# Supporting Information

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## SI Methods

**Synthesis of Ligand-Encoded Peptides and NWs.** Heterobifunctional ligand-encoded reporters R1–4 were synthesized by derivatizing the N terminus of glutamate-fibrinopeptide B-biotin (GluFib-biotin) (sequence, eGvndneeGffsar-biotin; lowercase, D-isomer; New England Peptide) with NHS-fluorescein (R1; Pierce), NHS-rhodamine (R3; Pierce), or NHS-Alexa Fluor 488 (R4; Invitrogen), in a 1:10 peptide:dye ratio, or were synthesized (R2; New England Peptide). Peptide structure details may be found in Fig. S2*F*. Alexa Fluor 488-PEG-biotin (R4, PEG) was synthesized by reaction of AF488 maleimide (Invitrogen) with NHS-biotin (Pierce) and $NH_2$-PEG-thiol (5 kDa; Laysan) in a 10:10:1 dye:biotin:PEG ratio and purified using illustra NAP-25 columns (GE Healthcare). The resultant conjugates were purified by HPLC (Gilson). Reporter concentration was quantified by absorbance according to dye-specific extinction coefficients in a 96-well plate by plate reader (Molecular Devices SpectraMax Plus).

Nanoworms (NWs) were formed by the reaction of iron(III) chloride hexahydrate and iron(II) chloride tetrahydrate (Sigma) with dextran ($M_r$, 15–25 kDa; Fluka) as previously described (1, 2). Mean hydrodynamic size by dynamic light scattering (Malvern Instruments Nano ZS90) was 60 nm.

Aminated NWs were reacted overnight with a 500-fold molar excess of *N*-succinimidyl iodoacetate (SIA) (Pierce) overnight at 20 °C in 50 mM sodium borate, pH 8.3, 5 mM EDTA to facilitate linkage to sulfhydryl-terminated peptides. Following purification by fast-performing liquid chromatography (FPLC) (GE Healthcare), SIA-derivatized NWs were reacted with substrate-conjugated reporters [Massachusetts Institute of Technology (MIT) Swanson Biotechnology Center, Tufts University Peptide Synthesis Core Facility, New England Peptide] and mPEG-thiol (20 kDa; Laysan) in a 1:95:20 NW:peptide:PEG ratio overnight at 20 °C in the same borate buffer. Reporter-encoded substrate-functionalized NWs were again purified and exchanged into 1× PBS by FPLC and stored at 4 °C. Substrate–reporter valency on NWs (typically 20–30) was quantified by absorbance or ELISA (described below).

**In Vitro Protease Activity Assays.** Fluorescein-functionalized matrix metalloproteinase (MMP)- or thrombin-sensitive NWs (2.5 μM by peptide) were mixed in 1% (wt/vol) BSA (Sigma) with recombinant thrombin (15 nM; Haematologic Technologies) or MMP9 (15 nM; R&D Systems) in 100-μL final volume in a 384-well plate per manufacturer's instructions, and fluorescent signal increase due to enzymatic release of homoquenched reporters was monitored at 37 °C (SpectroMax Gemini EM microplate reader). Argatroban (Sigma) or Marimastat (Tocris) were incubated with the NW–protease mixture at 100 μM final concentration. To assay proteolytic reporter release by lateral flow assay (LFA), reporter-functionalized enzyme-sensitive NWs were incubated with MMP9 or thrombin as above at 37 °C for 4 h and passed through a 30-kDa $M_r$ cutoff centrifugal filter. The filtered reporters were diluted to within LFA dynamic range and assayed by LFA as described below. Reporter stability experiments were performed using reporter 3 (1 μM) mixed in 1% (wt/vol) BSA with recombinant thrombin or MMP9 (both 15 nM) as above to 100-μL final volume and were incubated at 37 °C for 1 h. Following this, the reporters were passed through a 30-kDa $M_r$ cutoff centrifugal filter as above and assayed by R3 ELISA.

**In Vivo Imaging.** All animal studies were approved by MIT's committee on animal care (MIT protocol 0411-036-14). Synthetic biomarkers for in vivo imaging were prepared by reacting free amine groups on MMP- or thrombin-sensitive NWs (both on substrate N termini and on NWs) with VivoTag 750-NHS (Perkin-Elmer) and purified by FPLC.

