Supplementary Information

A radical $S$-adenosyl-L-methionine enzyme and a methyltransferase catalyze cyclopropane formation in natural product biosynthesis
1. Supplementary Figures

**Supplementary Figure 1** | The selected enzymatic cyclopropanation strategies in natural product biosynthesis

**Supplementary Figure 2** | Construction and verification of the Δc10Q mutant strain S. zelensis TG1405

**Supplementary Figure 3** | Purification and characterization of the radical SAM proteins and methyltransferase

**Supplementary Figure 4** | A cryptic biosynthetic gene cluster from *Shewanella woodyi* ATCC 51908 that shows high homology and synergy with that of CC-1065 from *Streptomyces zelensis* NRRL 11183

**Supplementary Figure 5** | HR-MS and MS/MS analysis of CC-1065 produced by enzymatic reactions

**Supplementary Figure 6** | HR-MS and MS/MS analysis of 7 produced by enzymatic reactions

**Supplementary Figure 7** | Comparison of the $^1$H NMR spectra of substrate 6 and product 7

**Supplementary Figure 8** | Comparison of the H-H COSY spectra of substrate 6 and product 7

**Supplementary Figure 9** | HPLC analysis of the product 7 incubated with C10P and/or C10Q

**Supplementary Figure 10** | Isothermal Titration Calorimetry (ITC) analysis of Swoo_2002 and C10Q

**Supplementary Figure 11** | Effect of different dithionite concentrations on the production of CC-1065 and 7

**Supplementary Figure 12** | Effect of pH on the production of CC-1065 and 7

**Supplementary Figure 13** | C10P/C10Q-catalyzed production of CC-1065 and 7 with different reduction systems

**Supplementary Figure 14** | Effect of the Swoo_2002/C10Q ratio on the production of CC-1065 and 7

**Supplementary Figure 15** | A time-course analysis of C10P/C10Q-catalyzed conversion of 6 into CC-1065 and 7

**Supplementary Figure 16** | Multiple sequence alignment of HemN-like radical SAM enzymes

**Supplementary Figure 17** | HPLC analysis of the substrate 6 incubated with C10Q and Swoo_2002 C57A

**Supplementary Figure 18** | HPLC analysis of the substrate 6 incubated with C10Q and Swoo_2002 mutant variants

**Supplementary Figure 19** | HR-MS analysis of SAH and 5'-dA produced by enzymatic reactions
Supplementary Figure 20 | A time-course analysis of the enzymatic production of 5’-dA, SAH, and 8

Supplementary Figure 21 | HR-MS analysis of 5’-dA and SAH produced in the enzyme assays using CD3-SAM instead of SAM

Supplementary Figure 22 | Detection of SAM in boiled C10P, boiled Swoo_2002 and boiled C10Q

Supplementary Fig. 23 | Multiple sequence alignment of selected methyltransferases identified a variant of the SAM-binding motif DxGxNxG for C10Q and a likely His residue for activation of the methyl acceptor group in the substrate 6

Supplementary Figure 24 | HPLC analysis of the substrate 6 incubated with Swoo_2002 and C10Q mutant

Supplementary Figure 25 | Proposed reaction mechanisms from the intermediate radical 9 to the off-pathway product 7

Supplementary Figure 26 | Sequence similarity network (SSN) analysis of 150 selected methyltransferases

2. Supplementary Tables

Supplementary Table 1 | Homologues of the pair of C10P and C10Q proteins are encoded by many other biosynthetic gene clusters (BGCs)

Supplementary Table 2 | Strains used in this study

Supplementary Table 3 | Plasmids used in this study

Supplementary Table 4 | Primers (shown from 5’ to 3’) used in this study

3. Supplementary References
1. Supplementary Figures

Supplementary Figure 1 | The selected enzymatic cyclopropanation strategies in natural product biosynthesis.

