



Development of a novel homogeneous immunoassay using the engineered luminescent enzyme NanoLuc for the quantification of the mycotoxin fumonisin B1

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ABSTRACT

Compared to the traditional heterogeneous assays, a homogeneous immunoassay is a preferred format for its simplicity. By cloning and isolating luminescent proteins from bioluminescent organisms, bioluminescence has been widely used for various biological applications. In this study, we present the development of a homogeneous luminescence immunoassay (FNanoBiT assay) for detection of fumonisin B1 (FB1), based on the binding of two subunits of an engineered luminescent protein (NanoLuc). For the detection of the mycotoxin FB1 in foods, the anti-fumonisin antibody was conjugated to the large subunit of NanoLuc (FLgBiT), and the FB1 was conjugated to the small subunit (FSmBiT). The conjugates were used for the detection of FB1 in a competitive immunoassay format without the need of a secondary antibody, or washing steps. The developed FNanoBiT assay revealed high specificity toward FB1 with no cross-reactivity with other mycotoxins, and it demonstrated acceptable recovery (higher than 94%) and relative standard deviation from spiked maize samples. Further, the assay was successfully applied for the detection of FB1 in naturally contaminated maize, with a dynamic range of 0.533–6.81 ng mL⁻¹ and a detection limit of 0.079 ng mL⁻¹. The results derived with FNanoBiT assay of all spiked samples showed a strong correlation to those obtained by the High-performance liquid chromatography method. Thus, the FNanoBiT based homogeneous immunoassay could be used as a rapid, and simple tool for the analysis of mycotoxin-contaminated foods.

1. Introduction

Fumonisin mycotoxins are toxic secondary metabolites produced by several *Fusarium* species such as *F. verticillioides* and *F. proliferatum*. These mycotoxins are causing a major concern in the United States and global agriculture (Kamle et al., 2019). Fumonisin B1 (FB1) is the most abundant naturally predominating form of the fumonisins in foods, mainly maize and maize-based products. It is also the most toxicologically important among all other fumonisins and has been classified as a group 2B carcinogen by the international agency for research on cancer (IARC) (International Agency for Research on Cancer, 2002). When consumed by animals, FB1 causes various adverse effects on multiple organs such as the brain in equine leukoencephalomalacia, or lungs in

porcine pulmonary edema syndrome. In addition, it shows carcinogenic effects for rodents and toxicity of liver and kidney in many species ((JECFA) Joint FAO/WHO Expert Committee on Food Additives, 2017). Human-health concerns associated with FB1 involve esophageal cancer, birth defects, growth impairment, and maybe other diseases ((JECFA) Joint FAO/WHO Expert Committee on Food Additives, 2017; Chen et al., 2018). Therefore, authorities, such as the United States Food and Drug Administration (USFDA) and European Commission, have set their guidelines and regulations for fumonisin levels in foods intended for human consumption (USFDA, 2001; Torelli et al., 2012).

To ensure food safety, several methods have been developed to determine fumonisins in foods such as chromatographic methods, including thin-layer chromatography (TLC), high-performance liquid

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chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), and Liquid chromatography-mass spectrometry (LC-MS) (Rottinghaus et al., 1992; Shephard et al., 1990; Silva et al., 2009; De Baere et al., 2018). HPLC is the only chromatographic method recommended by the association of official analytical chemists (AOAC) for the determination of fumonisins in maize and maize flakes (Visconti et al., 1994). However, these chromatographic methods are generally limited-throughput tools and labor-consuming, requiring trained personnel. On the other hand, enzyme linked immuno-sorbent assay (ELISA) is another method that is most frequently used for fumonisins detection because it is simple, sensitive, and can be used for high-throughput (Dang et al., 2016; Zheng et al., 2006). Even though it is preferred to chromatographic methods, ELISA is still performed in a heterogeneous mode requiring numerous incubations to immobilize the captured antibody and washing cycles to remove the unbound reagents resulting in prolonging analysis time and increasing the risk of contamination.

Unlike ELISAs, proximity based homogeneous immunoassay format has gained increasing attentions as it can quantitatively and sensitively distinguish antigen and antigen-antibody complexes in the immuno-reaction solution (Darwish, 2006). Homogeneous immunoassays have simple add-and-read format, require no washing steps, and can be easily automated for high throughput. To the best of our current knowledge, only a few homogeneous detection techniques such as fluorescence correlation spectroscopy (FCS), the FB1 antibody-coated gold nanoparticles (AuNPs) and a yellow fluorescent protein (YFP) -tagged FB1-mimotope (AuNP-YFP), a fluorescence polarization immunoassay (FPIA), and a multi-wavelength fluorescence polarization immunoassay (MWFPIA) have been developed for FB1 determination in foods (Bian et al., 2016; Peltomaa et al., 2018; Li et al., 2015, 2016). However, these immunoassays do not meet the desired levels of sensitivity and applicability.

Bioluminescence is a phenomenon that is found in a broad range of organisms, including bacteria, fungi, insects, and marine organisms. It is visible light produced when a photon-emitting substrate (luciferin) is oxidized by a generic class of enzymes named luciferases (Haddock et al., 2010). Unlike fluorescence and phosphorescence, bioluminescence production does not depend on light absorption or electron excitation to emit light; therefore, it provides much broader dynamic range and higher sensitivity than other luminescences (Widder and Falls, 2014; Hall et al., 2012). The great advantages of bioluminescence applications have driven researchers to investigate and develop new luciferases with higher brightness and sensitivity. Thus, an engineered luciferase (NanoLuc) was developed by improving the chemical structure of an *Oplophorus gracilirostris* (deep-sea shrimp) luciferase (Oluc) and developing a novel substrate (furimazine) (Hall et al., 2012).

