Supplementary data

Supplementary Figure 1. *C.neogracile* AFP isoform nucleotide and amino acid sequence. Under line indicated the signal peptide and star marks exhibit the possible glycosylation site. The red color letters show the N-myristoylation site.

Supplementary Figure 2. Alignment of *C.neogracile* AFP and AFP isoform. The alignment was carried out by ClustalW method. The Black squares show a consensus sequences. The identity of these two sequences is 74.8%.

Supplementary Figure 3. Genomic Southern blot analysis. The gDNA of *C.neogracile* digest with *EcoR*V, *Kpn*I and *Xba*I. The *Cn-isoAFP* ORF gene was used as probe. The DNA size markers are shown to left side. E; *EcoR*V, K; *Kpn*I, X, *Xba*I, U; Uncut gDNA.

Supplementary Figure 4. Multiple alignments of Cn-isoAFP with other AFP, IBP and IRIP of psychrophilic organisms. The multiple alignments were produced by ClustalW, and black squares revealed consensus regions. AFP; antifreeze protein, IBP; ice binding protein, IAFP; ice antifreeze protein, IRIP; ice recrystallization inhibition protein.

Supplementary Figure 5. Phylogenetic tree of selected AFPs, IAFP, IRIP, or IBP amino acid sequences from psychrophilic organisms. The phylogenetic tree produced by MEGA5 and Neighbor-joining method. Bootstrap values obtained with 5,000 repetitions. IBP; ice-binding protein, IAFP; ice antifreeze protein, IRIP; ice recrystallization inhibition protein.

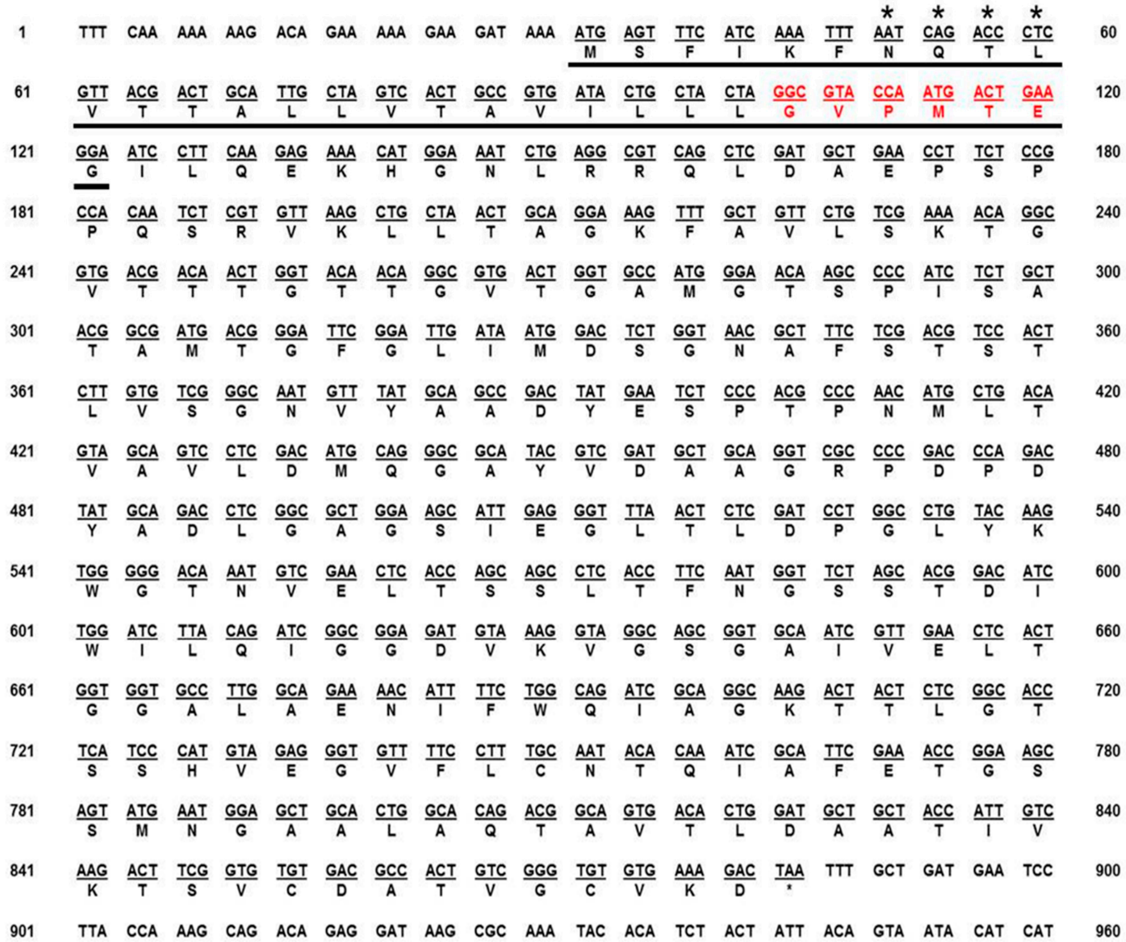
Supplementary Figure 6. Ice crystal morphology of Cn-isoAFP and its mutant proteins under various protein concentration. The scale bar indicated 100 μm.

