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Research Article

# Quantitative Analysis of γ-Aminobutyric Acid by Combined Cell-Free Protein Synthesis and Transamination Reactions

You Jin Lee, Hye Jin Lim, and Dong-Myung Kim*

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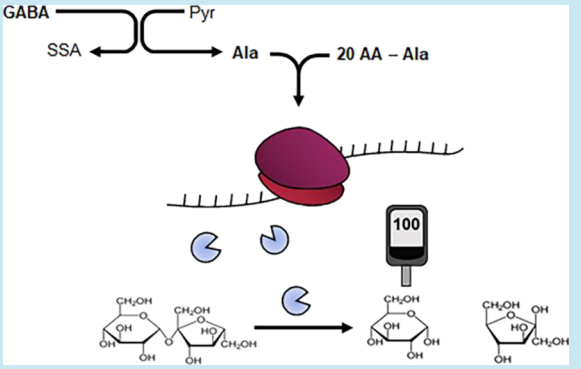
**ABSTRACT:** The synthetic power of cells can be harnessed for assaying important analytes, as well as for producing biomolecules. In particular, cell-free protein synthesis (CFPS) can be implemented as a signal amplification module for bioassays, while avoiding many problems associated with whole cell-based microbial biosensors. Here, we developed a method for analyzing γ-aminobutyric acid (GABA) by combining the enzymatic conversion of GABA and amino-acid-dependent CFPS. In this method, GABA molecules in the assay sample are used to generate alanine, which is incorporated into signal-generating proteins in the subsequent cell-free synthesis reaction. The activity of cell-free synthesized proteins was successfully used to estimate the GABA concentration in the assay sample. In principle, the developed method could be extended for the analyses of other important bioactive compounds.




**KEYWORDS:** *biosensor, cell-free synthetic biology, cell-free protein synthesis, synthetic biology, GABA, transaminase, alanine*



γ-Aminobutyric acid (GABA) is a major inhibitory neurotransmitter in the central nervous systems (CNS) of mammals. In addition to blocking impulses between nerve cells, many other functions have been reported for GABA including antihypertension,[1] antidiabetes,[2] anticancer,[3,4] anti-inflammation,[5] and intestinal protection activities.[6] Many medicines for neurological disorders have the mode of action that involves the modulation of GABA concentration in the brain.[7] Because of the potential health benefits, GABA-enriched food products are also actively investigated for the dietary therapeutic approach.[8] More recently, studies in mice and human cohorts have revealed that there is a strong correlation between the depression and GABA metabolism by the gut microbiota. For example, it has been documented that certain members of gut microbiota such as lactobacilli and biofidobacteria influence the CNS function by producing GABA.[9,10] The growing interest in GABA thus instigates the development of efficient tools to monitor its level in different assay samples. At present, GABA assay is commonly performed using high-performance liquid chromatography (HPLC) techniques. Although the HPLC assays can detect the micromolar concentrations of GABA,[11−13] they require chemical derivatization steps, long processing time, and expensive equipment with a large footprint. Compared with chromatographic methods, biological assays provide advantages including specific detection without physical separation of analytes. For example, different configurations of enzyme-linked immunosorbent assay (ELISA) have been developed to analyze GABA. Using highly specific antibodies, micromolar concentrations of GABA can be detected from crude samples by sandwich ELISA or competitive ELISA.[14,15] In addition, the speed and sensitivity of immunoassays can be improved by implementing electronic sensors to detect the interaction of GABA with antibodies. For example, by immobilizing anti-GABA antibodies on the electrode surfaces of quartz crystal or field-effect transistor sensors, the binding of GABA to antibodies can be monitored by the continuous measurement of impedance and conductance changes.[16−19] Nonetheless, these conventional or modified immunoassay methods are still subject to the intrinsic limitations of heterogeneous assays, including the requirement for multiple wash steps and sophisticated equipment to read electrical signals from the sensors.

