

Fluorescence-Quenching-Based Enzyme-Activity Assay by Using Photon Upconversion**

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Enzyme-activity assays are used, for example, for screening enzyme inhibitors and activators to discover novel drug candidates.^[1] A homogeneous assay principle for hydrolyzing enzymes based on a double-labeled fluorogenic substrate is commonly employed and is suitable for high-throughput screening. This separation-free assay concept relies on the strong distance dependency of fluorescence resonance energy transfer (FRET), which takes place only at distances below 10 nm.^[2,3] A synthetic internally quenched substrate for the enzyme is labeled with a fluorophore at one end and a quencher at the other end of the molecule. When the enzyme digests the substrate, the two labels are separated and fluorescence is recovered.

The performance of fluorescence-quenching-based homogeneous assays is still limited due to the autofluorescence originating from biological materials. This problem can be solved by a novel label technology based on upconverting phosphors (UCPs),^[4] which have the unique property of photoluminescence emission at visible wavelengths under near-infrared (NIR) excitation. No autofluorescence is detected at shorter wavelengths, because the upconversion phenomenon requires sequential multiphoton absorption not observed in nature. Due to the NIR excitation, UCP technology is also applicable to strongly colored samples (for example, whole blood),^[5] which absorb at ultraviolet and visible wavelengths, a process that interferes with other fluorescence technologies.

The aim of this study was to combine the advantageous features of UCP donors and fluorescence-quenching assays to construct a sensitive enzyme-activity assay. Wang et al.^[6] have quenched around 70 % of the emission of nanosized UCPs by using gold particles. More efficient quenching, however, is required for a practical assay as described above since the best theoretical signal-to-background ratio with these components would be as poor as 3:1. It is not possible to entirely quench the anti-Stokes photoluminescence originating from multiple dopant ions within submicrometer-sized UCPs because only

those emitter ions located near the surface of UCP can be quenched. We have now solved this problem with a sequential energy-transfer-based assay concept (Figure 1 a).

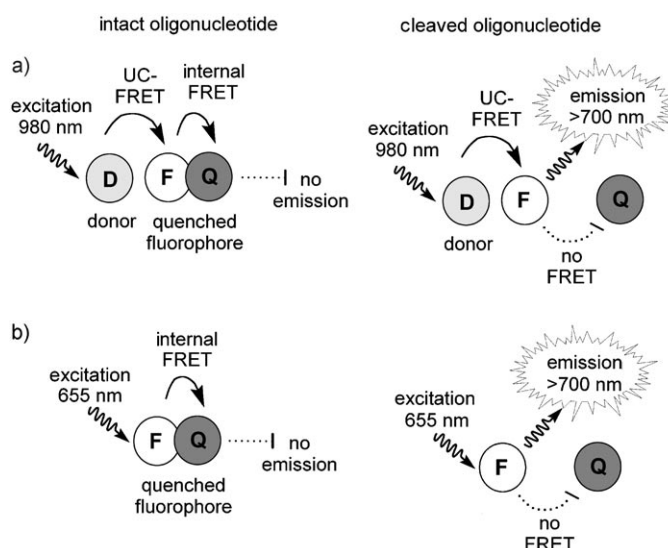


Figure 1. Principle of the homogeneous enzyme-activity assay based on an internally quenched double-labeled substrate a) with a UCP donor or b) without upconversion (conventional assay). The hydrolytic enzyme reaction separates the fluorophore (F) and quencher (Q) located at the different ends of the substrate molecule and so the emission of the fluorophore (measured at > 700 nm) is recovered. Intact substrates remain nonfluorescent. a) In the assay with an upconverting donor (D), the fluorophore is excited through upconversion fluorescence resonance energy transfer (UC-FRET) under continuous infrared (980 nm) excitation. b) In the conventional assay, the fluorophore is directly excited at 655 nm.

Benzonase endonuclease was chosen as a model enzyme for the upconversion FRET-based assay, because it efficiently degrades oligonucleotides to shorter fragments. The substrate oligonucleotide had three modifications: an Alexa Fluor 680 (AF680) fluorophore and biotin at the 5' end and a Black-Berry Quencher 650 quencher (BBQ650) at the 3' end. The quenching efficiency of BBQ650 in the double-labeled substrate was found to be very good (> 96 %). First, the enzyme reaction was carried out and this was followed by a second step in which all of the biotinylated oligonucleotide substrates (intact or cleaved) were collected on the streptavidin-coated UCPs. The energy-transfer-excited emission of the AF680 was directly proportional to the extent of substrate digestion until all of the substrates were degraded. Theoretically, all of the AF680 molecules were bound to the streptavidin-coated UCPs because the enzyme reaction

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cannot separate the biotin and AF680. A more detailed description of the assay can be found in the Supporting Information.

