SUPPORTING INFORMATION

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Experimental Section

Materials

Bovine serum albumin (fraction V, BSA), human serum albumin (fraction V, HSA), anti-HSA antibodies (rabbit polyclonal antiserum, affinity purified), casein, *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDAC), 3,3'5,5'-tetramethylbenzidine dihydrochloride (TMB), titanium isopropide (TIP) aminopropyltriethoxysilane (APTS), toluene, glutaraldehyde solution grade I (25% in water), methanol (HPLC grade) and Bradford reagent were purchased from Sigma- Aldrich (St. Louis, MO, USA). Acetic acid was purchased by Fisherbrand (ACS reagent 99.7%). Horse-radish peroxidase (HRP) was purchased from Roche Diagnostics (Bazel, Switzerland). Microtitre plates (Nunc maxi-sorp) were obtained from FisherScientific (Illkirch, France). Anti-BSA antibodies (rabbit polyclonal antiserum, immunoglobulin fraction obtained through ammonium sulphate precipitation) were an antiserum available in the lab and previously developed for internal purposes. Phosphate buffer (PB, phosphate 20mM, pH 7.4) and artificial urine used as diluents for the assays. Artificial urine contained following solutes: $CaCl_2 \cdot 2H_2O$ (0.65 g l^{-1}), $MgCl_2 \cdot H_2O$ (0.65 g l^{-1}), NaCl (4.6 g l^{-1}), Na_2SO_4 (2.3 g l^{-1}), Na_3 citrate$\cdot 2H_2O$ (0.65 g l^{-1}), KH_2PO_4 (2.8 g l^{-1}), KCl (1.6 g l^{-1}), NH_4Cl (1.0 g l^{-1}), urea (25 g l^{-1}), and creatine (1.1 g l^{-1}). The pH of the resulting solutions was adjusted to 6.5.
Ultrapure water was obtained by MilliQ SystemAcademic (Waters, Millipore) and used for TMB and H_2O_2 dilution and for washing microtitre plate wells.

TiO_2 nanoparticles preparation and characterization

TiO_2 was produced via hydrothermal synthesis starting from titanium isopropoxide (TIP) mixed with acetic acid [20]. Briefly, acetic acid (10 ml) was stirred for 10 minutes and TIP (5 ml) was added under stirring. The solution was brought to 50 ml with deionized water; a portion of the diluted solution (10 ml) was further diluted with deionized water (50 ml) and put in a Teflon autoclave. The hydrothermal treatment took place at 383 K for 24h and then at 418 K for 72h. The obtained TiO_2 was collected by centrifugation and dried overnight at 333 K. TiO_2 crystalline phase was attributed based on Raman spectra collected with a Bruker Senterra Raman Microscope using a 785 nm laser through a 40 objective (Fig S2). Particles dimension and morphology was determined by Transmission electron microscopy images taken on a Philips CM200 instrument operating at up to 200 kV, with line resolution of 0.14 nm and point resolution of 0.19 nm (Fig S1).

TiO_2 -NPs functionalization

TiO_2 particles were silanized to introduce coupling functionalities [10]. Briefly, TiO_2 (1 g) was dispersed in toluene (100 ml) under stirring and 3-aminopropyl-triethoxysilane (1 ml) was added. The reaction occurred for 24 h. After reaction, silanized TiO_2 NPs were collected by centrifugation and the powder was washed several times by using methanol and deionized water.

Labelling of antigens with functionalized TiO_2 -NPs

Conjugation with BSA and HSA was obtained by mixing a suspension of TiO_2 (4 ml, 0.25 mg ml^{-1}) with the protein solution (50 µl, 10 mg ml^{-1}) and glutaraldehyde (10 µl, 2.5% wt). All the solutions were prepared in phosphate buffer (pH 7.4). The reaction occurred overnight. TiO_2 nanoparticles conjugated to proteins (BSA-TiO_2 and HSA-TiO_2) were collected by centrifugation and washed with deionized water to eliminate non-bound proteins. To confirm binding of proteins to the functionalized TiO_2 nanoparticles, unconjugated and conjugated particles were stained with Comassie dye as follows: equal volumes of the nanoparticles suspension (0.25 mg ml^{-1}) and Bradford reagent were mixed and the colour of the solution was observed after 10 min incubation at room temperature in the dark. A blue colouring of the mixture was assumed as a confirmation of conjugation (Fig. S3).

