

CHAPTER 2

CONVERTING ANALOG SIGNAL TO DIGITAL BY INTRODUCING THRESHOLD ENABLES VISUAL READOUT FOR A CYSTATIN C QUANTIFICATION ASSAY

2.1 Converting analog-to-digital readout using pre-determined thresholds

As discussed in the previous chapter, the amount of information one can extract from a test is usually correlated to the complexity of the test. Qualitative tests such as pregnancy tests require only endpoint yes/no readout and are relatively robust to changes in environmental conditions, but they do not give information regarding the quantity of the target. Quantitative tests usually require instruments to resolve continuous signal intensity at an endpoint, such as a fluorometer or Ultraviolet–visible (UV/Vis) spectrometer, or require kinetic measurements such as ELISA and qPCR. For protein target quantification, the most widely used methodology is still to measure the change in output (color, voltage, precipitation) over time. Simple and quantitative assays have been developed¹⁷, but only with unsatisfactory resolution of an order of magnitude or higher.

We first asked the question of whether there is a way to convert the kinetic information to robust, reader-free endpoint information, and answered the question with the idea of “digitizing” the analog signal by introducing a threshold that inhibits the reaction with target concentration at or below a certain level¹⁸. Only when the target concentration exceeds the threshold will there be a positive reaction triggered by the excess target. By combining a series of ON (above threshold)/OFF (below threshold) bits, the concentration of target can be determined with the resolution set by the step size between thresholds. In the illustration given in Figure 2, a continuous analog signal is converted to a panel of digital bits that are either clear (ON) or colored (OFF). The input concentration can be determined by the position where bits transition from OFF to ON.

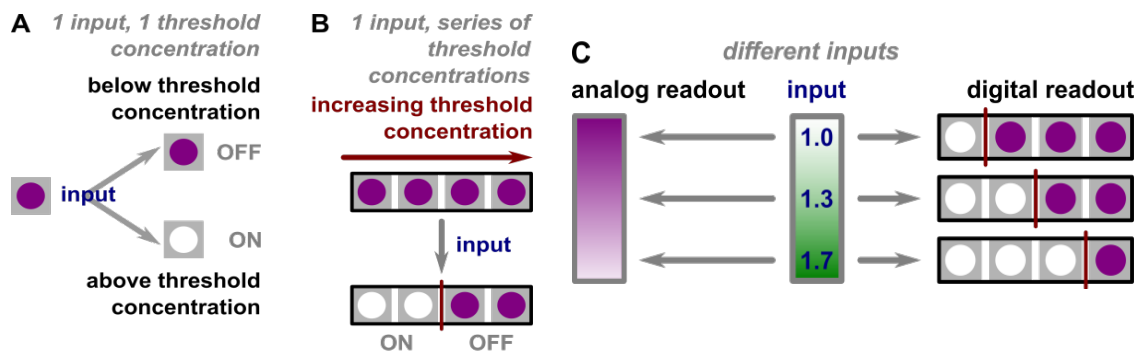


Figure 2 Illustration of analog-to-digital conversion based on thresholds. (A) ON (clear)/OFF (purple) of a single reaction depending on the relevant abundant of target to inhibitor. (B) A series of ON/OFF bits created by increasing threshold concentrations. (C) An illustration of analog readout versus digital readout. An analog readout gives a gradient change in intensity while a digital readout comprises a series of bits. Reprinted with permission from reference 18. Copyright 2013 American Chemical Society.

2.2 Applying the concept of threshold in the quantification assay of cystatin C

Glomerular filtration rate (GFR) describes the flow rate of filtered fluid through the kidney and is an indication of the state of kidney. Creatinine is the most widely used biomarker and its clearance rate is a useful measure for approximating the GFR. However, the level of creatinine varies with muscle mass and protein intake, and is not accurate at detecting mild renal impairment. A more accurate renal function indicator is the level of cystatin C, a low molecular weight protein removed from the bloodstream by glomerular filtration in the kidney. Its level is less dependent on age, gender, race and muscle mass compared with creatinine. It has been shown that cystatin C alone is superior to creatinine as a marker of kidney function in a meta-analysis¹⁹, and adding the measurement of cystatin C to that of creatinine improves assay accuracy²⁰. Therefore, here cystatin C is chosen as the model system to study. For cystatin C measurements, small changes as low as a 1.5 fold increase in concentration need to be detected²¹.

The principle here is based on enzymatic inhibition: the enzyme (linked to the cystatin C molecule via antibody-biotin-avidin conjugate) is allowed to be bound by different amounts of inhibitor before reacting with substrate. For threshold positions with higher inhibitor

concentration than the enzyme concentration, all enzymes are inhibited and the reaction stays negative. For threshold positions with lower inhibitor concentration than the enzyme concentration, uninhibited enzyme will turn the reaction to positive. The concentration of available active enzyme increases dramatically near the threshold position with comparable inhibitor and enzyme concentration and thus changes the enzymatic reaction from negative to positive. The enzyme Acetylcholinesterase (AChE) can hydrolyze acetylthiocholine to thiocholine, which then turns the purple I_3^- /starch solution to clear (Figure 3). The assay was successfully performed with two samples from patients and four standard solutions (solution of purified cystatin C dissolved in cystatin C-free serum) and showed good agreement between experimental result and pre-quantified values.