In our previous report, we demonstrated that the bacterial translational machinery can be harnessed as a signal generation module for bioassays.[20,21] In this method, dubbed complementary cell-free protein synthesis (CCFPS) assay, a reaction mixture for cell-free protein synthesis (CFPS) is prepared in the absence of an amino acid to be analyzed. While the lack of an amino acid keeps the reaction mixture from producing full-length proteins, the addition of an assay sample containing the missing amino acid completes the set of 20 amino acids to allow for the generation of the reporter protein encoded in the

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template DNA. Because the yield of the reporter protein linearly correlates the titer of amino acids, the CCFPS assay enables the precise measurement of amino acids without involving complicated chromatographic separation procedures. In the present study, we designed a versatile method that enables a facile and accurate measurement of GABA by combining the molecular mechanisms of the transaminase-mediated synthesis of amino acids and CCFPS assay. Specifically, GABA molecules in the assay sample are first consumed as substrates by a transaminase, which generates equimolar amounts of alanine by transferring the amine moiety of GABA to pyruvate. The generated alanine molecules are then used to produce signal-generating proteins in the subsequent CCFPS assay (Figure 1). The modular nature of

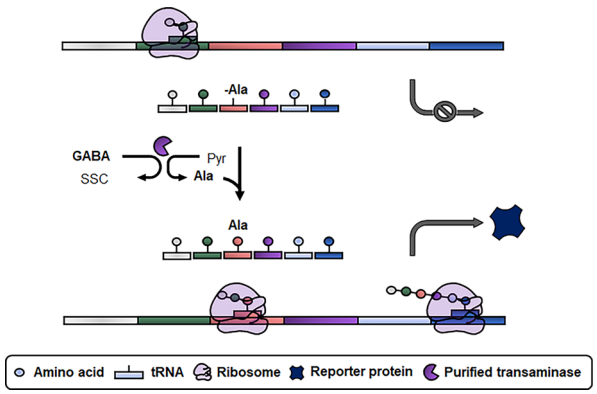


**Figure 1.** Scheme for generating reporter proteins by CCFPS combined with the enzymatic conversion of GABA. Transamination reaction between GABA and pyruvate produces alanine. Addition of the transamination reaction to the CCFPS reaction mixture completes the repertoire of 20 proteinogenic amino acids, leading to the production of reporter proteins encoded in the template DNA. SSA, succinic semialdehyde; Pyr, pyruvate; Ala, alanine.

this method enables the use of a variety of measuring instruments by programming the assay solution with genes encoding appropriate reporter proteins. Depending on the experimental settings, for example, the assay can be customized to measure the GABA concentration by fluorescence or electrical signals. Our results demonstrate the ability to harness the translational machinery to convert metabolic analytes into readily measurable reporter proteins.

## EXPERIMENTAL SECTION

**Materials.** Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, USA). DNA polymerase and other reagents for polymerase chain reaction (PCR) were purchased from Bioline (London, UK). Restriction enzyme and T4 ligase were obtained from Enzynomics (Daejeon, Korea). Luria-Bertani (LB) medium and ampicillin were purchased from Duchefa Biochemie (Haarlem, Netherlands). Ni-NTA agarose resin was from Qiagen (Hilden, Germany). Creatine phosphate and creatine kinase were purchased from Roche Diagnostics (Mannheim, Germany). All other chemical reagents and human serum were obtained from Sigma-Aldrich (St. Louis, MO, USA). The S12 extract for CFPS was prepared from *Escherichia coli* (*E. coli*) BL21star (DE3) cells (Invitrogen, Carlsbad, CA, USA) and diafiltered to remove residual amino acids, as described previously.[21,22]

**Preparation of Recombinant GABA Transaminase.** The gene-encoding GABA transaminase (GABA-TA) was PCR-amplified from the genomic DNA of *E. coli* and cloned into the pET-21a plasmid. Competent *E. coli* BL21 (DE3) cells were transformed with this plasmid and grown at 37 °C in 2.5 L baffled flasks containing 500 mL of LB medium. The expression of recombinant enzymes was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) when the $OD_{600}$ of the culture broth reached 0.6. After overnight culture at 30 °C following IPTG induction, cells were harvested by centrifugation (4500× *g*, 20 min) and washed thrice with deionized water. After the final washing step, cell pellets were resuspended in 20 mL of equilibrium buffer (300 mM NaCl, 50 mM $NaH_2PO_4$, pH 8.0) and disrupted by a single passage through a French Press (Thermo Fisher Scientific, Waltham, MA, USA) at 12,000 psi. After centrifugation at 12,000× *g* for 20 min, recombinant enzymes in the supernatant were purified using a Ni-NTA agarose column (Figure S1).