Catalytic activity of the TiO_2 probe covalently linked to antigens

Irradiation experiments were carried out in uncoated plates containing the TiO_2 probe (100 µl, 1 mg l^{-1}) and TMB (100 µl, 8mM) mixed with H_2O_2 (48 mM). All chemicals were prepared in MilliQ water. Samples were irradiated for variable times (from 10 minutes to 2 hours) by using a Philips TLK/05 lamp 40 Watt with maximum emission at 360 nm. After the addition of H_2SO_4 (50 µl, 2 M, stop solution), TMB absorbance was recorded

at 450 nm by means of a microplate reader (Multiskan FC, Thermo Fisher Scientific). To evaluate the auto photo-oxidation of TMB in the experimental conditions, the same experiment was also conducted on the mixture of TMB/H_2O_2 without adding BSA-TiO_2. The dependency of the catalytic activity of the TiO_2 probe (1 mg l^{-1}) on TMB and H_2O_2 amounts was studied for TMB diluted at 4 and 8 mM and mixed to H_2O_2 at different rates (TMB/H_2O_2: 1/0, 1/1, 1/3 and 1/6) (Fig. S4).

Stability of the TiO_2 probe

TiO_2-BSA long-term stability was studied by diluting the nanoparticles (1 mg l^{-1}) in phosphate buffer solution for 14 days at room temperature. Activity was checked after 1, 2, 3, 6, 10, and 14 days. Experiments were carried out by using the optimal concentrations of TMB (8 mM) and H_2O_2 (48 mM). After 2 weeks, the signals obtained with BSA-TiO_2 suspension were comparable to those achieved at day 0 (Fig. S6 B). Tolerance of BSA-TiO_2 to acidic and alkaline pH was studied by maintaining the nanoparticles (1 mg l^{-1}) in solutions kept at pH comprises from 2 to 10 for 1, 4 and 24 hours. After one day, the response of the photocatalytic system is comparable to the original (Fig S6 B).

Binding capability of the TiO_2 probe covalently linked to BSA towards immobilized anti-BSA antibodies

We prepared the immunoreactive solid phases by coating wells with 150 µl of anti-BSA (or anti-HSA) rabbit polyclonal antibodies diluted in carbonate/bicarbonate buffer pH 9.6 (overnight at 4°C) and washing them three times with Tween 20 (0.05%, washing solution). To assure complete saturation of well surface, we further incubated phosphate buffer supplied with 1% casein (250 µl) in immune-reactive wells for 1 hour at room temperature, followed by three washings with the washing solution. The rate of TiO_2-BSA binding to immobilized antibodies was studied by incubating BSA-TiO_2 (200 µl, 10 µg ml^{-1}) into immune-reactive wells coated with anti-BSA antibodies (1:2000) for times comprises between 15 minutes and 24h. After TiO_2-BSA binding to anchored antibodies, we removed the unbound fraction by washing wells three times with the washing solution, and five times with ultrapure water (this washing protocol was routinely applied in the following experiments). Colour development was carried out as described in the irradiation experiment and carried out for 1 hour.

Non-competitive immunoassay based on using TiO_2-labelled BSA and TiO_2-labelled HSA as the tracers

Checkerboard titrations to select the best combination of anchored antibody dilution and tracer amount for the competitive immunoassay were conducted by coating wells with increasingly diluted anti-HSA or anti-BSA antibodies (from 1: 2,000 to 1:40,000) and incubating into them HSA-TiO_2 or BSA-TiO_2 (200 µl), respectively. Tracers were diluted (4 and 10 µg ml^{-1} in phosphate buffer, PB) and reacted for 24h. After washing wells, we carried out the colour development as described for the irradiation experiment (irradiation time 1 hour).

Competitive immunoassay based on using TiO_2-labelled HSA as the tracer

The construction of calibration curves involved mixing HSA standard solution (100 µl diluted in PB, 0-1000 mg l^{-1}), and the TiO_2-labelled HSA as tracer (100 µl, 20 µg ml^{-1}) into immunoreactive wells for 1 hour. The unbound reagents were removed by washing with the above described washing protocol. The colour development was obtained by 1-hour irradiation with the mixture TMB/H_2O_2. Once the stop solution was added, we recorded TMB absorbance at 450 nm and fitted experimental data by the four-parameter logistic equation [1]. All standards were measured in duplicate.

