



Supporting Information

© Wiley-VCH 2008

69451 Weinheim, Germany

Fluorescence Quenching-Based Enzyme Activity Assay Using Photon Upconversion

*Terhi Rantanen**, *Marja-Leena Järvenpää*, *Johanna Vuojola*, *Katri Kuningas* and
Tero Soukka

University of Turku, Department of Biotechnology, Tykistökatu 6A, 20520 Turku,
Finland

Substrate oligonucleotide. The modified oligonucleotide (5'-biotin-dT AF680–GGGCGCGCGG–BBQ650-3', Biomers.net GmbH, Ulm, Germany) was purified by the reversed-phase high-performance liquid chromatography (HPLC) with a 150 x 4.6 mm Phenomex Luna C18 column (Phenomex, Torrance, CA, USA). We used a gradient from 95 % A and 5 % B to 100 % B in 35 min with flow rate of 1.0 mL min⁻¹ (A, aqueous 20 mM triethylammonium acetate (TEAA); B, 20 mM TEAA in 50 % acetonitrile). The liquid from dye containing fractions was evaporated in vacuum (Hetovac VR-1, Heto-Holten A/S, Allerød, Denmark) and then dissolved again in 10 mM Tris-HCl (pH 8.0). AF680 and oligonucleotide concentrations in fractions were determined by measuring absorbance readings at 679 nm and 260 nm, respectively. Fraction containing the right product was stored at –20 °C.

Coating of submicrometer-sized UCP particles. Commercial micrometer-sized (2.3–6.0 µm) UCP material (PTIR550/F; NaY_{0.77}Yb_{0.21}Er_{0.02}F₄, Phosphor Technology Ltd., Stevenage, UK) was ground in a planetary ball mill to gain colloidal particles with an average diameter under 400 nm.^[8] Originally there are no functional groups available on the surface of UCP particle, but adsorption of poly(acrylic acid) (Additol XW330, MW 30000–50000, Surface Specialties Austria GmbH, Werndorf, Austria) introduces carboxylic acid groups for conjugation of biomolecules.

UCPs (10 g L⁻¹) were treated with 0.1 % w/v Additol XW330 in an aqueous solution at 35 °C overnight. The excess of poly(acrylic acid) was washed away four times with water

and twice with 20 mM MES-buffer (pH 6.1) utilizing centrifugal forces (10 000 g) to separate the dense particles and the liquid phase. The newly introduced carboxylic acid groups were activated in MES-buffer containing 20 mM EDC (*N*-(3-dimethylaminopropyl)-*N*''-ethylcarbodiimide hydrochloride) and 30 mM sulfo-NHS (*N*-hydroxysulfosuccinimide sodium salt) for 45 minutes at RT. The activated UCPs were washed twice with MES-buffer to remove the activation reagents before addition of streptavidin. The conjugation reaction between the amino groups of streptavidin (0.75 g L⁻¹) and the activated UCPs (10 g L⁻¹) in MES-buffer was continued for 2.5 h at RT. The reaction was stopped with excess of amino groups (50 mM glycine, pH 11) and after 30 minutes additional incubation the unreacted streptavidin was removed with three final washes: twice with 10 mM borate buffer, pH 8.5, containing 1 g L⁻¹ Tween 20 and once with storage buffer (5 mM borate buffer, pH 8.5, containing 2 g L⁻¹ Tween 85, 5 g L⁻¹ BSA, and 0.5 g L⁻¹ NaN₃). The streptavidin-coated UCPs were stored at RT in slow rotation (4 rpm) in storage buffer.

Bath sonication (Finnsonic m03; Finnsonic Oy, Lahti, Finland) and tip sonication (Labsonic U, B. Braun Biotech International, Melsungen, Germany) were used to reduce aggregation of the particles and to help resuspension after centrifugation steps.

Fluorescence and absorption spectra. Fluorescence spectra were measured with a Varian Cary Eclipse fluorescence spectrophotometer (Varian Scientific Instruments, Mulgrave, Australia). UCP was diluted in assay buffer to concentration of 0.015 g L⁻¹ and AF680 in TSA (50 mM Tris, pH 7.75, 150 mM NaCl, 0.05 % NaN₃) to 37.5 nM. Absorbance spectrum of BBQ650 was measured with UV-1700 PharmaSpec Spectrophotometer (Shimadzu Corporation, Kyoto, Japan) and BBQ650 was diluted in 10 mM Tris-HCl (pH 8.0) to concentration of 6.89 μM. All spectra are illustrated in Figure S-1.

