

## Supporting information for

# Long-Lifetime Luminescent Europium(III) Complex as an Acceptor in an Upconversion Resonance Energy Transfer Based Homogeneous Assay

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## Preparation of assay reagents:

**Materials:** Cyclohexane (ACS Reagent,  $\geq 99\%$ ), Igepal CO-520, ammonium hydroxide (ACS Reagent 28-30%), tetraethyl orthosilicate (TEOS, 98% purity), (N-(3-trimethoxysilyl)propyl)ethylene diamine (TMED, 97% purity), acetonitrile (for HPLC,  $\geq 99.9\%$ ), triethylammonium acetate (TEAA, 1 M in  $\text{H}_2\text{O}$ ), sodium carbonate and sodium bicarbonate, Trizma base and  $\text{NaN}_3$  were purchased from Sigma-Aldrich (St. Louis, MO). Acetone was purchased from JT. Baker (Germany) and dimethylformamide (DMF) from Acros Organics (Belgium). The AF488 was purchased from Molecular probes (Carlsbad, CA) and EZ-Link Amino-PEG<sub>2</sub>-biotin used for biotinylation of  $\text{Eu}^{\text{III}}$ -chelates and AF488 from Thermo Fisher Scientific (Waltham, MA).

**UCNP synthesis, surface modification and streptavidin conjugation:** The  $\text{Na}_{0.8}\text{K}_{0.2}\text{YF}_4$ :  $\text{Yb}^{\text{III}}$ ,  $\text{Tm}^{\text{III}}$  ( $X_{\text{Yb}}$  0.2,  $X_{\text{Tm}}$  0.005) UCNP were synthesized in organic oils as described earlier.<sup>1</sup> The average size of 50 nm particles were calculated from transmission electron microscopy (TEM) image (Figure S1) by measuring the diameters of sixty particles with ImageJ software version 1.43s (<http://rsb.info.nih.gov/ij/>). The UCNP were silanized in order to obtain functional groups for biomolecule conjugation. The protocol was modified from a method presented earlier.<sup>2</sup> In short, the UCNP were transferred into cyclohexane by centrifuging (16 873 g, 30 min). A micro-emulsion was formed by mixing first 10 mL of cyclohexane, 1 mL of Igepal CO-520 and 10 mg of UCNP in cyclohexane and bath sonicating for 10 min. Then 200  $\mu\text{L}$  of ammonium hydroxide was added and the mixture was stirred at room temperature for 10 minutes. In order to form the silica shell, 15  $\mu\text{L}$  of TEOS was added to the mixture and then stirred as before for 3 hours after which 40  $\mu\text{L}$  of TMED was added to introduce primary amino groups. The mixture was stirred yet for 45 hours at room temperature before the amino modified particles were precipitated by adding 5 mL of acetone and centrifuged for 10 min (700 g). The particles were washed with MQ-water and finally suspended in ethanol. The amino groups were converted to carboxylic acid groups.<sup>3</sup> Streptavidin was conjugated covalently to carboxylic acid groups of silanized UCNP by using carbodiimide coupling chemistry.<sup>4</sup>

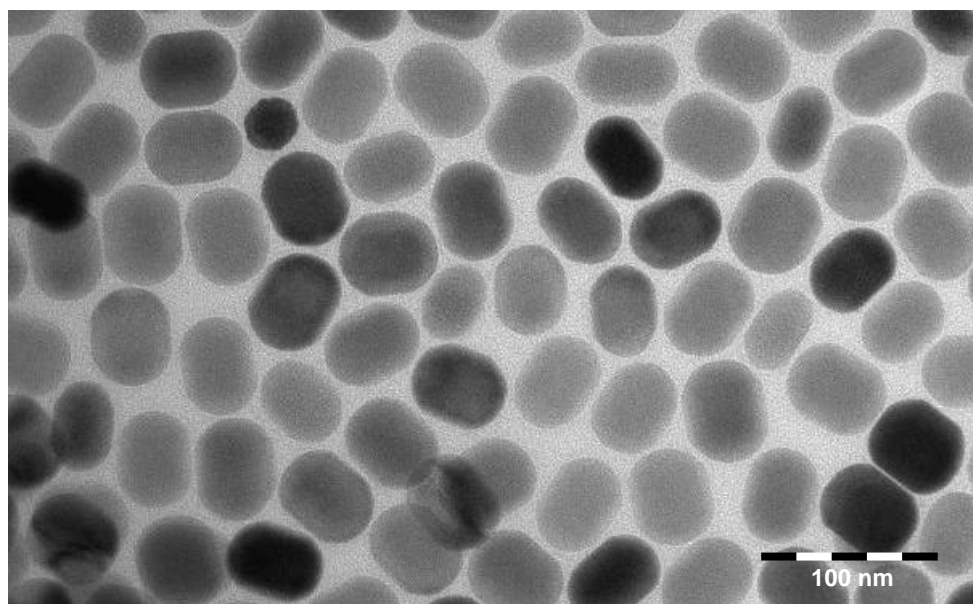
**Biotinylations of the  $\text{Eu}^{\text{III}}$ -chelates:** First the amino form of the intrinsically fluorescent non-adentate Europium(III) chelate ( $\text{Eu}^{\text{III}}$ -WN) of tetra-*tert*-butyl 2,2',2'',2'''-((((4-((4-aminophenyl)ethynyl)-pyridine-2,6-diyl)bis(methylene))bis((2-(*tert*-butoxy)-2-oxoethyl)azanediyl))bis(ethane-2,1-diyl))bis(azanetriyl))-tetraacetate<sup>5</sup> was activated with cyanuric chloride. The amino form of the  $\text{Eu}^{\text{III}}$ -WN (0.25 mmol) was dissolved in 2 mL of water. Cyanuric chloride (1.25 mmol) was dissolved in 2 mL of acetone and added to the  $\text{Eu}^{\text{III}}$ -chelate solution. During the 15 minute stirring, the pH was monitored and adjusted to 7.0 with 10% sodium carbonate and saturated sodium bicarbonate solutions. Then 46 mL of acetone was added and the precipitate was collected by centrifugation and washed twice with 50 mL of acetone. The formed product was dried by airflow and vacuum. Then 5.8  $\mu\text{mol}$  of the activated chelate and the EZ-Link Amine-PEG<sub>2</sub>-biotin were dissolved in 4 mL water in 1:1 molar ratio. The pH of the solution was adjusted to 8.0 with 10% sodium bicarbonate. The solution was stirred over night at room temperature after which the pH was adjusted to 7.0 with acetic acid prior high-performance liquid chromatography (HPLC). The final pure product was obtained by semi-preparative HPLC purification with a gradient of 20 mM TEAA buffer containing 5–30% acetonitrile. The Supelco Ascantis RP-AMIDE HPLC Column (25cm x 21.2mm, 5 $\mu\text{m}$ ; Sigma-Aldrich, St. Louis, MO) was used