Human LS174T colorectal cancer cells were grown in Dulbecco's modified Eagle's medium (ATCC) supplemented with 10% (vol/vol) FBS (Gibco) and 1% (vol/vol) penicillin-streptomycin (CellGro). Female NCr Nude mice (4–6 wk; Taconic) were inoculated s.c. with $5 \times 10^6$ LS174T cells per flank and allowed to grow to ~0.5-$cm^3$ total burden (volume = length*width*depth/2). Tumor-bearing and age-matched control mice were i.v. infused with 200 μL of VivoTag- and FAM-labeled MMP-sensitive NWs (1.67 μM by substrate), allowing visualization by an in vivo imaging system (IVIS) (Xenogen) 5–60 min postinfusion. For histology, mice were killed 1 h postinfusion. Tumors were removed, fixed in 4% paraformaldehyde, frozen in OCT (Tissue-Tek), sectioned, and stained with rat anti-CD31 (Santa Cruz), DAPI (Invitrogen), and goat anti-FAM (GeneTex) before imaging by fluorescence microscopy (Nikon Eclipse Ti).

To model thrombosis, female Swiss Webster (4–6 wk; Taconic) mice were coinfused with 200 μL of VivoTag- and FAM-labeled thrombin-sensitive NWs (0.84 μM by peptide), 10 μg/kg epinephrine (Sigma), and 280 μg/kg collagen (Chronolog). Fifteen minutes postinduction, mice were killed, and their lungs were inflated with PBS and excised. Infrared fluorescent imaging of lungs was taken using a LI-COR Odyssey infrared imager. Peptide substrates were PLGLRSW for thrombin and PLGVRGK for MMP (3).

**ELISA Characterization.** Mouse anti-fluorescein (GeneTex), rabbit anti-DNP and rabbit anti-AF488 (Invitrogen), and mouse anti-rhodamine (Rockland) antibodies were adsorbed to 96-well Bacti plates (Thermo) at concentrations of 0.4–0.8 μg/mL for 1 h in 1× PBS. Plates were then blocked for 1 h with 1× PBS with 1% (wt/vol) BSA (Sigma). Reporter standards were applied to blocked plates in twofold serial dilutions in 100-μL volume for 1 h to characterize assay linearity. To detect reporters, 100 μL of 0.4 μg/mL NeutrAvidin-HRP (Pierce) was applied for 1 h. Bound HRP was exposed with 50 μL of Ultra-TMB (Pierce) for 1–5 min followed by quenching with 50 μL of 1 M HCl. Between each step, plates were washed three times with 1× PBS with 0.5% (vol/vol) Tween 20 (Sigma). Absorbance at 450 nm was measured, plotted against known reporter concentration, and used to generate a linear fit over the assay's linear absorbance region. Assay limit of detection (LOD) was calculated as 3 SDs above mean background signal.

To test interference due to urine, urine from untreated mice was added to R1 standard at a 1:100 dilution. To quantify assay specificity, reporter concentrations at the peak of each reporter's linear region were applied to each of the four capture antibodies, and the ELISA was completed as normal. Signal for each of the four capture antibody types was quantified by comparison with a standard ladder and normalized to the maximal signal from reporters captured by their cognate antibody.

**Paper LFA Characterization.** Antibodies (same as above) were printed in lines spaced by 2 mm using 50-nL droplets at 0.5-mm pitch (Digilab MicroSys) onto HiFlow Plus cellulose ester membrane (240 s/4 cm flow rate; Millipore). Control lines were anti-streptavidin antibody (Abcam) at 0.5 mg/mL, whereas reporter capture antibodies were the same as for ELISA and were applied at 1 mg/mL (α-R1, α-R3, α-R4) or 2 mg/mL (α-R2). Cellulose membrane (Millipore) was laminated to a plastic backing. Ten-millimeter glass fiber conjugate pad (Millipore) was laminated to the sample side of the cellulose membrane, and 20-mm cellulose fiber pads were laminated to both the sample side of the conjugate pad and the run-off end of the cellulose membrane. The resultant construct was cut into 4-mm strips that were stored at 4 °C.

