A NON-HEME DIIRON MONOOXYGENASE FROM *SALMONELLA TYPHIMURIUM*: BIOCHEMICAL CHARACTERIZATION OF TRNA-MODIFYING MIAE

by

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ABSTRACT

A NON-HEME DIIRON MONOOXYGENASE FROM *SALMONELLA TYPHIMURIUM*: BIOCHEMICAL CHARACTERIZATION OF TRNA-MODIFYING MIAE PROTEIN

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MiaE is a carboxylate bridged, non-heme diiron monooxygenase which catalyzes the O2-dependent hydroxylation of a hypermodified tRNA nucleoside at position 37 (2-methylthio-N-6-isopentenyl adenosine (37)-tRNA) [designated $ms^2i^6A_{37}$] to produce $ms^2io^6A_{37}$. In Salmonella typhimurium, synthesis of $ms^2io^6A_{37}$ requires a multi-step enzyme-catalyzed process that includes two other enzymes, MiaA and MiaB. Therefore, to study the hydroxylation step in this reaction pathway, all the elements within this reaction pathway (tRNA substrate, MiaA, MiaB, and MiaE) are required. In this work, methods for in vitro tRNA synthesis were developed for the biosynthesis of viable MiaA/MiaB/MiaE substrates. Additionally, the enzymes MiaA and MiaB were recombinantly cloned, sequence verified, and demonstrated to express soluble maltose-binding protein (MBP) fusion proteins. Recombinant MiaA and MiaE (cloned previously) were demonstrated to be catalytically active.

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CHAPTER 1

INTRODUCTION

In the cell, transfer RNA (tRNA) is extensively post-transcriptionally modified. At present, more than 100 different modified nucleosides have been identified. These modifications are made to structurally diversify tRNA from the four canonical nucleoside building blocks [adenosine, (A); guanosine, (G); uridine, (U); and cytosine, (C)]. [20] Of these modifications, position 37 of tRNA is frequently modified. [1, 2] In some anaerobes, such as *S. typhimurium*, the extent of A_{37}-modification is believed to regulate aromatic amino acid uptake, enterochelin synthesis, iron transport, and aerobiosis. [7, 20, 21] Furthermore, it is also suggested that modifications in position 37 would stabilize the weak A_{36}-U pairing during translation. [3, 4] As a result, efficiency and fidelity of the anticodon-codon interaction are regulated by these modifications. [5]

Many of the tRNA-modification pathways utilize multiple enzyme-catalyzed chemical transformations to produce hypermodified nucleosides. **Figure 1.1** illustrates a particularly interesting hypermodification pathway which occurs at adenine-37 (A_{37}) within selected tRNA. In the first reaction shown in Scheme I, dimethylallyl (Δ^2-isopentyl) diphosphate tRNA transferase (designated MiaA) catalyzes the substitution of the dimethylallyl group from dimethylallyl pyrophosphate (DMAPP) to the exocyclic N^6-amino nitrogen of A_{37} to yield N^6-isopentenyladenosine (i^6A_{37}) with release of inorganic pyrophosphate. [22, 23] The second enzymatic transformation involves the C-H insertion of a sulfur atom and subsequent methylation of i^6A_{37} at the C2-position to produce 2-methylthio-N^6-isopentyladenosine ($ms^2i^6A_{37}$). The production of $ms^2i^6A_{37}$ is observed in both eukaryotic and bacterial tRNAs that read codons beginning with U except $tRNA_{I.V.}^{Ser}$. [6] The enzyme that catalyzes this transformation (Δ^2-isopentenylpyrophosphate tRNA-adenosine transferase, designated MiaB)

requires S-adenosylmethionine (SAM) as a co-substrate and is a member of the “radical-SAM” iron-sulfur super family of enzymes. [14, 24] In *S. typhimurium*, the $ms^2i^6A_{37}$ is then further modified into 2-methylthio-N^6-(4-hydroxyisopentenyl) adenosine ($ms^2io^6A_{37}$) during oxygen-dependent hydroxylation by the product of MiaE gene (2-methylthio-N-6-isopentenyl adenosine(37)-tRNA monooxygenase), which is the final step in this hypermodification pathway. [7] It has been reported that ΔMiaE knock-out cell strains, in which $ms^2i^6A_{37}$ hydroxylation is blocked, are unable to grow aerobically on the dicarboxylic acids, including succinate, fumarate or malate, of the citric acid cycle. This suggests the hydroxylation status of the isopentenyl group at adenosine 37 can be sensed by *S. typhimurium* and, as a result, may be involved in metabolic regulation of the bacteria, especially the gluconeogenesis process. [5]

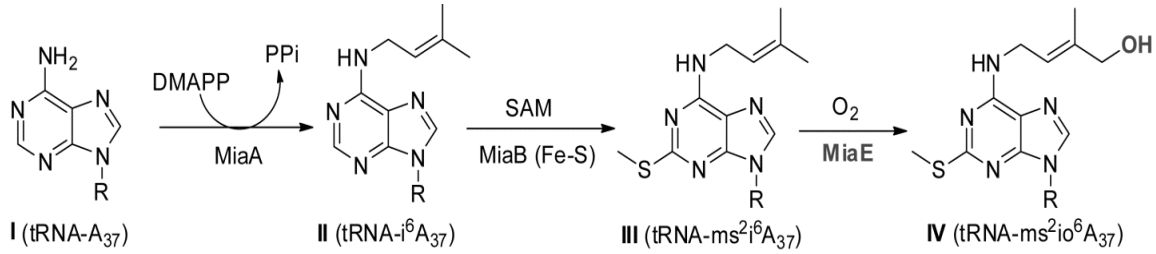


Figure 1.1 Biosynthetic pathway for ms^2io^6A in *S.typhimurium*.

The MiaE protein was demonstrated to be the first monooxygenase from the carboxylate-bridged non-heme diiron enzyme family that specifically targets tRNA. [1, 8] The mechanistic paradigm for this class of enzymes starts with the 2-electron reduction of the resting diferric cluster to produce a diferrous cluster. The diferrous active site can then reductively activate molecular oxygen to produce a high-valent Fe-oxo species capable of substrate (ms^2i^6A) oxidation. [1, 13, 25-26]

The overall goal of this project is to characterize the enzymatic activity and relevant protein-tRNA interactions between MiaE and its native hypermodified tRNA substrates (i^6A_{37}-tRNA and $ms^2i^6A_{37}$-tRNA) which regulate catalysis. As the cloning, expression, and purification of MiaE has already been accomplished, the next logical step is to develop methods for the *in*

vitro synthesis of viable tRNA substrates for MiaE. In this thesis, it was demonstrated that tRNA substrates were able to be synthesized *in vitro* by T7 RNA polymerase. Furthermore, in order to study the ms^2io^6A synthesis pathway, all enzymes involved in this hypomodification pathway are necessary. For steady-state kinetic measurements, MiaA and MiaB were consequently cloned into a T7 inducible expression vector (PF1K). Soluble expression of MiaA and enzymatic activity was confirmed, thus allowing isopentylation of suitable RNA oligomers for characterization of MiaE activity. [19] As to MiaE, it was further demonstrated to be catalytically active in live cell, as well, by another *in vivo* activity assay.

CHAPTER 2

MATERIALS AND METHODS

2.1 MiaE Expression and Purification

The vectors (PVP80K) cloned with MiaE gene were transformed into BL21 (DE3) *E. coli* cells by heat shock. After overnight incubation on LB agar plate with kanamycin (25 mg/L) at 37 °C, isolated colony was then grown in LB/kanamycin media at 37 °C for protein expression. Up on OD_{600} reached 0.6, 0.3 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used to induce maltose binding protein fused MiaE (MBP-MiaE) expression for 4 hours at 25 °C.

Approximately 25 g of cell paste was suspended in a 50 mL of lysis buffer (20 mM HEPES, 40mM NaCl, pH 8.0), and thawed in ice bath with 10 µg/mL each of lysozyme, deoxyribonuclease I, and ribonuclease with gentle stirring for 30 min. The cell suspension was sonicated in a 30 second on/off pulse cycle for a total of 10 min. The resulting cell free extract was centrifuged (JA 20 rotor) at 48,000 × g for 60 min at 4 °C. The supernatant was loaded onto a fast flow DEAE column pre-equilibrated in lysis buffer, and the protein was eluted by NaCl gradient (40 mM to 350 mM NaCl in 20 mM HEPES, 0.3 mM Tris [2-carboxyethyl] phosphine (TCEP), pH 8.0). The fractions containing the MBP-MiaE protein, determined by SDS-PAGE, were concentrated via Amicon N_2 stir cell equipped with an YM 30 ultrafiltration membrane. tobacco etch virus protease (TEV) was used to cleave the fusion protein by overnight storage at 4 °C. The cleaved fusion protein was desalted by dialysis in lysis buffer. To separate MBP from MiaE enzyme, the desalted protein was re-loaded through the DEAE Sepharose column equilibrated in lysis buffer. The protein was eluted on a linear NaCl gradient (0 mM to 350 mM NaCl in 20 mM HEPES, pH 8.0) and fractions containing only MiaE were identified by SDS PAGE.

2.2 *In Vitro* tRNA Transcription

Two DNA oligomers were needed: Primer 5'- TAA TAC GAC TCA CTA TAG -3'; Template of $tRNA^{phe}$ 5'- TGC CCG GAC TCG GAA TCG AAC CAA GGA CAC GGG GAT TTT CAA TCC CCT GCT CTA CCG ACT GAG CTA TCC GGG CTA TAG TGA GTC GTA TTA -3'. For annealing, a mixture of the promoter (25 μM) and template (25 μM) was incubated at 95 °C for 5 min and gradually cooled to 25 °C. Then transcription reaction (**Figure 2.1**) was carried out in 2.5 μM annealed oligonucleotides, 40 mM Tris-HCl, pH 8.0, 22 mM $MgCl_2$, 1 mM spermidine, 5 mM DTT, 0.5% Triton-X100, 4 mM each NTP, 5 mM GMP and 30 nM T7 RNA polymerase for 3 h at 37 °C. 10 μL of transcription reaction were run on a 2.3% agarose gel to check its product. [10]

Figure 2.1 Diagram of *in vitro* tRNA transcription. Red portion: primer (~20nt). Blue portion: tRNA template (~90nt). Black dash portion: promoter, where RNA polymerase binds. Green dash portion: tRNA transcription region.

2.3 Cloning, Expression and Purification of MiaA and MiaB

MiaA gene and MiaB gene were isolated from *Salmonella enterica* strain LT2 genomic DNA purchased from Integrated DNA Technologies. The two-step PCR amplification was carried out to isolate genes of MiaA and MiaB respectively for inserting into pVP80K vectors. The two groups of primers were designed to append restriction enzymes, SgfI and PmeI, sites and TEV protease cleavage site to the protein-coding region. First PCR reaction primers for MiaA: forward 5'- AAC CTG TAC TTC CAG TCC AAT GAT GTA AGC AAG GCG AGC CTG -3'; reverse 5'- GCT CGA ATT CGT TTA AAC TAG TCT GCG ATA GCA CCA ACA ACC -3'. First PCR reaction primers for MiaB: forward 5'- AAC CTG TAC TTC CAG TCC ACT AAA AAA CTC

CAT ATT AAA ACC -3'; reverse GCT CGA ATT CGT TTA AAC TAA GGC TGG TAG AAT CCT ACG CC. Second PCR reaction universal primers (TEV site insertion) for both MiaA and MiaB: forward 5'- GGT TGC GAT CGC CGA AAA CCT GTA CTT CCA GTC C -3'; reverse 5'- GGT TGC GAT CGC CGA AAA CCT GTA CTT CCA GTC C -3'. On the other hand, only one-step PCR amplification was needed for inserting either of them into pF1K vectors. The primers for this one were designed to append restriction enzymes, SgfI and PmeI, sites to the protein-coding region. PCR reaction primers for MiaA: forward 5'- AAA AGC GAT CGC CAT GAA TGA TGT AAG CAA GGC GAG CCT GCC TAA -3'; reverse 5'- AAA AGT TTA AAC TCA GTC TGC GAT AGC ACC AAC AAC CTG TAA TAC TT -3'. PCR reaction primers for MiaB: forward 5'- AAA AGC GAT CGC CAC TAA AAA ACT CCA TAT TAA AAC CTG G -3'; reverse 5'- AAA AGT TTA AAC AGG CTG GTA GAA TCC TAC GCC CAG CTC -3'. After the amplifications, the final PCR products (MiaA, MiaB templates) were purified by PCR purification kit (QIAGEN, 28704) to remove the DNA polymerase and primers. All the purified templates and acceptor vectors (pVP80K, pF1K) were digested with two rare-cutting restriction enzymes, SgfI and PmeI (Promega, R1852). Then digested templates were purified again to remove the small oligonucleotides released by the restriction enzymes. And the purified templates were ligated into the appropriate acceptor vectors by T4 ligase from Promega. After the ligation, all the vectors were transformed into Nova Blue *E.coli* cells, respectively. All the cell transformed with pVP80K based vectors were selected by using overnight incubation on the LB/kanamycin plates with 5% sucrose, while all the cell transformed with pF1K based vectors were selected on the LB/kanamycin plates. The isolated colonies were then incubated separately in 10 mL culture tubes overnight at 37 °C. The cells that show positive test in colony PCR were harvested by centrifuge and the target plasmids were collected from the cell pellets by using mini prep kit (QIAGEN). At this step, only the plasmids that were further confirmed by both vector digestion test and vector PCR test would be conserved for the following protein expression. In addition, sequence verification of MiaA gene was performed by Sequetech DNA Sequencing Service

(http://www.sequetech.com). Sequencing results and recombinant vectors (MIAAK and MIABK) were shown in Appendix.

The resulting plasmid pMIAAK was transformed to BL21(DE3) cells and its isolated colony from overnight incubated LB/kanamycin plate was cultured in LB/kanamycin medias at 37 °C until OD_{600} achieving 0.6. The expression of target protein (MiaA-MBP) was then induced by 0.3 mM IPTG and purified by immobilized metal affinity column (IMAL). The MBP tag was removed from MiaA by TEV cleavage. The expression, purification and TEV cleavage were examined by 12% SDS-PAGE, respectively.

