SUPPORTING INFORMATION FOR

Chemical Analog-to-Digital Signal Conversion Based on Robust Threshold Chemistry and Its Evaluation in the Context of Microfluidics-Based Quantitative Assays

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Derivation of the minimal working threshold (equation 1 in the main text)

The threshold concentration is set by the amount of inhibitor, the input is the total amount of enzyme, and the output is the amount of free enzyme. We have the equilibrium $E + I \rightleftharpoons EI$ where E is the enzyme, I is the inhibitor, and EI is the bound enzyme, with the initial concentrations of enzyme and inhibitor E_0 and I_0, respectively. If we call x the amount of EI at equilibrium and K_i the inhibition constant (or the dissociation constant), we have the equilibrium equation

(s1) $$K_i = \frac{(E_0 - x)(I_0 - x)}{x}.$$

Solving the quadratic equation, we have two roots

(s2) $$x_1 = \frac{1}{2}\left[E_0 + I_0 + K_i - \sqrt{-4E_0I_0 + (E_0 + I_0 + K_i)^2}\right]$$

(s3) $$x_2 = \frac{1}{2}\left[E_0 + I_0 + K_i + \sqrt{-4E_0I_0 + (E_0 + I_0 + K_i)^2}\right]$$

We now show that we can eliminate root x_2 by evaluating the inequality

$$x_2 \le E_0$$

(s4) $$\frac{1}{2}\left[E_0 + I_0 + K_i + \sqrt{-4E_0I_0 + (E_0 + I_0 + K_i)^2}\right] \le E_0$$

$$\sqrt{-4E_0I_0 + (E_0 + I_0 + K_i)^2} \le E_0 - I_0 - K_i$$

If the right hand side is negative, (s4) is false; if not, we can square both sides, expand and rearrange to yield $E_0K_D \le 0$, which is false. Therefore (s4) is false and x_1 is the only root of (s1). Now if we define $input \equiv E_0$, we have

(s5)
$$output\left(input \mid I_0, K_i\right) \equiv E_0 - x_1 = \frac{1}{2}\left[E_0 - I_0 - K_i + \sqrt{-4E_0 I_0 + \left(E_0 + I_0 + K_i\right)^2}\right]$$
$$output\left(input \mid I_0, K_i\right) = \frac{1}{2}\left[input - I_0 - K_i + \sqrt{-4I_0 input + \left(input + I_0 + K_i\right)^2}\right]$$

Plugging (s5) into equation (1), we have

(s6)
$$\gamma = 1 - \frac{output\left(I_0\left(1-\alpha\right)\right)}{output\left(I_0\left(1+\alpha\right)\right)}$$
$$\gamma = \frac{2I_0\alpha + \left[\sqrt{K_i^2 + 2I_0K_i\left(2+\alpha\right) + I_0^2\alpha^2} - \sqrt{K_i^2 + 2I_0K_i\left(2-\alpha\right) + I_0^2\alpha^2}\right]}{-K_i + I_0\alpha + \sqrt{K_i^2 + 2I_0K_i\left(2+\alpha\right) + I_0^2\alpha^2}}$$
$$\gamma = \frac{2I_0\alpha + A}{-K_i + I_0\alpha + \sqrt{K_i^2 + 2I_0K_i\left(2+\alpha\right) + I_0^2\alpha^2}}$$

Note that A is defined as in equation (s6) above. Because in practice $\alpha \ll 2$, we were motivated to make the approximation that $A \to 0$, which is shown to be reasonable in a separate section below. With that, we have

(s7)
$$\gamma \approx \frac{2I_0\alpha}{-K_i + I_0\alpha + \sqrt{K_i^2 + 2I_0K_i\left(2+\alpha\right) + I_0^2\alpha^2}}$$

and can solve for I_0 to give

(s8)
$$I_0 = \frac{\gamma(\gamma - \alpha + \gamma\alpha)}{\alpha^2(1-\gamma)}K_i = min.thresh.$$

which is equivalent to equation (2).

