Identification Of Vitamin B_{12} In Fermented Foods Using Riboswitch Sensor

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Abstract:
To determine the vitamin B_{12} content in fermented foods using Riboswitch gene based selective method. Riboswitch are organized metabolite binding domains which reside within the non-coding locales of mRNAs, where they tie metabolites and serve as precision sensors for their targets corresponding, and control gene expression. A *Propionibacterium freudenreichii* was cloned in p519NGFP vector in *Escherichia coli* BL21. Based on the Adenosylcobalamin concentration, it correlates the expression using green fluorescence protein. The conformational change of secondary structure of mRNA leads to bind directly Adenosylcobalamin and inhibits the expression. After various experiments, results obtained from standard curve from 10 to 1000 ng/mL of cyanocobalamin. Outcome of the study reveals that recovery percentage is 94.5% compared with microbiological assay, HPLC but Ca. 26% lower than in Riboswitch sensor method.

Keywords: Riboswitch, Green Fluorescence protein, Cobalamin.

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Introduction:

Vitamin B $_{12}$ is only integrated by certain microscopic organisms and archaea and is gathered in creature bodies by rumen bacteria. Hence animal based nourishments and few fermented plant based food sources are considered as the primary dietary wellsprings of nutrient B $_{12}$ for human. (Keuth & Bisping, 1994). Most of the vitamin B_{12} substance in fermented foods was procured by a microbiological assay (MA).The scientific techniques dominate in its affectability, yet experiences helpless selectivity, labor intensity and time consuming. Results got by microbiological test overestimate the substance of vitamin B_{12} in the fermented foods due to deoxyribonucleutide and different corrinoids .(Denter&Bisping, 1994) Not withstanding microbiological measure different techniques like HPLC based assurance strategies, normally Reversed-stage HPLC utilizing UV and fluorescence, are additionally created to look at vitamin B_{12} substance in supplements, nutrient enhancements and infant formula. (Campos-Gimenez, Fontannaz, Trisconi, Kilinc, Gimenez, Andrieux, et al., 2012; Kirchner, Degenhardt, Raffler, & Nelson, 2012; Schimpf, Spiegel, Thompson, & Dowell, 2012; Vyas, O'Kane, & Dowell, 2012).

In addition to the safety hazards of these analytical methods, these procedures have other drawbacks as well. The complicated nature of these methods limits their similarity with high output experiments, they measure association within the cell and not absorption into cytoplasm, and they need radio active compounds which are not easily available. In this the possibility of employing a genetically encoded molecular sensor is explored to conduct a study based on transport system within the living cells. (Flower et al., 2010). This molecular sensor employs an element of RNA known as Riboswitch, which binds directly to a target metabolite. (Winkler and Breaker, 2005). The riboswitch-based sensor engaged here detects an active form of vitamin B $_{12}$ (VB_{12}), which is adenosylcobalamin (AdoCbl), within *Escherichia coli* cells.

Adenosylcobalamin (AdoCbl) otherwise called coenzyme B_{12} is a cofactor needed for the catalysis of various isomerization reactions. (Roth et al., 1996). AdoCbl isn't significant for development under standard lab conditions for E.coli and related types of microscopic organisms. It is essential significance appears to be in metabolism of enzymes which are important means of carbon, nitrogen, and energy in explicit conditions. B_{12}-dependent compounds for using glycerol, propanediol, and ethanolamine are common in enteric microscopic organisms. (Abeles and Lee, 1961) E. coli does not have these catalysts for both glycerol and propanediol and in this way ethanolamine usage seems, to be the foremost B_{12}-requiring measure. A developing number of studies highlight a significant role for ethanolamine usage for microbial in vivo development and pathogenesis, giving an additional layer of interest in comprehension the metabolic and import measures responsible for giving cells with AdoCbl. (Bourgogne et al., 2006) The sensors portrayed in this examination of these cycles as they take into consideration for tactful perception of cellular AdoCbl levels utilizing quick and helpful measures. In this examination we mean to build up a riboswitch based technique for the investigation of vitamin B $_{12}$ in different foods.