Split-luciferase complementary assays are a type of assays where a luciferase is split into two halves and attached to two interacting proteins. The interaction of the two candidate proteins with each other allows the reconstitution of the actively complemented luciferase. A split-luciferase complementary system is a powerful tool for monitoring protein-protein interactions, protein-nucleic acid interactions, and many bioanalytes in vivo and in vitro (Azad et al., 2014). **NanoLuc Binary Technology (NanoBiT)** is a new protein complementation system based on NanoLuc. The NanoBiT is composed of two subunits, a large subunit (LgBiT 18 kDa) and a small subunit (SmBiT 11 amino acid peptides). The intrinsic affinity of those subunits to bind to each other is very low, resulting in very low nonspecific interaction and background signal (Dixon et al., 2016). However, when LgBiT and SmBiT are brought into close proximity, for example, by fusing them to two interacting proteins, a fully active bioluminescent enzyme re-forms. Recently, SmBiT-LgBiT complementation was used to develop sensitive immunoassays to probe cellular signaling (Hwang et al., 2020) and SARS-CoV-2 antibodies (Elledge et al., 2020).

In this study, we report the development and validation of a rapid, add-and-read homogeneous immunoassay using NanoBiT for FB1 analysis. This work is the first example of using the luminescent technique

for the quantification of fumonisin toxins in foods. The FNanoBiT assay is based on a competition assay format (Fig. 1a). The assay is comprised of two key elements, a 'tracer' which is FB1-SmBiT (FSmBiT), and an anti-fumonisin antibody labeled with LgBiT (FLgBiT). In solution FSmBiT and FLgBiT interact and generate a bioluminescent signal. When samples containing FB1 are added to the reaction, they compete with the FSmBiT for binding to the FLgBiT, which results in a concentration-dependent decrease in the bioluminescent signal. The optimized FNanoBiT immunoassay was used to detect FB1 in contaminated maize. Although not addressed in this paper, recent years have seen tremendous development in optical biosensors based on smartphones and it is easy to foresee a cartridge-based device incorporating FNanoBiT reagents interfaced with sensitive cameras for field-based detection of food toxins (Huang et al., 2019; Kim et al., 2017; Lee et al., 2017; Rateni et al., 2017).

2. Materials and methods

2.1. Chemicals and reagents

The common mycotoxins fumonisin B1 (FB1), aflatoxin B1 (AFB1), deoxynivalenol (DON), zearalenone (ZEA), ochratoxin A (OTA), zearalenone (ZEA), and patulin were purchased from Sigma-Aldrich. Fumonisin B2 (FB2) and maze naturally contaminated fumonisins (Certified Reference Material (CMS2)) were purchased from Trilogy analytical laboratories. All other ground maize samples were obtained from local stores in Madison, WI. The amine reactive HaloTag succinimidyl ester (O4) ligand, 10X NanoGlo buffer, and NanoGlo substrate (furimazine) were obtained from Promega, whereas SmBiT peptide with cysteine at N terminal (CVTGYRLFEEL) was purchased from Genscript Co. Monoclonal anti-fumonisin antibody came from Novus Biologicals, and serum-free protein blocking buffer was from ScyTek Laboratories. All aqueous solutions were prepared by using ultrapure water (18.2 MΩ cm), and other chemicals were analytical grade and used as received without further purification.

2.2. Apparatus

Zeba spin desalting columns were obtained from ThermoFisher scientific, and immunoaffinity columns (FumoniTest WB) were purchased from Vicam. All non-binding round-bottom 96-well white plates were purchased from Corning. GloMax® Navigator Microplate Luminometer was supplied by Promega Corporation, 280 nm wavelength was measured by Thermo Scientific NanoDrop. LC-MS was carried out on Waters Alliance, while high-resolution mass spectrometry (HRMS) was carried out on Sciex using Sciex 5600+ QTOF system. Ultra-performance liquid chromatography-tandem mass spectrometer (UPLC/MS/MS) was carried out on agricultural marketing services in the United States department of agriculture (USDA).

2.3. Preparation of FSmBiT

The conjugation of FB1 to SmBiT was performed in two steps. Firstly, FB1 standard was dissolved in N,N-Dimethylformamide, and then reacted with 10 M excess of diisopropylethylamine and 5 M excess of 4-N-Maleimidobenzoic acid-NHS to form FB1-maleimide compound. The resulted mixture was stirred for an hour and directly purified by prep-HPLC, and then identified by LC-MS. Secondly, an appropriate concentration of SmBiT peptide with cysteine at N terminal was dissolved in phosphate buffer (PBS) (10 mM, pH 7.4) and then added to an appropriate amount of fumonisin-maleimide mixture prepared in dimethyl sulfoxide. The resulted solution was stirred for 2 h and directly purified by prep-HPLC. The FB1 with SmBiT (FSmBiT) was identified and characterized by HRMS. The FSmBiT solution was stored at 4 °C until used.

