Successful synthesis of active human coagulation factor VII by co-expression of mammalian gamma-glutamyl carboxylase and modification of vit.K cycle in Drosophila Schneider S2 cells

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Abstract:	Mammalian gamma-glutamyl carboxylase and reduced vitamin K are indispensable for synthesis of mature mammalian vitamin K dependent proteins including some of blood coagulation factors (factors II, VII, IX, and X). It was well known that Drosophila melanogaster expressed gamma-glutamyl carboxylase and possessed a vit.K cycle although native substrates for them have not been identified yet. Despite the potential capability of gamma calboxylation in Drosophila melanogaster derived cells such as S2 cells, Drosophila gamma-glutamyl carboxylase failed to gamma carboxylate a peptide fused to the human coagulation factor IX propeptide. Thus, it had been believed that the Drosophila system was not adequate to synthesize mammalian vit.K dependent proteins. Indeed, we previously attempted to synthesize biologically active factor VII in S2 cells although we were not able to obtain it. However, recently, a successful transient expression of biologically active human factor IX from S2 cells were reported. In the present study, several expression vectors which enables to express mammalian GGCX, VKORC1, and/or PDIA2 along with F7 were developed. S2 cells transfected with pMKA85, pMAK86, and pMAK219 successfully synthesized active FVII. Thus, mammalian GGCX was indispensable to synthesize active FVII while mammalian VKORC1 and PDIA2 were not critical but supportive factors for S2 cells.
Response to Reviewers:	Reviewer #1 >(1). By demonstrating that the protein was indeed Factor VII using Western Blots, it is essential that procoagulant is observed in Factor VII deficient plasmas.

Response #1-(1). At the beginning of this study, clotting time-based FVII activity assays were carried out. In the assays, FVII deficient plasma and each supernatant was mixed and the clotting time was measured. The procoagulant was observed in the supernatant from S2/85, S2/86, or S2/219 cultured with vit.K in the media. We added this statement in the result section in p11.

>(2). The activity assays used in the current communication are not well described.
Response #1-(2). According to the reviewer's comment, we added the detail of the assay in P12.

>(3). It would also be nice to determine the levels of gamma-carboxylation of the current protein and the yield of active factor VII.
Response #1-(3). As the reviewer mentioned, it is very important to determine the levels of gamma-carboxylation. For this purpose, peptides containing the Gra domain need to be analyzed with Matrix-assisted laser desorption ionization (MALDI) mass spectrometry with time-of-flight (TOF). The cDNAs for all vit.K dependent proteins were already cloned, and hexa-Histidine tag and C3 protease recognition site were inserted to sandwich each Gra domein. We will analyze the levels of gamma-carboxylation for all vit.K dependent proteins and determine the yield of them in future communication. This comment was inserted in the discussion in p17.

Reviewer #2
>(1). The paper is written with a very basic english with numbers of grammatical mistake and lacked of technical writing skill. The introduction is weak and lacked of connectivity from one paragraph to another. The impact or contribution of research in cell culture is not clearly emphasised since the beginning and the reason of using Drosophila melanogaster over other cell lines is not clearly explained. Besides, the writing format is inconsistent throughout the paper.
Response #2-(1). According to the reviewer's comment, we asked Editage which provides English editing to improve this manuscript. We also emphasized the superior aspect of S2 cells to produce recombinant proteins in p17-19.

>(2). There are numbers of data that were not shown making the paper technically weak.
Response #2-(2). Actually, there were several "data not shown" in the manuscript.
>(2-1). The first in p4, "The secretions of these proteins were observed, however, activities were not detected (data not reported)."
Response #2-(2-1). We actually expressed FII, FIX, FX, Protin C and S.

Despite the successful expression of all vit.K dependent proteins with pMT based vectors, the supernatant did not contain their specific activities. Therefore, we mentioned "data not reported". In addition, these proteins were active with pCoVKE based vectors when the transformed cells were cultured with vit.K. We will report these results in future communication.

>(2-2). The second in p13, "All of the vital parts, such as the coding sequences for promoter (PCOPIA), poly A signal (pA), and each gene (PURO2G, Ggcx, Vkorc1, Pdia2) were verified by DNA sequencing (data not shown)."
Response #2-(2-2). We do not think it is necessary to show such sequence. Thus, we used "data not shown" in the revised manuscript again.

>(2-3). The third in p14, "The viability and growing rates of these transformed cells were similar to those of non-transfected S2 cells (data not shown)."
Response #2-(2-3). We extensively performed the experiments in previous papers. Thus, we added references instead of usage of "data not shown" in p13.

>(2-4). The fourth in p14, "On the other hand, no signals were detected in the culture supernatant from S2/132 (Fig.4C) but cellular accumulation was observed (data not shown)."
Response #2-(2-4). We realized that we do not need to mention "but cellular accumulation was observed (data not shown)." in this point. Thus, we deleted it.

>(2-5). The fifth in p14, "The FVII activity was not detected in the culture supernatant from all cells without vit.K (data not shown)."

Response #2-(2-5). We realized that we do not need to mention “data not shown” in this point. Thus, we deleted it.

>(3). Page 2 Para 2 "Many studies have indicated that expression levels of recombinant proteins from S2 cells reached to very high" - No references were cited for this statement.
Response #2-(3). According to the reviewer’s comment, we added several references in p4.

>(4). Page 2 Para 3 "It was well known that Drosophila melanogaster expressed gamma-glutamyl carboxylase"-This is rather a strong statement with only one cited reference.
Response #2-(4). According to the reviewer’s comment, we changed the sentence and added another reference in p4.

>(5). Page 9 Para 1 "When the expanded cells in a 25 cm2 flask were at 80% confluency, the media were replaced with 5 ml complete media containing 2.0 g/ml puromycin. The cells were continuously cultured for 72 hr. When the cells were at 80% confluency, the media were replaced with 5 ml complete media containing 2.0 g/ml puromycin. This step was repeated once."- the steps, including "...was repeated once" have been repeated thrice.
Response #2-(5). We apologize for the confusion. We rephrased the sentences in p9.

>(6). Page 9 Para 2 "Convert from FBS positive media to FBS negative media"
- Should be conversion of FBS-supplemented media to FBS-free media. Page 9 Para 2 "hereafter the media were referred as incomplete media"- the term incomplete media is not appropriate - FBS-free media sounds better.
Response #2-(6). According to the reviewer’s comment, we changed these terms in p10.

>(7). Some of the abbreviations should be mentioned in full before the abbreviation itself. e.g. ELISA
Response #2-(7). According to the reviewer’s comment, we described full terms before abbreviating them (e.g. ELISA, TF) in p11-p12.

>(8). Page 11 Para 2 "After the selection, these cells were almost 100% expressing EGFP (data not shown)."- This could be one of the interesting result to show how efficient the selection of clones was performed. Besides, no method of selection was explained in the procedure. Figures with flow cytometry image should be displayed to support the statement.
Response #2-(8). The selection was performed by puromycin. After the selection, we routinely performed flow cytometric analysis to detect EGFP signals from stably transformed cells. We added a section in Materials and Methods and explained in the result section with a new figure (New Fig 3).

Reviewer #3
>(1). 1) The writing needs editing for clarity, redundant phrases (e.g., "On the other hand" throughout the text and inappropriate words (reproductive instead of reproducible -last paragraph discussion).
Response #3-(1). According to the reviewer’s comment, we asked Editage which provides English editing to improve this manuscript.

>(2). 2) This is an interesting straightforward technique paper describing transfection systems to synthesize bioactive Factor VII in drosophila 2 cells.
Response #3-(2). We thank to the reviewer.

>(3). 3) The authors claimed that Methods are reliable and reproductive (reproducible?) but it is unclear how many separate consistent experiments were carried out to confirm their claims. It is also unclear what were the transfection rates and levels of stability.
Response #3-(3). The experiments with the vectors pMAK80, pMAK85, pMAK86, pAMK132, and pMAK219 were carried out four times. The positive cells were around 2-5% at the initial transfection (added in p13). The stability of the transfected cells were similar to those in previous report (added in p14). Similar results in each test was

obtained. Moreover, other active vit.K dependent proteins were successfully expressed although the data will be shown in future communication. (Please see in Response #2-(2-1)). We also changed “reproductive” to “reproducible”.

>(4). 4) In the Results section, clarification of what is being compared is needed and it would be informative if actual p values were provided rather than just showing the statistical table for figure 4.
Response #3-(4). We added the explanation of comparisons in the materials & methods section in p12 and the result section in p14. We added actual p values in new figure 5.

>(5). The discussion does not adequately explain the relevance, limitations and benefits of their study. Overall, this paper describes a technique that could facilitate synthesis of functional human Factor VII which would be beneficial for clinical use.
Response #3-(5). According to the reviewer’s comment, we added the relevance, limitations and benefits of their study were added to revised report in p16-p18.

>(6) 1). One of the major rationale behind this study was to improve the production of rHFVII. How one can make sure that the yield from the proposed method is better than the previously reported techniques?
Response #3-(6). From previous reports and our report, the concentrations of recombinant proteins synthesized in insect cells were higher than those in mammalian cells. Sf9 cells, which is other insect cell line, are often used to synthesize recombinant proteins by infecting baculovirus vectors. In the system, Sf9 cells were dead due to cell lysis induced by the infection of baculovirus. Thus, it is hard to maintain stable transformants. In contrast, exogenous genes are stably integrated into the genome of S2 cells, and stable transformed S2 cells are easily maintained. This aspect is a great advantage when a bioreactor such as CELLine Bioreactor (Wheaton, Millville, NJ, USA) is used. These points were emphasized in the discussion in p16-p18.

>(7). 2) What was the rationale behind using cDNA encoding human F7 without native signal sequence (hF7 δ SIg)?
Response #3-(7). Secretion of recombinant proteins from S2 cells is very important factor, thus, secretion signal peptide should be carefully considered. Addition of BiP signal peptide instead of native signal peptide typically enhanced the efficiency of secretion in S2 cells. However, it was reported that native signal peptide of tissue plasminogen activator was very functional, and BiP diminished the secretion of rabies virus glycoprotein although native signal peptide was functional in S2 cells. Recently, Vatandoost et. al. reported a successful transient expression of biologically active human coagulation factor IX (FIX) in S2 cells. Although we never attempted to express FIX with its native signal peptide, we expressed FVII in S2 cells using both BiP and its native signal peptide. We confirmed that Bip signal peptide was more functional than the native FVII signal in S2 cells. We added this rationale in p16.

>(8). 3) Sufficient gamma-carboxylation is necessary for tissue factor binding and enzymatic activity (Br J Haematol. 1996 May;93(2):445-50). There is no experiment conducted in this manuscript that confirms the gamma-carboxylation status of the synthesized FVII protein. Techniques such as Barium citrate precipitation can be performed (Br J Haematol. 1996 May;93(2):445-50); (Proc Natl Acad Sci U S A. 1991 Sep 15; 88(18): 8101-8105).
Response #3-(8). As the reviewer mentioned, we did not evaluate the levels of the levels of gamma-carboxylation. Although we performed Barium citrate precipitation and detected gamma calboxylated FVII by western blotting, this method did not tell us how many Glu residues converted to Gra residues in Gra domain. To asses this point, we need to perform, Matrix-assisted laser desorption ionization (MALDI) mass spectrometry with time-of-flight (TOF) is necessary. We are now planning to evaluate efficiency of gamma carboxylation in all vit.K dependent proteins. (Please refer to Response#1-(3)).

>(9). 4) Production of rHFVII by cotransfection of cDNA encoding human F7 and VKORC1 has been reported (Thromb Res. 2008;122(3):405-10). Furthermore, simultaneous expression of both human γ-carboxylase (hGC) and human FVII genes in insect sf9 cells has also been reported (Biotechnol Lett. 2010 Jun;32(6):803-9). How this study differs from the two mentioned studies should be discussed.

	Response #3-(9). According to the reviewer's comment, we added the statements in p17-p18. >(10). 1) The present title does not properly justify the work done in this manuscript. A better title could be given. Response #3-(10). According to the reviewer's comment, we changed the title as "Successful synthesis of active human coagulation factor VII by co-expression of mammalian gamma-glutamyl carboxylase and modification of vit.K cycle in Drosophila Schneider S2 cells"

Successful synthesis of active human coagulation factor VII by co-expression of mammalian gamma-glutamyl carboxylase and modification of vit.K cycle in *Drosophila* Schneider S2 cells

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Abstract

Mammalian gamma-glutamyl carboxylase and reduced vitamin K are indispensable for synthesis of mature mammalian vitamin K dependent proteins including some of blood coagulation factors (factors II, VII, IX, and X). It was well known that *Drosophila melanogaster* expressed gamma-glutamyl carboxylase and possessed a vit.K cycle although native substrates for them have not been identified yet. Despite the potential capability of gamma calboxylation in *Drosophila melanogaster* derived cells such as S2 cells, *Drosophila* gamma-glutamyl carboxylase failed to gamma carboxylate a peptide fused to the human coagulation factor IX propeptide. Thus, it had been believed that the *Drosophila* system was not adequate to synthesize mammalian vit.K dependent proteins. Indeed, we previously attempted to synthesize biologically active factor VII in S2 cells although we were not able to obtain it. However, recently, a successful transient expression of biologically active human factor IX from S2 cells were reported. In the present study, several expression vectors which enables to express mammalian *GGCX*, *VKORC1*, and/or *PDIA2* along with *F7* were developed. S2 cells transfected with pMKA85, pMAK86, and pMAK219 successfully synthesized active FVII. Thus, mammalian *GGCX* was indispensable to synthesize active FVII while mammalian *VKORC1* and *PDIA2* were not critical but supportive factors for S2 cells.

