7.2~7.4

DbD2 (http://cptweb.cpt.wayne.edu/DbD2/), BridgeD (http://biodev.cea.fr/bridged/), MODIP (http://caps.ncbs.res.in/iws/modip.html) were used to design disulfide bonds of SQR (pdb ID: 3T31). They predicted the pairs of amino acid residues that might form disulfide bonds were predicted.

Table 1. Some of the predicted results. The first two columns are the position number of pairs of residues that may form disulfide bonds if they were mutated to Cys. The third column is the prediction methods.

Res1.Seq	Res2.Seq	Method	Res1.Seq	Res2.Seq	Method
2	28	bridged	18	406	dbd2/modip
2	99	dbd2/modip	19	333	bridged
3	29	modip/bridged	20	30	bridged
4	100	modip/bridged	21	30	modip/bridged
5	31	bridged	22	407	bridged
5	98	dbd2/modip/bridged	23	337	bridged
5	101	bridged	24	28	dbd2/modip
6	16	bridged	31	72	dbd2/modip/bridged
6	32	bridged	33	74	modip/bridged
6	102	bridged	35	77	dbd2/modip/bridged
8	13	dbd2/bridged	37	75	dbd2/modip/bridged
8	104	dbd2/bridged	40	59	dbd2/modip/bridged
8	105	bridged	40	130	bridged
9	14	bridged	41	44	bridged
9	32	bridged	41	58	modip
9	34	dbd2/modip/bridged	44	58	dbd2/modip/bridged
10	13	bridged	45	133	modip/bridged
12	104	bridged	47	52	dbd2/modip/bridged
12	301	bridged	47	53	dbd2/modip
12	329	bridged	48	53	bridged
13	104	dbd2/bridged	48	133	dbd2/modip/bridged
16	329	modip/bridged	49	127	dbd2/bridged
17	32	dbd2/modip/bridged	49	133	dbd2
17	71	bridged	49	136	dbd2/bridged

See full results in supplements.

7.5~7.10

Rosetta ddg_monomer was used to predict the free energy change (ΔΔG) of amino acid residue pairs mutated to cysteine pairs.

Table 2. Mutant residue pairs with reduced ΔG

aa_replaced	ΔG(kcal/mol)	ΔΔG(kcal/mol)
MUT_156CYS_192CYS:	-2095.526667	-3.407333333
MUT_358CYS_365CYS:	-2094.832333	-2.713
MUT_40CYS_59CYS:	-2094.379667	-2.260333333
MUT_159CYS_317CYS:	-2093.859	-1.739666667
MUT_166CYS_358CYS:	-2093.547	-1.427666667
MUT_192CYS_314CYS:	-2093.477	-1.357666667
MUT_114CYS_117CYS:	-2092.612333	-0.493
MUT_162CYS_356CYS:	-2092.350333	-0.231
MUT_45CYS_133CYS:	-2092.153667	-0.034333333
WT:	-2092.119333	0

See complete calculation results in supplements.

7.11

For the 9 predicted structure of mutant residue pairs with a negative ΔΔG, PyMOL was used for visual analysis to observe whether disulfide bonds were formed.