According to the degree of dependence on SAM, these cyclopropanation strategies can be divided into three classes. Class I, a. formation of cyclopropane-containing terpenoids via inter- and intramolecular electrophilic addition; b. biosynthesis of the alkaloid cyclocitrulline through an α-ketoglutarate-dependent, non-heme iron oxygenase EasH-catalyzed oxidative rearrangement (three proposed mechanisms); and c. construction of cyclopropane-containing building blocks for nonribosomal peptides and hybrid nonribosomal peptide-polyketide compounds using halogenated carrier protein-linked intermediates as the substrates for SN2-like cyclopropanation. Class II, formation of cyclopropane fatty acids by cyclopropane fatty acid/mycolic acid synthases that catalyze direct transfer of the reactive one-carbon species from SAM to double bonds involving a mechanism of carbocationic intermediates (or transition states). Class III, biosynthesis of 1-aminocyclopropane-1-carboxylate as a precursor to the plant hormone ethylene, and of the cyclopropane warhead of colibactin through a carbanion-induced intramolecular SN2 reaction mechanism with elimination of methylthioadenosine.
Supplementary Figure 2 | Construction and verification of the Δc10Q mutant strain *S. zelensis* TG1405.

Verification was performed by PCR amplification using the genomic DNA from the mutant or wild type strain as the template. The PCR primers are labeled with their predicted sizes of the resulting products.
Supplementary Figure 3 | Purification and characterization of the radical SAM proteins and methyltransferase.

a, C10P (51.9 kDa); b, Swoo_2002 (51.8 kDa); c, C10Q (29.0 kDa); d, C10P/C10Q from co-expression; and e, ultraviolet-visible (UV-Vis) absorptions of the reconstituted Swoo_2002 (black line) and the protein reduced by sodium dithionite (red line). Proteins C10P and Swoo_2002 were purified under strictly anaerobic conditions and the resulting radical SAM enzymes were reconstituted therein to give dark brownish color. The UV-Vis spectrum exhibited an absorption with A280/A420 ratio of 3.4:1 and an apparent A420 decrease upon reduction by sodium dithionite.
Supplementary Figure 4 | A cryptic biosynthetic gene cluster from *Shewanella woodyi* ATCC 51908 that shows high homology and synergy with that of CC-1065 from *Streptomyces zelensis* NRRL 11183.

C10P shows 67% identity with Swoo_2002.
Supplementary Figure 5 | HR-MS and MS/MS analysis of CC-1065 produced by enzymatic reactions.

a, structure of CC-1065. b, HR-MS analysis of enzymatically produced CC-1065. c, HR-MS/MS analysis of CC-1065.
Supplementary Figure 6 | HR-MS and MS/MS analysis of 7 produced by enzymatic reactions.

a, structure of 7. b, HR-MS analysis of enzymatically produced 7. c, HR-MS/MS analysis of 7.
Supplementary Figure 7 | Comparison of the $^1$H NMR spectra of substrate 6 and product 7.
a. $^1$H NMR (600 MHz, DMSO-$d_6$) spectrum of substrate 6. The signal 6.96 (d, 1H, $J = 2.28$ Hz) is the 11-CH of substrate 6 coupling with the adjacent 12-CH. b. $^1$H NMR (600 MHz, DMSO-$d_6$) spectrum of product 7. The signal of 11-CH is missing while the signal of 12-CH remains, and the extra signal appears at 2.05 ppm corresponding to the 11-CH$_3$ connecting with the C=C (11-C).
Supplementary Figure 8 | Comparison of the H-H COSY spectra of substrate 6 and product 7. a, H-H COSY (600 MHz, DMSO-$d_6$) spectrum of substrate 6. There is an obvious signal of H-H coupling between 11-CH and 12-CH. b, H-H COSY (600 MHz, DMSO-$d_6$) spectrum of product 7. The signal of H-H coupling between 11-CH and 12-CH in substrate 6 is missing.
Supplementary Figure 9 | HPLC analysis of the product 7 incubated with C10P and/or C10Q.

