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Plasmid size: 4708 base pairs
Molecular weight: 2,908,890 daltons
(Molecular weight of RiG protein: 44.829 kg/mol)

Translated:

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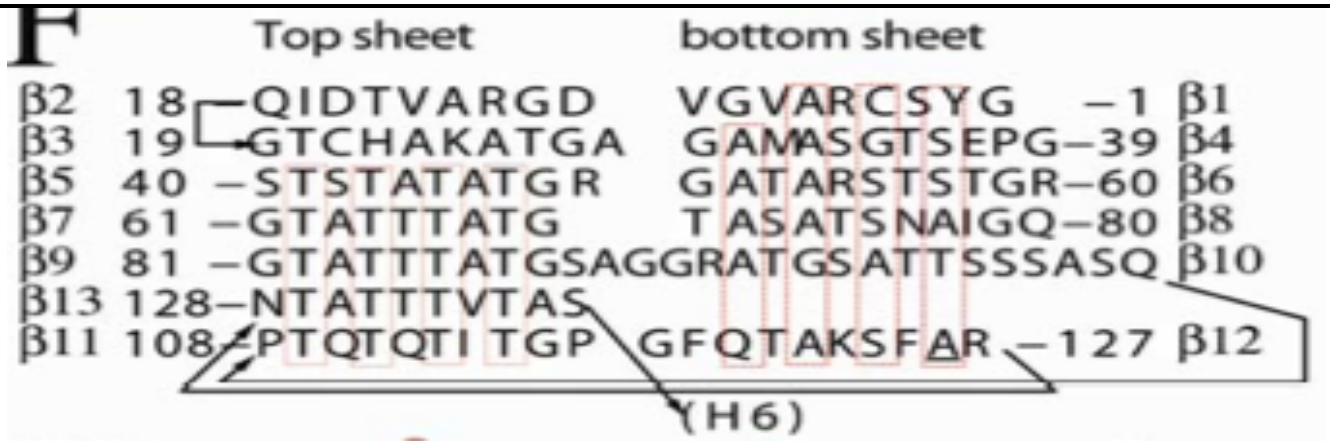
- start codon
- GFP
- RiAFP
- His tag



RiAFP Portion of interest:

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(B1)	Y	S	C	R	A	V	G	V	**D**	**G**	(b2)	R	A	V
	2	3	4	5	6	7	8	9	10	11		12	13	14

acg	gat	atc	cag	**ggt**	(B3)	acc	tgc	cac	gct	aag	gcc	acc	**ggt**	**gcg**
T	D	I	Q	**G**	(B3)	T	C	H	A	K	A	T	**G**	**A**
15	16	17	18	19		20	21	22	23	24	25	26	27	28

ggc	**gcc**	(b4)	atg	gcg	tcc	ggc	acc	tcc	gaa	**cca**	**ggt**	**tcc**	(B5)	acc
G	**A**	(b4)	M	A	S	G	T	S	E	**P**	**G**	**S**	(B5)	T
29	30		31	32	33	34	35	36	37	38	39	40		41

tcc	acc	gcg	acc	gca	acg	**ggc**	**cgt**	**ggc**	**gcc**	(b6)	act	gct	cgt	agc
S	T	A	T	A	T	**G**	**R**	**G**	**A**	(b6)	T	A	R	S
42	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**	**51**		**52**	**53**	**54**	**55**

acc	tcc	acc	**ggt**	**cgt**	**ggt**	(B7)	act	gct	acc	acc	act	gca	act	ggt
T	S	T	**G**	**R**	**G**	(B7)	T	A	T	T	T	A	T	G
56	57	58	59	60	61		62	63	64	65	66	67	68	69

acc	gca	tct	gcg	acc	tcc	aac	**gcc**	**atc**	**ggt**	**cag**	**ggt**	(B9)	acc	gca
T	A	S	A	T	S	N	**A**	**I**	**G**	**Q**	**G**	(B9)	T	A
70	71	72	73	74	75	76	77	78	79	80	81		82	83

acc	acg	act	gcg	acg	**ggt**	**tcc**	**gca**	**ggt**	**ggc**	**cgt**	**gca**	(b10)	acc	ggt
T	T	T	A	T	**G**	**S**	**A**	**G**	**G**	**R**	**A**	(b10)	T	G
84	85	86	87	88	89	90	91	92	93	94	95		96	97

agc	gcg	act	acg	agc	agc	tcc	**gcg**	**agc**	**cag**	**ccg**	(B11)	acc	cag	act
S	A	T	T	S	S	S	**A**	**S**	**Q**	**P**	(B11)	T	Q	T
98	99	100	101	102	103	104	105	106	107	108		109	110	111

