Video Article

Highly Sensitive and Rapid Fluorescence Detection with a Portable FRET Analyzer

Haseong Kim*[1], Gui Hwan Han*[1], Yaoyao Fu[1], Jongsik Gam[2], Seung Goo Lee[1,3]

[1]Synthetic Biology & Bioengineering Research Center, Korea Research Institute of Bioscience and Biotechnology

[2]College of Interdisciplinary & Creative Studies, Konyang University

[3]Biosystems and Bioengineering Program, University of Science and Technology

*These authors contributed equally

Correspondence to: Seung Goo Lee at sglee@kribb.re.kr

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Abstract

Recent improvements in Förster resonance energy transfer (FRET) sensors have enabled their use to detect various small molecules including ions and amino acids. However, the innate weak signal intensity of FRET sensors is a major challenge that prevents their application in various fields and makes the use of expensive, high-end fluorometers necessary. Previously, we built a cost-effective, high-performance FRET analyzer that can specifically measure the ratio of two emission wavelength bands (530 and 480 nm) to achieve high detection sensitivity. More recently, it was discovered that FRET sensors with bacterial periplasmic binding proteins detect ligands with maximum sensitivity in the critical temperature range of 50 - 55 °C. This report describes a protocol for assessing sugar content in commercially-available beverage samples using our portable FRET analyzer with a temperature-specific FRET sensor. Our results showed that the additional preheating process of the FRET sensor significantly increases the FRET ratio signal, to enable more accurate measurement of sugar content. The custom-made FRET analyzer and sensor were successfully applied to quantify the sugar content in three types of commercial beverages. We anticipate that further size reduction and performance enhancement of the equipment will facilitate the use of hand-held analyzers in environments where high-end equipment is not available.

Video Link

The video component of this article can be found at https://www.jove.com/video/54144/

Introduction

Förster resonance energy transfer (FRET) has been widely used as a biometric sensor to detect small molecules such as sugars, calcium ions, and amino acids[1-4]. FRET biosensors contain fluorescent proteins, cyan fluorescent proteins (CFPs), and yellow fluorescent proteins (YFPs), which are fused to both ends of periplasmic-binding proteins (PBPs). Sugars bind to PBPs located in the middle of the FRET sensor, causing structural changes to the sensor that subsequently alter the distance and transition dipole orientation of the two fluorescent proteins at either end of the PBPs. This change enables quantitative analysis of sugar content by measuring the ratio of the emission wavelengths of EYFP (530 nm) and ECFP (480 nm). Owing to the high sensitivity, specificity, real-time monitoring capacity, and fast response time of FRET biosensors, these sensors are widely used in environmental, industrial, and medical applications[5]. Moreover, ratiometric measurement using FRET biosensors has important practical benefits, as it can be used to measure components in complex biological samples where the sensor concentration cannot be easily controlled and background fluorescence is always present.

Despite these advantages of FRET-based sensors for quantitative visualization, small structural changes with incomplete domain motion-transfer to the fluorescent proteins produce inherently weak signal intensity. This weak signal limits the application of FRET-based sensors for *in vitro* or *in vivo* analysis[6]. Consequently, most FRET biosensors require the use of expensive and highly sensitive equipment. Previously, we developed an inexpensive and portable FRET analyzer with capabilities similar to those of the existing fluorescence analyzers[7]. In this device, inexpensive 405-nm band ultraviolet light-emitting diode (LED) was used as the light source to cause excitation of the fluorescence signal, replacing an expensive lamp or laser. The detection system of the analyzer efficiently focuses the dissipating fluorescence signal onto two photodetectors with a silicon photodiode. In a more recent study, we showed that optimization of detection temperature at 50 - 55 °C could significantly magnify the ratiometric FRET signal[8]. This temperature-specific signal enhancement, along with the custom-made FRET analyzer, enables the use of FRET sensors in more general diagnostic applications with rapid and high sensitivity.

In this protocol, we demonstrated the general applicability of the FRET analyzer under optimal FRET temperature conditions by quantifying the sugar content of commercially-available beverages. This protocol provides the details of the FRET device operation, as well as a brief description of sensor and sample preparation. We anticipate that this report will promote the potential application of the portable analyzer in

small-scale laboratory environments and provide a foundation for further development of an inexpensive on-site diagnostic device with FRET-based biosensors.