The results show that C10P and C10Q can not convert 7 into CC-1065, which indicates that 7 is not a reaction intermediate but an off-pathway product.
Supplementary Figure 10 | Isothermal Titration Calorimetry (ITC) analysis of Swoo_2002 and C10Q.

The result shows that the Ka value is $1.25 \times 10^5 \text{ M}^{-1}$, indicating that there is a strong protein-protein interaction between Swoo_2002 and C10Q.
Supplementary Figure 11 | Effect of different dithionite concentrations on the production of CC-1065 and 7.

Samples from a to h show the concentrations of dithionite at 100 μM (a), 200 μM (b), 500 μM (c), 1 mM (d), 2 mM (e), 5 mM (f), 10 mM (g) and 20 mM (h), respectively. 5 mM of dithionite is considered as an optimum condition.
Supplementary Figure 12 | Effect of pH on the production of CC-1065 and 7.
Samples from a to d show the different pH at 8.0 (a), 7.5 (b), 7.0 (c), and 6.5 (d), respectively. pH at 8.0 is considered as an optimum condition.
Supplementary Figure 13 | C10P/C10Q-catalyzed production of CC-1065 and 7 with different reduction systems. (a), in the presence of a natural reduction system (flavodoxin, flavodoxin reductase, and NADPH); (b), in the presence of the chemical reductant methyl viologen and NADPH; and (c), in the presence of the chemical reductant dithionite. Dithionite as the reduction system is considered as an optimum condition.
Supplementary Figure 14 | Effect of the Swoo_2002/C10Q ratio on the production of CC-1065 and 7.
Samples from a to e show the ratio of 3:1 (a), 2:1 (b), 1:1 (c), 1:2 (d), and 1:3 (e), respectively. The Swoo_2002/C10Q ratio has no detectable influence on the ratio of product CC-1065 and 7.
Supplementary Figure 15 | A time-course analysis of C10P/C10Q-catalyzed conversion of 6 into CC-1065 and 7.

Samples from a to i show the timing at 10 min (a), 30 min (b); 1 h (c), 2 h (d), 4 h (e), 6 h (f), 8 h (g), 10 h (h), and 12 h (i), respectively. Reaction time of 12 h is considered as an optimum condition.
Supplementary Figure 16 | Multiple sequence alignment of HemN-like radical SAM enzymes.

The selected sequences include HemN from *E. coli* (PDB: 1OLT_A), C10P from the biosynthetic gene cluster of CC-1065, WP_030684647.1 from *Streptomyces* sp. NRRL B-1347, Swoo_2002 from *Shewanella woodii* ATCC 51908, YtkT from the biosynthetic gene cluster of yatakemycin, SHJG_8494 from *Streptomyces hygroscopicus* subsp. jinggangensis 5008, AQJ11_07385 from...
Streptomyces corchorusii strain DSM 40340, and NosN from the biosynthetic gene cluster of nosiheptide. The alignment was carried out using CLUSTAL Omega (1.2.1). The conserved motif CxxxCxxC for binding [4Fe-4S] cluster is marked in green, the conserved glutamine and arginine residues for binding SAM₁ selected for mutation are marked in cyan, and the conserved tyrosine residue for binding SAM₂ selected for mutation is marked in yellow.
Supplementary Figure 17 | HPLC analysis of the substrate 6 incubated with C10Q and Swoo_2002 C57A.

The Cys-57 is one of the conserved cysteines for binding the iron-sulfur cluster in Swoo_2002. The result shows that the Swoo_2002 C57A mutant lost the ability to catalyze the transformation of 6 into CC-1065 and 7.
Supplementary Figure 18 | HPLC analysis of the substrate 6 incubated with C10Q and Swoo_2002 mutant variants.