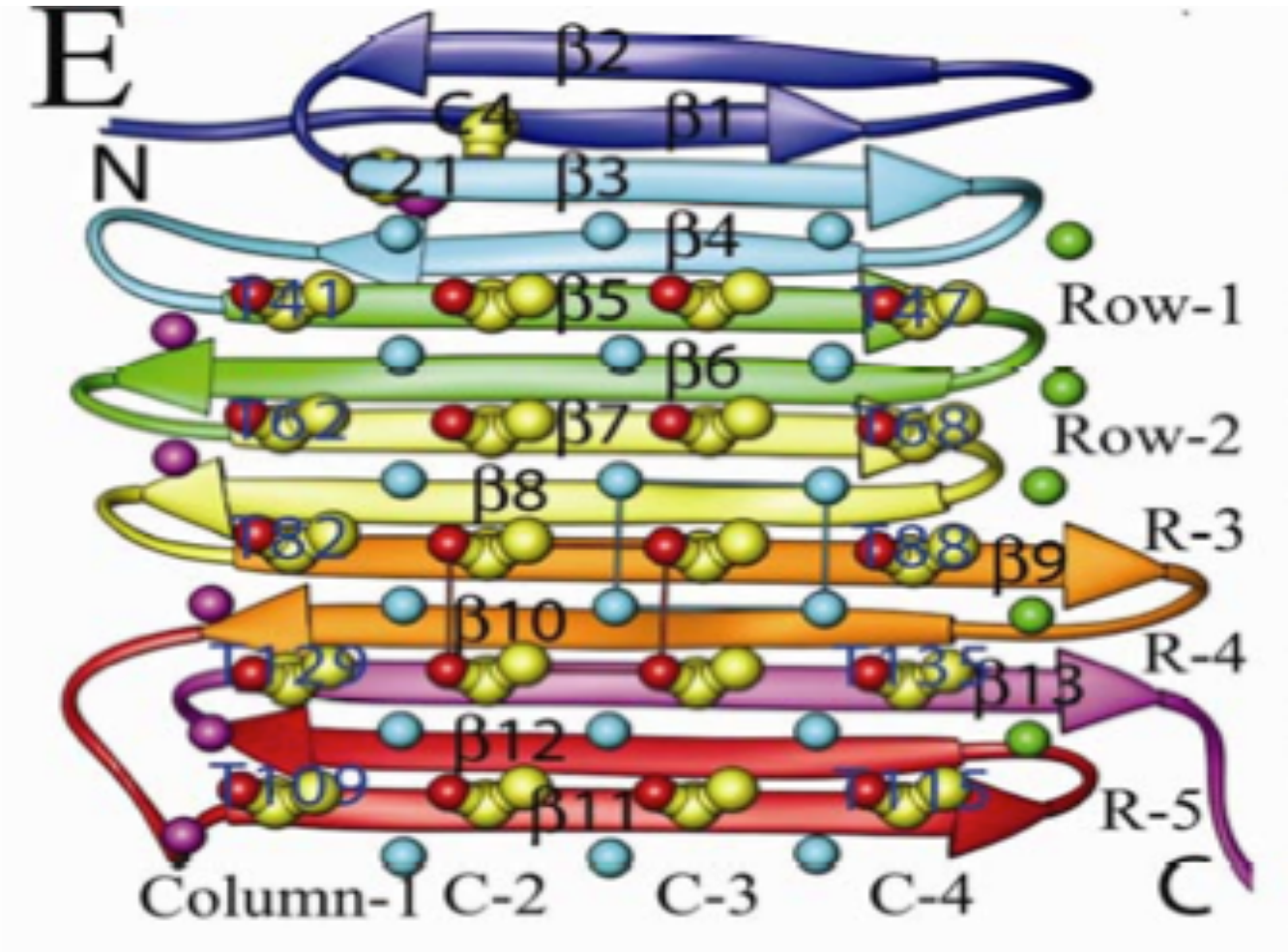
caa	acc	att	acc	**ggc**	**ccg**	**ggt**	(b12)	ttt	cag	act	gcg	aaa	agc	ttt
Q	T	I	T	**G**	**P**	**G**	(b12)	F	Q	T	A	K	S	F
112	113	114	115	116	117	118		119	120	121	122	123	124	125