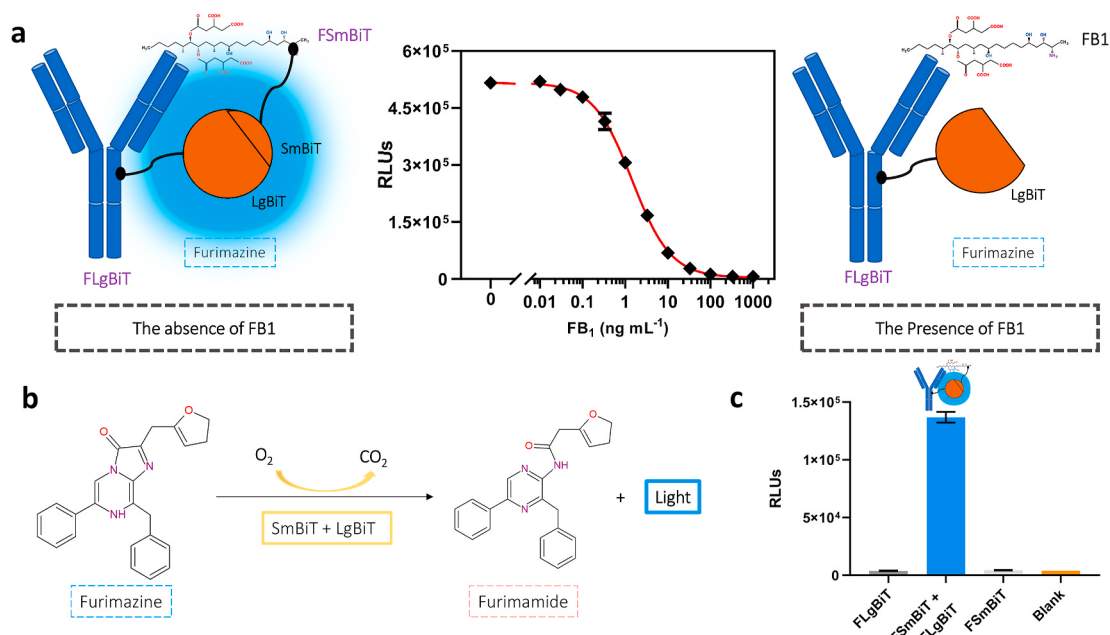


Fig. 1. Synopsis of the detection protocol. (a) Schematic representation of the principle of the homogeneous competitive FNanoBiT assay. (b) Oxidation of substrate by NanoLuc enzyme subunits. (c) Luminescence generation upon the binding of FSmBiT and FLgBiT.

2.4. Preparation of FLgBiT

The conjugation of the anti-fumonisin antibody to LgBiT was carried out in two steps according to the following protocol. A labeling kit (Lumit Immunoassay labeling kit) is now commercially available from Promega. First, the antibody was dialyzed into 10 mM bicarbonate buffer (pH 8.5) using zeba desalting columns, and then reacted with 20 M excess of amine reactive HaloTag® Succinimidyl Ester (O4) ligand (M.wt: 509 Da) for 90 min at 22–25 °C with gentle mixing. Free ligands were further removed by dialyzing antibody into PBS using Zeba desalting column followed by measuring absorbance at 280 nm to calculate the concentration of the labeled antibody. Subsequently, 4 M excess of HaloTag fused LgBiT (M.wt: 50 kDa) and 0.05% IGEPAL final concentration were added to the HaloTag labeled antibody and mixed gently at 4 °C for 16h. The anti-fumonisin antibody with LgBiT (FLgBiT) was stored at -20 °C until used.

2.5. Sample preparation and extraction of FB1

For artificially contaminated maize samples, various concentrations of FB1 standards ranging from 0.5 to 5 µg mL⁻¹ were added to each 1 g of ground maize, previously analyzed for FB1 background concentration, and left open in a fume hood for 30 min to allow the solvent to evaporate prior to extraction. The extraction of FB1 toxin from food samples was performed according to the AOAC protocol with slight modifications (Visconti et al., 1994). Briefly, 3 g of ground maize were mixed with 15 mL of acetonitrile-methanol-water (25 + 25 + 50, v/v/v) and shaken for 12 min. After centrifuging the mixture for 5 min at 5000 rpm, the extracted liquid was filtered with a 0.22 µm nylon filter, and then 5 mL of the filtered extract were diluted in 20 mL of PBS. Subsequently, the diluted extract was filtered for the second time through a glass fiber filter and collected in a 50 mL tube for FB1 analysis by FNanoBiT assay.

For HPLC analysis, the resulted filtrate was further cleaned up by transferring 5 mL to the immunoaffinity column and filtering at 1–2 drops/s. The elute was discarded, and the column was washed with 5 mL PBS at a rate of 1–2 drops/s. FB1 toxin was eluted with 3 mL of methanol-acetic acid (99 + 1, v/v), and the elute was evaporated to dryness under the fume hood. The dry samples were dissolved in 1 mL

acetonitrile-water (50 + 50, v/v) and stored at 4 °C until used.

2.6. Detection procedure

To perform FNanoBiT assay, different solutions including FSmBiT, FLgBiT, and substrate solutions were prepared separately and mixed in the 96-well microplate. First, the optimal concentration of FSmBiT solution (10 nM) that contained 4.5% non-serum protein blocking buffer (SBB) was prepared in acetonitrile-water (50 + 50, v/v). For developing FB1 calibration curve, 10 µL of each FB1 standards (0.01, 0.033, 0.1, 0.33, 1, 3.3, 10, 33, 100, 333, and 1000 ng mL⁻¹) was added to 1 mL of the FSmBiT solution and mixed well. For analyzing food samples, 10 µL of the maize extract was added to 1 mL of the FSmBiT solution and mixed well. Afterward, 10 µL of the test solutions (the FB1 standards and 1% maize extract-containing solutions) were added per well in white non-binding 96-well microplate.

Second, FLgBiT solution was prepared in 10 mM PBS containing 0.1 mg mL⁻¹ bovine serum albumin (BSA) and then diluted in 7.5% (w/v) 2-Hydroxypropyl β-cyclodextrin in PBS (PH 8) to obtain the optimal concentration (0.33 nM). Subsequently, 10 µL of the prepared FLgBiT solution was added to the test solutions in the microplate, followed by the addition of 10 µL per well of the substrate solution (furimazine). After 5 min of the substrate addition, luminescence intensity (RLUs) values were recorded using a GloMax® Navigator Microplate Luminometer. For the toxin dose–response plots, the luminescence readings were analyzed with GraphPad software (GraphPad Prism 8) using a four-parameter logistic regression (4-PL) function. HPLC was performed according to the AOAC procedure (details are shown in Supplementary Material).

3. Results and discussion

FB1 is the most toxicologically important toxin among all other fumonisin forms due to its toxicity and prevalence in agricultural commodities especially maize, and it has been found to cause a variety of diseases in animals as well as humans (Fig. S1). Since the recognition of FB1 as a carcinogenic agent, many analytical methods utilizing different technologies have been developed to facilitate the determination of FB1 in foods.

