Interactions in the native state of monellin, which play a protective role against aggregation

Supplementary information

Materials, chemicals and buffers

Oligonucleotides and PCR primers were purchased from DNA- Technology (Aarhus, Denmark). The nucleic acid gel stain, GelStar® was from FMC (Vallensbaek Strand, Denmark). Restriction enzymes were obtained from New England Biolabs (MA, USA). The NHS-LC-biotinylation kit, avidin and streptavidin were from Pierce (Rockford, Illinois). Dynabeads coated with streptavidin (Dynabeads M-280) were from Dynal (Oslo, Norway). High Fidelity DNA polymerase and the PCR nucleotide mix were obtained from Boehringer Mannheim (Mannheim, Germany). Sequencing reagents and TRR-mix were from Perkin Elmer Biosystems (Foster City, California). T4 DNA ligase was obtained from USB (Cleveland, Ohio). The Pet 3a vector was from Novagen (Canada). The XL-blue MRF´Kan cells were from Stratagene (La Jolla, CA). ER2566 cells were from New England Biolabs (MA, USA). We obtained the K91Kan from George Smith.

Qiagen plasmid Midi Kit was from Qiagen, (Germany). GFX purification kit for gel bands and PCR products and the kit for plasmid preparation were from Amersham Pharmacia Biotech (Uppsala, Sweden). NAP10 gel filtration columns (G25) and Agarose were from FMC BioProducts (Rockland, Maine). PCR was performed using a Perkin Elmer 2400 PCR instrument. A BioRad E.coli Pulser electroporator was used for transformation. Flexiprep columns were from Amersham Bioscience.

Phage display library design of MNA

We have used phage display to generate a large amount of degenerate MNA fragments for selection against the MNB-fragment. The oligonucleotide corresponding to MNA (monellin A chain) was chosen to be degenerated and expressed in a phage display library (Figure S1a). To obtain a degenerated oligonucleotide encoding for the entire amino acid sequence of a

MNA-fragment (132 base pairs) two overlapping oligonucleotides (A1, 115 bases, and A2, 103 bases) were used, each covering a little more than half of the MNA oligonucleotide fragment, thereby overlapping in the middle of this fragment (Figure S1b). Both A1 and A2 contain flanking 5′ and 3′ DNA regions which aloud primers, PGlibFor and PGlibRev, to anneal in the PCR amplification. A1 and A2 also contain a unique internal *Dde* I restriction site (C/TnAG) (Figure S1b) in the overlapping region, which aloud the two fragments to ligate after cleavage with *Dde* I, yet yielding a whole MNA fragment with the desired amino acid variation. The flanking 5′ and 3′ DNA regions in the whole MNA are again used for primer annealing in PCR amplification as above and they also encode potential *Bgl* I restriction sites (GCCnnnn/nGGC) (Figure S1c). The oligonucleotides were modified so that no *Dde* I or *Bgl* I sites were present in any of the possible degenerate DNA sequences in the library at any other DNA position. The final goal was to obtain a degenerated PCR product of MNA which after cutting with *Bgl* I contained overhangs that allow for directional cloning into *Sfi* I digested fUSE5 vector (*Sfi* I sites: GGCCnnnn/nGGCC) (Figure S1d). All oligonucleotides were designed to minimize secondary structure formation through conservative base changes that did not alter the degenerate amino acid sequence.

Preparation of degenerated MNA inserts

Inserts were produced and amplified using polymerase chain reaction, PCR on a Perkin Elmer 2400 PCR thermal cycler. The High fidelity polymerase was used according to the manufacturer's guidelines in terms of buffer, primer and dNTP concentrations.

First, oligonucleotides of monellin fragments A1 and A2 were used as templates in separate polymerase chain reactions consisting of five cycles: one minute at 95 °C, four minutes at 62 °C, 1.5 minutes at 72 °C. Around 20 μg (corresponding to 4×10^{14} DNA molecules) of A1 and A2 was used as the single-stranded DNA template. The reaction volume of A1 and A2 was 0.6 ml, and was for each template divided into 12 separate PCR tubes. The products had the expected size (115 and 103 bp) as verified by agarose gel electrophoresis (2.5 % gel). Each product was purified by ethanol precipitation and digested by *Dde* I for 4 hours at 37 °C. The digestion mixtures were run on a 2.5% agarose gel, the gel bands containing the digested fragments were cut out and placed in a dialysis bag (cut off MW 3500), released from the gel

