ELECTRONIC SUPPLEMENTARY INFORMATION
Bioconjugates of photon-upconversion nanoparticles with antibodies for the detection of prostate-specific antigen and p53 in heterogeneous and homogeneous immunoassays

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1 Materials and methods

1.1 Chemicals and materials

Anhydrous *N*,*N*-dimethylformamide (DMF), azido-dPEG$_8$-NHS ester, L-ascorbic acid sodium salt, neridronate (Ner), Float-A-Lyzer G2 dialysis device (molecular weight cut-off, MWCO, of 100 and 300 kDa), and Amicon Ultra centrifugal filters (0.5 mL, MWCO of 30 and 100 kDa) were obtained from Merck/Sigma-Aldrich (Germany). Streptavidin was purchased from Thermo Fisher Scientific (USA). Argon (purity ≥ 99.999%) was supplied by Messer (Germany). All other chemicals were obtained in the highest quality available from Carl Roth (Germany) or Penta (Czech Republic).

Buffers included phosphate buffer (PB; 50 mM NaH_2PO_4/Na_2HPO_4; pH 7.4), washing buffer (50 mM NaH_2PO_4/Na_2HPO_4, 0.05% NaN_3, 0.01% Tween 20; pH 7.5), and dialysis buffer (50 mM Tris, 1 mM KF, 0.05% NaN_3; pH 7.5).

1.2 UCNP surface modification

The $NaYF_4$:Yb^{3+},Er^{3+} and $NaYF_4$:Yb^{3+},Tm^{3+} UCNPs were synthesized by high-temperature co-precipitation according to our previously reported protocols.[1,2] The Ner-PEG-alkyne linker was synthesized according to the published protocol.[3]

UCNP ligand exchange reaction was carried out utilizing two procedures, based on HCl and $NOBF_4$. In the HCl-based approach, UCNPs (10 mg, 500 µL) dispersed in cyclohexane were mixed with an equivalent volume of 200 mM HCl. The mixture was incubated under shaking for 30 min at 38 °C, followed by sonication for 30 min, facilitating the phase transfer from cyclohexane to water. The upper phase was then transferred to a separate vial, and an excess of acetone was added. Afterward, the dispersion was centrifuged (1,000 *g*, 40 min), and the resulting UCNP pellet was redispersed in 500 µL of double-distilled water and sonicated for 5 min.

In the $NOBF_4$-based procedure, UCNPs (10 mg, 500 µL) dispersed in cyclohexane were mixed with an equivalent volume of 20 mg/mL $NOBF_4$ in anhydrous DMF. The resulting mixture was incubated for 30 min under shaking at laboratory temperature, followed by 30 min of sonication. Afterward, the upper phase was transferred to a separate vial, followed by the addition of an excess of chloroform. The dispersion was then centrifuged (1,000 *g*, 40 min), and the resulting UCNP pellet was redispersed in 500 µL of double-distilled water and sonicated for 5 min.

After the ligand exchange, the UCNP-Cl^- and UCNP-BF_4^- dispersions in water were mixed with Ner-PEG-alkyne linker (3 mg in 500 µL of double-distilled water) and incubated overnight at 38 °C under shaking. The UCNP-Ner-PEG-alkyne conjugates were purified by dialysis in a Float-A-Lyzer G2 (100 kDa MWCO) at 4 °C against double-distilled water containing 1 mM KF, which was exchanged nine times, and stored at 4 °C.