Gln-161 and Arg-173 are two of the conserved residues for binding SAM1 and Tyr-44 is one of the conserved residues for binding SAM2 in Swoo_2002. The results show that Swoo_2002 mutants without SAM1 or SAM2 binding site lost the ability to catalyze the transformation of 6 into CC-1065 and 7.
Supplementary Figure 19 | HR-MS analysis of SAH and 5'-dA produced by enzymatic reactions.

a, HR-MS analysis of enzymatically produced SAH. b, HR-MS analysis of enzymatically produced 5'-dA.
Supplementary Figure 20 | A time-course analysis of the enzymatic production of 5’-dA, SAH, and 8.

a, the profile of the production of 5’-dA and SAH as the reaction proceeded. b, the profile of the production of the critical intermediate 8 as the reaction proceeded. The detection wavelength is 260 nm.
Supplementary Figure 21 | HR-MS analysis of 5’-dA and SAH produced in the enzyme assays using CD$_3$-SAM instead of SAM.

a, the majority of product 5’-dA shows a mass shift of +1 m/z and the minority remains unchanged.
b, the molecular mass of product SAH remains unchanged.
Supplementary Figure 22 | Detection of SAM in boiled C10P, boiled Swoo_2002 and boiled C10Q.

a. boiled C10P, b. boiled Swoo_2002, c. boiled C10Q and d. standard SAM. SAM is detected in boiled C10P and Swoo_2002, but not detected in boiled C10Q.
C10Q       -------------------------------------------MTTEAPLLDLAE-RVPL 16
DnrK       DRLSPQ---AVADKLNSPL------RSVEQMLIALRAMYLDQ----------GECYHL 76
MmcR       PRTA--------TALAEATGA---HEQTLRLLRLATVGFDDL-------GHDLDFAQ 92
ChOMT      TPPGAFXS---IPSKPASTQHSDLPNRLDRXLRLASYSVLTSTTTR1EDGARVLY 107
IOMT       ------------MASSINGRKPSEIFKAQALLYKHIYAFIDSMSLKWAVEMNIPNIIQNH 48
6OMT       ---------------GAMVMINKENLSQAKLWNTYGFADSLVLKSAVQLDLANI1HNIH 45
C10Q       -------------------------------------------MTTEAPLLDLAE-RVPL 16
DnrK       DRLSPQ---AVADKLNSPL------RSVEQMLIALRAMYLDQ----------GECYHL 76
MmcR       PRTA--------TALAEATGA---HEQTLRLLRLATVGFDDL-------GHDLDFAQ 92
ChOMT      TPPGAFXS---IPSKPASTQHSDLPNRLDRXLRLASYSVLTSTTTR1EDGARVLY 107
IOMT       ------------MASSINGRKPSEIFKAQALLYKHIYAFIDSMSLKWAVEMNIPNIIQNH 48
6OMT       ---------------GAMVMINKENLSQAKLWNTYGFADSLVLKSAVQLDLANI1HNIH 45
Supplementary Fig. 23 | Multiple sequence alignment of selected methyltransferases identified a variant of the SAM-binding motif DxGxNxG for C10Q and a likely His residue for activation of the methyl acceptor group in the substrate 6.

The sequences used are C10Q from the biosynthetic gene cluster of CC-1065, DnrK for carminomycin O-methyltransferase from Streptomyces peucetius, MmcR for Mitomycin 7-O-methyltransferase from Streptomyces lavendulae, IOMT for isoflavone-O-methyltransferase from Medicago sativa, ChOMT for Chalcone O-Methyltransferase from Medicago sativa, and 6OMT for (S)-norcoclaurine 6-O-methyltransferase from Thalictrum flavum subsp. Glaucum. Sequences are aligned using CLUSTAL Omega (1.2.1), the conserved motif DxGxGxG for binding SAM are marked in green (in C10Q the conserved motif is DxGxNxG instead), and the conserved histidine (His) residue proposed to deprotonate the substrate hydroxyl group is marked in yellow.
Supplementary Figure 24 | HPLC analysis of the substrate 6 incubated with Swoo_2002 and C10Q mutant.
Asp-61 is one of the conserved amino acid residues for binding SAM in C10Q. The result shows that C10Q mutant without the SAM binding site lost the ability to catalyze the transformation of 6 into CC-1065 and 7.
Supplementary Figure 25 | Proposed reaction mechanisms from the intermediate radical 9 to the off-pathway product 7.

