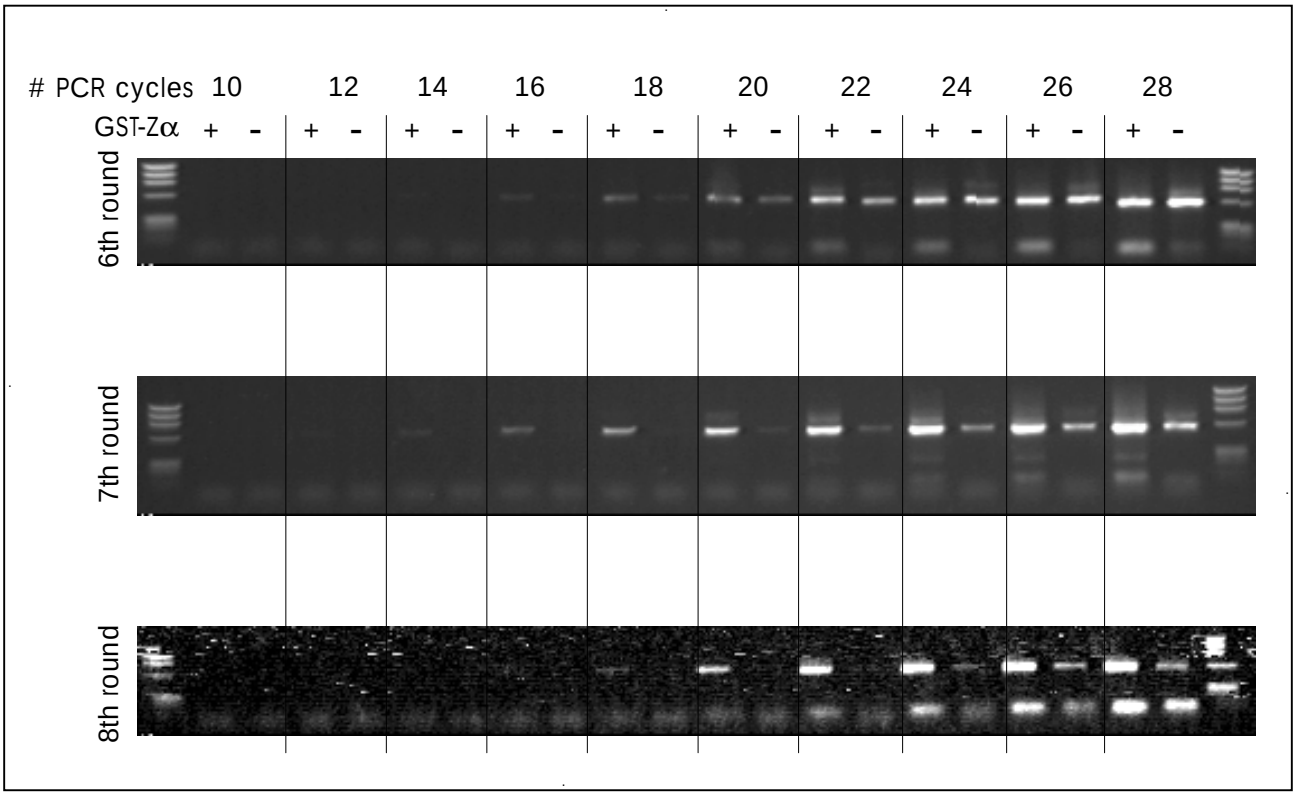


Figure 6.21 PCR amplification of the eluates from the minicircle selection with GST-Zα peptide. The eluates of both the sample incubated with and without GST-Zα peptide were used as templates in a PCR reaction. Aliquots were removed after every other PCR cycle and resolved on a 2 % (w/v) agarose gel. Results are shown for selection round 6 to 8. The number of PCR cycles and the presence or absence of GST-Zα peptide during the selection step is indicated.

Several limiting factors prevented the use of even more stringent conditions. First of all it was not possible to ascertain the precise concentrations of active Zα peptide nor of covalently closed minicircle DNA. While the protein concentration could be measured this gave no indication whether the protein was fully active. Possible reasons for inactive protein could be misfolding or degradation during storage. However, since long term storage did not lead to the loss of activity in bandshift assays, it was assumed that essentially all the protein was active. As to the DNA concentration uncertainty arose since only the amount of DNA used for the ligation reaction was measured. The success of the ligation was checked using PAGE and the dye Sybr Green. This, however, did not permit a quantitative determination of the ligation success. In principle, two different options exist to change the protein:DNA ratio. Either the amount of DNA can be increased, or the amount of protein decreased. However, an increase in the amount of minicircles was not