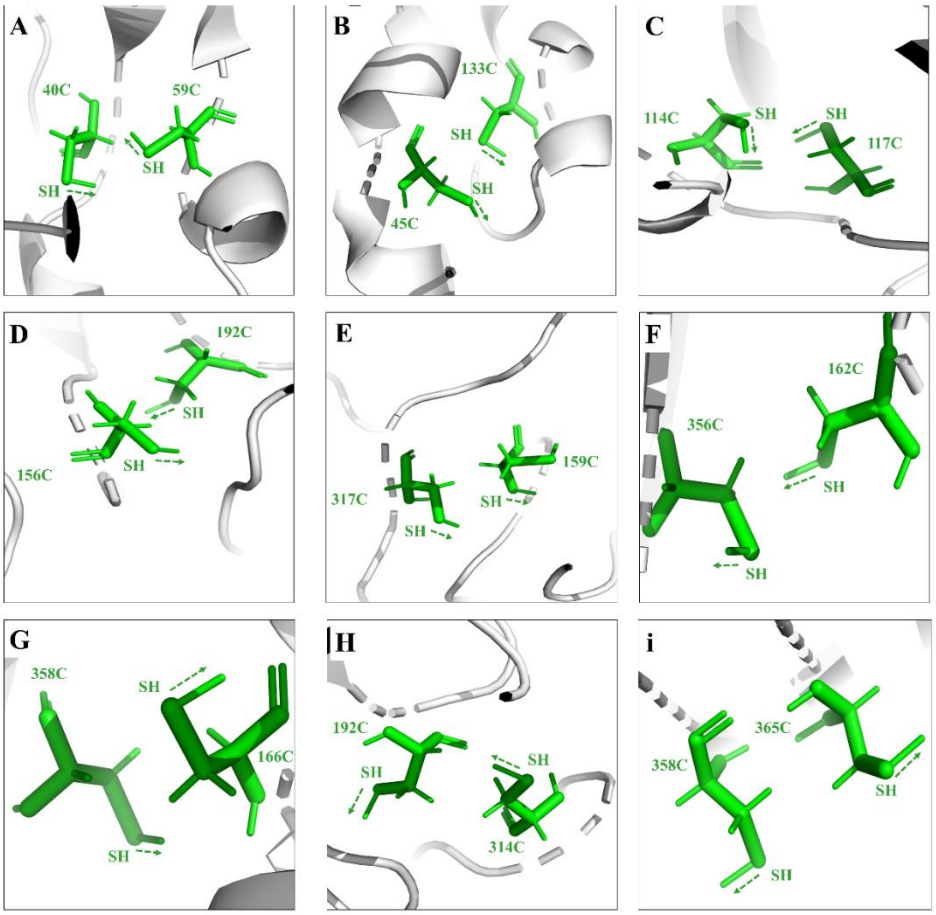


Figure 1. Structures of Cys pairs with a negative ΔΔG. A:mut_40cys_59cys. B:mut_45cys_133cys. C:mut_114cys_117cys. D:mut_156cys_192cys. E:mut_159cys_317cys. F:mut_162cys_356cys. G:mut_166cys_358cys. H:mut_192cys_314cys. I:mut_358cys_365cys.

See pdb files in supplements.

All the 9 mutation groups are not matched in the sulphydryls' orientation and cannot form disulfide bonds.

7.12~7.13

Rosetta ddg_monomer was used to calculate the ΔΔG of cysteine 226, 277 mutated to other amino acids.

Table 3. The ΔΔG values of 226Cys and 277Cys mutants

mutants	ΔΔG(kcal/mol)	mutants	ΔΔG(kcal/mol)
C226L	-4.093	C277V	-2.533
C226I	-3.018	C277I	-0.512
C226V	-2.462	C277L	-0.251
C226W	-0.881	C277C	0
C226M	-0.596	C277T	0.901
C226C	0	C277F	1.249
C226F	1.113	C277W	2.627
C226T	1.791	C277Y	3.332
C226Y	3.107	C277M	3.571
C226A	3.453	C277A	3.998
C226Q	4.928	C277R	6.023
C226N	5.642	C277N	6.669
C226S	6.836	C277S	6.894
C226R	7.147	C277Q	7.353
C226H	7.191	C277G	7.981
C226H	7.191	C277H	8.263
C226G	8.216	C277H	8.263
C226K	8.816	C277D	11.584
C226E	9.033	C277E	12.46
C226D	10.447	C277K	13.073
C226P	34.653	C277P	28.38

7.14

ConSurf-DB (https://consurfdb.tau.ac.il/) was used to predict the conservation of each amino acid residue in SQR (pdb ID: 3T31).

Table 4. Conserved amino acid residues. COLOR is a value (from 1 to 9) that characterizes the conservation of amino acids. Larger number represents higher conservation, and the residues valued 8 or 9 is considered conserved.

Position number	Amino acid	COLOR	Position number	Amino acid	COLOR
11	G	9	37	S	8
13	G	9	42	F	8
16	G	9	44	F	8
20	A	9	49	P	8
46	P	9	107	A	8
101	Y	9	129	S	8
102	D	9	131	C	8
104	L	9	136	A	8
109	G	9	156	G	8
119	G	9	163	C	8
162	S	9	167	A	8
165	G	9	194	T	8
166	P	9	202	L	8
168	Y	9	207	V	8
169	E	9	223	I	8
200	G	9	262	M	8
201	H	9	267	F	8
206	G	9	288	V	8
265	P	9	289	D	8
284	G	9	292	Q	8
304	G	9	294	S	8
305	I	9	315	T	8
322	P	9	318	P	8
323	K	9	325	G	8
324	T	9	326	Y	8
328	I	9	327	M	8
331	M	9	329	E	8
338	N	9	357	A	8
359	C	9	361	A	8
362	D	9	364	G	8
378	P	9	372	A	8
379	R	9	391	H	8
394	K	9	397	F	8

398	E	9	401	F	8
415	E	9	404	K	8
1	M	8	408	G	8
9	I	8	423	G	8

See the whole conservation results in supplements

7.15~7.20

Rosetta ddg_monomer was used to calculate the ΔΔG of the full sequence saturation mutation of SQR (pdb ID: 3T31).