for the purification. HR-MS for the purified product  $C_{46}H_{54}ClEuN_{12}O_{14}S^2$ : required 609.1257 and 608.1251, found 609.1194 and 608.1185. The intrinsically fluorescent heptadentate Europium(III) chelate ( $Eu^{III}$ -Tekes) of 2,2',2'',2'''-((4-((4-isothiocyanatophenyl)ethynyl)pyridine-2,6-diyl)bis(methylenenitrilo))tetrakis (acetic acid)<sup>6</sup> and nonfluorescent heptadentate Europium(III) chelate ( $Eu^{III}$ -N1) of (N1-(4-isothiocyanatobenzyl)diethylenetriamine-N1,N2,N3,N3-tetrakisacetate)<sup>7</sup> were biotinylated with EZ-Link Amine-PEG<sub>2</sub>-biotin according to the protocol described earlier.<sup>8</sup> The structures of the biotinylated chelates are depicted in figure S2. The  $Eu^{III}$  concentrations of the conjugates were measured by comparing time-resolved luminescence of the conjugate to a known  $Eu^{III}$  standard.

*Biotinylation of AF488:* The amino reactive form of AF488 succinimidyl ester dye was biotinylated with EZ-Link Amine-PEG<sub>2</sub>-biotin according to the protocol described earlier.<sup>9</sup> The molar excess of the dye in the reaction was 2.5. The concentration of the dye in the conjugate was determined by measurement of absorbance at 495 nm.

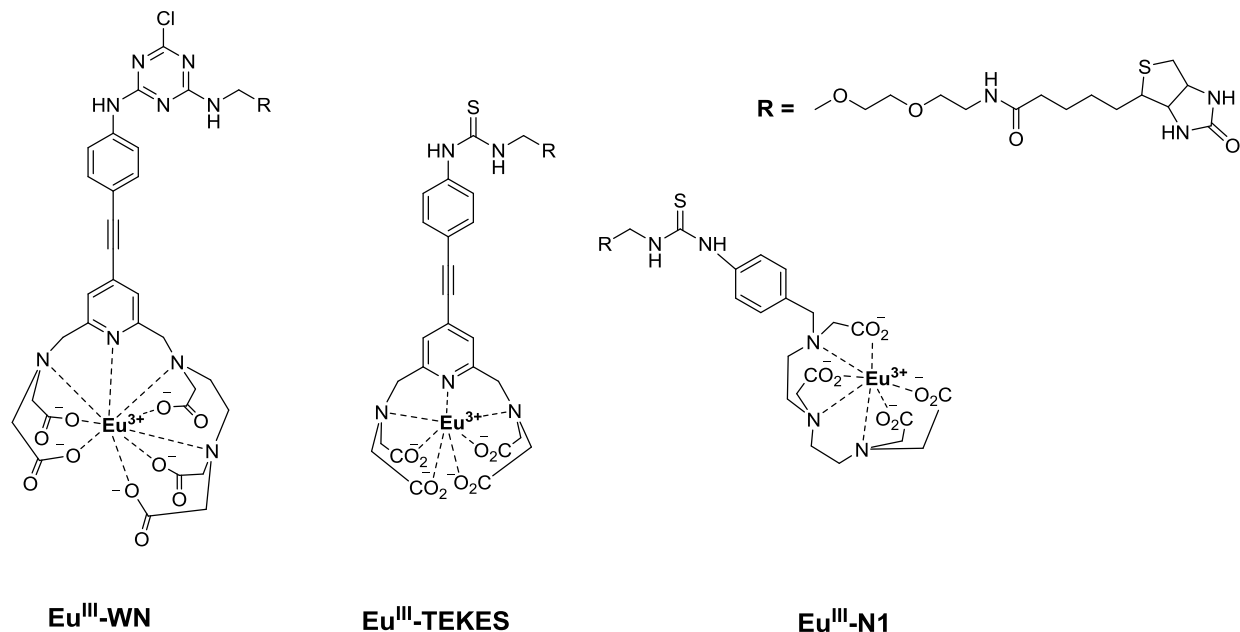
### Transmission electron microscopy image of UCNPs



**Figure S1.** Transmission electron microscopy image of the upconverting nanoparticles with an average size of 38 x 50 nm (JEM-1400 Plus TEM, 80 kV, JEOL, Massachusetts, USA).