In pathway a, the carbon-centered radical at C-12 in 9 abstracts a solvent-exchangeable proton to produce the intermediate 8. The intermediate 8 may be non-enzymatically converted to the intermediate 10 containing an exocyclic double bond via release of SAH, followed by rapid and thermodynamic driving isomerization to give a methylated off-pathway compound 7. Pathway b is an alternative proposal for the conversion from 9 to 7, that is, the carbon-centered radical at C-12 in 9 triggers the removal of a proton at C-11 by an unknown base with elimination of SAH, and then the formed allylic radical is quenched at the C-11M position. However, pathway b is in conflict with our labeling experiments result that the D atom in the produced D-7 is either located in the C-12 or C-11M position but not merely in C-11M position when using D₂O instead of H₂O in the enzymatic assay.
Supplementary Figure 26 | Sequence similarity network (SSN) analysis of 150 selected methyltransferases.

The 150 sequences including O-methyltransferases (contain the conserved D\textsubscript{x}G\textsubscript{x}G\textsubscript{x}G for SAM-binding and the conserved histidine (His) residue for activating the hydroxy group), C-methyltransferases, N-methyltransferases, and methyltransferases from bacterial strains containing the homologs of C10P and C10Q. Sequences were used for the construction of sequence similarity network (SSN) from the website (http://efi.igb.illinois.edu/efi-est/stepa.php)\textsuperscript{1}. The network parameters used were: (1) E-value, 5; (2) fraction, 1; and (3) alignment score, 30.
## 2. Supplementary Tables

**Supplementary Table 1** | Homologues of the pair of C10P and C10Q proteins are encoded by many other biosynthetic gene clusters (BGCs).

| GeneBank accession number | Open reading frames (ORFs) | ORF corresponding to C10P | ORF corresponding to C10Q | Product |
|---------------------------|---------------------------|--------------------------|--------------------------|---------|
| JF429418                  | From ytkA to ytkX         | YtkT                     | YtkU                     | Yatakemycin |
| NZ_JOJM01000053.1         | From 32187 to 60425 bp    | WP_030684647.1           | WP_030684646.1           | Gilvusmycin |
| NZ_KQ948212.1             | From 11372 to 40755 bp    | WP_067124173.1           | WP_067124175.1           | Gilvusmycin |
| NC_010506                 | From Swoo_1990 to Swoo_2028 | Swoo_2002              | Swoo_2001              | Unknown |
| NC_017765                 | From SHJG_8481 to SHJG_8515 | SHJG_8494              | SHJG_8495              | Unknown |
| LMWP01000006              | From AQJ11_07275 to AQJ11_07450 | AQJ11_07385         | AQJ11_07380         | Unknown |
| NZ_KB891296.1             | From 958720 to 989508 bp  | WP_018510354.1           | WP_018510355.1           | Potential producer of CC-1065 |
| NZ_CP015098.1             | From 269837 to 299239 bp  | WP_062924735.1           | WP_062924734.1           | Potential producer of CC-1065 |
| NZ_KB898279.1             | From 26872 to 56268 bp    | WP_018891269.1           | WP_018891268.1           | Potential producer of CC-1065 |
| NZ_JOJB01000017.1         | From 77503 to 106904 bp   | WP_030847869.1           | WP_030847876.1           | Potential producer of CC-1065 |
| NZ_KI911520.1             | From 163217 to 192616 bp  | WP_027735188.1           | WP_027735189.1           | Potential producer of CC-1065 |
| Accession Number | Method/Region | Genes | Potential Producer |
|------------------|---------------|-------|--------------------|
| NZ_JOAW01000206.1 | Discontinuous sequencing | WP_031106456.1 | WP_031106457.1 | Potential producer of CC-1065 |
| NZ_JOAW01000693.1 | From 26856 to 56252 bp | WP_017946733.1 | WP_017946732.1 | Potential producer of CC-1065 |
| NZ_JOAW01000131.1 | From 1889 to 31303 bp | OSC71384.1 | OSC71383.1 | Potential producer of CC-1065 |