See the whole ΔΔG calculation results in supplements.

PyMOL was used to screen out the amino acid residues around active sites (pdb ID: 3T31).

Table 5. Active Sites of SQR.

Amino acid residues within 5Å around FAD						
7	8	9	10	11	12	13
33	34	35	36	42	43	45
46	76	77	78	104	105	106
107	127	128	160	163	264	267
269	300	301	302	303	320	321
322	323	325	355	356	357	391
Amino acid residues within 5Å around DUQ						
41	43	322	323	353	355	357
368	370	390	391	394	411	413
Amino acid residues within 5Å around H_2S						
159	160	161	162	163	199	319
321	355	356	357	367		

7.21~7.25

The RMSF of each amino acid residue of wild-type SQR protein (pdb ID: 3T31) was calculated by GROMACS at 298K and 333K.

Table 6. Some amino acids with larger Δrmsf

position number	amino acid	rmsf_298K	rmsf_333K	Δrmsf
52	W	0.1227	0.5748	0.4521
418	M	0.4942	0.7946	0.3004
352	W	0.1145	0.3908	0.2763
121	E	0.2938	0.4633	0.1695
195	Y	0.0955	0.2638	0.1683
417	K	0.4073	0.5724	0.1651

406	V	0.1312	0.2861	0.1549
158	A	0.0725	0.2263	0.1538
122	G	0.1619	0.3094	0.1475
178	R	0.1613	0.3082	0.1469
159	S	0.0748	0.2211	0.1463
347	Q	0.1857	0.3307	0.145
177	K	0.1862	0.3289	0.1427
416	F	0.3245	0.4672	0.1427
123	P	0.1407	0.277	0.1363
413	K	0.2371	0.3664	0.1293
43	P	0.1523	0.2802	0.1279
315	P	0.1387	0.2602	0.1215
321	T	0.1036	0.2249	0.1213
346	E	0.282	0.4032	0.1212
372	Q	0.1118	0.2309	0.1191
279	P	0.1694	0.2866	0.1172
47	W	0.1011	0.2183	0.1172
115	P	0.1457	0.2627	0.117
348	T	0.1148	0.227	0.1122
120	H	0.3897	0.4998	0.1101
157	G	0.0864	0.1945	0.1081
313	P	0.1861	0.2892	0.1031
343	R	0.33	0.4315	0.1015
311	T	0.1762	0.2773	0.1011

See the whole RMSF calculation results in supplements.

7.26

We comprehensively considered RMSF value, ΔΔG value of each mutation, screened out key sites, and we give preference to residues in loop structure rather than in helix or sheet. Finally we chose 31 mutations.

Table 7. Selected mutations by I-Mutant 2.0 and Rosetta

E121F	T311I	Q372L	Y195F
E121Y	T311V	Q372I	Q347L
E121L	D118Y	Q372W	E346M
E121W	D118L	Q372V	E346L
E121T	D118W	H120Y	E346T
E121V	E243T	H120C	E346V
C277V	E243L	H120T	K344L
Q86L	E243M	H120L	

7.27

We selected five mutations first and designed whole plasmid PCR primers as follows.

Table 8. Primers first designed

mutation	F-primer(5'-3')	R-primer(5'-3')
D118L	GAATGTACCGGGTTCCCTACCCCATGAAGGCCCGG	CCGGGCCTTCATGGGGTAGGGAACCCGGTACATTC
D118W	GAATGTACCGGGTTCCTGGCCCCATGAAGGCCCGG	CCGGGCCTTCATGGGGCCAGGAACCCGGTACATTC
E121V	CCGATCCCCATGTAGGCCCGGTGCA	TGCACCGGGCCTACATGGGGATCGG
E346T	AGGCCGCAAGGGCACGCAGACCATGGGC	GCCCATGGTCTGCGTGCCCTTGCGGCCT
Q372L	TCGCCTTGCCCCTGTTGAAGCCCCG	CGGGGCTTCAACAGGGGCAAGGCGA

5μL E. coli BL21 with plasmid pET-11a-SQR was cultured into 5mL LB+A medium, and was incubated in the shaker at 37 ° C.

7.28

After the bacteria grew overnight, we extracted the plasmid pET-11a-SQR, and it was used as template for whole plasmid PCR.