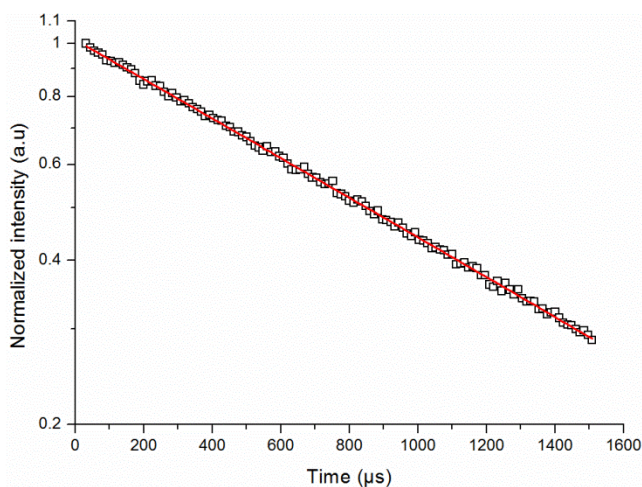
## Structures of biotinylated $\text{Eu}^{\text{III}}$ -WN, $\text{Eu}^{\text{III}}$ -Tekes, and $\text{Eu}^{\text{III}}$ -N1



**Figure S2.** Structures of the biotinylated nonadentate intrinsically fluorescent  $\text{Eu}^{\text{III}}$ -WN, heptadentate intrinsically fluorescent  $\text{Eu}^{\text{III}}$ -Tekes, and heptadentate nonfluorescent  $\text{Eu}^{\text{III}}$ -N1

## Lifetime of the $\text{Eu}^{\text{III}}$ -WN

The direct UV-excited luminescence lifetime of  $\text{Eu}^{\text{III}}$ -WN was measured with Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara CA) lifetime programme. The  $\text{Eu}^{\text{III}}$ -WN was diluted in TSA (50 mM Tris-HCl, pH 7.75, 150 mM NaCl, 0.5 g L<sup>-1</sup> NaN<sub>3</sub>) to a final concentration of 50 nM. The lifetime was measured at 615 nm after 360 nm excitation. The data was analyzed with Origin 8 (Origin Lab, Northampton, MA) using single order exponential decay fitting. The lifetime was determined to be 1206 ± 15 μs (Figure S3).



**Figure S3.** UV-excited (360 nm) luminescence decay curve of  $\text{Eu}^{\text{III}}$ -WN measured at 615 nm. a.u.= arbitrary units