using electrophoresis and concentrated by ethanol precipitation. The cleaved A1 and A2 were then ligated for 16 hours at 16 °C and the ligation product of the oligonucleotide of MNA had the expected size (147bp) as verified by agarose gel electrophoresis (2.5 % gel). The ligation product was concentrated by ethanol precipitation and was then used as a template in a second PCR as described above. The amplified ligation product was digested with *Bgl* I for 1 hour at 37 °C. The digested ligation product was purified by using elecrophoresis as described above, ethanol precipitated and redisolved in TE-buffer to a concentration of about 1 μg/μl.

Preparation of vector and ligation with insert

The glycerol stock of fUSE5 phage vector in the strain DH5α (a gift from Johan Salomonsson) was used to infect LB with 20μg/ml tetracycline and the bacteria with the vector was grown over night at 37°C and vigorous shaking. The cells were harvested by centrifugation, and the vector was isolated using QIAGEN tips and flexiprep columns, followed by isopropanol-precipitation. The vector was then dissolved in 150 μl of TE buffer resulting in an approximate concentration of around 1 μg/μl (or 1×10^{10} vector molecules per μl) as judged from the absorbance at 260 nm and the intensity of the gel bands. The vector was digested by *Sfi* I for 4 hours at 50 °C and after that another portion of *Sfi* I was added and the reaction was continued for another 4 hours at 50 °C. Cleaved vector was purified from uncleaved by agarose gel electrophoresis (2.5 % gel), and extracted from the gel band using the GFX kit. The cleaved vector was dissolved in TE-buffer resulting in an approximate concentration of around 1 μg/μl (or 1×10^{10} vector molecules per μl) as judged from the absorbance at 260 nm and the intensity of the gel bands. The vector and the insert were heat treated (70 °C for 10 minutes in TE-buffer) to destroy any possible contaminating Dnase. Cleaved vector was ligated with the degenerated MNA inserts for 16 hours at 16 °C. Different concentrations of vector and insert were tested and the best conditions for the ligation were found out after transformation. For the ligation reaction around 9×10^{12} vector molecules and 30×10^{12} insert molecules were used.

Transformation, scraping, pooling, culturing, and purification of libraries

Electrocompetent XL-blue MRF' Kan cells were produced as described in the Bio-Rad

manual. The ligation product, vector with insert, was electroporated into XL-blue MRF' Kan cells. The primary transformants were spread on LB plates with 20 μg/ml tetracycline. The number of primary transformants was around 7.5×10^5. The colonies were scraped from the plates and divided into 50 pools and each pool was suspended in 20 ml of LB medium with 20 μg/ml tetracycline and cultured over night in 37°C. The cells were centrifuged and the phage display libraries were purified from the medium by repeated PEG precipitations according to the manual from George Smith. After the final precipitation step the phages were resuspended in TBS with 0.02 % sodium azide, yielding an approximate final concentration of 2×10^{13} physical phage particles per ml according to the manual by Smith. A small volume of the library was used to infect *E. coli* for DNA sequencing.

Biotinylation and immobilization of MNB peptide

The peptide MNB1 (monellin B1 chain) was a gift from Wei-Feng Xue (Xue et al., 2006). This mutant contains a mutation Cys41Ser compared with the wild type MNB in order to avoid unwanted interference due to disulfide bond formation and/or deprotonation of the thiol group. The peptide was suspended in 100 mM sodium phosphate buffer with 150 mM NaCl (pH 7.5) to a concentration of 4 mg/ml. NHS-LC-biotin was first dissolved in water and then added to the peptide. A five molar excess of NHS-LC-biotin was added to the peptide solution. The mixture was allowed to react for 30 minutes at room temperature, and the unreacted NHS-LC-biotin was removed by gel filtration on a NAP10 column. The degree of biotinylation was 0.4 as judged by using [2-(4´-hydroxyazobenzene) benzoic acid] reagent included in the NHS-LC-biotinylation kit. The reaction was set up to achieve below 1 biotin per protein to avoid protein molecules with more than one biotin. There are five lysine residues in the MNB1 peptide. All the lysine residues are surface exposed and the coupling is not likely to greatly perturb the interactions between the fragments MNA and MNB.