feasible. The ligation scale of 1 µg linear DNA in a 10 ml reaction volume was estimated to yield somewhere between 10 ng and 300 ng of minicircle DNA. A 10 fold larger production scale would have led to problems during the precipitation of the ligation products. The use of lower amounts of protein was limited by unspecific binding of DNA during the selection step. Since the amount of specifically bound DNA, recovered after a selection cycle is dependent on the amount of protein used, whereas the amount of unspecifically bound DNA is not protein dependent, the lowest feasible amount of protein was determined by the level of unspecific binding. It turned out that the largest contributor to unspecific binding was the glutathione-sepharose resin. Since less protein was used the amount of resin could be safely reduced without risking to loose protein due to lack of binding sites on the resin. However, a practical limit was encountered since resin volumes of less than 10 µl resulted in high losses of beads during the wash steps. An attempt was made to reduce the problem of unspecific binding by using a different elution method. The elution using SDS probably elutes DNA specifically bound to protein equally well as DNA bound

| | Sequence | Z-score |
|---|---|---|
| G81-9 | CGCTCAG**GCGCGCCCGCGCTTAC..**GAAGGG | 10,8,3 |
| G81-10 | CGCTCAG**GCACGCGCGCCTGCG...**CAAGGG | 16,8 |
| G81-11 | CGCTCAG**GTCTTTCTTCTGGTCA..**GAAGGG | 0 |
| G81-12 | CGCTCAG**AGAGCTCGCGGGGTACA.**GAAGGG | 6,3 |
| G81-14 | CGCTCAG**GCGCGCATGCGGCGG...**GAAGGG | 19 |
| G81-15 | CGCTCAG**ACGGGCGCAGGCGGGC..**GAAGGG | 7 |
| G81-16 | CGCTCAG**CTCGCACTATTACCCA..**GAAGGG | 5 |
| G81-17 | CGCTCAG**GCGGGCCCGCGCGCTT..**GAAGGG | 12 |
| G81-21 | CGCTCAG**GCGCGCGCGTGCGTGCG.**GAAGGG | 28 |
| G81-22 | CGCTCAG**CGCGCGCGATCGTGTG..**GGAGGG | 15,6 |
| G81-23 | CGCTCAG**GTGCGAGTGTGCGTGC..**GAAGGG | 6,14 |
| G81-24 | CGCTCAG**GCCGGGGCAAACGCGGG.**GAAGGG | 7 |
| G81-27 | CGCTCAG**GCGTGCGCGCCTTACG..**GAAGGG | 16,3 |
| G81-29 | CGCTCAG**GTGCATGCGCGCGCG...**TAAGGG | 23 |
| G81-32 | CGCTCAG**CGCGCGATGCGCGTAAGG**GAAGGG | 21 |

Table 6.10 Alignment of the sequences obtained after eight rounds of selection. Stretches of five or more alternating purines and pyrimidines are underlined. The nucleotides originating from the randomized region are shown in bold characters. The Z-scores were determined using the Interpret program of the MacMolly program package.

to the resin or the plastic surfaces. Incubation with the protease Factor Xa should cleave the fusion protein and thereby specifically elute the DNA bound to the protein. In practice the amount of unspecifically bound DNA recovered by Factor Xa treatment was even higher than by using SDS (Data not shown). The reasons for this are not clear, but might be related to the long incubation time necessary to achieve cleavage of the protein.

After the 8$^{th}$ round of selection, an aliquot of the eluted DNA was subcloned and individual clones sequenced. An alignment of the sequences is shown in Table 6.10 . The sequences obtained after 8 rounds of selection were analyzed further for the represented sequence words and their frequencies (Appendix).

### 6.3.3. Analysis of the selected sequences

In order to investigate whether the selected sequences are capable of forming Z-DNA three of the sequences obtained after the fifth round of selection were chosen for further analysis. The sequences are referred to as G51-5, G51-17 and G51-22 :

| | |
|---|---|
| G51-5 | TACGTGCATGCGCACG |
| G51-17 | CCGGATTGGCAGTCTA |
| G51-22 | GCGCGCGCGTCGCGAG |

Whereas sequence G51-17 does not contain any sequence generally assumed to easily form Z-DNA, the sequence G51-22 is rich in GC dinucleotides and the sequence G51-5 is a mixture of different alternating purine and pyrimidine dinucleotides.

#### 6.3.3.1. Bandshift assays

Hairpin DNA oligonucleotides containing the sequences G51-5, G51-17 and G51-22 and a loop consisting of four thymidine bases were synthesized, radioactively end labeled with T4-DNA polynucleotide kinase and gel purified on 15 % (w/v) acrylamide gels. Upon addition of Zα peptide, both G51-5 and G51-22 gave rise to a strong shifted band (Figure 6.22). In the case of G51-5 most of the DNA is actually bound and shifted.