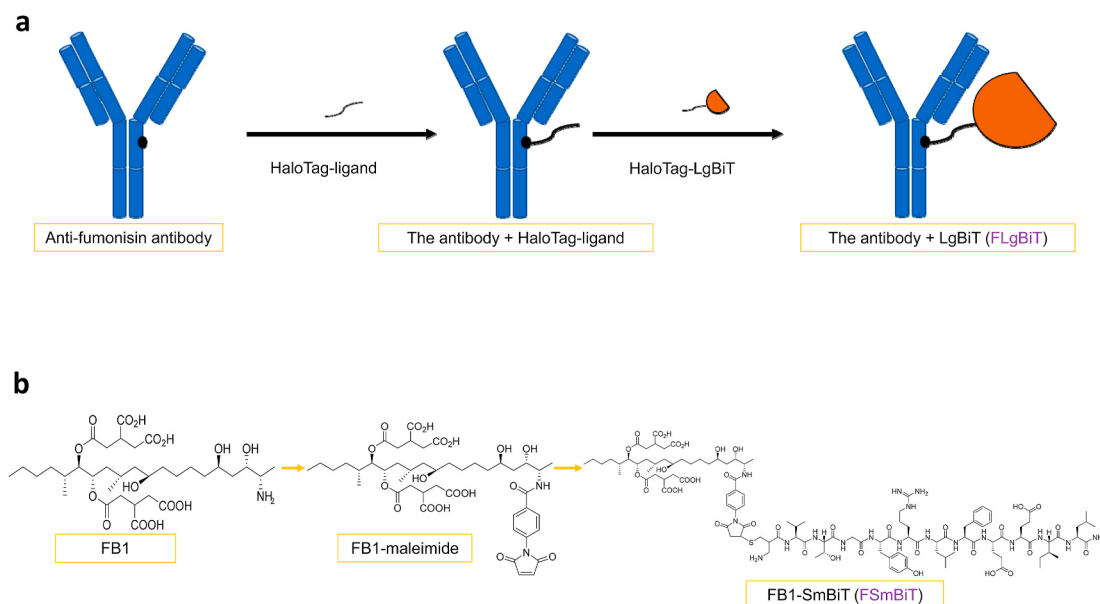


Fig. 2. A schematic presentation of two key conjugants. (a) Conjugation of the anti-fumonisin antibody with the large subunit of NanoLuc enzyme. (b) Conjugation of FB1 toxin with the small subunits of NanoLuc enzyme.

3.1. Labeling and characterization of FLgBiT and FSmBiT

The homogeneous FNanoBiT assay is a competition immunoassay (Fig. 1a) and uses an anti-fumonisin antibody labeled with LgBiT (FLgBiT), and FB1 labeled with SmBiT (FSmBiT) as a tracer molecule. In the FNanoBiT system, the tracer binds to FLgBiT to produce luminescence upon the oxidation of furimazine to furimamide (Fig. 1b and c). In the presence of FB1 toxin, the competition between the tracer and FB1 hampered the binding event of FLgBiT and FSmBiT, thus reducing the luminescence signal. The more FB1 toxin presented in the sample the

lower light signal (RLUs) was measured; therefore, the FB1 typical sigmoidal standard curve was developed for the measurement of the unknown FB1 content in the test solution (Fig. 1a). As shown in Fig. 2a, the conjugation of the antibody with LgBiT was done using HaloTag chemistry (Los et al., 2008) in two steps. Firstly, the amine reactive HaloTag succinimidyl ester ligand, comprising a reactive succinimidyl ester group connected to chloroalkane linker, was reacted with primary amines on lysine amino acids on the antibody to form stable amide bond linkages. After removing the unreacted ligand using Zeba desalting column, the antibody-HaloTag ligand was incubated with

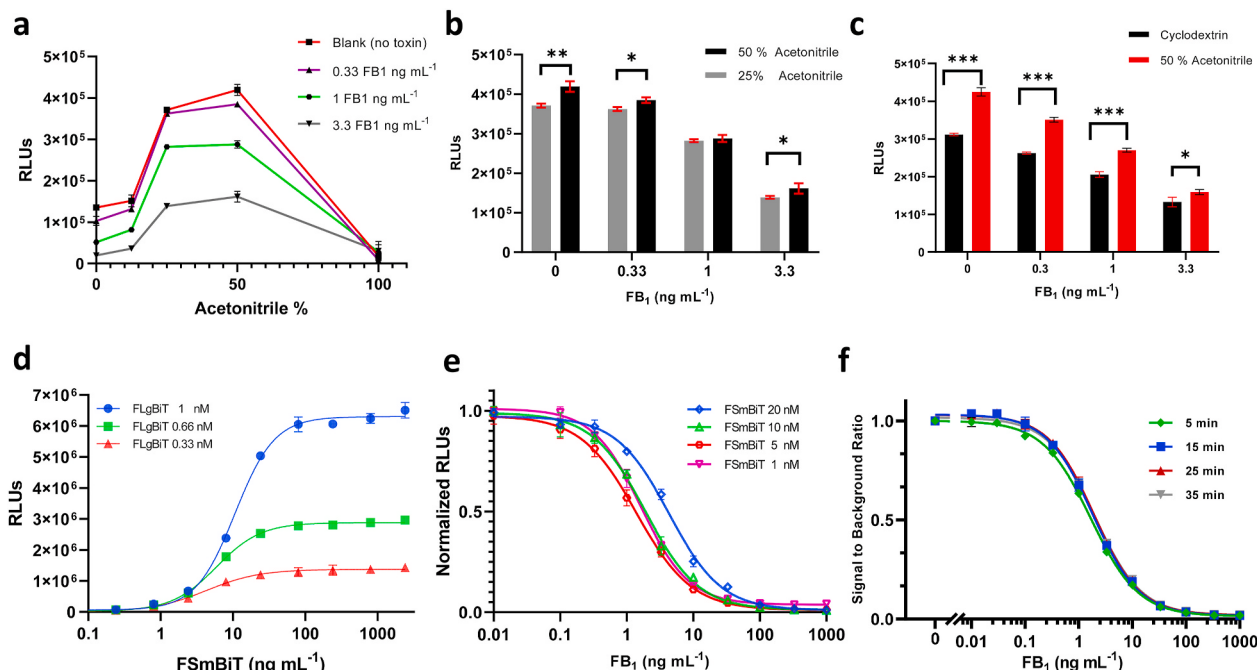


Fig. 3. Validation and optimization of the assay. (a) Acetonitrile solvent at different concentrations were used as FSmBiT diluent for the FNanoBiT assay in the absence and presence of FB1. (b) The difference of luminescence signal with 25 and 50% acetonitrile solvent in the absence and presence of FB1. (c) The difference of luminescence signal with 50% acetonitrile solvent and 7.5% (w/v) β -cyclodextrin in the absence and presence of FB1 concentrations. (d) The production of luminescence at various concentrations of FSmBiT and FLgBiT (e) FB1 calibration curves of FNanoBiT assay using various FSmBiT concentrations and a fixed FLgBiT concentration (0.33 nM). (f) Signal to background ratio of FB1 calibration curves at different incubations times.

