Rational Engineering of *Mesorhizobium* Imine Reductase for Improved Synthesis of *N*-Benzyl Cyclo-tertiary Amines

Zi-Han Zhang, An-Qi Wang, Bao-Di Ma and Yi Xu *

School of Chemical and Environmental Engineering, Shanghai Institute of Technology, Shanghai 201418, China; 206061251@mail.sit.edu.cn (Z.-H.Z.); 226061271@mail.sit.edu.cn (A.-Q.W.); mbd2966@sit.edu.cn (B.-D.M.)

* Correspondence: xuyi@sit.edu.cn; Tel.: +86-21-60873024

Supporting Information

Contents   Page

Figure S1. SDS-PAGE assay of recombinant *Mes*IREDs and GDH. M: protein marker; 1: supernatant of cell extract from *E. coli* (*Mes*IRED-GDH); 2: precipitation of cell extract from *E. coli* (*Mes*IRED-GDH); 3: supernatant of cell extract from *E. coli* (*Mes*IRED$^{V212/I213V}$-GDH); 4: precipitation of cell extract from *E. coli* (*Mes*IRED$^{V212/I213V}$-GDH); 5: supernatant of cell extract from *E. coli* (*Mes*IRED$^{V212A/I177A/A241I}$-GDH); 6: precipitation of cell extract from *E. coli* (*Mes*IRED$^{V212A/I177A/A241I}$-GDH).
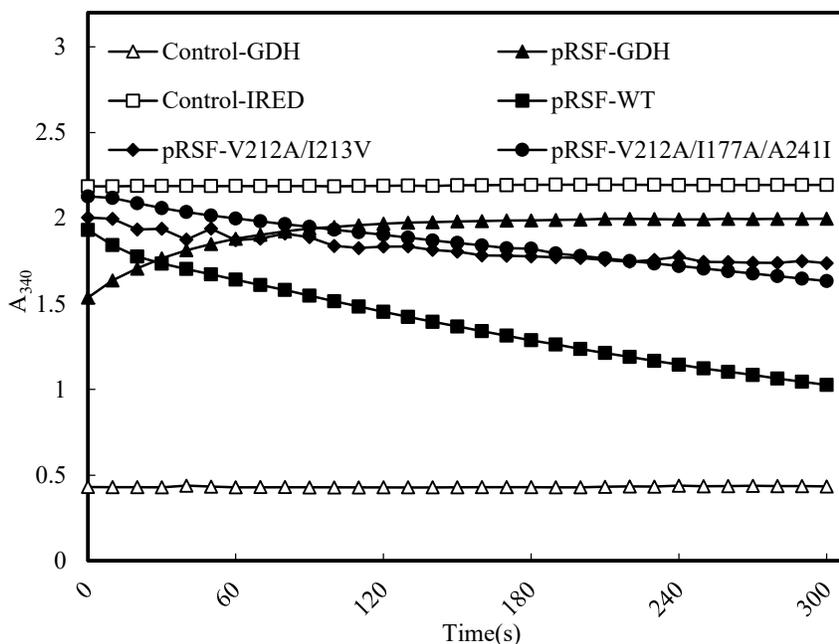
Figure S2. Enzyme activity determination of GDH, *Mes*IRED and *Mes*IRED's mutants. A_{340} over time to NADPH or NADP$^+$ was measured with microplate reader. Conditions for IRED: 15 mM cyclohexanone, 60 mM methylamine, 100 μL cell free lysate, 0.3 mM NADPH. Conditions for GDH: 200 mM glucose, 100 μL cell free lysate, 0.3 mM