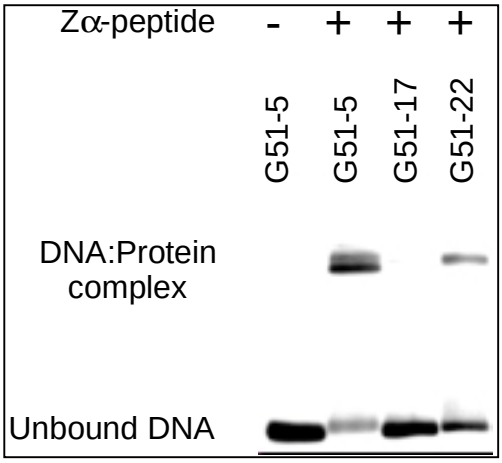


Figure 6.22 Hairpin oligonucleotides were radioactively end labeled and identical amounts of radioactivity incubated with or without 500 ng Zα peptide. The samples were resolved on a 4 % (w/v) polyacrylamide gel and visualized by autoradiography. The type of the sequence and the presence or absence of GST-Zα peptide is indicated above each lane. The positions of the gel origin, DNA:protein complex and free DNA is indicated on the left.

### 6.3.3.2. DEPC footprinting

In order to show that the selected sequences do actually form Z-DNA at normal bacterial negative supercoiling, DEPC footprinting studies were done. Minicircles containing the sequences G51-5, G51-17 and G51-22 were subcloned into the pCR2.1-TA vector and plasmid DNA with bacterial supercoiling isolated. Plasmid DNA was treated with DEPC for different lengths of time, the reaction quenched by the addition of tRNA and the DNA precipitated. Afterwards the plasmid DNA was linearized. In order to detect chemical modification by DEPC, primer extension reactions were done using the taq DNA polymerase and DNA primers labeled with an infrared marker. Taq DNA Polymerase cannot easily pass modified bases and will stop transcription at such sites, leading to products whose lengths correspond to the positions where the DNA had been modified. Gel electrophoresis of the products alongside a sequencing reaction enabled the mapping the modifications to the sequence .

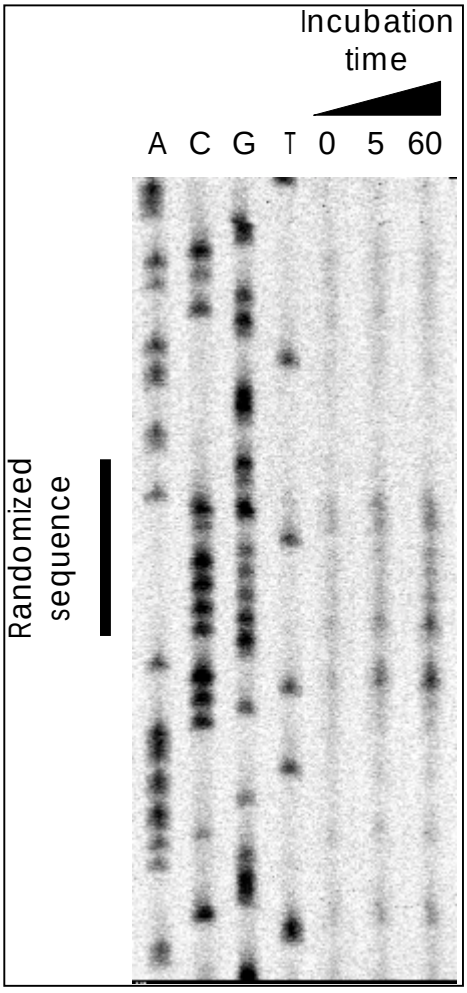


Figure 6.23 Plasmid DNA pCR2.1-G51-22 (1.5 µg) was incubated in 200 µl 50 mM sodium cacodylate buffer pH 8.0 and treated for 5 or 60 minutes with 3 µl DEPC. A control reaction in which the DEPC was omitted is shown as zero incubation time. The reaction was stopped by the addition of 3 µg tRNA and the DNA precipitated. The DEPC treated DNA was used as template in a primer extension reaction. The reaction products were resolved and detected on a LiCor sequencer. A sequencing reaction of the pCR2.1-G51-22 plasmid is shown next to the DEPC reactions for comparison. The position of the bases originating from the randomized sequence is indicated on the left.

Treatment of the plasmid pCR2.1-G51-22 with DEPC resulted in bands throughout the stretch of alternating purine/pyrimidine bases of the selected sequence (Figure 6.23). The