Introduction

Mammalian gamma-glutamyl carboxylase (GGCX, encoded by *GGCX*) is indispensable for the synthesis of mature mammalian vitamin (vit.)K-dependent proteins; e.g., coagulation factors II, VII, IX, and X (FII, FVII, FIX, and FX, respectively) and anti coagulation factors such as protein (P)C and protein (P)S (Suttie, 1980). These proteins have a so-called Gra domain at the N terminus; gamma-glutamyl carboxylation of glutamic acids in the Gra domain is necessary for protein maturation (Stenflo and Suttie, 1977), a reaction that is catalyzed by GGCX with reduced vit.K acting as a cofactor. Reduced vit.K is produced by the vit.K cycle, in which vit.K 2,3-epoxide reductase (VKOR) plays a major role. Although the enzyme itself has not yet been homogeneously purified, the gene has been identified as vitamin K epoxide reductase complex, subunit 1 (*VKROC1*). *GGCX* and/or *VKORC1* deficiency result in the synthesis of proteins induced by vit.K absence or antagonist, which do not bind negatively charged phospholipids with calcium ions and are thus unable to function as mature proteins.

Recombinant activated FVII (rFVIIa) has been approved for the treatment of hemophilia and congenital FVII deficiency, and can also restore coagulopathy in traumatic or postoperative bleeding and postpartum hemorrhage (Dutta and Verma, 2014). Several mammalian cell lines including HEK293 human embryonic kidney, Chinese hamster ovary, and baby hamster kidney cells have been used to synthesize recombinant FVII and other vit.K-dependent proteins (Bohm et al., 2015); however, as the yield is low, a more efficient large-scale culture system is needed.

The *Drosophila melanogaster* Schneider (S)2 cell line was established in 1972 (Schneider, 1972). These cells grow rapidly at room temperature, without a need for CO_2. Many studies have demonstrated that recombinant proteins can be highly expressed in S2 cells (Bernard et al., 1994; Hill et al., 2001; Lehr et al., 2000; Li et al., 1996; Nilsen and Castellino, 1999; Park et al., 2001). Our group has successfully expressed several human and murine proteins related to coagulation and fibrinolysis in S2 cells using the pMT-PURO2G expression vector, including plasminogen, urokinase-type plasminogen activator, FXII, high-molecular weight kininogen, and prekallikrein along with many variants of these proteins (Iwaki and Castellino, 2008). We then subcloned the coding sequences of several human and murine vit.K.-dependent proteins into the pMT-PURO2G vector and analyzed their expression. Although the proteins were secreted, they had no demonstrable activity (data not shown).

D. melanogaster expresses GGCX (Li et al., 2000; Walker et al., 2001) and has a vit.K cycle (Robertson, 2004), although the endogenous substrates have yet to be identified. However, *Drosophila* GGCX failed to gamma carboxylate a peptide fused to human FIX propeptide. It was therefore concluded that the *Drosophila* system is unable to synthesize mammalian vit.K-dependent proteins.

A recent study reported the transient expression of biologically active human FIX in S2 cells; full-length human FIX cDNA (*F9*) was subcloned into the pMT-V5 HisA vector and protein expression was driven by metallothionein promoter (P_{MT}), while secretion was initiated by the *F9* native signal peptide. Since these observations contradicted our unpublished data, in the present study

we established a new expression system that enabled the generation of stable transformants in S2 cells with gamma carboxylation activity.

Materials and methods

Construction of pCoPGE, pCoPGKE, and pCoVKE

A co-expression vector (pCoPURO, Fig.1A) (Iwaki et al., 2003) was used for an inverse polymerase chain reaction (PCR) using two primers: pMT-PURO2.tagF; 5'-GAGGCCCACCGACTCTAGATCAAGC, and pMT-PURO2.KozakR;

5'-GGTGGCGGCGCAAGCTATCGAATTCCTGCAGCCCG

(the Kozak sequence was underlined). The resulted amplicon was used as a backbone for pCoPURO2G, pCoGGCX, pCoVKORC1, and pCoPDAI2 described later. All PCRs in this study were carried out using PrimeSTAR® HS DNA Polymerase (TAKARA BIO, Japan) according to manufacturer's instruction.

A coding sequence of a fusion protein of puromycin resistant marker (*pac*) and enhanced green fluorescent protein (EGFP) was taken from pMT-PURO2G (Iwaki and Castellino, 2008) by a PCR using two primers: PURO.F; 5'-ATGACCGAGTACAAGCCCACGGTG and 5'-EGFP.R; TTACTTGTACAGCTCGTCCATGC. The amplicon was phosphorylated by T4 polynucleotide kinase (NEB Japan, Tokyo, Japan), and then was ligated to the backbone using Quick Ligation Kit (NEB Japan). The resulting plasmid was named pCoPURO2G (Fig.1B).

A coding sequence of murine gamma-glutamyl carboxylase (*Ggcx*) was taken from a FANTOM clone (ID#6820418L05, DANAFORM, Japan) by a PCR using two primers: Ggcx.F; 5'- ATGGCTGTGCACCGCGGCTCCGC and Ggcx.R; 5'- TCAGAACTCAGAGTGAACATGCTCAGAATCTGG. A coding sequence of murine vitamin K epoxide reductase complex, subunit 1 (*Vkorc1*) was taken from a FANTOM clone (ID#1110001K05, DANAFORM) by a PCR using two primers: Vkorc1.F; 5'- ATGGGCACCACCTGGAGGAGCCC and Vkorc1.R; 5'- TCAGTGCTTTTTGGTCTTGTGTTCTGGTACCTTCTG. A coding sequence of murine protein disulfide isomerase family A, member 2 (*Pdia2*) was taken from a FANTOM clone (ID#1810041F13, DANAFORM) by a PCR using two primers: Pdia2.F; 5'- ATGGACAAGCAGCTTCTGCCAGTGTTGCTGC and Pdia2.R; 5'- CTACAGCTCCTCCTTGGGACCCAAGG. Each amplicon was phosphorylated and ligated to the backbone. The resulting plasmids were named pCoGGCX, pCoVKORC1, and pCoPDAI2 (Fig.1C, 1D, and 1E, respectively).

The new co-expression vector (pCoPURO2G, Fig.1B) was used for an inverse PCR using two primers VKBinvF; 5'-AGCTGCGGCCGCAAGGCGCGCCCCACAGAATCAGGGGATAACGCAGGA AAGAAC (The underlined parts; *Not*I recognition site and *Asc*I recognition site, respectively) and VKB/MinvR; 5'-CCTTGCGGCCGCAGCTTGTACATTCCTAGGCCGTATTACCGCCTTTGAGT GAGCTGATACC (The underlined parts; *Not*I recognition site and *Avr*II recognition site, respectively) to introduce new restriction enzyme recognition sites. The resulting amplicon was digested by *Not*I (NEB Japan) and *Avr*II (NEB

Japan), and then the fragment was used to generate pCoPGE described in the next step.

An expression unit for murine *Ggcx* driven by Copia promoter (P_{COPIA}) and poly A signal (pA) was taken from pCoGGCX by a PCR using two primers: VKMinvF; 5'- GCGCACTAGTTTTCCCCGAAAAGTGCCACCTGACGTC (The underlined part; *Spe*I recognition site) and VKB/MinvR. This amplicon was digested by *Spe*I (NEB Japan) and *Not*I, and then ligated to the previous fragment. The resulting plasmid was pCoPGE (Fig.1F). An expression unit for murine *Vkorc1* driven by Copia promoter (P_{COPIA}) and poly A signal (pA) was taken from pCoGGCX by a PCR using two primers: VKMinvF and VKB/MinvR. This amplicon was digested by *Spe*I and *Not*I, and then ligated to pCoPGE digested by *Not*I and *Avr*II. The resulting plasmid was pCoPGKE (Fig.1G). An expression unit for murine *Pdia2* driven by Copia promoter (P_{COPIA}) and poly A signal (pA) was taken from pCoGGCX by a PCR using two primers: VKMinvF and VKB/MinvR. This amplicon was digested by *Spe*I and *Not*I, and then ligated to pCoPGKE digested by *Not*I and *Avr*II. The resulting plasmid was pCoVKE (Fig.1H).

Construction of pMAK80, pMAK85, pMAK86, pMKA132, and pMAK219

cDNA encoding human *F7,* which is the gene for human coagulation factor VII (FVII) was taken from a cDNA clone (MGC:163340, IMAGE:40146499) by PCR using following primers: MAK80F; 5'-CTCGCTCGGGAGATCTGCAGTCTTCGTAACCCAGGAGGAAGCC (The

underlined part; *Bgl*II recognition site), and MAK80R; 5'-CGAAGGGCCCTCTAGACTAGGGAAATGGGGCTCGCAGG (The underlined part; *Xba*I recognition site). This fragment was subcloned to pMT-PURO2G (Fig.2A) (Iwaki and Castellino, 2008) digested by *Bgl*II (NEB Japan) and *Xba*I (NEB Japan) using In-Fusion® HD Cloning Kit (TAKARA BIO) according to manufacturer's instruction (pMAK80, Fig.2B).

An expression unit for human *F7* driven by P_{MT} and poly A signal (pA) was taken from MAK80 by a PCR using two primers: MAK80F-LIC; 5'-GGTAATACGGCCTAGGCTGCAAGGCGATTAAGTTGGGTAACGCCAG (The underlined part; *Avr*II recognition site) and MAK80R-LIC; 5'-GCGCGCCTTGCGGCCGCCGCAGCGAGTCAGTGAGCGAGGAAG (The underlined part; *Not*I recognition site). The amplicon was subcloned to pCoPGE digested by *Avr*II and *Not*I, pCoVKE digested by *Avr*II and *Not*I, and pCoPGKE digested by *Avr*II and *Not*I, and then pMAK85 (Fig.2C), pMAK86 (Fig.2D), and pMAK219 (Fig.2E) were generated, respectively. In order to replace BiP secretion signal in pMAK86 to native *F7* signal, a fragment containing *F7* signal sequence was taken from the cDNA clone (MGC:163340, IMAGE:40146499) by PCR using following primers: MAK132F; 5'- ATGGTCTCCCAGGCCCTCAGGC (The underlined part; the initial codon), and MAK132R; 5'-GGCACCGACAGGAGCGCTTGG (The underlined part; *Afe*I recognition site). For an overlapped and extension PCR, an additional fragment was taken from pMAK80 by a PCR using following primers: MAK80F-LIC and MAK132OLR; 5'-GGCCTGGGAGACCATATTGAGATCGGATCCCCCCTTTAG. These

amplicons were subcloned to pMAK86 digested by *Avr*II and *Afe*I (NEB Japan) using In-Fusion® HD Cloning Kit (TAKARA BIO) (pMAK132, Fig.2F).

Transfection of pMAK80, pMAK85, pMAK86, pMAK132, and pMAK219 to S2 cells

Non-transfected S2 cells (RIKEN BioResource Center, Tsukuba, Japan) were maintained at 27°C in ExpressFive SFM (Life Technologies Japan) supplemented with 10% fetal bovine serum (FBS, Life Technologies Japan) and 1 x antibiotic antimycotic solution (Sigma-Aldrich Japan, Japan) (hereafter the media were referred as FBS-supplemented media). S2 cells (2 x 10^5) were seeded to each well of a 24-well plate containing 1 ml of FBS-supplemented media, and the cells were grown for 16 hr at 27°C. A lipofection was employed to produce stable transformants using HillyMax transfection reagent kit (Dojindo, Japan). Briefly, 1 μg pMAK80, pMAK85, pMAK86, pMAK132, or pMAK219 was mixed with 30 μl ExpressFive SFM, and then 5 μl HillyMAx reagent was mixed. The mixture was incubated at RT for 15 min, and the added to each well. After 4 h, the media were changed, and the cells were incubated for 48 h.

Puromycin selection

When the expanded cells in a 25 cm^2 flask were at 80% confluency, the media were replaced with 5 ml FBS-supplemented media containing 2.0 μg/ml puromycin, and the cells were continuously cultured for 72 hr. These procedures were repeated twice.

Flow cytometric analysis

Flow cytometric analysis was performed to detect the fluorescent signals generated by enhanced green fluorescent protein (EGFP) in stably transformed cells. This analysis was performed using a FACS Aria cytometer (BD, Franklin Lakes, NJ, USA).