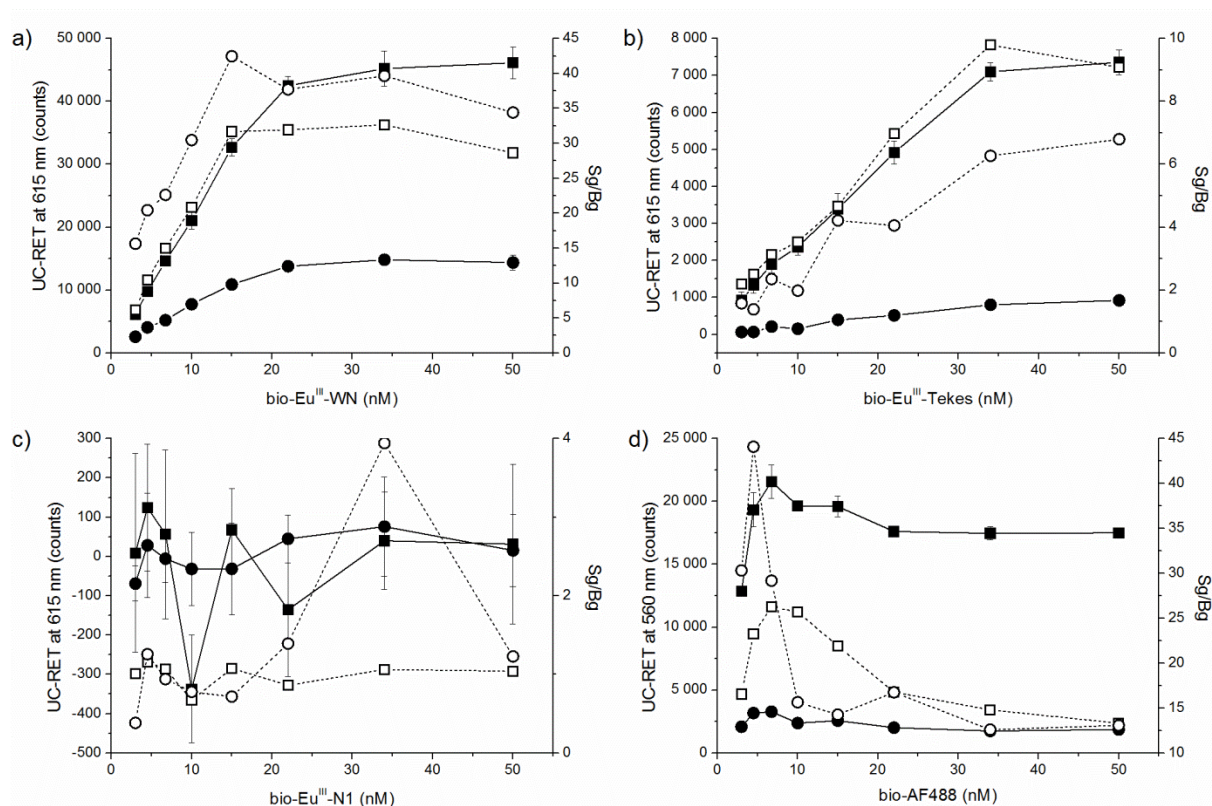


## Optimization of the homogeneous assay

*Titration of biotinylated acceptors:* The optimal acceptor concentration in homogeneous back titration assay for biotin was determined by titrating the acceptor.<sup>4</sup> The assay was performed in black, half area microtitration wells and all the assay components were diluted in assay buffer. The total reaction volume was 80  $\mu\text{L}$  which contained 32  $\mu\text{L}$  of assay buffer or biotin in a final concentration of 5  $\mu\text{M}$ , 24  $\mu\text{L}$  of UCNP-SAv in a final concentration of 7.5  $\mu\text{g mL}^{-1}$  and 24  $\mu\text{L}$  of biotinylated acceptor in a final concentration range from 0 to 50 nM. Four replicates of each acceptor concentration were utilized. The maximum UC-RET signals were obtained when no free biotin was added. The background levels originating from nonspecific binding of the acceptor and radiative energy transfer were obtained when high excess amount of biotin (5  $\mu\text{M}$ ) was added to block all the binding sites of the acceptor. First, the assay buffer with or without biotin was added to the wells with the UCNPs. The wells were incubated for 15 minutes in slow shaking after which the different concentrations of acceptors were added. The wells were protected from light with aluminum foil and incubated for 15 minutes in slow shaking. The sensitized steady-state and time-resolved luminescence of biotinylated acceptors were measured with modified Plate Chameleon. In steady-state measurement the emission light was collected for 2000 ms under continuous excitation at 980 nm. Time-resolved luminescence was measured using 10 ms cycle time with 2900 cycles, 3 ms wide pulsed excitation, 1 ms delay and 2 ms measurement window. The sensitized emissions of  $\text{Eu}^{\text{III}}$ -chelates were measured at 615 nm (615 nm centered bandpass filter with half width of 15 nm) and of AF488 at 565 nm (565 nm centered bandpass filter with half width of 20 nm).

The acceptor concentrations of 34 nM and 6.8 nM were selected for bio- $\text{Eu}^{\text{III}}$ -chelates and bio-AF488, respectively, when the UCNP concentration was 7.5  $\mu\text{g mL}^{-1}$  (Figure S4). The sensitized emission of bio- $\text{Eu}^{\text{III}}$ -N1 did not rise with concentration increase, but the same concentration of acceptor was chosen as for other  $\text{Eu}^{\text{III}}$ -chelates.





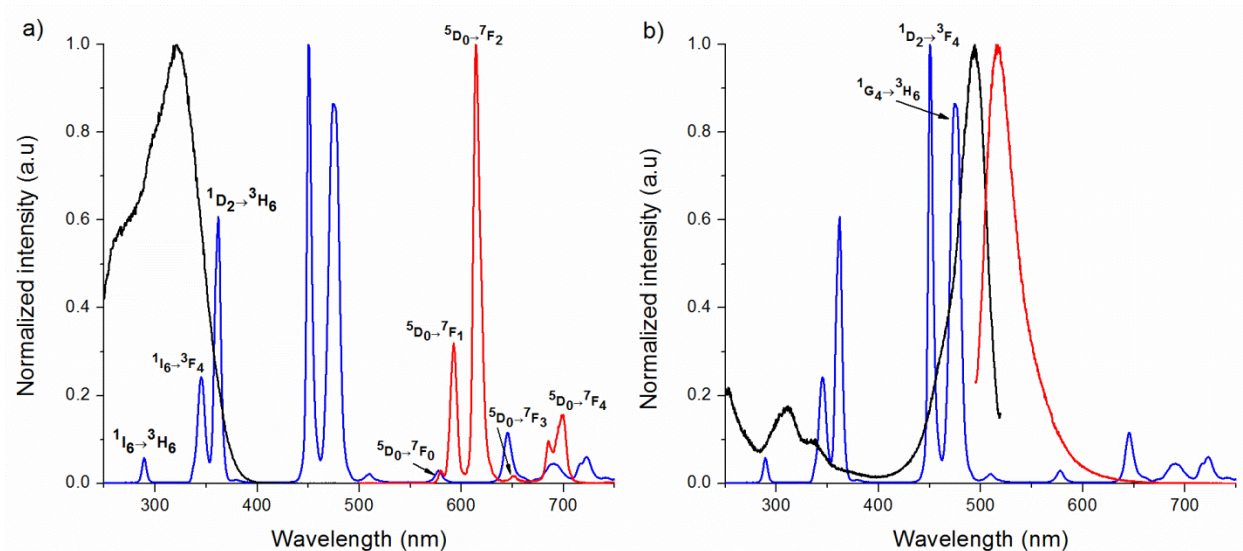
**Figure S4.** Titration of a) bio-Eu<sup>III</sup>-WN, b) bio-Eu<sup>III</sup>-Tekes, c) bio-Eu<sup>III</sup>-N1 or d) bio-AF488. The upconversion resonance energy transfer (UC-RET) at 615 nm or 565 nm is depicted as solid line and signal-to-background ratios as dashed line. The signal was measured by using steady-state (square) or temporal resolution (circle) mode.

### Excitation and emission spectra of Eu<sup>III</sup>-Tekes and AF488

The excitation and emission spectra of Eu<sup>III</sup>-Tekes and Eu<sup>III</sup>-N1 were measured as the spectra of Eu<sup>III</sup>-WN. AF488 was diluted in TSA to 30 nM concentration and the spectra were measured using the fluorescence mode of Cary Eclipse fluorescence spectrophotometer. The spectra of Eu<sup>III</sup>-Tekes and AF488 are depicted in Figure S5 with the emission spectra of NaKYF<sub>4</sub>: Yb<sup>III</sup>, Tm<sup>III</sup> UCNPs. The excitation and emission spectra of Eu<sup>III</sup>-N1 could not be presented because it is not intrinsically fluorescent and therefore could not be excited with the spectrofluorometer.