Human urine analysis

Samples of human urine were kindly provided by the Laboratory of Clinical Chemistry and Microbiology of the Umberto I Hospital (Turin, Italy). The reference content of albumin was determined by means of their routine method of analysis based on an Architect Ci8200® analyzer (Abbott, Abbot Park, IL, USA). Urine albumin was also measured by the developed TiO_2-based immunoassay, according to a two-step protocol, and diluting the HSA standard in artificial urine [18] instead of PB: HSA standard solution (200 µl, 0-1000 mg l^{-1}) were allowed to react into immunoreactive wells for 30 min, the unbound reagents were removed by three washing with a washing solution composed of Tween 20 (0.05%) and NaCl (0.3M) and further three washings with ultrapure water, then the TiO_2-labelled HSA (200 µl, 20 µg ml^{-1}) were allowed to react for additional 30 min, followed by three washing with water. Colour development and data analysis was carried out as above described. The limit of detection (LOD) and the dynamic range were evaluated as the HSA concentration that inhibits the maximum signal at 90%, and between the 80 and 20%, respectively [21]. Within- and between-day precision was calculated by repeating HSA measurement in four replicates within the same day and on three different days for samples 7, 8, and 9, which concentrations corresponded to low (21 mg l^{-1}), medium (253 mg l^{-1}) and high (1223 mg l^{-1}) HSA levels.

Analytical parameters for the reference method for measuring urinary albumin

The reference method for quantitatively measure HSA content in human urine was the Multigent Microalbumin assay (Abbott, Abbot Park, IL, USA), carried out on an Architect Ci8200® analyzer from the same producer. This is a turbidimetric immunoassay that uses polyclonal antibodies against human albumin. The reportable range of the assay (defined as the interval in which the mean results of recovery experiments were within 10% of the theoretical concentration) is 5-500 mg l^{-1}. The limit of detection of the assay is 1 mg l^{-1} and the precision (measured as the coefficient of variation at two levels of microalbumin controls for the within run, between run and between days reproducibility) was below 5%. According to manufacturer instructions, samples with urine albumin content above 500 mg l^{-1} were re-tested after proper dilution to comply with assay specifications.