Supplementary Figure 7. Circular dichroism spectroscopy of purified Cn-isoAFP and its mutants. Each spectrum is the average of five scans. A correction was made by subtracting the spectra obtained in the presence of buffer only.

Supplementary Table 1. The primer information used in this study. The underline showed a restriction enzyme site.

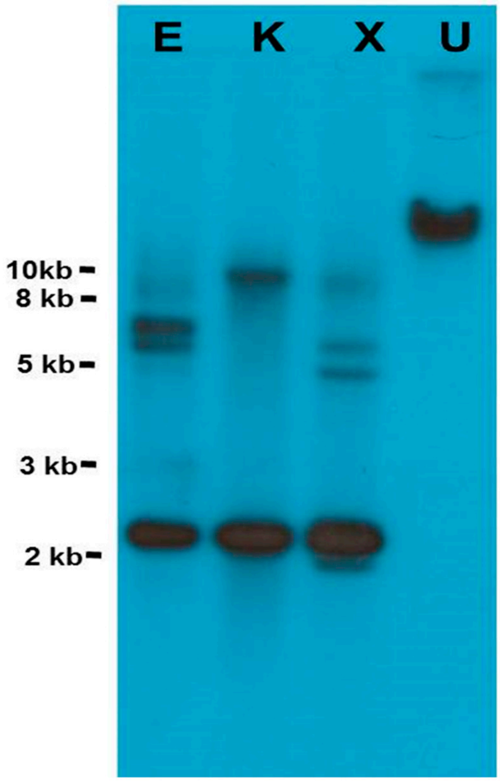
Supplementary Table 2. Information of site-directed mutagenesis primers. The bold letters indicated the site-directed mutation sequences.

Supplementary Figure 1.



Supplementary Figure 2.

Supplementary Figure 3.



Supplementary Figure 4.