Validation of the approximation to get equation (s7) from equation (s6)

We now evaluate the value of A relative to $2I_0\alpha$. If we define β as the upper limit of this ratio, we have

(s9)
$$\frac{A}{2I_0\alpha} = \frac{\sqrt{K_i^2 + 2I_0K_i\left(2+\alpha\right) + I_0^2\alpha^2} - \sqrt{K_i^2 + 2I_0K_i\left(2-\alpha\right) + I_0^2\alpha^2}}{2I_0\alpha} \le \beta$$

After proper manipulation of this inequality (including squaring both sides when they are positive) and the change of variables $y \equiv \frac{I_0}{K_i}$ we have

(s10)
$$\frac{K_i}{I_0} - 4\beta^2 - \frac{K_i}{I_0}\beta^2 - \frac{I_0}{K_i}\alpha^2\beta^2 + \frac{I_0}{K_i}\alpha^2\beta^4 \le 0$$
$$\alpha^2\beta^2\left(\beta^2 - 1\right)y^2 - 4\beta^2 y - \left(\beta^2 - 1\right) \le 0$$

The two roots of the second order polynomial on the left hand side are

(s11)
$$y1 = \frac{2\beta^2 - \sqrt{4\beta^2 + \alpha^2\beta^2\left(\beta^2 - 1\right)^2}}{\alpha^2\beta^2\left(\beta^2 - 1\right)}$$
$$y2 = \frac{2\beta^2 + \sqrt{4\beta^2 + \alpha^2\beta^2\left(\beta^2 - 1\right)^2}}{\alpha^2\beta^2\left(\beta^2 - 1\right)}$$

In the relevant case of $0 < \beta < 1$, we have $y2 < 0 < y1$, and the coefficient for y^2 in (s10) is negative. Inequality (s10) is then equivalent to

(s12) $(y - y1)(y2 - y) \le 0$

This inequality is true when $y \ge y1$ or $y \le y2$. The latter is unphysical, so we have the constraint

(s13) $$y \ge y1 \Leftrightarrow \frac{I_0}{K_i} \ge \frac{2\beta^2 - \sqrt{4\beta^2 + \alpha^2\beta^2\left(\beta^2 - 1\right)^2}}{\alpha^2\beta^2\left(\beta^2 - 1\right)}$$

Within reasonable ranges $0.1 \le \beta < 0.2$ (corresponding to 10%-20% effective increase in required γ (equation s6) and $0.01 \le \alpha < 0.2$ (even with stringently small differences in input), the value of $y1$ is just above 20 (Figure S1), smaller than practical values of $\frac{I_0}{K_i}$ in the scope of setting up the threshold (~ 1000). Therefore, the approximation to get equation (s7) from equation (s6) is valid in most practical cases.

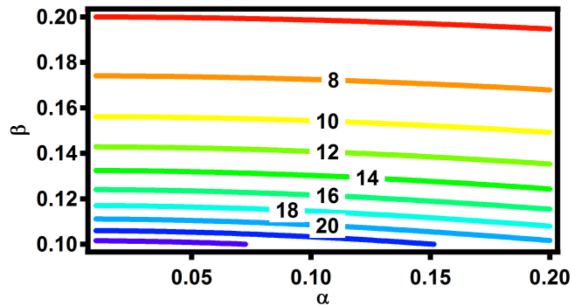

Figure S1. Contour plot of $y1$ versus α and β following equation (s11). Note that all values are unitless because they are relative quantities.

Correlation between stages of chronic kidney disease (CKD) and cystatin C concentration

CKD STAGE	**GFR (mL/min/ 1.73 m^2)**[1]	**Upper-bound [cyst C] (mg/L)** (calculated from Stevens *et al.*[2])	**ACTION** (quoting Levey *et al.*[1])
1	120	0.7	Diagnosis and treatment; treatment of comorbid conditions; slowing progression; CVD risk reduction
2	90	0.9	Estimating progression

3	60	1.2	Evaluating and treating complications
4	30	2.2	Preparation for kidney replacement therapy
5	15	3.9	Kidney replacement (if uremia present)

Table S1. Concentrations of serum cystatin C ([cys C]) and recommended actions for different stages of chronic kidney disease (CKD)

Reactions and Assays

Buffers and solutions

Phosphate buffer (PB): PB is a solution of sodium phosphate 0.1 M, pH 7 with Pluronic F127 Prill (BASF) 1 mg/mL. This buffer was made from NaH_2PO_4 (Fisher BP329) and Na_2HPO_4 (Fisher BP332).