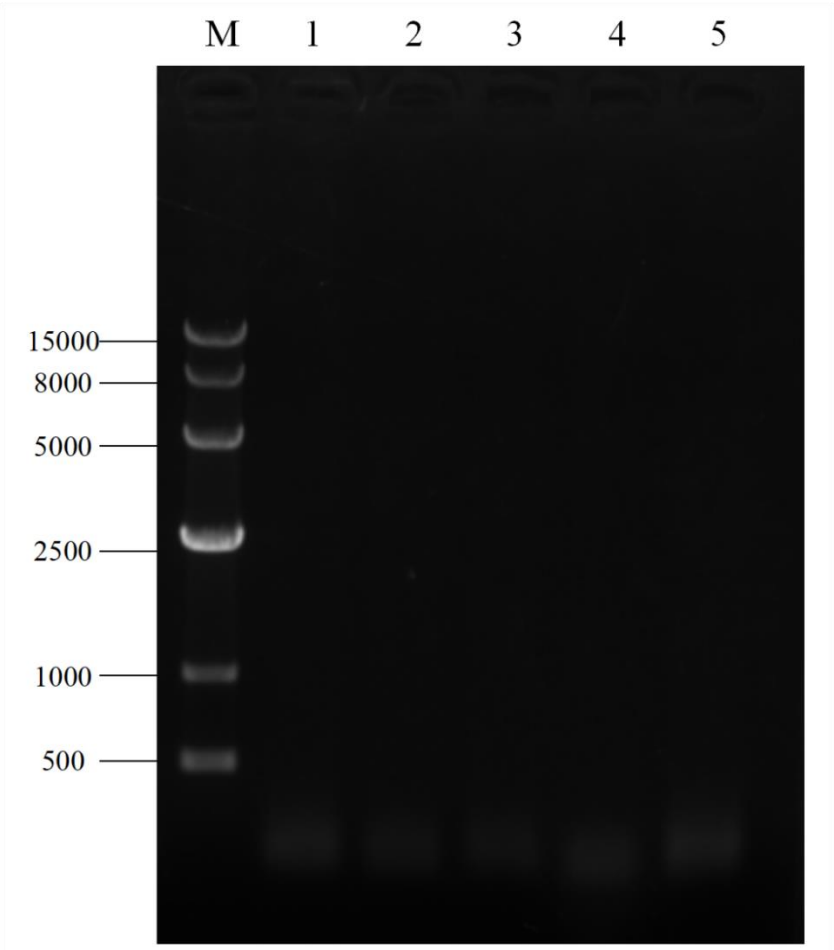


Figure 2. Electrophoresis. M: marker. 1: D118L. 2: D118W. 3: E121V. 4: E346T 5:Q372L