Protocol

1. Preparation of Biosensor

1. Construct the plasmid pET21a(+)-CFP-MBP-YFP-His6 by following the previously-established protocol[2].
2. Inoculate 5 ml of Luria broth (LB) with a single colony of an *Escherichia coli* DE3 strain and incubate at 37 °C for 16 hr with shaking.
3. Transfer 1 ml of the O/N culture into a 500-ml flask containing 100 ml LB and incubate at 37 °C in a shaking incubator until the optical density at 600 nm (OD_{600}) reaches 0.5 (about 3 hr).
4. Harvest the cells in a 50-ml conical tube by centrifugation at 1,000 × g for 20 min at 4 °C.
5. Resuspend the pellet quickly in each tube with 50 ml ice-cold distilled water (DW) and centrifuge at 1,000 × *g* for 20 min at 4 °C.
6. Resuspend the pellet in 50 µl of ice-cold DW with 10% (v/v) glycerol by gently swirling until the solution (electrocompetent cells) reaches an OD_{600} of 100.
7. Place the mixture of electrocompetent cells (50 µl of the cells at an OD_{600} of 100) and 10 ng of the plasmid pET21a(+)-CFP-MBP-YFP-His6 in an ice-cold electroporation cuvette in an electroporation device and electroporate the mixture (18 kV/cm, 25 µF).
8. Quickly add 1 ml SOC medium to the cuvette and resuspend the cells gently, followed by recovery at 37 °C for 1 hr with gentle shaking in a 15 ml round-bottom tube.
9. Spread the cells on an LB plate containing 100 µg/ml ampicillin and incubate at 37 °C for 12 hr.
10. Isolate a single colony using a loop and inoculate the colony in 10 ml of LB containing 100 µg/ml ampicillin at 37 °C in a shaker for 12 hr.
11. Add 5 ml of the seed culture to 500 ml of LB containing 100 µg/ml ampicillin and incubate the culture in a 37 °C shaking incubator.
12. Add 0.5 mM isopropyl β-d-thiogalactoside (IPTG) when the OD_{600} reaches 0.5 and incubate the culture in a 37 °C shaking incubator for 24 hr.
13. Centrifuge the cells at 4,500 × g for 20 min (4 °C) and gently remove supernatant.
14. Resuspend the pellet in 5 mL binding buffer (20 mM Tris-HCl, pH 8.0, 1 mM PMSF, 0.5 mM EDTA, and 1 mM DTT).
15. Sonicate the cells on ice with six 10-sec bursts at 200-300 W, following each burst with 10 sec of cooling.
16. Centrifuge the lysate at 10,000 × g for 30 min at 4 °C to pellet the cellular debris. Transfer supernatant (soluble protein) into a new collection tube.
17. To achieve affinity purification of the FRET sensor proteins, load 4 ml of the cleared cell lysate onto a Ni-NTA affinity column (5-ml volume) and perform a chromatography assay using fast protein liquid chromatography (FPLC)[18].
18. Wash the column once with five column volumes of wash buffer I (50 mM phosphate buffer, 300 mM sodium chloride, 10 mM imidazole, pH 7.0).
19. Repeat the wash step with five column volumes of wash buffer II (50 mM phosphate buffer, 300 mM sodium chloride, 20 mM imidazole, pH 7.0).
20. Elute the sensor protein with five column volumes of elution buffer (50 mM phosphate buffer, 300 mM sodium chloride, 500 mM imidazole, pH 7.0).
21. To concentrate and desalt the eluted sample, fill concentrator (membrane size of 10,000 MW) with up to 20 ml of sample and centrifuge for 10 min at 3,000 × g. Refill concentrator with 0.8% phosphate-buffered saline (PBS). Repeat this step twice, first filling the concentrator with 20 ml of sample, and then refilling with PBS.
22. Recover the concentrated and de-salted sensor protein and store it at -80 °C.

2. Measurement of Sugar Content using the FRET Analyzer

NOTE: The details of the FRET analyzer construction were described in our previous work[7].