1.3 Preparation of click-reactive biomolecules

Click-reactive antibody-azide and streptavidin-azide conjugates were prepared by using the NHS-dPEG$_8$-azide linker. In the case of polyclonal anti-HSA antibody, NHS-dPEG$_8$-azide (100 mM, 5 µL) in DMF was added to 46 µL of antibody (10.9 mg/mL) in 350 µL of PB. In the cases of polyclonal and monoclonal anti-PSA and anti-p53 antibodies, NHS-dPEG$_8$-azide

(100 mM, 0.5 μL) in DMF was added to 100 μL of antibody (0.5 mg/mL) in 300 μL of PB. In the case of streptavidin, NHS-dPEG$_8$-azide (100 mM, 62.5 μL) in DMF was mixed with streptavidin (1.8 mg/mL, 337.5 μL) in PB. The mixtures were incubated overnight at 4 °C under mild shaking, followed by stopping the reaction by the addition of 50 μL of washing buffer. The conjugates were purified by centrifuge filtration (14,000 *g*, 6 × 20 min) using Amicon ultra (30 kDa MWCO in the case of streptavidin and 100 kDa MWCO in the cases of antibodies) and stored in PB at 4 °C in the concentration of 1 mg/mL in the case of streptavidin and 0.5 mg/mL in the cases of antibodies.

1.4 Preparation of Er-doped UCNP-PEG-anti-HSA conjugate

Er-doped UCNP-Ner-PEG-alkyne was conjugated with click-reactive polyclonal anti-HSA antibody. The reaction was carried out in an Ar atmosphere. First, 50 μL of Tris-HCl (375 mM in water; pH 7.5), 10 μL of aqueous $CuSO_4$/THPTA solution (25 mM/125 mM), and 5 mg of UCNP-Ner-PEG-alkyne in 1 mM KF were added to the reaction flask. The mixture was purged with Ar for 30 min, and different volumes (20, 50, 100, 200, 500, or 1000 μL) of azide-modified polyclonal anti-HSA antibody (0.5 mg/mL) in PB were added. After purging the mixture with Ar for another 15 min, 10 μL of aqueous solution of sodium ascorbate (50 mM) were added to initiate the click reaction, and the mixture was incubated for 40 min under Ar purging. Finally, the mixture was dialyzed in a Float-A-Lyzer G2 device (300 kDa MWCO) against 5 L of dialysis buffer, which was exchanged nine times. The purified conjugate was stored at 4 °C.

1.5 Characterization of UCNPs and their conjugates

To measure their emission spectra, the oleic acid-capped Er- and Tm-doped UCNPs were dispersed in cyclohexane to a concentration of 1 mg/mL. A 976 nm laser at an intensity of 50 W/cm^2 was used for the excitation. The emission spectra were recorded by a QE65 Pro spectrometer (Ocean Optics, USA).

For the transmission electron microscopy (TEM) analysis, a small amount (~5 μL) of dispersion of oleic acid-capped UCNPs in cyclohexane was deposited onto a copper grid coated with 12 nm continual carbon foil, and the dispersant was allowed to evaporate at laboratory temperature. The TEM images were taken by Titan Themis (FEI, Czech Republic) with FEI Ceta 16-megapixel CMOS camera.

The bioconjugates of UCNPs were characterized by dynamic light scattering (DLS) utilizing Zetasizer Nano ZS (Malvern, UK). All the bioconjugates and bioconjugation intermediates were diluted to 10 μg/mL using dialysis buffer, and the measurements were conducted in a ZEN0112 plastic cuvette (Malvern, UK) at 25 °C using a scattering angle of 173°.

1.6 Processing massively parallel spectroscopy data

The ImageJ distribution FIJI[4] was used for the visualization and manual evaluation of the images, while a custom laboratory-developed software employing a convolutional neural network was used for the automated processing of massively parallel spectroscopy (MPS) data. This software was implemented in Python using the Keras deep learning framework. MPS images were recorded at a 16-bit pixel depth with intensity values ranging from approximately 100 to 65535. Before neural network analysis, these images were transformed by applying a

base-2 logarithm and scaling by a factor of 16, resulting in values between approximately 0.4 and 1.0.