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C. neogracile isoAFP     MSFIKFNQILVTTALLVTAVILLLGVPMTEGILTEKHGNLRRQLDAEPSPPQSRVKLLTAGKFAVLSKTG------VTTT 74
C. neogracile AFP        MSLITINHILVVTALLFAAVA-LLGVPMAEGHRQEKRGGLRRQLDDEP---LSAVKLLTAGRFAILTKTG------VTTT 70
N. glaciei IBP           ----MMAKTVTVAVASSVAAS-------------AVDG-------------------TAG--DAVSKAG------VSTT 35
T. ishikariensis AFP    -----MSASSAVAASSVSAAG-------------SAVG-------------------TAG--NYVASTG------VSTV 34
P. ingrahamii IBP        MNTKNYSKKSSSGVGNAVAG--------------YSVN-------------------SAD--NAVSKSG--------TN 36
F. curta IAFP            --------GMSVASASSTAVN-------------RTAG--------------------------DVAKAG-------TNV 26
F. populicola IBP        --------MTSSVGSSSVAG---------------AVG-------------------KAN--ASTGVSN--------VD 27
Colwellia sp. IBP        MKTSN-SKKVMGSAGNVMAAG-------------YAVG-------------------AG----TTSKSG--------TD 34
L. edodes IBP            MSTTN----TSGAVVSVVAAG-------------GSVG-------------------SGG--GSGGGSGGGNGGGGGAN 41
Luecosporidium sp. AFP  --------MSSTGAGGVNGR--------------DSVG-------------------VASN--AAKAGSS--------VD 30
L. antarcticum AFP       --------MRSNHAASVRCAH----------------------------------------SRRTDSS-------SST 23
Chlamydomonas sp. IBP   MSSSMKAAACMATSMAAVVCK------------MDADG-------------------NTTAACSVGVSG------GGSG 42
D. antarctica IRIP      -----MAKCGSAAASATACHSR-------------DRAGARNGG---------------VGCVRAAWSGDG-------CCD 41
L. perenne IRIP         ------MKSWAAASVAVSCHD--------------DARGAGNS----------------NGGVHAKW--DN-------CCS 36

C. neogracile isoAFP     GTTGVTGAMGTSPISATAMTGFGLIMD-SGNAFSTSTLVSGNVYAADYESPTPNML---TVAVLDMQGAYVDAAGRPDPD 150
C. neogracile AFP        GPTDLKGDMGTSPITGAAITGFGLITDPSDTTFSTSSLVIGQVFASDYTSPTPNML---TVAVLDMQAAYVDAAGRPDPD 147
N. glaciei IBP           GTVTGDGTSASTAIGAKDSS--N----------TSTSSVTGK-YAADYTATSK------MTTASDVSTAT-DAAGRSDD- 94
T. ishikariensis AFP    SVTGAVGVSGTAASTGSSGT--G----------TSTSSVTG--TGADYGTTS-------TTAGDMGTAY-NAATRSGD- 90
P. ingrahamii IBP        VSDSVTGDVG-----------------ASTGAATTCSVVG-DYATTANGACSNAG---TGSAGDMGAYN-DAAGRVSD- 92
F. curta IAFP            GGVTGDGVSAASAMTGDMD------------SSNSTSSVVDGSVYANYMSTGTKT------TAVSDVTAYN-DAAARVTS- 86
F. populicola IBP        SSVNCDGVSGASGVTGSTG---------DSGGSSTSKVTGRVYASTYGDTAS-------TTAVDMNAYK-DARDDTNH- 88
Colwellia sp. IBP        VYSTVTGNVG-----------------TSTGAAN-CDVTG-AMYTVDSAGCSNS-----YAVSDVGAYN-DAAGRVADH 88
L. edodes IBP            AGAAVNGTAGNYAAKSGSTVSSG--NGVSSTTATGSTDSTGKATSVVGAASAATTT---TTAVSDVTA-N-DATGRVTDT 114
Luecosporidium sp. AFP  SAG--DGVSAAATYTGGT----------DSSTTYATSVTG--YAADYSTTNY-------AAVANATAY-NAAGVDDG- 86
L. antarcticum AFP       SAATADGVAGYDVVAR-----------------SATGAATG--NVGSGSTATG--------TVVDHG------------ 63
Chlamydomonas sp. IBP   GSVSYTDANNRSTCGDDD----------VDSSYSSTNNCRGCSNASNTTSTRSTACTSKYGCMAVVGAATTDWSSSANTT 112
D. antarctica IRIP      WGVGCDGASGRVTTT----------------RGAGG-ASAGVHVKGNRRTANRSG------TNNSVR--------GR---- 87
L. perenne IRIP         WGVGCDGGSGRVTTWG---------------HGAGHTASAGARSNANNKVGTSWGVDHCYDSNNSVGKNRRCNVGRSGMA 101