Table 1

Analytical parameters of the FNanoBiT assay using a fixed concentration of FLgBiT and various concentrations of FSmBiT.

FLgBiT(nM)	FSmBiT(nM)	Parameters (ng mL ⁻¹) ^a		<i>r</i> ²
		Dynamic Range	LOD	
0.33	20	1.22–15.22	0.144	0.99
	10	0.53–6.81	0.079	0.99
	5	0.37–5.28	0.114	0.99
	1	0.51–5.19	0.995	0.98

^a The analytical parameters were obtained by plotting the RLUs against FB1 concentrations. The dynamic range values were determined as that corresponding to the 20%–80% inhibition (IC₂₀ – IC₈₀) of luminescence production. The LOD was determined by calculating the average blank luminescence signal - 3 × the standard deviation of the blank. Data represent the average of three replicates.

HaloTag-LgBiT to make a covalent conjugate of antibody-HaloTag-LgBiT. The covalent bond formation between the HaloTag-LgBiT and the chloroalkane linker of the antibody-HaloTag ligand is fundamentally irreversible, highly specific, and kinetically fast under physiological conditions. Fig. 2b illustrates the labeling of FB1 to SmBiT via the formation of FB1-maleimide followed by the addition of SmBiT peptide containing cysteine at N terminal. The formation of FSmBiT was identified and confirmed by HRMS (Fig. S2). In fact, this homogeneous FNanoBiT assay made it much easier to differentiate the antibody-bound tracer (FLgBiT-FSmBiT) from the antibody-bound toxin (FLgBiT-FB1) in shorter analysis time, without washing steps, and with less risk of contamination from exogenous interferences.

3.2. Optimization of the FNanoBiT assay

Some experimental conditions involved in the detection procedure were evaluated and optimized to enhance the sensitivity of the FNanoBiT assay. Organic solvents are required for the extraction and/or

solubilization of the mycotoxins, and acetonitrile was found to be a suitable solvent for fumonisins stability (Visconti et al., 1994). However, the organic solvent can have a significant impact on the catalytic activity of enzymes and the binding ability of antibodies (Lu et al., 1997). Therefore, the effect of acetonitrile concentrations on the enzymatic activity and the binding of FSmBiT-FLgBiT was studied to obtain high assay sensitivity. Among 12.5, 25, 50 and 100% acetonitrile, the higher luminescence intensity (RLUs) was observed at acetonitrile-water (50 + 50, v/v) as shown in (Fig. 3a and b). This observation gave an indication that the best solubility and binding of FSmBiT to FLgBiT occurred when 50% acetonitrile was used as FSmBiT diluent. In addition, acetonitrile-water (50 + 50, v/v) was compared to 7.5% (w/v) β-cyclodextrin in PBS (PH 8) since the latter was used to stabilize FLgBiT. Fig. 3c showed that acetonitrile solvent enhanced the luminescence signal more than cyclodextrin did with and without the presence of FB1 in the solution. Therefore, acetonitrile-water (50 + 50, v/v) was chosen as the optimal diluent for FSmBiT solution in the subsequent experiments.

The ratio of a tracer to an antibody in homogeneous assays plays an important role on the assay sensitivity and the immunocomplex formation (Bian et al., 2016). In this study, FSmBiT to FLgBiT ratio was evaluated based on the half-maximal response (EC₅₀) and lower limit of detection (LOD), calculated by taking the average luminescence of the blank (no FB1) minus 3 times the standard deviation of the blank according to the method reported in the literature (Peltomaa et al., 2018). As shown in Fig. 3d, FLgBiT (1 nM) produced much larger luminescence intensity; however, FLgBiT (0.33 nM) showed higher sensitivity as it required lower concentrations of FSmBiT to give EC₅₀ (Table S1). Also, when the concentration of FSmBiT reached 50 ng mL⁻¹, the RLUs of FLgBiT (0.33 nM) did not significantly increase, indicating that almost all antibodies were occupied by tracers at 50 ng mL⁻¹, and any other addition of FSmBiT would be an excess reducing the assay sensitivity. Therefore, the molar concentrations of FSmBiT (1, 5, 10, and 20 nM) were added to a fixed amount of FLgBiT (0.33 nM) to develop FB1 calibration curves. The developed curves were used to obtain the LOD