Convert from FBS-supplemented media to FBS-free media

After the selection, the cells were subcultured in ExpressFive SFM supplemented with 7.5% FBS and 1 x antibiotic antimycotic solution. The reduction of FBS was sequentially performed at each passage. After 5-6 passage, the media were totally free of FBS with 1 x antibiotic antimycotic solution (hereafter the media were referred as FBS-free media).

Western blot for recombinant human FVII

After establishing stable transformants with FBS-free media, the cells (1 x 10^7) were seeded onto a 6-well plate containing 3 ml FBS-free media. In order to induce the expression of recombinant FVII, 3 μL of 500 mM $CuSO_4$ with or without 3.0 μL of 10 mg/mL phytonadione (vit.K; Vitamin K1 injection, Isei-Pharma, Japan) was added to each well, and the cells were incubated for 48 hr at 27°C. The culture supernatants (5 μl) were electrophoresed on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE). The gels were used to transfer the recombinant proteins to a PVDF membrane (Immobilo Western, Nihon Millipore,

Japan) for western blots. The membranes were exposed to a rabbit anti-human FVII polyclonal antibody (Cat.# ab97614; Abcam, Japan) and a goat anti-rabbit IgG-HRP conjugate (Cell Signaling Technology Japan, Japan), and visualized with a SuperSignal West Pico kit (Thermo Fisher Scientific, Japan) according to manufacturer's instructions.

Quantification of recombinant human FVII

The quantity of recombinant human FVII in the culture supernatant (N = 4) was measured by Enzyme-Linked Immuno Sorbent Assay (ELISA) using F7 (Human) ELISA kit (Abnova, Taipei City, Taiwan). Briefly, the culture supernatant (from 0.5 μl to 2.0 μl) was applied to a well of 96-well plate coated by polyclonal anti human FVII antibody, and then incubated for 2 h at RT. After washing, biotinylated polyclonal anti human FVII antibody was added to each well, and the mixture was incubated for 1 h at RT. After washing, streptavidin-peroxidase conjugate was added to each well, and then chromogenic substrate was added to determine the concentration of FVII.

Activity assay for recombinant human FVII

The FVII activity in all culture supernatant was evaluated by a clotting-time based method. In the assays, FVII deficient plasma and the supernatant was mixed and the clotting time was measured in a clinical laboratory center (SRL, Tokyo, Japan). The procoagulant was observed in the supernatant from S2/85, S2/86, or S2/219 cultured with vit.K in the media. Then, the

recombinant human FVII activity in the culture supernatant (N = 4) was measured by F7 (Human) Chromogenic Activity kit (Abnova). The assay measured the ability of lipoprotein tissue factor (TF)/FVIIa complex to activate factor X (FX) to FXa. The amidolytic activity of the TF/FVIIa complex was quantified by the amount of FXa produced using a highly specific FXa substrate releasing a para-nitroaniline (pNA) chromophore. The culture supernatant (0.5 μl to 2.0 μl) was applied to a well of 96-well plate coated by monoclonal antibody specific for human FVII. After washing, recombinant human TF and FX was added to each well, and then a highly specific substrate for FXa was added. The FVII activity was measured the change in absorbance of a yellow para-nitroaniline (pNA) chromophore released from the FXa substrate.

Statistical analyses

Data are expressed as mean ± SD. FVII antigen levels with and without vit.K were compared by two-way factorial analysis of variance (ANOVA). FVII antigen and activity levels with vit.K were evaluated by one-way factorial ANOVA followed by Tukey's post-hoc test. Analyses were performed using EZR (Kanda, 2013), and the null hypothesis was voided at $P < 0.05$.

Results

Construction of pCoPGE, pCoPGKE, and pCoVKE

We were able to successfully assemble pCoPGE, pCoPGKE, and pCoVKE (Fig. 1F, G and H, respectively). All of the vital parts, such as the

coding sequences for promoter (P_{COPIA}), poly A signal (pA), and each gene (PURO2G, *Ggcx*, *Vkorc1*, *Pdia2*) were verified by DNA sequencing (data not shown).

Construction of pMAK80, pMAK85, pMAK86, pMAK132, and pMAK219

pMAK80 was generated by insertion of cDNA encoding human *F7* without native signal sequence (h*F7*δSIg) into *Bgl*II and *Xba*I sites in the multiple cloning sites (MCS) of pMT-PURO2G (Fig. 2B). The signal sequence in pMAK80 was BiP, which enables to secrete the recombinant protein from S2 cells efficiently. The expression units were successfully transferred into pCoPGE (pMAK85, Fig.2C), pCoVKE (pMAK86, Fig.2D), and pCoPGKE (pMAK219, Fig.2E). The replacement of BiP signal sequence to *F7* native signal sequence was successfully performed (pMAK132, Fig.2F).

Establishment of stable transformants by puromycin selection

The positive cells were around 2-5% at the initial transfection. The S2 cells transformed with pMAK80, pMAK85, pMAK86, pMAK132, or pMAK219 were selected by puromycin. After the selection, these cells were almost 100% expressing EGFP (Fig.3). The viability and growing rates of these transformed cells were similar to those of non-transfected S2 cells, which was similar to previous reports (Iwaki et al., 2003). Hereafter, these cells were referred as S2/80, S2/85, S2/86, S2/132, and S2/219.

Western blots for recombinant human FVII

After the induction of the stable transformants by addition of $CuSO_4$ with or without vit.K, the recombinant FVII in the culture supernatant was detected by western blots. The expression of recombinant FVII in the culture supernatant with or without vit.K from S2/80, S2/85, S2/86, and S2/219 was clearly detected (Fig.4A, B). On the other hand, no signals were detected in the culture supernatant from S2/132 (Fig.4C).

Antigen and Activity assay for recombinant human FVII

There were no significant differences in FVII antigen levels by two-way factorial ANOVA when vit.K+/− and type of vector were used as independent variables. However, a significant difference was detected by one-way factorial ANOVA when vector type was an independent variable. The Tukey post-hoc analysis revealed that FVII antigen levels were lower in S2/132 than in S2/80, S2/85, S2/86, and S2/219 (Fig. 5A). Thus, vit.K supplementation did not affect the synthesis of FVII antigen in S2 cells; however, the native FVII signal peptide compromised FVII secretion from these cells. FVII activity was detected in the culture supernatant of S2/85, S2/86, and S2/219 with vit.K but not in that of cells cultured without vit.K; a significant difference was detected by one-way factorial ANOVA when vector type was an independent variable, and the Tukey post-hoc analysis revealed that FVII activity level was higher in S2/86 than in S2/219, which was in turn higher than that in S2/85 (Fig. 5B).

Discussion

We successfully expressed several human and murine proteins related to coagulation and fibrinolysis using the pMT-PURO2G vector (Iwaki and Castellino, 2008). Gene expression was induced by addition of $CuSO_4$ to the culture medium, and secretion of recombinant proteins was controlled by means of the BiP signal peptide.

In a recent study, biologically active human FIX was expressed in S2 cells by transient transfection of the pMT-hFIX expression vector without addition of vit.K. Moreover, co-expression of human *GGCX* further enhanced human FIX expression (Vatandoost et al., 2012). However, some caveats of their study were the presence of FBS in the culture supernatant, transient expression, and absence of vit.K in the medium. To address these issues, we developed a new expression system that enabled the generation of stable transformants without FBS.

S2/80 generated and secreted recombinant human FVII with or without vit.K; however, no activity was detected under either condition, indicating that *Drosophila Ggcx* and *Vkor* do not contribute to the gamma calboxylation of recombinant human FVII. Additionally, S2/85 and S2/86 generated and secreted recombinant human FVII with or without vit.K; however, the absence of vit.K was critical for the synthesis of active recombinant human FVII in S2 cells. On the other hand, S2/85, S2/86, and S2/219 produced active recombinant human FVII in the presence of vit.K. These results indicate that co-expression of mammalian *Ggcx* and vit.K supplementation are indispensable for the synthesis of active FVII

in S2 cells. *Drosophila Vkor* in S2 cells was functional, but co-expression of mammalian *Vkorc1* and *Pdia2* synergistically enhanced FVII activity, while native signal peptide inhibited FVII secretion in S2 cells (summarized in Fig. 6). On the other hand, these were unnecessary for the synthesis of biologically active recombinant human FIX (Vatandoost et al., 2012).

Secretion of recombinant proteins is an important aspect of expression systems. Addition of BiP instead of native signal peptide enhanced secretion efficiency in S2 cells (Kirkpatrick et al., 1995). On the contrary, it was reported that native signal peptide of tissue plasminogen activator—which was functional in S2 cells—was highly effective (Ivey-Hoyle et al., 1991a; Ivey-Hoyle et al., 1991b) whereas BiP reduced secretion of rabies virus glycoprotein (Dos Santos et al., 2009). Protein disulfide isomerase (PDI) is a thioredoxin-like oxidoreductase and chaperone that is present at high concentrations in the endoplasmic reticulum (Wajih et al., 2008) and provides electrons for reduction of the CXXC center in VKORC1 to enable the reduction of vit.K epoxide to vit.K hydroquinone by VKOR (Garcia and Reitsma, 2008). In this study, mammalian VKORC1 enhanced active FVII synthesis; thus, exogenous PDIA2 facilitated vit.K cycling in S2 cells. The signal peptide and Gra region of FVII differed from those of FIX; such differences may complicate gamma calboxylation and GGCX secretion. To evaluate the functional significance of these differences, we are currently carrying out experiments in which other vit.K-dependent proteins are expressed from the pCoVKE vector.

Precise determination of the number of Glu residues that are changed to Gra residues in the Gra domain is important. We performed barium citrate precipitation of recombinant FVII in preliminary experiments and confirmed the expression of FVII by western blotting; however, these experiments revealed only some Glu were converted to Gra residues. We have cloned the cDNAs for all vit.K-dependent proteins such that a hexa-histidine tag and C3 protease recognition site flank the Gra domain. We intend to analyze the expressed proteins by matrix-assisted laser desorption ionization–time-of-flight spectroscopy to determine the levels of gamma-carboxylation.

Bioactive recombinant FVII was expressed in HEK293 cells—which endogenously express human *GGCX*—by co-transfecting rat *Vkorc1* (Wajih et al., 2008); it was also expressed in Sf9 cells (another insect cell line) by co-transfecting human *GGCX* (Masroori et al., 2010). Mammalian cells are used to express bioactive vit.K-dependent proteins as therapeutic compounds, including recombinant FVII (Novo Nordisk, Princeton, NJ, USA), recombinant FIX (CSL Behring, King of Prussia, PA, USA), and recombinant activated protein C (Eli Lilly and Company, Indianapolis, IN, USA). These cells have a vit.K cycle and are capable of gamma carboxylation. However, given that the yield of recombinants proteins is relatively low, cost is major concern when using mammalian cell-based expression systems. Sf9 cells are useful for expressing various types of recombinant protein, although they require baculovirus infection and die during protein generation, precluding continuous production. In contrast, S2 cells can be maintained as stable transformants once properly selected; this is

an important consideration in large-scale culture systems, for instance when producing large amounts of recombinant protein using bioreactors.

The new recombinant mammalian vit.K-dependent protein expression system described herein yielded reliable and reproducible results. FVII is an essential factor for the coagulation cascade; our system can be easily expanded for mass production so that recombinant FVII can be produced in large quantities for clinical applications, for instance disseminated intravascular coagulation in trauma control units. The pMAK80, pMAK85, pMAK86, pAMK132, and pMAK219 vectors will be made available from the Riken BioResource Center (http://www.brc.riken.jp/lab/dna/).

Acknowledgments

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Figure Legend

Figure 1. Map of (A) pCoPURO, (B) pCoPURO2G, (C) pCoGGCX, (D) pCoVKORC1, (E) pCoPDIA2, (F) pCoPGE, (G) pCoPGKE, and (G) pCoVKE. PMT; Metallothionein promoter, PCOPIA; Copia promoter pA; SV40 late polyadenylation signal, AMP; ampicillin resistant gene, pUCori; pUC origin, PURO; puromycin N-acetyl-transferase (*pac*)、PURO2G; enhanced green fluorescent protein fused to *pac* gene. Small red and blue arrows indicate primers for inverse PCRs (A-B) and primers for excision of an expression casset for CoGGCX (C), CoVKORC1 (D), and CoPDIA2 (E). *Avr*II, *Not*I, and *Asc*I are restrction enzyme sites (F-H).

Figure 2. Map of (A) pMT-PURO2G, (B) pMAK80, (C) pMKA85, (D) pMAK86, (E) pMAK219, and, (F) pMAK132. PMT; Metallothionein promoter, PCOPIA; Copia promoter pA; SV40 late polyadenylation signal, AMP; ampicillin resistant gene, pUCori; pUC origin, BiP; secretion signal, MCS; multiple cloning site.

Figure 3. Fluorescent signals from the stably transformed cells. This showed fluorescent intensity of S2/86. Non-transfected cells (black line) and the cells after puromycin selection (red line).

Figure 4. Western blotting of FVII antigen in S2/80, S2/85, and S2/86 (A), S2/86 and S2/219 (B), and S2/86 and S2/132 (C).

Figure 5. (A) FVII antgen levels in S2, S2/80, S2/85, S2/86, S2/132, and S2/219. (B) FVII activity levels in S2, S2/80, S2/85, S2/86, S2/132, and S2/219.