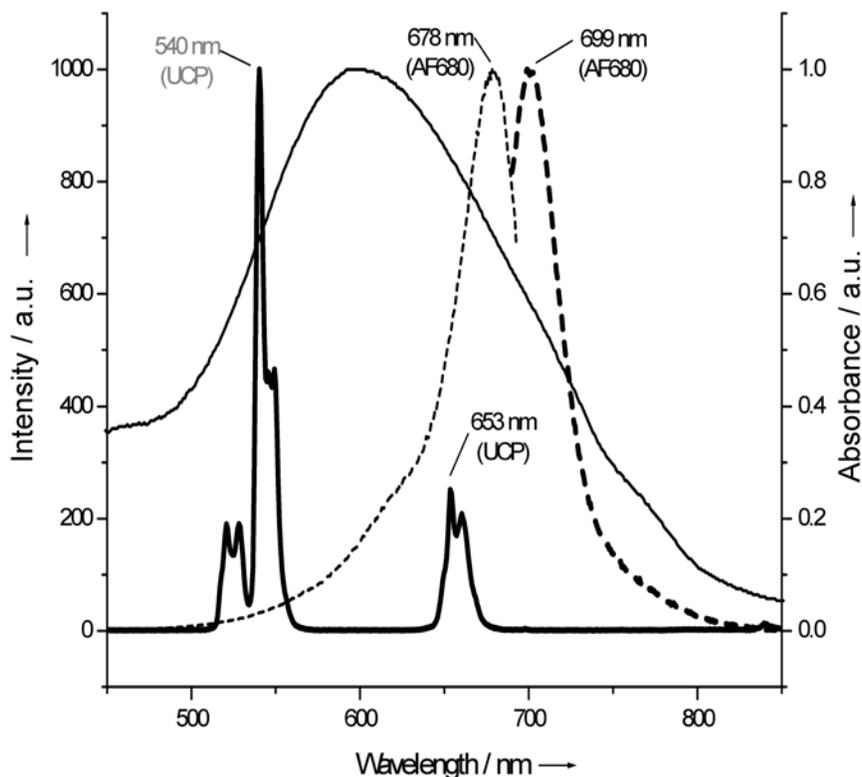


Figure S-1. Fluorescence emission spectra of UCP (solid thick line) and AF680 (dashed thick line), fluorescence excitation spectrum of AF680 (dashed thin line) and absorption spectrum of BBQ650 (solid thin line; axis at right). *UCP*, upconverting phosphor; *AF680*, Alexa Fluor 680; *BBQ650*, BlackBerry Quencher 650; *a.u.*, arbitrary unit.

The UCP particles generated intense and sharp emission at 653 nm under IR excitation. The AF680 fluorophore has an overlapping excitation spectrum and is a suitable acceptor molecule. BBQ650 quencher has a broad absorption spectrum reaching to the emission wavelengths of AF680. Although the absorption is also efficient at the emission wavelengths of UCP, no quenching was detected even with high concentrations of BBQ650. Direct quenching of submicrometer-sized UCP was not possible.

Principle of the enzyme activity assay. Detailed principle of the homogeneous enzyme activity assay is described in figure S-2. The energy transfer-excited emission of AF680 rises with increasing enzyme activity.