Figures

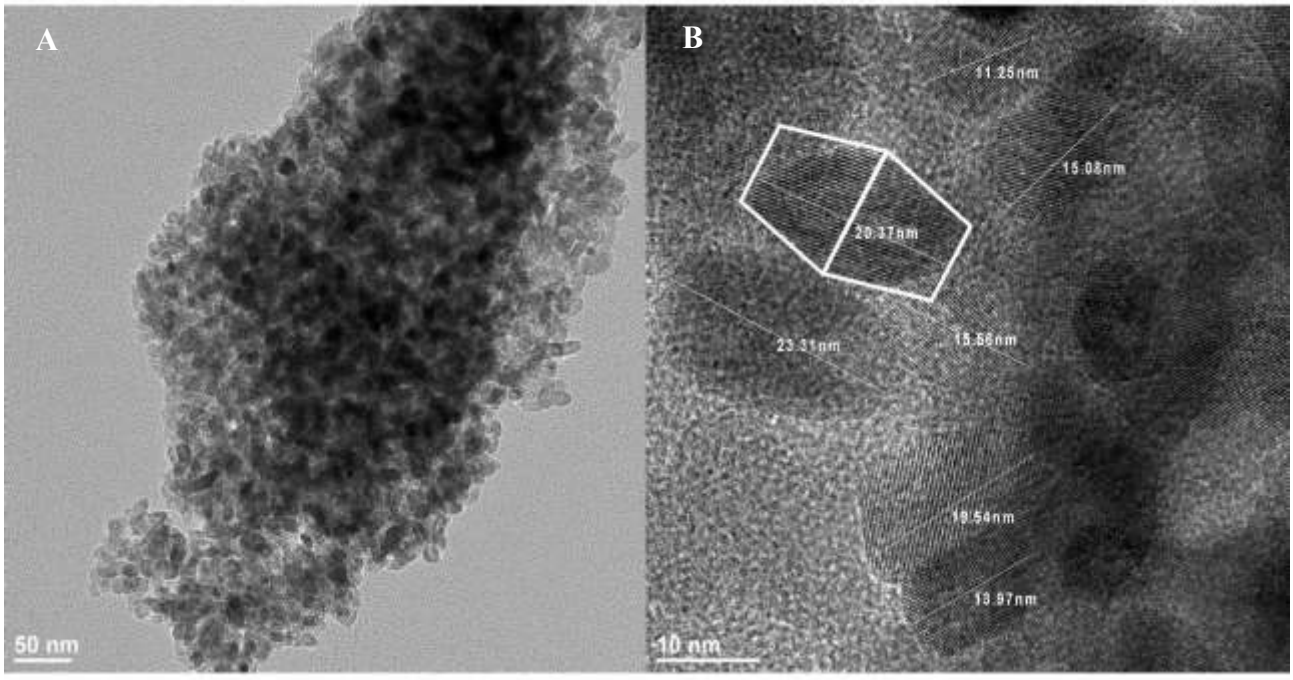


Figure S1 TEM images of the TiO_2 nanoparticles: the low magnification image shows a homogeneous sample (A); the high magnification image (B) shows particles shapes, both of truncated bipyramids and ellipsoidal.

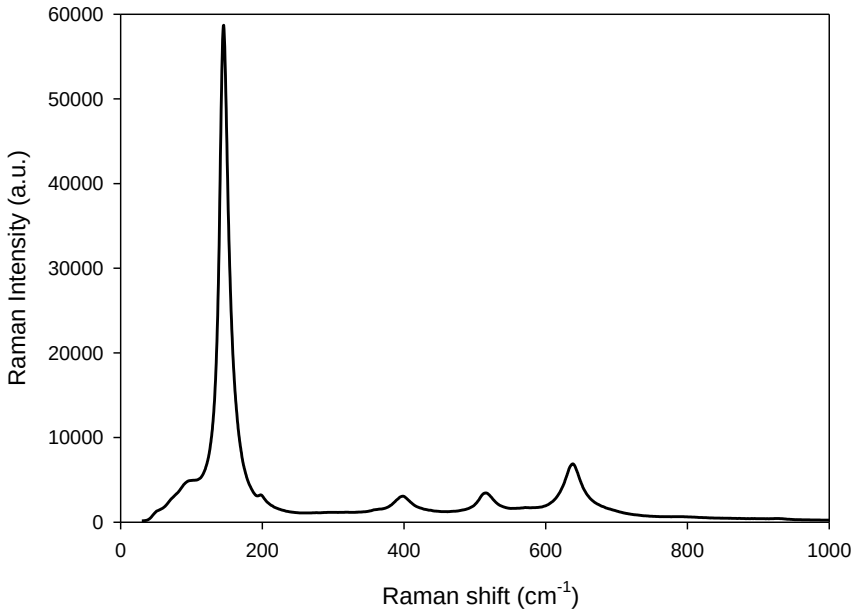


Figure S2 Raman spectrum of the TiO_2 nanoparticles. The crystalline phase was attributed to anatase, based on signals at 197 cm^{-1}, 639 cm^{-1} 399 cm^{-1}, and at 519 cm^{-1}

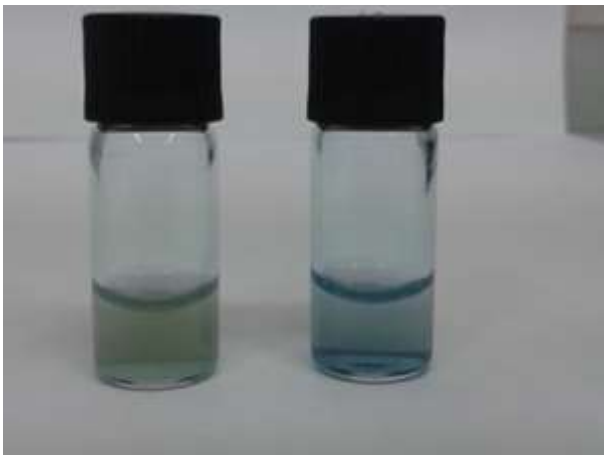


Figure S3 Functionalized TiO_2 nanoparticles before (left) and after covalent linking to BSA (right), both stained with Bradford reagent. The blue color of the suspension containing BSA-TiO_2 confirmed successful conjugation of the protein.