2.4 *In Vivo* MiaE Activity Assay

E.coli BL21(DE3) was transformed with pMHT∆238 (TEV) vector (as a control) and pMIAE80K vector. Cells were grown respectively in LB media with 25 mg/L kanamycin at 37 °C until OD_{600} reached 0.8 and induced by 0.3 mM IPTG. TEV transformed Cells with IPTG induction and MiaE transformed cells with/without IPTG induction were harvested by centrifuge. Total RNA was recovered by phenol extraction and ethanol precipitation as described previously. Briefly, RNA was extracted from the cell free extract solution by addition of an equal volume of phenol: chloroform (5:1). Following vortex mixing, the aqueous phase containing RNA was recovered. This process was repeated 3-times. The pooled aqueous extracts were then mixed with 2.5-volumes of ethanol and stored on ice to precipitate RNA. After 2 hours on ice, the RNA pellet was recovered, dried, and reconstituted in autoclaved MQ H_2O. tRNA was not fractionated from rRNA with $LiCl_2$ washes, because ms^2i^6A and ms^2io^6A are relatively hydrophobic and appear at the end of the elution gradient. [17] Total RNA were digested hydrolyzed by nuclease P1 and then dephosphorylated by alkaline phosphatase. The resulting samples were analyzed by HPLC. [1, 9] To determine the positions of ms^2i^6A and ms^2io^6A, one of our group mates synthesized the ms^2i^6A and made an *in vitro* MiaE assay by using synthetic ms^2io^6A, both of which can be used as the standards for our *in vivo* assay. [17]

2.5 *In Vitro* MiaA-MiaE Assay

This assay comprises two steps: MiaA modification and MiaE modification. The MiaA reaction mixtures (50 µL for each sample) contained TMD buffer (30 mM Tris-HCl (pH 7.5 at 24 °C), 10 mM $MgCl_2$, and 1 mM dithiothreitol (DTT), 100 µg of bovine serum albumin (BSA) per mL, 2.0 mM DMAPP (Sigma), 0.4 µM ASL^{Leu} (IDT) and 11.4 nM MiaA. [11]Reactions were carried out at room temperature and quenched at various times, 0 min, 10 min, 30 min, and 60 min. The MiaE reaction mixtures (50 µL for each sample) contained 0.4 µM ASL^{Leu}, which was obtained from the product of MiaA reaction, 20 µM MiaE, 20mM H_2O_2, in TMD buffer. [1] The reactions were carried out at 37 °C for 0 min, 10 min, 30 min, and 60 min in air. All the ASL^{Leu} samples from both MiaA assay and MiaE assay were digested, respectively, into nucleotides by nuclease P1 and bacteria alkaline phosphatase, and then analyzed by HPLC.

2.6 HPLC Method

The C18 reverse phase column (phenomenex, 150 cm × 4.6 mm) connected to Shimadzu HPLC system was used to analyze the samples. The HPLC elution buffers were as follows: (A) 2.5% methanol in 0.01 M $NH_4H_2PO_4$; PH 5.1, (B) 20% methanol in 0.01 M $NH_4H_2PO_4$; PH 5.3, (C) 35% acetonitrile in 0.01 M $NH_4H_2PO_4$; PH 4.9. The ternary gradient elution program was carried out at room temperature with a flow rate of 1 ml/min as described in **table 2.1** and absorbance was detected at 254 nm. [9]

Table 2.1 the Gradient Elution Program for HPLC

Step (NO.)	Step time (min)	Buffers composition (%) A	B	C	Gradient type
1	12.0	100.0	0.0	0.0	Isocratic
2	8.0	90.0	10.0	0.0	Linear
3	5.0	75.0	25.0	0.0	Linear
4	7.0	40.0	60.0	0.0	Linear
5	4.0	38.0	62.0	0.0	Linear
6	9.0	0.0	100.0	0.0	Linear
7	35.0	0.0	0.0	100.0	Linear
8	10.0	0.0	0.0	100.0	Isocratic

CHAPTER 3

RESULTS

3.1 MiaE Expression and Purification

As indicated in **Figure 3.1**, the IPTG-inducible MBP-MiaE fusion protein exhibits an apparent molecular weight of ~70 kDa as observed by SDS PAGE. Overnight cleavage with TEV protease resulted in two protein bands ~40 kDa and ~25 kDa, for MBP and MiaE, respectively (Figure 3.1, Lane 6). Following buffer exchange by dialysis, MiaE and MBP were easily resolved by a secondary DEAE Sepharose column as shown in (Figure 3.1, Lane 7). The molecular weight of MiaE observed by SDS PAGE is consistent with the expected value based on its amino acid sequence (25 kDa) [1]

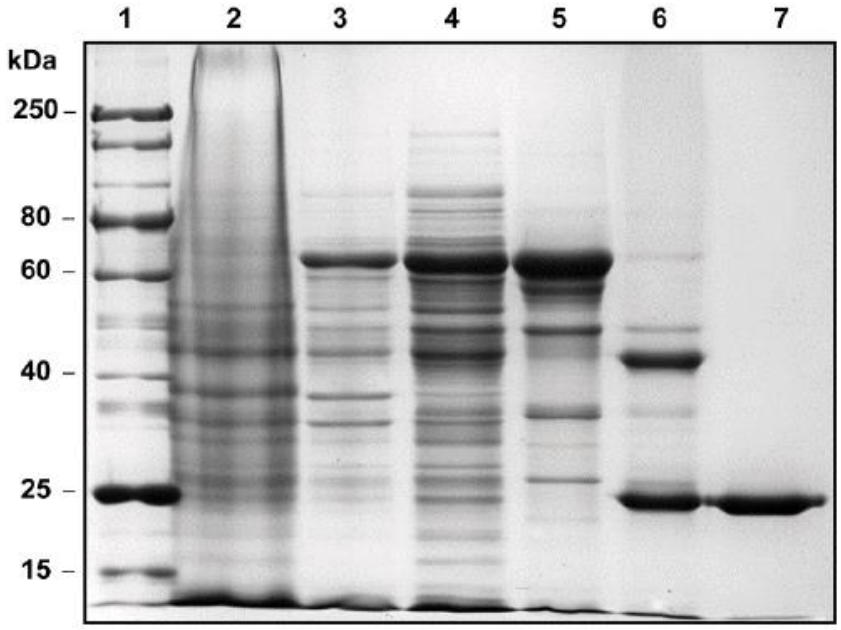


Figure 3.1 SDS PAGE (10%) for MiaE expression and purification. Lane 1, protein markers; Lane 2, before IPTG induction; Lane 3, after IPTG induction; Lane 4, cell free extract; Lane 5,

MBP-MiaE fusion protein isolated from DEAE AX column A, Lane 6, TEV cleaved fusion protein; Lane 7, isolated MiaE following anion exchange DEAE column B.

3.2 *In Vitro* tRNA Transcription.

Shown in **Figure 3.2** is a 2.3% agarose electrophoresis gel demonstrating tRNA molecule synthesis *in vitro*. In lane 6, a band, which width is about two times of the template's, can be seen at ~70bp. Since tRNA (76 bases) and template (~90 bases) cannot be resolved by agarose gel due to similar sizes, the band on last lane should be the mixture of tRNA and template. In addition, a slight shift of this band can also be seen compared to the template band, indicating that the tRNA product and template could be bound together as a duplex. Regardless, the tRNA was clearly successfully synthesized by PCR. This work demonstrates custom a series of custom designed MiaA substrates, which contain a suitable ASL hairpin-loop and an appropriately positioned adenoside ribonucleosides for insertion of the isopentenyl group, would be able to be generated in our lab.

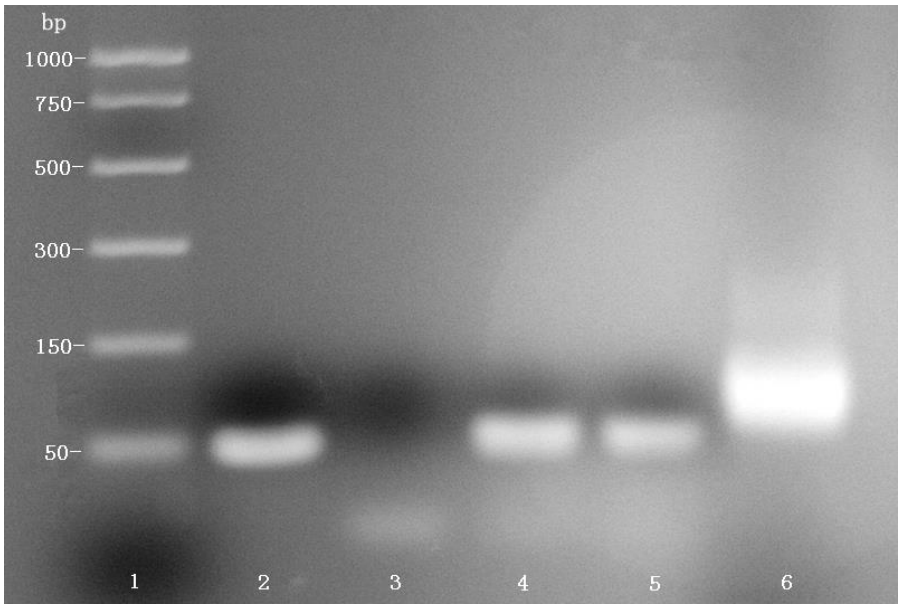


Figure 3.2 2.3% Agarose gel for *in vitro* tRNA transcription. Lane 1, Benchtop PCR markers (promega); Lane 2, tRNA template; Lane 3, primer; Lane 4, tRNA template and primer before annealing; Lane 5, tRNA template and primer after annealing; Lane 6, transcription reaction.

3.3 MiaA and MiaB Cloning

The MiaA was successfully cloned in both pVP80K and pF1K vectors. pVP80K-MiaA expresses recombinant histidine tagged MBP-MiaA fusion protein; pF1K-MiaA, on the other hand, produces untagged MiaA. And eventually, pVP80K based recombinant MiaA vector was chosen to be the default vector for MiaA protein expression due to the reason that, under the same conditions, pF1K-MiaB transformed cells growed much slower than pVP80K transformed cells. SDS-PAGE in **Figure 3.3** shows MBP-MiaA (~73.5kDa) was overexpressed by IPTG induction and this fusion protein was purified by IMAL column (**Figure 3.4**). After TEV cleavage, as shown in **Figure 3.5**, most of the fusion protein was cleaved into MBP (~40 kDa) and MiaA (~33.5 kDa), which indicates that MiaA protein had been successfully obtained in our lab.[17] And its activity was examined in the following MiaA-MiaE activity assay.

While not complete, a similar strategy for cloning MiaA was applied to MiaB. At this point, MiaB has been ligated into both pVP80K and pF1K vectors, however, PCR screening and sequence verification is still required.

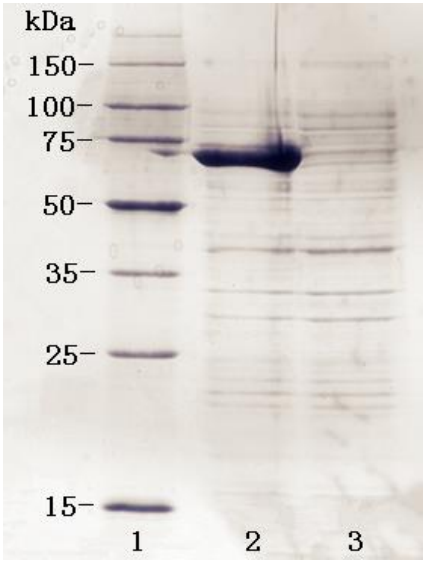


Figure 3.3 12% SDS-PAGE for expression test of MBP-MiaA fusion protein. Lane 1, protein markers; Lane 2, after IPTG induction; Lane 3, before IPTG induction.

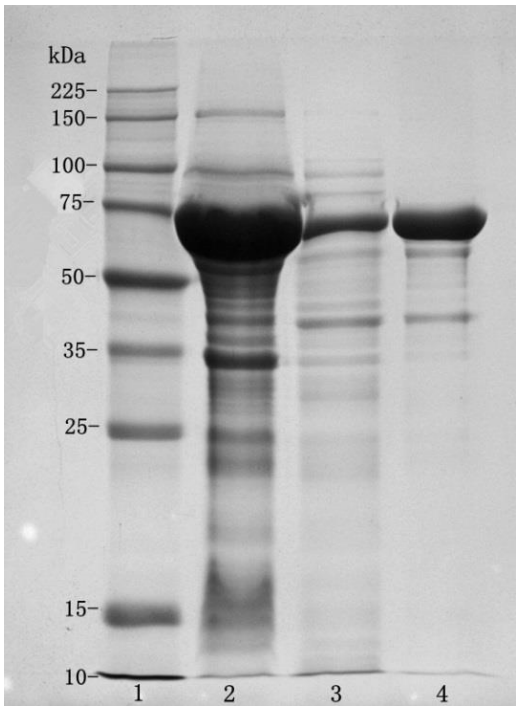


Figure 3.4 12% SDS-PAGE for MBP-MiaA purification. Lane 1, protein markers; Lane 2, cell free extract; Lane 3, flow through of IMAL column; Lane 4; MBP-MiaA fusion protein isolated from IMAL column.

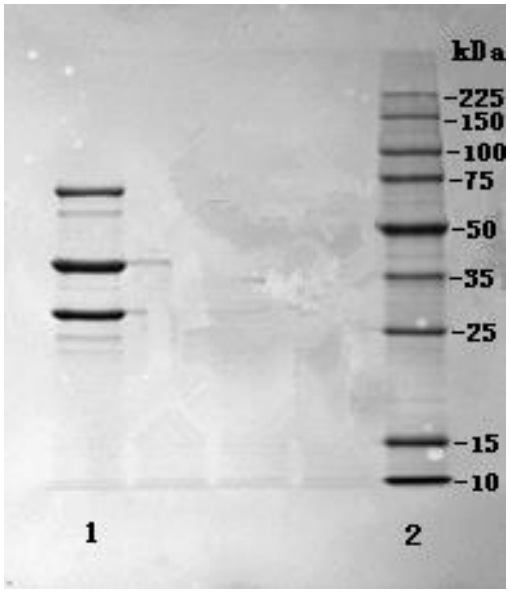


Figure 3.5 12% SDS-PAGE for TEV protease cleavage. Lane 1, after TEV protease cleavage; Lane 2, protein markers.

