SUPPORTING INFORMATION

Disease Detection by Ultrasensitive Quantification of Microdosed Synthetic Urinary Biomarkers

Andrew D. Warren,[†,‡] Shonda T. Gaylord,[§,‡] Kevin C. Ngan,[§] Milena Dumont Milutinovic,[§] Gabriel A. Kwong,[†] Sangeeta N. Bhatia,[†,∥,*] and David R. Walt[§,*]

[†]Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Building 76-453, Cambridge, Massachusetts 02139, United States

[§]Department of Chemistry, Tufts University, 62 Talbot Avenue, Medford, Massachusetts 02155, United States

[∥]Institute for Medical Engineering & Science at MIT, Department of Electrical Engineering & Computer Science, David H. Koch Institute at MIT, and the Howard Hughes Medical Institute, 77 Massachusetts Avenue, Building 76-453, Cambridge, Massachusetts 02139, United States. Division of Medicine, Brigham and Women's Hospital, Boston, Massachusetts 02155, United States.

Text S1

Reporter/peptide synthesis. Thrombin-sensitive reporter 1 (R1) was synthesized by CPC Scientific, with the sequence Biotin-PEG_{5kDa}-Lys(5FAM)-Gly-Gly-DPhe-Pro-Arg-Ser-Gly-Gly-Gly-Cys, where PEG_{5kDa} is 5 kDa poly(ethylene glycol). Biotin-PEG_{5kDa}-Lys(5FAM) serves as a ligand-encoded reporter (R1) that is released upon proteolytic cleavage of the peptide substrate. Standard (non-protease-sensitive) ligand-encoded reporters R1-2 were synthesized by derivatizing biotin-poly(ethylene glycol) 5 kDa-amine (Laysan) with NHS-fluorescein (R1; Sigma) or NHS-Alexa Fluor 488 (R2; Invitrogen) and were purified by illustra NAP-25 Sephadex columns (GE Healthcare). Reporters were quantified by extinction coefficient (Molecular Devices SpectraMAX Plus).

Protease-sensitive R1-NW synthesis. Dextran-crosslinked nanoworms (NWs) were synthesized as previously described.[1] Briefly, NWs were formed by the reaction of Fe(III) chloride hexahydrate and Fe(II) chloride tetrahydrate (both Sigma) with dextran (M_r 15-25 kDa; Fluka) and were aminated by treatment with ammonia. Mean hydrodynamic size by dynamic light scattering (Malvern Instruments Nano ZS90) was 60 nm.

Aminated NWs (approximately 115 kDa each) were activated with N-succinimidyl iodoacetate (SIA; Pierce) in a 1:500 (NW:SIA) molar excess for 1 hour in 50 mM borate, 5 mM ethylenediaminetetraacetic acid (EDTA) buffer, pH 8.3. Activated NWs were purified from excess SIA by fast protein liquid chromatography (FPLC; GE Healthcare) and reacted at a 1:95:20 (NW:peptide:mPEG_{20kDa}-SH) ratio with sulfhydryl-terminated protease-sensitive peptide reporters (thrombin-sensitive R1 is terminated with a cysteine) and mPEG_{20kDa}-SH (Laysan) for 1 hour in the same buffer. Remaining free succinimidyl groups were quenched by the addition of cysteine (Sigma) and protease-sensitive R1-NWs were purified by FPLC into 1x phosphate buffered saline (PBS; Thermo Scientific). Protease-sensitive peptide valency on NWs was quantified by extinction coefficient (Molecular Devices SpectraMAX Plus) as 50-70 per NW. R1-NW concentrations refer to the concentration of thrombin-sensitive R1-substrate peptides on the surface of NWs.

Single molecule array (SiMoA) assay

Preparation of magnetic beads coated with capture antibody. Carboxylic acid functionalized paramagnetic beads (2.7-µm in diameter; Agilent Technologies) were covalently coupled with capture antibody (same as for ELISA) using an in-house 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC; Thermo Scientific) coupling protocol. Briefly, beads (100 µL) were washed prior to antibody addition with PBS containing 1%

Tween 20 (Sigma) (wash buffer) and 2-(N-morpholino)ethanesulfonic acid (MES; Thermo Scientific) buffer.

