SUPPLEMENTARY INFORMATION

Upconverting phosphors in a dual-parameter LRET-based hybridization assay

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Preparation of donor conjugates

Reagents. Upconverting phosphor (UCP) particles (PTIR550/F; $NaYF_4$:Yb^{3+},Er^{3+}) were purchased from Phosphor Technology Ltd. (Stevenage, UK). Tetraethyl orthosilicate (TEOS; purity 98%) and Triton X-100 were from Acros Organics (Geel, Belgium) and 3-aminopropyl triethoxysilane (APTS; purity 98%) and glutaric anhydride (purity 95%) from Sigma-Aldrich (St. Louis, MO). *N*-(3-Dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC) and *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS) were obtained from Fluka (Buchs, Switzerland). Bovine serum albumin fraction V was purchased from Bioreba (Nyon, Switzerland). Synthetic single-stranded oligonucleotides (Table 1 and Table S-1) with desired modifications for conjugation purposes were from Biomers.net GmbH (Ulm, Germany). The washing buffer contained 10 mM borate, pH 8.5, and 1 g L^{-1} Tween 20 (E. Merck, Darmstadt, Germany) and the storage buffer contained 5 mM borate, pH 8.5, 2 g L^{-1} Tween 85 (E. Merck), 50 µM ethylenediaminetetraacetic acid and 0.5 g L^{-1} NaN_3.

Table S-1. Oligonucleotide conjugates and their sequences.

Name	Sequence 5'→ 3'
β-actin test probe (**TP1**)	**AF546** – $T^{\#}$ TCC TGT CGG CAA TG
HLA-B27 test probe (**TP2**)	CTG CTC TAT CCA CGG $T^{\#}$ – **AF700**

$T^{\#}$ stands for thymine with C6 aminolink for label conjugation.

Colloidal UCP suspension. The grinding of commercially available UCP material (original diameter 2.3–6.0 µm) was mainly performed as described earlier.[1] Briefly, ~600 mg of UCP particles in 3 mL of deionized water were ground in a planetary ball mill (Planetary Mono Mill pulverisette, Fritsch GmbH, Idar-Oberstein, Germany) reversing the direction of rotation (600 rpm) after every 5 min and repeating this procedure for 60 times to achieve particles with average diameter of ~300 nm. Abrasion resistant smooth zirconia balls (~400 pieces) with a diameter of 2 mm (Retch GmbH, Haan, Germany) in a 12-mL zirconia bowl (Fritsch GmbH)

were used for milling. Ground UCP material was collected and diluted in deionized water with a total volume of 12 mL. The mass concentration of UCPs was determined by weighing the dried solids from a known volume of UCP suspension.

Surface modification of UCP particles. The UCP particles do not originally have any functional groups suitable for bioconjugation. Therefore, the UCPs were encapsulated into a silica shell following for the most part the protocol published by Tan *et al.*[2]. Monomeric TEOS and APTS were copolymerized in water-in-oil emulsion containing the UCP particles. TEOS formed a 3-dimensionally growing silica polymer, which was functionalized with a TEOS derivative, APTS, providing amino groups for bioconjugation purposes.

Suspension containing 8 mg of colloidal UCPs in 580 µL of deionized water was vigorously sonicated (VialTweeter, Hielscher Ultrasonics GmbH, Teltow, Germany) for 15 pulses using 0.5 repeating duty cycle to avoid particle aggregates. Other components of water-in-oil emulsion except water were mixed (15 mL of cyclohexane, 3.6 mL of n-hexanol and 3.54 mL of Triton X-100) and divided into two vials. The aqueous UCP suspension (580 µL) and catalytic ammonium hydroxide (120 µL) were added into the first vial and the emulsion was further bath sonicated (Finnsonic m03; Finnsonic Oy, Lahti, Finland) for 10 min. The other vial was completed by adding 466 µL of water and the two silanes: 100 µL of TEOS and 200 µL of APTS. Actual reaction was initiated when the contents of the two vials were combined and incubation was continued for 2 h at room temperature in slow rotation (6 rpm). In the beginning and once during the incubation, the reaction vial was bath sonicated for 10 min.