3.4 *In Vitro* MiaA-MiaE Assay

It has been previously demonstrated that the minimal substrate for MiaA is the anti-codon stem loop of tRNA (ASL), thus ASL^{Leu} (5'-rGrUrUrGrArUrUrCrArArArArUrCrArArC-3', IDT DNA), which has an appropriately positioned adenoside ribonucleoside was used as the initial substrate for this MiaA-MiaE assay for insertion of the isopentyl group. [11, 12] In addition, the isopentylated-ASL^{Leu} molecules, product of MiaA reaction, being used as the substrates for the following MiaE catalyzed O_2-dependent hydroxylation has been reported as well. [19] The results (**Figure 3.6**) show that the MiaA protein is active *in vitro* by forming i^6A and the i^6A here was confirmed by coeluting with the synthetic i^6A standard (**Figure 3.7**).Moreover, the decreasing of i^6A in MiaE catalysis step (**Figure 3.6**) indicates that MiaE protein is enzymatically active *in vitro* as well, by consuming the i^6A produced in MiaA catalysis step. In addition, both i^6A production and i^6A consumption can be applied to first-order reaction rate law, which is exactly the reaction type for both of the enzymes, MiaA and MiaE, under the conditions of excessive DMAPP and H_2O_2, respectively.

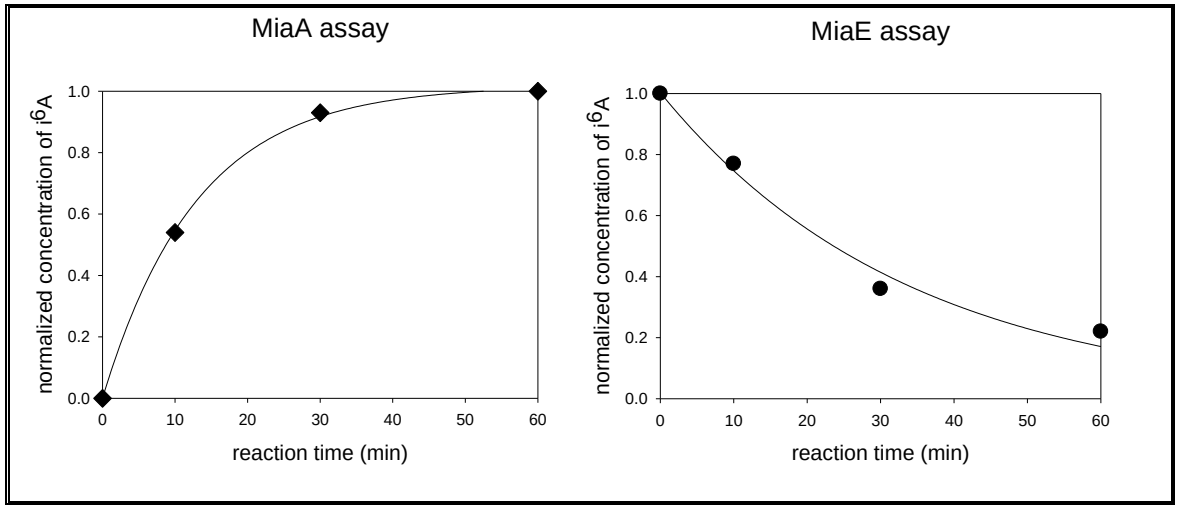


Figure 3.6 i^6A amount change as a function of reaction time in MiaA-MiaE assay. First step (left plot), i^6A production under MiaA catalysis, $y = a*(1 - e^{-kt})$, k = 0.0772; second step (right plot), i^6A consumption under MiaE catalysis, $y = a*e^{-kt}$, k = 0.0295.

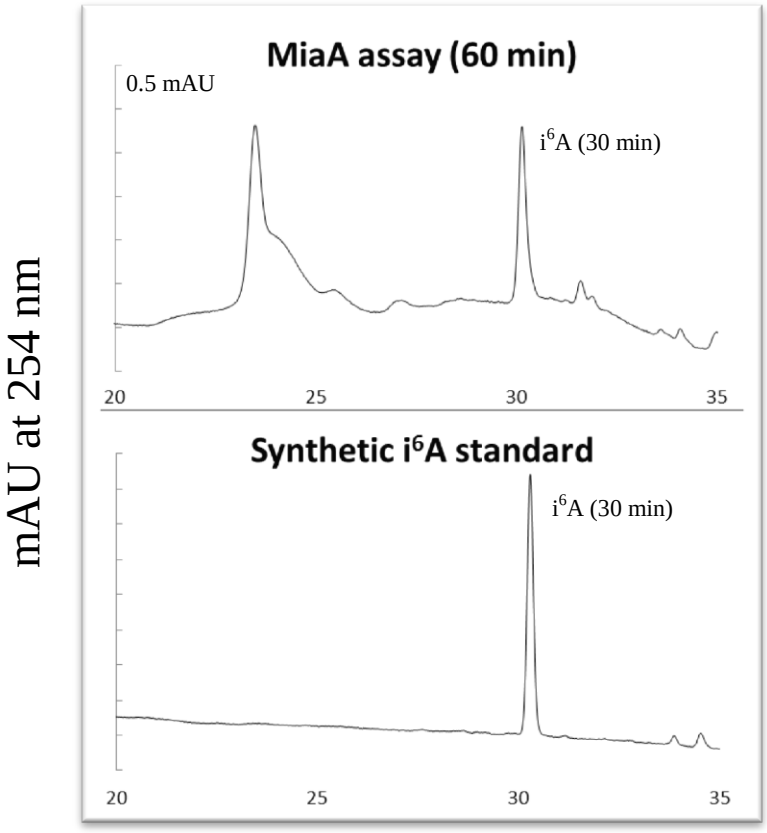


Figure 3.7 Selected HPLC chromatograms for MiaA reaction at 60 min (upper) and synthetic i^6A standard (lower).

3.5 *In Vivo* MiaE Activity Assay

Both pMIAEK (untagged MiaE) and pMHTΔ238 (TEV) vectors have the same Promega flexi vector backbone and antibiotic resistance, therefore RNA extracts isolated from cells transformed with pMHTΔ238 offer a reasonable baseline for nucleoside distribution in the absence of MiaE. The chromatograms shown in Figure 9 represent the nucleosides obtained from *E.coli* post-IPTG induction (4-hrs) of TEV (trace A) and untagged MiaE (trace B). The peak at 81 minutes (●) is observed in both of the nucleoside samples collected from with/without IPTG induction of pMIAEK, as well as cells transformed with pMHTΔ238, and a consumption of this peak can be observed in pMIAEK cells sample. On the basis of its retention time, UV-visible spectra, and coelution with synthetic standards (trace C), this peak is assigned to ms^2i^6A.

Alternatively, the 67 min. peak (○) is only observed in both IPTG induction (-) and IPTG induction (+) of pMIAEK, and coelutes with synthetic ms^2io^6A standard and the hydroxylated product generated by MiaE peroxide-shunt assay. On the basis of these experiments, it is clear that recombinant MiaE is catalytically active *in vivo*. And MiaE hydroxylation appears not to be affected by IPTG induction due to the "leaky" expression in pET system. [18]

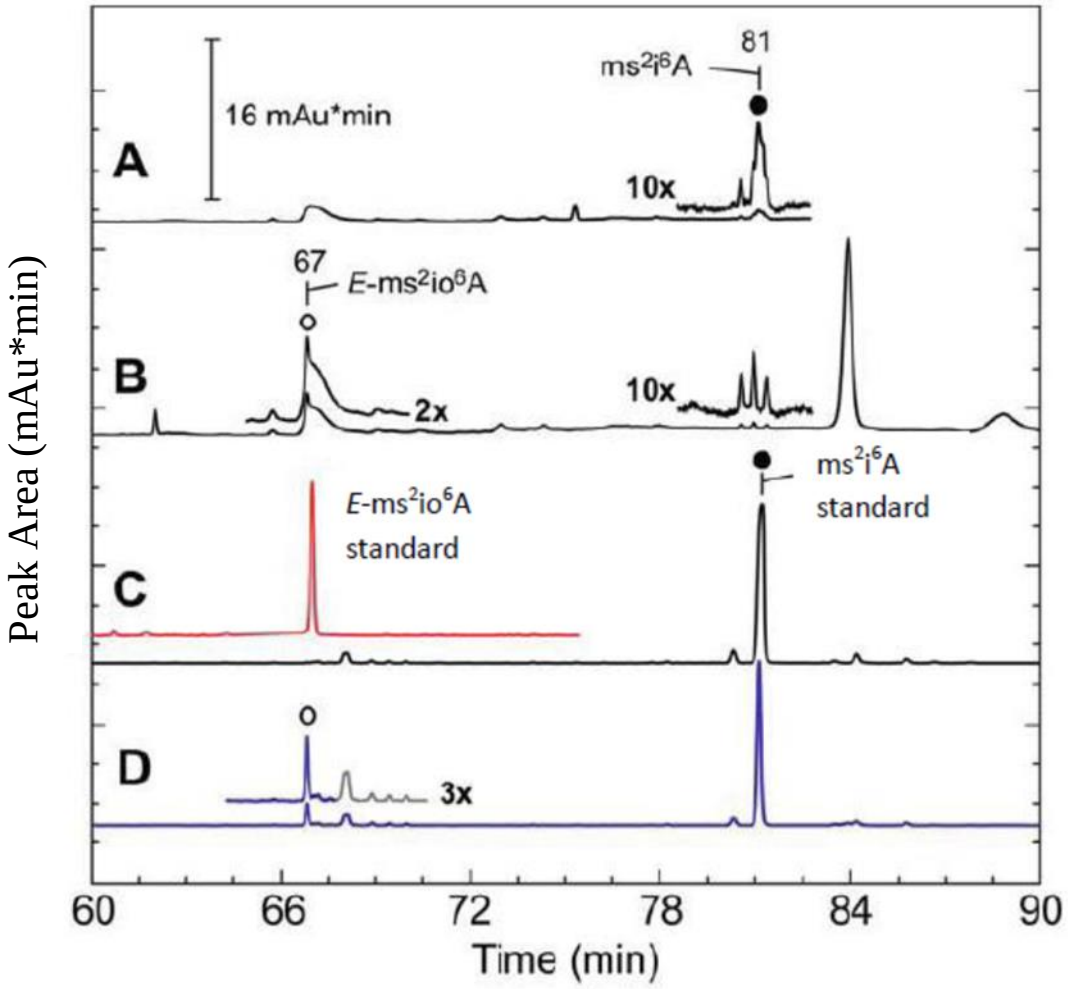


Figure 3.8 Reverse phase HPLC chromatogram of hydrolyzed tRNA nucleosides extracted from IPTG-induced *E.coli* BL21 (DE3) transformed with (A) pMHTΔ238 and (B) pMIAEK. Assignment of (E)-ms^2io^6A (67 min) and ms^2i^6A (81 min) β-epimers were confirmed by comparison of retention times and UV-visible spectra to synthetic standards (C). For clarity, a selected time point (30 min) is shown in trace D for the MiaE peroxide-shunt catalyzed hydroxylation of synthetic i^6A.

CHAPTER 4

DISCUSSION

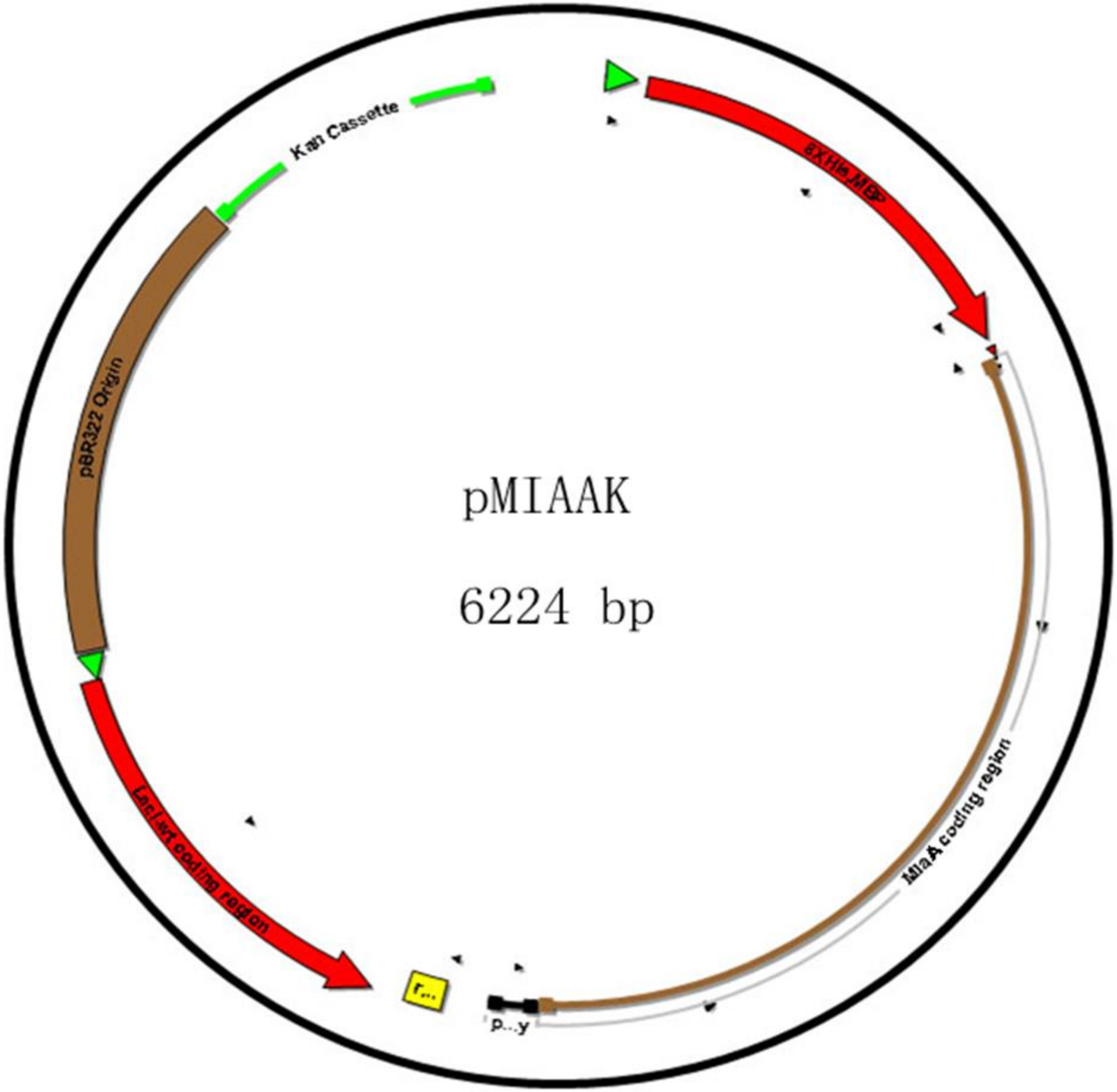
While still in its initial stage of development, the work provided here demonstrate the possibility of generating viable tRNA substrates for mechanistic characterization of the enzymes involved in A_{37}-hypermodification (MiaA, MiaB and MiaE). The only limiting factor in this RNA oligomers synthesis is the cost of tRNA polymerase; however, we have recently overcame this problem by obtaining an IPTG inducible tRNA polymerase expression vector from Dr. Studier (Brookhaven National Laboratory). Using the methods described here, a library of isopentenylated-tRNA molecules can be generated to use as potential substrates for MiaE catalyzed O_2-dependent hydroxylation. Once MiaB is successfully cloned, expressed and its activity verified, all the significant segments that participate in this modification process, RNA oligomers, MiaA, MiaB and MiaE, will be ready for a comprehensive study.