The biotinylated target peptide (0.20 mg) was bound to 10 mg Dynabeads coated with streptavidin in PBS and the solutions (1ml) were rocked for 30 minutes at room temperature. The beads were then washed five times with PBS to remove unbound peptide. 10μl 10 mM biotin (ten-fold excess) was added to decrease the possibility of unspecific binding to the

biotin binding sites on streptavidin. The binding capacity of the beads is about 60 pmol biotinylated peptide per mg beads.

Phage selection

Selection was done by exposing the library to MNB target that was immobilized on Dynabeads. 200 µl corresponding to approximately 4×10^{12} phage particles of the original library and 200 µl of the biotinylated MNB-fragment on Dynabeads in TBS/0.1% Tween were used in the first round and the mixture was rocked over night at 20 °C in 1.5 ml eppendorf tubes. In each subsequent round, the input was 100 µl of an amplified eluate from the previous round. The amount of Dynabeads in the first round was 2 mg, carrying about 0.7 µg of biotinylated MNB (or 7.2×10^{13} molecules), in the second 0.5 mg and in the third round the amount was 0.25 mg of beads. Parallel experiments were performed at 60°C. After ten washes with TBS/0.1% Tween, to remove weak and unbound phages, the strong binders were eluted with acid (400 µl of 0.2 M glycine buffer at pH 2.2). After neutralization (75 µl of 1 M Tris-HCl, pH 9.1) the phages were propagated in *E. coli* for amplification and purification using two PEG precipitation steps, or tittered on plates for DNA sequencing. A total of three rounds of enrichment were carried out. Parallel to the selections at different temperatures, negative controls and blanks were performed to screen for unspecific binding. The negative control experiments were based on selection of the input phage display material against the magnetic beads coated with streptavidin and biotin. 10 µl phage display library was used in the negative control experiments. A blank test was made by incubating phages in the test tube without any beads. 10 µl phage display library was used as blank 1 and 10 µl of phages prepared for third selection was used as blank 3. The negative control and blank samples were treated as other selection samples above.

Phage titering and DNA sequencing

Phages were prepared according to the manual from George Smith. *E. coli* terrific broth (TB) culture was prepared as follows. On the night before cells are needed, 5 ml of LB containing 100 µg/ml kanamycin was inoculated with K91Kan cells and shaken overnight at 37°C. The overnight culture was used to inoculate 25 ml of TB with which was then shaken vigorously

at 37°C for a few hours until the OD of a 1/10 dilution reached 0.1-0.2. 100µl of the terrific broth culture was then transfected with 100µl of phages from each selection round. The transfected mixture was then added to 25ml LB containing 0.2µg/ml tetracycline and cultured in 37°C for 30-60min and then a small amount of the culture was used to make a dilution series on LB agar plates containing 20 µg/ml tetracycline such that isolated colonies were obtained and the rest of the culture was cultured over night in 37°C after an addition of 25µl of a 20mg/ml tetracycline stock. The next day, cells were centrifuged and the phage display libraries were purified from the medium by repeated PEG precipitations according to the manual from George Smith. After the final precipitation step the phages were resuspended in TBS with 0.02 % sodium azide, yielding an approximate final concentration of 2×10^{13} physical phage particles per ml according to the manual by Smith.

The amino acid sequences of selected phage bound peptides were derived by sequencing the DNA corresponding to the MNA insert. A DNA fragment containing the MNA insert was obtained by PCR amplification. Two oligonucleotides (Nprimer, 5′ HO-TAAAGGCTCCTTTTGGA-GCC-OH; and Cprimer, 5′ HO-CCCTCATAGTTAGCGTAACG
-OH 3′) that bind to complementary DNA sequences, flanking the MNA DNA sequence were used to generate a 343 base-pair fragment, and a fraction of an isolated colony was used as template for each reaction. The PCR product was purified by ethanol-precipitation and sequenced using standard procedures. The sequencing reactions were analyzed by BM-unit, Lund University. Alternatively colonies were shipped to Agowa in Germany who performed plasmid preparations and sequencing reactions.