NADP$^+$. The control used buffer instead of substrate. Reactions were incubated at 25°C, 100 mM pH 9.0 Tris-HCl buffer.
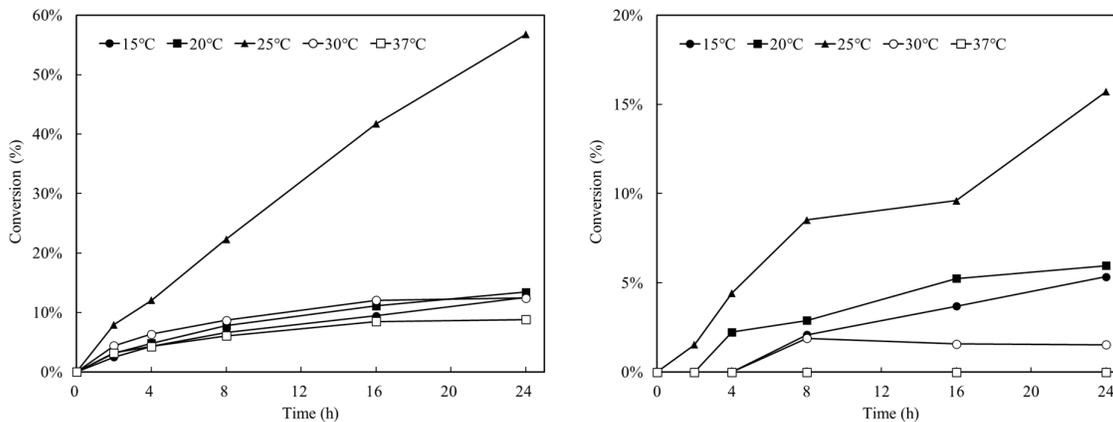
Figure S3. Effect of temperature on the conversion of benzyl alcohol (left) and *N*-benzylpyrrolidine (right) by whole cells of *E. coli* (*Mes*IRED-GDH).
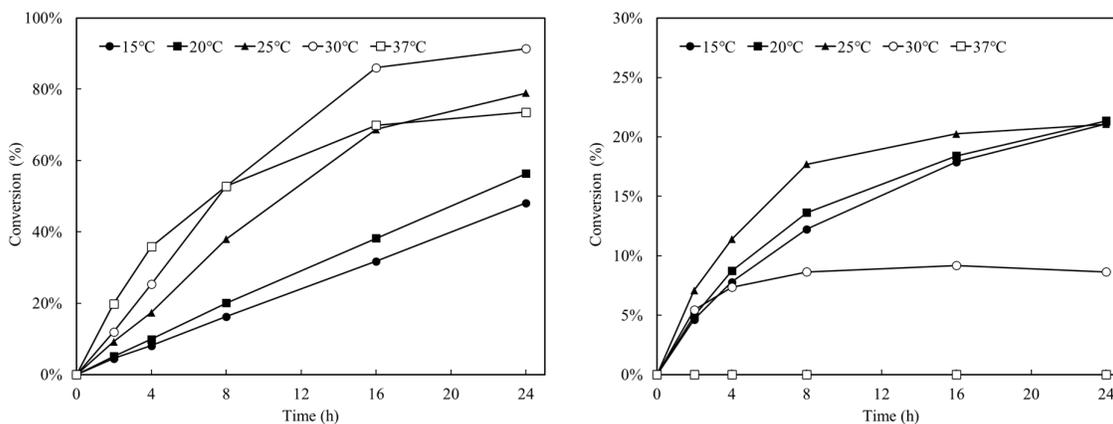
Figure S4. Effect of temperature on the conversion of benzyl alcohol (left) and *N*-benzylpiperidine (right) by whole cells of *E. coli* (*Mes*IRED-GDH).
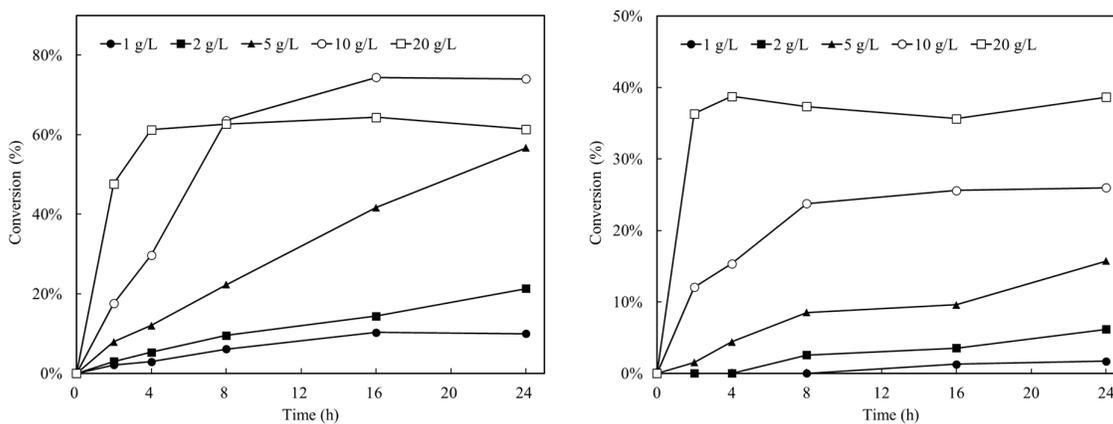
Figure S5. Effect of cell amounts on the conversion of benzyl alcohol (left) and *N*-benzylpyrrolidine (right) by whole cells of *E. coli* (*Mes*IRED-GDH).