While basic, verification of enzymatic activity is the most fundamentally important first step in the characterization of an enzyme. Based on the results of both of the assays, the recombinant MiaE protein has been proved to be catalytically active both *in vivo* and *in vitro.* At its current stage, MiaE hydroxylation of i^6A_{37}-tRNA substrate-analogues by MiaE has been achieved by peroxide shunt. However, future work will focus on the use of a native electron-transport chain (ferredoxin (Fd), ferredoxin reductase (FdR), and NADPH) for simulating the physiological catalysis. [15] Cloning, soluble expression, and confirmation of Fd and FdR activity as reported earlier and Fd has been obtained by now. [16] Moreover, since each tRNA substrate analogue is synthesized *in vitro*, its actual nucleoside sequence (and secondary structure) can be completely controled over. Therefore, the ribonucleoside-protein interactions can be investigated through characterization of the steady-state kinetics of some specifically designed

RNA oligomers hydroxylation by MiaE. Additional work may involve formation of MiaE mutants to develop a structure-function model for MiaE O_2-dependent tRNA hydroxylation.

APPENDIX A

VECTOR MAPS OF PMIAAK AND PMIABK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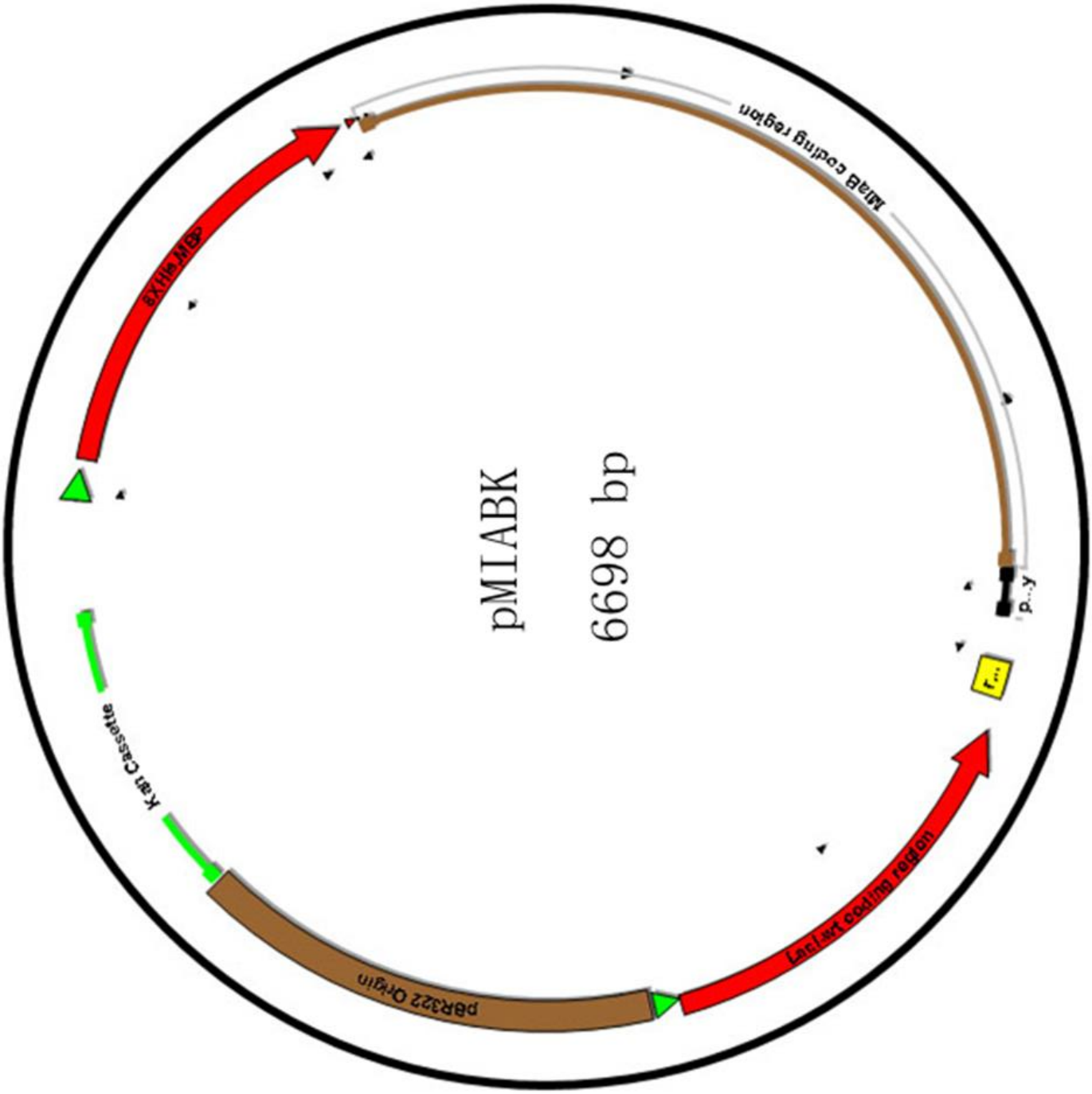
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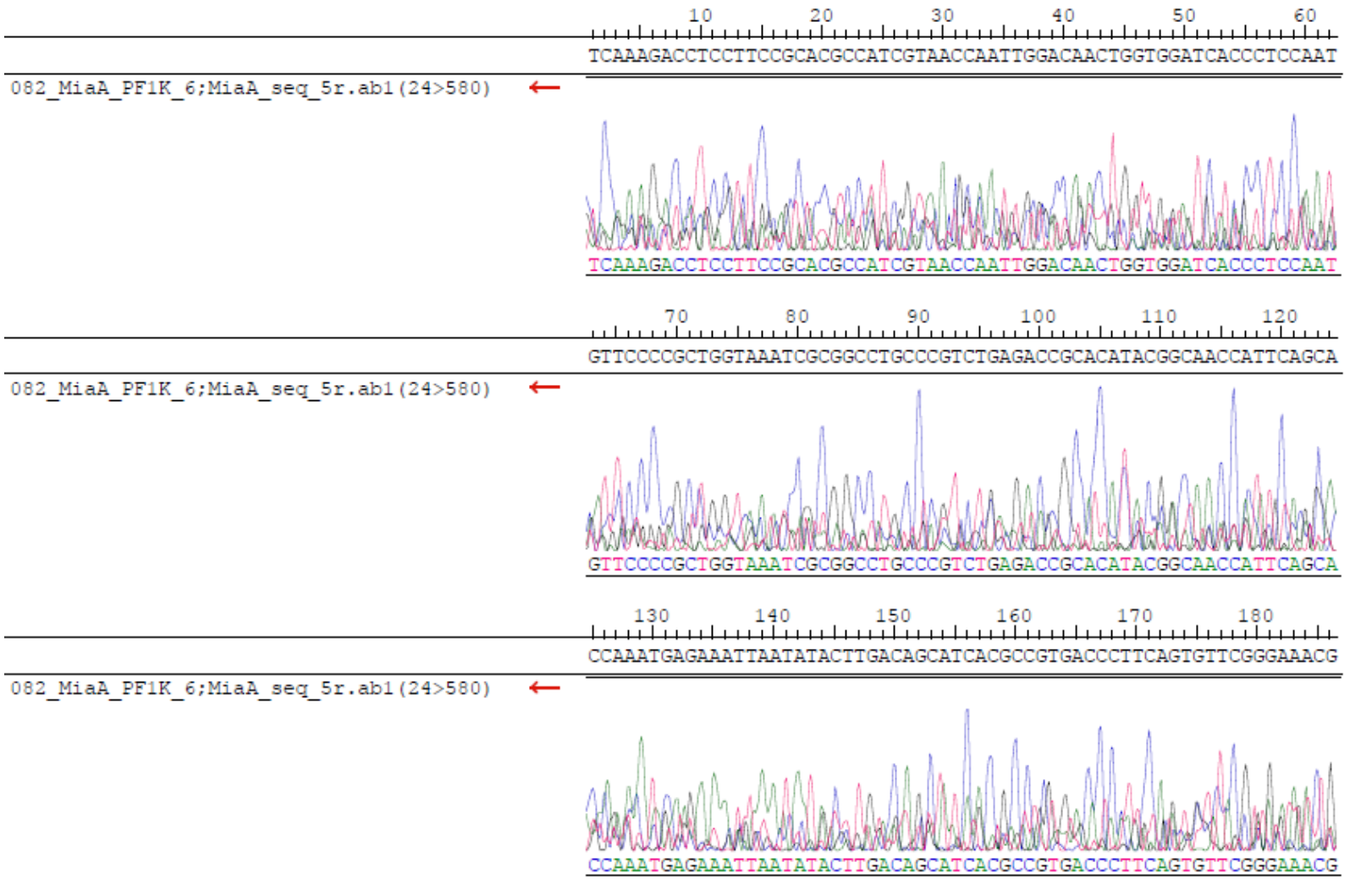
APPENDIX B