The gene sequence of *cbiB* in *Propionibacterium shermanii* was predicted as a cobalamin riboswitch, which has different sequences from *btuB* riboswitch in *Escherichia coli*. (Fowler et al., 2008) We have reported an engineered *btuB* riboswitch based on *E. coli* riboswitches in detecting low concentrations vitamin B_{12} and the expression of green fluorescence protein (GFP) was also inhibited by other corrinoids. (Zhu et al., 2015). Primary outcome of the study is to develop a sensitive riboswitch based method (RB) for the analysis of vitamin B12 in various fermented foods. The novel approach requires simple sample preparation and takes 5 h. A 2nd curve range of the assay is 10 ng/mL to 1000 ng/ nL. Based on the comparison with microbiological assay (MA) were minimally influenced by deoxyribonucleotide and corrinoids. Our aim of the study is to be find the vitamin B_{12}

content in fermented foods using Riboswitch gene based selective method.

Materials and Methods:

Inoculating Bacterial culture

Transformants of E. coli were cultivated in Luria-Bertnai (LB) medium supplemented with Kanamycin (10 mg/mL) at 37 ℃ and 200 rpm. *Lactobacillus delbrueckii* spp. *Lactis DSM 20355* was used in the microbiological assay of vitamin B12 which act as indicator strain. *Propionibacterium freudenreichii* spp. *shermanii* DSM 20270, *Propionibacterium shermani, Propionibacterium denitrificans* and were cultured in de Man, Rogosa, and Sharpe (MRS) broth at 37℃. The construction of recombinant plasmid carrying cobalamin riboswitch gene using plasmid p519ngfp. The source of bacterial strains were listed in Table 1.

Construction and validation of Riboswitch gene

Based on the manufacturer's instructions, the Himedia DNA isolation kit was used to extract and purify the whole genome. The template for PCR was isolated DNA. RBS from *P. freudenreichii spp. shermanii* DSM 20270 was amplified using primers created based on the sequence in the Gene Bank database (NC 014215.1 from 1368045 to 1368257). The fragment containing the riboswitch gene is 213 bp in size (Table 1). The following PCR conditions were used: 5 min at 94 °C, then 30 cycles of 30 s each at 94 °C, 50 °C, and 72 °C. Ten minutes at 72°C are the final extension. Subcloning of the fragment into the p519ngfp vector.

E. Coli DH5a amplified the resultant plasmid. For expression, E. coli BL21 (DE3) was employed. The p519-switch-ngfp plasmid-containing E. coli was multiplied in LB medium at 37 °C overnight, centrifuged at 4000g for 5 min, and then washed three times in 0.9% sodium chloride. At 37°C for 5 hours, 107 bacteria were added to 10 mL of vitamin B_{12} test broth that also included 0.01, 1, and 10 mg/L of adenosylcobalamin. GFP expression was analyzed using a fluorescent microscope set at 1000x. (Sambrook, Fritsh, & Maniatis, 2001).

Construction of samples and Vitamin B_{12} determination:

10 g of samples were used to extract vitamin B_{12}, which was then placed in 100 mL of sodium acetate buffer (pH 6.0) containing KCN and treated in a water bath for 30 minutes at 70°C. Since CNCBL is the most stable form of cobalamin, it was adopted as a standard material. 106 cfu/mL of p519-switch-ngfp-carrying *E. coli* were added to 9 mL of vitamin B_{12} test broth along with 1 mL of 0.01, 0.25, 0.5, 0.75, 1, and 1.25 mg/L of cyanocobalamin (CNCBL) and incubated at 37°C for 5 hours. Using a Fluorescence Spectrophotometer with an excitation emission set at 420 nm/470 nm, the intensity of GFP was measured. For all samples, the results were standardized using the absorbance at 600 nm (OD 600). A standard curve was determined by plotting the concentration of CNCBL on the horizontal axis and the fluorescence intensity on the vertical axis. Responses were calculated as second-order polynomial regressions. Fluorescence intensity was expressed as a percentage of the activity of 0.01 mg/L cyanocobalamin. Hydroxycobalamin (HOCBL), methylcobalamin (MCBL), adenosylcobalamin (ADCBL), intrinsic factor protein-bound CNCBL (IF-CNCBL), haptocholine-bound CNCBL (HC-CMCBL), deoxyribonucleotides, pseudovitamin B_{12}, and vitamin B_{12} are , was used at various concentrations that are degraded by light. We evaluate the impact of cobalamin analogues on the sensitivity and selectivity of this riboswitch sensor.