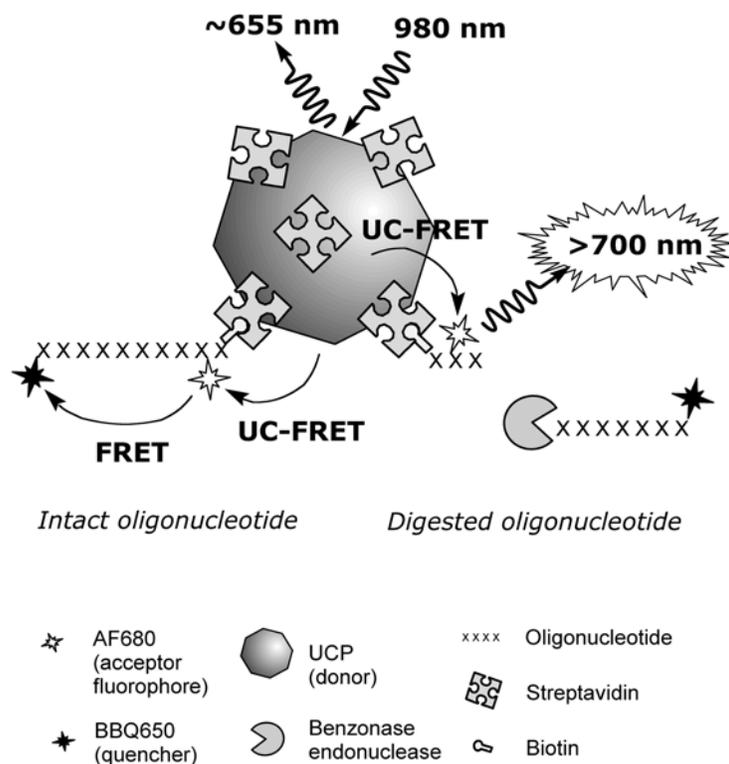


Figure S-2. Principle of the homogeneous enzyme activity assay. AF680 fluorescence (>700 nm) is only detected after hydrolytic enzyme reaction when the two labels of the synthetic substrate oligonucleotide are separated. Infrared laser diode (980 nm) is used as an excitation source. UC, upconversion; FRET, fluorescence resonance energy transfer; UCP, upconverting phosphor; AF680, Alexa Fluor 680; BBQ650, BlackBerry Quencher 650.

Optimal substrate oligonucleotide concentration in the enzyme activity assay. The binding capacity of UCP particles determines the optimal substrate oligonucleotide concentration in the enzyme activity assay. Streptavidin-coated UCPs (25, 50 or 100 ng) and intact or totally digested substrate oligonucleotide (3.125–400 fmol) were added to black half area microtitration wells (Corning Inc., Corning, NY) in 50 μL of enzyme buffer (50 mM Tris-HCl, pH 8, 1 mM MgCl_2 , 0.1 % BSA). After 15-minutes incubation at RT in 6-rpm rotation the sensitized emission of AF680 was measured with modified PlateChameleon as described in Experimental section. The substrate oligonucleotide

digestion was done in advance with 1 U of Benzonase per 1 pmol of oligonucleotide for 2 hours at +37 °C.

As a result, optimized amounts of substrate oligonucleotide were determined to be 25, 50 or 100 fmol for 25, 50 or 100 ng of UCP, respectively (Figure S-3; 50 ng of UCP). The optimized amount of totally digested biotinylated substrate oligonucleotide did not saturate all the binding sites on the surface of UCP particles in the reaction, but still the concentration was high enough to generate intense AF680 emission. Too dense binding of the substrate oligonucleotide might cause self-quenching of AF680 fluorescence. In the case of undigested substrate oligonucleotide, the AF680 emission was only slightly above the background due to the efficient quenching (>96 %) by BBQ650, as expected.

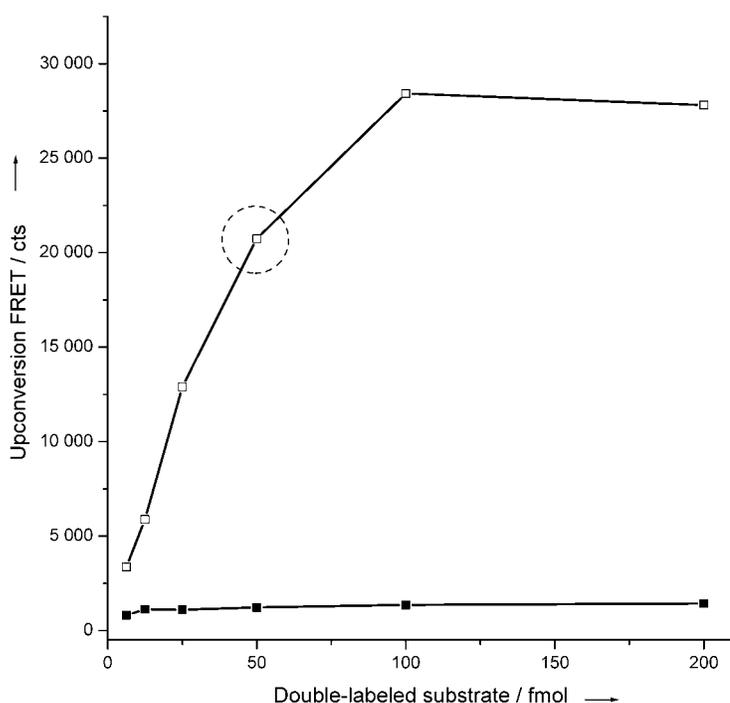


Figure S-3. Determination of proper amount of double-labeled substrate oligonucleotide for 50 ng of UCP. With a constant amount of UCPs and varying concentration of digested (open square) substrate in a reaction well, the UC-FRET increased until it reached a saturation level as a consequence of fully occupied binding sites. 50-fmol amount of substrate (circled) was selected for further applications. The quenching efficiency of BBQ650 in double-labeled substrate seemed to be very good, as the fluorescence of the intact substrate (filled square) was almost undetectable. *cts*, counts.