1. Prepare a detection solution of 0.8% PBS containing 0.2 µM of the sensor proteins.
2. Turn on the FRET analyzer. Press the "UP" button for 2 sec to calibrate the optimal temperature. Set the temperature to 53 °C using the "UP" and "DOWN" buttons and press the "SET" button.
3. For the calibration, press and hold the "UP" and "DOWN" buttons simultaneously for 2 sec. Confirm that the LED panel displays "CALIB" and press the "SET" button.
4. Place a 12.5 × 12.5 × 45 mm (length × width × height) rectangular parallelepiped vessel (cuvette) containing only PBS buffer into a cuvette holder of the analyzer and press the "SET" button.
5. Replace the cuvette with one containing only the detection solution (see 2.1) without sugar (maltose/sucrose) and press the "SET" button to calibrate the baseline.
6. Replace the cuvette with one containing the detection solution with 10 mM sugar and press the "SET" button.
7. To determine the sugar content of a beverage sample, put 1 ml beverage sample in a 1.5-ml microcentrifuge tube and centrifuge at 16,000 × g for 1 min.
   NOTE: FRET sensor-based fluorescence measurement has the advantage of not requiring special pre-treatment of the sample because only 1% (v/v) of the sample is included in the total volume. However, we recommend removing any material that may affect the fluorescence measurement (*e.g.,* cells, insoluble particles, lipid, fat, or any material with autofluorescence). In addition, if a strong acid, strong base, cleaning agent (detergent), or emulsifying agent (emulsifier) is present at a high concentration and may affect the properties of the FRET biological sensor, it should be removed using an organic solvent or a neutralizer. For example, when dairy fat and emulsifiers are eliminated from frozen snacks, the samples are centrifuged in a microfuge tube at 16,000 × g for 30 min, and the liquid between the bottom sediment and the top layer of dairy fat is extracted. An equal amount of hexane is then added, followed by centrifugation at 15,000 × g for 30 min to eliminate lipids.
8. Remove the supernatant with a 1-ml syringe and filter it through a syringe filter (pore size 0.2 µm).
9. Place 0.1 ml filtered beverage sample in a 1.5-ml microcentrifuge tube containing 0.9 ml PBS and vortex gently.

NOTE: It is critical to dilute the beverage sample properly. In this case, 1,000-fold dilution was performed so that the sugar concentration would fall within the dynamic range of the device. We recommend estimating the target sugar concentration in advance by referring to the sugar content in the label of the beverage.

10. Add 5 µl of the diluted beverage sample (1%, v/v) to a cuvette containing 0.495 ml of the detection solution.
11. Place the cuvette in a cuvette holder of the FRET analyzer and preheat the sample solution to 53 °C.
12. Press the "SET" button to measure the sugar content.
    Note It is possible to evaluate the FRET measurement using a multilabel plate reader or a fluorescence spectrophotometer equipped with a Peltier device for temperature control by reading the ratio at 488/535 nm[7,8]. For sucrose detection, follow the steps from 1.1 to 2.12 with a CSY-LH sensor[2].

Representative Results

To perform quantitative analysis of sugar content using the FRET analyzer, it is necessary to build a fitted curve estimating the target sugar concentration from the observed FRET ratio. Let *r* define the ratio of the emission intensity of CFP at 480 nm and the emission intensity of YFP generated at 530 nm (Eq. 1).

$$r = \frac{I_{530nm}}{I_{480nm}} \qquad (1)$$

The dose-response curve of the FRET biosensor (CMY-BII at 53 °C) can be generated by observing the FRET ratio, *r*, at different sugar concentrations. The curve can then be expressed as an S-shaped sigmoidal curve as follows:

$$\hat{r} = r_{min} + \frac{r_{max} - r_{min}}{1 + 10^{p(log_{10}x_0 - log_{10}x)}} \qquad (2)$$

where r_{max} and r_{min} represent the signal ratio with sugar concentrations of 0 and saturated (1,000 µM), respectively; x_0 represents the sugar concentration at 50% response; and *p* represents the slope of the response, which is close to 1 or -1. In the present study, r_{max}, r_{min}, x_0, and *p* are 4.256, 2.672, 71.779, and 1, respectively. The concentration range from 1 µM to 1000 µM was used in the model fitting.

