**TITLE:**

**High sensitive and rapid fluorescence detection with a portable FRET analyzer**

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**KEYWORDS:**

Fluorescence resonance energy, portable device, point-of-care testing, sugar contents, fluorometers, food assessment

**SHORT ABSTRACT:**

This protocol describes the rapid and highly sensitive quantification of Föerster resonance energy transfer (FRET) sensor using custom made portable FRET analyzer. FRET sensors detecting maltose were examined as examples at critical temperatures that maximize the sugar detection sensitivity, which enables practically efficient sugar content assessment.

**LONG ABSTRACT:**

Recent developments in Föerster Resonance Energy Transfer (FRET) sensors have enabled the detection of various small molecules, including ions and amino acids. However, the innate weak signal intensity of FRET sensors is a major challenge preventing the application of FRET sensors in various fields; this limitation necessitates expensive, high-end fluorometers, further limiting the potential applications of this technology. Previously we built a cost-effective, high-performance FRET analyzer which can specifically measure the ratio of two emission wavelength bands (530 and 480 nm) for the high detection sensitivity. More recently it was discovered that FRET sensors with bacterial periplasmic binding proteins has a critical temperature, 50-55 °C detecting ligands with maximum sensitivity. This report describes a protocol of the sugar content assessment including the portable FRET analyzer operation with the preparation of temperature specific FRET sensor including commercially available beverage samples. As results, additional preheating process of FRET sensor significantly increases the FRET ratio signal in measuring sugar contents using the custom made FRET device and the device with the FRET sensor was successfully applied to quantify the sugar content in commercial beverages. We anticipate that further size reduction and performance enhancement of the equipment will facilitate the practical use of hand-held analyzers in environments where high-end equipment is not available.

**INTRODUCTION:**

Föerster resonance energy transfer (FRET) has been widely used as a biometric sensor to detect small molecules, such as sugars, calcium ions, or amino acids1-4. FRET biosensors contain fluorescent proteins, enhanced cyan fluorescent proteins (ECFPs), and enhanced yellow fluorescent proteins (EYFPs), which are fused to both ends of periplasmic-binding proteins (PBPs). The mechanism of FRET biosensors involves the binding of sugars to PBPs located in the middle of the sensor; this causes structural changes to the sensor and subsequently alters the distance and transition dipole orientation of the two fluorescent proteins at either end of the PBPs. This change enables quantitative analysis of sugar contents by measuring the ratio of the emission wavelengths of EYFP (530 nm) and ECFP (480 nm). Due 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 applications5. Moreover, the ratiometric measurement of FRET biosensors has important practical benefits when measuring 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, the innate weak signal intensity originating from the small structural changes with incomplete domain motion transfer to the fluorescent proteins limits the application of FRET-based sensors for in vitro or in vivo analysis6. 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 analyzers7. A recently developed, inexpensive 405-nm band ultraviolet (UV) light-emitting diode (LED) was used as the light source to replace the expensive lamp or laser for excitation of the fluorescence signal. 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 optimization of detection temperature (50~55°C) could significantly magnify the ratiometric FRET signal8. This temperature specific signal enhancement along with the custom made FRET analyzer enables one to apply FRET sensors to more general diagnostic applications with rapid and high sensitivity.

In this protocol, we evaluated the general applicability of the FRET analyzer with optimal FRET temperature by quantifying the sugar contents of commercially available beverages. This protocol includes the details of the FRET device operation with 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 an inspiration for further development of an inexpensive on-site diagnostic device with FRET based biosensors.