The BGC of yatakemycin has been previously cloned in our group\(^2\). The gilvusmycin has been connected with two potential bacterial strains\(^2\). Eight of cryptic BGCs have been assigned potential producer of CC-1065\(^3\).
### Supplementary Table 2 | Strains used in this study.

| Strains        | Characteristics                                                                 | Reference   |
|----------------|---------------------------------------------------------------------------------|-------------|
| **Streptomyces** |                                                                                   |             |
| *S. zelensis* NRRL 11183 | Wild type strain, CC-1065 producing                                               | NRRL        |
| *S. zelensis* TG1402 | *C10P* in-frame deletion mutant, CC-1065 non-producing                           | 4           |
| *S. zelensis* TG1405 | *C10Q* in-frame deletion mutant, CC-1065 non-producing                           | This study  |
| *S. zelensis* TG1406 | Mutant TG1405 containing *c10Q* complementary plasmid pTG1406, CC-1065 producing | This study  |
| *S. zelensis* TG1407 | Mutant TG1402 containing *swoo_2002* expression plasmid pTG1407, CC-1065 producing | This study  |
| *S. zelensis* TG1408 | Mutant TG1402 containing *c10P* complementary plasmid pTG1408, CC-1065 producing | This study  |
| *S. lividans* 1326 | Host for gene cluster heterologous expression                                     |             |
| **E. coli**    |                                                                                   |             |
| *E. coli* DH5α | Host for general cloning                                                          | Invitrogen  |
| *E. coli* S17-1 | Donor strain for conjugation between *E. coli* and *Streptomyces*                | 5           |
| *E. coli* BL21(DE3) | Host for protein expression                                                       | Invitrogen  |
| *E. coli* Rosetta(DE3) | Host for protein expression                                                       | Invitrogen  |
| *E. coli* Ro28-P | Rosetta derivative with pTG1409 for expression of *C10P*                           | This study  |
| *E. coli* Ro37-Q | Rosetta derivative with pTG1410 for expression of *C10Q*                          | This study  |
| *E. coli* RoDue-PQ | Rosetta derivative with pTG1411 for co-expression of *C10P/C10Q*                 | This study  |
| *E. coli* RoTB-SW | Rosetta derivative with pTG1412 for producing *Swoo_2002*                         | This study  |
| *E. coli* RoDue-PQm | Rosetta derivative with pTG1413 for producing *C10Q-H138A*                       | This study  |
### Supplementary Table 3 | Plasmids used in this study.