Figure 6. Schema of recombinant FVII produced in S2/80, S2/85, S2/86, S2/132, and S2/219 with various parameters.

Figure1

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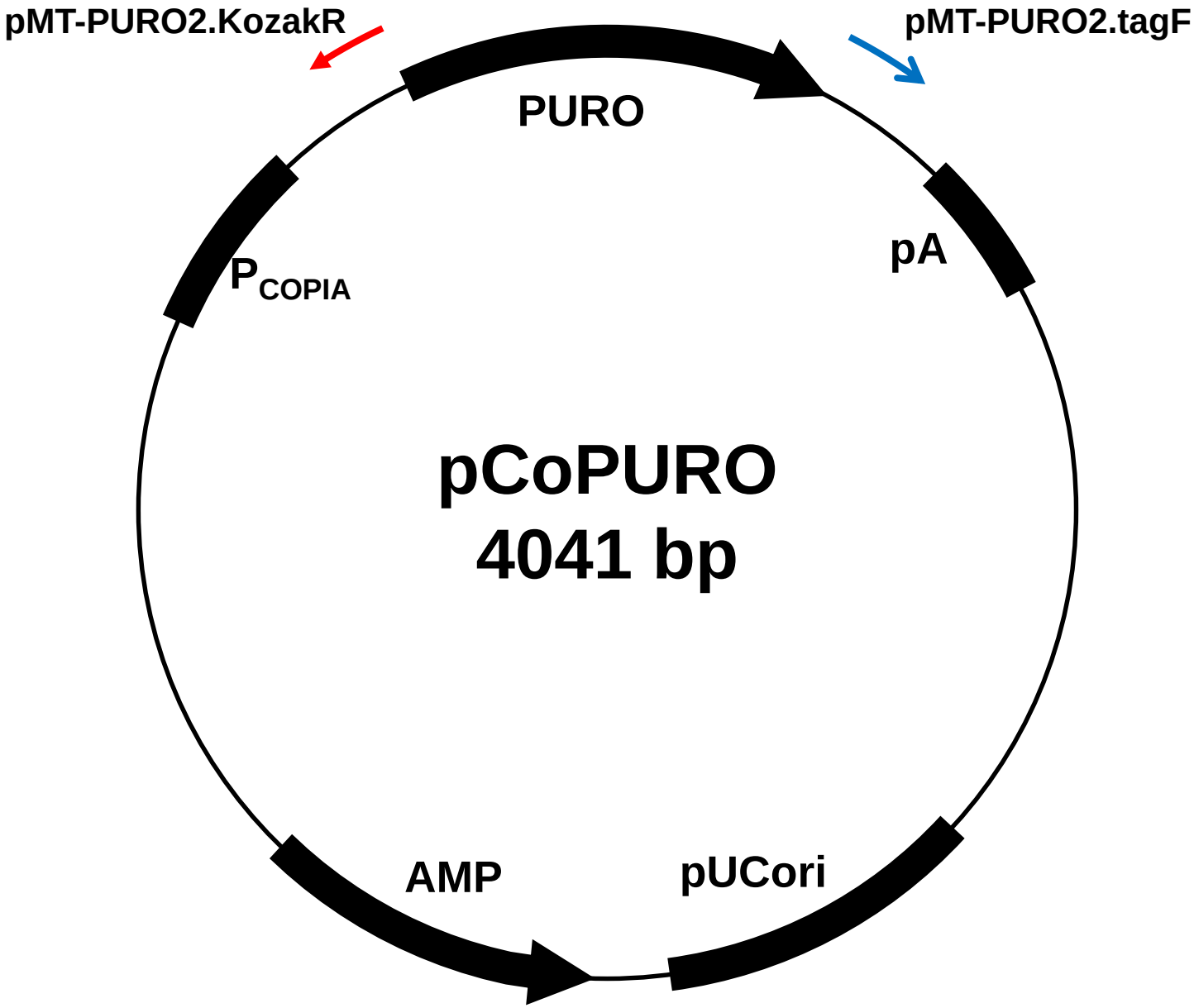