Results of DNA sequencing and mutant design

Several colonies were sequenced; 45 colonies from the library pool, 41 colonies from the selection at 20C pool and 94 colonies from the negative control. The occurrences of different amino acids at different positions are summarized in Figure S2 a-c. Several sequences were found in both the 20C-selection and the negative control. This indicates that surface active MNA variants dominate not only in the negative control but also in the selection against MNB. MnA10 was found in 3 copies in the 20C selection and in 4 copies in the negative

control. MNA12 was found in 2 copies in the 20C selection and in 2 copies in the negative control. MnA14 was found in 4 copies in the 20C library and in 1 copy in the negative control. MNA15 was found in 15 copies in the 20C selection and in 9 copies in the negative control. MNA10, 12, 14 and 15 were chosen to be produced for further investigations (Figure S3). Beside those variants, two additional variants were produced; MNA11 which is a combination of MNA12-AI and MNA10-AII and MNA13 which is a combination of MNA12-AI and MNA15-AII (Figure S3 and S5).

Production of genes for the selected mutants

The oligonucleotide of the MNB fragment was obtained by a PCR reaction with the scMN2 gene in a Pet 3a vector as the template and the primers MNB1 and MNB2 (Figure S4 a and b). The oligonucleotides of the chosen mutants were obtained by PCR of two overlapping sequences AI and AII (Figure S5) to avoid errors that can occur in the synthesis of to long oligonucleotides. The region which enable MNA to anneal with MNB is added to the AI sequence and the region allowing cleavage with *BamH* I is added to the AII sequence (Figure 5S). The oligonucleotides of those 6 MNA sequences were obtained by PCR reaction with different MNA-AI and MNA-AII sequences as the template and the primers PrimerMNA1 and PrimerMNA2 (Figure S6) The six MNA oligonucleotide fragments were purified using GFX kit. To get an oligonucleotide sequence of scMN10, 11, 12, 13, 14 and 15 (single chain monellin), oligonucleotides of MNA1 and MNB were used in a PCR together with the primers PrimerMNB1 and PrimerMNA2 (Figure S7). The product was GFX-purified. The oligonucleotide sequences of scMN10, 11, 12, 13, 14 and 15 were then cleaved with *Nde* I and *BamH* I yielding a product of 295 bp. This product was then ligated into a dubble cleaved *Nde* I and *BamH* I Pet 3a vector which was then transformed into ER2566 and spread on LB plates with 50 μg/ml ampicillin. 5 colonies of each construct were checked for the size by running a PCR with Pet I and Pet II primers and colonies which seemed to have the right size of insert corresponding to scMN10, 11, 12, 13, 14 and 15 were used for sequencing and plasmid preparations.

Figure captions

Figure S1. Construction of phage display library of the MNA fragment. a) Secondary structure of monellin, degenerate oligonucleotide sequence corresponding to the 45-residue MNA peptide and the resulting peptide library. b) Degenerate sequences of monellin fragments A1 and A2, oligonucleotide flanking regions and the corresponding primers PGlibFor and PGlibrev in bold. Restriction site for *Dde* I is marked as cursive region and a line. c) The MNA is digested with the restriction enzyme *Bgl* I. d)The fUSE5 vector is digested with the restriction enzyme *Sfi* I.

Figure S2. Sequencing results. Frequency (in %) of each amino acid at each position in the a) phage display library, b) selection at 20ºC and c) negative control. "-" –
means that there is a gap in the sequence, in bold are shown the non-designed amino acids found. In the case where there is no number below an amino acid, a 100% occurrence was observed. "x" represents an unidentified amino acid according to the sequence analysis.

Figure S3. Six variants that were chosen to be produced. The regions in MNA10-MNA15 that are identical are bolded. Underlined are the regions of the MNA oligonucleotides which are used as overlapping sequences in the production of each MNA oligonucleotide from two fragments.