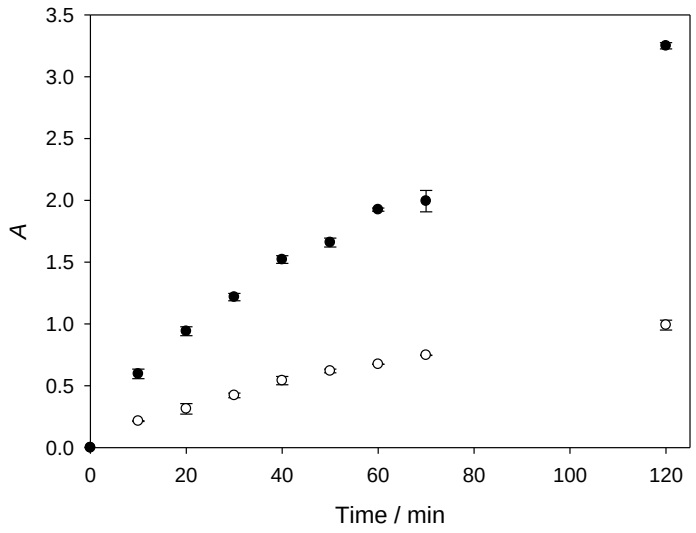


Figure S4 TMB oxidation by BSA-TiO_2 as a function of irradiation time (●, solid circle), measured by UV-vis spectroscopy (absorption at 450 nm). Auto-oxidation under UV irradiation of TMB in the absence of the catalyst was also evaluated (○, open circle).

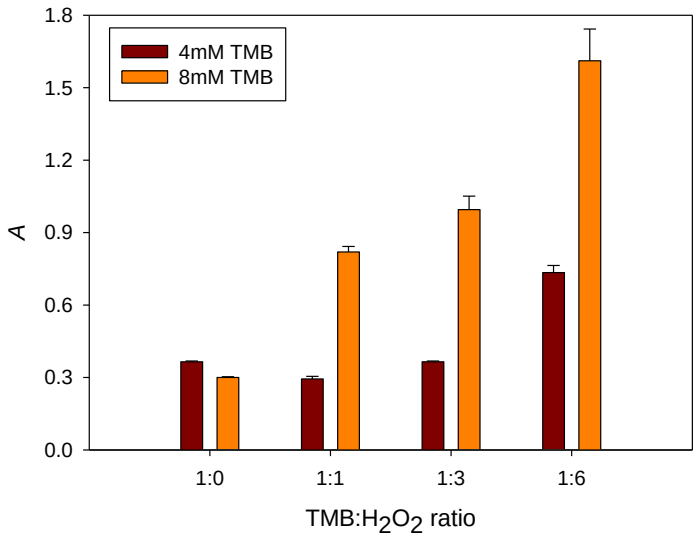
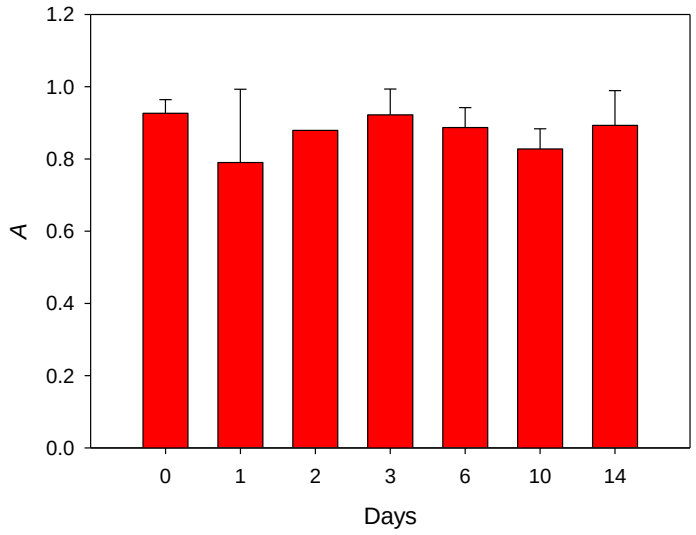


Figure S5 Catalytic activity of BSA-TiO_2 probe as a function of TMB concentration (4 and 8 mM) and TMB:H_2O_2 ratio (from no H_2O_2 to 6 times as much H_2O_2 as TMB).