**PROTOCOL:**

**1. Preparation of biosensor, CMY-BII**

* 1. Construct the plasmid pET21a(+)-CFP-MBP-YFP-His6 by following the previously established protocol2.
  2. Inoculate 5mL of Luria broth (LB) with a single colony of an *Escherichia coli* DE3 strainand incubate at 37 °C for 16 hr with shaking.
  3. Transfer 1mL of the overnight culture in a 500 mL flask containing 100 mL LB and incubate at 37 °C in a shaking incubator until an optical density at a wavelength of 600nm (OD600) reaches 0.5 (It will take 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 again.
  6. Resuspend the pellet in 50 L of ice-cold DW with 10% (v/v) glycerol by gently swirling until the solution (electrocompetent cells) reaches an OD600 of 100.
  7. Place the mixture of the electrocompetent cells (50 L of the cells at an OD600 of 100) and the plasmid pET21a(+)-CFP-MBP-YFP-His6 (10 ng) in an ice-cold electroporation cuvette in an electroporation device and electroporate (18 kV/cm, 25 μF) the mixture.
  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.
  9. Spread the cells on an LB plate containing 100 mg/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 g/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 g/mL ampicillin.
  12. Add 0.5 mM isopropyl β-D-thiogalactoside(IPTG) when the OD600 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-second bursts at 200–300 W and 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) in a new collection tube.
  17. For affinity purification of the FRET sensor proteins, load the 4 mL of cleared cell lysate on to Ni-NTA affinity column (5 mL volume) and chromatography assay using a fast protein liquid chromatography (FPLC)18.
  18. Wash the column once with 5 column volume the wash buffer I (50 mM phosphate buffer, 300 mM sodium chloride, 10 mM imidazole, pH 7.0).
  19. Repeated wash step with 5 column volume the wash buffer II (50 mM phosphate buffer, 300 mM sodium chloride, 20 mM imidazole, pH 7.0).
  20. Elute the CMY-BII sensor protein with 5 column volumes of elution buffer (50 mM phosphate buffer, 300 mM sodium chloride, 500 mM imidazole, pH 7.0).
  21. For concentration and desalting of eluted sample, fill concentrator (membrane size of 10,000 MW) with up to the 20 mL of sample, centrifuge for 10 min at 3,000 X g. Refilled 0.8% phosphate-buffered saline (PBS) into concentrator, repeated 2 times same step.
  22. Recover the concentrated and de-salted CMY-BII sensor, storage of purified sensor at -80°C.

1. **Measurement of sugar content using the FRET analyzer**

Note: The details of the FRET analyzer construction method were described in our previous work7.

* 1. Prepare a detection solution of 0.8% phosphate-buffered saline (PBS) containing 0.2 M CMY-BII sensor proteins.
  2. Turn on the FRET analyzer. Press the “UP” button for 2 sec for the calibration of optimal temperature. Set the temperature to 53 °C using the “UP” and “DOWN” buttons, and press the “SET” button.
  3. For 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 rectangular parallelepiped vessel (cuvette) containing only PBS buffer into a cuvette holder of the analyzer and press the “SET” button.
  5. Replace with a cuvette containing only the detection solution (see 2.1) without sugar (maltose/sucrose) and press the “SET” button for baseline calibration.
  6. Replace with a cuvette containing detection solution with 10 mM sugar and press the “SET” button.
  7. For determination of sugar contents from beverage sample, put 1 mL beverage sample in a 1.5 mL micro centrifuge tube and centrifuge at 16,000 x g for 1 min to remove.

Note: FRET sensor based fluorescence measurement has an advantage that it does not require a special pre-treatment to the sample since only 1% (v/v) of the sample is included to the total volume. However, it is preferred to remove any material that may affect the fluorescence measurement (e.g cells, insoluble particles, lipid, fat, any material with auto fluorescence). In addition, such a high concentration of strong acid, strong base, the cleaning agent (detergent), emulsifying agent (emulsifier) which may affect the properties of the FRET biological sensor made ​​of a protein is preferably measured after removal using an organic solvent or a neutralizer. For example, with frozen snacks subjected to elimination of dairy fat and emulsifiers, the samples are centrifuged in a microfuge tube at a speed of 15,000 rpm for 30 min, and the liquid between the bottom sediment and the top layer of dairy fat should be extracted. An equal amount of hexane is then added, followed by centrifugation at 15,000 rpm for 30 min for lipid elimination.