100 µg of either αR1 or αR2 were incubated with beads for 15 min. 1 mL of MES buffer and 100 µL of EDC (10

mg/mL) were added to the bead-antibody solution and incubated for an additional 30 min. After incubation, S2

beads were washed with 1200 µL of wash buffer. A second 40 minute incubation with PBS containing 1% wt/vol BSA was performed to cap any remaining active surface groups. The beads were then washed three times with wash buffer and stored in 200 µL of bead storage buffer.

Formation of reporter-enzyme complex on beads. Serial dilutions of target nanoworms or clearance marker were prepared in 1% urine solutions to generate standard curves. Experimental and control urine containing cleaved nanoworms and clearance marker were diluted 100x in PBS buffer containing 25% FBS (blocking buffer). Each sample was incubated for 2 h in the presence of approximately 200,000 beads. Beads were washed six times with 7.5x PBS containing 0.1% Tween 20 (wash buffer). The bead-protein complex was then incubated with 20 pM of streptavidin-β-galactosidase (SβG; Quanterix) for 30 minutes. Beads were then washed 12 times with wash buffer, once with sucrose buffer and re-suspended in 10 µL of sucrose buffer for loading onto the fiber optic arrays (SCHOTT North America) containing ~50,000 46-fL reaction wells.[2] Cross reactivity experiments were performed as detailed above with 1% vol/vol urine in blocking buffer in the presence of non-target analyte (αR1 beads with R2 or αR2 beads with R1).

Preparation of fiber optic arrays for imaging. Preparation of fiber optic arrays has been previously described.[2] Briefly, optical fiber bundles were polished using 3- and 1-µm diamond lapping films (Allied High Tech Products). To generate wells 4.5 µm in diameter and ~3.25 µm in depth, fibers were etched with 0.025 M hydrochloric acid for 130 seconds, sonicated in water for 10 seconds and dipped in ethanol. To contain beads during loading, etched fibers were wrapped with PVC tubing. Fibers were then loaded with 10 µL of beads and centrifuged at 10,000 x g for 5 min to trap individual beads into wells. Imaging and analysis using a custom-built imaging system has been previously described using fluorescent substrate resorufin-β-D-galactopyranoside (RDG; Life Technologies) and a custom imaging apparatus.[2, 3]

Concentration determination for nanoworm and clearance marker in mice. Urine samples from control or thrombosis-induced mice were diluted 1:10^2 in blocking buffer resulting in a 1% vol/vol urine solution. Capture of R1 and R2 was performed as described above. The equation generated from the linear regression from the standard curves was used to extrapolate the concentration of R1 and R2 present in each sample. Each value was multiplied by 100 to determine the total concentration.

Inulin and R3 plasma and renal kinetics. To compare renal clearance of our reporters to the clinical standard for GFR measurement (fractionated inulin, a 5-10 kDa polysaccharide), we redesigned a third reporter (R3; PEG_{5kDa}-VT750) that is kinetically similar to R1-2, unaffected by proteases, and may be detected from plasma by near-infrared fluorescence. Inulin (10 mg/kg; BioPAL) and R3 (5 µM) were coinjected into female Swiss

Webster mice (n=7) and plasma concentration of each was quantified at approximately 5 min intervals for 60 min. At 60 min, urine was collected. Inulin was quantified by competitive inulin ELISA (BioPAL) and R3 was quantified by near-infrared fluorescence (LI-COR Odyssey). Plasma half-life was calculated using a single exponential decay fit on all lumped data points and renal concentration factor was calculated as urine concentration normalized to peak plasma concentration. Statistical tests were paired Student's *t* test.

Thromboplastin-induced model of venous thrombosis. Thrombin-sensitive R1-NWs and renal clearance control R2 were IV coinjected in equimolar amounts at 0.2-200 pmol in 200 µL PBS into female Swiss Webster mice (n=5-10 per concentration) and all urine 0-30 min post-injection was collected to obtain control clearance values. 3-5 days later (to allow synthetic biomarker clearance), mice were again coinjected with R1-NWs and R2 along with 20 U/g body weight thromboplastin (Biopharm Laboratories) to induce intravascular coagulation as previously described[4] and all urine 0-30 min post-injection was collected. R1 and R2 concentrations were quantified by ELISA (20-200 pmol) or SiMoA (0.2-2 pmol). This model of pulmonary embolism initiated by the extrinsic clotting cascade has been characterized in the hematology literature by our group and others to produce a rapid formation of blood clots that embolize to the lungs to recapitulate this life-threatening condition.[4] This model has been used to characterize the roles played by different vascular receptors in thrombosis development and to understand the function of new antithrombotic agents. To ensure accurate use of this model, we have used injected doses of thromboplastin similar to these studies and consequently expect similar physiological levels of clotting.