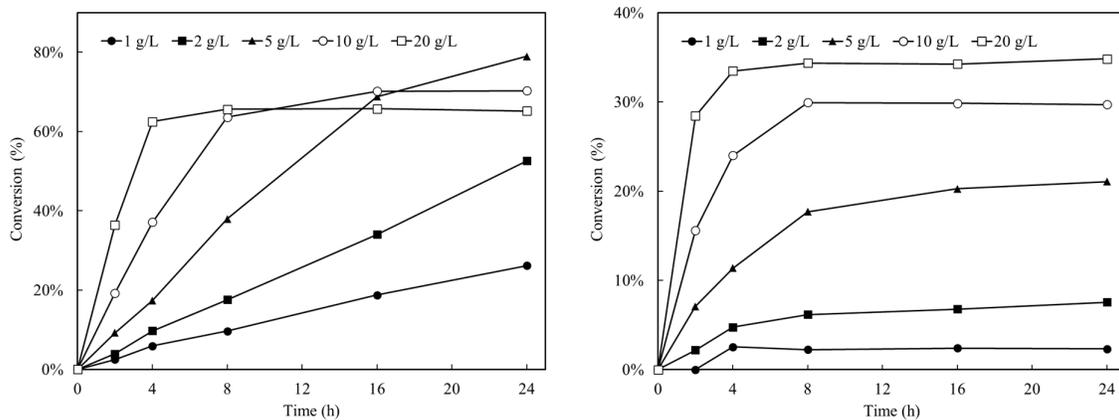
Figure S6. Effect of cell amounts on the conversion of benzyl alcohol (left) and *N*-benzylpiperidine (right) by whole cells of *E. coli* (*Mes*IRED-GDH).
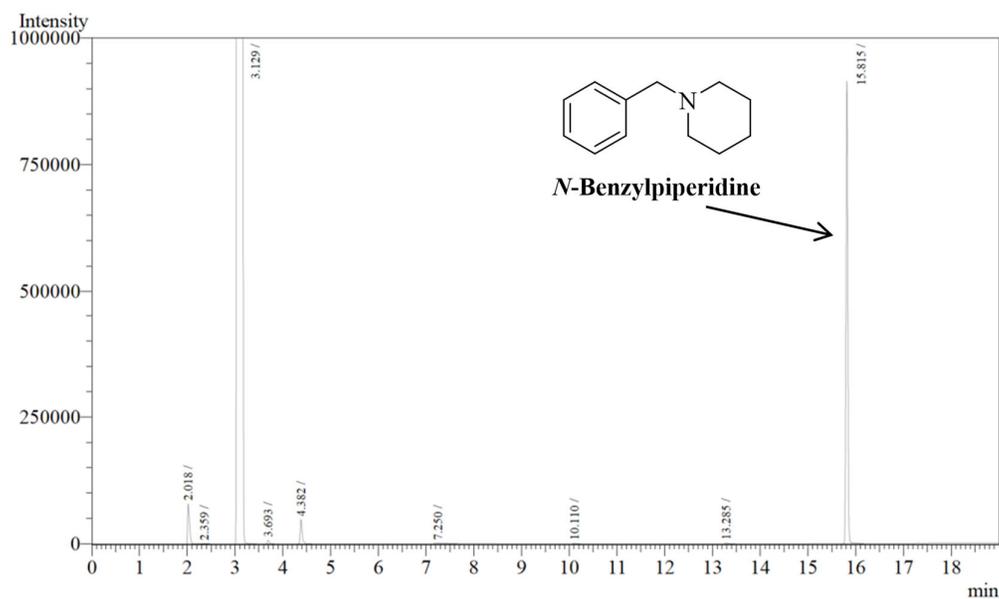
Figure S7. The gas chromatograph of *N*-benzylpiperidine isolated from preparative-scale biotransformation.
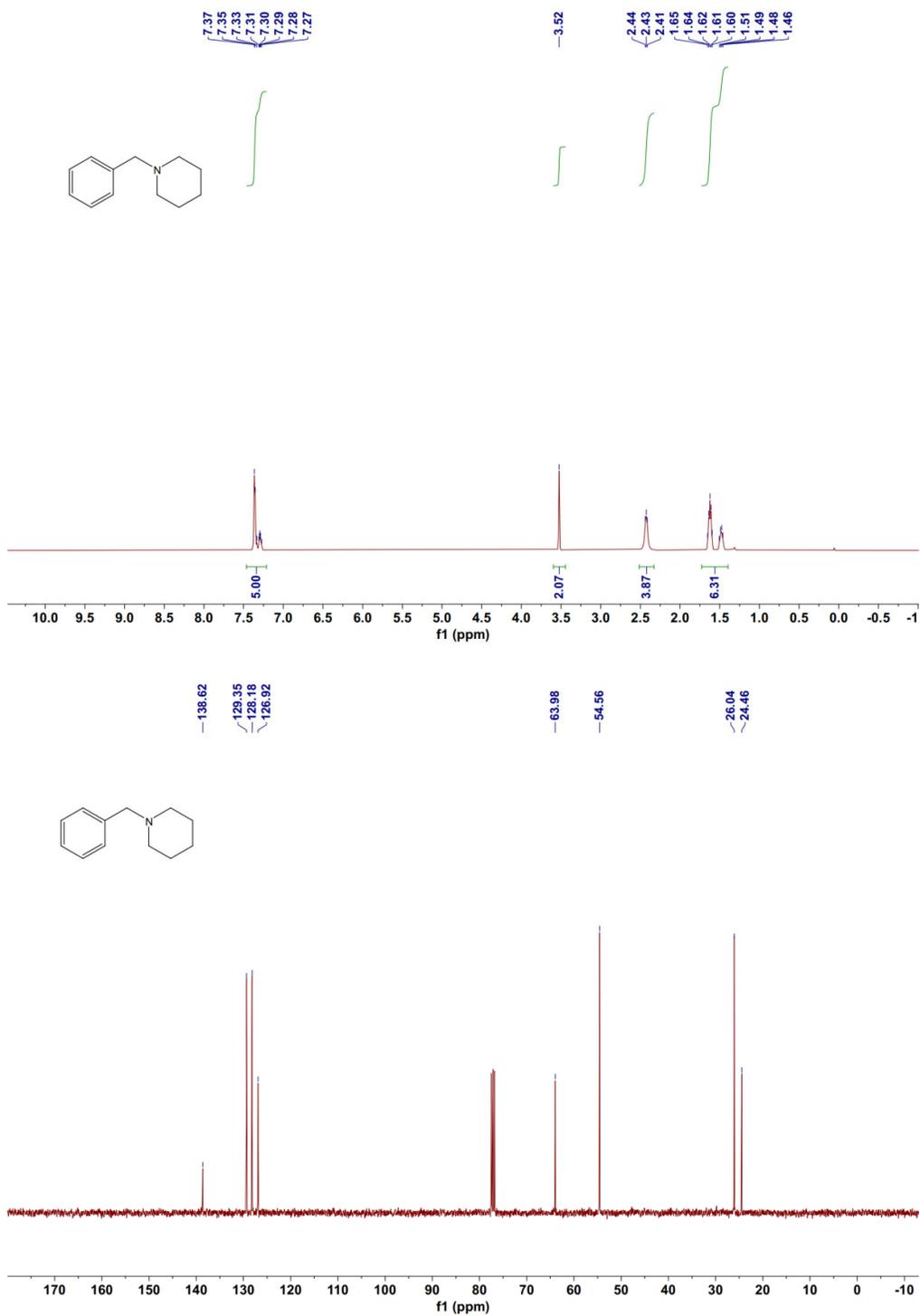
Figure S8. NMR spectrums of *N*-benzylpiperidine isolated from preparative-scale biotransformation.
¹H NMR (400 MHz, CDCl₃): δ 7.37-7.21 (m, 5H), 3.52 (s, 2H), 2.44-2.41 (m, 4H), 1.65-1.46 (m, 6H).
¹³C NMR (100 MHz, CDCl₃): δ 138.62, 129.35, 128.18, 126.92, 63.98, 54.56, 26.04, 24.46.