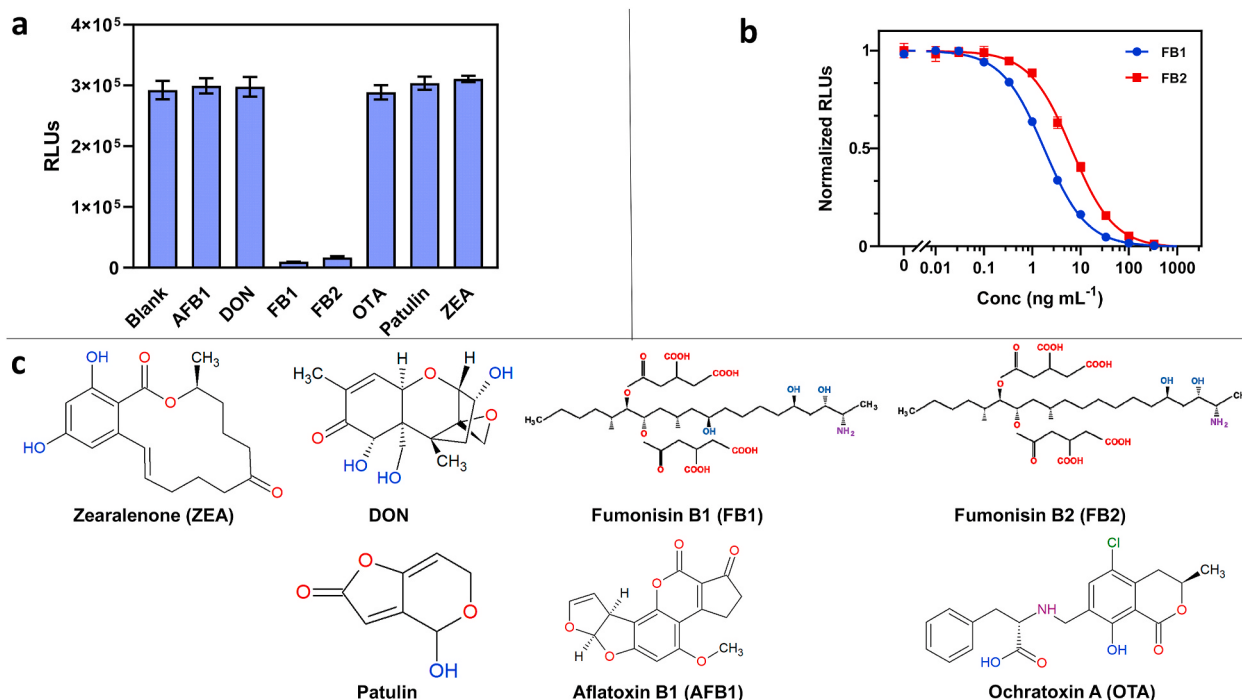


Fig. 4. Specificity of the assay toward FB1 and FB2. (a) Specificity of the FNanoBiT assay assessed by testing FB1 and FB2 with different mycotoxins such as aflatoxin B1 (AFB1), deoxynivalenol (DON), ochratoxin A (OTA), patulin, and zearalenone (ZEA) at 100 ng mL⁻¹ concentration for each (b) FB1 and FB2 calibration curves developed by FNanoBiT assay (FB1 blue, FB2 red) (c) The chemical structures of tested mycotoxins. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

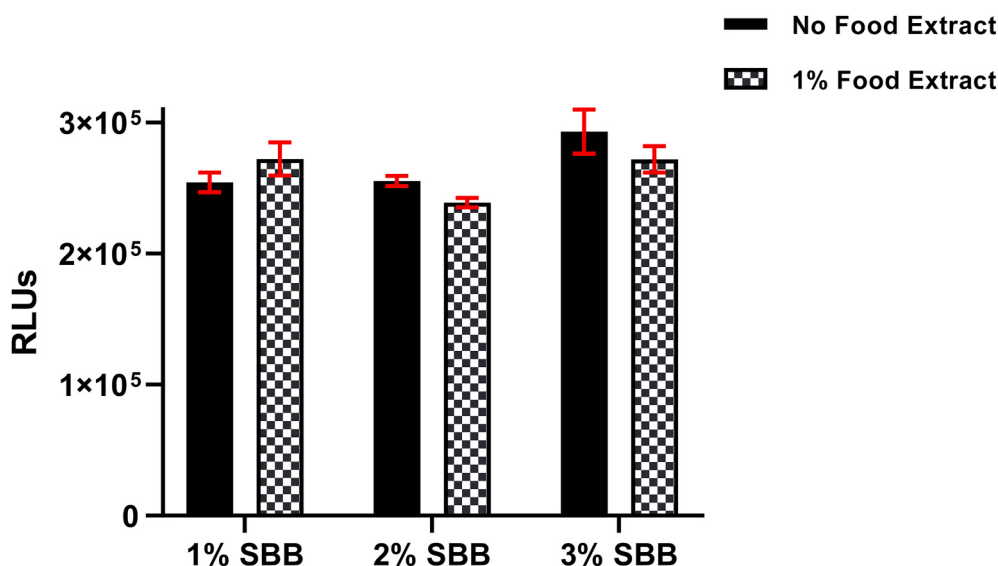


Fig. 5. Different concentrations of super blocking buffers (SSB) added to the FNanoBiT system to block the non-specific interaction of FLgBiT to FSmBiT.

and dynamic range, which was calculated from the 20%–80% inhibition (IC₂₀ and IC₈₀) according to the method reported in the literature (Li et al., 2015; Peltomaa et al., 2018) (Fig. 3e). The results showed that the higher assay sensitivity, LOD of 0.079 ng mL⁻¹ and dynamic range from 0.533 to 6.81 ng mL⁻¹, were obtained when 10 nM FSmBiT and 0.33 nM FLgBiT were used (Table 1). Thus, the ratio of 10 nM FSmBiT to 0.33 nM FLgBiT was selected for further assay development.

Further, the binding kinetic between FLgBiT and FSmBiT was evaluated in this study. The calibration curves of FB1 were developed at different time points of adding the substrate solution (different incubation times). In fact, the highest luminescence signal was observed at 15 min of incubation, and longer incubation times somehow decreased RLUs (Fig. S4). However, the lower signal-to-background ratio, calculated by dividing the luminescence in the presence of FB1 by the luminescence obtained in the absence of FB1, was observed at 5 min yielding to higher sensitivity (lower IC₅₀) and higher accuracy (Fig. 3; Table S2). Therefore, a 5 min incubation time was chosen for further assay development as it provided a shorter analysis time and better assay accuracy.

The specificity of the FNanoBiT assay was assessed by determining the cross-reactivity with other mycotoxins. Fig. 4a illustrated that mycotoxins such as aflatoxin (AFB1), deoxynivalenol (DON), ochratoxin A (OTA), zearalenone (ZEA), or alternariol (AOH) did not bind to FLgBiT and did not reduce luminescence signal in the assay when tested at 100 ng mL⁻¹ concentration as the luminescence readings were comparable to the background (no toxin). In fact, while FLgBiT bound specifically to both FB1 and FB2, which are structurally related to each other, the assay showed much more affinity toward FB1 than FB2 form (Fig. 4b and c). Thus, the cross reactivity toward other mycotoxins was ruled out, and

the FNanoBiT assay was developed for FB1 determination only.