Mouse-human scaling and microdose calculations.

Microdosing calculations. Though interspecies protease kinetics are similar, blood volume and renal clearance differences should be considered to obtain a reasonable microdose estimate. Blood volume varies approximately linearly with weight;[5] an average 62 kg human[6] therefore has approximately 2200 times the blood volume of the average 28 g mouse used in this study. Our synthetic biomarker injection has an adjusted molecular weight of 19.7 kDa (see below for calculations). Adjusting for blood volume, a 100 µg microdose in humans is approximately 2.3 pmol for mice (100 µg / 19.7 kDa / 2200 blood volume difference). Therefore, our synthetic biomarker doses of 0.2-2 pmol are beneath the approximate microdose threshold for humans. Here, consideration of renal clearance differences (~16x slower GFR in humans as quantified by inulinn clearance[7]) is neglected: decreased rate of reporter reporter clearance results in a less than 4x difference in urine concentration after 30 min, which may be easily resolved by the SiMoA assay sensitivity. Minor interspecies

variation in clotting cascade regulation and function are also ignored, as key hemostatic coagulation metrics are very similar between mouse and human (Table S1).

Molecular weight calculations. The nanoworms used in these studies have an average molecular weight of 115 kDa. During synthesis, nanoworms were reacted with a 20-fold excess of 20 kDa PEG-SH (total MW 400 kDa), and average valency of 6.6 kDa peptide-reporter per nanoworm was measured to be approximately 70. Therefore, the total molecular weight of a thrombin-responsive R1-NW was approximately 975 kDa (115 + 20*20 + 6.6*70). As we considered concentration by functional substrate available, this is an effective molecular weight of ~14 kDa per peptide-reporter. Adding the molecular weight contribution of free R2 (approximately 5.8 kDa), the adjusted molecular weight per reporter is 19.7 kDa.