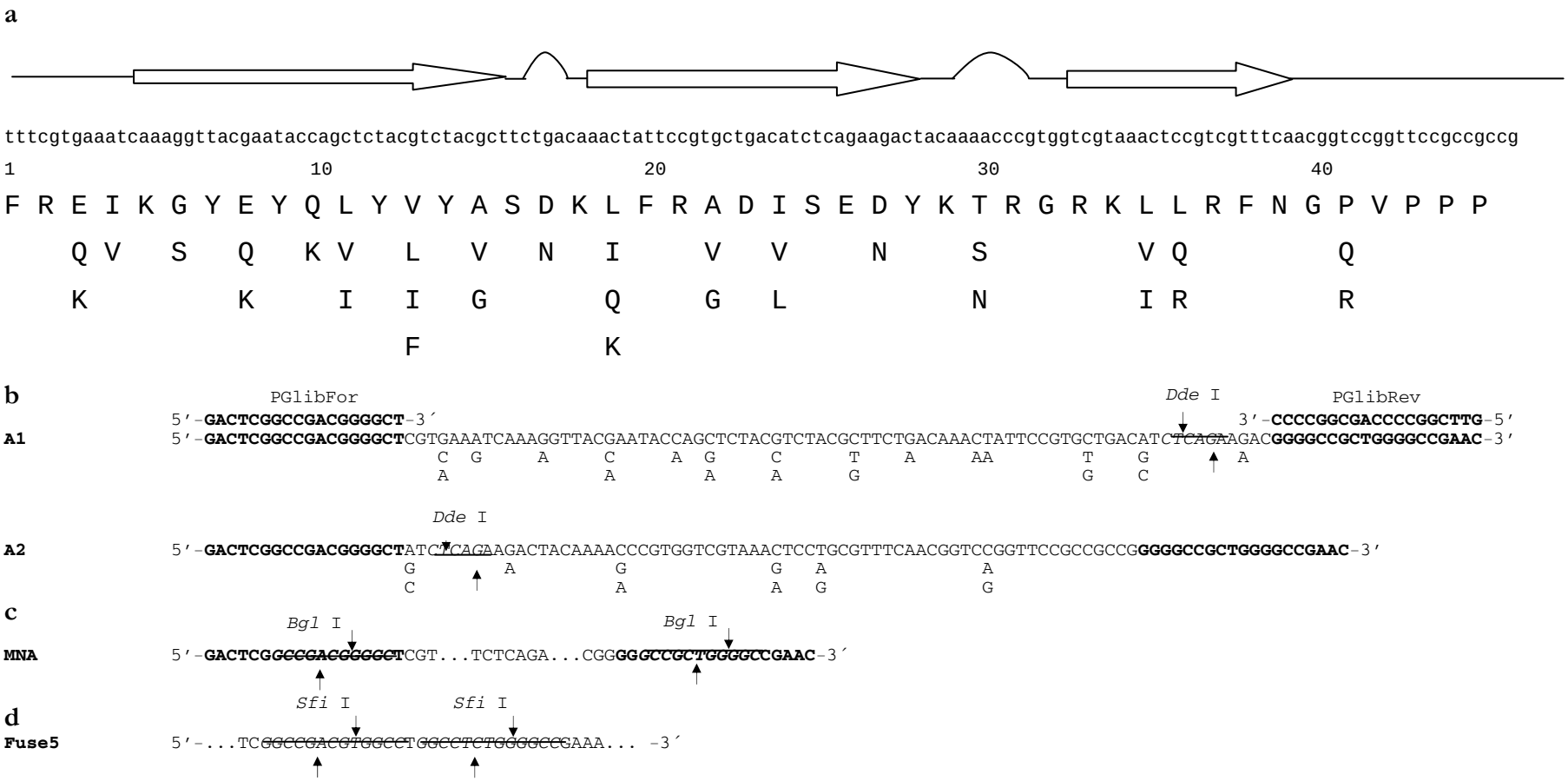
Figure S4. a). Parent scMN in a Pet 3a vector. In bold are marked the regions where primers PrimerMNB1 and PrimerMNB2 anneal. The restriction sites of *Nde* I and *Bam*H I are

underlined. b). MNB is produced by using primers PrimerMNB1 and PrimerMNB2.

Figure S5. The two fragments AI and AII for each variant that was chosen to be produced. In bold are the regions which enable overlap with MNB-chain. Underlined are the regions that are used as the overlap between the two MNA fragments AI and AII. In bold and underlined are regions to enable cleavage with *BamH* I.

Figure S6. PCR reaction of sequence MNA10-AI and MNA1-AII in the process of production of MNA10 oligonucleotide. I bold, the overlapping sequence with MNB fragment. Also in bold, the primers PrimerMNA1 and PrimerMNA2. Underlined, the overlapping sequence between Sequence MNA10-AI and MNA10-AII used in the process of production of MNA10 oligonucleotide.