**Transamination Reaction to Generate Alanine from GABA.** The reaction mixture for the generation of alanine from GABA consists of 10 mM sodium pyruvate, 20 μM pyridoxal phosphate, 50 mM Tris−HCl (pH 7.5), 200 μg/mL purified transaminase, and sample solutions containing varying concentrations of GABA. After adjusting the final volume to 100 μL with water, the reaction mixture was incubated at 37 °C for 1 h.

**CCFPS Assay of Alanine Produced by Transamination Reaction.** The reaction mixture for CCFPS assay consisted of the following components in a final volume of 30 μL: 57 mM hydroxyethyl piperazineethanesulfonic acid (HEPES)-KOH (pH 8.2), 1.2 mM adenosine triphosphate (ATP), 0.85 mM each of cytidine triphosphate (CTP), guanosine triphophate (GTP), and uridine triphosphate (UTP), 2 mM dithiothreitol, 0.17 mg/mL *E. coli* total tRNA mixture (from the MRE600 strain), 0.64 mM cyclic adenosine monophosphate (cAMP), 90 mM potassium glutamate, 80 mM ammonium acetate, 12 mM magnesium acetate, 34 μg/mL l-5-formyl-5,6,7,8-tetrahydrofolic acid, 2 mM each of 19 amino acids without alanine, 2% polyethylene glycol 8000, 67 mM creatine phosphate, 3.2 μg/mL creatine kinase, 27% (v/v) of diafiltered S12 extract, and 6.7 μg of template DNA encoding reporter proteins between the T7 promoter and the T7 terminator.[21,22] After the addition of 30% (v/v) terminated transamination reaction, the assay mixture was incubated at 30 °C for 2 h. In the experiments using superfolder green fluorescence protein (sfGFP) as a reporter protein, 15 μL of the reaction sample was withdrawn and diluted 20-fold in phosphate-buffered saline (PBS) to measure fluorescence using a Victor X2 microplate reader (PerkinElmer, Waltham, MA, USA). In CCFPS assays programmed with the invertase gene, 15 μL of the reaction mixture was mixed with the same volume of 100 mM sucrose and further incubated for 10 min. A 5 μL sample of the reaction mixture was then used to measure the glucose titer using an Accu-Check Inform II personal glucose meter (PGM; Roche Diagnostics). In the experiments where human serum was used as a sample matrix, assay samples were diluted fourfold in water and heated to 90 °C for 10 min to inactivate nucleases. SPSS version 22.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses. $P > 0.05$ was considered statistically indifferent.


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## RESULTS AND DISCUSSION

**Measurement of Alanine by CCFPS Assay.** The CFPS approach allows mix-and-match formulation of individual components involved in the gene expression. Previously, we demonstrated that this feature of CFPS can be harnessed for the rapid analysis of proteinogenic amino acids.[20] In the CCFPS assay, a reaction mixture for CFPS is prepared in the absence of an amino acid to be analyzed. For example, the CCFPS assay of alanine (Ala-CCFPS) can be performed using a reaction mixture for cell-free synthesis of sfGFP prepared without alanine. When tested using standard solutions, the fluorescence intensity of sfGFP measured after 2 h incubation linearly correlated with the concentration of alanine within the range of 0 to 100 $\mu$M (Figures S2A and S2B).