C. neogracile isoAFP     Y-----ADLGAGSIEGLTLDPGLYKWGT------------NVELTSSLTFNGSSIDIWI-L-QIGGDVKVGSGAIVELT 210
C. neogracile AFP        Y-----VELGAGNIEGLTLEPGLYKWGT------------DVGFTNSLTFDGSDIDIWI-L-QIDGDVTAGSGAKVKLI 207
N. glaciei IBP           ------G-AGSGTVAG-------YKWGT------------DVSTSS---VDGSAIDVWV-A-KDVGNGAMYTGTAKAN- 141
T. ishikariensis AFP    ------Y-TGAGGTTG-------YKWTS------------SVGASAD--TSGTSTDTWD-G-TGAAGKKTVGGAAKNVW 139
P. ingrahamii IBP        --------TGAGGGT------GYKWS--------------TDVGTSDVTKGNATDVWA-G-TSAAYKNVTAGGAANVW 141
F. curta IAFP            ---------GGAGAG-------ATYIN------------GAGGTTGVYTYTDVRTDSDVTDGKNDSSVKTSKNAANT 137
F. populicola IBP        --------TGAGGAVG-------YKTIG------------VSTADVT--GGTDIYAG-----TSAAGVKNVGGANVVW 132
Colwellia sp. IBP        --------TGTGGGTG------VYKWS--------------SDVNSTDVTNGTMDDVWMSG-NNANAKRVTTGGAAKNW 138
L. edodes IBP            --------NGGGGGVT------GYKWTG------------AVSVNSTGVTAGTDH-----A-TGAAASRVTVGGASNVW 161
Luecosporidium sp. AFP  --------AGR-DTVG-------YKWTS------------SVSVTDTG--NGDATWVAG----GSADGVATAGGANSTN 131
L. antarcticum AFP       --------TCSAG----------VKYCG------------ADSSTSATSVKGRGADS--------------SAGAATDV 98
Chlamydomonas sp. IBP   STCASSATSGRVGGAARNDSGAMTAVVKGSTTSTACDGGAHSANASTTDSTTGTAVDGGAVVGTNATVASVKSSTTSSTA 192
D. antarctica IRIP      -------NNAAGN-------------DN------------TVSGNNNTVS-GSNTVVGSDN---TGSKHVVSGRKHVTD 130
L. perenne IRIP         STNMTVKHNASGNTTG-TNNYVRSGVNN------------VVSGNHNTVTSGNNNVVSGNHNTVSGTNHVVTGNNHVVT 167