3.3. Real food sample analysis

Due to the lack of blank maize quality control, the concentration of FB1 in contaminated maize samples was determined relative to the assay buffer-based calibration curve. BSA as a common blocking agent for immunoassay was used to prevent the antibody non-specific binding (Xiao and Isaacs, 2012). In addition, non-serum protein super-blocking buffer (SBB) was added with BSA to synergistically suppress the non-specific reaction of the FLgBiT and minimize the maize matrix effect. As presented in Fig. 5, the addition of 1% SBB of the total assay solutions partially prevented the non-specific reaction of test solutions containing 1% extract maize, previously tested for FB1 background concentration by UHPLC-MS/MS. However, 1% maize extract still interfered with the assay showing higher luminescence intensity than test solutions containing no food matrix (1% PBS buffer). Even though this difference in luminescence measurement was insignificant, it could underestimate the actual FB1 amount in the food sample. Interestingly, 2 and 3% SBB added to the system somehow decreased the RLUs of the test solution that contained 1% maize extract compared to the test solution that contained assay buffer. This could lead to overestimating the actual FB1 content in the food. Therefore, the addition of 1.5% SBB of the total assay solutions was found to effectively block the non-specific interaction of FLgBiT, showing a negligible food matrix effect (Table 2).

The FNanoBiT assay was applied for determining FB1 content in contaminated foods. For artificially FB1 contaminated maize, ground maize sample (CMS1), which was analyzed for FB1 background concentration by UHPLC-MS/MS, was spiked with certain amounts of FB1 standards and tested using FNanoBiT and HPLC methods. As illustrated in (Table 3), the results showed recoveries of 94% (RSD 5.1%) and 103% (RSD 5.4%) for CMS1 spiked with 5 and 1 mg kg⁻¹ FB1, respectively. For naturally FB1 contaminated food, a naturally contaminated fumonisins maize (certified reference material (CMS2)) was analyzed by the FNanoBiT assay. The detected FB1 was 2.8 mg kg⁻¹ showing a good agreement with the results provided by Trilogy analytical laboratory (Table 4). Another ground maize sample (CMS3) was also analyzed by the FNanoBiT assay and then validated by HPLC in our laboratory and UPLC/MS/MS in a different laboratory (Fig. S5). The results obtained by the FNanoBiT method for FB1 analysis highly agreed with those obtained by HPLC and UPLC/MS/MS (Table 4). To determine the correlation between FNanoBiT and HPLC methods, a total of 20 ground maize samples, which were previously analyzed for FB1 background

Table 2

Detection of FB1 in maize samples tested by 1.5% SBB FNanoBiT assay before and after the removal of interferences.

Sample No	Food Sample Extract (before cleanup)		Food Sample Extract (after cleanup) ^a	
	Detected Concentration(mg kg ⁻¹)	RSD%	Detected Concentration(mg kg ⁻¹)	RSD%
1	1.5	5.4	1.5	14.7
2	1.9	3.6	1.9	7.8
3	2.8	5.49	2.9	3.7
4	1.2	9.6	1	5.6

concentration by UPLC/MS/MS, were spiked with different quantities of FB1 standard ranging from 0.5 to 5 mg kg⁻¹. All spiked samples were analyzed by FNanoBiT and HPLC, and FNanoBiT results were compared with those of HPLC. As shown in Fig. 6a, the developed linear relation showed a strong positive correlation of 0.9699 between the two methods. Therefore, the reported homogeneous luminescent assay might be used as a rapid, high throughput and highly sensitive method for mycotoxins analysis in foods.

Stability of the FNanoBiT assay was determined by measuring

luminescence to the blank (no toxin) and FB1 standards after storing the FLgBiT and FSmbiT solutions at 4 °C for up to 12 days Fig. 6b illustrates a significant decrease in RLUs over the first four days and a slight decrease during the next 8 days in response to the blank, 1 and 10 ng mL⁻¹ FB1. In fact, this decrease in RLUs did not significantly affect the detection of FB1 toxin in the maize sample in the first 4 days (Fig. 6c). Therefore, the determination of FB1 in the maize extract and the RSD obtained indicated good stability over the first 4 days (Table S3). It is desirable to have an assay with a long shelf life at 4 or room temperature

Table 3

Detection of FB1 in artificially contaminated maize samples by FNanoBiT and HPLC.

Sample ID	FB1 Background Concentration (mg kg ⁻¹) ^a	FB1 Spiked Concentration (mg kg ⁻¹)	FNanoBiT Assay			HPLC		
			Detected Concentration (mg kg ⁻¹) ^b ± SD	RSD% (RSD ^c %)	Recovery %	Detected Concentration (mg kg ⁻¹) ^b ± SD	RSD% (RSD ^c %)	Recovery %
CMS1	0.07	5	4.8 ± 0.24	5.1 (12.79)	94.7	4.1 ± 0.23	5.6 (13.08)	80.8
		1	1.1 ± 0.06	5.4 (15.81)	102.8	1.1 ± 0.03	2.9 (15.81)	102.8

^a Samples tested by UPLC/MS/MS in a different laboratory.

^b The number was rounded up when the third digit is 5 or more.

^c The maximum RSD allowed by United States Department of Agriculture Grain Inspection (GIPSA) for developing rapid fumonisin test kits.

Table 4

Detection of FB1 naturally contaminated maize samples by FNanoBiT, HPLC and/or UPLC/MS/MS.

Sample ID	FNanoBiT Assay		HPLC		UPLC/MS/MS ^d	
	Detected Concentration (mg kg ⁻¹) ^b ± SD	RSD%(RSD ^c %)	Detected Concentration (mg kg ⁻¹) ^b ± SD	RSD%(RSD ^c %)	Detected Concentration (mg kg ⁻¹)	RSD% (RSD ^c %)
CMS2 ^a	2.8 ± 0.24	10.7 (13.82)	3.0 ^e			
CMS3	1.5 ± 0.08	5.4 (15.12)	1.3 ± 0.16	12.2 (15.44)	1.5	4.9 (15.12)

^a Naturally Contaminated Fumonisin maize (Certified Reference Material).