A)



B)

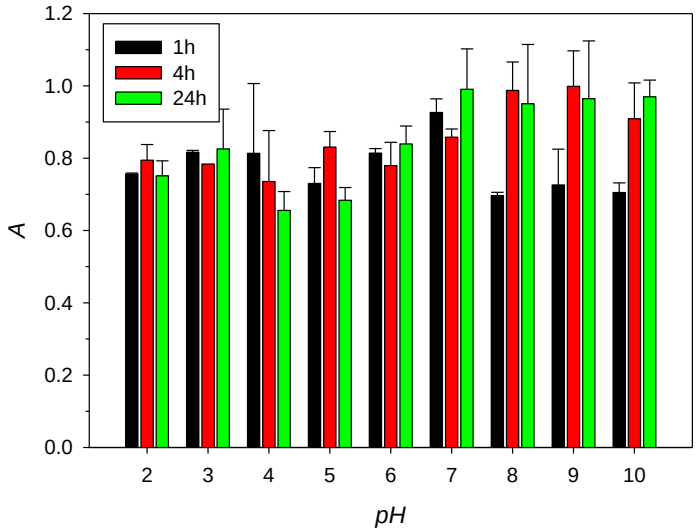


Figure S6 BSA-TiO_2 stability at room temperature (A) and as a function of pH (B). The NPs were stored in phosphate buffer for up to 14 days at room temperature and their catalytic activity was tested on different days for assessing long-term stability. The pH stability was evaluated by testing the catalytic activity after 1, 4 and 24 hours of storage in the solutions at different pHs.The concentration of the NPs was 1 mg/l in all cases. To measure the catalytic activity, the BSA-TiO_2 was mixed with the TMB/H_2O_2 mixture (8 mM / 48 mM) and irradiated for 1 hour. The developed colour was measured at 450 nm and compared to that of the fresh NPs diluted with buffer at pH 7.

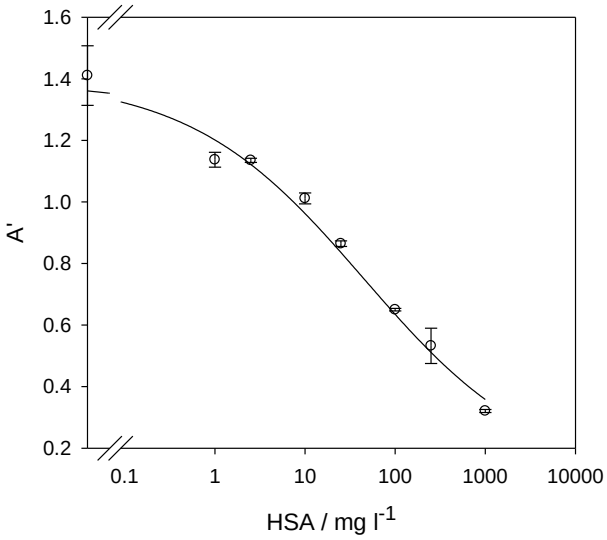


Figure S7 Typical inhibition curve for measuring HSA obtained by using the HSA-TiO_2 probe as the tracer in the direct competitive immunoassay. The curve was obtained from the competition between the analyte (HSA) and the tracer (HSA-TiO_2) for binding to anti-HSA antibodies immobilized into wells of a microtitre plate. Competition was carried out for 1 hour, followed by washings to remove unbound HSA-TiO_2. After color development, the absorbance of oxidized TMB was recorded. Photo-oxidation of TMB in the absence of the catalyst was also measured and subtracted to signals measured for each analyte concentration.

Tables

Table S1 Precision of the TiO_2-based immunoassay for measuring HSA in human urine. We tested three urine samples chosen to contain low, medium, and high HSA concentrations according to the reference method on three days.

HSA [mg l^{-1}] measured by the reference turbidimetric immunoassay [a]	HSA $\pm$ SD [mg l^{-1}] [b] measured by the TiO_2-based immunoassay		
	Day 1	Day 2	Day 3
21	17 $\pm$ 1	24 $\pm$ 1	25 $\pm$ 0.2
253	241 $\pm$ 43	223 $\pm$ 4	225 $\pm$ 8
1223	1084 $\pm$ 105	1138 $\pm$ 212	1236 $\pm$ 257

[a] Multigen Microalbuminuria assay (Abbott), CV% ≤ 5%

[b] n=4