Table 1: construction of recombinant plasmid carrying cobalamin riboswitch gene containing bacterial strains

Strains	Genotype	Reference Source
***Lactobacillus delbrueckii spp. lactis* DSM 20355**	Indicator organism	Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)
***Propionibacterium freudenreichii spp. Shermanii* DSM 20270**	Organism including vitamin B12 riboswitches	DSMZ
Propionibacterium shermani	Vitamin B12 synthesis organism	DSMZ
Propionibacterium denitrificans	Vitamin B12 synthesis organism	DSMZ
E. coli DH5a	F-,u80dlacZ DM15, D(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17 (rK-, mK+), phoA , supE44, λ-, thi-1, gyrA96, relA1	Zhejiang Gongshang Uni, China
***E. coli BL21* (DE3)**	hsdSB(rB_mB_)k(DE3[lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Zhejiang Gongshang Uni, China
Plasmid		
p519ngfp	nptII promoter in front of gfp; IncQ replicon; Kmr	Zhejiang Gongshang Uni, China
Primer (5'-3')	Restriction enzyme sites are underlined	Restriction enzyme
Riboswitch-F	GCTCTAGATGTACTAGGGTCAATGTGCTGG	XbaI
Riboswitch-R	GGAATTCCATATGGGACAAGAACCTCAAATCCACG	NdeI

Results:

Construction of Riboswtich sensor:

Construct a 213bp fragment cobalamin ribosensor switch and an RBS from *P. freudenreichii* spp. *shermanii* DSM 20270 was cloned into p519ngfp between the pnpt2 promoter and GFP (Figure 1). Recombinant plasmids were transferred to E. coli BL21 (DE3) strain for heterologous expression and riboswitch analysis. Large-scale cultures were performed at 0.01, 1, and 10 mg/L adenosylcobalamin. The inverted region of this fragment was 100% similar to that reported by (Vitreschak et al. 2003). Fluorescence intensity decreased with increasing adenosylcobalamin concentration. In contrast, the fluorescence intensity of E. coli harboring p519ngfp remained stable during increasing concentrations of adenosylcobalamin. These outcomes indicated that this predicted riboswitch was successfully cloned into her p519ngfp plasmid and could inhibit her GFP expression as hypothesized.

Table 2: Vitamin B12 test broth fermented by *P. freudenreichii, P. shermani, P.denitrificans* and photolysis of Cyanocobalamin (1000ng/mL)

Strains	HPLC ng/g	MA ng/g	RB ng/g	RB/HPLC ng/g	RB/MA ng/g
P. freudenreichii	604.1±30.56^a	869.2±26.9^a	764.35±28.5^a	1.07	0.64
P. shermani	438.5±8.6^a	568.21±4.8^b	437.91±23.4^c	1.04	0.87
P. denitrificans	375.6±4.3^a	476.51±21.32^b	423.52±24.6^a	0.85	0.69
Cyanocobalamin by light	N/A	437.3±32.4	N/A	N/A	N/A