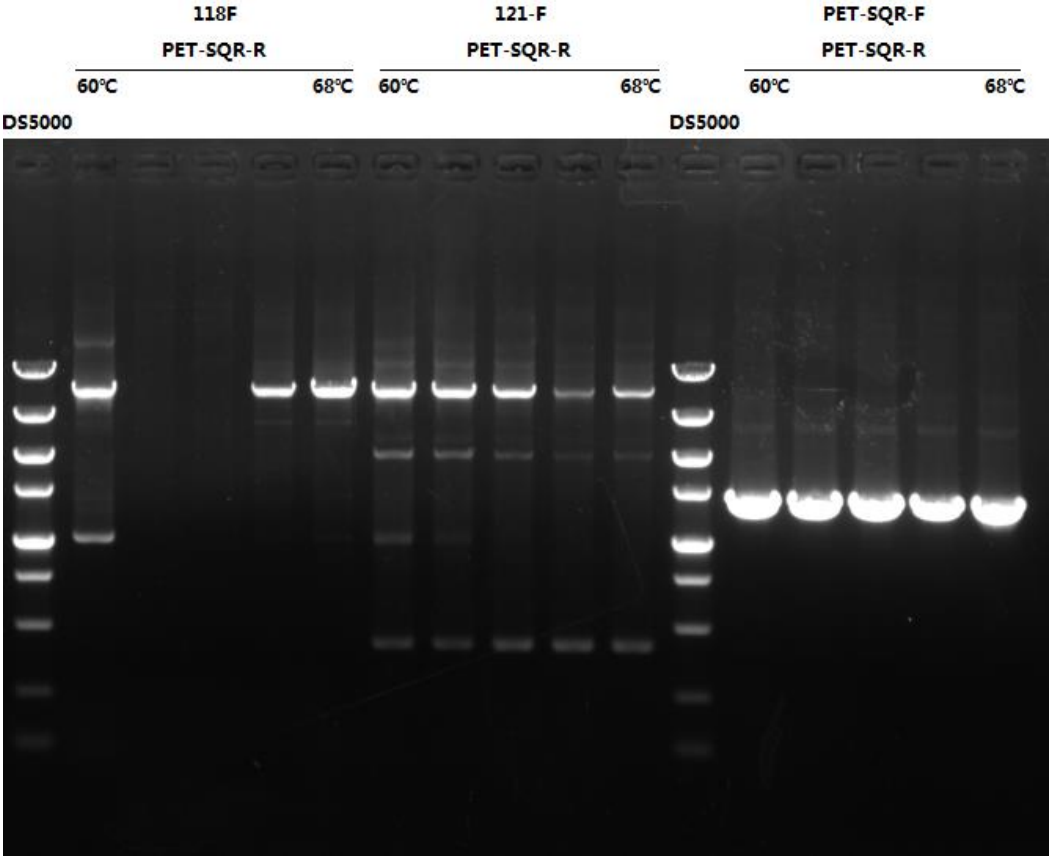
7.29~7.31

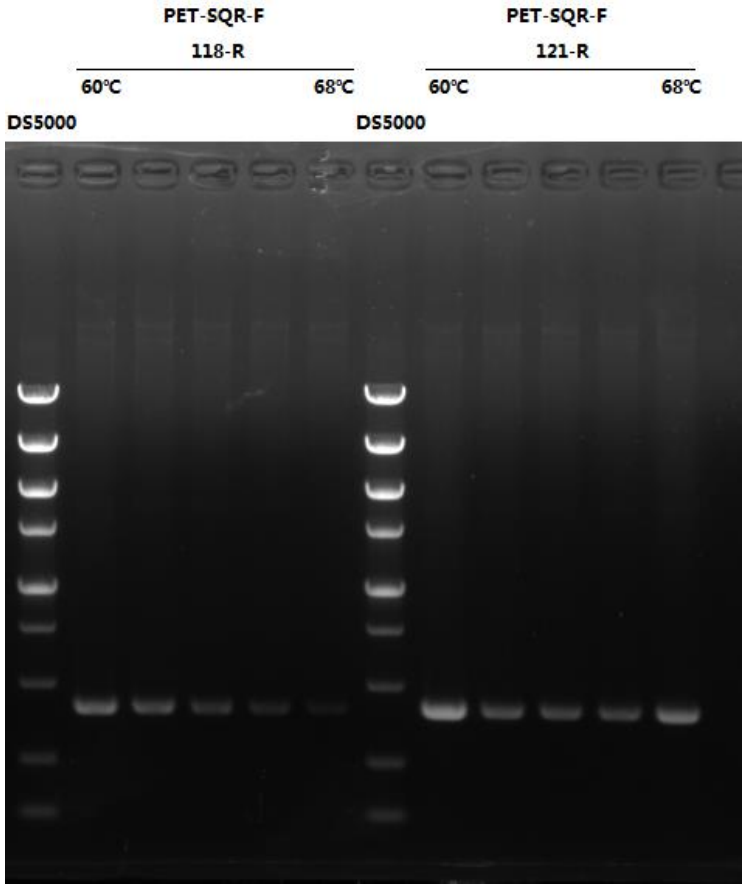
We designed primers before and after the gene.

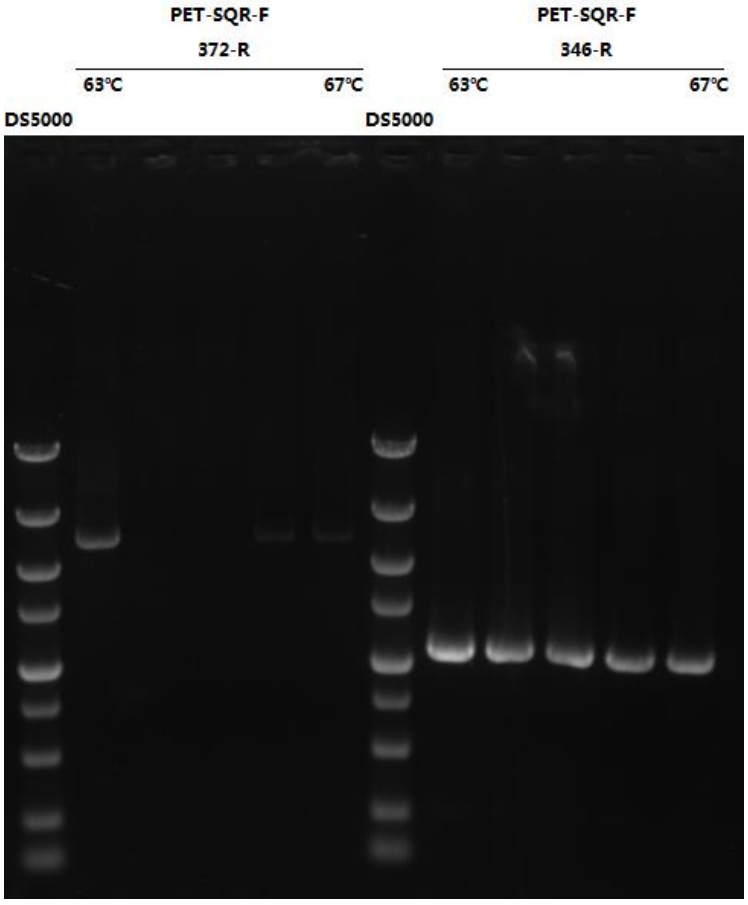
Table 8. Primers before and after the gene