MIAA GENE SEQUENCING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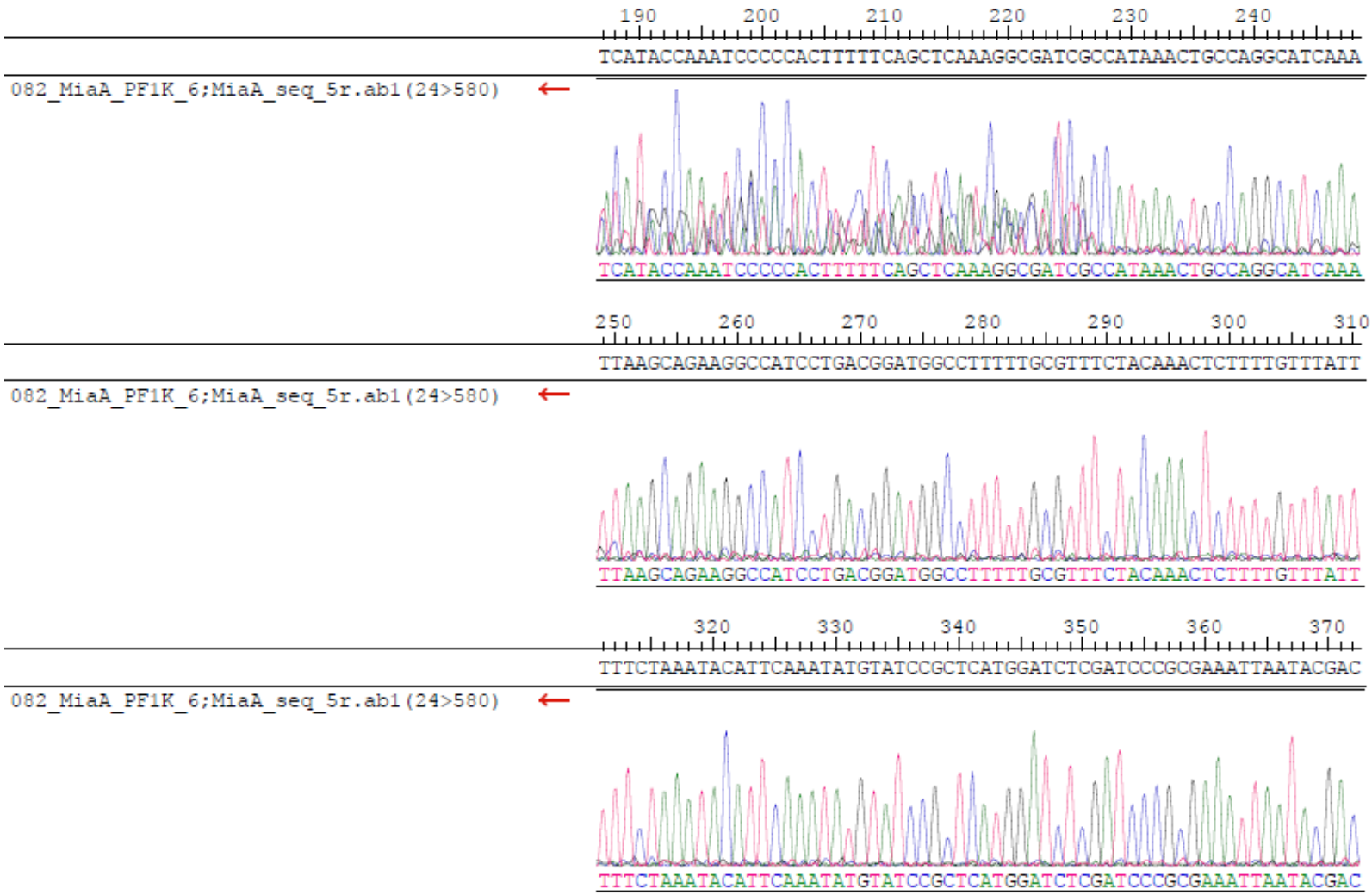
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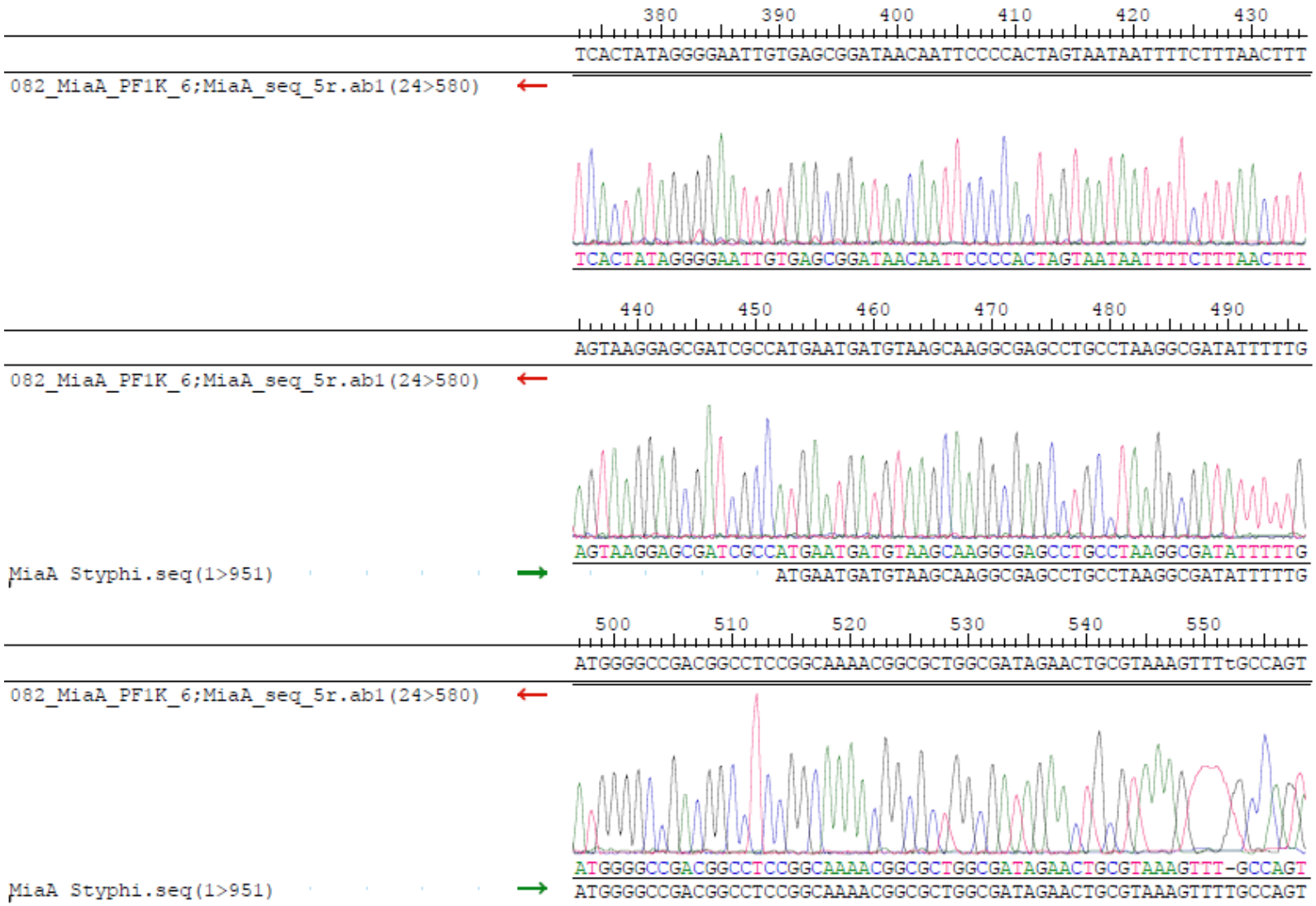
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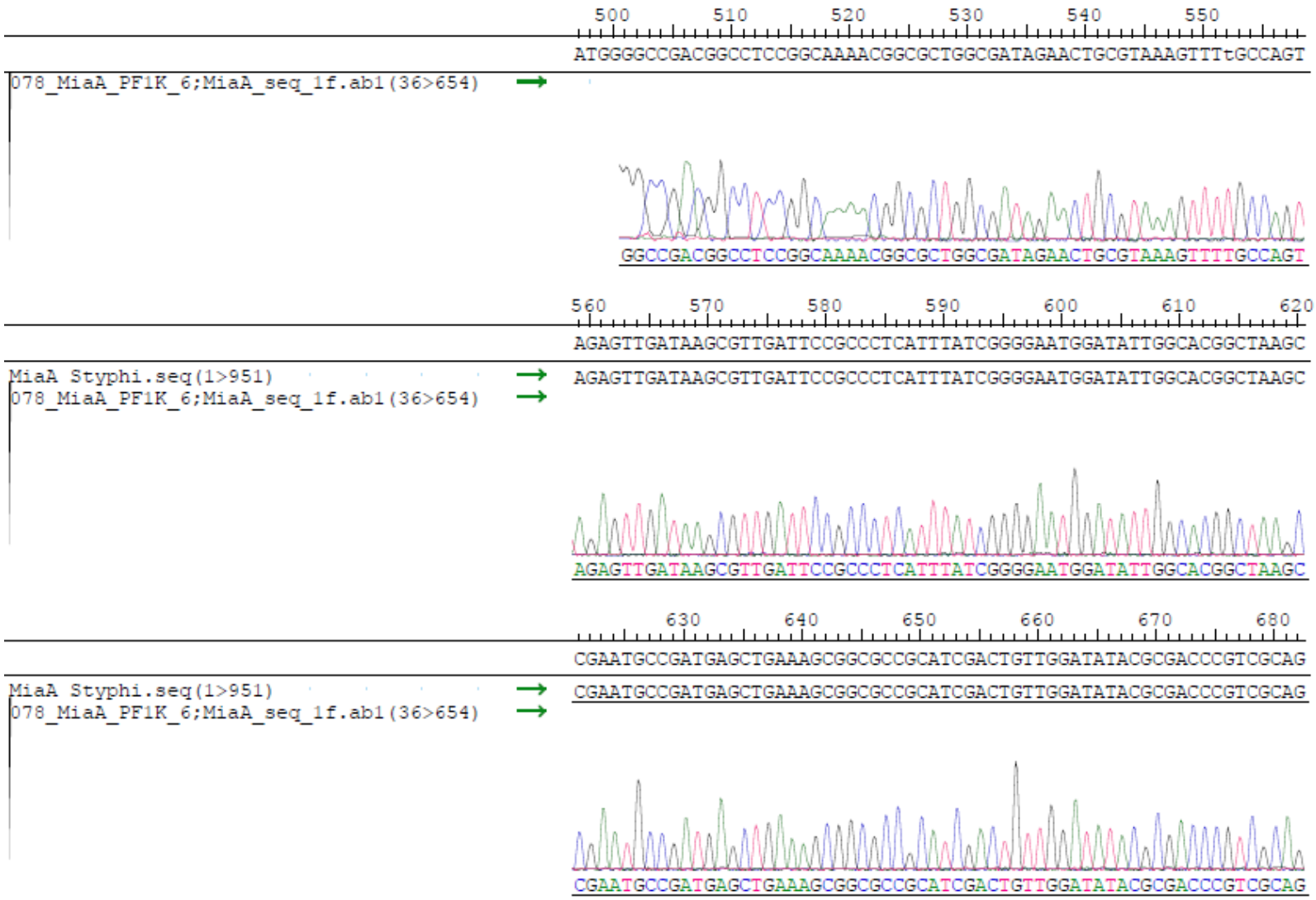
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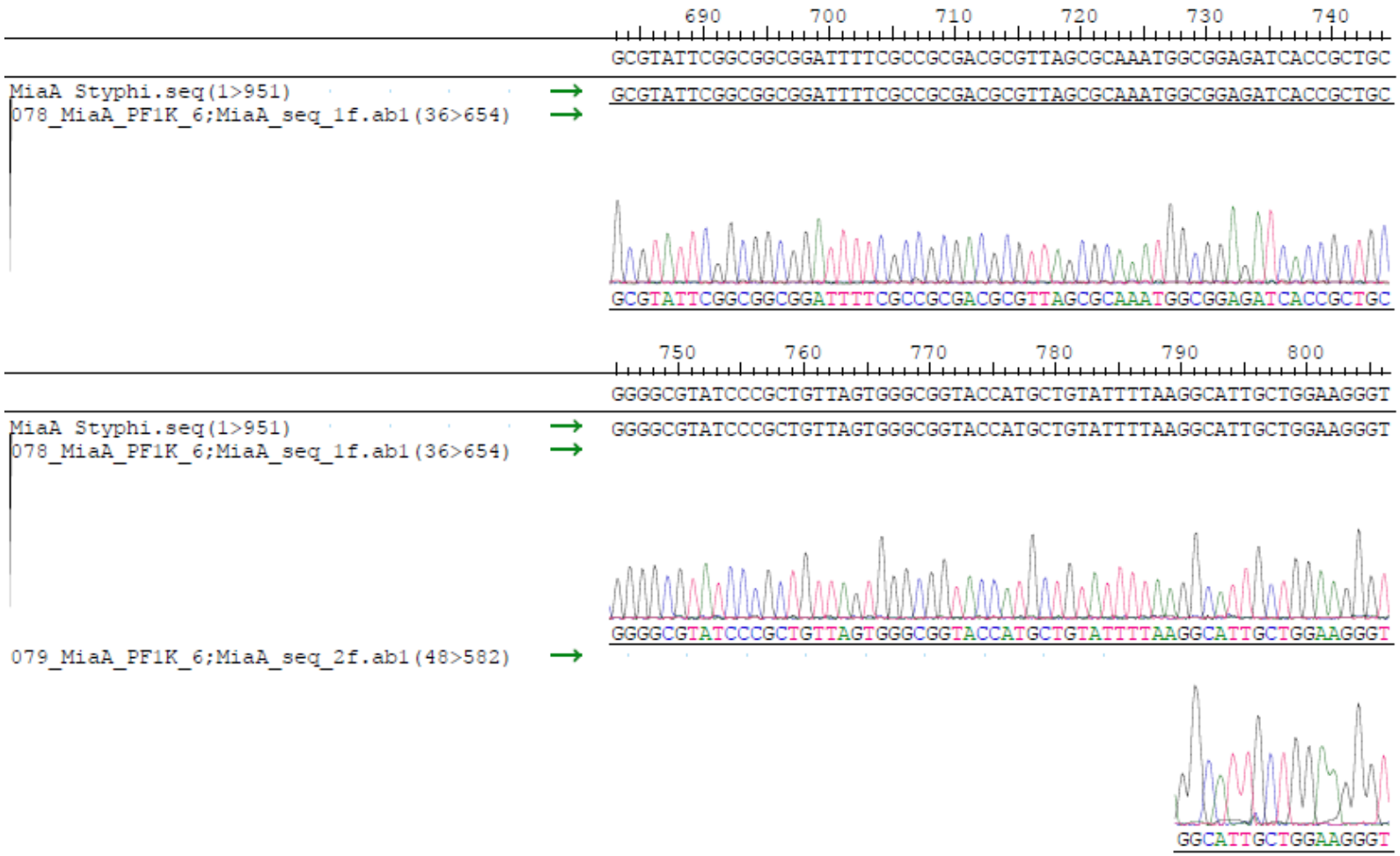
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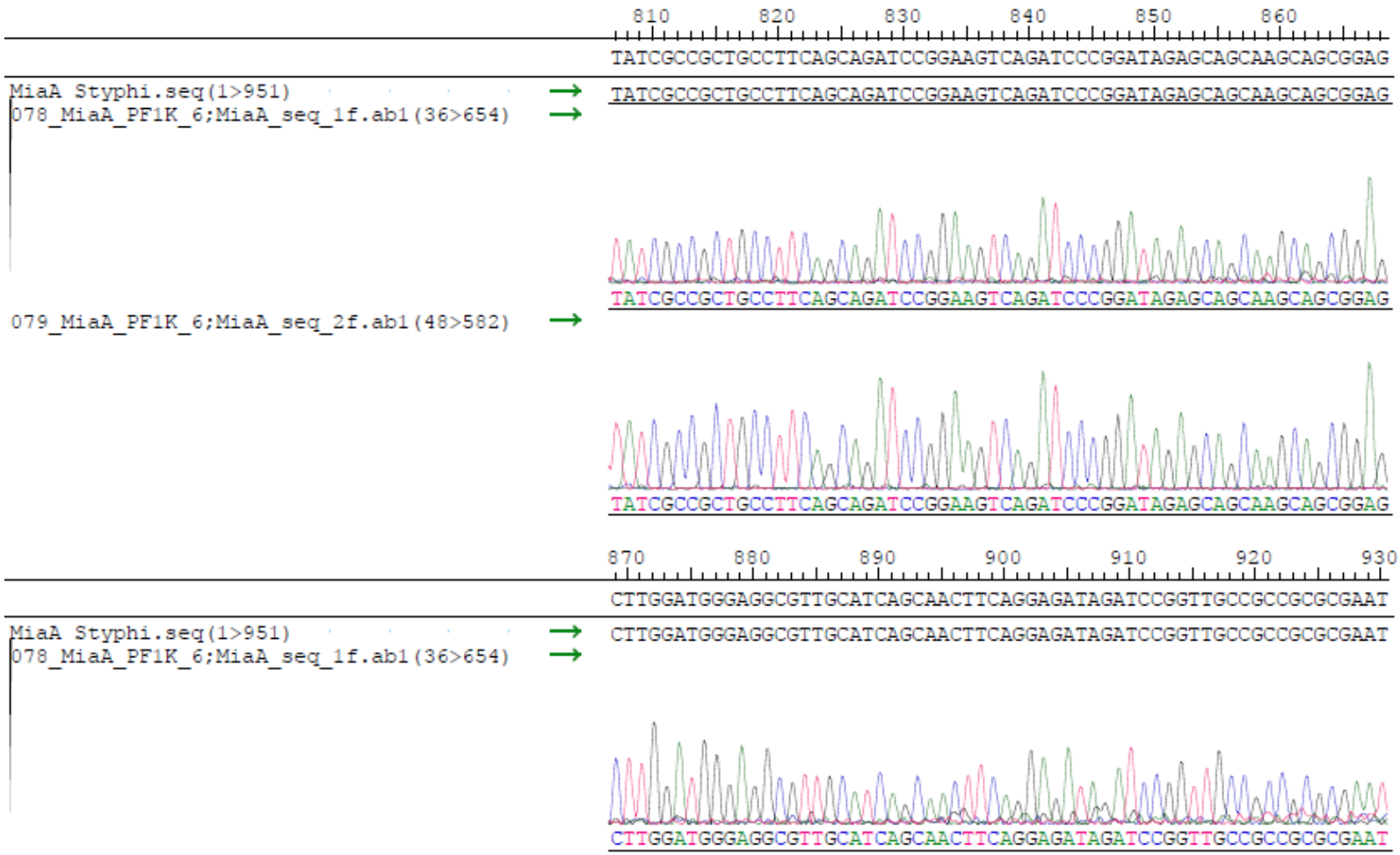
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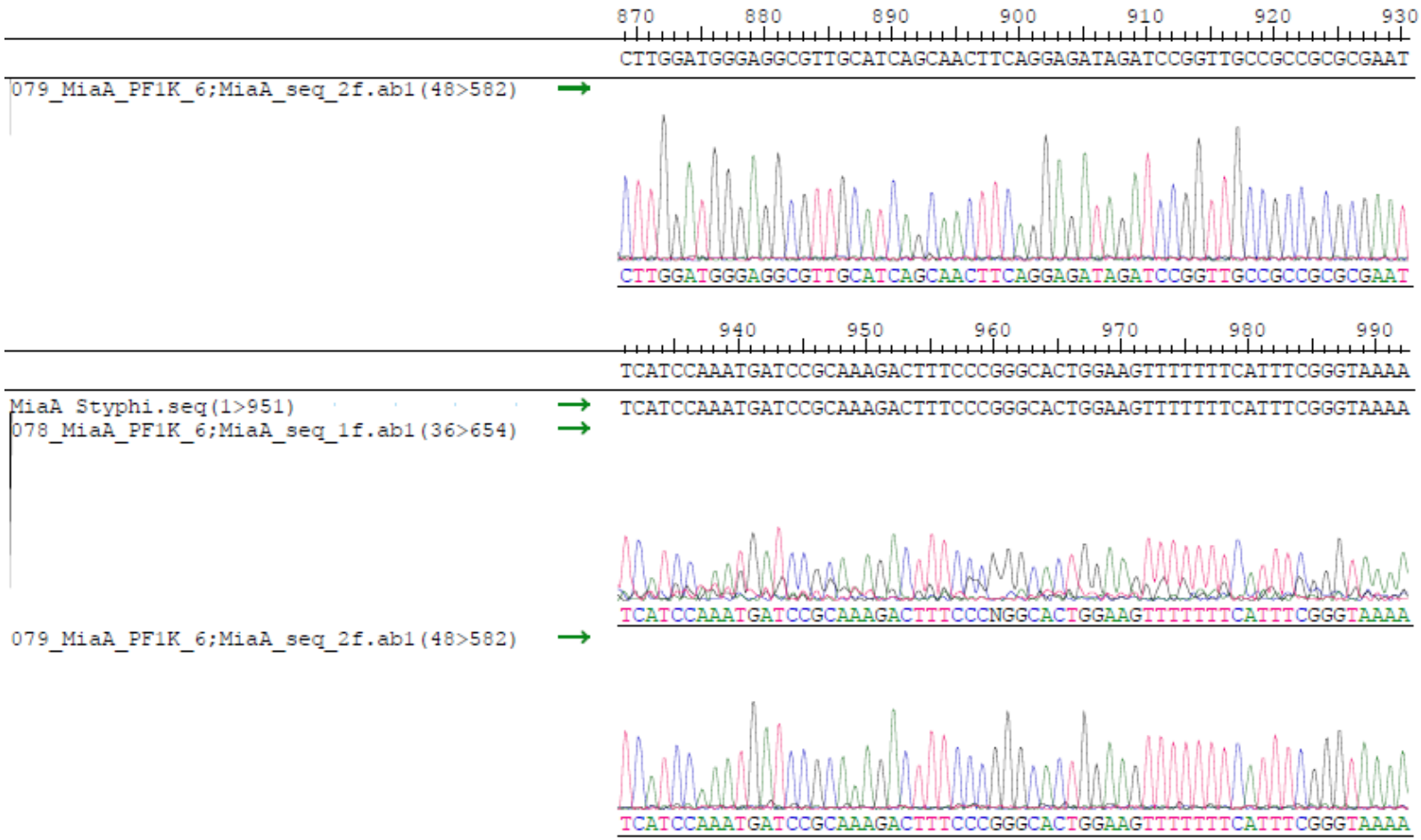