Modified UCPs were collected by adding 6.5 mL of acetone to the reaction vial prior to centrifugation (1 600 g, 1 min). The UCP pellet was recovered, transferred to a smaller tube (2 mL capacity) and washed twice with 1.5 mL of ethanol and twice with 1.5 mL of deionized water utilizing centrifugal forces (4 500 g, 5–7 min) in separation step. After every centrifugation, the UCPs were resuspended and bath sonicated for 1 min. Finally, the UCPs were suspended into 1 mL of deionized water and vigorously sonicated with VialTweeter (20 pulses). The amino groups of the silica-coating were further converted into carboxylic acid groups. The UCPs were collected by centrifugation (10 000 g, 7 min) and the liquid phase was replaced with 400 µL of dry pyridine. Precautionary handling is recommended due to an unpleasant odor. Glutaric anhydride (~65 mg) was dissolved into 100 µL of pyridine, added instantly to the UCP suspension and the suspension was bath sonicated for 5 min. Reaction was incubated for 6 h at 50 °C. Because UCPs do not form a decent pellet in pyridine, 500 µL of ethanol was added prior to centrifugation (10 000 g, 7 min). Pyridine and excess glutaric anhydride were removed by washing twice with 400 µL of ethanol and twice with 400 µL of deionized water. Finally, the modified UCPs ready for bioconjugation were dissolved into deionized water and stored at room temperature in slow rotation.

Conjugation of capture oligonucleotides to UCP particles. Two conjugates of UCPs were prepared; one with β-actin capture (C1) and one with HLA-B27 capture (C2) oligonucleotides. In the reaction between a carboxylic acid group on the surface of the coated UCP and a primary amino group incorporated into the oligonucleotide an amide bond was formed.

The surface modified UCP particles (2.5 mg) were washed once with 20 mM MES (2-(*N*-morpholino)ethanesulfonic acid), pH 6.5, using centrifugation (10 000 g, 7 min) and resuspended into 205 µL of MES-buffer. To avoid possible aggregates, the UCP suspension was bath sonicated for 1 min following the addition of 3 nmol of capture oligonucleotide (C1 or C2) in 15 µL of deionized water. Conjugation reaction with total volume of 250 µL in MES-buffer was continued for 2.5 h in the presence of activating reagents 50 mM EDC and 25 mM sulfo-NHS (final concentrations). The activating reagents were freshly dissolved in MES buffer and added to the reaction in 30 µLvolume. The reaction was stopped with excess of amino groups by adding 6.3 µL of 2 M glycine (pH 11). After 30 min, the UCP conjugates were washed twice with 500 µL of washing buffer and once with 250 µL of storage buffer utilizing centrifugal forces (10 000 g, 7 min) in separation step and bath sonication in resuspension. After over night storage in slow rotation the UCP conjugates were once more centrifuged and resuspended into 250 µL of storage buffer containing 5 g L^{-1} bovine serum albumin. They were stored at room temperature in slow rotation to avoid sedimentation.

The concentration of UCPs was determined by comparing the luminescence of the conjugate suspension to a UCP standard with known concentration. UCPs were diluted in washing buffer and the luminescence was measured with modified Plate Chameleon at 665 nm using a band-pass filter (665/10 nm, peak transmittance ≥ 80%; Spectra-Physics, Mountain View, CA) under continuous laser excitation at 980 nm.

Preparation of acceptor conjugates

Labeling of oligonucleotide probes. The probes and the test probes for β-actin sequence (P1 and TP1) or HLA-B27 sequence (P2 and TP2) were labeled with Alexa Fluor 546 or Alexa Fluor 700, respectively. The total reaction volume was 60 µL containing 10 nmol of amino-modified oligonucleotide in 100 mM carbonate buffer (pH 9.2) and 10-fold molar excess of the succinimidyl ester modified fluorophore freshly dissolved in small volume of dry dimethylformamide (10% vol/vol in reaction). The fluorophore was protected from light and the reaction was incubated over night at room temperature in slow rotation (6 rpm). All reaction mixtures were stored in –20 °C before purification by the reversed-phase high-performance liquid chromatography (HPLC).

For purification we used a 150 x 4.6 mm, 3-µm Hypersil-Keystone C18 (Thermo Electron, Waltham, MA) HPLC-column with 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; B, 20 mM triethylammonium acetate in 50% acetonitrile). Absorbance of the oligonucleotides at 260 nm was recorded together with emission of the fluorophore at 580 nm (AF546) or 720 nm (AF700). Fractions containing the labeled oligonucleotides were collected for further analysis. The concentrations of the AF546 or AF700 and oligonucleotide were determined spectrophotometrically (NanoDrop Spectrophotometer ND-1000; Thermo Fisher Scientific, Waltham, MA) by reading the absorbance at 556 nm or 702 nm and 260 nm, respectively. Molar absorption coefficients are 104 000 M^{-1} cm^{-1} for AF546 and 192 000 M^{-1} cm^{-1} for

AF700. The concentration of the single-stranded oligonucleotide was calculated based on the information that absorbance reading of 1 corresponds concentration of 0.033 g L^{-1}. For the AF546-labeled oligonucleotides the absorbance reading at 260 nm was corrected because of the overlapping absorbance of AF546 (21% of the absorbance at 556 nm). Due to the monofunctional reagents, the concentrations of the label and the oligonucleotide were practically equal in the fraction containing the conjugate.