Twofold dilutions of marker standards in 1× PBS with 1% (wt/vol) BSA with 1:1 control urine spiked in were applied to the conjugate pad and washed with 200 μL of wash buffer [1× PBS with 1% (wt/vol) Tween 80] on the sample pad. To detect the markers, 5 μL of streptavidin-conjugated gold nanoparticles (40 nm; BBI International) were applied to the conjugate pad and washed with an additional 200 μL of wash buffer. Test strips were allowed to dry and could be visualized by eye or applied to a scaling template and scanned (600 dpi; Epson V330 Photo) or imaged by cell phone (Fig. S3*G* only; Samsung Galaxy Nexus). Resultant images were loaded into MATLAB (MathWorks) and processed by a custom script that integrated signal over background across each antibody line. Marker orthogonality was characterized by comparing reporter capture by each antibody by applying a single reporter and quantifying signal over background noise across each antibody line. All strips were performed in at least triplicate.

**Collection and Analysis of Urinary Peptides.** Mice were i.v. infused with 200 μL of PBS with R4 [AF488-PEG-biotin; thrombosis model: 0.125 μM; colorectal cancer (CRC) model: 1 μM] as an injection control and either R2-functionalized MMP-sensitive NWs (1.67 μM by peptide; tumor volume, ∼0.5 $cm^3$) or R3-functionalized thrombin-sensitive NWs (0.84 μM by peptide; thrombosis model). Immediately following infusion, mice were placed over 96-well plates enclosed by a cylindrical tube to collect urine for 30 min (thrombosis model) or 1 h (tumor model). Urine collection times were optimized from previous studies using these disease models (3, 4) and are dependent on site of disease and rate of enzymatic substrate cleavage. Urine was stored at −80 °C directly following collection.

Unprocessed urine was diluted (1:100–1:10,000) in 1× PBS with 1% (wt/vol) BSA, and reporters were quantified by ELISA (at least two replicates) using standards as described above. Urine was applied to lateral flow test strips in 5-μL volume, at 1:4 (thrombosis model) or 1:5 (CRC model) dilution. Lateral flow tests were performed in triplicate as described above and test strips were allowed to dry and were quantified by automated script as described above. ELISA and LFA data were analyzed using a Wilcoxon signed-rank test (CRC) and a Mann–Whitney test (thrombosis).

**Companion Diagnostic Cost Analyses.** Approximate costs for materials and labor costs to produce LFAs were based on estimates from a technical document by LFA materials' manufacturer Bangs Laboratories (5). The majority of costs are packaging and assembly, and the major variable costs are due to the specific antibodies used and region of manufacture, resulting in a raw material cost of roughly $0.60 and an assembled product cost of less than $2.

1. Park J-H, et al. (2008) Magnetic iron oxide nanoworms for tumor targeting and imaging. *Adv Mater* 20(9):1630–1635.
2. Park J-H, et al. (2009) Systematic surface engineering of magnetic nanoworms for in vivo tumor targeting. *Small* 5(6):694–700.
3. Kwong GA, et al. (2013) Mass-encoded synthetic biomarkers for multiplexed urinary monitoring of disease. *Nat Biotechnol* 31(1):63–70.
4. Lin KY, Kwong GA, Warren AD, Wood DK, Bhatia SN (2013) Nanoparticles that sense thrombin activity as synthetic urinary biomarkers of thrombosis. *ACS Nano* 7(10): 9001–9009.
5. Bangs Laboratories (1999) TechNote 303 (Bangs Laboratories, Inc., Fishers, IN). Available at http://www.bangslabs.com/sites/default/files/bangs/docs/pdf/303.pdf. Accessed February 2, 2014.