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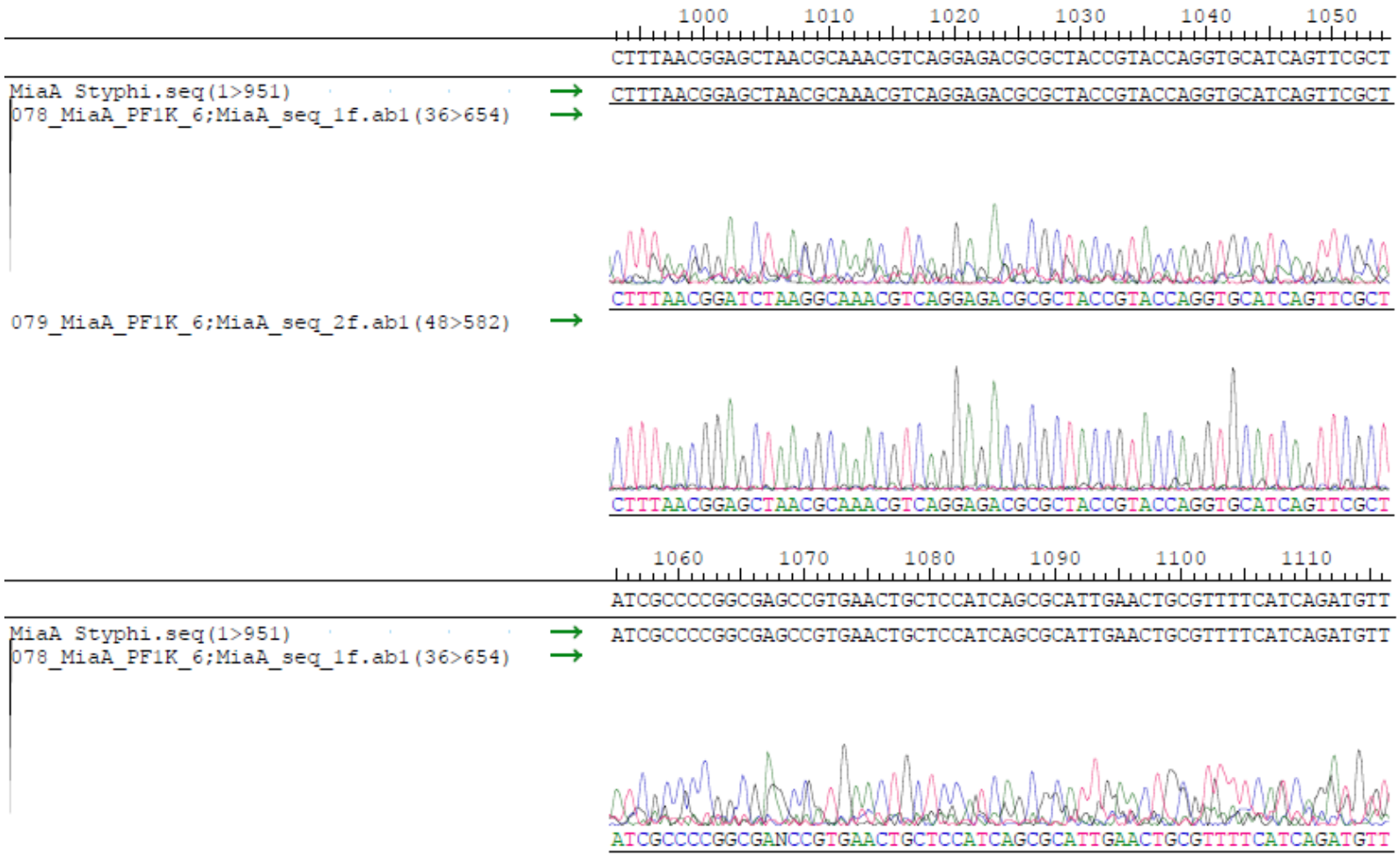
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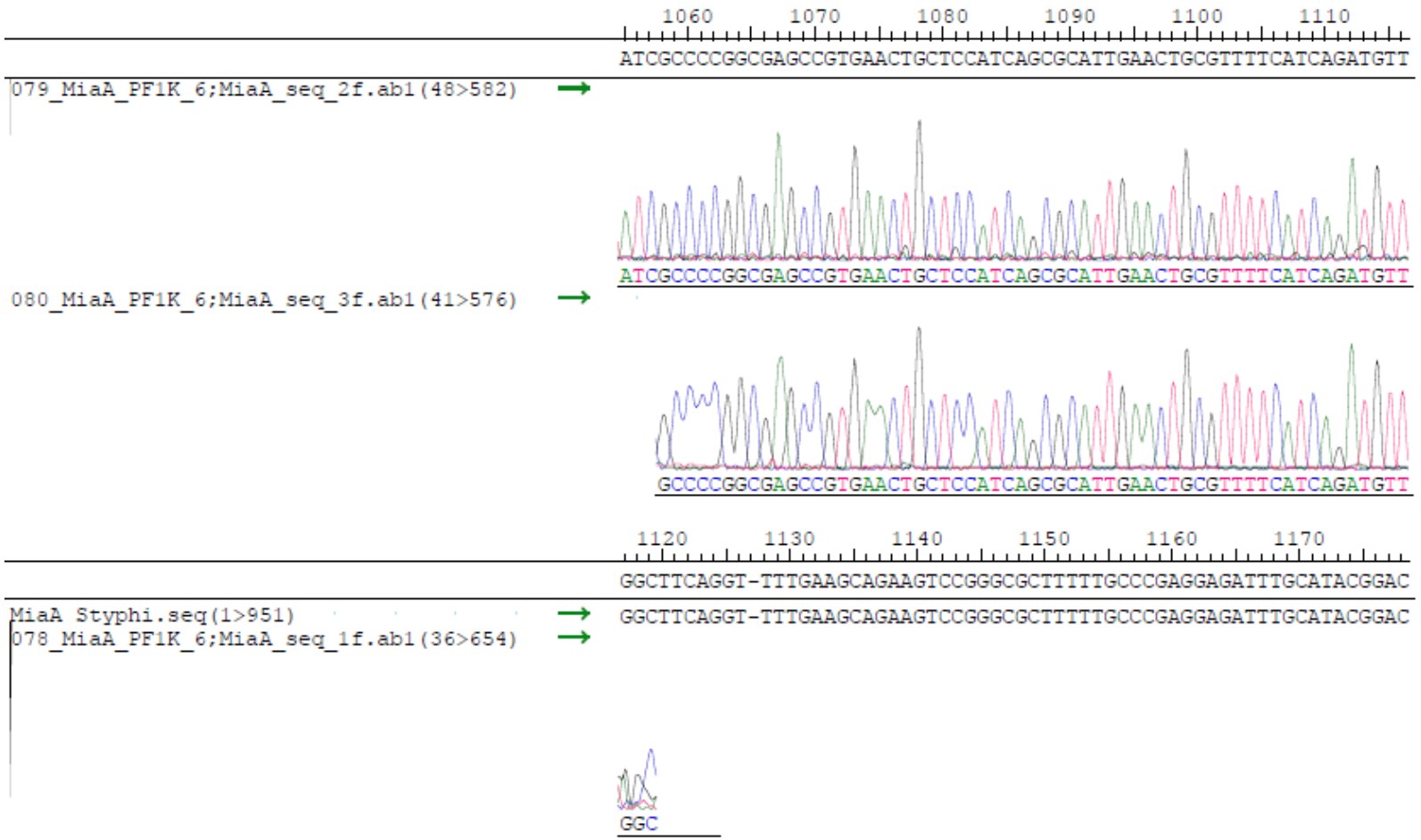
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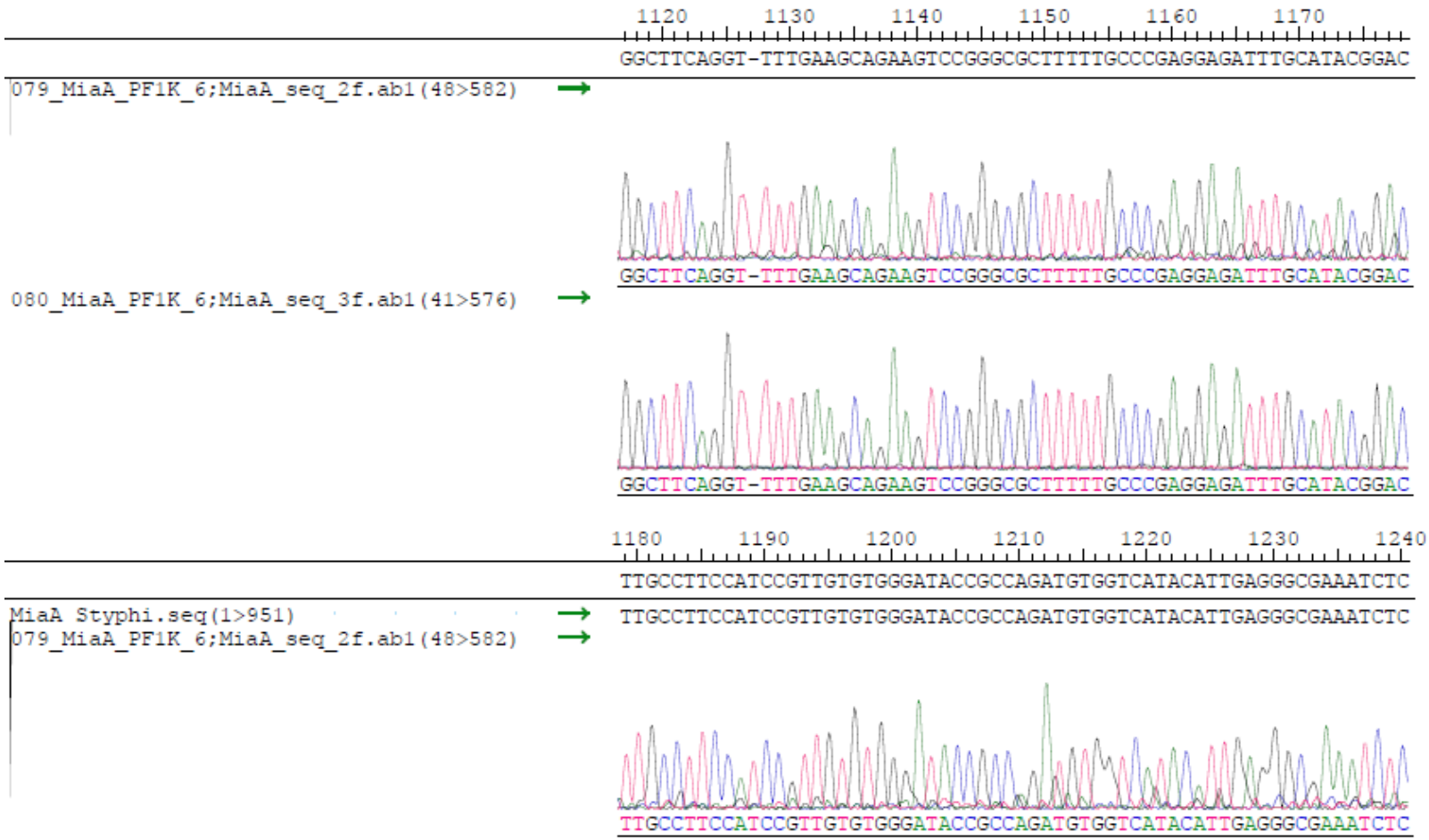
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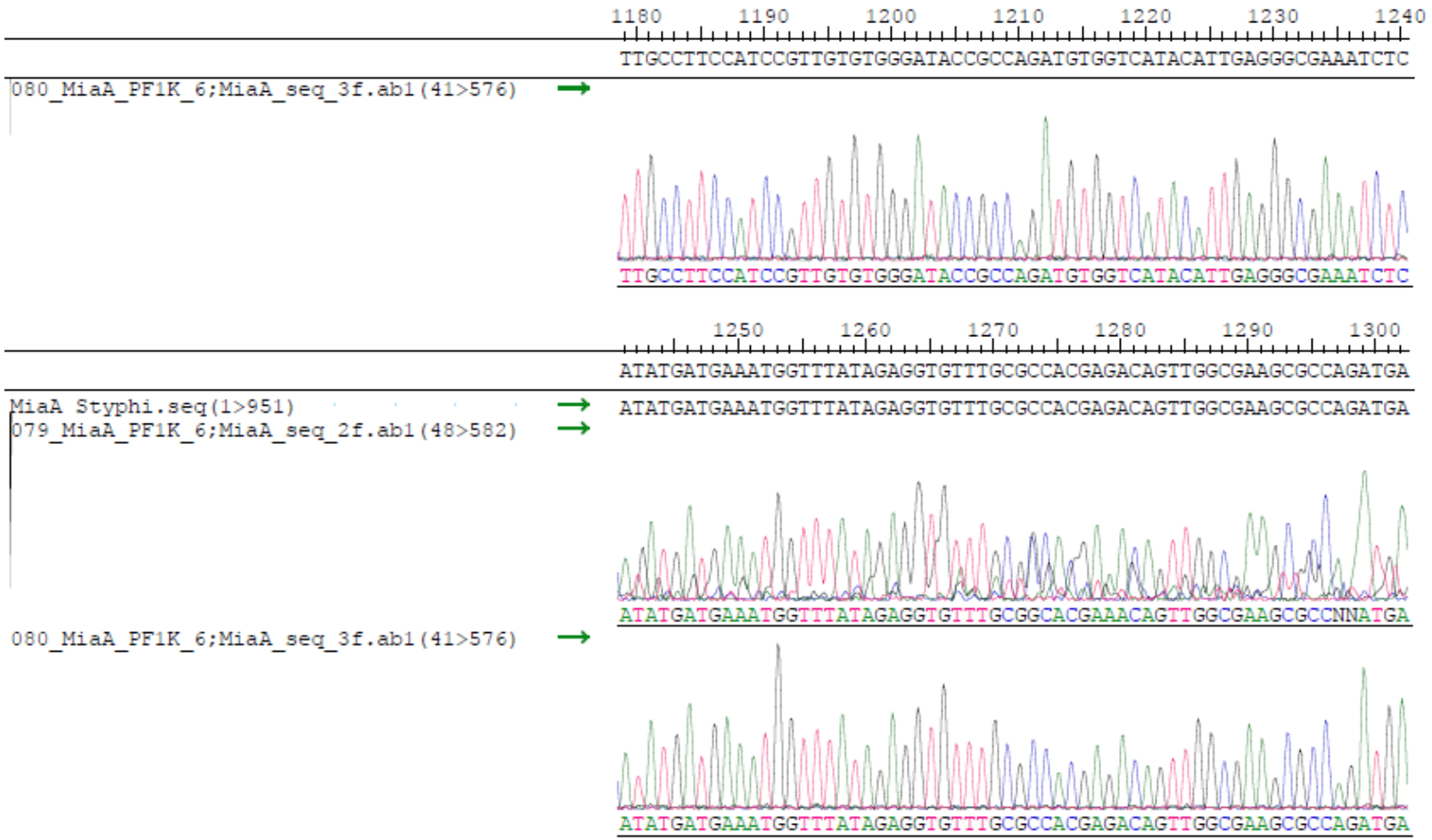
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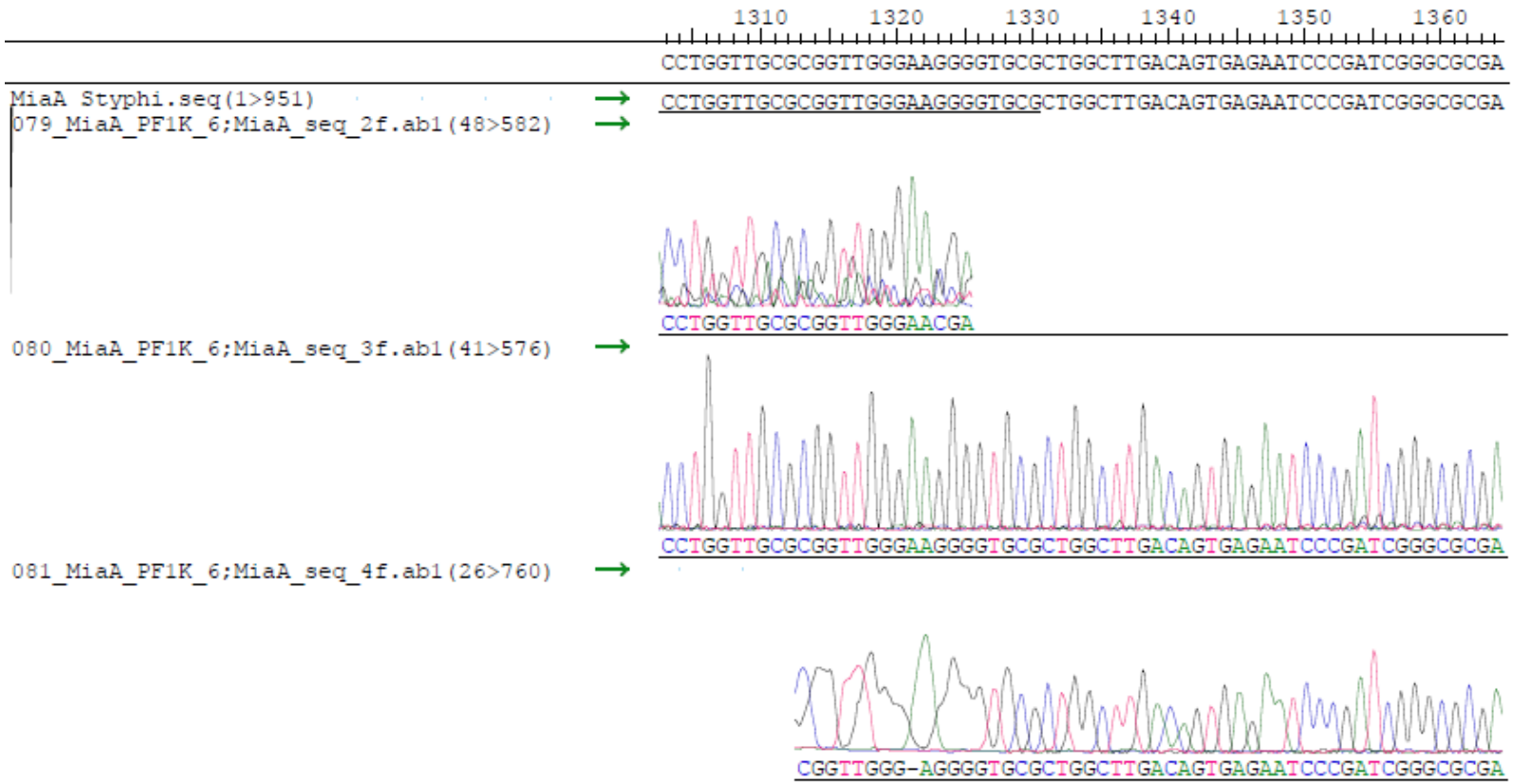
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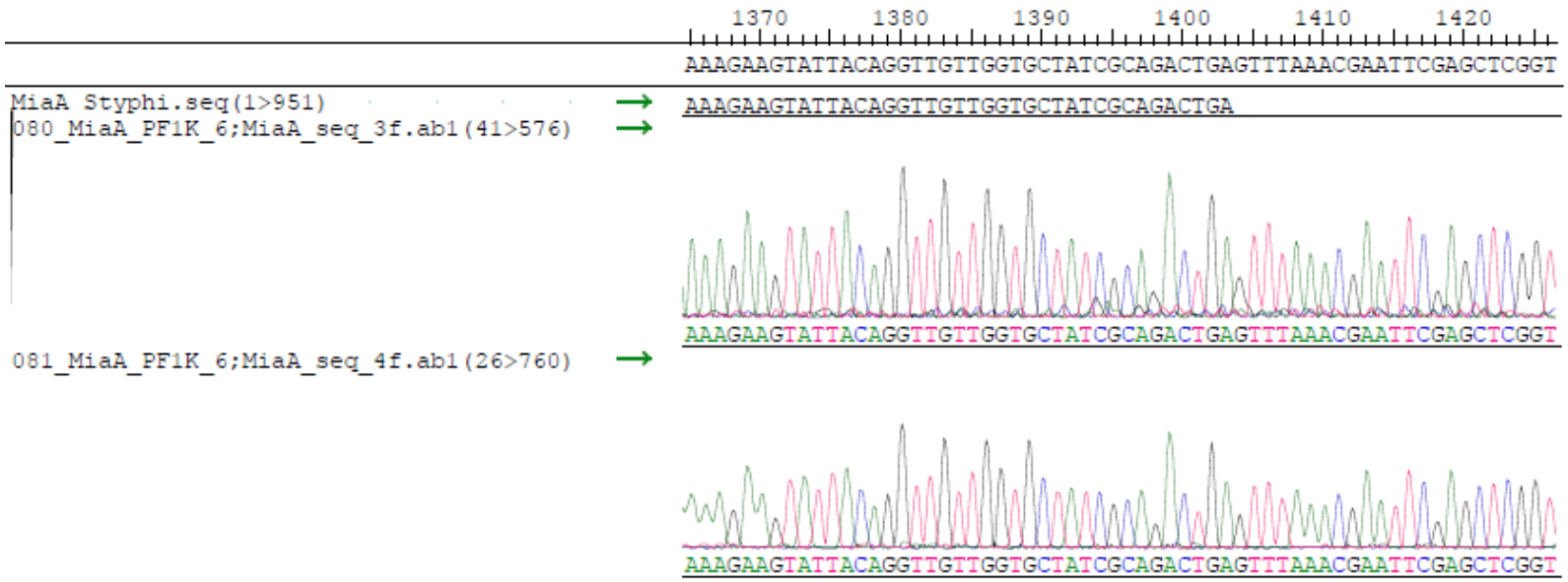
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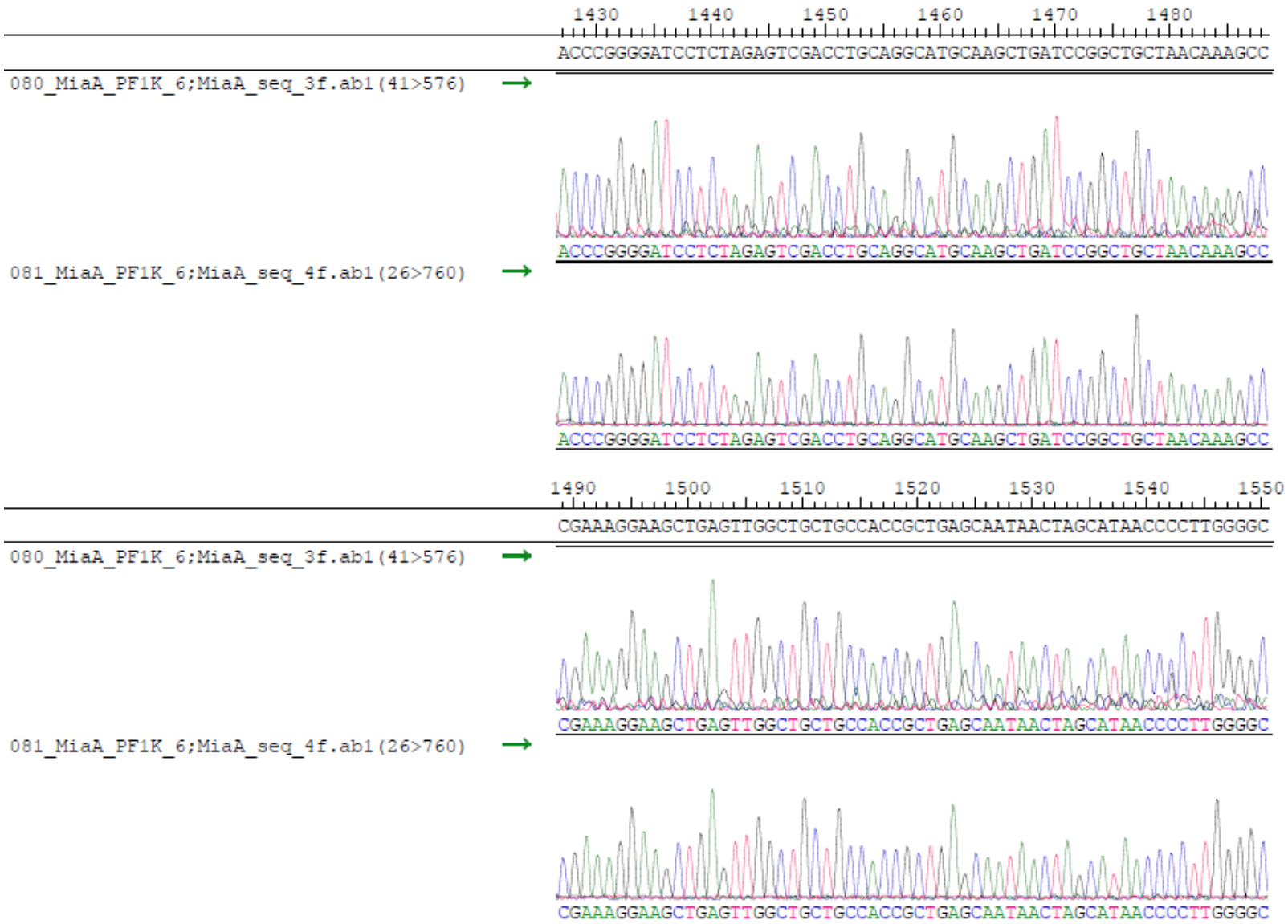