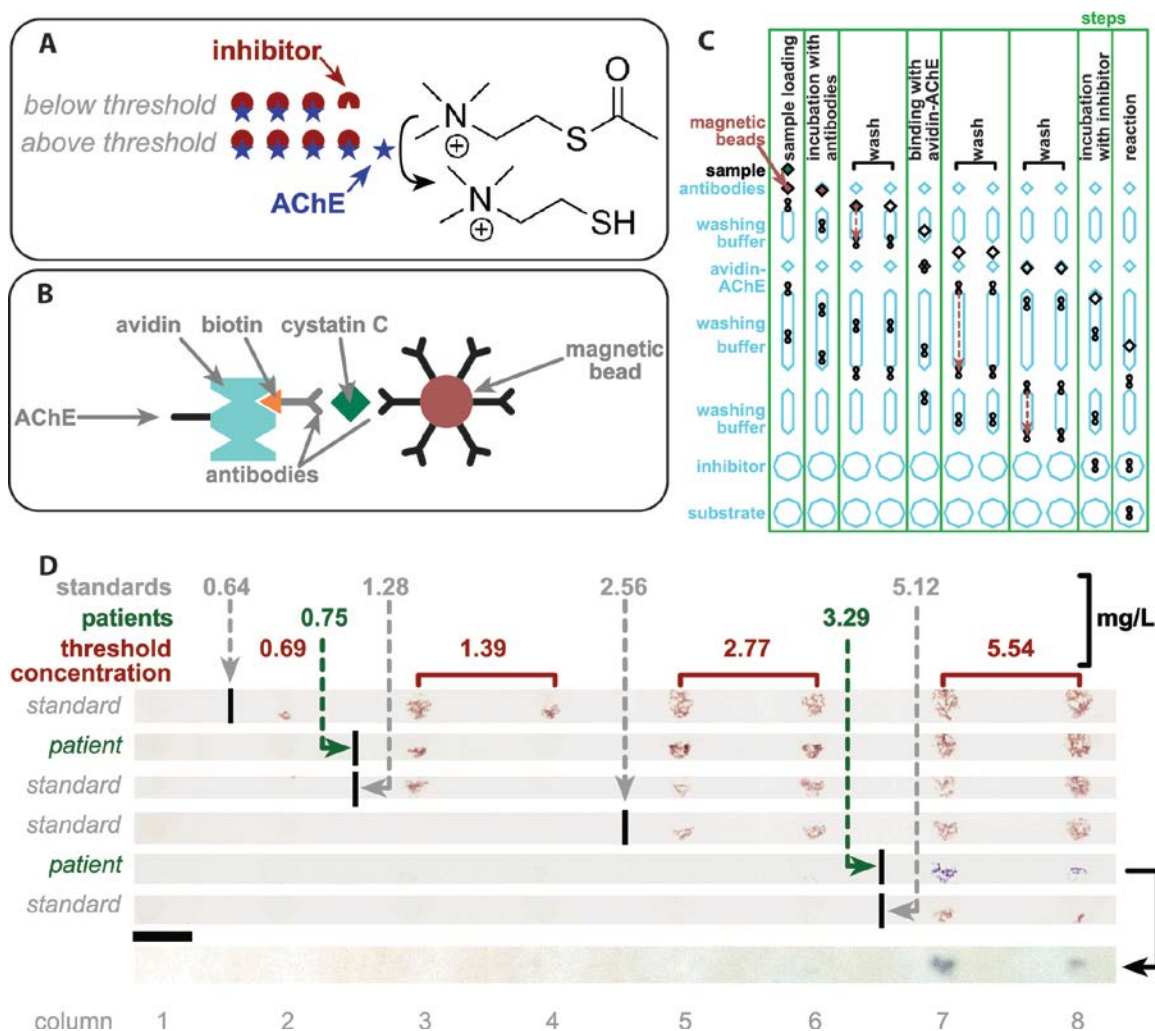


Figure 3 Schematic illustration and experimental results of the threshold-based cystatin C quantification assay. (A) A schematic drawing of the threshold chemistry and detection reactions. When the concentration of the enzyme AChE (star shape) exceeds that of the inhibitor (hemispherical shape), the enzyme hydrolyzes acetylthiocholine to give thiocholine. (B) A drawing of the complex used in the magnetic bead-based immunoassay for cystatin C, where cystatin C is conjugated with capture antibody on the magnetic bead and detection antibody linked to AChE via the biotin-avidin structure. (C) A drawing of the overall SlipChip design and assay procedures. One of the 12 columns is shown (11 times), illustrating the 8 steps of the assay controlled by the movement of the top (black) plate. The movement of magnetic beads is shown with red dashed arrows. (D) Photographs of results from standard solutions and clinical serum samples over a wide dynamic range, with each row corresponding to one assay (one SlipChip device). The very bottom row is a photograph of the result of the 3.29 mg/L patient sample taken by an iPhone 4 camera. Columns 3–4, 5–6, 7–8, 9–10, and 11–12 were set up as duplicates such that each pair contained the same inhibitor concentration. Scale bar: 2 mm. Reprinted with permission from reference 18. Copyright 2013 American Chemical Society.