Figure S7. a) Annealed MNA and MNB olgonucleotides in the first PCR reaction. The arrows show the direction of the prolongation process. b) Following PCR reactions to amplify the MNA-MNB oligonucleotide sequences. In bold are primer sequences and underlined.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1 10 20 30 40
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K K I I G Q G L N I R R
F K
b
PGlibFor
Dde I
PGlibRev
5′-GACTCGGCCGACGGGGCT-3′
3′-CCCCGGCGACCCCGGCTTG-5′
A1
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Dde I
A2
5′-GACTCGGCCGACGGGGCTATCTCAGAAGACTACAAAACCCGTGGTCGTAAACTCCTGCGTTTCAACGGTCCGGTTCCGCCGCCGGGGGCCGCTGGGGCCGAAC-3′
c
Bgl I
Bgl I
MNA
5′-GACTCGGCCGACGGGGCTCGT...TCTCAGA...CGGGGCCGCTGGGGCCGAAC-3′
Sfi I
Sfi I
d
Fuse5
5′-...TCGGCCGACGTGGCCTGGCCTCTGGGGCCGAAA... -3′

Figure S1

a

F	R	E	I	K	G	Y	E	Y	Q	L	Y	V	Y	A	S	D	K	L	F	R	A	D	I	S	E	D	Y	K	T	R	G	R	K	L	L	R	F	N	G	P	V	P	P	P
		47	58	96	62	96	44	98	71	20		53	98	14	98	69		25	94	98	11		38		98	73			22	98		98		29	36	98		98		31	96		98	98
		Q	V	**Q**	S	**N**	Q	**-**	K	V		L	**T**	V	**L**	N		I	**I**	**G**	V		V		**K**	N			S	**K**		**C**		V	Q	**K**		**-**		Q	**Y**		**S**	**–**
		40	42	2	36	2	38	2	27	49		12	2	13	2	27		29	4	2	25		36		2	27			51	2		2		49	33	2		2		33	2		2	2
		K		**T**	**K**	**-**	K		**H**	I		I		G		**Q**		Q	**S**		G		L						N					I	R					R	**L**			
		13		2	2	2	16		2	31		13		71		2		22	2		64		24						27					22	31					36	2			
							-					F		**F**		**-**		K					**T**																					
							2					22		2		2		24					2																					