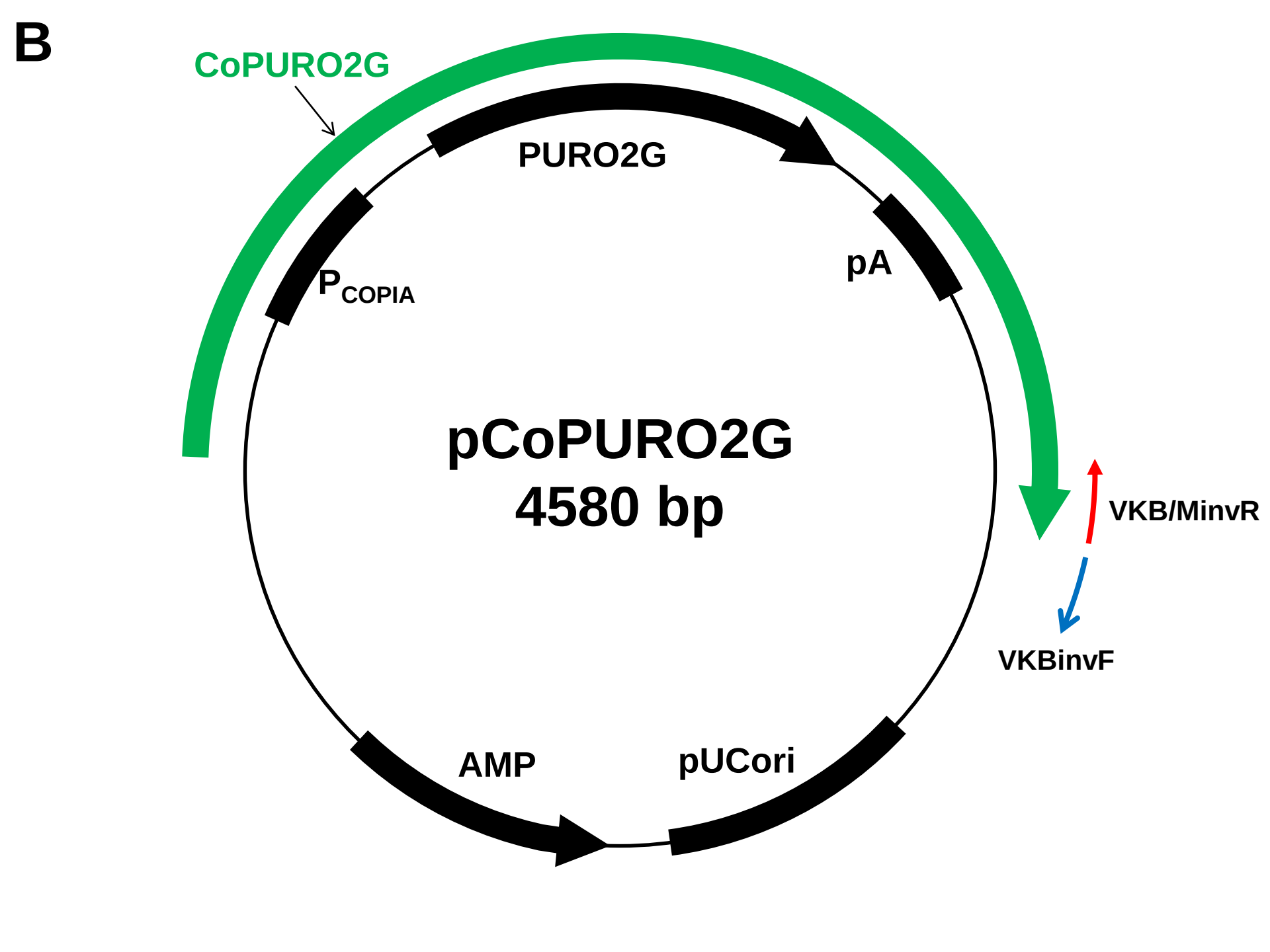
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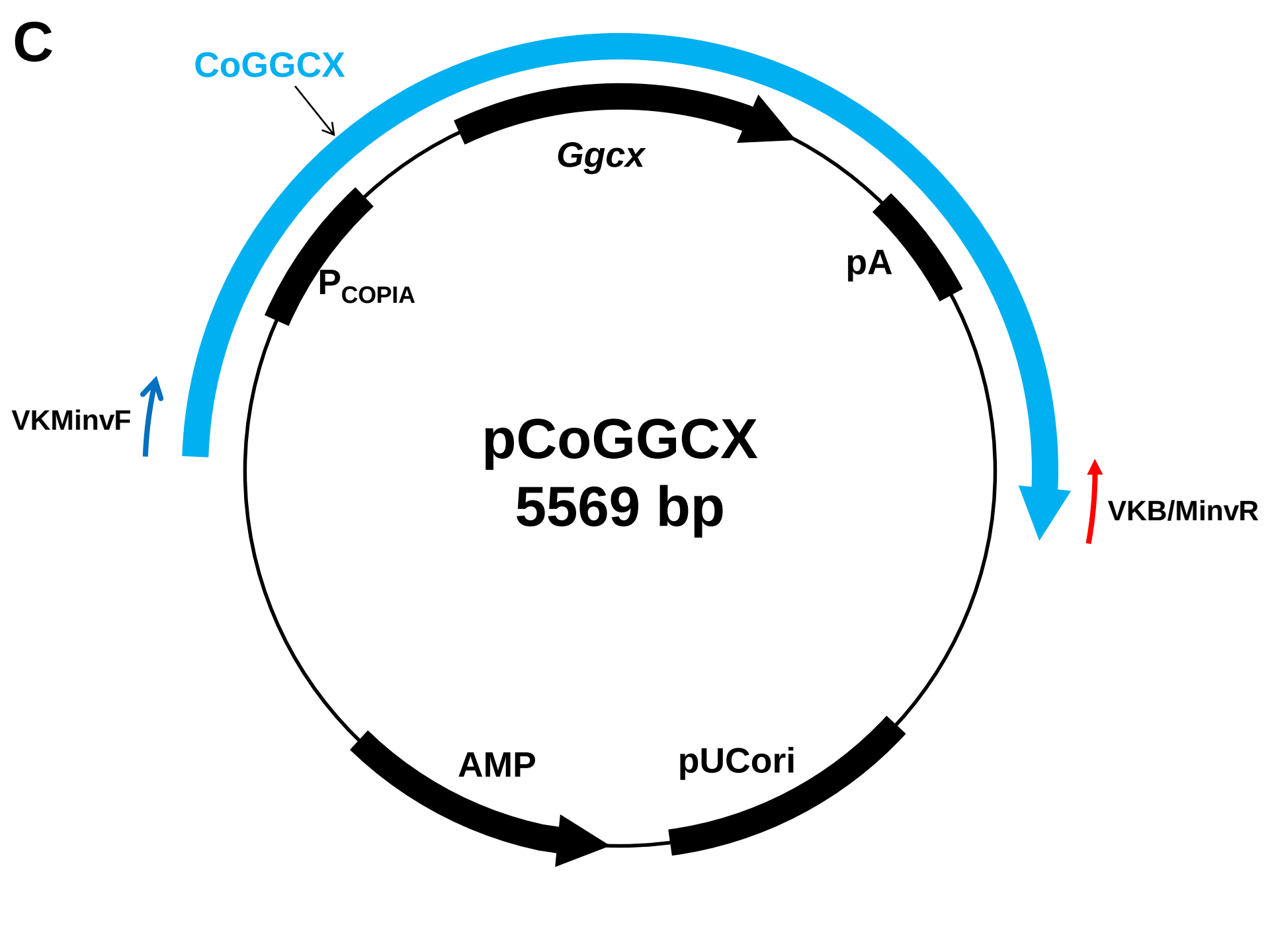
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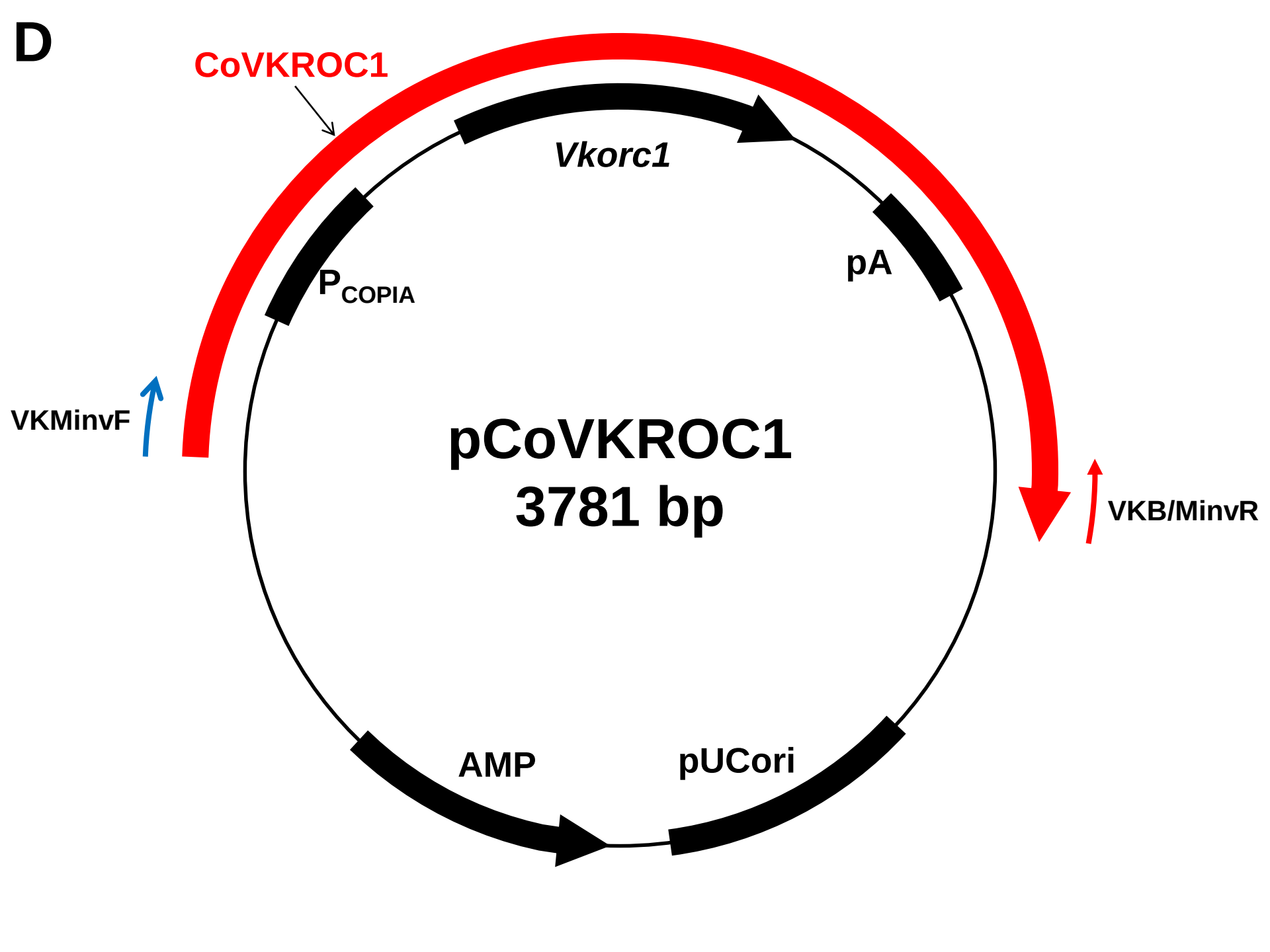
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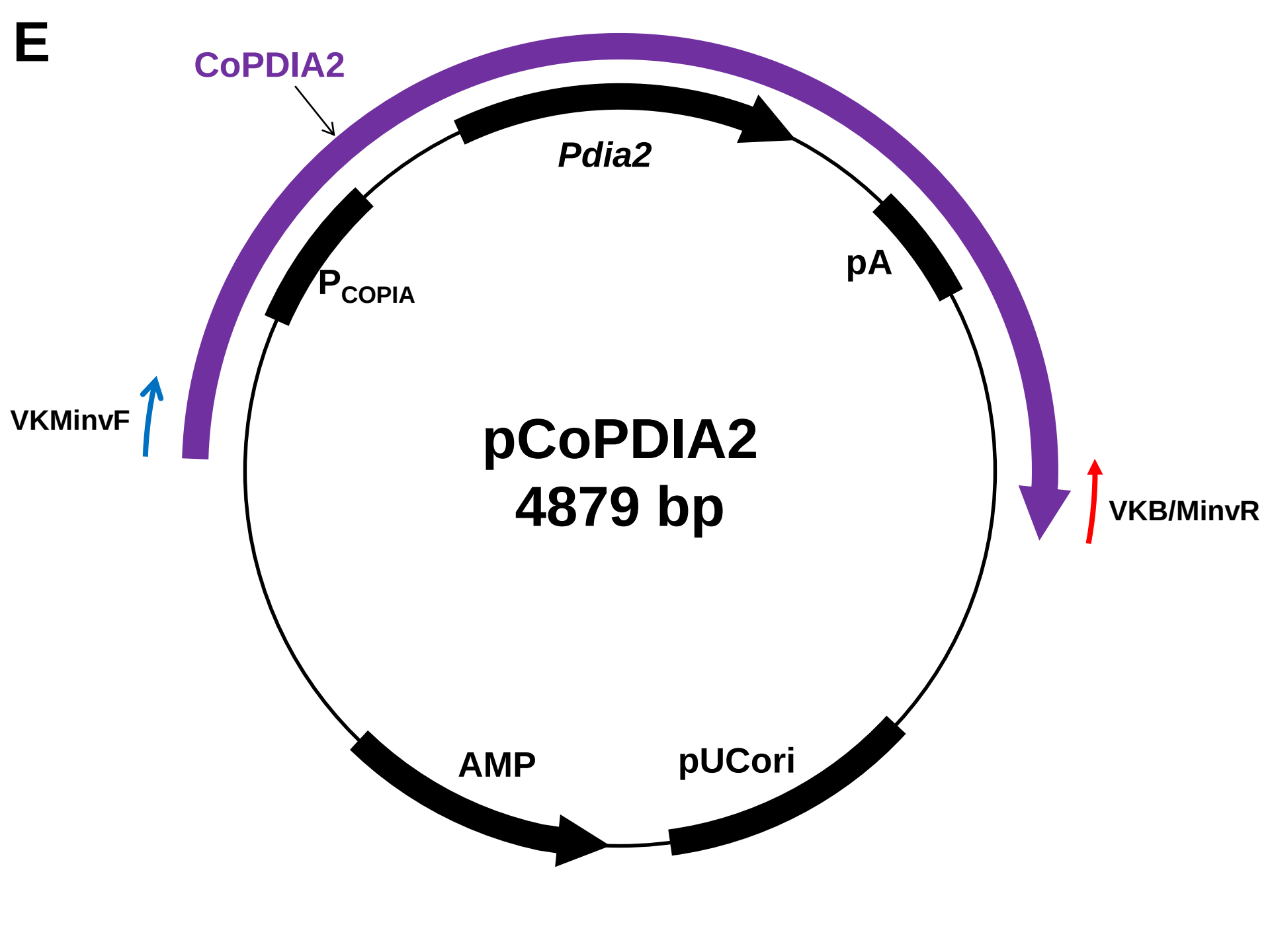
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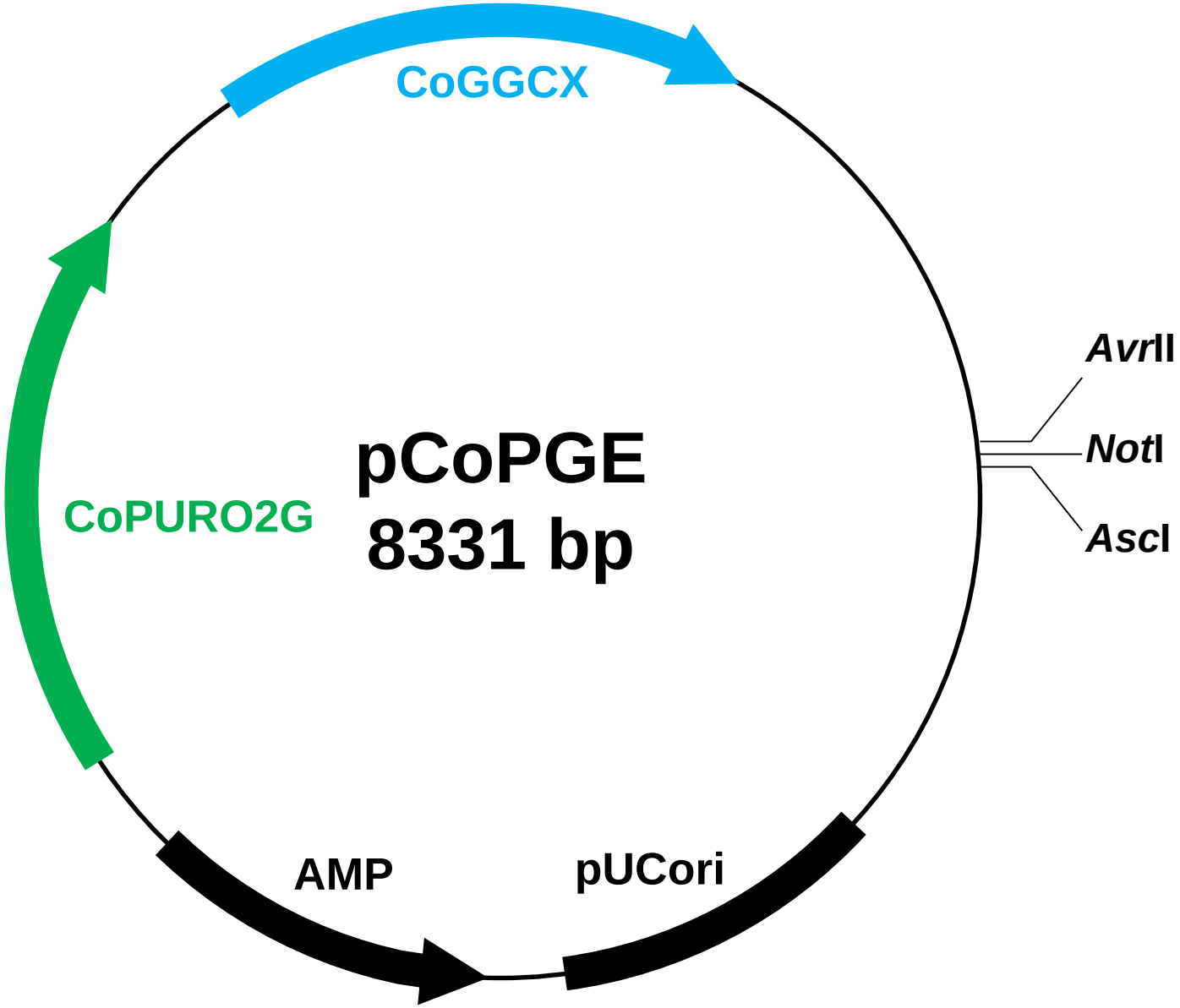