The upconversion-based technology for enzyme-activity measurement was compared with the conventional method by excluding the UCPs from the protocol and measuring the AF680 directly with excitation at 655 nm instead of IR excitation (Figure 1b). More favorable results were achieved with the upconversion-based technology, which could detect benzonase activities at around 0.01 U (Figure 2). The signal levels were higher and the signal-to-background ratios (from 10:1 to 20:1 depending on the substrate amount; increased with concentration) were 8 times better than with the conventional method without the UCPs and infrared excitation. Due to the inadequate blocking properties of the emission filter, some UCP emission was detected in the measurement window of AF680 and this influenced the

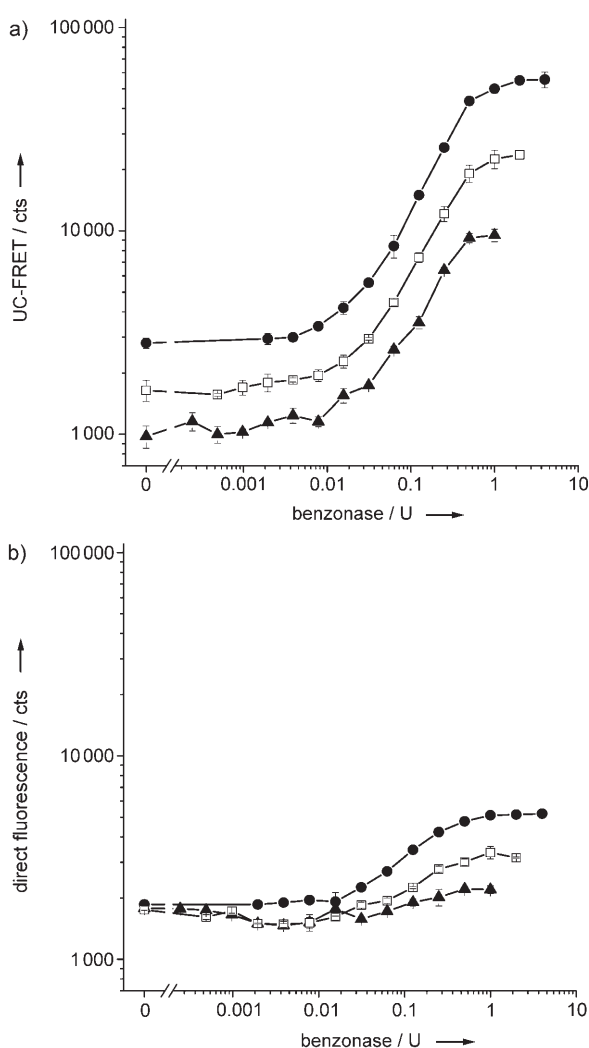


Figure 2. Standard curves of the benzonase-activity assay a) with upconversion or b) without upconversion (conventional method). Emission of the AF680 was measured from buffer by using different amounts of double-labeled substrate oligonucleotide (25 fmol: $\blacktriangle$; 50 fmol: $\square$; 100 fmol: $\bullet$) and optimized quantities of donor UCP particles in the upper figure (25, 50, or 100 ng, respectively). U: activity units; cts: counts.

background signal. However, the upconversion-based technology did not suffer from poor specific signals as the conventional method did, which allowed the amount of reagents and the background signal at the same time to be reduced.

Addition of whole blood (20 % v/v) into the reaction wells before the fluorescence measurement affected the signal levels (Figure 3). The conventional method suffered from