Supplementary Figures

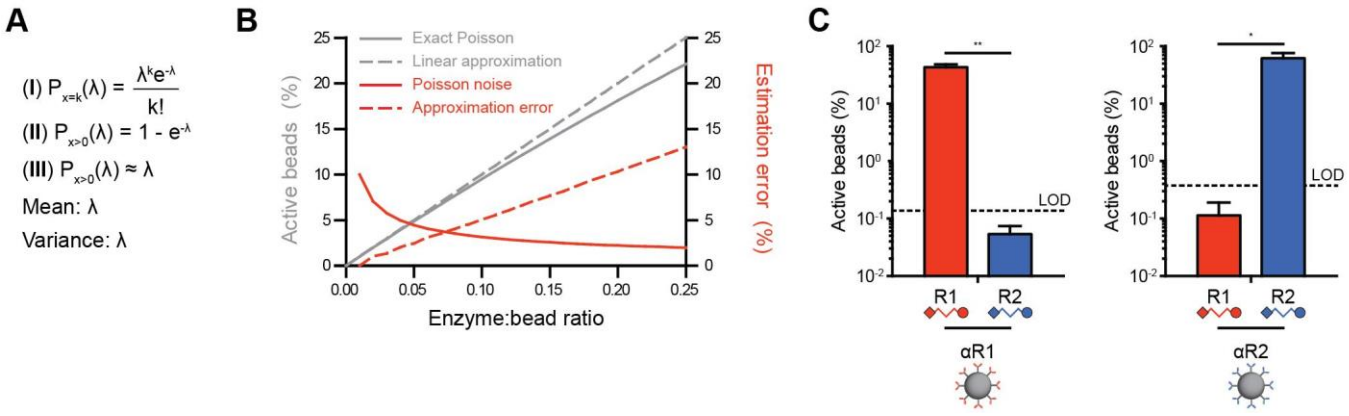

Figure S1. (A) The Poisson probability distribution function (I) describes the frequency at which *x* events occur with an average of λ. In our digital ELISA system, we determine the probability of a particular well being "on" – that is, having one or more active enzymes. For all λ (ratio of enzymes to beads), this may be expressed as (II). A first order Taylor series approximation of (II) around λ=0 demonstrates that the probability of a bead being active is linear with the enzyme:bead ratio (III) for small λ. (B) Our SiMoA assay enables concentration quantification by counting the proportion of rare events. Even with perfect reporter capture efficiency, assay sensitivity is limited by Poisson noise ("shot noise" due to distribution variance $\sigma^2=\lambda$) more than approximation error. (C) Assay specificity was quantified using saturating concentrations of R1 and R2 on either αR1 (*Left*; *P*=0.0051) or αR2 (*Right*; *P*=0.018) capture beads. Here, capture efficiency of non-cognate reporters (e.g., R1 by αR2) was below the limit of detection (LOD; defined as $\mu_{BG}+3\sigma_{BG}$) of each assay.

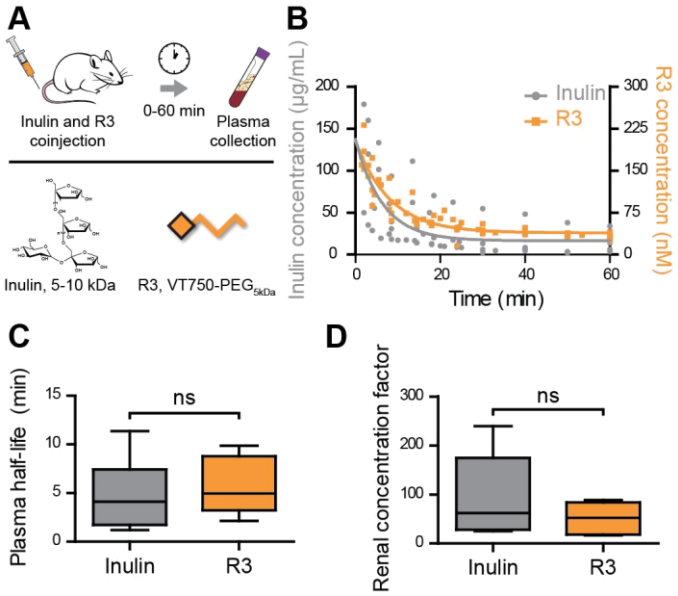

Figure S2. (A) Inulin is a 5-10 kDa polysaccharide used in the clinic to assess glomerular filtration rate (GFR) as it is small, stable, and not actively handled by the nephron. To compare pharmacokinetics of our reporters to inulin, we coinjected inulin and a near-infrared fluorescent derivative of our reporters (R3; VivoTag750-PEG_{5kDa}) in female Swiss Webster mice and observed plasma kinetics and renal handling. (B) Analysis of plasma concentration of inulin and R3 over the course of 1 hour (n=7; timepoints taken approximately every 5 min) demonstrated a first-order decay process. (C) Blood half-lives for both compounds using these same data points were statistically indistinguishable (P=0.57). (D) Renal concentration factor (urine concentration after 1h normalized to peak plasma concentration) was also not significantly different between gold standard inulin and our reporter R3, suggesting rapid renal clearance and a lack of active handling by the nephron (P=0.17). Statistical tests were paired Student's t test.