C. neogracile isoAFP     GGALAENIFWQIAG-KTILGTSSHVEGVFLCNTQIAFETGSSMNGAALAQTAVTLDAATIVKTSVCDATVGCVKD.    285
C. neogracile AFP        NDAKAENIFWQIAG-KTDLGTTSHVEGVFLCSTAITFKTGSSMNGAALAQTAVTLDSATIVKESVCDVDVGCVAPN.   283
N. glaciei IBP           -----------VSG-AVNGTTAHVGN-------SATAATGSSNGKASTATDSVTVS.                       179
T. ishikariensis AFP    -----V-----VAG-AVNVGAKGTAK-------TAVTKTGSSNGRA-TAVASATVK.                       177
P. ingrahamii IBP        -----------VAD--SVTGTGAHGG--------KAVNTGATVKGRATAVTKNKTAVSHSVNNS.               185
F. curta IAFP            ---------DVTNGAKANWSVAVNGAGAHMKGVATAVKTGSNVGRVAATAVTATTDTAATSSTR.               193
F. populicola IBP        -----------AVADSVTIVAATSSGG------KTVVVNTNASVGRATAVVKATVVGVCGA.                  176
Colwellia sp. IBP        -----------VAG--YTAGTYASGVS------KTSVNTGTTVNGRATAVTKNTNATYA.                    179