**Cell-Free Protein Synthesis Combined with Transaminase-Mediated Amino-Acid Synthesis.** Transaminases catalyze the transfer of primary amines to carbonyl groups; hence, they are frequently employed for the production of chiral amino acids using an $\alpha$-keto acid as an amine acceptor. For example, transaminases can catalyze the synthesis of L-alanine by transferring an amine group to pyruvate from a variety of compounds. Based on this, we envisioned combining GABA−pyruvate transamination with Ala-CCFPS assay to estimate the GABA concentration in an assay sample by measuring the signal from the cell-free synthesized reporter protein. We used GABA-TA from *Escherichia coli* for this purpose. It was examined whether the concentration of GABA added to the transamination reaction could be estimated by the subsequent Ala-CCFPS assay. Transamination reactions were performed with varying concentrations of GABA and subsequently transferred to the Ala-CCFPS assay mixture containing the template DNA encoding sfGFP. In contrast to expectation, however, fluorescence from the Ala-CCFPS assay was not significantly correlated with the concentration of GABA used for the transamination reaction. Specifically, the Ala-CCFPS assay generated strong sfGFP fluorescence even when the prior transamination step was performed in the absence of GABA, and transamination reactions containing different GABA concentrations did not alter the fluorescence intensity (Figure 2A). Moreover, supplementation of additional alanine during the Ala-CCFPS assay did not markedly affect the final sfGFP fluorescence (Figure 2B). These results imply that GABA-TA and/or pyruvate transferred from the transamination reaction generates sufficient alanine to saturate the Ala-CCFPS assay. Because the GABA-independent sfGFP fluorescence remained high even after GABA-TA was heat-inactivated (Figure 2A), we suspected that residual pyruvate transferred from the transamination reaction was responsible for the synthesis of alanine during the CCFPS assay.

The cytoplasm of *E. coli* contains numerous transaminases,[23] and because transaminases generally exhibit broad substrate specificity, we reasoned that these *E. coli* transaminases likely catalyze transamination between some of the 19 amino acids in the CCFPS mixture and the transferred pyruvate. This assumption was supported by the observation that the reaction mixture for the Ala-CCFPS assay generated a high fluorescence signal when supplied with pyruvate alone. Compared with the control reaction in which the Ala-CCFPS step was performed without any additions, the presence of 3 mM pyruvate resulted in a more than twofold increase in sfGFP fluorescence to a level similar to that achieved when the Ala-CCFPS mixture was directly supplied with alanine (Figure 3A). Therefore, to prevent the generation of alanine during the Ala-CCFPS step, we decided to use a pan-transaminase inhibitor. We chose aminooxyacetate (AOA), a potent transaminase inhibitor possessing a broad inhibitory spectrum,[24−26] to inactivate endogenous transaminases in the S12 extract, and thereby reduce GABA-independent alanine generation. As expected, the addition of AOA (2 mM) to the Ala-CCFPS reaction markedly reduced the GABA-independent synthesis of sfGFP (Figure 3A). Although AOA slightly impaired the translational activity at this concentration (Figure S3A), by inhibiting endogenous transaminases, we anticipated the use of AOA would allow for the Ala-CCFPS assay to discriminate alanine generated by GABA−pyruvate transamination. Indeed, as

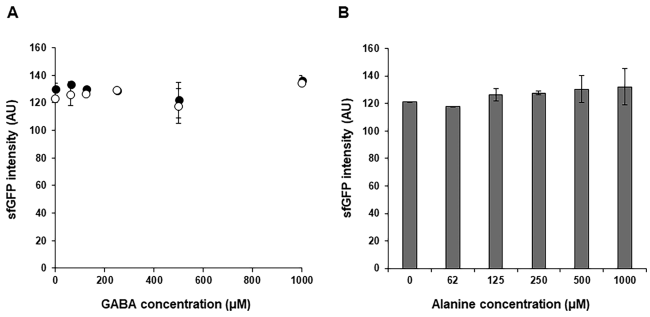


**Figure 2.** sfGFP intensity from CCFPS assay following transamination reaction. (A) Varying concentrations of GABA were used for transamination reaction with 3 mM pyruvate. When the aliquots of the transamination reaction were added, because of the high level of the background signal (0 mM GABA), no significant changes in the fluorescence intensity of sfGFP were observed with increasing concentrations of GABA used for the transamination reaction (blank circles). Inactivation of the transaminase by heat treatment (90 °C, 10 min) after the transamination reaction did not improve the response to the GABA concentrations (filled circles). (B) Supplementation of alanine to the transamination reaction did not increase the intensity of sfGFP fluorescence from the following CCFPS assay. Taken together, these results imply that the high background resulted from the transfer of pyruvate to the CCFPS reaction, rather than GABA-TA.