BAB: BAB is a solution of pluronic F127 1 mg/mL in 1xDPBS (10x Gibco 14200 2/2010) pH 7.

Starch solution: A suspension of cornmeal in phosphate buffer was heated in a boiling water bath for 10 minutes and cooled down to room temperature. The supernatant was then filtered through a syringe filter with a 5-µm membrane to give the starch solution.

Inhibitor: The solid generously given by Roman Manetsch and Jordany Maignan (University of South Florida) was dissolved in DMSO (Fisher D128) to make a 1 mM solution, which was diluted in PB into lower concentrations. The aliquots were stored at -80 ^{o}C.

Substrate mixture: A mixture of 98 µL starch solution, 1 µL acetylthiocholine (AcSCh) (Sigma A5626) solution (0.4 M in PB), and 1 µL of the 4.016 mL solution of NaI (798.07 mg) (Fisher S324) and I_2 (101.93 M) (Fisher I37) in PB were vortexed in a 600-µL microcentrifuge tube. The substrate mixture was made freshly for each assay.

Antibody mixture: 0.709 µL bead-antibody (141 mg/mL), 0.500 µL antibody-biotin (4 OD/cm), and 18.8 µL BAB were mixed by pipetting at the time of the assay.

Avidin-AChE solution: 1 µL of avidin-AChE (3.36 OD/cm) was mixed with 66.2 µL BAB, and stored at 0–4°C.

Conjugation

Bead-antibody: Cystatin C antibody clone 24 (Genway, cat#20-511-242278) was conjugated to tosylated paramagnetic beads (Invitrogen, cat#65501) following the manufacturer's instructions. The approximated final concentration was 141 mg/mL (beads) in PBS (pH 7 with BSA 0.1%, Tween 20 0.05%, and sodium azide 0.02%). The mass of antibody was approximated at 1/8 the mass of beads.

Antibody-biotin: Cystatin C antibody clone 10 (Genway, cat#20-511-242277) was conjugated to biotin using Lightning Link (Novus Biological, cat#704-0015) following the manufacturer's instructions. The buffer was PB, and the absorbance at 280 nm was 4 OD/cm.

Avidin-acetylcholinesterase (avidin-AChE): acetylcholinesterase (AChE) (SigmaAldrich, cat#C2888) (1000 units, 1210 units/mg protein) was conjugated to avidin using Lightning Link (Novus Biological, cat#717-0015) (1 mg avidin). The buffer was sodium phosphate 25 mM, pH 7.2 with Pluronic F127 1 mg/mL. The absorbance at 280 nm was 3.36 OD/cm

The conjugates were stored at 0–4°C.

Robustness with respect to temperature and detection time (Figure 3)

We checked the working ranges of temperature and reaction time of the threshold chemistry using absorbance readout with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)[3] (Sigma-Aldrich D8130) (to react with thiocholine, the product of the enzymatic reaction) (Figure 3). In each well in the 96-well plates, acetylcholinesterase (AChE) was incubated with the inhibitor for 30 minutes, followed by the addition of a solution of DNTB and acetylthiocholine (AcSCh) and absorbance measurement at 412 nm by a plate reader (BMG LABTECH, POLARstar Omega). The total volume in each well was 80 μL, and the final concentrations of AcSCh and DTNB were 0.2 mM and 0.15 mM, respectively. The inhibitor concentrations used in each set of experiments were 13, 16, 21, 27, 36, and 46 nM (Figure 3A). We also investigated the robustness when the concentrations of the enzyme and the inhibitor were 10 times smaller (Figure 3B). The temperatures were 22, 25, 28, 31, and 34 °C.