L. edodes IBP            -----------AATSVVTAGAGSHGVVA------KTAVTTGATMNGRATVASATVG.                       201
Luecosporidium sp. AFP  -----------AVGDDVTVGKGAHG-------VAKRVTTGSSNGRVSTVAKATVNSVAVVKRSNARW.            181
L. antarcticum AFP       ----------------VAAWKSASS----------ADYTKRDAGGSDTAG.                             123
Chlamydomonas sp. IBP   VTTG-----GMVAGGAVSDDSTTNGDAKGKCVWDKCSYTDKTNNTWTGCAKSSWCKAHDGNKWTTCGMGRCY.      260
D. antarctica IRIP      -----------NNNKVSGNDNNVSGSH-------TVSGSHNTVSGSNNTVSGSNHVVSGSNKVVTGG.            180
L. perenne IRIP         -----------RNNTVSGSHHKVSGGHN------TVSGSHNTVSGSHNTVSGSNHVH-GNNKVVTGG.            217
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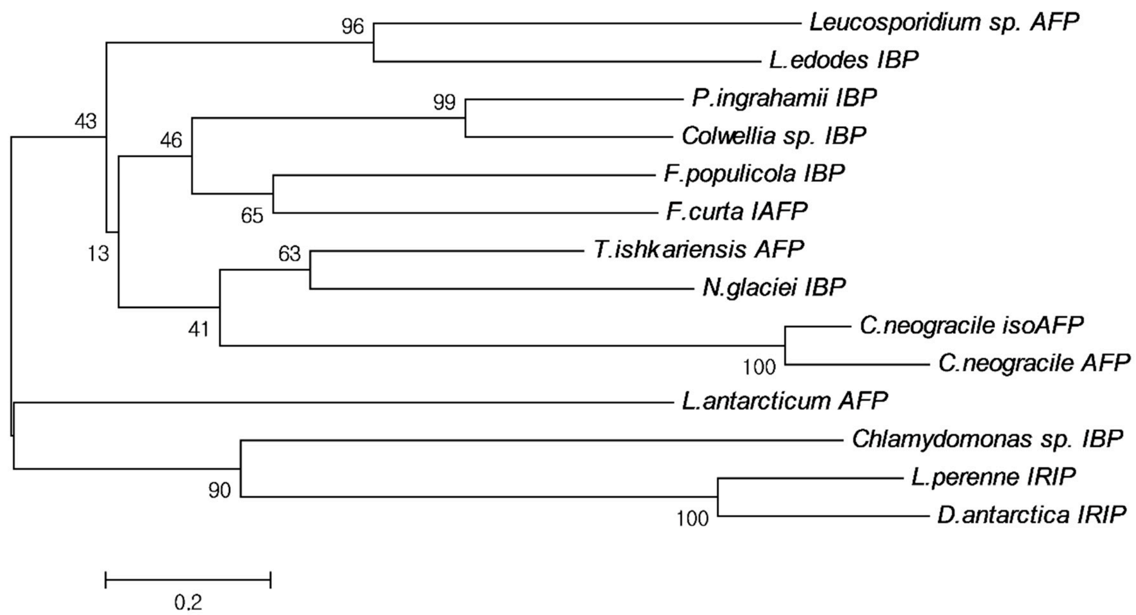
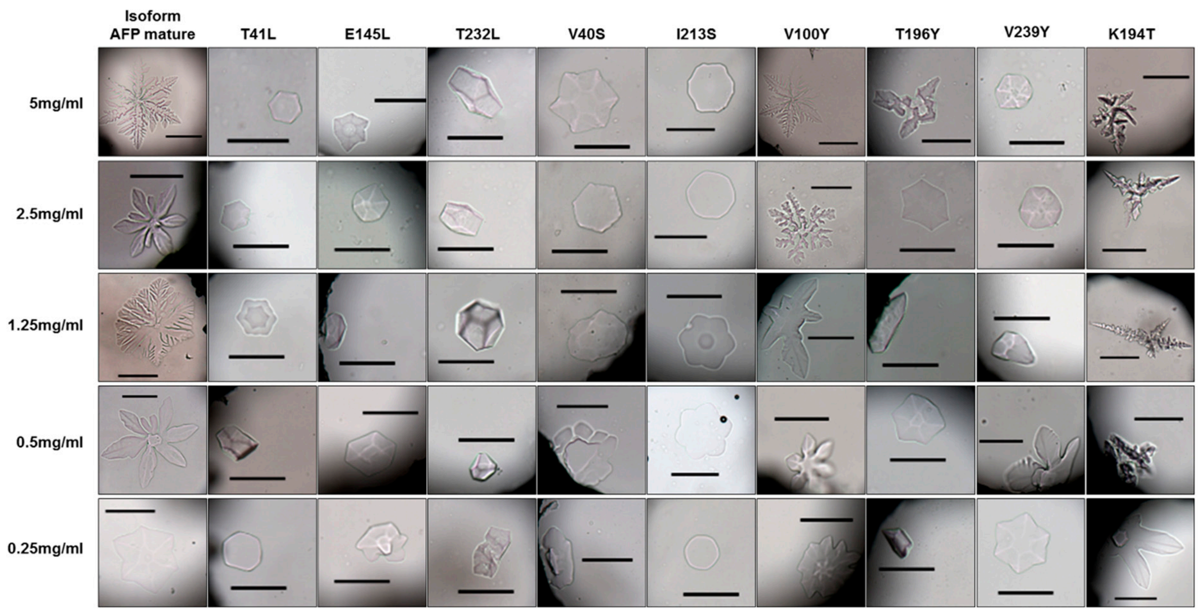
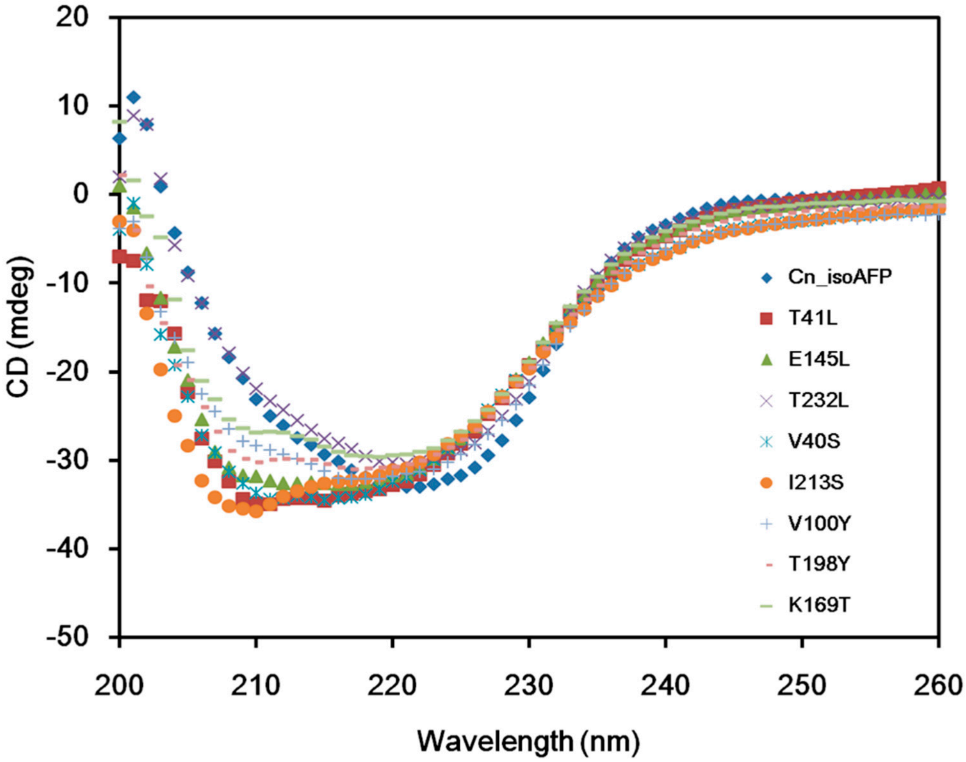
Supplementary Figure 5.