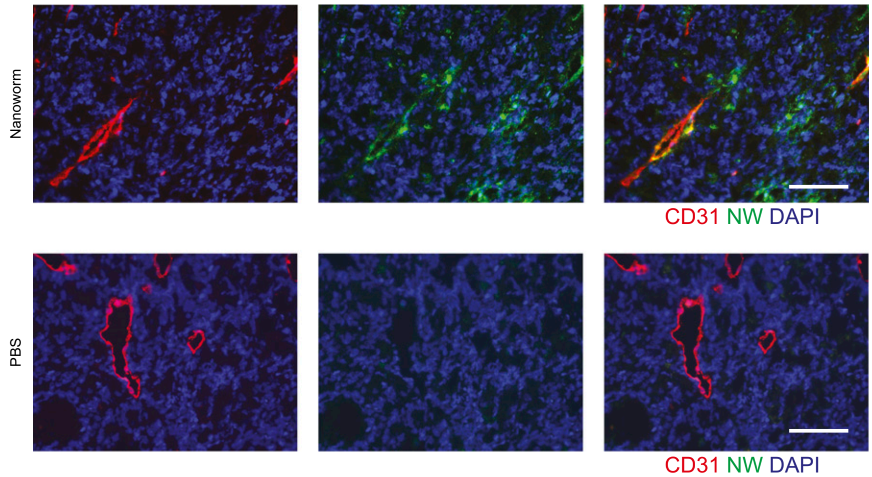
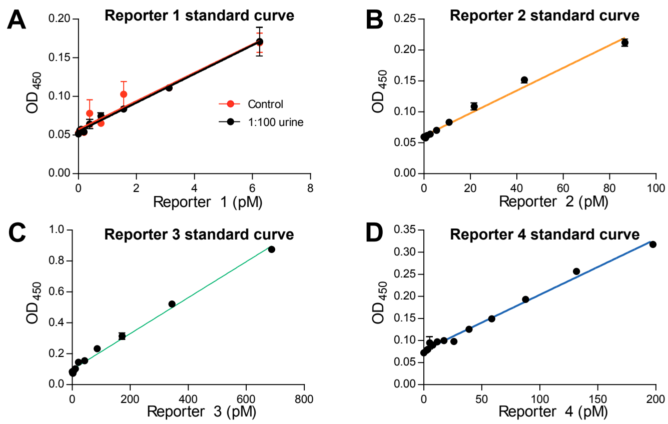
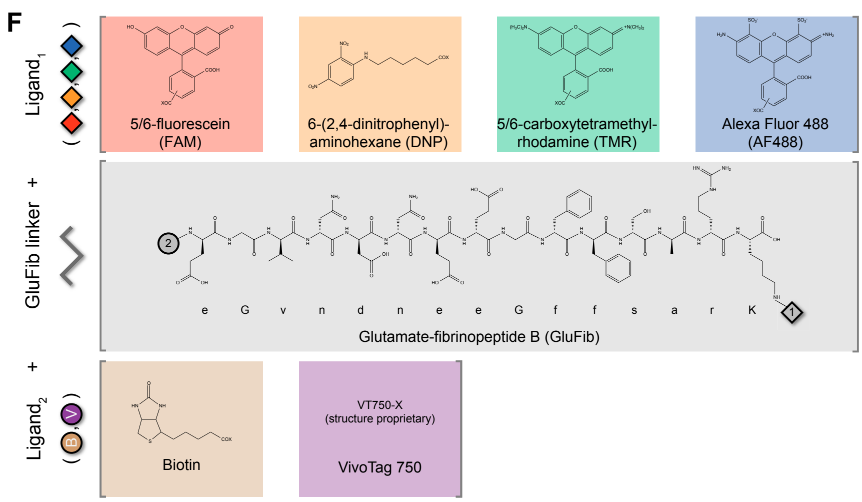



**Fig. S1.** Model system validation. Immunohistochemical staining of LS147T flank tumors 1 h postinjection of FAM-labeled NWs (*Upper*) or PBS (*Lower*). Staining of endothelial cells (CD31; red) indicates both colocalization of injected NWs (green) with blood vessels as well as extravasation of NWs into the tumor interstitium. Nuclei are counterstained blue with DAPI. (Scale bar: 100 μm.)




**E**

| Reporter | Reporter structure | Linear range (pM) | Slope | Intercept | $R^2$ |
|---|---|---|---|---|---|
| 1 | FAM-GluFib-Biotin | 0.09 - 6.25 | 0.01832 +/- 0.001611 | 0.05723 +/- 0.003875 | 0.9486 |
| 2 | DNP-GluFib-Biotin | 7.35 - 87 | 0.001832 +/- 0.00007243 | 0.06115 +/- 0.002290 | 0.9877 |
| 3 | TMR-GluFib-Biotin | 54.5 - 700 | 0.001163 +/- 0.00003436 | 0.09762 +/- 0.008225 | 0.9922 |
| 4 | AF488-GluFib-Biotin | 23 - 200 | 0.001259 +/- 0.00003604 | 0.07760 +/- 0.002649 | 0.9911 |