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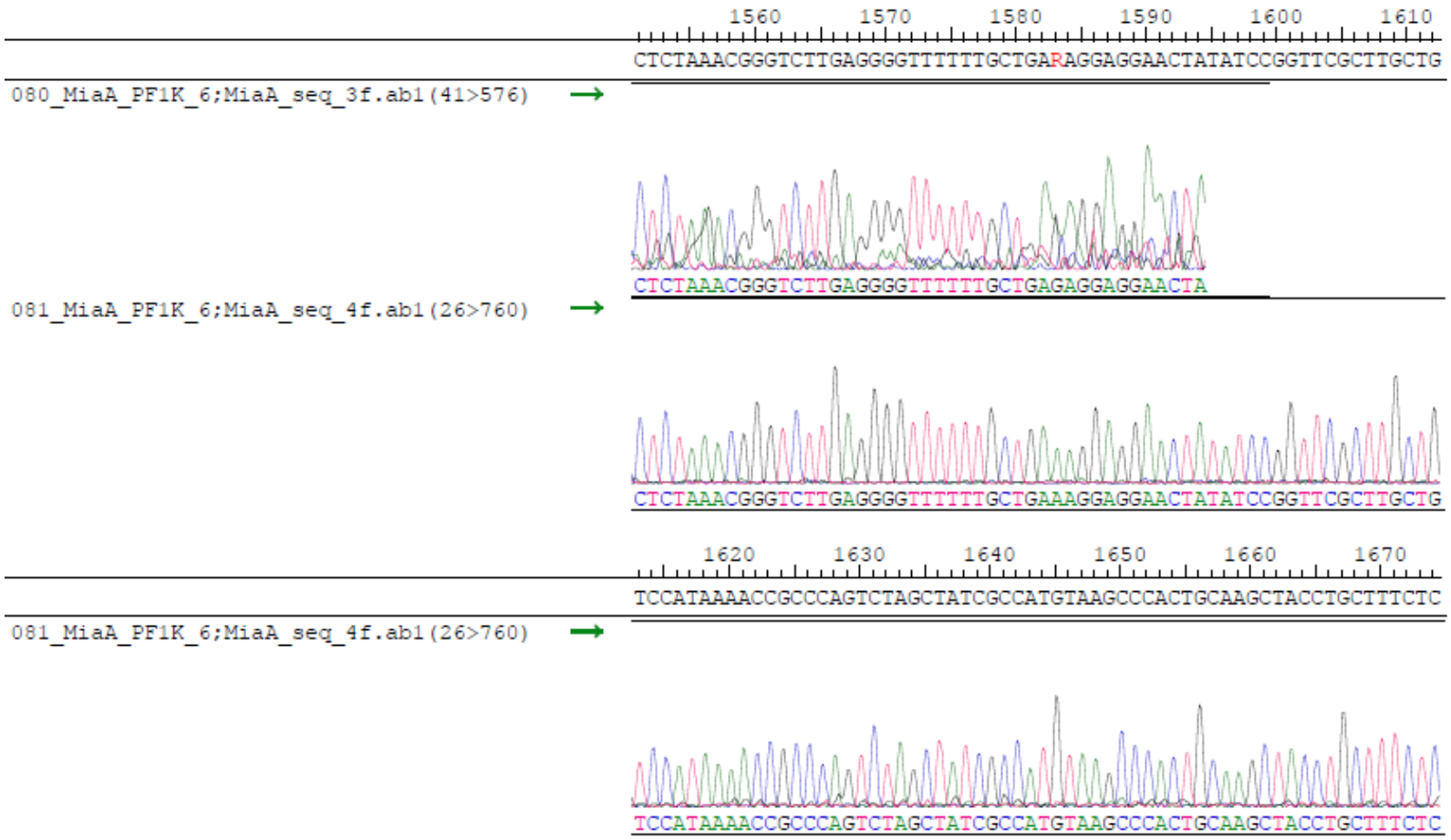
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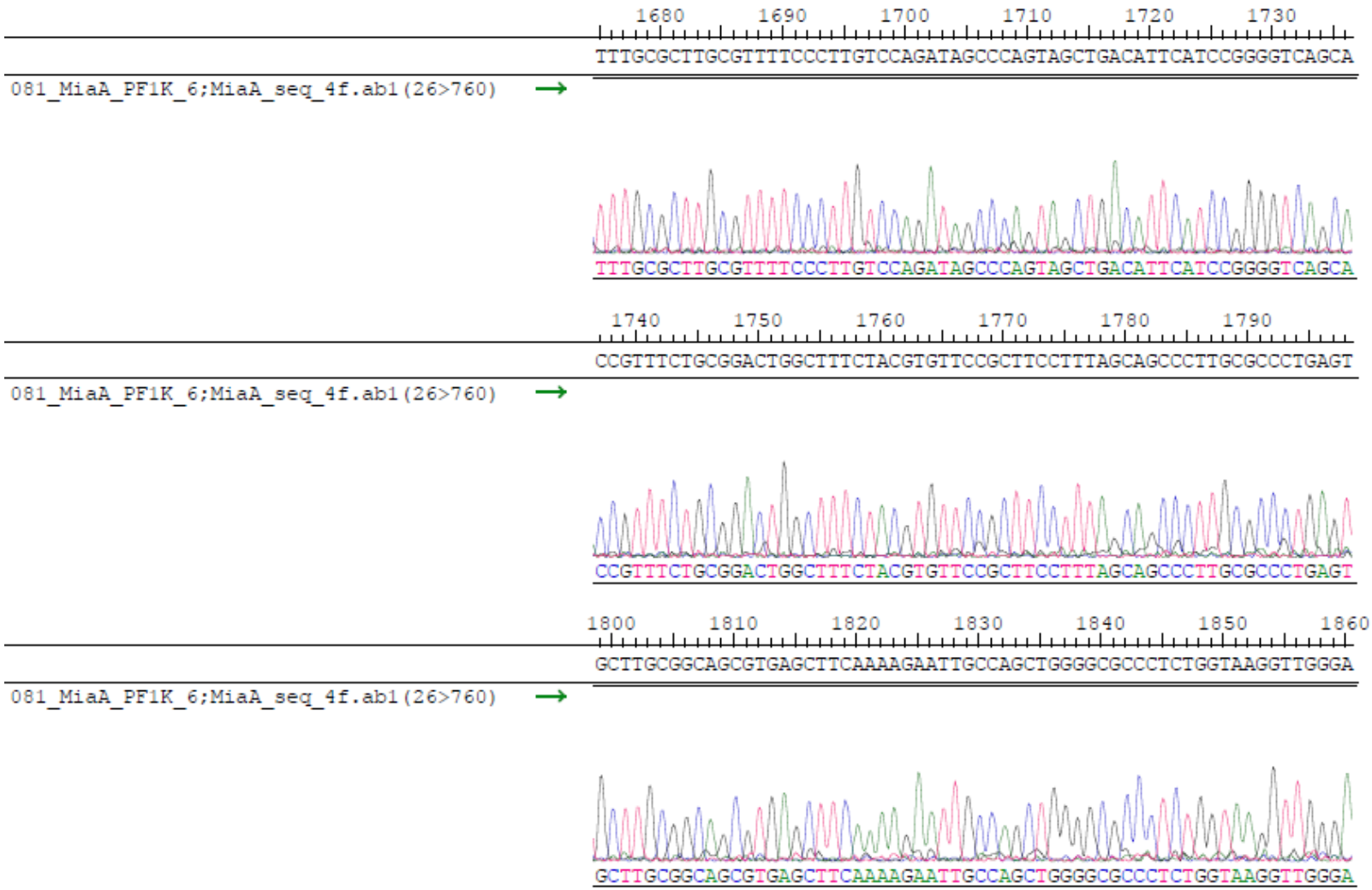
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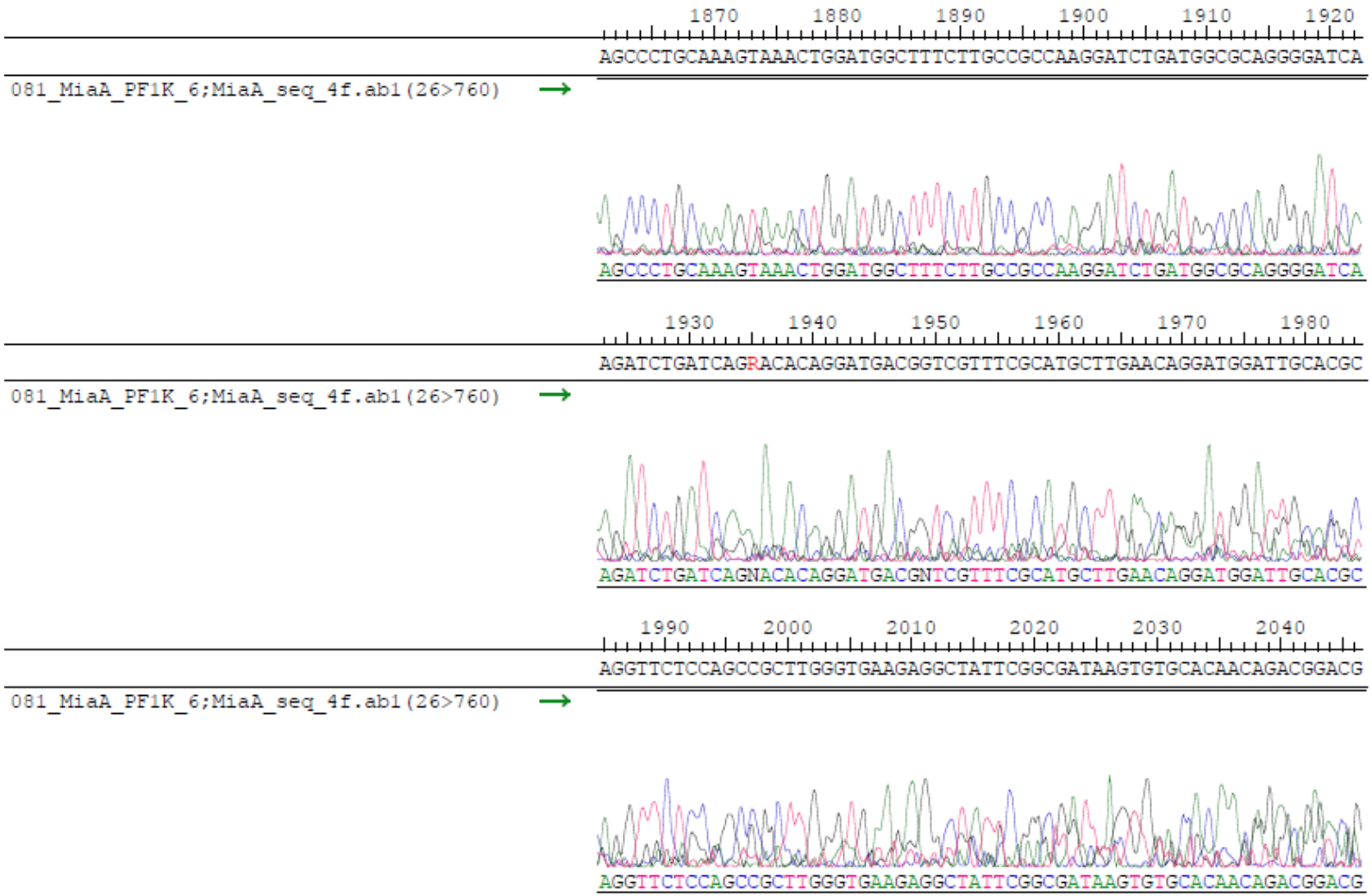
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1880
1890
1900
1910
1920
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1930
1940
1950
1960
1970
1980
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1990
2000
2010
2020
2030
2040