Using Equations 1 and 2, the sugar content of commercially- available beverages was quantified with the FRET analyzer. Two maltose FRET sensors were examined to test the signal, *r*, depending on various temperatures[2,8]. The first FRET sensor, CMY-0, is a basic FRET-based sensor consisting of CFP, maltose-binding protein (MBP), and YFP, with no linker peptides. The second sensor, CMY-BII, has a Ser-Arg linker between MBP and the two fluorescence proteins[2]. As **Figure 1A** shows, CMY-0 is not observed at measurement temperatures below 50 °C, as there is no signal difference between 0 and 1 mM maltose concentration. The signal differences of both FRET sensors were maximized between 50 and 55 °C (**Figure 1**)[8]. In order to quantify the sugar content of the three types of commercially-available beverages, a dose-response curve of the CMY-BII sensor at 53-°C was generated (**Figure 2A**) and the maltose content of the three samples was identified by converting the FRET ratio into the maltose concentration.

As sample A is made of grains such as rice and barley, which are important maltose sources, the sample was expected to contain relatively high maltose content (average 11.892 g/235 ml) (**Figure 2A**). In contrast, sample C is a sports drink that had the lowest maltose (0.29 g/250 ml) among the three beverages. These results suggest that the FRET analyzer can be used at optimal temperatures to maximize the quantification of sugar content, eliminating the need for an expensive high-end FRET-detection device.

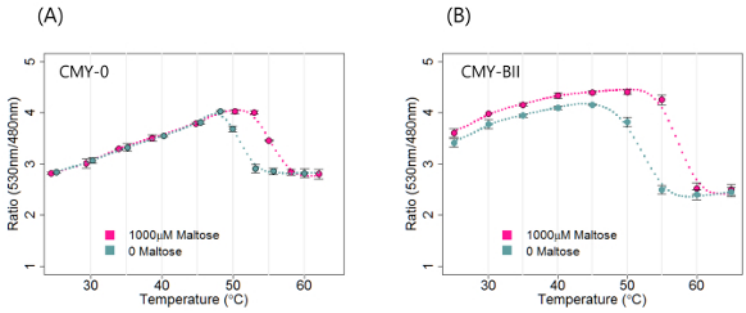

Figure 1. FRET Signal Difference Between 0 and 1 mM Maltose using the FRET Analyzer at Various Temperatures. (A) The CMY-0 sensor showed no signal difference at different maltose concentrations at temperatures below 50 °C. **(B)** The CMY-BII sensor was able to distinguish the FRET signal difference between 0 and 1 mM maltose in a wide range of temperatures. In both cases, the signal difference dramatically increased in a specific temperature range (50 - 55 °C). The error bars represent standard deviation. Please click here to view a larger version of this figure.

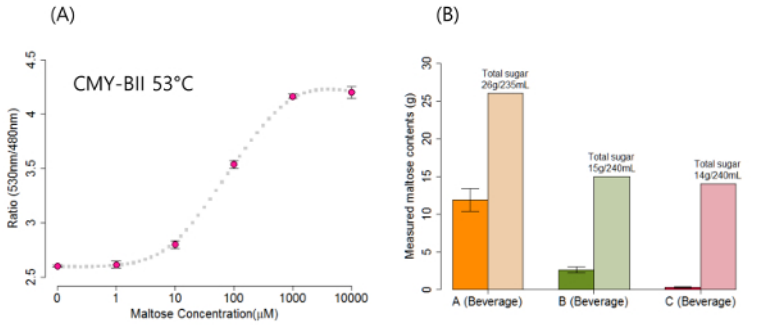

Figure 2. Maltose Content Quantification in Three Commercially-available Beverages. (A) A dose-response curve for CMY-BII. **(B)** Maltose content of three beverage samples was quantified. Note that "Total sugar" indicates the quantity of all sugar (including maltose) reported by the beverage manufacturer on the beverage label. The error bar indicates the standard deviation. Please click here to view a larger version of this figure.

Discussion

This protocol allows rapid and efficient quantification of the sugar content in beverage samples, using a custom-made FRET analyzer[7] at an optimal temperature for FRET sensors. The analyzer was designed with a recently-developed, inexpensive 405-nm band ultraviolet-LED as the light source and two photodetectors with a silicon photodiode. This device is more cost-effective than other comparable fluorometers. The device showed high detection sensitivity, specifically when measuring the ratio of two emission wavelength bands (530 nm and 480 nm) in an optimal temperature range for FRET sensors. Its sensitivity and intensity in detecting various sugars were superior to those of a fluorescence spectrophotometer device[7].