The Eu<sup>III</sup>-Tekes has excitation at UV-wavelengths centered at 321 nm overlapping with emission bands of the UCNPs centered at 345 and 362 nm and sharp emission peak at 613 nm characteristic for Eu<sup>III</sup> ions. The excitation spectrum of AF488 centered at 495 nm overlaps with emission bands of UCNPs centered at 450 and 475 nm. The emission of AF488 is centered at 519 nm.





**Figure S5.** Excitation (black) and emission (red) spectra of a) Eu<sup>III</sup>-Tekes and b) AF488 depicted with the emission spectrum of UCNP (blue). a.u.= arbitrary units

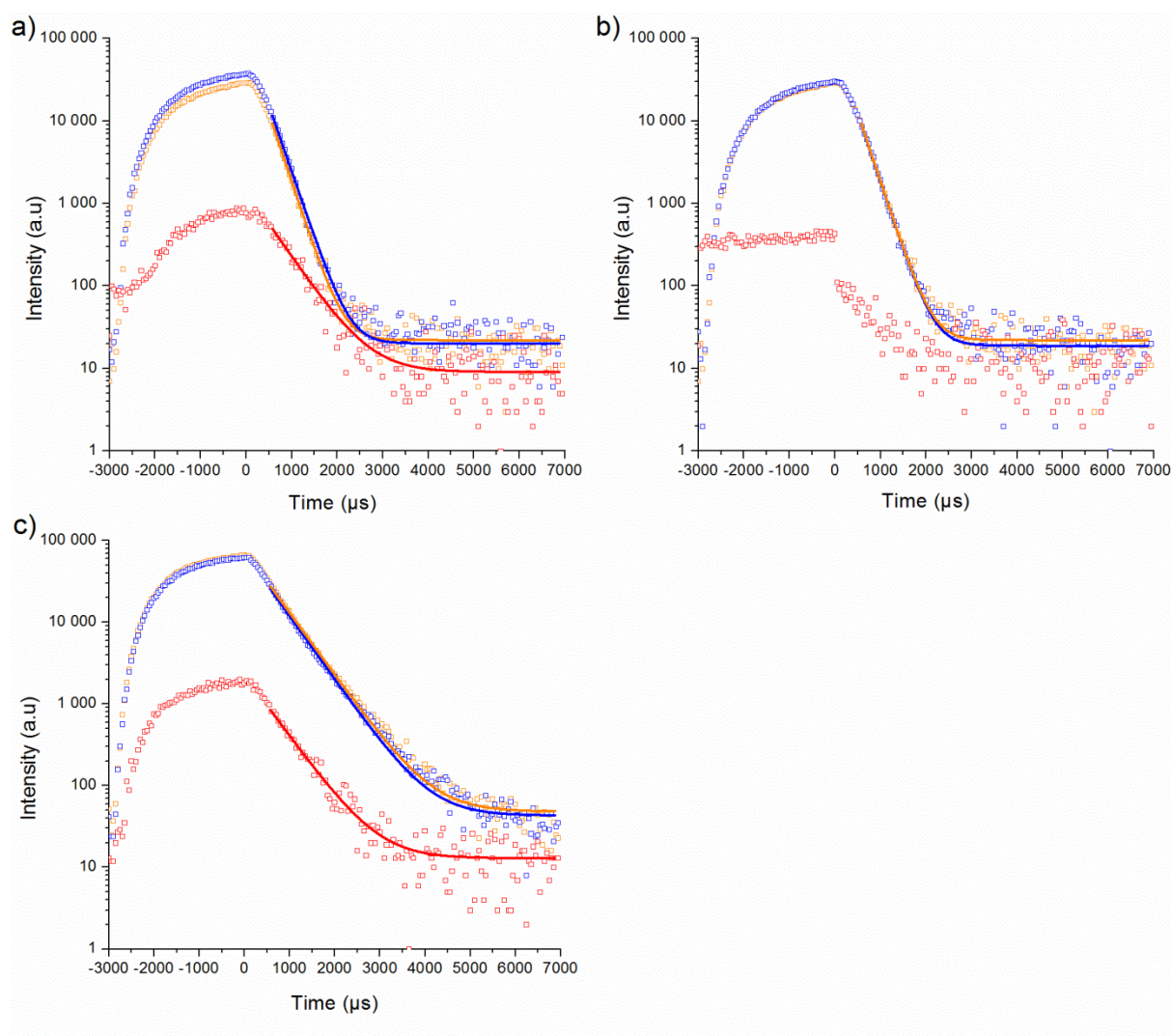
### Luminescence lifetimes of upconverting photoluminescence and sensitized Eu<sup>III</sup>-Tekes, Eu<sup>III</sup>-N1 and AF488 emissions

The lifetimes of upconversion photoluminescence and sensitized acceptor emission were measured from UCNP-SAVs with or without the acceptor collected on the particle surface as described in the article. The concentrations in the measurements were 50 nM and 24 nM for Eu<sup>III</sup>-chelates and AF488, respectively, when the UCNP concentration was 15 mg mL<sup>-1</sup>. After a 3 ms wide pulsed excitation at 980 nm the emissions were collected at 365 ± 10 nm and at 615 ± 7.5 nm if the acceptor was Eu<sup>III</sup>-Tekes or Eu<sup>III</sup>-N1. When AF488 was utilized as an acceptor the emissions were collected at 470 ± 10 nm and 565 ± 8 nm.