gct	cgt	aac	(B13)	act	gca	act	acc	acc	gtt	acc	gca	agc	cat	cat
A	**R**	**N**	(B13)	T	A	T	T	T	V	T	A	S	H	H
126	**127**	**128**		**129**	**130**	**131**	**132**	**133**	**134**	**135**	**136**	**137**	**138**	**139**

cat	cat	cat	cac	ggc	gta	***gac***	***ggt***	cgt	gca	gta	acg	gat	atc	cag	***ggt***
H	H	H	H	G	V	D	G	R	A	V	T	D	I	Q	G
140	141	142	143	144	145	146	147	148	150	151	152	153	154	155	156

acc	tgc	cac	gct
T	C	H	A

- number of RiAFP protein sequence and further amino acids
Bold - turns
b – hydrophilic beta stands
B – hydrophobic “ice-binding” beta strands
X – TXTXTXT motifs
- Histidine tag



PRIMER DESIGNS:

- primer sequence
A/ - site of mutation
F – forward primer
R – revers primer
(#) – Tm
[#] – primer ID

1. Expand the number of TxTxTxT rows:
   A. Change the H22 and K24 in B3 to T's by substitution:

acc	tgc	cac	gct	aag	gcc	acc
T	C	H	A	K	A	T
20	21	22	23	24	25	26

To:

acc	tgc	acc	gct	acg	gcc	acc
T	C	T	A	T	A	T
20	21	22	23	24	25	26

F: gc acc gct acg g cca ccg gtg (64)[7]

R: ggc cgt agc ggt gca ggt acc c (64) [8]

   B. insertion of another hydrophilic and hydrophobic strands (16+ amino acids, 48 base pairs)
   - use 3 SDM reactions using Gene Art?
   http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/Mutagenesis.html ($32.50/rxn; up to 25 bp insertions/deletions/rxn)
   - or synthesize the best possible mutant based on results of further SDM experiments

2. Disable various numbers of rows (and combinations if time permits) of TxTxTxT by substituting all T's with A's:
   A. Disable B5 Tx's by substitution to Ax's:

(B5)	acc	tcc	acc	gcg	acc	gca	acg
(B5)	T	S	T	A	T	A	T
	41	42	43	44	45	46	47

To:

(B5)	gcc	tcc	gcc	gcg	gcc	gca	gcg
(B5)	A	S	A	A	A	A	A
	41	42	43	44	45	46	47

F: cc gaa cca ggt tcc gcc tcc gcc gcg gcc gca gcg ggc cg (83) [31]
R: ggc gcc acg gcc cgc tgc ggc cgc ggc gga ggc gga acc tg (85) [32]

   B. Disable B7 Tx's by substitution to Ax's:

(B7)	act	gct	acc	acc	act	gca	act
(B7)	T	A	T	T	T	A	T
	62	63	64	65	66	67	68

To:

(B7) gct gct gcc acc gct gca gct
(B7) A A A T A A A
62 63 64 65 66 67 68

F: cc tcc acc ***ggt cgt ggt*** gct gct gcc acc gct gca gct ggt acc (78) [33]
R: cgc aga tgc ggt acc agc tgc agc ggt ggc agc agc acc acg ac (78) [34]

C. Disable B9 Tx's by substitution to Ax's:

(B9) acc gca acc acg act gcg acg
(B9) T A T T T A T
82 83 84 85 86 87 88

To:

(B9) gcc gca gcc acg gct gcg gcg
(B9) A A A T A A A
82 83 84 85 86 87 88

F: c ***gcc atc ggt cag ggt*** gcc gca gcc acg gct gcg gcg ***ggt*** tcc (82) [35]
R: gcc acc tgc gga acc cgc cgc agc cgt ggc tgc ggc acc ctg acc (83) [36]

D. Disable B11 Tx's by substitution to Ax's:

(B11) acc cag act caa acc att acc
(B11) T Q T Q T I T
109 110 111 112 113 114 115

To:

(B11) gcc cag gct caa gcc att gcc
(B11) A Q A Q A I A
109 110 111 112 113 114 115

F: **gc tcc gcg agc cag ccg** gcc cag gct caa gcc att gcc **ggc ccg** (82)[37]
R: g aaa acc cgg gcc ggc aat ggc ttg agc ctg ggc cgg ctg gc(80)[38]

E. Disable B13 Tx's by substitution to Ax's:

(B13) act gca act acc acc gtt acc
(B13) T A T T T V T
129 130 131 132 133 134 135