The main goal of this protocol is to support the wide applicability of the FRET-based sensors with the custom-made FRET analyzer. While the analyzer indirectly measures sugar content via FRET sensors, it is clear that the device incorporates a number of the benefits of FRET sensors, including widely extendable genetically engineered ligand specificity, modular design, sensor concentration-independent signals, and accurate targeting of subcellular small molecules. FRET sensors are actually used to detect a wide range of small molecules, including ions[9], heme[10], and others. Moreover, more than 20 types of FRET constructs can be easily found and ordered through the nonprofit depository AddGene[11].

Despite the wide applicability of the FRET analyzer, there are two main issues with the operation of the device. First, because operation of the device is relatively simple, sample preprocessing is the critical step that affects quality of detection, except in cases of device malfunction. In this protocol, one step (sample dilution) was sufficient to process liquid samples that were clearly transparent and contained no insoluble particles. However, other samples may require additional processing to remove insoluble materials, such as cellular or lipid components. Any autofluorescent particles that can affect the FRET signal should also be removed, as noted following Step 2.7. Secondly, quality control and connectivity interfacing with hospital information systems need to be addressed, as with all types of point-of-care testing (POCT) tools[12]. Since the signal quality of the FRET analyzer largely depends on the quality of the FRET sensor and on the preprocessing steps, regular quality control checks are required to ensure that measurements remain within the standard signal range for regular quality control data analysis. The FRET sensor stability and storage period, both of which are essential for further reliable applications, should be investigated during quality control checks. Creating guidelines and developing appropriate software can also address the connectivity limitation. Current versions of the FRET analyzer are equipped with RS232 connectivity for remote command line control, but wireless communication may be a feature of the next version of the analyzer, which will have an improved interface for hospital information systems.

However, FRET sensors have been engineered for substrate specificity, an approach that normally allows broader specificity[2]. Consequently, the FRET signal may encounter unintended interference from other ingredients, including other types of sugars in commercial beverages. Further investigations should explore how FRET sensors respond to various sugar mixtures to accurately quantify the amount of sugar. Collaboration with companies that produce the beverages will help confirm the sugar content to calibrate of the FRET analyzer.

It is anticipated that the proposed portable FRET device with various FRET sensors will be used in POCT applications. POCT is used for assessing pregnancy, blood glucose levels, biomarker proteins, infectious bacteria, and infectious viruses. POCT methods have rapid turnaround times and generally exhibit low error rates owing to the small number of processing steps. These are important advantages of POCT over the central laboratory testing approach. Hand-held portable POCT devices, such as the device described herein, have attracted increasing attention because of their potential applications in food assessment and blood sugar monitoring. In particular, glucose monitoring of blood samples in patients with diabetes requires a rapid, accurate, and cost-effective POCT method[13]. After the Ames research team developed the first blood glucose test strip in 1965 (using a strip that contains glucose oxidase), several technologies were proposed for blood glucose monitoring purposes[12]. The FRET analyzer is also available to detect glucose in blood samples with appropriate preprocessing of blood and periplasmic glucose-binding protein (MglB)[14]-based FRET protein.

Simple, rapid methods for food quality assessment are needed. The consumption of sugar-containing beverages is associated with a variety of diseases and syndromes, such as increased body mass index in childhood[15], pediatric obesity[16], and risk of stroke[17]. Understanding this connection necessitates accurate measurement of sugar components in beverages. Therefore, the glucose and fructose concentrations of beverages are of interest to scientists concerned with human health. This protocol demonstrates the highly sensitive performance of the FRET analyzer with optimal temperature control. The device may be used with various FRET sensors to detect various small molecules- including glucose and fructose[14,15]. Our portable and rechargeable device, which has a battery life of 10 - 20 hr, depending on the heating protocol, is applicable for POCT. Its simple operational protocol makes the device easy to use and eliminates the need for complicated staff training. With

technical improvements, including reduction of equipment size, minimization of pretreatment steps, and identification of practical requirements for field use, this device will promote FRET-based research development in small-scale laboratory environments.

Disclosures

The authors have nothing to disclose.

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