Threshold chemistry in bulk with visual readout (Figure 2D)

Solutions of 23 μL of enzyme and inhibitor (in PB) were incubated in the wells of a 96-well plate at room temperature for 30 minutes. Then 20 μL of the substrate mixture was added in each of the wells. The concentrations shown in Figure 2D were the final concentrations. The image was taken after 39 minutes of reaction using a stereoscope with incident light. The image was subtracted from an image taken after 151 minutes of reaction, when all wells had reacted (as a background subtraction), and then corrected with Photoshop (contrast+95).

Device (SlipChip) fabrication

The AutoCad file of the masks used to make the SlipChip is available upon request.

The design of the SlipChip used for these experiments (Figure 5B, D) is similar to a previously published design used for immunoassays[4]. We improved the washing step by moving the magnetic beads through a channel containing buffer (Figure 5C).

The SlipChip was fabricated by wet-etching glass plates coated with chromium and photoresist (Telic, soda lime 0.7 mm thick, positive photoresist) with exposed areas from photolithography (mask printed by CadArt), as previously described[5], with a twice-concentrated etching mixture (666 mL water, 30 g NH_4F, 78 mL HNO_3 70%, 56 mL HF mix (SigmaAldrich 01066). To etch features of different depths on the same chips, features with shallower depths were covered with packing tape at the beginning and the tape was later removed to expose the features during the process.

The depths of the features measured by a contact profiler (Dektak 150) were 142 μm for the analyte wells of the top plate, 22 μm for the rest of the top plate, and 130 μm for the bottom plate.

The inlets (on the top plate) and the alignment holes (on both plates) were formed by drilling the chips using 0.035 in drill bits (Harvey Tool 74335-C4).

Treatment of chip surface

The chips were coated with FEP to prevent wetting of the surface by aqueous solutions in areas that do not contain any features (wells or ducts). The glass chips were cleaned in H_2SO_4 98% : H_2O_2 30% (3:1 v/v) for 1 hour, then sonicated in NaOH (concentrated, ~ 50 g/L). They were then dip-coated in FEP emulsion (Fuel Cell Earth LLC, cat#TE9568-250) diluted 3 times with Millipore water (advancing and withdrawing speeds of 10.8 and 1.8 cm/min, respectively). The coated chips were baked on a hot plate at 250 ^{o}C (30 min total time, including heating up

from room temperature and baking at at 250 ^{o}C) and at 340 ^{o}C for 1 minute, then cooled in air at room temperature. The chips were then incubated in deionized water for 2.5 hours, and then dried with a N_2 stream.

The features designed to contain aqueous droplets had their FEP layer removed either completely (in the case of the top plate) or partially (in the case of the bottom plate) to ensure retention of the droplets during droplet deposition and transfer during the assay.

The FEP layer in wells and washing channels on the bottom plate were partially removed by laser machining (Resonetics RapidX250 system, with demagnification of 10, constant energy mode of 50 mJ with 50 % attenuator, 75-mm lens, and fluence of 9.32 J/cm^2. The ablated areas were approximately half of those of the features and were centered in the features.

The FEP coating of wells on the top plate were removed manually and completely with a needle (27G1/2, Beckton-Dickinson 305109) under deionized water. The process was performed under a stereoscope. Another purpose of the removal of FEP in the analyte wells was so that the analyte solutions would wet and fill the wells completely.

Setting up the assay

The bottom plate was put in a square petri dish (Fisher 08-757-11A) filled with FC-40 (3M). The side with features was facing up, and the plate was separated from the bottom of the petridish by the ends of insect pins inserted into the alignment holes.

Washing solutions were deposited into the washing channels by pipetting with volumes of 0.5 µL (BAB), 1.2 µL (BAB), and 0.7 µL (PB), following the progress of the assay (Figure 5).

Solutions (1 µL) of inhibitor were deposited into inhibitor wells by pipetting with low-retention pipette tips.

Solutions of antibody mixture and substrate mixture were made at the time of the experiment.