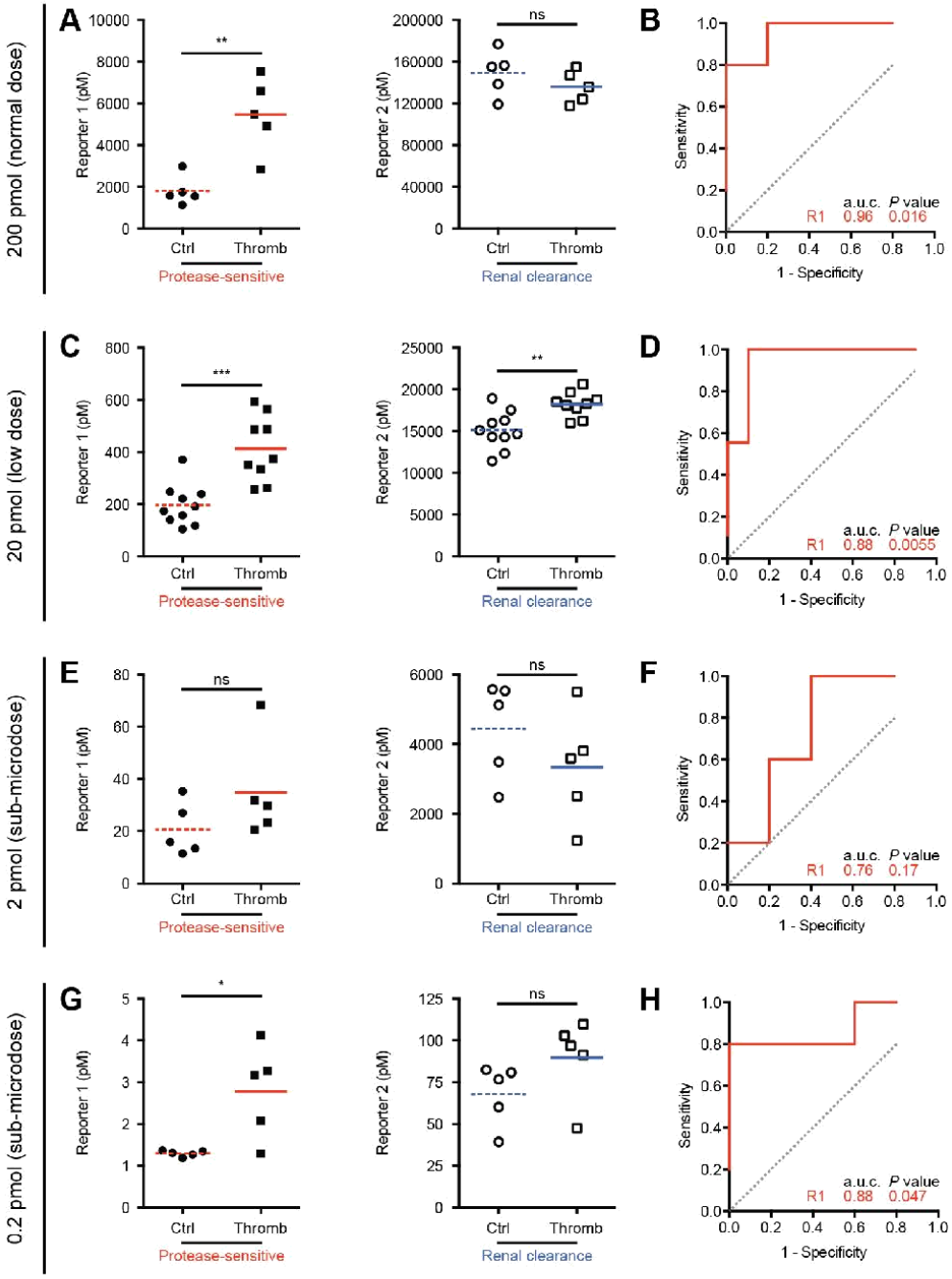

Figure S3. 0.2-200 pmol thrombin-sensitive NW-R1 and renal clearance control R2 were coinjected in female Swiss Webster mice (n=5-10) with either thromboplastin (to induce intravascular coagulation) or PBS. (A,C,E,G) At 200, 20, and 0.2 pmol injected doses, disease resulted in higher renal clearance of thrombin-sensitive R1 as detected by ELISA or SiMoA (*Left*; *P* values are 0.0027, 0.0003, 0.1837, 0.017 from high to low injected dose), while R2 remained relatively constant with fold changes between groups of 0.75-1.32 (*Right*; *P*

values are 0.30, 0.0025, 0.27, 0.15 from high to low injected dose). (B,D,F,H) Receiver-operating characteristic (ROC) curves for each injected dose indicate the diagnostic ability of R1 concentration in urine.