| Plasmids       | Characteristics                                                                 | Reference |
|----------------|---------------------------------------------------------------------------------|-----------|
| pKC1139        | E. coli-Streptomyces shuttle vector for gene inactivation with apramycin resistance |           |
| pSET152        | gene complementary vector                                                        |           |
| pTG1402        | pKC1139 derivative for gene replacement of c10P                                 |           |
| pTG1405        | pKC1139 derivative for gene replacement of c10Q                                  | This study|
| pTG1406        | pSET152 derivative for gene complementary of c10Q                                | This study|
| pTG1407        | pSET152 derivative for gene heterologous complementary of c10P using swoo_2002   | This study|
| pTG1408        | pSET152 derivative for gene complementary of c10P                                | This study|
| pET28a         | vector for expression of protein in E. coli                                       | Novagen   |
| pET37b         | vector for expression of protein in E. coli                                       | Novagen   |
| pRSFDuet       | vector for co-expression of protein in E. coli                                    | Novagen   |
| pRSETB         | vector for expression of protein in E. coli                                       | Novagen   |
| pTG1409        | pET28a derivative for encoding C10P                                              | This study|
| pTG1410        | pET37b derivative for encoding C10Q                                               | This study|
| pTG1411        | pRSFDuet derivative for encoding C10P and C10Q                                   | This study|
| pTG1412        | pRSETB derivative for encoding Swoo_2002                                         | This study|
| pTG1413        | pRSFDuet derivative for encoding C10Q H138A                                      | This study|
**Supplementary Table 4** | Primers (shown from 5’ to 3’) used in this study.

| Primers | Sequence | Usage |
|---------|----------|-------|
| c10Q-L-for | ATAAAGCTTGGCTCCTCGAAACCCA | c10Q in-frame deletion |
| c10Q-L-rev | ATACTAGAGATCCCAGGTCGTAAGGCC | c10Q in-frame deletion |
| c10Q-R-for | ATACTAGAGCCCGGTACTCGCTCGACC | c10Q in-frame deletion |
| c10Q-R-rev | ATAGAATTCCACGAACCTCGGAAGTCAC | c10Q in-frame deletion |
| c10Q-gt-for | CACGGCGTCAACTTCTGG | \( \Delta c10Q \) genotype verification |
| c10Q-gt-rev | TTTGACTCCATCGGACCACC | \( \Delta c10Q \) genotype verification |
| C10P-com-for | GTTTCTAGAGGAGCGACATGAGCATCA | C10P complementation |
| C10P-com-rev | GTTGAATCTTTGTCATGCGCCCTCC | C10P complementation |
| C10Q-com-for | CATATGCTCTCGCTACCGAC | C10Q complementation |
| C10Q-com-rev | GAATTCTCACATGCTACCCGGGT | C10Q complementation |
| swoo_2002-com-for | CATATGCTCTTGATCTGCTGTTA | swoo_2002 complementation |
| swoo_2002-com-rev | GAATTCTACAGCAGTCTGCTTAT | swoo_2002 complementation |
| c10P-for | ATAGAATTCCATATGAGCATCACCACACCAG | expression of C10P |
| c10P-rev | ATAAAGCTTTACTCGAGGTTGTACATGGCACGCCTCAC | expression of C10P |
| c10Q-for | ATAGAATTCTAGGACCACCGTACAGCC | expression of C10Q |
| c10Q-rev | ATAGAATTCTTCTACGCTACCCGCGG | expression of C10Q |
| swoo_2002-for | ATAGAATTCCATATGAGCATCACCACACCAG | expression of swoo_2002 |
| swoo_2002-rev | ATAGAATTCTAAAGCTTTGCTCGAGTTGTGATTTCAA | expression of swoo_2002 |
| c10P-gong-For | ATAGAATTCCATATGAGCATCACCACACCAG | co-expression of C10P/C10Q |
| c10P-gong-Rev | ATAAAGCTTTACTCGAGGTTGTACATGGCACGCCTCAC | co-expression of C10P/C10Q |
| c10Q-gong-For | ATAGAATTCTAGGACCACCGTACAGCC | co-expression of C10P/C10Q |
| c10Q-gong-Rev | ATAAAGCTTTACTGCTACCCGCGG | co-expression of C10P/C10Q |
| c10P-Q H138A for | ATCTGCTACTCTGTCGCTCGGCGCGCTGGC | expression of C10Q H138A |
| c10P-Q H138A rev | TCTCCACGGCCCGCCGCAGCGGACGAGCA | expression of C10Q H138A |

Restriction sites are underlined.
3. Supplementary References

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