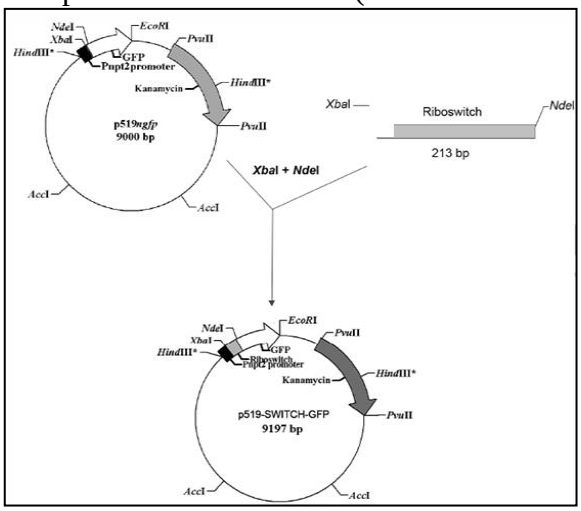
1. Values are mean±SD
2. NA- Not detected
3. Analytical replicates =2
4. MA means microbiological assay, RS means Riboswitch sensor
5. Values with dissimilar superscript letters (a, b, c) along each row indicate significant difference (P<0.05)

Vitamin B_{12} estimation using the riboswitch sensor:

We aim to determine the standard curve of this experiment after finding that GFP intensity reduces when vitamin B_{12} is present. We looked at the ideal cell density for measuring vitamin B_{12}. The slope was steep and the assay's sensitivity was strong when cells were planted at a low density (Ca. 10^3 CFU/mL). But conducting a test takes longer than 48 hours. In contrast, the range of the vitamin B_{12} to be evaluated is restricted when

more than 10^7 CFU/mL cells were implanted. For measuring vitamin B_{12} concentrations between 10 and 1000 ng/mL, 10^6 CFU/mL cells were utilized due to the significance of measurement precision and variance.

Fig 1 Genetic Map for Restriction sites: (Note : General for reference)



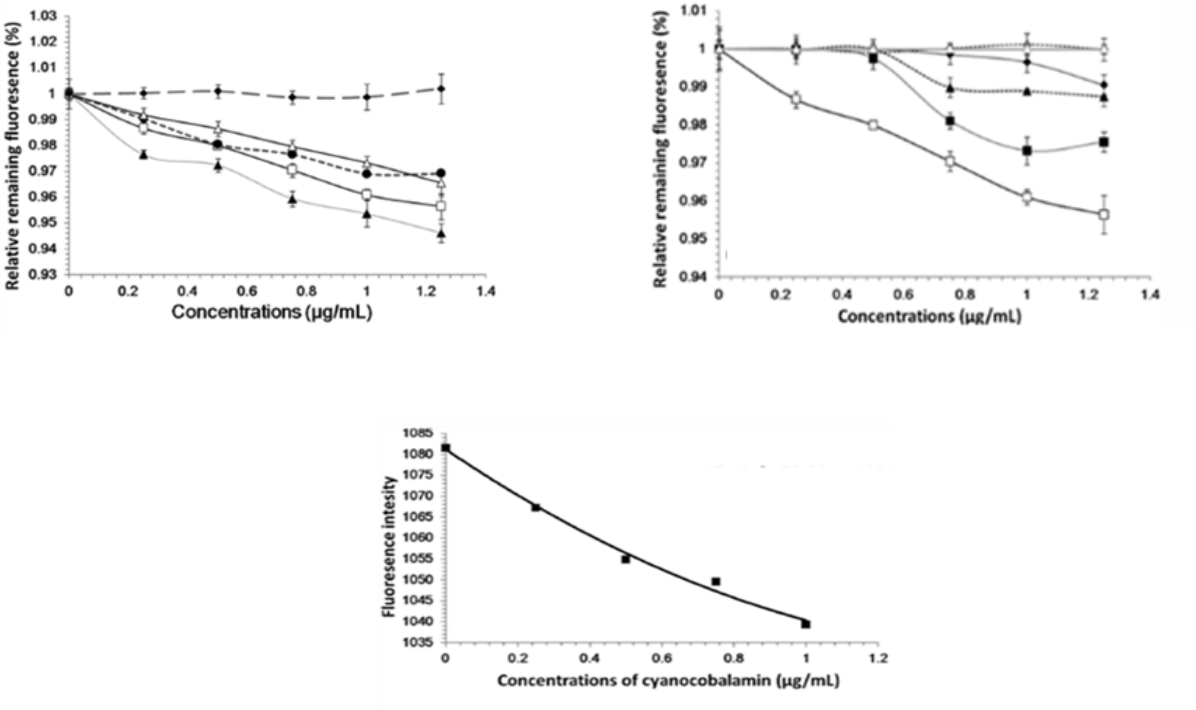
Quantification of vitamin B_{12} by riboswitch sensor:

Vitamin B_{12} cannot be naturally produced by *E. coli* (Fowler, Sugiman- Marangos, Junop, Brown, & Li, 2013). Thus, it needs additional supplements in order to survive in the vitamin B_{12} test broth. We discovered that the bacteria expanded in culture when given 5 ng/mL of vitamin B_{12}. As a result, at a concentration of 5 ng/mL vitamin B_{12}, the average and standard deviation of the GFP intensity were determined. When the concentration of vitamin B_{12} was three times more than the standard deviation of GFP intensity at 5 ng/mL vitamin B_{12}, the limit of detection of vitamin B_{12} (i.e. 10 ng/mL) was taken into consideration. The curve could not be fitted with a second order polynomial curve when the vitamin B_{12} concentration was higher than 1200 ng/mL. Therefore, the range between 10 and 1000 ng/mL can be covered by the 2nd order polynomial curve. By analyzing the inter- and intra-assay coefficients of variation of the results, the measurement accuracy was assessed. The Vitamin B_{12} test broth fermented by *P. freudenreichii*, *P. shermani*, and *P. denitrificans* all had coefficients of variation respectively. This method's recovery rate was 93.6%, which showed that the extraction and measuring techniques were appropriate.

Investigated were the riboswitch sensor's reactions to corrinoids and other compounds. As seen in Fig. 2c, the inhibition of GFP expression by MCBL and OHCBL was similar and had a gradual slope. However, compared to CNCBL, ADCBL significantly reduced GFP expression. Therefore, when a sample is tested using the riboswitch assay, all bioactive cobalamin should be removed by heating in an acetate buffer with KCN to create CNCBL prior to the test. Additionally, CNCBL is the form that is most thermally stable. Deoxyribonucleotide and broken-down vitamin B_{12} cannot stop GFP expression by turning off the riboswitch sensor, as seen in the Fig 2a. In contrast, the microbiological assay revealed that these were vitamin B_{12} molecules.

Complex forms of cobalamin have also been tested. As shown in Figure 2b, only high concentrations of IF-CNCBL (750 ng/mL) and HCCNCBL (750 ng/mL) were able to disable the riboswitch sensor. Therefore, the release of vitamin B_{12} from several proteins prior to measurement is essential for this riboswitch sensing method.

Fig 2 a, b,c : A standard curve for determination of Vitamin B_{12} by Riboswitch method:



Comparison of vitamin B_{12} calculation in samples with other methods:

When compare our experiment with other methods. The results of *P. freudenreichii* cells through MA, HPLC and RB were similar. When we analyzed the results obtained from RB showed that the vitamin B_{12} content was 25.4% lower than that of MA obtained, while the same result was obtained by HPLC. The vitamin B_{12} content as determined by RB was 91.5% HPLC and 83.0% MA, respectively. For fermentation media with *A. pasteurianus* and degraded cyanocobalamin, no detectable levels of cyanocobalamin by HPLC and RB were found. However, the result of MA remains high. This phenomenon may be due to the inherent disadvantages of AD.