Plugs were made and deposited onto the bottom piece.[4] Plugs were made with a cross channel (Idex Health & Science P-634). FC-40 flowed into two opposite inlets with rate of 80 µL/min each (B-D plastic syringe 1 mL (B-D REF 309602), needle 23G1½, B-D 305194, tubing FEP 1/16x0.20x50ft (Idex Health & Science 1548L)). The aqueous solution flowed into the remaining inlet at 40 µL/min (B-D plastic syringe 1 mL (B-D REF 309602), needle 18G1½, B-D 305196, tubing FEP 1/16x0.20x50ft (Idex Health & Science 1548L)). The syringe for the aqueous inlet was filled with FC-40, then with a small air bubble, then the actual aqueous solution so that the aqueous solution stayed in the FEP tubing only. The pumps used to make plugs were Harvard PHD 2000. Plugs were put into FEP tubing 1/16x0.20x50ft (Idex Health & Science 1548L). The tip of the outlet tubing was cleaned using a piece of Kimwipe (Kimberly-Clark) wetted with D.I. water prior to making each set of plugs.

After the series of plugs were formed in the outlet tubing, the flow rate was slowed down to 20 µL/min during deposition, and to 2 µL/min during the movement to another bottom plate. The sequence of solutions that were deposited was as follows: substrate mixture, avidin-AChE, and antibody mixture. The plug-making apparatus was washed by making plugs of BAB between uses with different solutions.

After all the solutions were loaded onto the bottom plate, the top plate was placed onto the bottom plate with its alignment holes going over the insect pins. The chips were then taken out of the petri dish with a pair of tweezers, clamped with plastic clothespins (3 on each of two opposite sides of the chip), and aligned with better accuracy under a stereoscope.

Running the assay

The assays were run at room temperature (21–23°C).

Loading: The sample (10 μL) was loaded into the inlet with a pipette tip.

Incubation: When the analyte wells were completely filled with the sample, they were slipped to overlap with the wells containing the antibody mixture. The magnet (long enough to cover the whole row of wells) was used to pull the beads up to the top plate, then to the bottom plate, for a total of 2–3 iterations of back-and-forth movement in the direction of slipping (Figure 5), and the magnet was pulled away from the chip when it was at the middle of the row. The 6 clothespins were replaced with 2 clamps (plastic clamp grips for paper, cut into pieces that fit the chip) on opposite sides of the chip. The chip was incubated in an inverted position (bottom plate up) in a petri dish containing FC-40 at room temperature for 1 hour.

End of incubation: After the incubation, the chip was taken out of the petri dish and held by hand in the proper position (top plate up). The magnet was used to pull the beads to the top plate. The magnet was then held on the top plate during slipping to move the wells containing the beads in contact with the first washing channel.

Washing: The magnet was used to move the beads through the washing phase (Figure 5C). The magnet was then held on the top plate near the beads during slipping to move the beads to the next step.

The beads underwent incubation and washing as described above. Each of the incubations with avidin-AChE and the inhibitor also lasted 1 hour at room temperature with the chip in the inverted position in the petri dish containing FC-40.

Initiation of reaction: After the incubation with the inhibitor, the chips were taken out of the petri dish, held by hand in the proper position. The magnet was used to bring the beads into the dumbbell-shaped wells in the top plate. The magnet was held onto the top plate during slipping to bring the wells that contained the beads into contact with the substrate wells. The magnet was used to move the beads to the direction of slipping, then immediately opposite. This moment was set as 0, the beginning of reaction time. During the reaction, the chip was left in air at room temperature. Images of assay results were taken at 70 minutes after the initiation of the reactions.

Handling of images of assay results

Images taken with a digital SLR camera (Figures 6B and 7A): We used a Canon RebelXT with EFS18-55mm lens. The images were cropped and processed in Photoshop (selective color: hue 0, saturation -100, lightness +50; brightness -5, contrast +50).

Images taken with a camera phone (Figure 7B): We used an iPhone4 (Apple Inc.). The images were cropped and processed in Photoshop (brightness +50, contrast +50).

Estimation of threshold concentration from assay results of standards and patient samples (Figure 7)

The smallest increase of [cystatin C] that led to a change in the position of ON/OFF transition was 0.64–0.75 mg/L (1.2 fold). Therefore, we estimated the threshold concentration in column 2 as $0.69 = (0.64*0.75)^{0.5}$ and used this value to estimated the threshold concentrations in other columns, based on 2-fold steps in inhibitor concentrations.

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