Table S1. Conversions of benzyl alcohol and *N*-benzyl cyclo-tertiary amines by wild-type and single-site mutants of *Mes*IRED.

1 + **2a-c** → *Mes*IRED → **3** + **4a-c**

NADPH / NADP⁺ ; D-Gluconic acid ← GDH ← D-Glucose ; n=0-1; X=NH or CH₂

	IREDs	Conversion (%)			
		3	3, 4a	3, 4b	3, 4c
	WT	>99.9	76.3, 23.7	77.2, 22.8	3.1, 0.0
Alanine Scanning	M121A	>99.9	>99.9, 0.0	93.9, 0.0	0.0, 0.0
	D171A	>99.9	>99.9, 0.0	>99.9, 0.0	2.9, 0.0
	L174A	>99.9	69.1, 30.9	84.7, 15.3	0.5, 0.0
	L175A	>99.9	>99.9, 0.0	>99.9, 0.0	1.2, 0.0
	I177A	>99.9	43.2, 56.8	62.2, 37.8	1.3, 0.0
	M178A	>99.9	55.9, 44.1	60.8, 39.2	5.5, 0.0
	T209A	>99.9	58.5, 41.5	79.7, 20.3	3.2, 0.0
	V212A	>99.9	40.8, 59.2	54.7, 45.3	0.0, 0.0
	I213A	>99.9	53.1, 46.9	70.1, 29.9	2.4, 0.0
	L216A	>99.9	51.6, 48.4	86.4, 13.6	1.3, 0.0
Conserved but Different Sites	I120M	>99.9	88.9, 11.1	82.4, 17.6	15.3, 0.0
	M121L	>99.9	58.9, 41.1	77.6, 22.4	32.0, 0.0
	L174V	>99.9	71.6, 28.4	78.6, 21.4	19.9, 0.0
	I177L	>99.9	58.1, 41.9	75.5, 24.5	5.1, 0.0
	I177V	>99.9	65.6, 34.4	74.0, 26.0	0.0, 0.0
	T209L	>99.9	62.7, 37.3	51.5, 48.5	2.6, 0.0
	I213V	>99.9	60.2, 39.8	72.6, 27.4	1.9, 0.0
	L216V	>99.9	50.9, 49.1	69.1, 30.9	1.9, 0.0
	A241T	>99.9	65.2, 34.8	70.0, 30.0	16.6, 0.0
	A241I	>99.9	60.3, 39.7	63.1, 36.9	11.2, 0.0
	A241P	>99.9	60.8, 39.2	74.3, 25.7	6.1, 0.0
	A241V	>99.9	59.4, 40.6	80.8, 19.2	7.0, 0.0
	A245S	>99.9	83.0, 17.0	89.1, 10.9	0.0, 0.0

Table S2. Primers for site-directed mutangenesis.