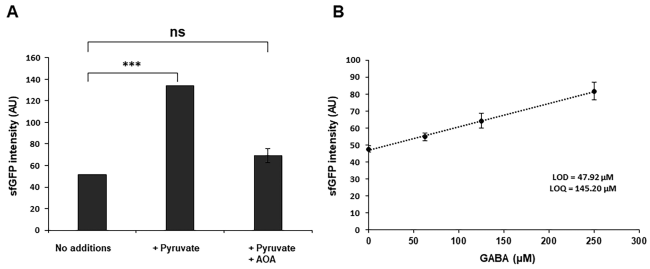


**Figure 3.** Inhibition of endogenous transaminases by AOA. (A) Assay mixture for Ala-CCFPS was supplemented with 3 mM pyruvate or 3 mM pyruvate plus 2 mM AOA. While the addition of pyruvate alone increased the sfGFP synthesis by the transaminase activity in the S12 extract, 2 mM AOA effectively inhibited the conversion of pyruvate to alanine, lowering the background level of sfGFP synthesis. (B) After solving the background problem by AOA addition, the CCFPS assay combined with transamination reaction produced a linear standard curve, depending on the GABA concentrations in the transamination reaction. Measurements were made in three independent experiments. Error bars represent standard deviations. LOD and LOQ were determined by the formulas: LOD = 3.3 × (Sy/S) and LOQ = 10 × (Sy/S), where Sy and S represent the standard deviations of y-intercepts of regression lines and the slopes of the curves, respectively.


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shown in Figure 3B, a linear standard curve of sfGFP fluorescence was obtained for different GABA concentrations in the transamination reaction when the reaction mixture for the Ala-CCFPS assay was supplemented with AOA. When supplied with GABA−pyruvate transamination reaction, the sfGFP fluorescence from the Ala-CCFPS assay displayed a linear response to the GABA concentration over the range of 0 to 1 mM. The limit of detection (LOD) and the limit of quantification (LOQ) were estimated to be approximately 47. and 145.2 $\mu$M, respectively (Figure 3B). The use of higher concentrations of AOA was not desirable as it severely decreased the translational activity (Figure S3B).

**GABA Analysis Using a PGM.** We predicted that the GABA assay could be made more accessible if the signal it generates could be read using commonly available devices. One advantage of the developed assay is that the analyte can be measured by the activity of various reporter proteins by switching the template DNA for the CCFPS assay step. To measure GABA using a PGM, the most widely distributed analytical device, the Ala-CCFPS reaction was programmed with the gene-encoding bacterial invertase instead of sfGFP. In this case, alanine generated from the transamination reaction is incorporated into an invertase, which in turn generates glucose from sucrose. As a result of the streamlined synthesis of alanine, invertase, and glucose, GABA could be analyzed by a PGM (Figure 4A). The LOD of GABA measured by the PGM

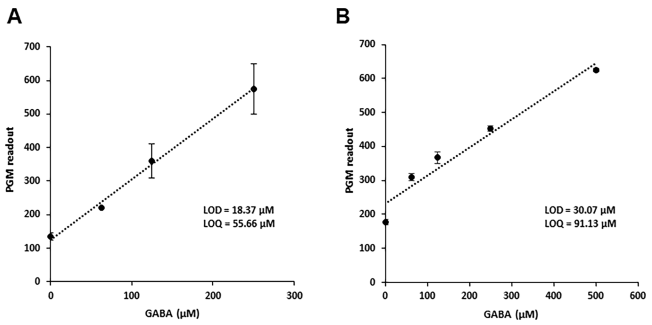


**Figure 4.** Measurement of GABA by a PGM. (A) PGM readout by the function of invertase synthesized by CCFPS after transamination reactions with varying concentrations of GABA. AOA (1 mM) was included in the CCFPS reaction to inhibit the GAGA-independent generation of alanine. (B) Measurement of GABA spiked in human serum using a PGM. Pretreated serum (50 $\mu$L) was added to the transamination mixture with a final volume of 100 $\mu$L. The concentrations of alanine were estimated by the activity of invertase synthesized during the subsequent Ala-CCFPS assay. Measurements were made in three independent experiments. Error bars represent standard deviations.