b

F	R	E	I	K	G	Y	E	Y	Q	L	Y	V	Y	A	S	D	K	L	F	R	A	D	I	S	E	D	Y	K	T	R	G	R	K	L	L	R	F	N	G	P	V	P	P	P
		85	80		78		27		42	32		7		7		85		20			5		10			73	98	98	20	98	98	59	42	2		22	84	88	98	98	57	98	98	98	98
		Q	V		S		Q		K	V		L		V		N		I			V		V			N	**-**	**-**	S	**-**	**-**	**S**	**Q**	V	Q	**A**	**-**	**-**	**-**	Q	**-**	**-**	**-**	**-**
		12	20		22		7		56	56		47		3		15		20			7		68			27	2	2	54	2	2	12	2	27	54	14	12	2	2	22	2	2	2	2
		K					K		**N**	I		I		G				Q			G		L						N			**P**	**E**	I	R	**-**				R				
		3					66		2	12		7		90				55			88		22						24			10	2	17	7	2				17				
												F						K											**-**			-	-	**S**	**P**					**S**				
												39						5											2			19	54	2	2					2				
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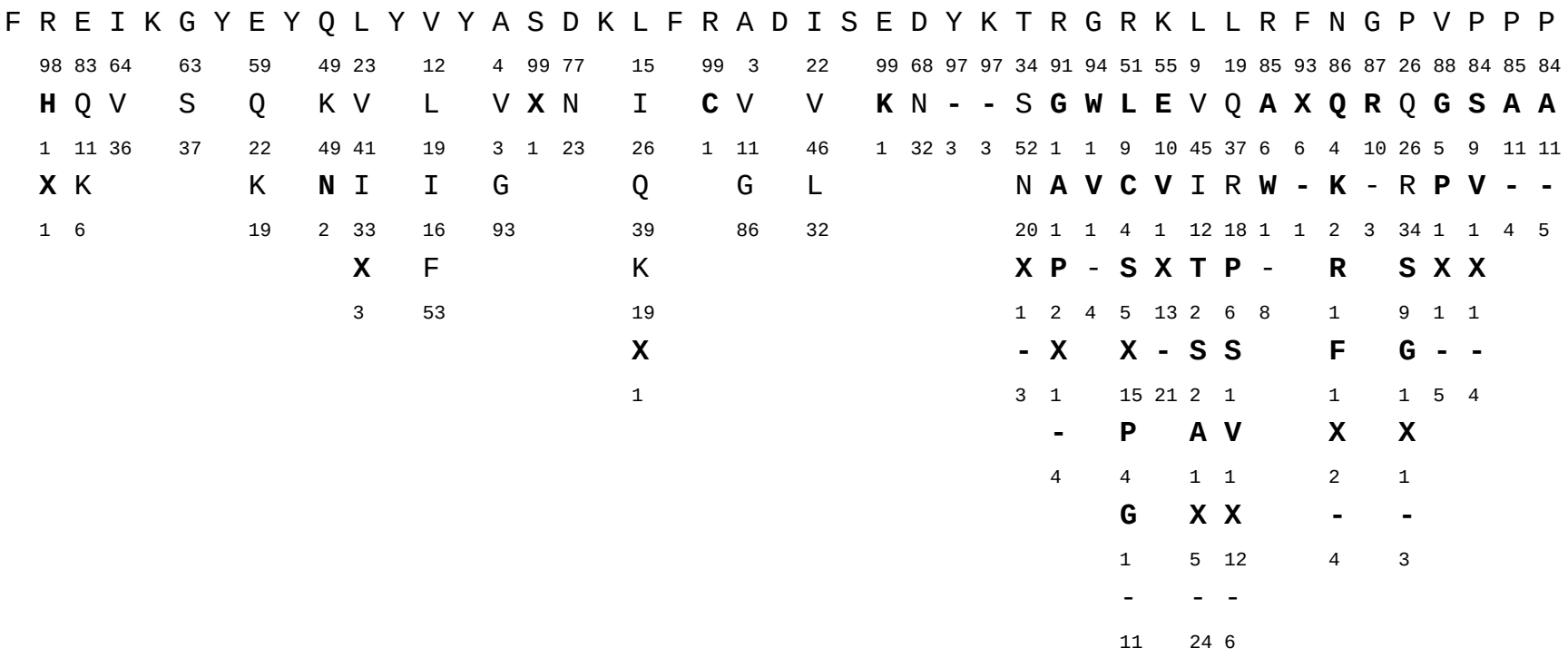
																																			52	15						2				

c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X F K X P - S X T P - R S X X
3 53 19 1 2 4 5 13 2 6 8 1 9 1 1
X - X - S S F G - -
1 3 1 15 21 2 1 1 1 5 4
- P A V X X
4 4 1 1 1 2
G X X - -
1 5 12 4 3
- - -
11 24 6

Figure S2

MNA10
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F R E I K G Y E Y K V Y F Y G S N K Q F R G D V S E D Y K T R G K V Q R F N G Q V P P P

MNA11
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F R E V K S Y K Y Q L Y F Y G S D K I F R G D V S E D Y K T R G K V Q R F N G Q V P P P

MNA12
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MN13
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MNA14
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F R E V K S Y K Y Q L Y F Y G S D K L F R G D L S E D Y K N R G N G P V P P P

MNA15
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F R E V K G Y K Y K V Y L Y G S D K Q F R G D V S E D Y K S R G R Q R F N G P V P P P

Figure S3

a

NdeI

catatgggcgagtgggaaatcatcgatatcggtccattcactcaaaacttgggtaaattcgctgttgatgaagaaaacaagattggccaatacggtagattgacctttaacaaggttatca**gaccatccctgaagaag**