The data collected were fitted with the single-exponential decay function (Figure S6). The luminescence lifetimes were determined to be 261 ± 0.70 μs for UCNP-SAV at 365 nm, 270 ± 0.70 μs for UCNP-SAV-bio-Eu<sup>III</sup>-Tekes complex at 365 nm and 534 ± 7.6 μs for sensitized Eu<sup>III</sup>-Tekes emission at 615 nm. When Eu<sup>III</sup>-N1 was used as acceptor the luminescence lifetimes were 261 ± 0.70 μs for UCNP-SAV at 365 nm and 262 ± 0.76 μs for UCNP-SAV-bio-Eu<sup>III</sup>-N1 complex at 365 nm. The intensity of sensitized Eu<sup>III</sup>-N1 emission was so low that the luminescence lifetime could not be determined due to nonexistent absorption at 365 nm. The luminescence lifetimes at 470 nm for UCNP-SAV was 568 ± 1.9 μs and for UCNP-SAV-bio-AF488 complex 556 ± 1.9 μs. The luminescence lifetime of sensitized AF488 emission at 560 nm was 571 ± 5.9 μs.

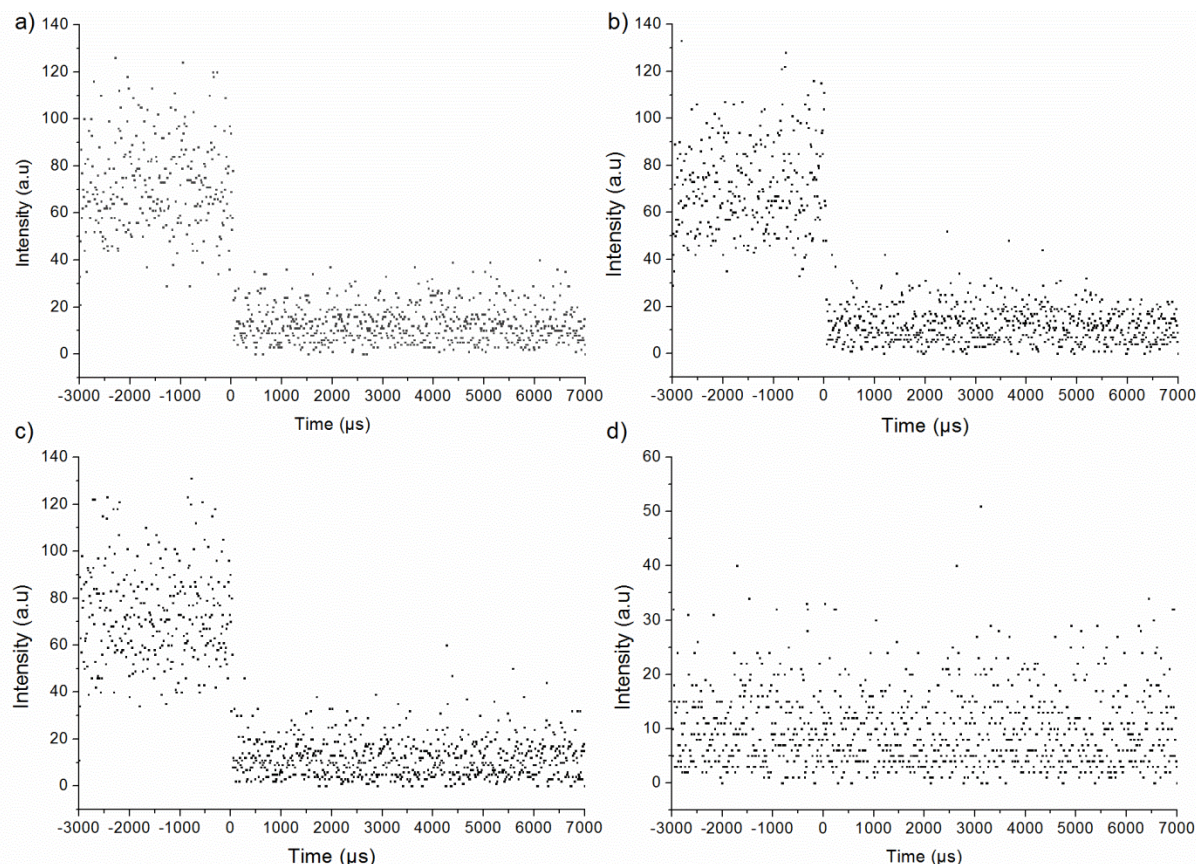
The luminescence lifetimes of the acceptors without the UCNP were measured with 980 nm excitation in order to conclude that the acceptors are sensitized through upconversion (Figure S7). When the lifetimes of bio-Eu<sup>III</sup>-chelates were measured at 615 nm, there is a small signal rise due to inadequate laser blocking. However, the signal is insignificant in intensity and doesn't affect the results.





**Figure S6.** Luminescence decay curves for UCNP-SAv (orange), UCNP-SAv-biotinylated acceptor conjugate (blue) and sensitized acceptor emission (red) when a) bio-Eu<sup>III</sup>-Tekes, b) bio-Eu<sup>III</sup>-N1 or c) bio-AF488 was used as an acceptor. The data were measured at 365 and 615 nm when Eu<sup>III</sup>-chelates were used as acceptors or at 470 and 565 nm when AF488 was the acceptor. a.u.= arbitrary units.