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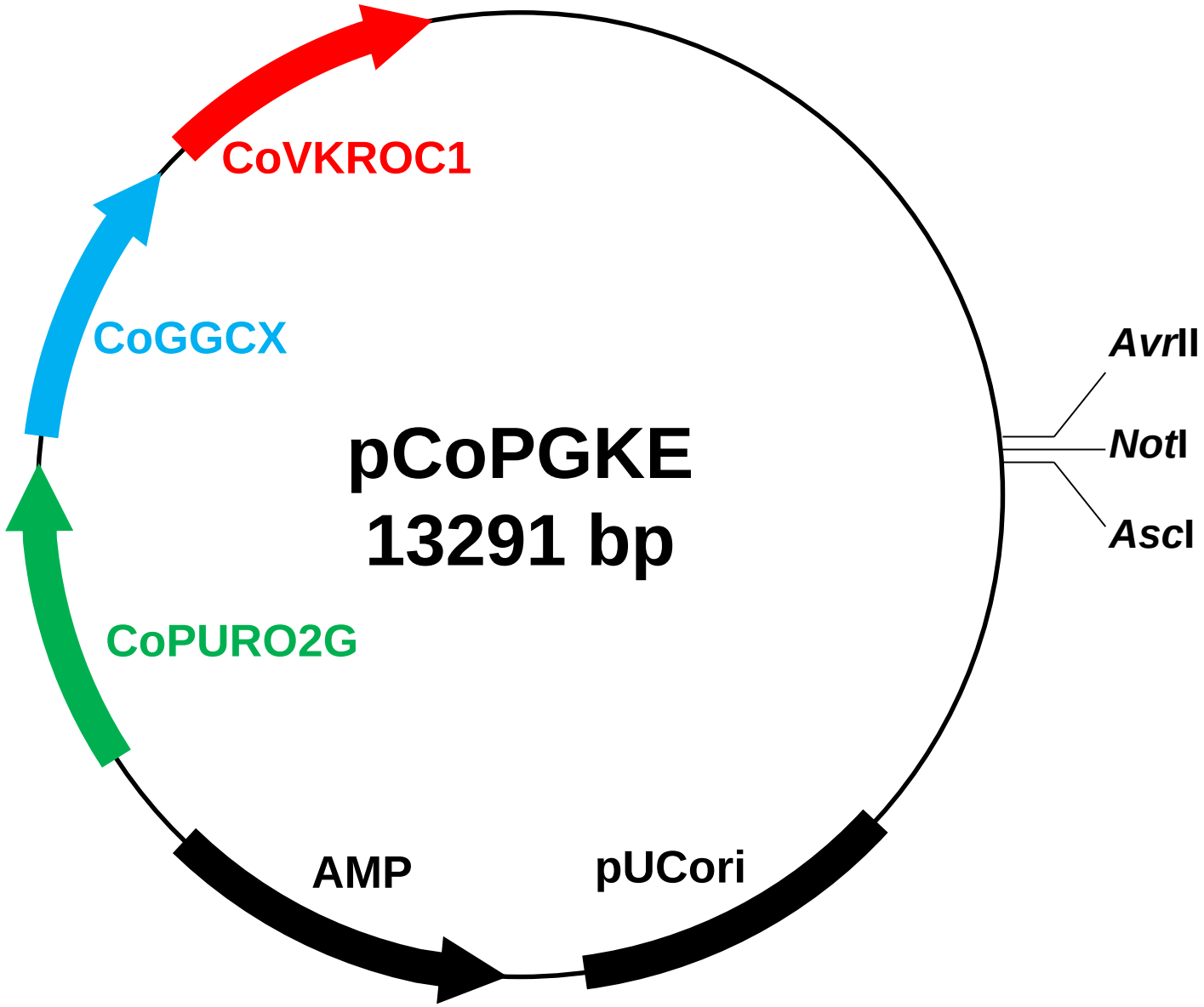
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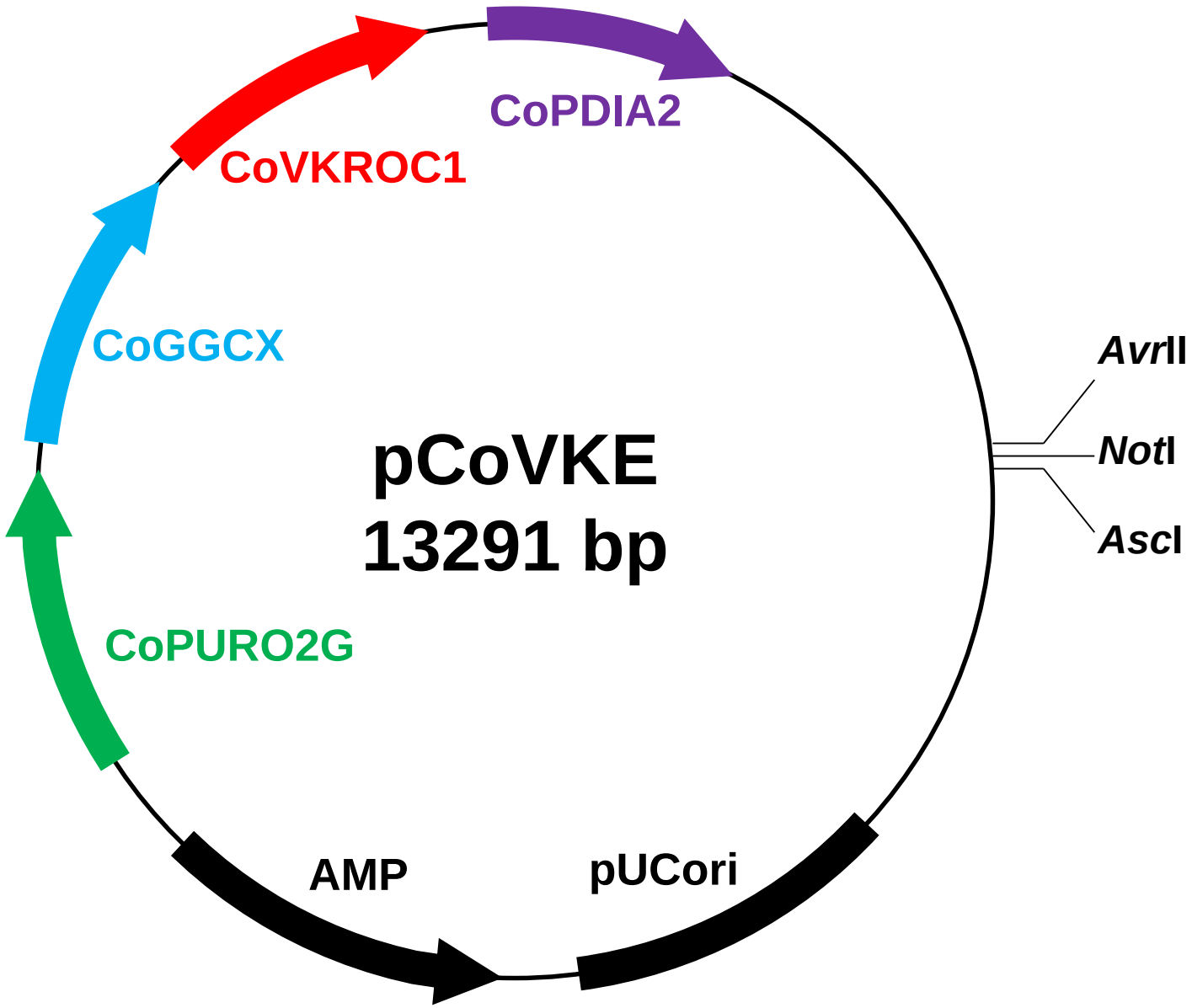
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Figure2

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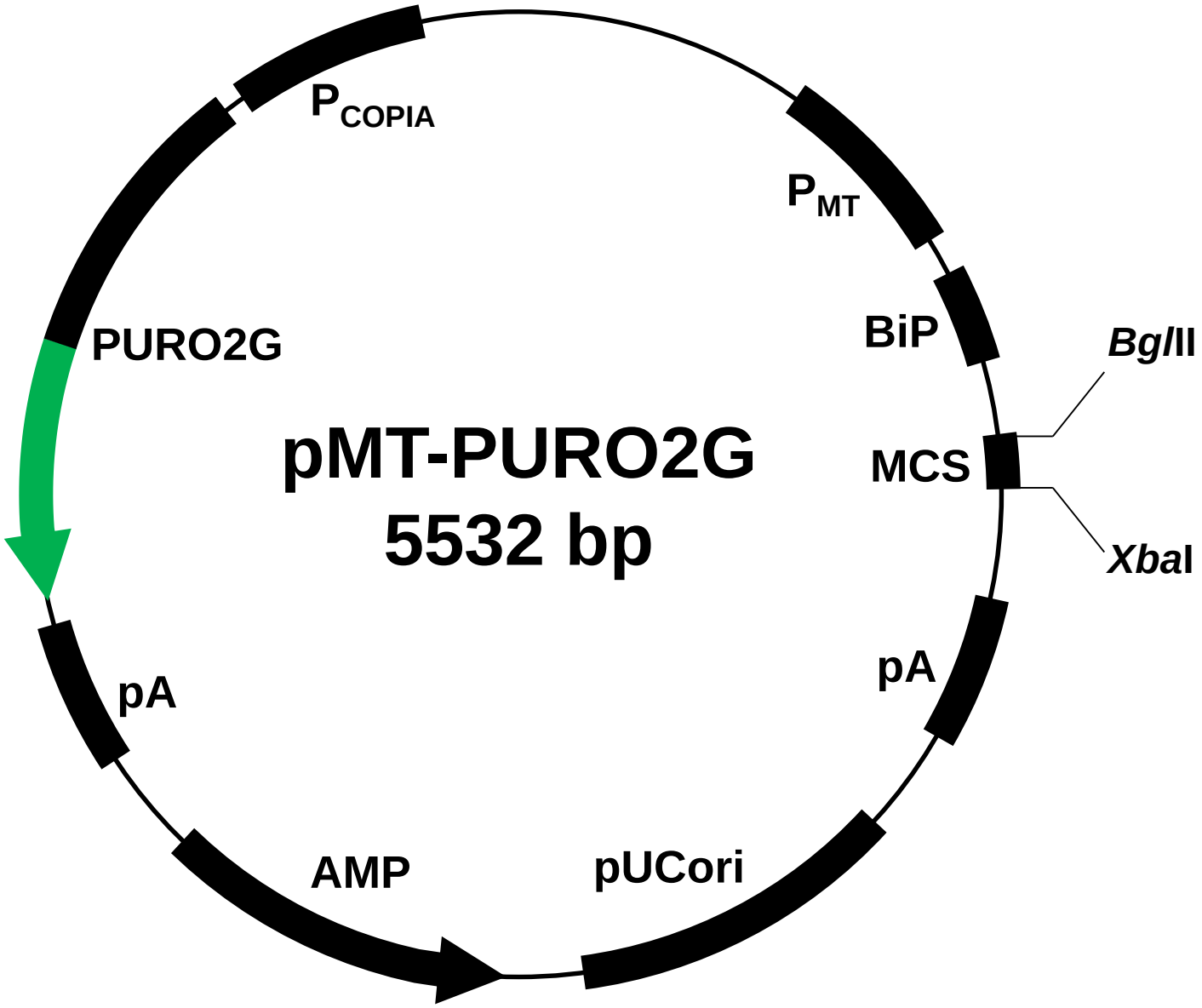