G E W E I I D I G P F T Q N L G K F A V D E E N K I G Q Y G R L T F N K V I R P S L K K

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T I Y E E N

B45 B50

b

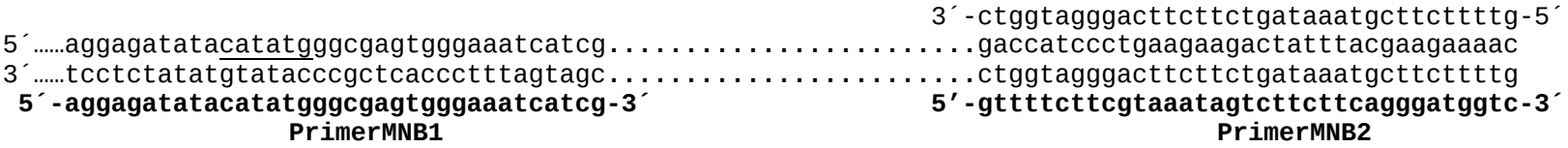

Figure S4

MNA10-AI
5´-**gaccatccctgaagaagactatttacgaagaaaacatgttt**cgtgaaatcaaaggttacgaatacaaggtctacttctacggttctaacaaacaattccgtggtgatgtctcagaagac-3´

MNA10-AII
5´-**gttagcagccggatcctcatta**cggcggcggaacctgaccgttgaaacgctggactttaccacgggttttgtagtcttctgagacatcaccacggaa-3´

MNA11-AI
5´-**gaccatccctgaagaagactatttacgaagaaaacatgttt**cgtgaagtcaaaagttacaaataccagctctacttctacggttctgacaaaatattccgtggtgatgtctcagaagac-3´

MNA11-AII
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MNA12-AI
5´-**gaccatccctgaagaagactatttacgaagaaaacatgttt**cgtgaagtcaaaagttacaaataccagctctacttctacggttctgacaaaatattccgtggtgatgtctcagaagac-3´

MNA12-AII
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MNA13-AI
5´-**gaccatccctgaagaagactatttacgaagaaaacatgttt**cgtgaagtcaaaagttacaaataccagctctacttctacggttctgacaaaatattccgtggtgatgtctcagaagac-3´

MNA13-AII
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MNA14-AI
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MNA14-AII
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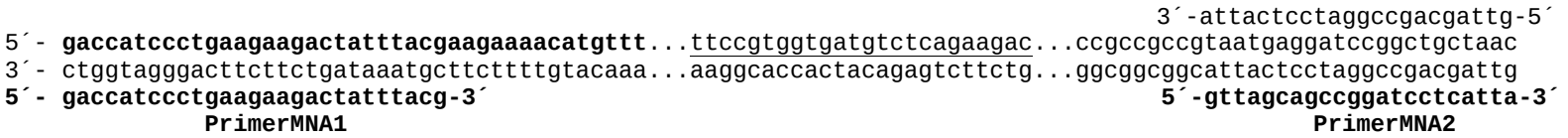
Figure S5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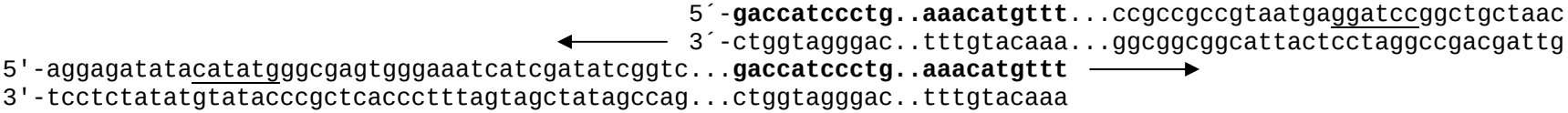
PrimerMNA1
PrimerMNA2

Figure S6

a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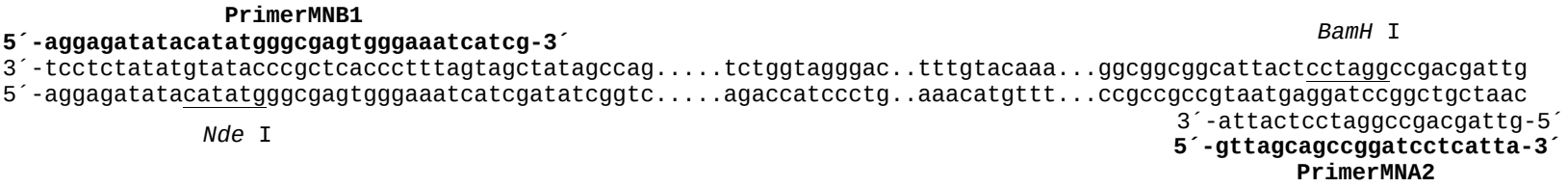
b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Nde I
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PrimerMNA2

Figure S7