Direct hybridization assay

Before constructing the actual sandwich assay, the hybridization capability of capture oligonucleotides coupled with UCPs and the feasibility of the labels for upconversion LRET were tested in a simple single-parameter homogeneous direct hybridization assay (Figure S-1). All assays were performed in black, half-area microtitration wells (Costar; Corning Inc., Corning, NY) in total volume of 80 µL and all reagents were diluted in the assay buffer (50 mM Tris-HCl, pH 7.8, 1 M NaCl, 0.5 g L^{-1} NaN_3, 5 g L^{-1} bovine serum albumin, 0.1 g L^{-1} Tween 40, 0.5 g L^{-1} bovine γ-globulin, and 20 µM diethylenetriaminepentaacetic acid). Different amounts of the AF546-labeled β-actin test probe (TP1) or AF700-labeled HLA-B27 test probe (TP2) were directly hybridized with complementary capture oligonucleotides conjugated with the UCPs. The extent of background fluorescence originating from the acceptor fluorophore was quantified in separate reactions by blocking the test probes with excess of free, unlabeled capture oligonucleotides before introducing the UCP conjugates. In these blocked reactions the formation of LRET-pairs was prevented.

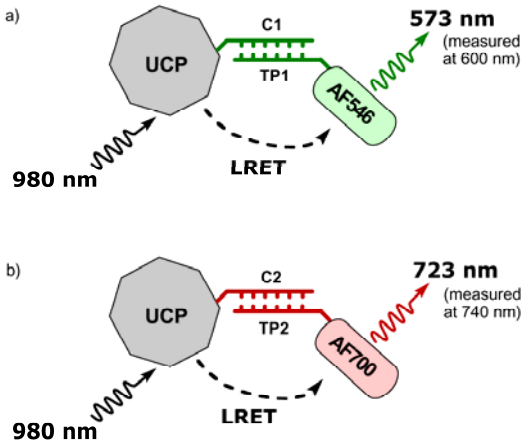

Figure S-1. Assay principle of the homogeneous direct hybridization assay with (**a**) AF546-labeled β-actin test probe and (**b**) AF700-labeled HLA-B27 test probe. The capture oligonucleotides complementary to the single-stranded test probe sequence are covalently conjugated with the UCP particles. After hybridization, the upconversion LRET signal is measured under continuous infrared excitation (980 nm).

First, the labeled test probes (0–0.8 pmol of TP1 or TP2) with (1.6 pmol) or without the blocking capture oligonucleotides (C1 or C2) were incubated in 48 µL-volume with shaking

(900 rpm; Thermomix 1415, Labsystems, Helsinki, Finland) at 30 °C for 20 min. Capture oligonucleotides coupled with UCPs (0.6 µg of UCP-C1 or 1 µg of UCP-C2) were added in 32 µL and incubation was continued as previously for 20 min before measuring the upconversion LRET with the modified Plate Chameleon.

The conjugation to the particle did not affect the capture oligonucleotide and it hybridized readily with the complementary sequence. The upconversion LRET emission of both acceptors increased almost linearly with the amount of the labeled test probe until saturation of the capture oligonucleotides (Figure S-2). The specific upconversion LRET signals in Figure S-2 were calculated by subtracting the background signal (excess of unlabeled complementary oligonucleotide present to block the hybridization of the test probe) from the signals without blocking (normal hybridization of the test probe). According to this test, upconversion LRET -based principle and the label conjugates were suitable for designed assay purposes reaching the signal level of about 100 000 counts with low-nanomolar test probe concentrations.