was ∼18.4 $\mu$M, and the accuracy of the GABA assay using a PGM was almost comparable with the results of the conventional assay performed using an amino-acid analyzer (Figure 5). We successfully used this method to determine the concentration of GABA in human serum. Using the transamination reaction solution with serum added, a linear graph was obtained using a glucose meter; hence, GABA in serum could be accurately quantified (Figure 4B).

## CONCLUSIONS

This study demonstrates that CFPS can be harnessed as a signal generator for the biological assay of metabolic compounds. The presented method is built by wiring

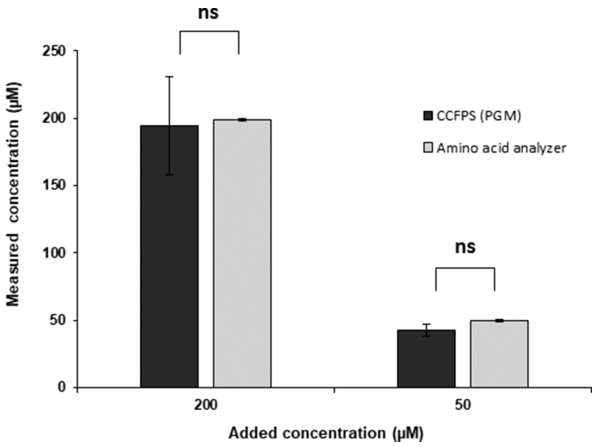


**Figure 5.** Comparison of GABA measurements by a PGM and an amino-acid analyzer. Measurements were made in three independent experiments. Error bars represent standard deviations.

enzymatic conversion of the target compound with the supply of amino acids required for CCFPS reaction, which produces proteins having measurable activities. In particular, the genetic programmability of this approach enables the conversion of target molecules into various reporter proteins that can be flexibly measured by a wide array of analytical instruments, including those that allow on-site analysis. Compared to the present HPLC or ELISA techniques, this method provides superior speed and convenience of operation. However, we also note that there remain several issues to be investigated to put this method into practice. For example, the use of liquid cell extract substantially limits the storage and shipment of reagents because the liquid extract needs to be stored at −80 °C to maintain protein synthesis activity. Fortunately, many recent reports indicate that the cell extract can be lyophilized and stored at room temperature without losing protein synthesis activity.[27−30] The use of lyophilized extract will substantially improve the feasibility of the presented method, and it thus needs to be confirmed to exhibit similar performance when conducted by rehydrating lyophilized extracts. We also expect that the detection limit of this assay will be further improved by programming the assay with more sensitive reporter genes (e.g., luciferases). By designing a mechanism that connects the molecules of interest to the key components of protein synthesis, we anticipate this strategy to be extended to the analysis of various metabolites having industrial and clinical implications.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.1c00501.

Purification of recombinant GABA transaminase (Figure S1), CCPFS assay of alanine (Figure S2), and inactivation of endogenous transaminases by AOA (Figure S3) (PDF)

## AUTHOR INFORMATION

### Corresponding Author

**Dong-Myung Kim** − *Department of Chemical Engineering and Applied Chemistry, Chungnam National University, Daejeon 34134, Korea;* orcid.org/0000-0001-7875-0694; Email: dmkim@cnu.ac.kr



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### Authors

**You Jin Lee** − *Department of Chemical Engineering and Applied Chemistry, Chungnam National University, Daejeon 34134, Korea*

**Hye Jin Lim** − *Department of Chemical Engineering and Applied Chemistry, Chungnam National University, Daejeon 34134, Korea*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssynbio.1c00501


### Author Contributions

D.M.K. conceived the project and supervised the work. Y.J.L. and H.J.L. performed the experiments. D.M.K. and Y.J.L. drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

### Notes

The authors declare no competing financial interest.

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