A convolutional neural network with a U-Net architecture (**Figure S1**) was employed to localize double spots. The network was trained using simulated data where double spots were modeled as two-dimensional Gaussian peaks with added Poisson noise. The noise was introduced by replacing each simulated intensity value with a random sample from a Poisson distribution centered on that intensity. These simulated double spots were overlaid onto real MPS images incorporating realistic backgrounds. A mask indicating the positions of simulated double spots was generated for each image. Training data consisted of 6000 simulated image-mask pairs, each 256 × 256 pixels in size and containing between 1 and 50 double spots per image. A validation split of 25% was used for training the neural network. After manually testing the trained U-net on real MPS images, the network was used to process the measured data, generating double-spot localization maps that were thresholded to produce binary masks. These masks identified double spot locations, which were subsequently counted. To handle larger images, 256 × 256 pixel sections were processed individually, and the results were combined after localization. For ease of manual verification, the double spots were highlighted with green rectangles in overlays of the original images.

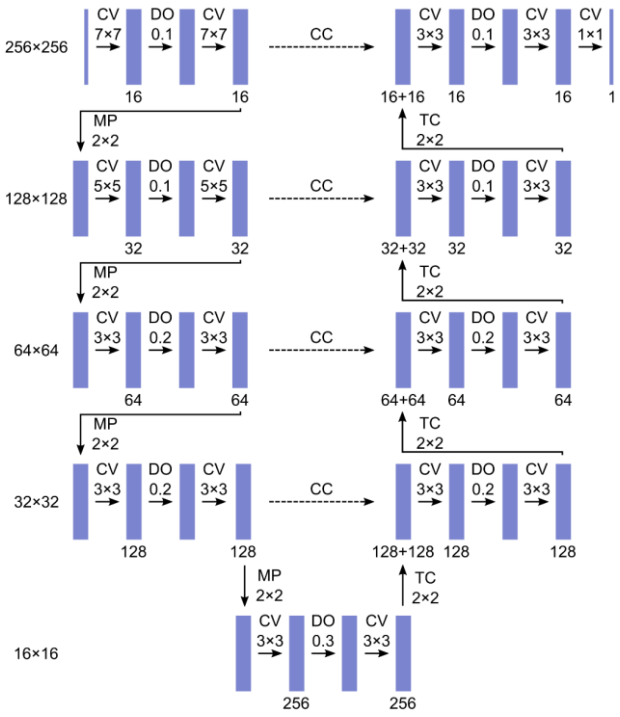

Figure S1: Schematic representation of the utilized U-Net. The network consists of several layers, including convolution (CV), dropout (DO), max pooling (MP), transposed convolution (TC), and concatenation (CC). In the first stage, the input image (256 × 256 pixels) is progressively downsampled, reducing its spatial dimensions while increasing the number of feature maps. By the final downsampling step, the image is represented as 256 feature maps, each with a reduced resolution of 16 × 16 pixels. In the second stage, the network reconstructs the spatial information, generating a final output map (256 × 256 pixels) that identifies the double spot locations.