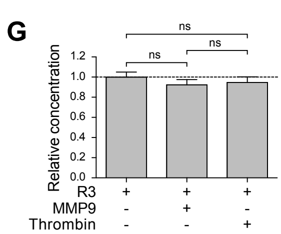
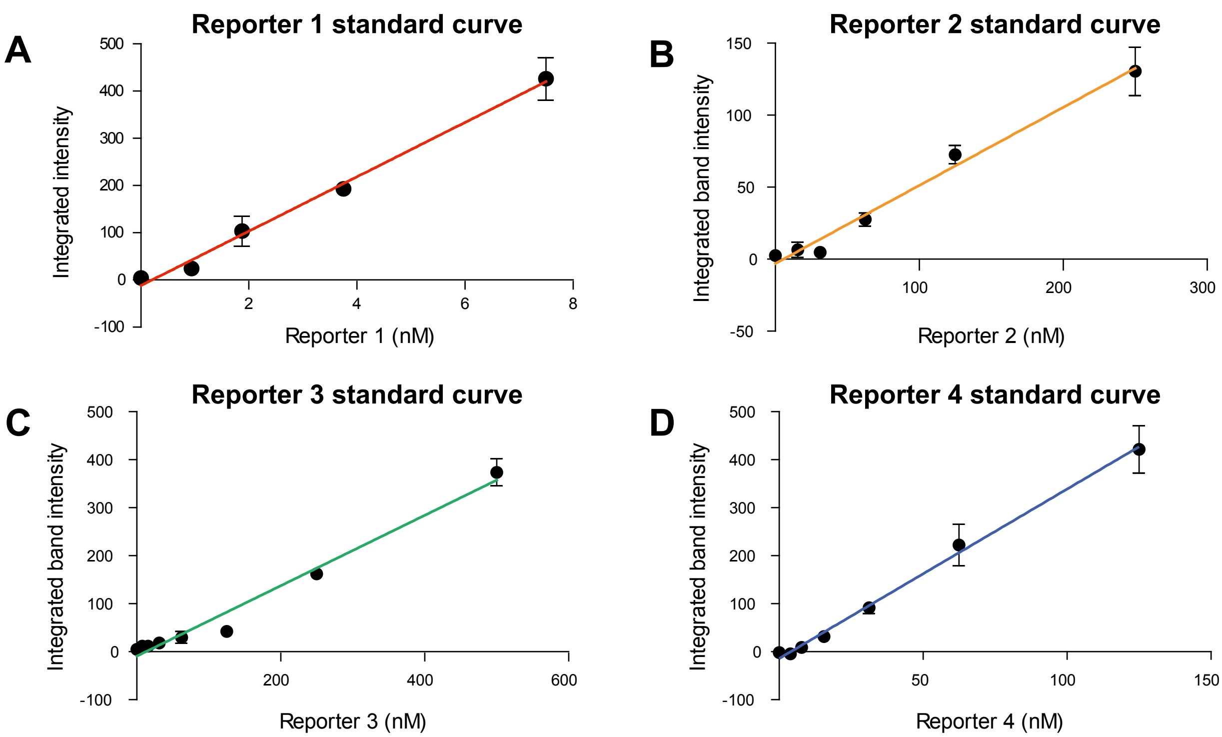




**Fig. S2.** Conventional sandwich ELISA validation. (*A*) Standard curve for reporter 1 (R1) diluted in 1:100 urine (black) or PBS (red). (*B–D*) Standard curves for R2–R4 diluted in PBS. (*E*) Analyses of linear regions of R1–4 standard curves. (*F*) Ligand-encoded reporter structures. All reporters were comprised of heterobifunctionalized peptides with the structure biotin-eGvndneeGffsarK(ligand$_2$) in which ligand$_2$ comprised one of four capture ligands (fluorescein, dinitrophenyl, tetramethylrhodamine, or Alexa Fluor 488). We chose the D-stereoisomer peptide glutamate-fibrinopeptide B (eGvndneeGffsar) as a linker because it is biologically inert, stable, and efficiently filters into the urine (1, 2). Near-infrared reporters used for in vivo biodistribution studies (Fig. 2 *C–E*) made use of a modified reporter with VivoTag 750 substituted for biotin [VT750-eGvndneeGffsarK(FAM)]. (*G*) Stability of reporters to protease activity was assayed by exposing reporter 3 to MMP9 or thrombin for 1 h at 37 °C. ELISA quantification of recovered reporter demonstrated no significant differences between controls and enzyme-exposed reporters. Limit of detection (LOD) is defined as 3 SDs above mean background. Error bars are SEM. Means were compared using a nonparametric multiple-comparisons ANOVA (Kruskal–Wallis).