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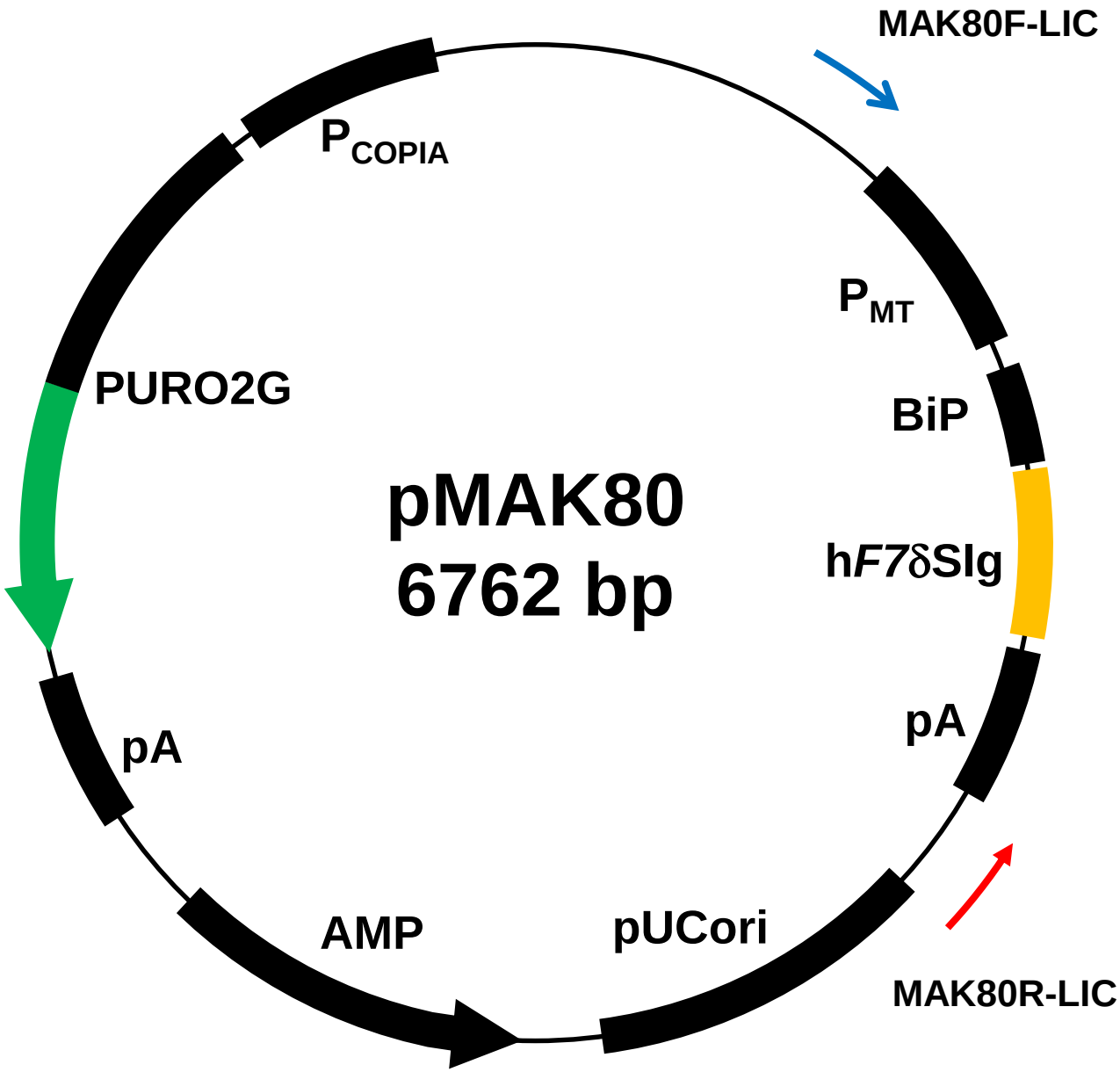
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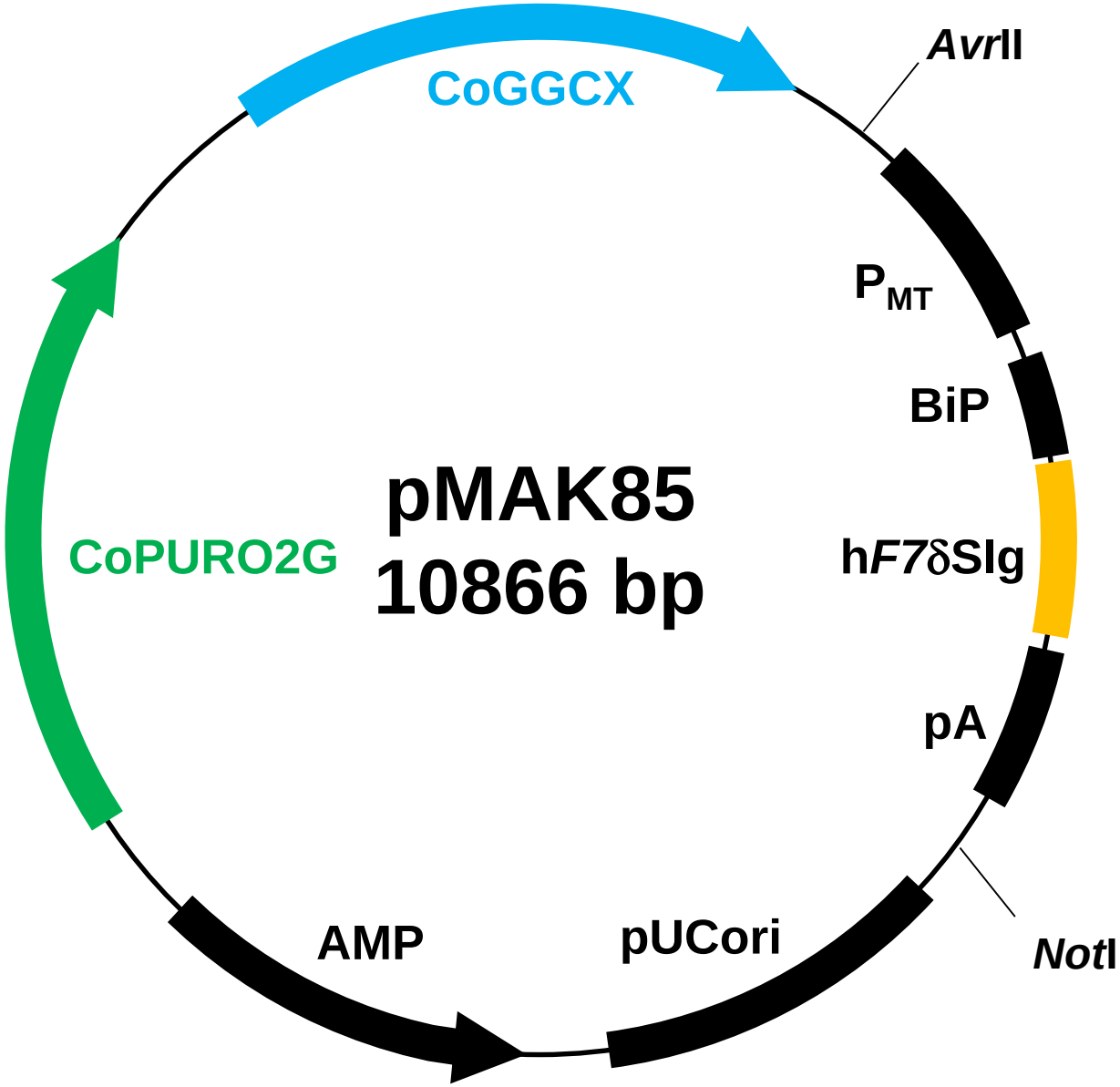
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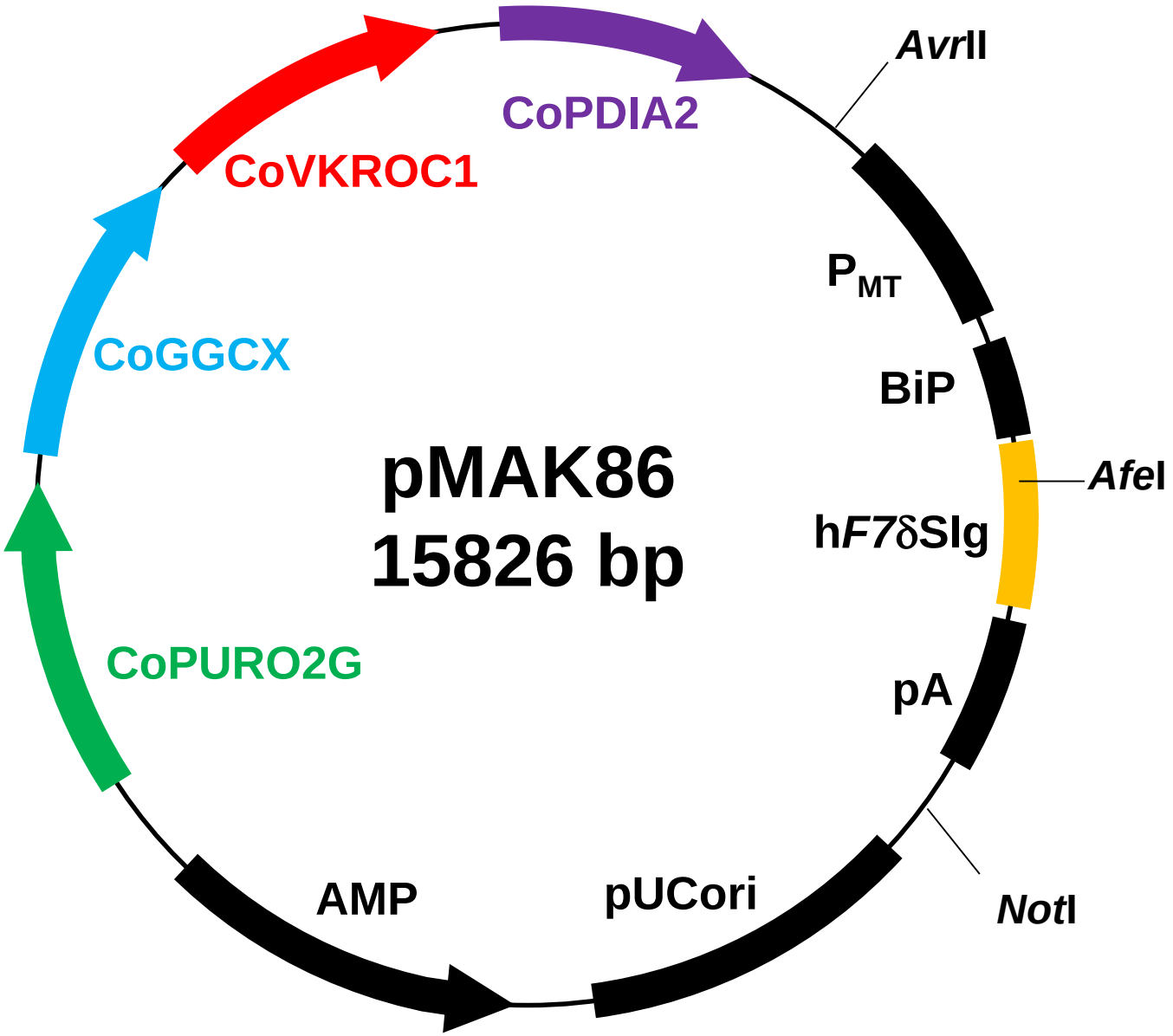
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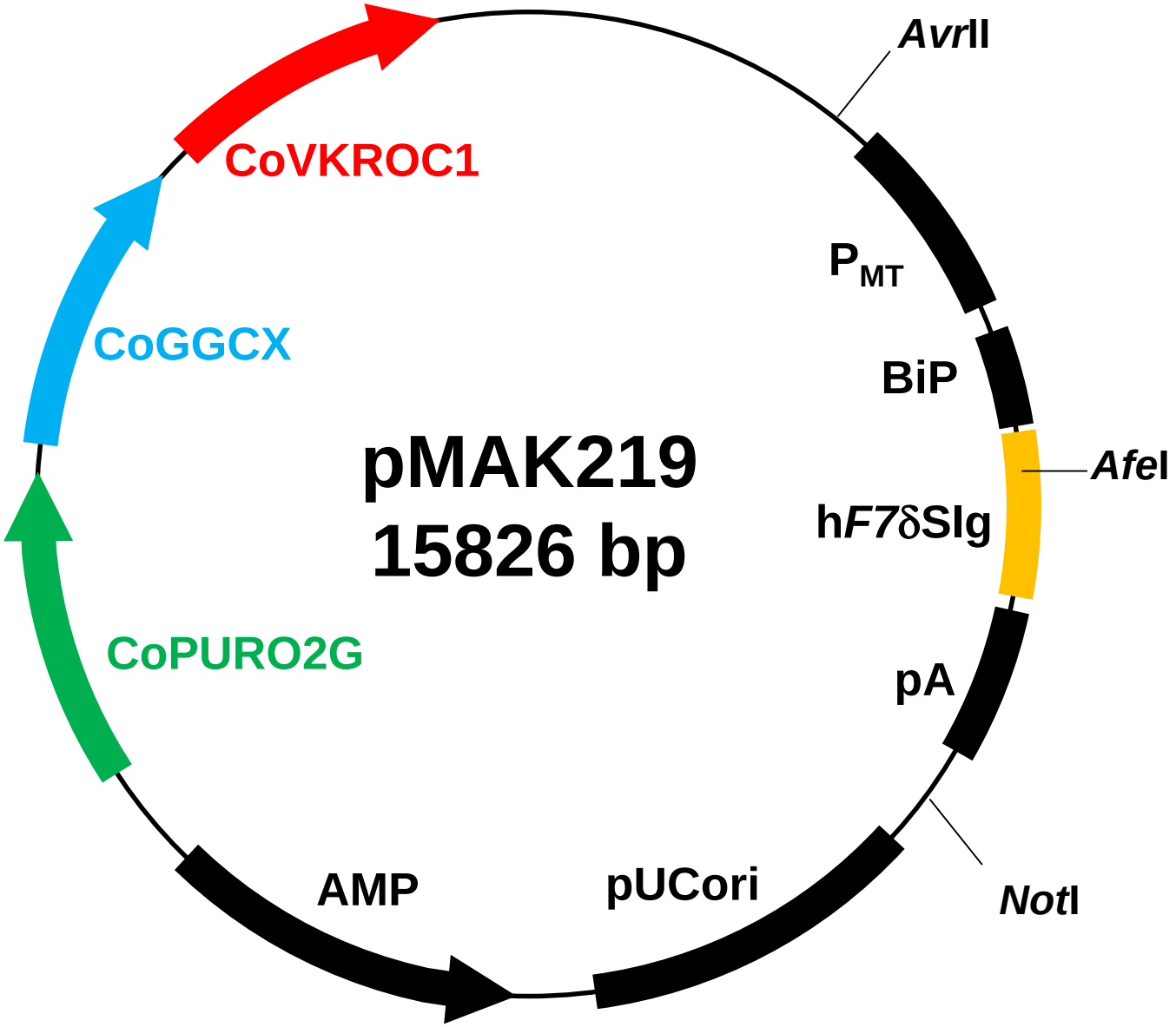
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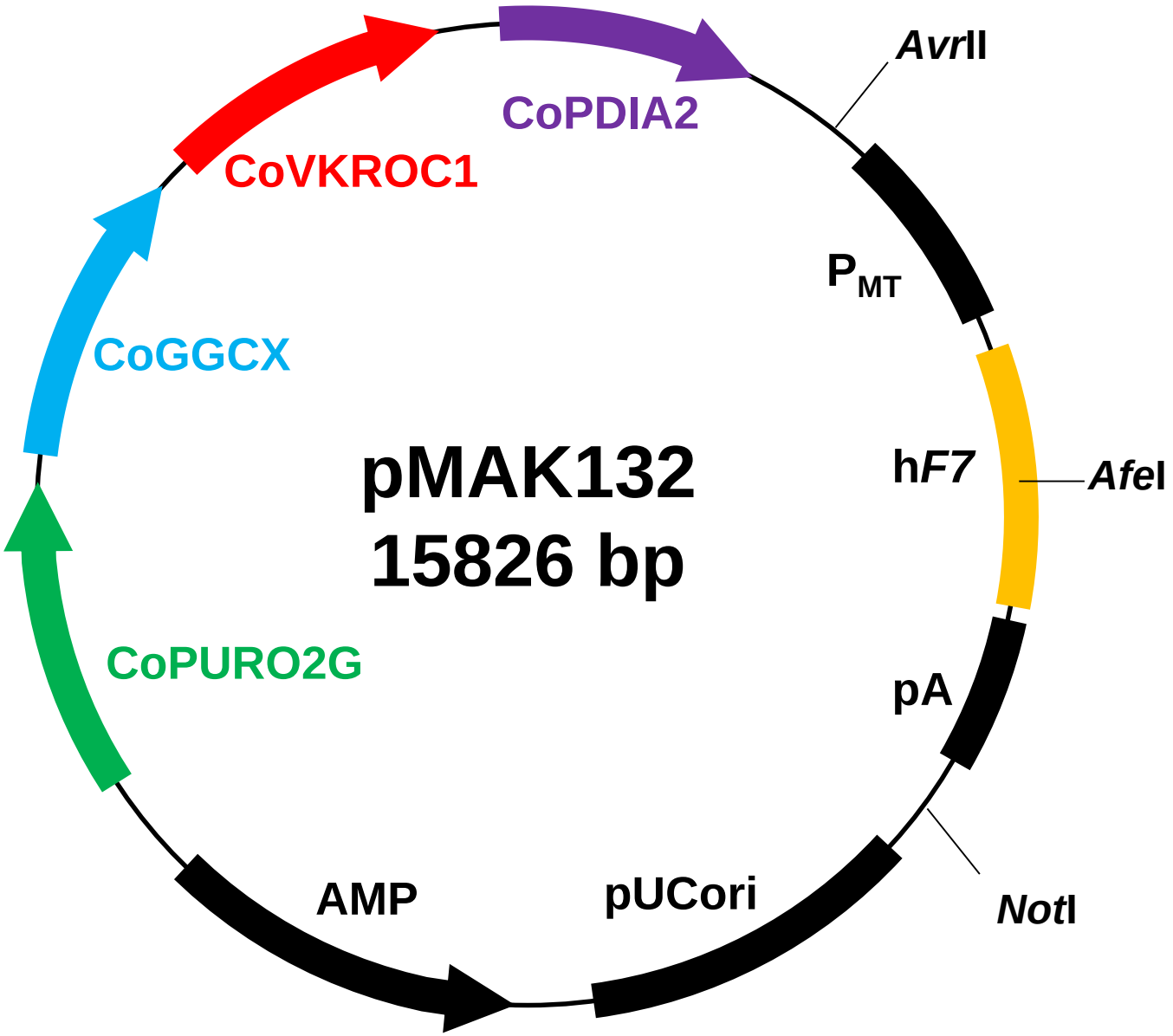
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Figure3