2 Results and discussion

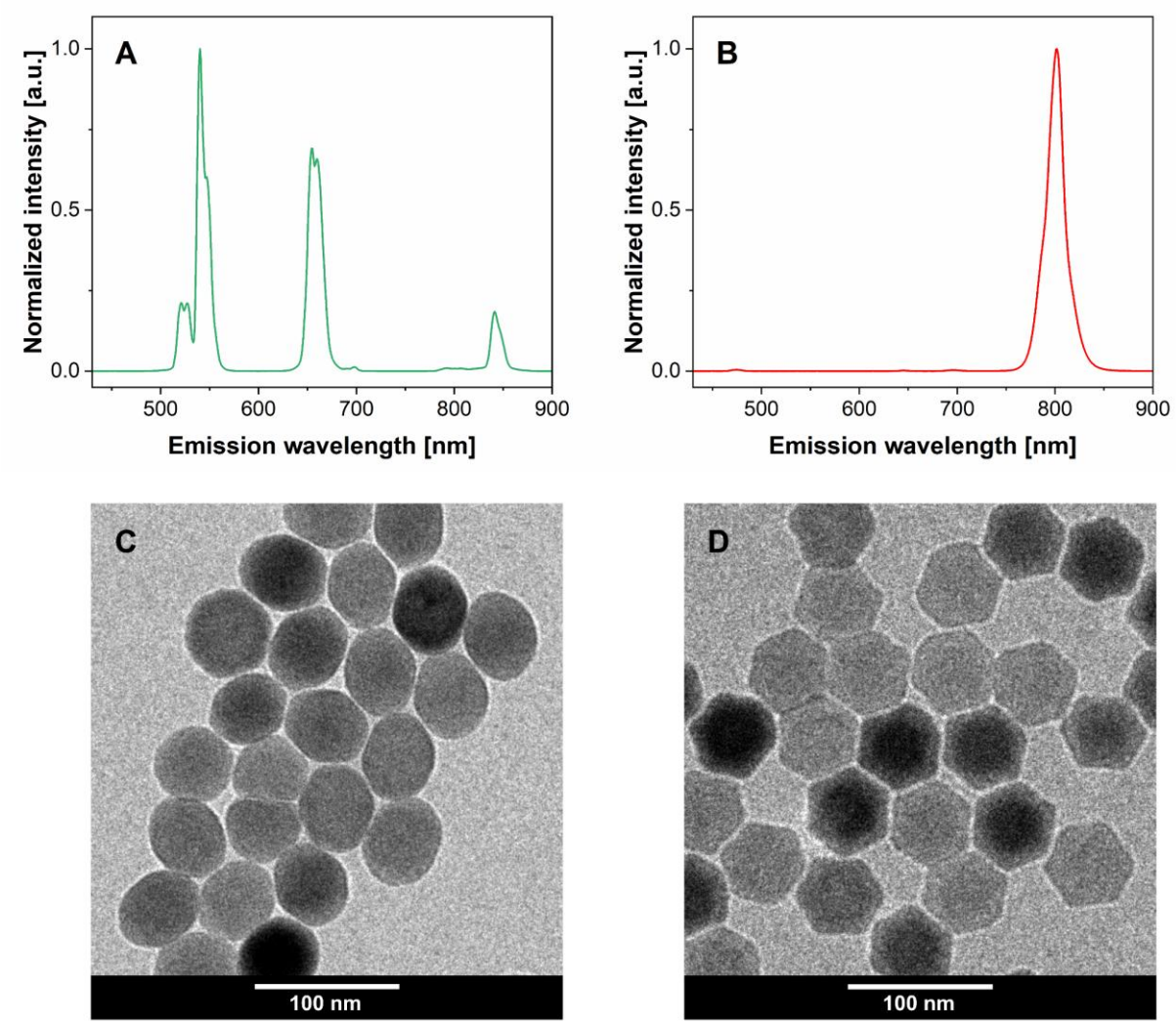

Figure S2: Emission spectra of oleic acid-capped UCNPs under 976 nm laser excitation at an intensity of 50 W/cm^2. (**A**) $NaYF_4$:Yb^{3+},Er^{3+} (Er-doped UCNPs) normalized at 540 nm and (**B**) $NaYF_4$:Yb^{3+},Tm^{3+} (Tm-doped UCNPs) normalized at 802 nm. TEM images of (**C**) Er-doped and (**D**) Tm-doped UCNPs.