1. Kwong GA, et al. (2013) Mass-encoded synthetic biomarkers for multiplexed urinary monitoring of disease. *Nat Biotechnol* 31(1):63–70.
2. Morris TA, et al. (2003) Urine and plasma levels of fibrinopeptide B in patients with deep vein thrombosis and pulmonary embolism. *Thromb Res* 110(2-3):159–165.




**E**

| Reporter | Reporter structure | Linear range (nM) | Slope | Intercept | $R^2$ |
|---|---|---|---|---|---|
| 1 | FAM-GluFib-Biotin | 0.8423 - 7.5 | 57.59 +/- 2.750 | -11.92 +/- 10.63 | 0.9932 |
| 2 | DNP-GluFib-Biotin | 27.54 - 250 | 0.5423 +/- 0.03311 | -3.031 +/- 3.900 | 0.9853 |
| 3 | TMR-GluFib-Biotin | 44.92 - 500 | 0.7336 +/- 0.04559 | -9.186 +/- 9.307 | 0.9773 |
| 4 | AF488-GluFib-Biotin | 12.45 - 125 | 3.530 +/- 0.09818 | -14.12 +/- 5.355 | 0.9961 |

**F**

| Material | Cost ($) |
|---|---|
| Antibodies | 0.200 |
| Membranes | 0.020 |
| Absorbent pad | 0.001 |
| Sample pad | 0.002 |
| Streptavidin gold NPs | 0.050 |
| Membrane backing | 0.010 |
| Desiccant | 0.020 |
| Pouch | 0.080 |
| Plastic housing | 0.200 |
| **Raw material cost** | **0.583** |
| Labor costs | 1.166 |
| **Assembled product cost** | **1.749** |

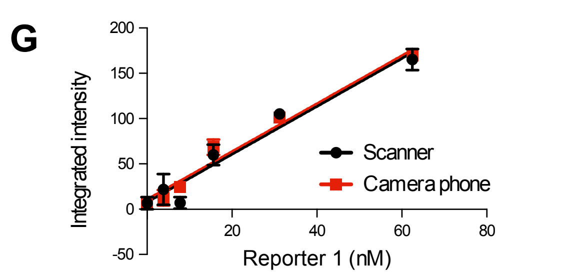



**Fig. S3.** Paper LFA validation. (*A*–*D*) Standard curves for detection of R1–4 by paper LFA. Increasing dilutions of each reporter spiked 1:1 in urine were applied to each LFA, and test strips were imaged using a flatbed scanner and analyzed by an automated MATLAB script. (*E*) Statistics for linear regions of R1–4 standard curves. Limit of detection (LOD) is defined as 3 SDs above mean background signal. (*F*) Approximate costs for materials and labor costs to produce LFAs based on estimates from a technical document by LFA materials' manufacturer Bangs Laboratories (1). The majority of costs are packaging and assembly, and the major variable costs are due to the specific antibodies used and region of manufacture. (*G*) The same automated analysis script was modified to quantify LFA band intensity using a 5-megapixel cellular phone camera (Samsung Galaxy Nexus). Analyses of decreasing concentrations of R1 on paper LFA performed using images from a scanner (black) or camera phone (red) produced indistinguishable results. Error bars are SEM.

1. Bangs Laboratories (1999) TechNote 303 (Bangs Laboratories, Inc., Fishers, IN). Available at http://www.bangslabs.com/sites/default/files/bangs/docs/pdf/303.pdf. Accessed February 2, 2014.