To:

(B13) gct gca gct acc gcc gtt gcc
(B13) A A A T A V A
129 130 131 132 133 134 135

F: gc ttt ***gct cgt aac*** gct gca gct acc gcc gtt gcc gca agc (75)[39]
R: g atg atg gct tgc ggc aac ggc ggt agc tgc agc gtt acg agc(75)[40]

3. Disable the number of columns 1, 2, and 3 by reusing mutants with 2 disabled columns and the reverse 1 column primers (*):

I. Disable one column (the 4th T):

A. Disabling 4th T47 in B5:

(B5) acc tcc acc gcg acc gca acg
(B5) T S T A T A T
41 42 43 44 45 46 47

To:

(B5) acc tcc acc gcg acc gca gcg
(B5) T S T A T A A
41 42 43 44 45 46 47

F: **c** acc tcc acc gcg acc gca gcg **Ggc cgt gg (77)** [51]
*alpha R: g agc agt ggc gcc acg gcc cgc tgc ggt cgc (78)[52]

B. Disabling 4th T68 in B7:

(B7) act gct acc acc act gca act
(B7) T A T T T A T
62 63 64 65 66 67 68

To:

(B7) act gct acc acc act gca gct
(B7) T A T T T A A
62 63 64 65 66 67 68

F: **gt** act gct acc acc act gca gct ggt acc gc (69) [53]
*beta **R:** **gga ggt cgc aga tgc ggt acc agc tgc agt ggt g** (72) [54]

C. Disabling 4th T88 in B9:

(B9) acc gca acc acg act gcg acg
(B9) T A T T T A T
82 83 84 85 86 87 88

To:

(B9) acc gca acc acg act gcg gcg
(B9) T A T T T A A
82 83 84 85 86 87 88

F: ***g ggt*** acc gca acc acg act gcg gcg ***ggt*** tcc gc (76)[55]
*gamma R: gc acg gcc acc tgc gga acc cgc cgc agt cgt gg (78)[56]

D. Disabling 4th T115 in B11:

(B11) acc cag act caa acc att acc
(B11) T Q T Q T I T
109 110 111 112 113 114 115

To:

(B11) acc cag act caa acc att gcc
(B11) T Q T Q T I A
109 110 111 112 113 114 115

F: **g ccg** acc cag act caa acc att gcc **ggc ccg g** (74)[57]
*delta R: gc agt ctg aaa acc cgg gcc ggc aat ggt ttg ag (71)[58]

E. Disabling 4th T135 in B13:
(B13) act gca act acc acc gtt acc
(B13) T A T T T V T
129 130 131 132 133 134 135
To:
(B13) act gca act acc acc gtt gcc
(B13) T A T T T V A
129 130 131 132 133 134 135

F: ***c*** act gca act acc acc gtt gcc gca agc cat c (69)[59]
* epsilon R: atg atg atg atg gct tgc ggc aac ggt ggt ag (67)[60]

II. Disable 2 columns (the 1st and 2nd T):

A. Disabling first 2 T's (T41 and T43) in B5:
(B5) acc tcc acc gcg acc gca acg
(B5) T S T A T A T
41 42 43 44 45 46 47
To:
(B5) gcc tcc gcc gcg acc gca acg
(B5) A S A A T A T
41 42 43 44 45 46 47

F: ggc acc tcc gaa ***cca ggt tcc*** gcc tcc gcc gcg acc gc(79)[41]
R: g gcc gct tgc ggt cgc ggc gga ggc gga acc tgg ttc (79)[42]

B. Disabling first 2 T's (T62 and T64) in B7:
(B7) act gct acc acc act gca act
(B7) T A T T T A T
62 63 64 65 66 67 68
To:
(B7) gct gct gcc acc act gca act
(B7) A A A T T A T
62 63 64 65 66 67 68

F: gc acc tcc acc ***ggt cgt ggt*** gct gct gcc acc act gc (77) [43]
R: gc ggt acc agt tgc agt ggt ggc agc agc acc acg acc gg (76) [44]

C. Disabling first 2 T's (T82 and T84) in B9:

(B9) acc gca acc acg act gcg acg
(B9) T A T T T A T
82 83 84 85 86 87 88

To:

(B9) gcc gca gcc acg act gcg acg
(B9) A A A T T A T
82 83 84 85 86 87 88

F: cc tcc aac ***gcc atc ggt cag ggt*** gcc gca gcc acg act gcg (78) [45]
R: gc gga acc cgt cgc agt cgt ggc tgc ggc acc ctg acc g (79) [46]