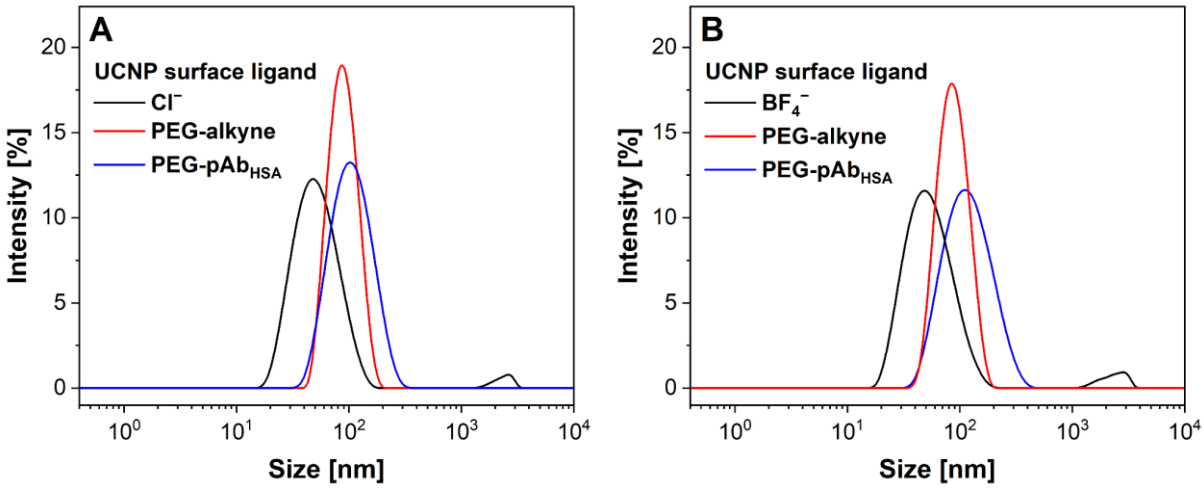

Figure S3: Particle size distributions of Er-doped UCNPs during the conjugation using different ligand exchange agents (HCl and $NOBF_4$). Both procedures provided similar average hydrodynamic diameters and polydispersity indexes (PDI). (**A**) HCl-based ligand exchange procedure: UCNP-Cl^- (d = 54 ± 5 nm, PDI = 0.209 ± 0.06), UCNP-PEG-alkyne after HCl ligand exchange (d = 91 ± 2 nm, PDI = 0.07 ± 0.01), and UCNP-PEG-anti-HSA after HCl ligand exchange (d = 105 ± 2 nm, PDI = 0.12 ± 0.01). (**B**) $NOBF_4$-based ligand exchange procedure: UCNP-BF_4^- (d = 55 ± 6 nm, PDI = 0.203 ± 0.05), UCNP-PEG-alkyne after $NOBF_4$ ligand exchange (d = 90 ± 2 nm, PDI = 0.08 ± 0.01), and UCNP-PEG-anti-HSA after $NOBF_4$ ligand exchange (d = 109 ± 3 nm, PDI = 0.13 ± 0.02). For these conjugates, the ratio of 10 µg of azide-modified antibody per 1 mg of UCNPs was used in the click reaction. The measured data points were connected using a B-spline function.

Table S1: Summary of the analytical parameters of ULISA assays for HSA detection using 6.5 µg/mL of UCNPs modified with polyclonal anti-HSA antibody.

Ligand exchange agent	**Amount of azide-modified antibody in click reaction [µg/1 mg of UCNPs]**	**LOD [pg/mL]**	**Working range [ng/mL]**	***S/B***
HCl	2	270	16–160	171
HCl	5	90	12–100	203
HCl	10	65 78	12–100 12–100	256 240
HCl	20	57	11–99	168
HCl	50	34	9.3–100	175
HCl	100	46	5.8–61	112
$NOBF_4$	10	76	15–140	316