Primers	Sequences(5' to 3')	GC/%	Tm/°C
I120M-F	TTAGATGGTGCTATGATGGCAACCCCGGATT	48	62
I120M-R	TATAATCCGGGGGTTGCCATCATAGCACCAT	47	59
M121L-F	TTAGATGGTGCTATCCTGGCAACCCCGGATT	52	61
M121L-R	TATAATCCGGGGGTTGCCAGGATAGCACCAT	50	62
L174V-F	GCCTTAGATAGCGCTGTACTGGCCATTATG	50	61
L174V-R	CCCCACATAATGGCCAGTACAGCGCTATC	55	61
I177L-F	CGCTTTACTGGCCCTTATGTGGGGCGC	63	63
I177L-R	CCCACATAAGGGCCAGTAAAGCGCTATC	54	61
I177V-F	CGCTTTACTGGCCGTTATGTGGGGCGC	63	65
I177V-R	CCCACATAACGGCCAGTAAAGCGCTATC	54	59
T209L-F	GGAGTGCTCTGGCTCCAGTTATCGATGG	57	58
T209L-R	CGATAACTGGAGCCAGAGCACTCCACTGGC	60	61
I213V-F	GGCTCCAGTTGTCGATGGCTTAGTGACGG	59	63
I213V-R	CTAAGCCATCGACAACTGGAGCCGTAGC	57	61
L216V-F	GGCTCCAGTTATCGATGGCGTAGTGACGG	59	62
L216V-R	GATCAGATCCGTCACTACGCCATCGATAACTG	50	60
A241T-F	GAGTAGTATCTCAACACATCATGGCGCAATGCAG	47	62
A241T-R	GCGCCATGATGTGTGTTGAGATACTACTCAGGG	52	63
A241I-F	GAGTAGTATCTCAATACATCATGGCGCAATGCAG	44	59
A241I-R	GCGCCATGATGTATTGAGATACTACTCAGGG	48	58
A241P-F	GAGTAGTATCTCACCACATCATGGCGCAATGCAG	50	61
A241P-R	GCGCCATGATGTGGTGAGATACTACTCAGGG	55	64
A241V-F	GAGTAGTATCTCAGTACATCATGGCGCAATGCAG	44	59
A241V-R	GCGCCATGATGTACTGAGATACTACTCAGGG	48	58
A245S-F	CAGCACATCATGGCTCAATGCAGCATTTAC	47	61
A245S-R	RCTGCATTGAGCCATGATGTGCTGAGATAC	48	60
M121A-F	GGTGCTATCGCGGCAACCCCGGATTATATTGG	56	60
M121A-R	AATCCGGGGGTTGCCGCGATAGCACCATCTAA	55	59
D171A-F	GTGCTAATGCCTTAGCTAGCGCTTTACTGG	50	58
D171A-R	ATAATGGCCAGTAAAGCGCTAGCTAAGGCATTA	42	61
L174A-F	CTTAGATAGCGCTGCACTGGCCATTATGTGGGGC	56	61
L174A-R	CCACATAATGGCCAGTGCAGCGCTATCTAAGG	53	58
L175A-F	CTTAGATAGCGCTTTAGCGGCCATTATGTGGGGC	53	59

