†S-1 Experimental section Fluorogenic and chromogenic assay correlations

Absorbance and fluorescence measurements were performed in ⁵ an Infinite M200 spectrophotometric microplate reader (Tecan Group Ltd, Männedorf, Switzerland) equipped with a UV Xenon flashlamp. Flat, black-bottom 96-well polystyrol FluorNunc[™] microplates from Thermo Fisher Scientific (Roskilde, Denmark) were used for fluorescence measurements. Flat, transparent 96-

well Greiner® microplates from Greiner Bio-One (Gloucestershire, United Kingdom) were used for absorbance measurements. All measurements for the fluorogenic anti-FXa assay and the chromogenic assay were carried out in reconstituted citrated human pooled plasma. Pooled commercial plasma
samples were spiked with pharmacologically relevant concentrations (0–0.8 U/ml) of therapeutic anticoagulants

including UFH, enoxaparin, and tinzaparin. For the fluorogenic assay, FXa and Pefafluor[™] FXa

fluorogenic substrate concentrations were previously optimized

- ²⁰ as 0.004 μ M and 0.9 μ M, respectively.⁹ Each well contained 6 μ l of 100 mM CaCl₂, 44 μ l of pooled plasma, and 50 μ l of FXa. The reaction was started by adding 50 μ l of PefafluorTM FXa fluorogenic substrate. Samples within wells were mixed with the aid of orbital shaking at 37 °C for 30 s. Immediately after
- ²⁵ shaking, fluorescence measurements were recorded at 37 °C for 60 min, with a 20 μs integration time. Fluorescence excitation was at 342 nm and emission was monitored at 440 nm, corresponding to the excitation/emission wavelengths of the 7amino-4-methylcoumarin (AMC) fluorophore. All the ³⁰ measurements were carried out in triplicate.

The Biophen® Heparin chromogenic assay from Hyphen BioMed (Neuville-Sur-Oise, France) was carried out according to the manufacturer's instructions as follows: each well contained 50 μ l of plasma and 50 μ l of antithrombin (AT). To this, 50 μ l of

- ³⁵ FXa was added. The reaction was started by adding 50 μl of FXa specific chromogenic substrate. Samples within wells were mixed within the spectrophotometer by orbital shaking at 37 °C for 30 s. Immediately after shaking, absorbance measurements were recorded at 37 °C for 60 min, at 10 s intervals. Absorbance was ⁴⁰ measured at 405 nm and all measurements were performed in
- triplicate.

S-2 Results and discussion

Optimization of substrate deposition

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- A range of buffers (10 mM HEPES, 0.01 mM HEPES, StabilCoat buffer, 2% Tween 20, 10% PEG 3400, 1% Triton X-100) were tested for their effect on the dissolution, deposition, and drying characteristics of the fluorogenic substrate. The morphology of ⁵⁰ the dried substrate spots can be seen in **Fig. S-1** (A-F). It can
- immediately be seen From **Fig. S-1** (A-F) that the matrices based on HEPES, StabilCoat and PEG produced uniform spots. However, the mixtures containing surfactant showed spot spreading and irregularity. Both the 10 mM HEPES and
- 55 StabilCoat buffers showed a classical coffee-ring or doughnut morphology due to the movement of material to the edge of the droplet during drying. The dried polymer could also be seen in

the sample containing PEG. However, 0.01 mM HEPES buffer resulted in uniform spots with few defects. Horizontal spot of diameters measured on average 845 μ m ± 59 μ m (n=10; CV = 6.9%) and vertical spot diameters measured 877 μ m ± 51 μ m (n=10; CV = 5.8%).



⁶⁵ Fig. S-1: Morphology of fluorogenic substrate deposited in a range of buffers (x 100 magnification): (a) 10 mM HEPES buffer (b) 0.01 mM HEPES buffer (c) StabilCoat buffer (d) 2 % Tween 20 (e) 10 % PEG 3400 (f) 1 % Triton X-100.

⁷⁰ Based on these morphological characteristics and fluorescent assay responses, HEPES buffer was found to be the most suitable matrix for the fluorogenic substrate. The impact of HEPES concentration on the performance of the substrate was further assessed. Three concentrations of HEPES buffer were ⁷⁵ subsequently tested for optimal performance in the assay using plasma or plasma with 0.5 U/ml UFH, 0.26 μ M FXa and 150 μ M substrate in 0.01, 0.05, and 0.1 mM HEPES (**Fig. S-2**). HEPES at 0.01 mM was selected as the optimal concentration as it returned the maximum fluorescence signal at 0 U/ml heparin and showed ⁸⁰ the greatest potential signal range with the smallest errors (1,896 AU between 0 U/ml and 0.5 U/ml).



Fig. S-2: Comparison of the fluorescence responses of unheparinised (0 U/ml) and heparinised (0.5 U/ml) plasmas in microfluidic devices with fluorogenic substrate prepared in 0.01, 0.05, and 1 mM HEPES buffer ⁸⁵ (n=3).

The effect of temperature on the drying of the fluorogenic substrate was also assessed. Fluorogenic substrate prepared in 0.01 mM HEPES was dried onto the microfluidic devices using a ⁵ range of temperatures and humidities. The most reliable and reproducible method of drying as determined from the morphology of the deposited spots proved to be drying at room temperature, at 10% RH, in a glass desiccator, which was subsequently adopted for preparation of assay devices.

Anticoagulant calibrations

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Fig. S-3: Fluorescence response profiles of human plasmas in the anti-FXa assay device supplemented with concentrations of UFH from 0 to 0.8 U/ml (n=3).

30 Table S-1: Comparison of data analysis methodologies based

on linear regression

Time of fluorescence	Regression	R² value
measurement (s)	equation	
30	y = -0.915x + 3.427	0.99
60	y = -1.124x + 3.665	0.97
90	y = -1.110x + 3.728	0.93