Discussion:

We describe a sensitive and selective method for the determination of vitamin B_{12} content in fermented foods using a riboswitch sensor. 200 riboswitch B_{12} elements from 66 bacterial genomes were found by calculating multiple linkages (Vitreschak et al., 2003). In addition, on the Genome Net site, a riboswitch B_{12} element responsible for the expression of the ABC transporter substrate-binding protein was found in the genome of *Probionibacterium propionicum* F0230a (16287 to 16468 bp). . Fowler et al. used a FACS-based approach to recognize engineered artificial riboswitches (Campos-Gimenez et al., 2012). In addition, these riboswitch tools have been used to explore the intermolecular interactions of a vitamin B_{12}-binding protein (Fowler et al., 2012). Even very low concentrations of ADCBL resulted in a strong repression of reporter expression. These man-made elements are so sensitive that they have a very narrow detection range, limiting their application in quantitative methods. *E. coli* is an ideal host for the riboswitch B_{12} sensor because it cannot produce vitamin B12 de novo. In our study, the riboswitch factor B_{12} of *P. freudenreichii* spp. *shermanii* DSM 20270 is structurally distinct from the riboswitch in *E. coli* (Fowler et al., 2008), although the riboswitch is regulated by a similar mechanism including the RBS fraction by a counter-sequencing. Our riboswitch has a short right arm and a "CCCC" sequence end which is responsible for folding the RNA structure.

Reporter gene expression due to the presence of vitamin B_{12} is also associated with the efficiency of ADCBL transport proteins or precursors into the cytoplasm (Kirchner et al., 2012). The degree of GFP inhibition was affected by the transport proteins, as they have different affinity for different cobaminds. The precursors or cobaminds of

ADCBL are rapidly converted to ADCBL by metabolic enzymes after transport (Kirchner et al., 2012). Therefore, these substances can also be measured. Furthermore, the affinity of the riboswitch is also an important factor for the inhibition of GFP. Several riboswitches have recognized MCBL and OHCBL with an affinity 500 times higher than that of ADCBL (Johnson, Reyes, Polaski, & Batey, 2012). This short right-arm container structure is highly sensitive to derivatives with small upper axial fractions Unlike E. coli btuB riboswitch selectively binds to adenosylcobalamin which reacts with various bioactive cobalamins such as CNCBL, OHCBL and MCBL in addition to ADCBL. GFP expression is partially inhibited by IF-binding vitamin B_{12}. One is dimethylbenzimidazole, which is bound to cobalt on the lower axis. The remainder is part of the A and B rings of the porphyrin ring. As a result, the upper corrin ring ligand was not affected by IF. In contrast, HC binds to cobalamin via a single site in the porphyrin ring. Although the upper and lower ligands are not covered by HC, steric hindrance occurs between a large group and the HC molecule. The binding strain could be the reason for the lower response of HCCBL in the RB method. For determining low amounts of vitamin B_{12} in foods and biological material, the MA is accepted as an official standard method. However, compared to HPLC and other techniques, it was consistently observed to produce greater vitamin B12 readings. Due to their reaction to corrinoids that are inactive for humans, such as phony vitamin B_{12} and nucleic acid, this aberration became apparent (Santos et al., 2007). In earlier research, we discovered that *Acetobacter pasteuriamus* was capable of producing corrinoids, which allowed *L. delbrueckii* to survive in vitamin B_{12} test broth. However, using the HPLC approach, no functional vitamin B12 could be discovered. (1987; Schneider & Stroinski). According to several researchers, MA measurement of vitamin B_{12} concentration in food was 35% higher than HPLC (Martens et al. 2002). In the current study, results from the RB approach were comparable to those from the HPLC method (Table 2), and no cobalamin analogs could be found. The collection of free vitamin B_{12} liberated from protein, however, needs a time-consuming preparatory process. As a result, vitamin B_{12} attached to protein may occasionally go undetected.

Conclusion:

In our study, the riboswitch sensors were used as a quick and sensitive way to measure the amount of vitamin B_{12} in fermented foods. By allowing for the studies were conducted of bioactive vitamin B_{12}, the approach was able to eliminate the MA's. MA's effect from nucleic acid and other inactive corrinoids. Similar to the HPLC procedure, this one allowed for the elimination of the impact of proteins.

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