^b The number was rounded up when the third digit is 5 or more.

^c The maximum RSD allowed by United States Department of Agriculture Grain Inspection (GIPSA) for developing rapid fumonisin test kits.

^d Performed in a different laboratory.

^e Results were Provided by Trilogy analytical laboratories.

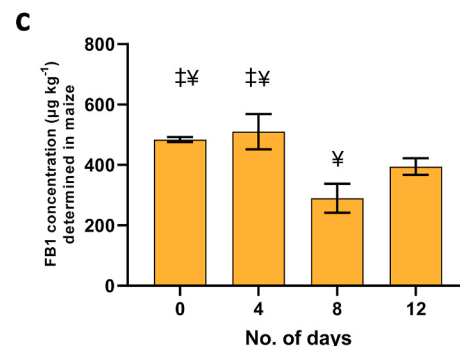
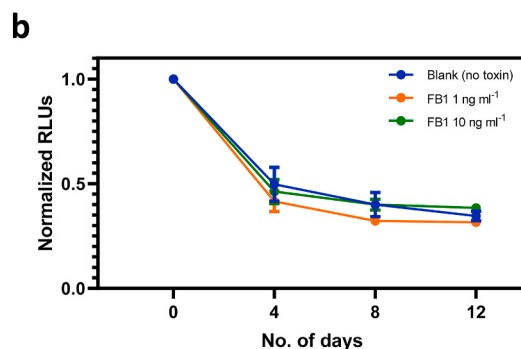
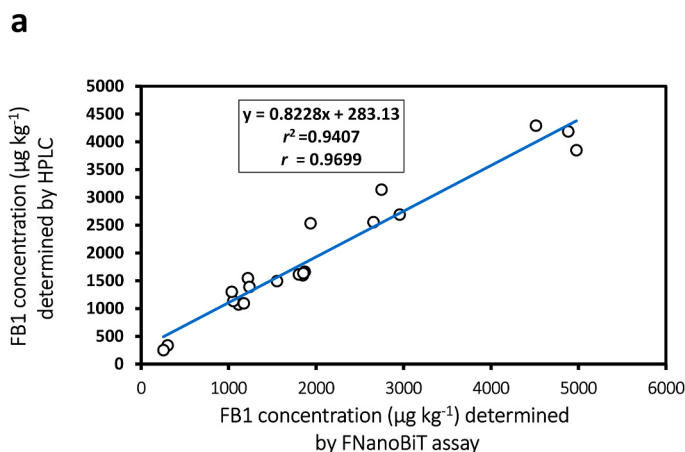


Fig. 6. (a) Correlation between FNanoBiT and HPLC methods for FB1 analysis of contaminated maize samples. (b) Luminescence of the FNanoBiT assay within 12 days after storing FSmbiT and FLgBiT solutions at 4 °C. (c) Determination of FB1 toxin in contaminated maize over 12 days after storing FSmbiT and FLgBiT solutions at 4 °C. ‡ Results are significantly different than the results of 8 days. ¥ Results are significantly different than results of 12 days. *P* value < 0.05.

to enable off-site food testing. Freeze drying of the antibodies in the presence of excipients has been reported to improve their room temperature stability of therapeutic antibodies (Haeuser et al., 2020), and similar formulations may be developed for the FNanoBiT assay to make it better suitable for field use.

4. Conclusion

This study reveals the applicability of bioluminescence to sensitive immunoassays for FB1 analysis in food. In fact, the homogeneous luminescent assay based on binding of the FLgBiT and FSmBiT pair we have developed for FB1 analysis is more sensitive than the previously reported homogeneous assays, and shows better analytical features including a simplified assay protocol and lower detection limit (Table S4). In contrast to the heterogeneous immunoassays, there was no need for secondary antibodies, washing and a further conjugation reaction because FSmBiT (SmBiT-FB1 conjugate) was directly used as the tracer to bind to FLgBiT (LgBiT-anti fumonisin antibody). The luminescence production of NanoLuc enzyme via the oxidation of furimazine provides an excellent feature to develop a homogeneous assay, and the use of LgBiT- HaloTag-antibodies and SmBiT-toxin provides a versatile strategy that can readily be applied to different targets of interest. The high sensitivity of the FNanoBiT assay allows detecting FB1 after multiple sample dilutions (solid to liquid ratio), which reduce the effects of the food matrix with no need for sample cleanup steps. In general, the analytical features and ease of the NanoBiT assay provide a powerful tool for rapid and sensitive analysis of contaminants in food samples.

It is worth noting that in case of FB1, a primary amine side chain allowed an easy handle to synthesize FSmBiT tracer. However, such reactive groups may not be available with other small molecules, and therefore alternate chemistries may be needed. Chemistry used for the design of tracer may also have an implication for antibody selection as it is possible that antibody that binds to an analyte with high affinity may have lower affinity for the tracer. Ideally, a panel of high-affinity antibodies with low cross-reactivity may have to be screened to obtain assay performance for a specific analyte.

Finally, recent years have seen a convergence of two technologies- smartphones with high quality cameras and fluorescent and luminescence-based assays-creating new opportunities for field based portable diagnostics devices as well as for food testing. Several biosensors designs that combine bright luminescent enzyme reporters and smartphone have been described for detection of microRNAs, bio-samples, and other analytes (Chen et al., 2021; Kim et al., 2017; Zhou et al., 2020). We believe approaches used in these reports can be incorporated into FNanoBiT assay offering a potential for field deployable biosensor for food testing.

Compliance with ethical standards

The authors declare that there were no ethical implications or research involving human or animals.

CRediT authorship contribution statement

Tawfiq Alsulami: Conceptualization, Methodology, Validation, Investigation, Formal analysis, Writing - original draft, Visualization. **Nidhi Nath:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing. **Rod Flemming:** Conceptualization, Methodology, Investigation, Resources, Writing - review & editing. **Hui Wang:** Conceptualization, Methodology, Investigation. **Wenhui Zhou:** Conceptualization, Methodology, Investigation. **Jae-Hyuk Yu:** Supervision, Resources, Funding acquisition, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bios.2020.112939>.

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