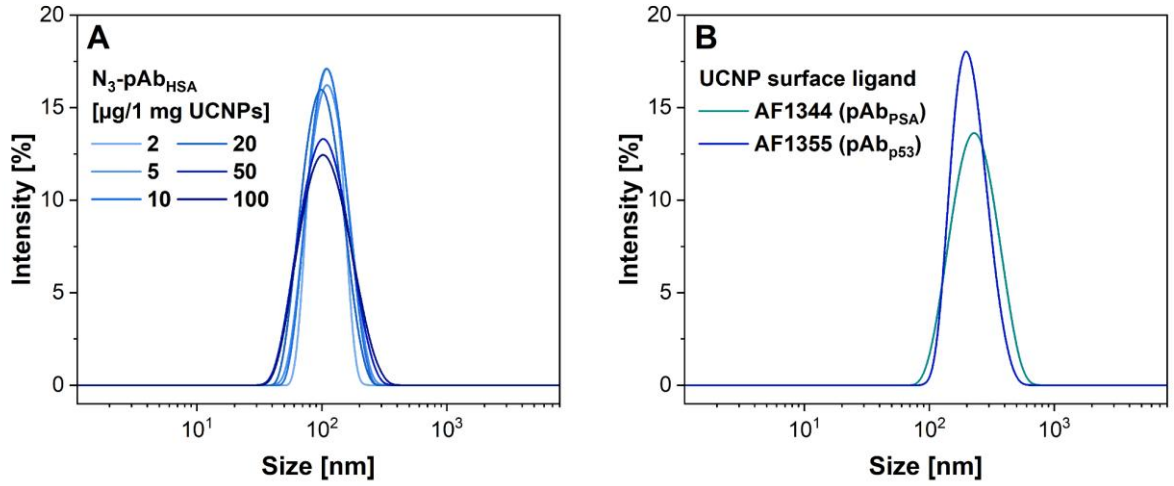

Figure S4: Particle size distribution of Er-doped UCNPs conjugated with polyclonal antibodies. (**A**) UCNP-anti-HSA conjugates prepared using different amounts of azide-modified anti-HSA antibody during the click reaction [µg of Ab-azide/1 mg of UCNPs]: 2 (d = 111 ± 1 nm, PDI = 0.07 ± 0.01), 5 (d = 110 ± 2 nm, PDI = 0.08 ± 0.02), 10 (d = 108 ± 3 nm, PDI = 0.12 ± 0.01), 20 (d = 102 ± 2 nm, PDI = 0.12 ± 0.02), 50 (d = 104 ± 3 nm, PDI = 0.14 ± 0.03), and 100 (d = 102 ± 4 nm, PDI = 0.15 ± 0.02). (**B**) UCNPs modified with polyclonal anti-PSA antibody (AF1344; d = 240 ± 6 nm, PDI = 0.26 ± 0.04) and polyclonal anti-p53 antibody (AF1355; d = 225 ± 8 nm, PDI = 0.28 ± 0.05). The measured data points were connected using a B-spline function.

Table S2: Summary of the analytical parameters of ULISA assays for PSA and p53 detection based on Er-doped UCNPs modified with streptavidin and polyclonal antibodies.

Analyte	**Label**	**Label concentration [µg/mL]**	**LOD [pg/mL]**	**Working range [ng/mL]**	***S/B***
PSA	UCNP-SA	3.3	1.9	0.2–2.1	206
		6.5	1.3	0.3–3.2	272
	UCNP-AF1344	3.3	36	1.8–28	39
		6.5	17	1.1–14	61
p53	UCNP-SA	3.3	520	94–980	512
		6.5	330	120–370	427
	UCNP-AF1355	3.3	1700	33–420	27
		6.5	840	28–270	50