* 1. Take the supernatant with a 1 mL syringe and filtered it through a syringe filter (pore size 0.2 m).
  2. Put 0.1 mL filtered beverage sample in a 1.5 mL micro centrifuge tube containing 0.9 mL PBS and vortex gently.

Note: It is critical to dilute the beverage sample properly. In this case, 1000 fold dilution was carried out so that the sugar concentration falls within the dynamic rage of the device. It is suggested to estimate the target sugar concentration in advance by referring the sugar contents labeled on the beverage products.

* 1. Add 5 L of the diluted beverage sample (1%, v/v) into to a cuvette containing 0.495 mL of the detection solution.
  2. Place the cuvette into a cuvette holder of the FRET analyzer and preheat the sample solution to 53 °C.
  3. Press the “SET” button to measure sugar contents.

Note**:** It is possible to evaluate the FRET measurement with a multilabel plate reader or a fluorescence spectrophotometer equipped with a Peltier device for temperature control by reading the ratio at 488/535 nm7,8. In the case of sucrose detection, one can follow the steps from 1.1 to 2.14 with CSY-LH sensor2.

**REPRESENTATIVE RESULTS:**

For the 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 ECFP at 480 nm and the emission intensity of EYFP generated at 530 nm in **Equation 1**.

(1)

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

(2)

where *rmax* and *rmin* represent the signal ratio with sugar concentrations of 0 and saturated (1000 M), 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 this study, *rmax*, *rmin*, *x*0, and *p* are 2.672, 4.256, 71.779, and 1, respectively. The concentration range from 1 to 1000 μM was used in the model fitting.

Based on Equation 1 and 2, the sugar contents in commercially available beverages were quantified using the FRET analyzer. Two maltose FRET sensors were examined to test their signal, *r*, depending on various temperatures2,8. The first one, CMY-0, is a basic FRET based sensor consisting of CFP, MBP, and YFP with no linker peptides while the second one, CMY-BII, has a Ser-Arg linker between MBP and the two fluorescence proteins2. As Figure 1A shows CMY-0 is not available in less than 50 °C of measurement temperature as there is no signal difference between 0 and 1mM maltose conditions. The signal differences of both FRET sensors were maximized between 50 and 55 °C (Figure 1)8. For the quantification of the sugar contents in the three types of commercially available beverages, a dose response curve of the CMY-BII sensor in 53 °C was built (Figure 2A) and maltose contents were identified in the three samples by converting the FRET ratio into maltose concentration. In Figure 2A, since sample A is made of grains such as rice and barley which are one of main maltose sources, it is reasonable the sample contained relatively high maltose content (average 11.892 g/235 mL). On the other hand, sports drink (sample C) had the lowest maltose (0.29 g/250 mL) among the three beverages. These results suggest that FRET analyzer with optimal temperature control can be used by maximizing the efficiency of sugar contents quantification without expensive high-end FRET detection devices.

**FIGURE LEGENDS:**

**Figure 1: Measuring FRET signal difference between 0 and 1mM maltose concentrations using the FRET analyzer in various temperatures.** (A) YMC-0 sensor showed no signal difference based on maltose concentration in less than 50 °C (B) YMC-BII sensor was available in a wide range of temperature as the FRET signal in 1mL maltose was distinguished from that of 0 maltose concentration. In both cases, the signal difference dramatically increases in a specific temperature range (50~ 55 °C).

**Figure 2:** **Maltose content quantification in commercially available three beverages.** (A) A dose response curve of YMC-BII. (B) Three beverage samples were examined to their quantify maltose contents. Note that Total sugar indicates the quantity of the all sugar contents (including maltose) by the beverage providers. The error bar indicates the standard deviation.