Name	Sequence		
L175A-R	ACATAATGGCCGCTAAAGCGCTATCTAAGGCATTAGC	46	59
I177A-F	CGCTTTACTGGCCGCTATGTGGGGCGC	67	60
I177A-R	CCCCACATAGCGGCCAGTAAAGCGCTATC	59	57
M178A-F	GGCCATTGCGTGGGGCGCTCTGTTTGG	67	61
M178A-R	GCGCCCCACGCAATGGCCAGTAAAGCG	67	61
T209A-F	CCAGTGGAGTGCTGCGGCTCC	71	61
T209A-R	CCATCGATAACTGGAGCCGCAGCACTCC	61	62
V212A-F	GGAGTGCTACGGCTCCAGCTATCGATGG	61	62
V212A-R	RCCGTCACTAAGCCATCGATAGCTGGAGCC	59	63
I213A-F	GGCTCCAGTTGCCGATGGCTTAGTGACGG	62	58
I213A-R	CCATCGGCAACTGGAGCCGTAGCACTCC	64	59
L216A-F	CGATGGCGCAGTGACGGATCTGATCAAACGTACAA	51	60
L216A-R	GATCAGATCCGTCACTGCGCCATCGATAACTG	53	57
M121L/I120M-F	TTAGATGGTGCTATGCTGGCAACCCCGGATT	52	64
M121L/I120M-R	TATAATCCGGGGGTTGCCAGCATAGCACCAT	50	60
T209L/I213V-F	GGCTCCAGTTGTCGATGGCTTAGTGACGG	59	64
T209L/I213V-R	CTAAGCCATCGACAACTGGAGCCAGAGC	57	61
T209L/V212A-F	CTCTGGCTCCAGCTATCGATGGCTTAGTGAC	55	60
T209L/V212A-R	CCATCGATAGCTGGAGCCAGAGCACTCC	61	63
T209L/V212G-F	CTCTGGCTCCAGGTATCGATGGCTTAGTGAC	55	63
T209L/V212G-R	CCATCGATACCTGGAGCCAGAGCACTCC	61	61
V212A/I213V-F	GGCTCCAGCTGTCGATGGCTTAGTGACGG	62	64
V212A/I213V-R	CTAAGCCATCGACAGCTGGAGCCGTAGC	61	63
V212A/L216V-F	CGATGGCGTAGTGACGGATCTGATCAAAC	52	60
V212A/L216V-R	GATCCGTCACTACGCCATCGATAGCTGG	57	60
V212A/T209I-F	CAGTGGAGTGCTATCGCTCCAGCTATCGATGGC	58	61
V212A/T209I-R	CGATAGCTGGAGCGATAGCACTCCACTGGCGGGC	65	67
V212A/T209V-F	CAGTGGAGTGCTGTGGCTCCAGCTATCGATGGC	61	64
V212A/T209V-R	CGATAGCTGGAGCCACAGCACTCCACTGGCGGGC	68	68
T209L/L216V/I177A-F	GCTTTACTGGCCGCTATGTGGGGCGCTCTGTTTG	59	62
T209L/L216V/I177A-R	CGCCCCACATAGCGGCCAGTAAAGCGCTATCTAAG	57	62
T209L/L216V/V212A-F	TCTGGCTCCAGCTATCGATGGCGTAGTGAC	57	61
T209L/L216V/V212A-R	CCATCGATAGCTGGAGCCAGAGCACTC	59	61
T209L/L216V/I213V-F	TGGCTCCAGTTGTCGATGGCGTAGTGACG	59	64
T209L/L216V/I213V-R	TACGCCATCGACAACTGGAGCCAGAGCAC	59	64

T209L/L216V/A241I-F	GAGTAGTATCTCA<u>ATA</u>CATCATGGCGCAATGCAG	44	59
T209L/L216V/A241I-R	GCGCCATGATG<u>TAT</u>TGAGATACTACTCAGGG	48	58
V212A/I177A/L216V-F	GCTATCGATGGC<u>GTA</u>GTGACGGATCTGATC	53	60
V212A/I177A/L216V-R	TCCGTCAC<u>TAC</u>GCCATCGATAGCTGGAG	57	60
V212A/I177A/T209L-F	AGTGGAGTGCT<u>CTG</u>GCTCCAGCTATCGATGG	58	62
V212A/I177A/T209L-R	TAGCTGGAGC<u>CAG</u>AGCACTCCACTGGCG	64	61
V212A/I177A/A241I-F	GAGTAGTATCTCA<u>ATA</u>CATCATGGCGCAATGCAG	44	59
V212A/I177A/A241I-R	GCGCCATGATG<u>TAT</u>TGAGATACTACTCAGGG	48	58
V212A/I213V/I177A-F	GCTTTACTGGCC<u>GCT</u>ATGTGGGGCGCTCTGTTTG	59	62
V212A/I213V/I177A-R	CGCCCCACAT<u>AGC</u>GGCCAGTAAAGCGCTATCTAAG	57	62
V212A/I213V/L216V-F	GCTGTCGATGGC<u>GTA</u>GTGACGGATCTGATC	57	62
V212A/I213V/L216V-R	ATCCGTCAC<u>TAC</u>GCCATCGACAGCTGG	59	62
V212A/I213V/T209L-F	AGTGGAGTGCT<u>CTG</u>GCTCCAGCTGTCG	63	61
V212A/I213V/T209L-R	AGCTGGAGC<u>CAG</u>AGCACTCCACTGGC	65	60
V212A/I213V/A241I-F	GAGTAGTATCTCA<u>ATA</u>CATCATGGCGCAATGCAG	44	59
V212A/I213V/A241I-R	GCGCCATGATG<u>TAT</u>TGAGATACTACTCAGGG	48	58

*mutated bases were underlined