Hemostatic coagulation index	**Human**[8] (healthy clinical range)	**Male Swiss Webster mice**[9] (mean ± SD)	**Female CD-1 mice**[10] (mean ± SEM)	**Female CD-1 mice**[11] (interquartile range)
Prothrombin time (PT; s)	11.5 – 13.5	10.2 ± 1.0	11.9 ± 0.30	14.0 – 14.6
International normalized ratio (INR; unitless)	0.8 – 1.4	0.88 ± 0.09	Not given	Not given
Activated partial thromboplastin time (aPTT; s)	27 – 38	19.4 ± 1.4	22.5 ± 0.60	33.1 – 37.2
Thrombin time (TT; s)	10 – 14	16.6 ± 1.5	12.8 ± 0.40	22.0 – 25.5
Fibrinogen (mg/dL)	123 – 370	266 ± 54	188 ± 6.4	Not given

Table S1. Overview of standard values for five hemostatic coagulation indices regularly used in the clinic. Overall, the clotting cascade is highly conserved between mice and humans in terms of both sequence homology and functional clotting characteristics. Our pulmonary embolism model uses thromboplastin to trigger the extrinsic clotting cascade via the complexation of factor VII and tissue factor; consequently, prothrombin time (PT; a measure of extrinsic clotting cascade function) is the most relevant parameter to consider between organisms. A survey of three studies that quantify PT in healthy mice report values similar to those expected in healthy humans, indicating no large interspecies differences in relevant measures of clotting function.[8-11]

References

1. Park, J. H.; von Maltzahn, G.; Zhang, L.; Schwartz, M. P.; Ruoslahti, E.; Bhatia, S. N.; Sailor, M. J., *Adv Mater* **2008,** *20* (9), 1630-1635; Park, J. H.; von Maltzahn, G.; Zhang, L.; Derfus, A. M.; Simberg, D.; Harris, T. J.; Ruoslahti, E.; Bhatia, S. N.; Sailor, M. J., *Small* **2009,** *5* (6), 694-700.
2. Rissin, D. M.; Kan, C. W.; Campbell, T. G.; Howes, S. C.; Fournier, D. R.; Song, L.; Piech, T.; Patel, P. P.; Chang, L.; Rivnak, A. J.; Ferrell, E. P.; Randall, J. D.; Provuncher, G. K.; Walt, D. R.; Duffy, D. C., *Nat Biotechnol* **2010,** *28* (6), 595-9.
3. Rissin, D. M.; Fournier, D. R.; Piech, T.; Kan, C. W.; Campbell, T. G.; Song, L.; Chang, L.; Rivnak, A. J.; Patel, P. P.; Provuncher, G. K.; Ferrell, E. P.; Howes, S. C.; Pink, B. A.; Minnehan, K. A.; Wilson, D. H.; Duffy, D. C., *Anal Chem* **2011,** *83* (6), 2279-85.
4. Lin, K. Y.; Kwong, G. A.; Warren, A. D.; Wood, D. K.; Bhatia, S. N., *ACS Nano* **2013,** *7* (10), 9001-9; Smyth, S. S.; Reis, E. D.; Väänänen, H.; Zhang, W.; Coller, B. S., *Blood* **2001,** *98* (4), 1055-62; Weiss, E. J.; Hamilton, J. R.; Lease, K. E.; Coughlin, S. R., *Blood* **2002,** *100* (9), 3240-4.
5. Sharma, V.; McNeill, J. H., *Br J Pharmacol* **2009,** *157* (6), 907-21.
6. Walpole, S. C.; Prieto-Merino, D.; Edwards, P.; Cleland, J.; Stevens, G.; Roberts, I., *BMC Public Health* **2012,** *12*, 439.
7. Prescott, L. F.; McAuslane, J. A.; Freestone, S., *Eur J Clin Pharmacol* **1991,** *40* (6), 619-24.
8. Gomella, L.; Haist, S., *Clinician's Pocket Reference*. 11 ed.; McGraw-Hill Medical: 2006.
9. Liu, J. Y.; Li, N.; Yang, J.; Qiu, H.; Ai, D.; Chiamvimonvat, N.; Zhu, Y.; Hammock, B. D., *Proc Natl Acad Sci U S A* **2010,** *107* (39), 17017-22.
10. Lemini, C.; Jaimez, R.; Franco, Y., *Thromb Res* **2007,** *120* (3), 415-9.
11. Palm, M.; Frankenberg, L.; Johansson, M.; Jalkesten, E., *Comparative Haematology International* **1997,** *7* (4), 243-246.