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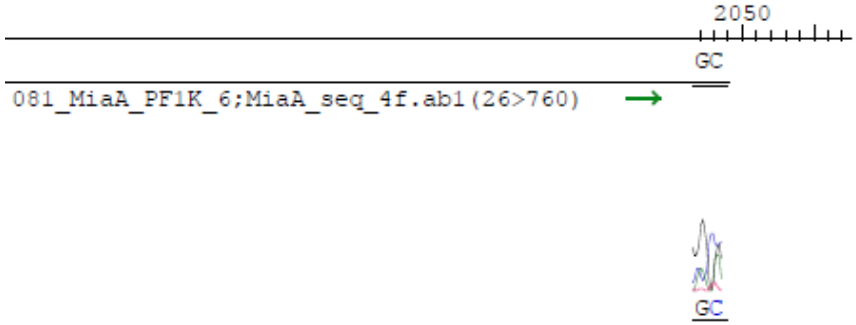
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GC

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BIOGRAPHICAL INFORMATION

Siai Zhang joined group of Dr. Brad Pierce in 2011 and focused on utilizing a variety of biochemistry and biophysical techniques to investigate a non-heme dirrion monooxygenase (MiaE). Prior to her graduate study at University of Texas at Arlington, Siai Zhang received her B.S, in Pharmaceutical Science from Tianjin University, China. Later, Siai Zhang is going to Germany for her Ph.D study.