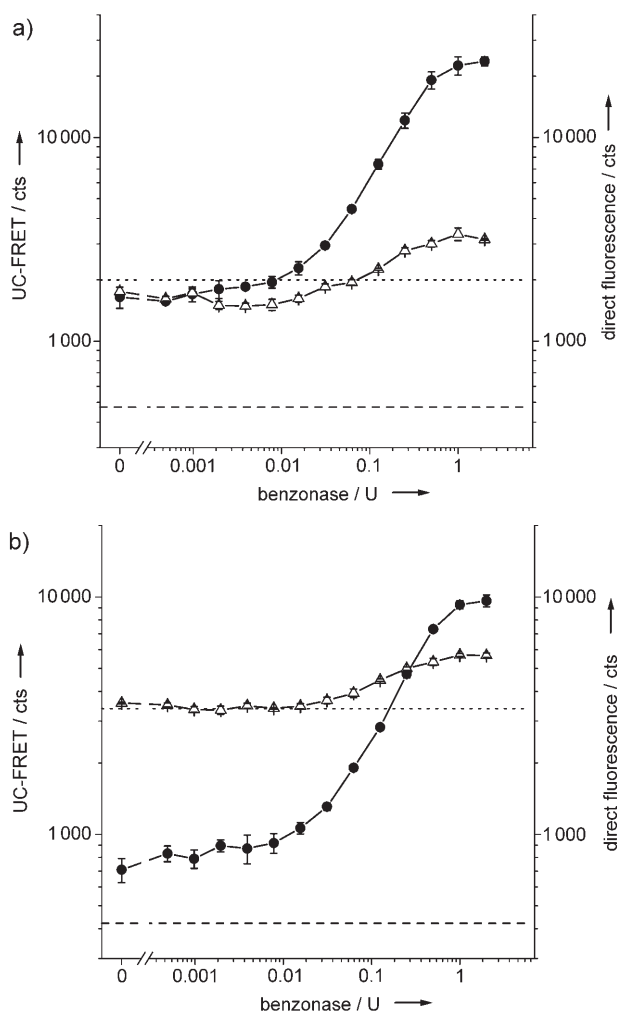


Figure 3. Standard curves of the benzonase-activity assay measured from a) buffer or b) 20% whole blood. Both the upconversion-based ($\bullet$; left y axis) and conventional ($\triangle$; right y axis) methods were tested. The horizontal lines (----: UCP-based;: conventional) represent the background-signal levels measured without any fluorescent component. The quantities of substrate oligonucleotide and UCP in the reaction were 50 fmol and 50 ng, respectively.

significantly elevated autofluorescence, which doubled the background signal. The upconversion-based method showed a lower overall signal level due to the scattering of excitation light in blood. The effect of scattering is significant in upconversion as the emission intensity is proportional to the square of the excitation-light intensity.^[7]

As a conclusion, the quenching-based assay principle introduced in this communication is suitable for particulate

UCP labels although no efficient quenching of the phosphor particles is possible. A more sensitive assay was achieved by exploiting UCP particles compatible with colorful sample matrices than by using a comparable conventional fluorescence-based system. The same technology should be applicable for measuring the activity of other hydrolyzing enzymes by just changing the internally quenched fluorogenic substrate.

Experimental Section

Reagents: Benzonase endonuclease was purchased from Merck KGaA (Darmstadt, Germany) and the substrate oligonucleotide (5'-biotin-dT AF680-GGGCGCGCGG-BBQ650-3') from Biomers.net GmbH (Ulm, Germany). UCP particles (PTIR550/F; NaY_{0.77}Yb_{0.21}Er_{0.02}F₄) from Phosphor Technology Ltd. (Stevenage, UK) were ground and conjugated with streptavidin (see the Supporting Information).^[8] The average particle size in water suspension was 340 nm with a large size distribution. The assay buffer (50 mM tris(hydroxymethyl)aminomethane/HCl (Tris-HCl; pH 7.8), 9 g L⁻¹ NaCl, 0.5 g L⁻¹ NaN₃, 5 g L⁻¹ bovine serum albumin (BSA), 0.1 g L⁻¹ Tween 40, 0.5 g L⁻¹ bovine γ -globulin, 20 μ M diethylenetriaminepentaacetic acid) was from Innotracs Diagnostics (Turku, Finland).

Upconversion-based enzyme-activity assay: The assay was performed in a total volume of 50 μ L by using black half-area microtitration wells (Corning Inc., Corning, NY). The substrate oligonucleotide (25, 50, or 100 fmol) was first degraded with benzonase (0–4 U) in enzyme buffer (26.6 μ L; 50 mM Tris-HCl (pH 8), 1 mM MgCl₂, 0.1% BSA) for 20 min at +37 °C and 6 rpm rotation. Streptavidin-coated UCP particles (25, 50, or 100 ng) were added to the reaction well in assay buffer (13.3 μ L) and the incubation in rotation was continued for 15 min at room temperature. Whole blood or assay buffer (10 μ L) was added and, after a short period of shaking, the sensitized emission of AF680 was measured with a modified

PlateChameleon^[4] (Hidex Oy, Turku, Finland). The fluorescence was measured for 2 s by using a band-pass filter of 740/40 nm (center wavelength = 740 nm; half width = 40 nm; Chroma Technology Corp., Rockingham, VT) under continuous IR laser excitation (980 nm).

Conventional enzyme-activity assay: The same protocol as above was used, but assay buffer was added with no UCP particles and the fluorescence was measured for 2 s with a Victor 1420 Multilabel Counter (PerkinElmer LAS, Wallac Oy, Turku, Finland; equipped with a red-sensitive photomultiplier tube) by using an excitation filter of 655/22 nm and an emission filter of 720/45 nm (Chroma Technology Corp., Rockingham, VT).

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