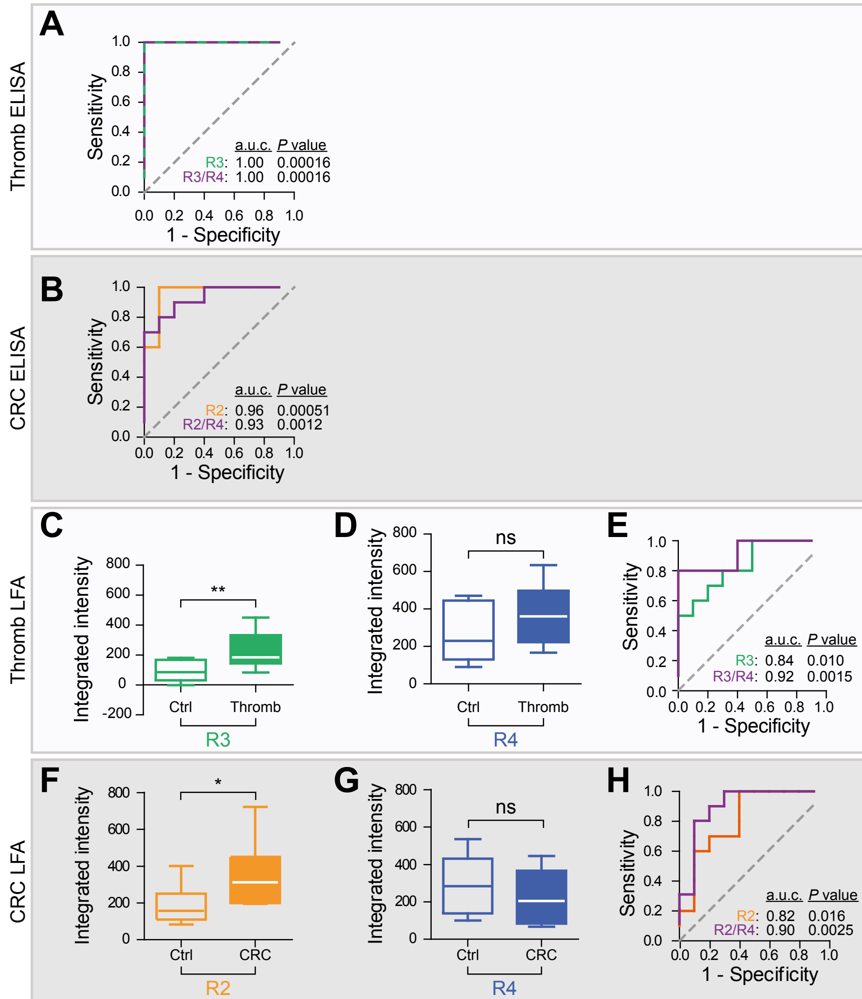



**Fig. S4.** Conventional ELISA and paper-based detection of synthetic urinary biomarkers. (*A*) ROC curves for urinary clearance of synthetic biomarkers in our murine model of platelet-mediated thrombosis, as detected by ELISA. We coinjected mice ($n = 10$) with R3-encoded thrombin-sensitive NWs, free R4, and either PBS or collagen/epinephrine and collected urine after 30 min. Both R3 (green, $P = 0.00016$) and the ratio R3/R4 (purple, $P = 0.00016$) demonstrated perfect discrimination (a.u.c. = 1) of diseased from healthy animals by urine ELISA. (*B*) Receiver-operating characteristic (ROC) curves for urinary clearance of synthetic biomarkers in a flank tumor model of colorectal cancer. Urinary concentration of liberated R3 from MMP-sensitive NWs by ELISA discriminated disease with an a.u.c. of 0.96 ($P = 0.00051$). Normalization of liberated R2 to free R4 resulted in an a.u.c. of 0.93 ($P = 0.0012$). (*C*) Upon LFA interrogation of urine from mice with or without thrombosis, integrated intensity of R3 capture was higher in animals with thrombosis compared with controls ($P = 0.0089$). (*D*) LFA band intensity of urinary free reporter R4 was not significantly different between control and thrombosis cohorts ($P = 0.12$). (*E*) ROC curve analysis of discriminatory ability of our synthetic biomarkers detected in urine by LFA. Cleaved reporter R3 discriminated with an a.u.c. of 0.84 ($P = 0.010$). Normalization of liberated R3 to free reporter R4 achieved an a.u.c. of 0.92 ($P = 0.0015$). (*F*) LFA analysis of urine from the CRC model demonstrated increased urinary concentration of cleaved reporter R2 ($P = 0.027$) in diseased animals versus controls, but no significant difference in urinary concentration of free reporter R4 ($P = 0.16$, *G*) as expected from ELISA analysis of the same urine cohort. (*H*) ROC curve analysis of LFA quantification demonstrated an improved ability of urinary R2 (a.u.c. = 0.82, $P = 0.016$) and the ratio R3/R4 (a.u.c. = 0.90, $P = 0.0025$) to discriminate control from diseased animals compared with a random classifier. Box-and-whisker plots show extremes, quartiles, and median.