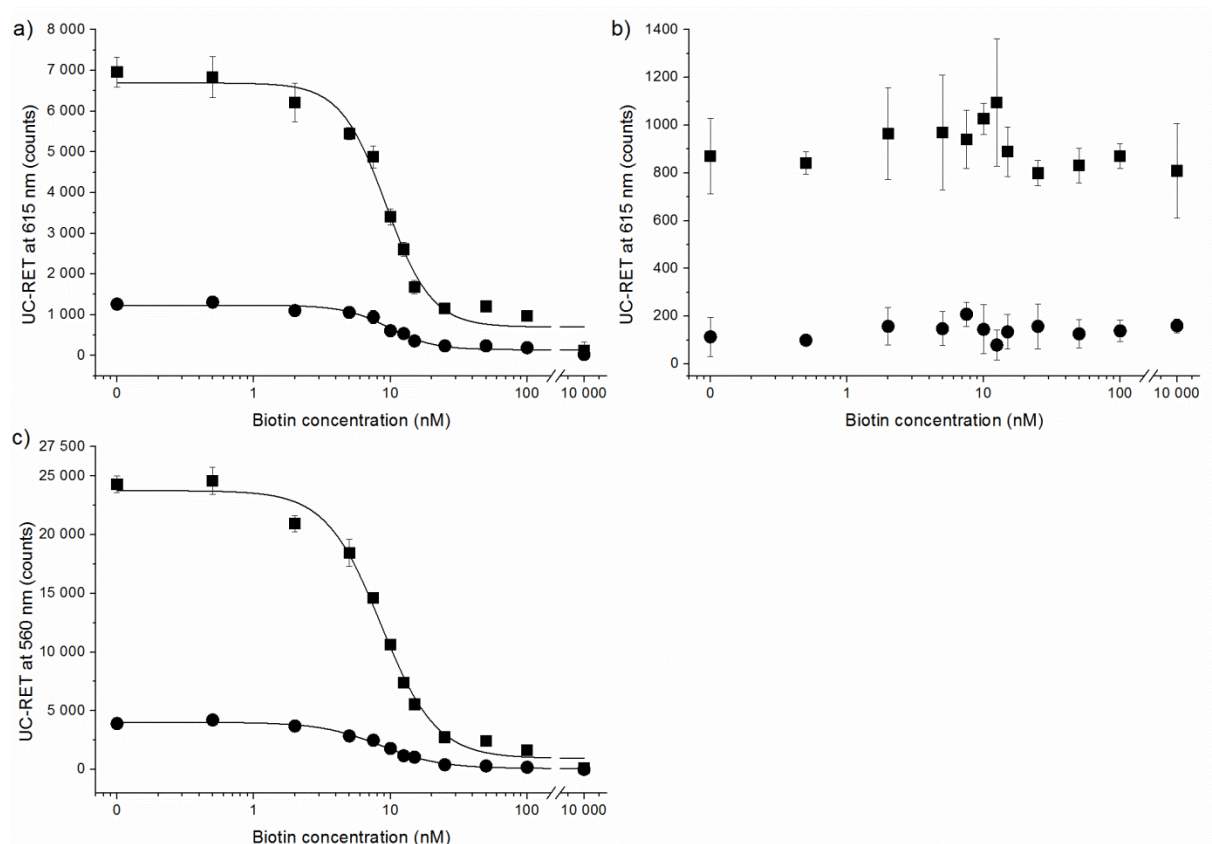
**Figure S7.** Luminescence decay curves for emissions of a)  $\text{Eu}^{\text{III}}$ -WN, b)  $\text{Eu}^{\text{III}}$ -Tekes, c)  $\text{Eu}^{\text{III}}$ -N1 or d) AF488 after pulsed excitation at 980 nm. The data were measured at 615 nm when  $\text{Eu}^{\text{III}}$ -chelates were measured and at 565 nm when AF488 was measured. a.u.,= arbitrary units.

### Homogeneous back-titration assay for biotin

The UC-RET system was employed in a competitive homogeneous back-titration assay for biotin (Figure S8). The signals from the sensitized acceptors were measured with steady-state and temporal resolution mode. The limits of detection (LODs) were 5.2 and 5.9 nM in steady-state and TR mode, respectively, when bio- $\text{Eu}^{\text{III}}$ -Tekes was utilized as an acceptor. The signal-to-background ratios were equivalent in both measurements (9.6 in steady-state and 8.7 in TR mode). However, the intensity of sensitized emission of the  $\text{Eu}^{\text{III}}$ -Tekes is low in TR measurement due to shorter intrinsic fluorescence lifetime of the  $\text{Eu}^{\text{III}}$ -Tekes than that of the  $\text{Eu}^{\text{III}}$ -WN. The emission of the bio- $\text{Eu}^{\text{III}}$ -Tekes has already significantly decayed when the emission is measured.

When bio-AF488 was employed as an acceptor the LODs were 3.0 and 4.3 nM in steady-state and TR mode, respectively. The signal-to-background ratios were two times higher when TR mode was utilized (25 and 50 in steady-state and TR mode, respectively) but the obtained signals were six times lower compared to steady-state mode. The LODs could not be calculated for bio- $\text{Eu}^{\text{III}}$ -N1 based assay because of the inefficient energy transfer from the UCNPs to the chelate.



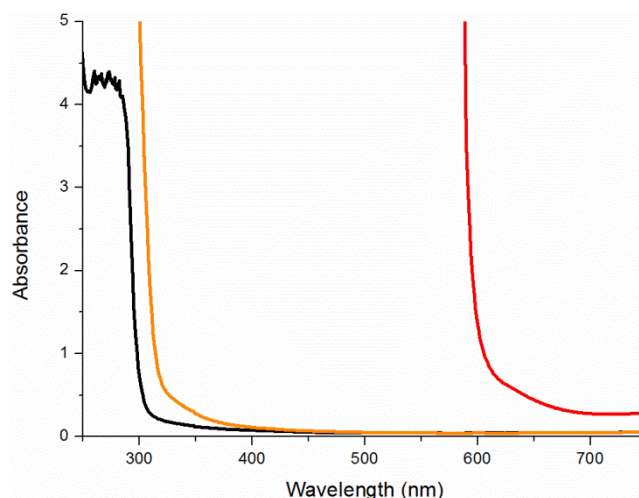


**Figure S8.** Standard curves for competitive homogeneous upconversion resonance energy (UC-RET) assay for biotin utilizing a) bio-Eu<sup>III</sup>-Tekes, b) bio-Eu<sup>III</sup>-N1 or c) bio-AF488 as an acceptor. The UC-RET was measured with steady-state (square) or temporal resolution (circle) mode after 980 nm excitation.