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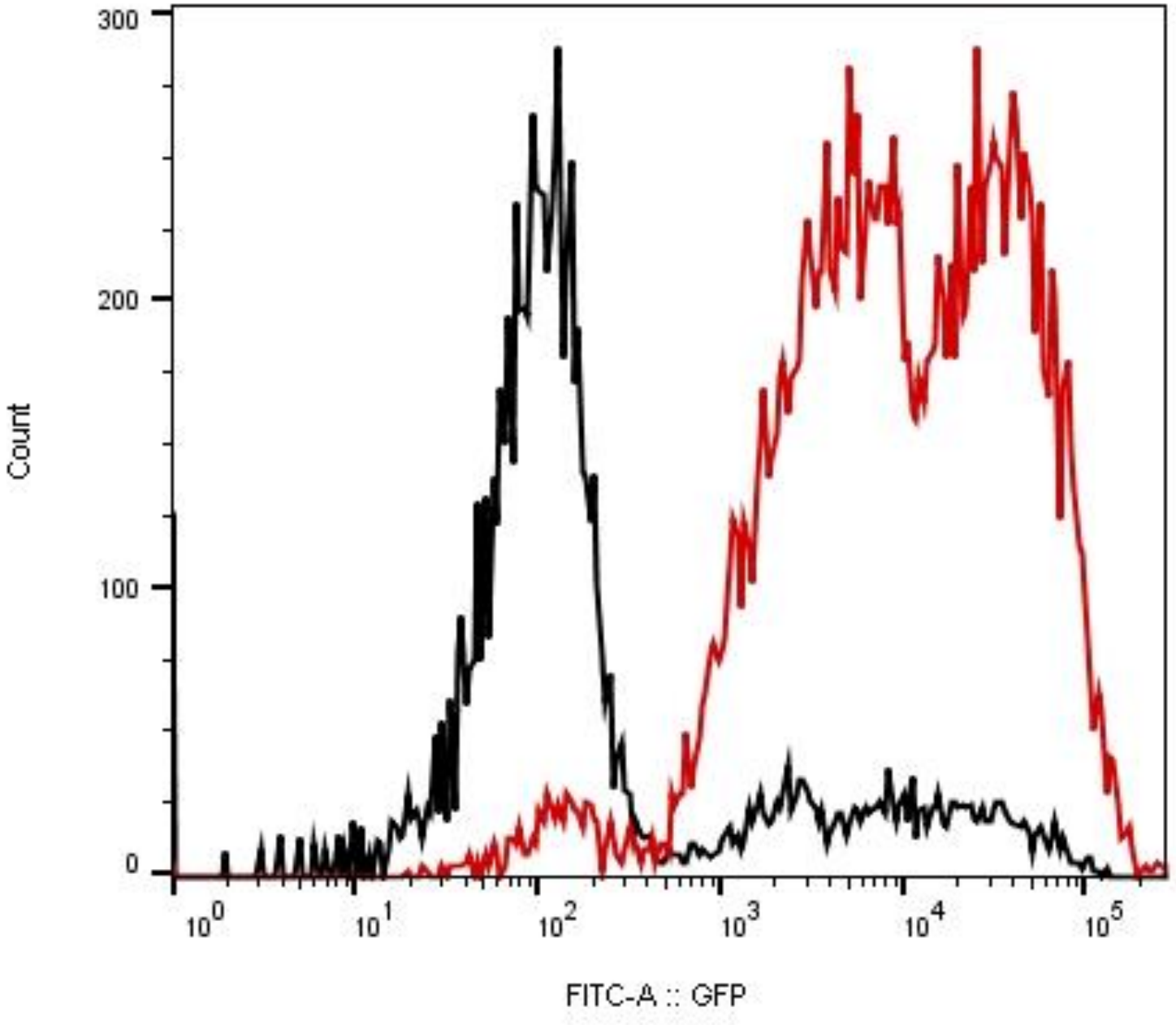

Figure4

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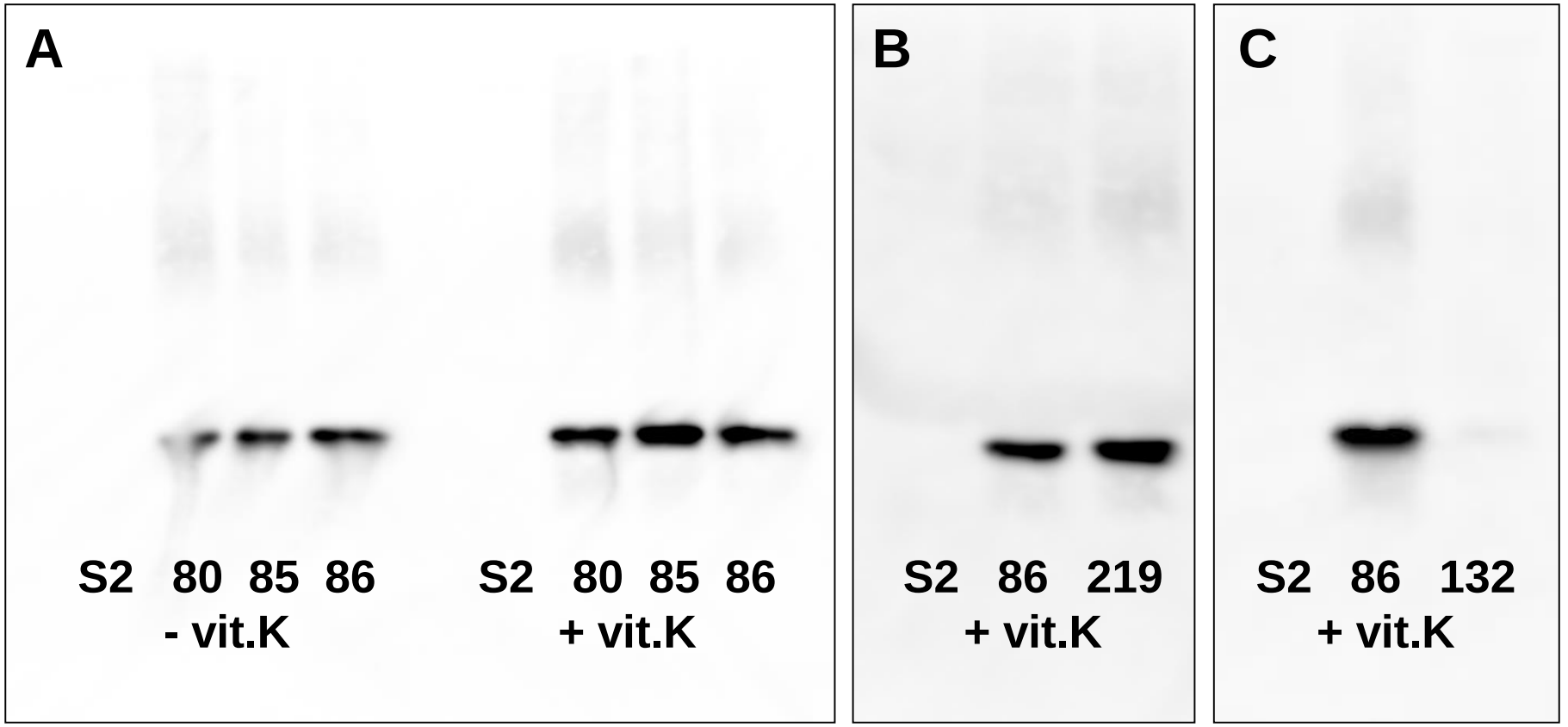

Figure5

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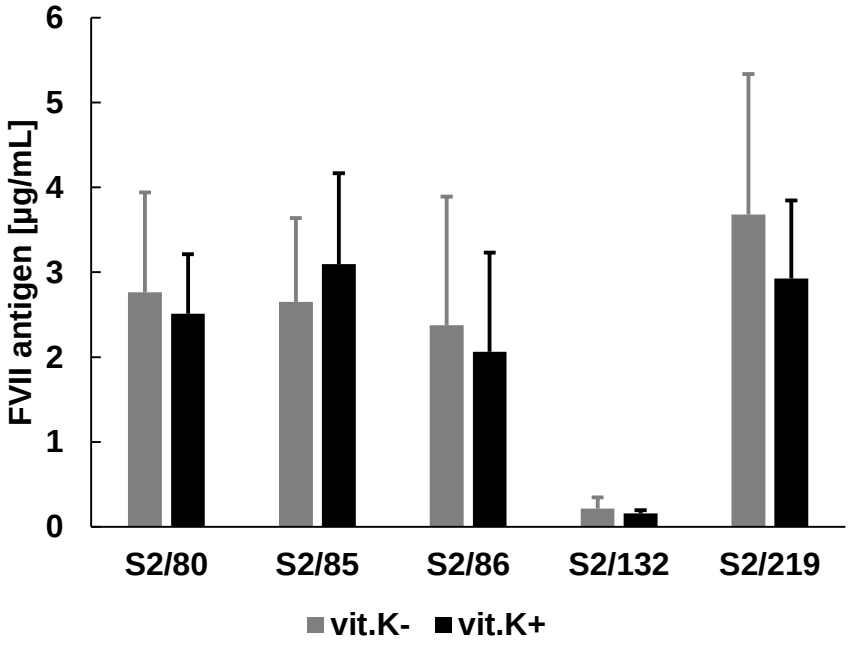

Post-hoc (Tukey) vit.K+

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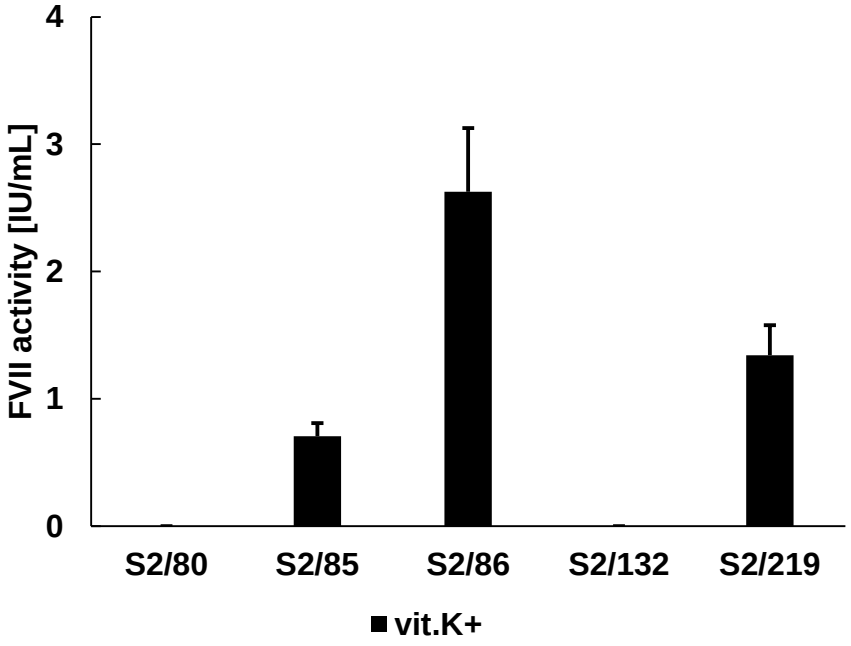
80		0.8750	0.9480	0.0130	0.9598
85			0.4811	0.0021	0.9986
86				0.0497	0.6410
132					0.0036
219					

B



Post-hoc (Tukey) vit.K+

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85			0.0000	0.0089	0.0198
86				0.0000	0.0000
132					0.0000
219					

Figure6

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