**DISCUSSION:**

This protocol shows the rapid and efficient quantification of the sugar contents in beverage samples using a custom made FRET analyzer7 with optimal FRET sensor temperature control. The analyzer was designed with a recently developed, inexpensive 405-nm band UV-LED as the light source and two photodetectors with a silicon photodiode; this device is cost effective in comparison with other expensive fluorometers. For the high detection sensitivity, the device specifically measures the ratio of two emission wavelength bands (530 and 480 nm) in an optimal temperature of FRET sensors and showed better sensitivity and intensity than a fluorescence spectrophotometer device in detecting various sugars7. 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 contents via FRET sensors, it is clear that the device incorporates a number of 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 ions9, heme10, and others. Moreover, more than 20 types of FRET constructs can be easily found and ordered through the nonprofit depository, AddGene11.

Despite the wide applicability of the FRET analyzer, there are two main issues with the operation of the device. First, since operation of the device is rather simple, sample preprocessing is the critical step for quality of detection, except in cases of device malfunctioning. In this protocol, one step (sample dilution) was sufficient for processing of liquid samples that were clearly transparent without any insoluble particles. However, it is necessary to remove any insoluble materials, such as cellular or lipid components. Any auto-fluorescent particles that can affect the FRET signal should be also removed as noted in Step 2.7. Secondly, quality control and connectivity interfacing with hospital information systems (HISs) need to be addressed, as with all types of Point-of-care testing (POCT) tools12. Because the signal quality of the FRET analyzer is largely dependent on the quality of the FRET sensor and the preprocessing steps, regular quality control checks are required to ensure that measurement are within the standard signal range for regular quality control data analysis. The quality control analysis includes the investigation of FRET sensor stability and storage period which are essential for further reliable applications. These can be partly achieved by well-documented guidelines and appropriate software development, which can also address the connectivity limitation. Current versions of the FRET analyzer equipped RS232 connectivity for remote command line control but wireless communication are possible in the next version of the analyzer, which should have an improved interface for the HIS. On the other hand, FRET sensors have been engineered for substrate specificity but it normally resulted in broaden the specificity2. Consequently, the FRET signal can be interfered by the unintended ingredients including other types of sugars in the commercial beverages. It is required further to investigate FRET sensors responding to various cases of sugar mixtures for the accurate sugar quantification. Also collaboration with companies that produce the beverages will be helpful to confirm and to adjust the calibration of the FRET analyzer.

The proposed portable FRET device with various FRET sensors is anticipated to be used to POCT applications. POCT is currently used for assessing pregnancy, blood glucose levels, biomarker proteins, infectious bacteria, and infectious viruses. POCT methods have rapid turn-around times and generally exhibit low error rates due to the reduced number of processing steps. In terms of turn-around time and errors caused by multiple processing steps, POCT is more competitive than the central laboratory testing (CLT) approach. Hand-held portable devices for POCT, such as the device described herein, have attracted increasing attention due to its 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 method13. After the Ames research team developed a blood glucose detecting strip method, which contains glucose oxidase, as the first blood glucose test strip in 1965, several technologies were proposed for blood glucose monitoring purposes12. 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.

Importantly, simple, rapid methods for food quality assessment, particularly measurement of components of beverages, are needed; indeed, sugar-containing beverage consumption is associated with a variety of diseases and syndromes, such as increased body mass index in childhood15, pediatric obesity16, and risk of stroke17, necessitating measurement of sugar components in beverages. In this sense, glucose and fructose concentration in beverages could be a kin interest of people for the reason of healthcare.

Despite this protocol focus on the simple and high sensitive performance of the FRET analyzer with optimal temperature control, it is clear that the device with various FRET sensors detecting various small molecules including glucose and fructose14, 15. In terms of POCT, our portable and rechargeable device is beneficial. The battery life ranges from 10 to 40 hr depending on the heating protocol. The simple operational protocol of the analyzer will make the device easy to use, without complicated staff training requirements, as shown in the protocol session.

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.

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**DISCLOSURES:**

The authors have nothing to disclose.

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