Supplementary Figure 6.



Supplementary Figure 7.



Supplementary Table 1.

Number	Primer name	Sequences (5' → 3')	Tm (°C)
1	AFP degenerated forward primer	AAR CAN GGN GTN CAN CAN AC	54
2	AFP degenerated reverse primer	ARN GTN CAN GCN GTY TGN GC	56
3	Isoform AFP DNA walking target specific primer 1	AGA GTA GTC TTG CCT GCG AT	57
4	Isoform AFP DNA walking target specific primer 2	GGA TCG AGA GTT AAA CCC TC	57
5	Isoform AFP DNA walking target specific primer 3	CCA TTA TCA ATC CGA ATC CCG T	58
6	Isoform AFP 3' race primer	AAC ATT TTC TGG CAG ATC GC	56
7	Isoform AFP inverse primer 1	CAC GGC AGT GAC TAG CAA TGC	63
8	Isoform AFP inverse primer 2	ATG GAG CTG CAC TGG CAC AGA	63
9	Isoform AFP 5' UTR probe forward primer	TGA GTT TAG GTC CAG CGT CCG	63
10	Isoform AFP 5' UTR probe reverse primer	GGT ACG CCT AGT AGC AGT ATC	63
11	Isoform AFP pCold pre-mature forward primer	<u>GGT ACC</u> ATC CTT CAG GAG AAA	59
12	Isoform AFP pCold mature forward primer	<u>GGT ACC</u> ATG AGT TTC ATC AAA TTT	58
13	Isoform AFP pCold reverse primer	<u>AAG CTT</u> TTA GTC TTT CAC ACA CCC	61

Supplementary Table 2.

Number	Primer name	Sequences (5' → 3')	Tm (°C)
1	V100Y forward	CCA CGC CCA ACA TGC TGA CA**T AC**G CAG TCC TCG ACA TGC AGG G	84
2	V100Y reverse	CCC TGC ATG TCG AGG ACT GC**G TA**T GTC AGC ATG TTG GGC GTG G	84
3	T196Y forward	GGC AGA TCG CAG GCA AGA CT**T AT**C TCG GCA CCT CAT CCC ATG	93
4	T196Y reverse	CAT GGG ATG AGG TGC CGA G**AT A**AG TCT TGC CTG CGA TCT GCC	83
5	V239Y forward	CAC TGG ATG CTG CTA CCA TT**T AC**A AGA CTT CGG TGT GTG ACG CC	82
6	V239Y reverse	GGC GTC ACA CAC CGA AGT CTT **GTA** AAT GGT AGC AGC ATC CAG TG	82
7	T41L forward	TTC TGT CGA AAA CAG GCG TG**C TG**A CAA CTG GTA CAA CAG GCG T	81
8	T41L reverse	ACG CCT GTT GTA CCA GTT GT**C AG**C ACG CCT GTT TTC GAC AGA A	81
9	E145L forward	ACA AGT GGG GGA CAA ATG TC**C TA**T TCA CCA GCA GCC TCA CCT T	81
10	E145L reverse	AAG GTG AGG CTG CTG GTG AA**T AG**G ACA TTT GTC CCC CAC TTG T	81
11	T232L forward	CAC TGG CAC AGA CGG CAG TG**C TA**C TGG ATG CTG CTA CCA TTG T	83
12	T232L reverse	ACA ATG GTA GCA GCA TCC AG**T AG**C ACT GCC GTC TGT GCC AGT G	83
13	V40S forward	CTG TTC TGT CGA AAA CAG GC**T CG**A CGA CAA CTG GTA CAA CAG GCG	82
14	V40S reverse	CGC CTG TTG TAC CAG TTG TC**G TC**G AGC CTG TTT TCG ACA GAA CAG	82
15	I213S forward	GGG TGT TTT CCT TTG CAA TAC ACA A**TC C**GC ATT CGA AAC CGG AAG CAG	81
16	I213S reverse	CTG CTT CCG GTT TCG AAT GC**G GA**T TGT GTA TTG CAA AGG AAA ACA CCC	81