primer	sequence (5'→3')
PET-SQR-F	GTGAGCGGATAACAATTCCCCTCTAGAAGGAGG
PET-SQR-R	CGGGCTTTGTTAGCAGCCGGATCCTC

PCR was performed with primer at one end of the gene and primer at intermediate mutation sites to test whether the primers could combine to the template. And this time, we added 5% DMSO to prevent DNA from forming secondary structures, and we also set a series of annealing temperature gradient.




PET-SQR-F
118-R
60℃
68℃
PET-SQR-F
121-R
60℃
68℃
DS5000
DS5000


PET-SQR-F
372-R
63℃
67℃
PET-SQR-F
346-R
63℃
67℃
DS5000
DS5000

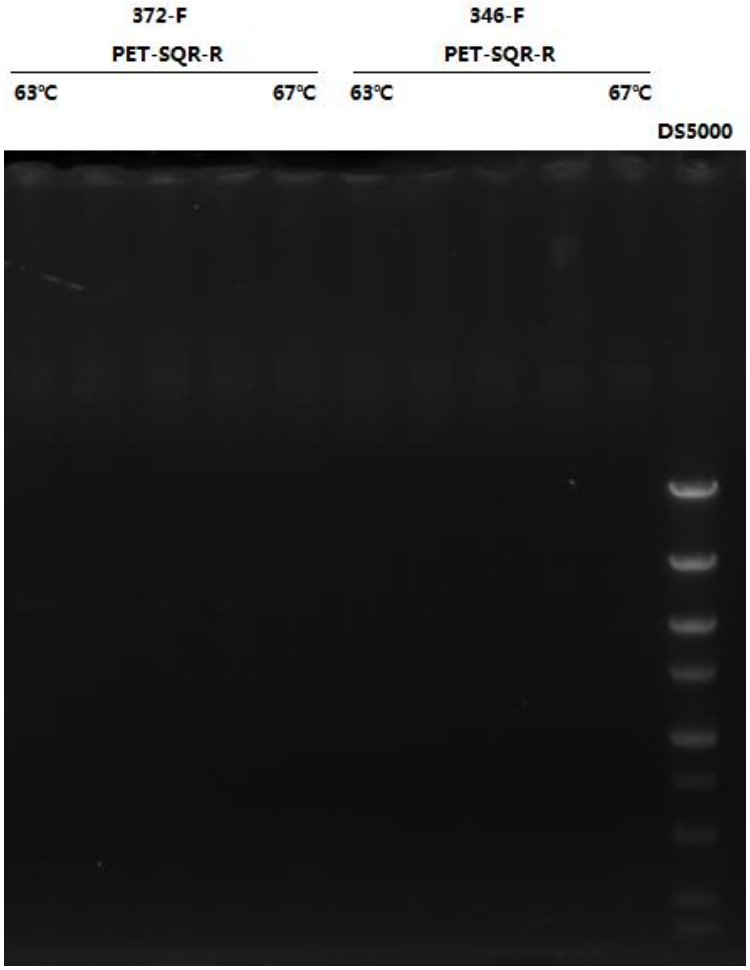


Figure 3. PCR of anterior and posterior segments of *sqr*.

However, the results was still strange.

8.1~8.5

We extracted the plasmid again and sent it for sequencing. Then we found out that the sequence was wrong: we used the original sequence to design primers, but the *sqr* gene in the BL21 in our lab had been codon-optimized.

8.6~8.31

We designed primers based on the correct sequence, and tried to create these mutants. Finally, there were 24 mutants were confirmed by sequencing.