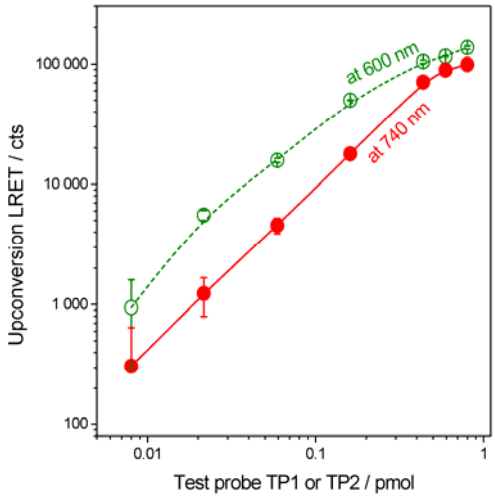

Figure S-2. Potential of the upconversion LRET -based assay in terms of signal level. Titration of the labeled test probes for β-actin (green open circle; Alexa Fluor 546 -labeled TP1) or for HLA-B27 (red filled circle, Alexa Fluor 700 -labeled TP2) in the direct hybridization assay (see Figure S-1). Data points are means from three independent replicas; error bars show s.d. *cts, counts.*

Sandwich hybridization assay

Sandwich hybridization assay principle was carried out both in a single-parameter format exploiting two separate reactions for quantifying the amount of the two target oligonucleotide sequences (T1 and T2) and in multiplexed dual-parameter format reported in the Research Paper.

Compared to the direct hybridization assay, about 60–70% of the signal level was achieved in the sandwich assay taking into account the used probe concentrations (Figure S-3 vs. Figure S-2). The distance between the donor and the acceptor was increased by 4 or

5 nucleotides in the sandwich assay, which might decrease the energy transfer efficiency and result in lower signal level.

Multiplexing the sandwich hybridization assay did not affect significantly the specific signals compared to separate reactions for each analyte (Figure S-3). The specific upconversion LRET signals presented in the Figure S-3 were obtained by subtracting the background signals (the assay with zero concentration of target oligonucleotide) from raw signals obtained with different concentrations of the target. However, multiplexing caused somewhat elevated background signal (14 000 counts for T1 and 8 500 counts for T2) compared to separate quantification (~6 000 counts for both T1 and T2). The background could originate from the mis-hybridization of the oligonucleotides, which could probably be reduced with a slightly higher temperature during the fluorescence measurement (not possible with the current instrumentation) together with lower salt concentration in the assay buffer. Another source of background is the acceptor emission generated through the reabsorption of the donor emission (radiative energy transfer), which is directly proportional to the concentration of both the donor and the acceptor itself. The leaking of the intense donor emission to the measurement wavelengths of the acceptor causes also small background fluorescence. With high-quality emission filters and selection of acceptor fluorophore it is possible to impact on the donor cross-talk. The increase in the background signal was related to the higher donor concentration in the dual-parameter assay format. The background in the case of target sequence T1 is more susceptible to donor concentration due to the more intense green emission peak of the UCP at 540 nm compared to the minor red peak at 653 nm.

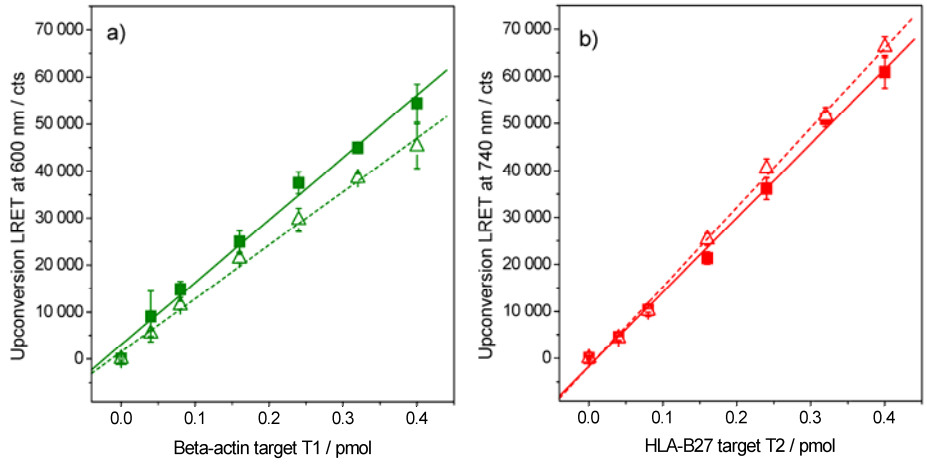

Figure S-3. Standard curves of the sandwich assays. Results from both the dual-parameter assay format (filled square; simultaneous detection of two target sequences) and the single-parameter assay format (open triangle; separate reactions for the two targets) are illustrated. **(a)** The β-actin sequence (T1) was quantified using an Alexa Fluor 546 labeled probe P1 and **(b)** the HLA-B27 sequence (T2) using an Alexa Fluor 700 labeled probe P2. Data points are means from three independent replicas; error bars show s.d. *cts, counts.*

References

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2 M. Tan, Z. Ye, G. Wang and J. Yuan, *Chem. Mater.*, 2004, **16**, 2494-2498.