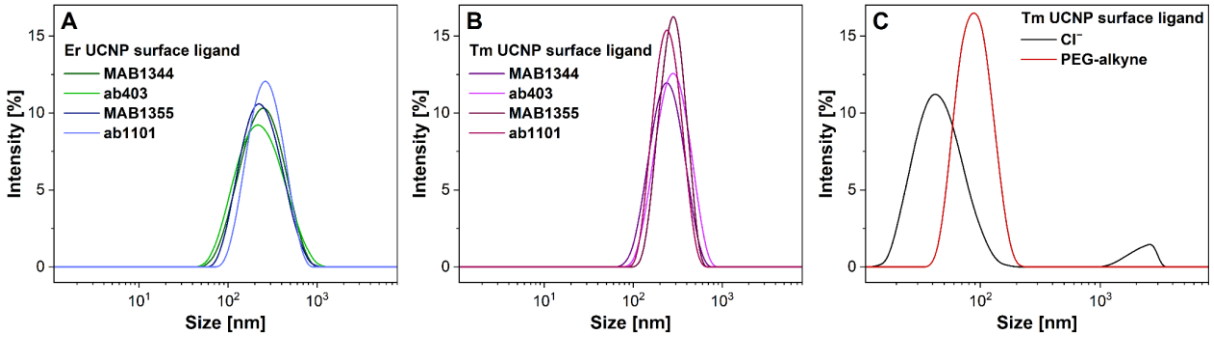

Figure S5: Particle size distribution of Er- and Tm-doped UCNPs. (**A**) Er-doped and (**B**) Tm-doped UCNPs conjugated with different monoclonal antibodies: MAB1344, ab403, MAB1355, and ab1101. (**C**) Tm-doped UCNP conjugation intermediates: UCNP-Cl^- (d = 51 ± 4 nm, PDI = 0.28 ± 0.05) and UCNP-PEG-alkyne (d = 84 ± 2 nm, PDI = 0.09 ± 0.01) The measured data points were connected using a B-spline function.

Table S3: Summary of the analytical parameters of ULISA assays for the detection of PSA and p53 using 6.5 µg/mL of Er- and Tm-doped UCNPs modified with monoclonal antibodies and DLS characterization of the conjugates (average hydrodynamic diameters and polydispersity indexes calculated from 3 measurements).

Label (UCNP dopant – antibody)	**LOD [pg/mL]**	**Working range [ng/mL]**	***S/B***	***d* [nm]**	**PDI**
PSA detection					
Er – MAB1344	16	1.1–15.0	35	203 ± 4	0.28 ± 0.03
Tm – MAB1344	32	0.6–10.1	30	227 ± 3	0.24 ± 0.04
Er – ab403	38	1.2–14.8	23	190 ± 5	0.32 ± 0.04
Tm – ab403	33	0.5–4.7	42	253 ± 5	0.28 ± 0.03
p53 detection					
Er – MAB1355	580	17.8–132	36	197 ± 6	0.34 ± 0.04
Tm – MAB1355	750	17.1–208	34	225 ± 4	0.22 ± 0.03
Er – ab1101	780	17.0–105	26	204 ± 4	0.35 ± 0.05
Tm – ab1101	480	30.1–321	34	264 ± 5	0.29 ± 0.04

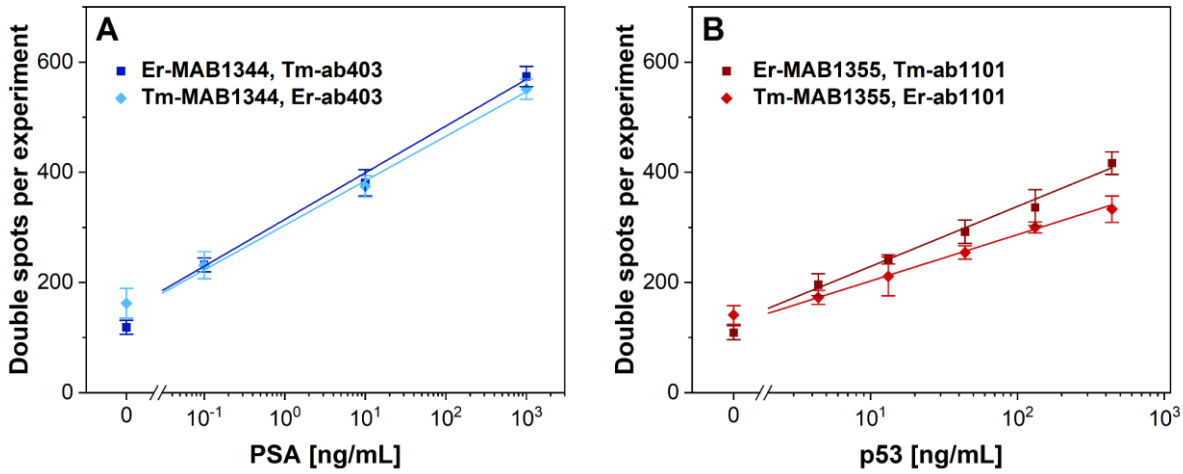

Figure S6: Immunoassay based on massively parallel spectroscopy utilizing different combinations of monoclonal antibody-modified Er- and Tm-doped UCNPs for the detection of (**A**) PSA and (**B**) p53. Error bars represent the standard deviations of 3 independent MPS measurements.

3 References

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