D. Disabling first 2 T's (T109 and T111) in B11:

(B11) acc cag act caa acc att acc
(B11) T Q T Q T I T
109 110 111 112 113 114 115

To:

(B11) gcc cag gct caa acc att acc
(B11) A Q A Q T I T
109 110 111 112 113 114 115

F: gc ***agc tcc gcg agc cag ccg*** gcc cag gct caa acc att acc (77)[47]
R: c cgg gcc ggt aat ggt ttg agc ctg ggc cgg ctg gct cgc (79) [48]

E. Disabling the first 2 T's (T129 and T131) in b13:

(B13) act gca act acc acc gtt acc
(B13) T A T T T V T
129 130 131 132 133 134 135

To:

(B13) gct gca gct acc acc gtt acc
(B13) A A A T T V T
129 130 131 132 133 134 135

F: gcg aaa agc ttt ***gct cgt aac*** gct gca gct acc acc g (71) [49]
R: g gct tgc ggt aac ggt ggt agc tgc agc gtt acg agc (72) [50]

III. Disabling 1st, 2nd, and 4th rows from mutant with disabled 1st and 2nd row:

- Reuse alpha-epsilon reverse primers for disabling the fourth column T's.

A. Disable the 1st, 2nd,and 4th T's in B5:

(B5) gcc tcc gcc gcg acc gca acg

(B5) A S A A T A T

41 42 43 44 45 46 47

To:

(B5) gcc tcc gcc gcg acc gca gcg

(B5) A S A A T A A

41 42 43 44 45 46 47

F: gcc tcc gcc gcg acc gca gcg ***Ggc cgt ggc*** (81) [61]

B. Disable the 1st, 2nd,and 4th T's in B7:

(B7) gct gct gcc acc act gca act

(B7) A A A T T A T

62 63 64 65 66 67 68

To:

(B7) gct gct gcc acc act gca gct

(B7) A A A T T A A

62 63 64 65 66 67 68

F: ***gt ggt*** gct gct gcc acc act gca gct ggt acc gc (74) [62]

C. A. Disable the 1st, 2nd,and 4th T's in B9:

(B9) gcc gca gcc acg act gcg acg

(B9) A A A T T A T

82 83 84 85 86 87 88

To:

(B9) gcc gca gcc acg act gcg gcg

(B9) A A A T T A A

82 83 84 85 86 87 88

F: ***g ggt*** gcc gca gcc acg act gcg gcg ***ggt*** tcc gc (79) [63]

D. Disable the 1st, 2nd,and 4th T's in B11:

(B11) gcc cag gct caa acc att acc

(B11) A Q A Q T I T

109 110 111 112 113 114 115

To:

(B11) gcc cag gct caa acc att gcc

(B11) A Q A Q T I A

109 110 111 112 113 114 115

F: ***g*** gcc cag gct caa acc att gcc ***ggc ccg gg*** (76) [64]

E. Disable the 1st, 2nd,and 4th T's in B13:

(B13) gct gca gct acc acc gtt acc
(B13) A A A T T V T
129 130 131 132 133 134 135

To:

(B13) gct gca gct acc acc gtt gcc
(B13) A A A T T V A
129 130 131 132 133 134 135

F: gct gca gct acc acc gtt gcc gca agc cat c (72) [65]

4. Deletion of 4th T's to make the ice binding face concave:

A. Deletion of last xT (A46 and T47) in B5:

(B5) acc tcc acc gcg acc gca acg
(B5) T S T A T A T
41 42 43 44 45 46 47

To:

(B5) acc tcc acc gcg acc _____
(B5) T S T A T _____
41 42 43 44 45 46 47

F: ggt tcc acc tcc acc gcg acc [redacted] ggc cg (71) [11]
R: gct acg agc agt ggc gcc acg gcc [redacted] ggt cgc (74) [12]

B. Deletion of last xT (A67 and T68) in B7:

(B7) act gct acc acc act gca act
(B7) T A T T T A T
62 63 64 65 66 67 68

To:

(B7) act gct acc acc act _____
(B7) T A T T T _____
62 63 64 65 66 67 68

F: gt ggt act gct acc acc act gca act [redacted] gca tc (66)[13]
R: ggc gtt gga ggt cgc aga tgc [redacted] agt tgc (66)[14]

C. Deletion of last xT (A87 and T88) in B9:

(B9) acc gca acc acg act gcg acg
(B9) T A T T T A T
82 83 84 85 86 87 88

To:

(B9) acc gca acc acg act _____
(B9) T A T T T _____
82 83 84 85 86 87 88

F: gcc atc ggt cag ggt acc acg act ggt tcc (68)[15]
R: ggt tgc acg gcc acc tgc gga acc agt cgt gg (72) [16]

D. Deletion of last xT (I114 and T115) in B11:

(B11) acc cag act caa acc att acc
(B11) T Q T Q T I T
109 110 111 112 113 114 115

To:

(B11) acc cag act caa acc _____
(B11) T Q T Q T _____
109 110 111 112 113 114 115

F: gc cag ccg acc cag act caa acc ggc ccg (71)[17]
R: gct ttt cgc agt ctg aaa acc cgg gcc ggt ttg (68)[18]

E. Deletion of last xT (V134 and T135) in B13:

(B13) act gca act acc acc gtt acc
(B13) T A T T T V T
129 130 131 132 133 134 135

To:

(B13) act gca act acc acc _____
(B13) T A T T T _____
129 130 131 132 133 134 135

F: gt aac act gca act acc acc gca agc c (63) [19]
R: g atg atg atg atg gct tgc ggt ggt ag (61) [20]

5. Insertion of xT (AT) repeats ater current TxTxTxT motif to create a convex ice-binding face:

A. Insertion after B5 TxTxTxT motif:

(B5) acc tcc acc gcg acc gca acg
(B5) T S T A T A T
41 42 43 44 45 46 47

To:

(B5) acc tcc acc gcg acc gca acg gct acc
(B5) T S T A T A T A T
41 42 43 44 45 46 47

F: ***ggt tcc*** acc tcc acc gcg acc gca acg gct acc ***Ggc cgt ggc*** (77) [21]
R: gct acg agc agt ggc gcc acg gcc ggt agc cgt tgc ggt c (78) [22]

B. Insertion after B7 TxTxTxT motif:

(B7) act gct acc acc act gca act
(B7) T A T T T A T

62 63 64 65 66 67 68

To:

(B7) act gct acc acc act gca act gcc acg

(B7) T A T T T A T A T

62 63 64 65 66 67 68

F: gct acc acc act gca act ggt acc gcc acg gca tct gc (73)[23]
R: ggc gtt gga ggt cgc aga tgc cgt ggc ggt acc (73) [24]

C. Insertion after B9 TxTxTxT motif:

(B9) acc gca acc acg act gcg acg

(B9) T A T T T A T

82 83 84 85 86 87 88

To:

(B9) acc gca acc acg act gcg acg gct aca

(B9) T A T T T A T A T

82 83 84 85 86 87 88

F: ***g ggt*** acc gca acc acg act gcg Acg gct aca ***ggt*** tcc (73)[25]
R: ggt tgc acg gcc acc tgc gga acc tgt agc cgt cgc (75) [26]

D. Insertion after B11 TxTxTxT motif:

(B11) acc cag act caa acc att acc

(B11) T Q T Q T I T

109 110 111 112 113 114 115

To:

(B11) acc cag act caa acc att acc gct aca

(B11) T Q T Q T I T A T

109 110 111 112 113 114 115

F: **g ccg** acc cag act caa acc att acc gct aca **ggc ccg** (72) [27]
R: gct ttt cgc agt ctg aaa acc cgg gcc tgt agc ggt aat g(71) [28]

E. Insertion after B13 TxTxTxT motif:

(B13) act gca act acc acc gtt acc

(B13) T A T T T V T

129 130 131 132 133 134 135

To:

(B13) act gca act acc acc gtt acc gca act

(B13) T A T T T V T A T

129 130 131 132 133 134 135

F: ***gct cgt aac*** act gca act acc acc gtt acc gca act gca agc c (72) [29]
R: g atg atg atg gct tgc cgt tgc ggt aac ggt ggt agt tgc (71) [30]

4. (Fun/Random idea) Explore role/importance of Alanine as the identity of the “X” residue

5. Change Q110 and Q112 in B11 to A’s by substitution:

```
(B11) acc cag act caa acc att acc
(B11)  T   Q   T   Q   T   I   T
      109 110 111 112 113 114 115
```

To:

```
(B11) acc gcg act gca acc att acc
(B11)  T   A   T   A   T   I   T
      109 110 111 112 113 114 115
```

F: ccg acc gcg act gca acc att acc (63) [9]
R: ggt tgc agt cgc ggt cgg ctg c (64) [10]