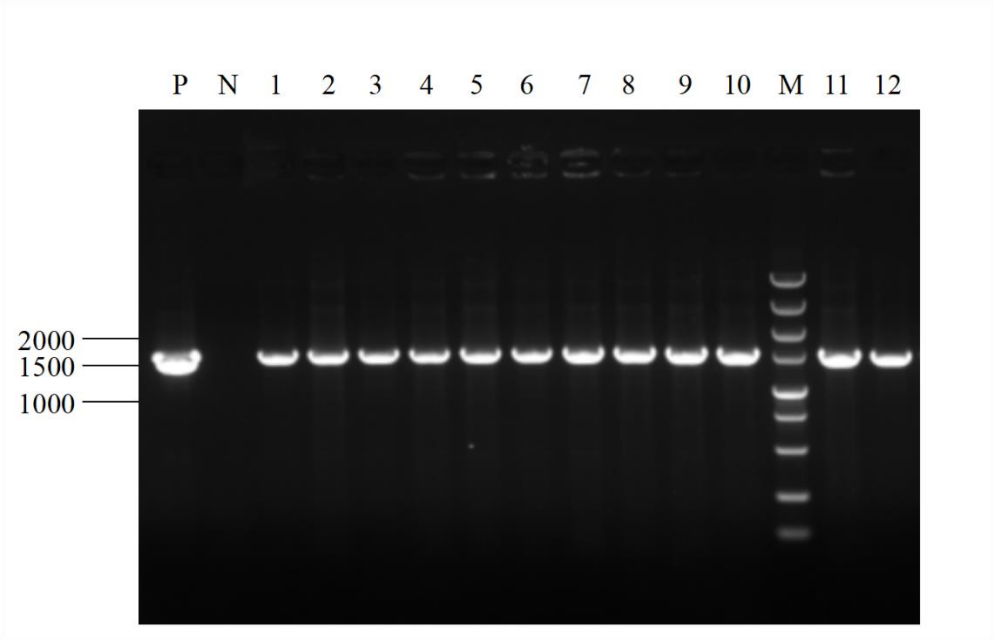
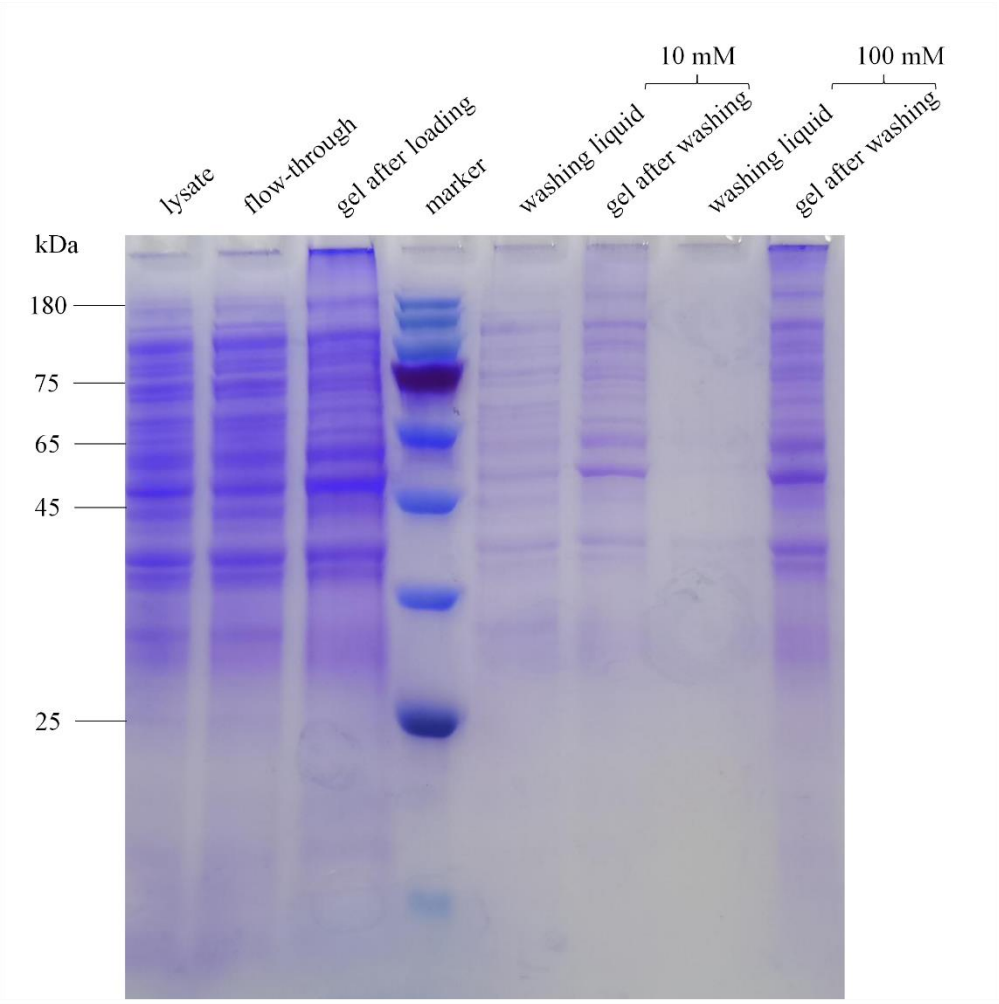
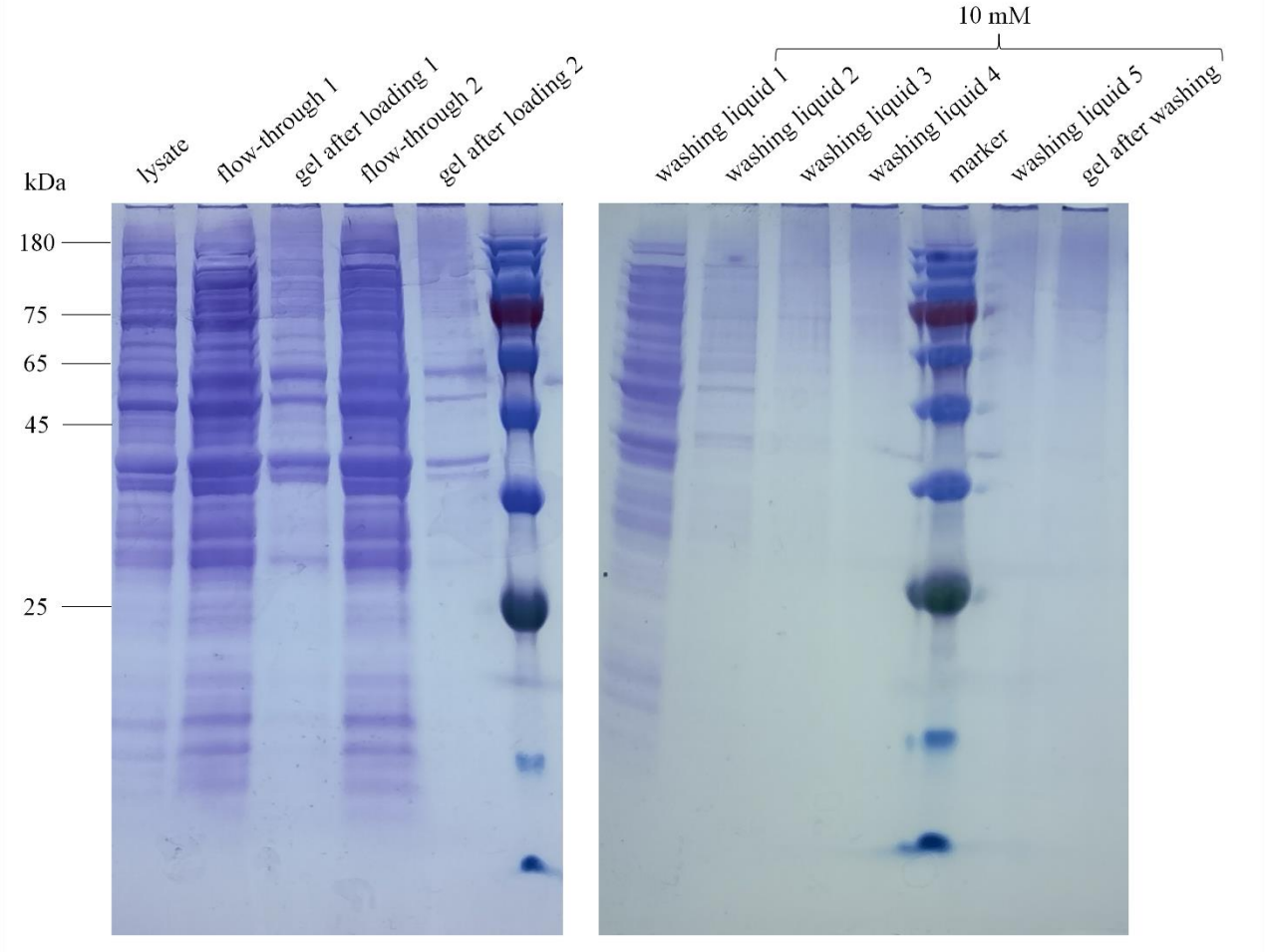


Figure 4. Colony PCR of BL21 P: positive control, N: negative control M: marker, 1~3: T311I, 4~6:T311V, 7~9: Y195F, 10~12: Q347L

9.1~9.26

We did enzyme fermentation and purification many times, but got strange results. Sometimes there were still many proteins left on the nickel column even after washed with 300 mM imidazole, but sometimes they had all been washed down after washed by 10 mM imidazole.


10 mM
100 mM
lysate
flow-through
gel after loading
marker
washing liquid
gel after washing
washing liquid
gel after washing
kDa
180
75
65
45
25


10 mM
lysate
flow-through 1
gel after loading 1
flow-through 2
gel after loading 2
washing liquid 1
washing liquid 2
washing liquid 3
washing liquid 4
marker
washing liquid 5
gel after washing
kDa
180
75
65
45
25