### Absorbance spectra of sample matrices

The lysed blood, urine, and TSA buffer with 7.5 % bovine serum albumin (BSA) were diluted to assay buffer to comprise 10 % of the total volume. The absorbance spectra were measured with Cary Eclipse spectrophotometer (Figure S9). A cuvette with 1 cm light path length was used in the measurement.

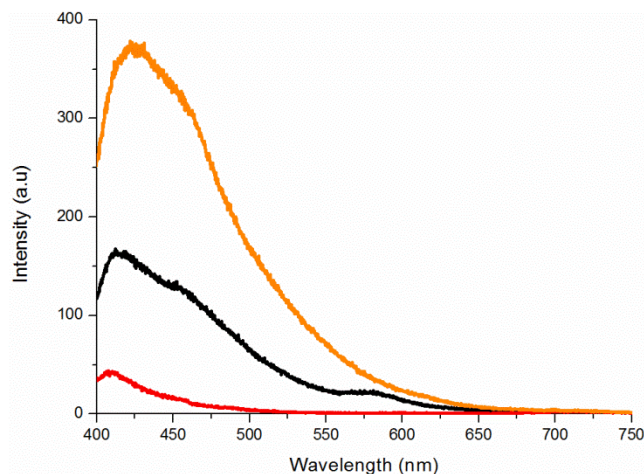




**Figure S9.** Absorbance spectra of the 10 % sample matrices in assay buffer. Lysed blood (red), urine (orange), and TSA buffer with 7.5 % BSA (black).

### Steady-state emission spectra of matrices to determine autofluorescence

The lysed blood, urine, and TSA buffer with 7.5 % BSA were diluted to assay buffer to comprise 10 % of the total volume. The steady-state emission spectra were measured with Cary Eclipse fluorescence spectrophotometer with fluorescence mode (Figure S10). The matrices were excited with 360 nm. A cuvette with 0.3 cm light path length was used in order to minimize the absorbance of the sample matrices. Nonetheless, the emission spectrum of lysed blood has low intensity because the blood has high absorbance below 600 nm and therefore absorbs most of the excitation light.

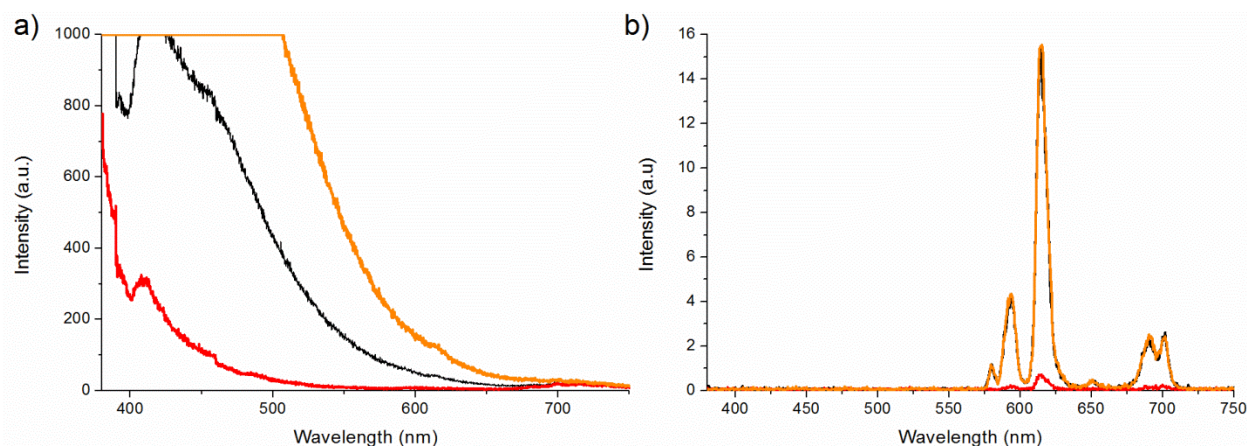


**Figure S10.** Steady-state emission spectra of 10 % sample matrices in assay buffer upon 360 nm excitation. Lysed blood (red), urine (orange), and TSA buffer with 7.5 % BSA (black). a.u.= arbitrary units



### UV-excited emission spectra of bio-Eu<sup>III</sup>-WN in sample matrices

The bio-Eu<sup>III</sup>-WN was diluted in an assay buffer, which contained 10 % lysed blood, urine, or TSA with 7.5 % BSA of the final volume, to a final concentration of 34 nM. The steady-state and TR emission spectra were measured with Cary Eclipse fluorescence spectrophotometer using fluorescence mode and phosphorescence mode, respectively (Figure S11). The solutions were excited with 360 nm. The emission of the bio-Eu<sup>III</sup>-WN cannot be detected from the autofluorescence in steady-state measurement in any of the matrices. The emission spectrum of lysed blood has low intensity in TR measurement because the blood has high absorbance below 600 nm and therefore absorbs most of the excitation light.



**Figure S11.** a) Steady-state and b) TR emission spectra of bio-Eu<sup>III</sup>-WN in 10 % matrices. Lysed blood (red), urine (orange), and TSA buffer with 7.5 % BSA (black). a.u.= arbitrary units

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