2.3 Evaluation of the robustness of the threshold position to temperature variation

How an assay performs under various environmental conditions needs to be tested rigorously for any POC test. Here, we evaluated the robustness of the developed assay to changes in temperature and readout time (Figure 4). The temperature was controlled by a plate reader and the absorbance was measured periodically. To simplify the system, a chromogenic substrate, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), together with acetylthiocholine were adopted instead of the substrate used for visual readout, and the concentration of enzyme was kept constant when quantified with multiple thresholds determined by inhibitor concentration. We found that when the enzyme and inhibitor concentrations were higher (13 to 46 nM), the threshold was robust with a consistent contrast between ON and OFF reactions over a range of readout times (3-30 min), a range of temperatures (22–34 °C), and triplicates (Figure 4A). Reactions with inhibitor concentrations that were lower than the enzyme concentration gave ON results (13, 16, 21 nM), while the rest gave OFF results (27, 36, 46 nM). When the enzyme and inhibitor concentrations were lower (1.3 to 4.6 nM), even though the resolution was kept constant (1.3X change per threshold step), the working range of the assay became narrower. Increasing temperature shifts the enzyme/inhibitor equilibrium, and also influences the rate of the overall enzyme/substrate/DTNB reaction. The threshold position and working range depend on the combination of the two. In the case of high concentration, the two effects either cancel each other out or the influence is not significant enough; in the case of low concentration, the shift of equilibrium leads to the change in the threshold position.

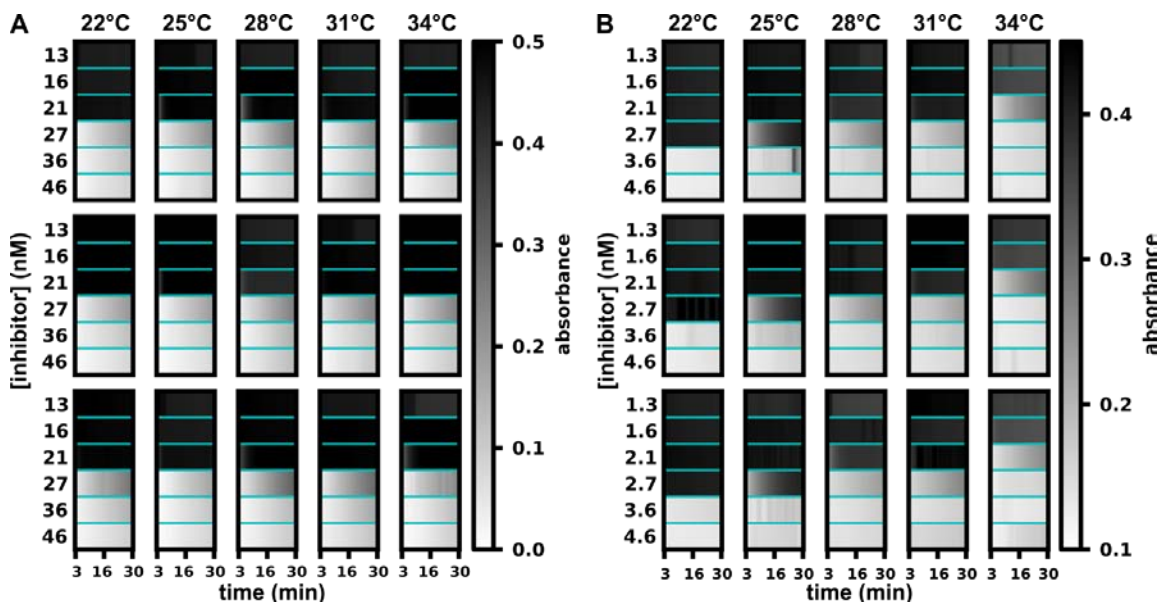


Figure 4 Robustness test results at different temperatures and reaction times for threshold chemistry. (A) False-color maps showing the results of threshold chemistry over a 1.3x dilutions of inhibitor concentration (13, 16, 21, 27, 36, and 46 nM) across a 12-degree range of temperatures (22 °C, 25 °C, 28 °C, 31 °C, and 34 °C). Three repeats are shown in different rows. (B) False-color maps showing the results of threshold chemistry over a 1.3x dilutions of inhibitor concentration (1.3, 1.6, 2.1, 2.7, 3.6, and 4.6 nM) across a 12-degree range of temperatures (22 °C, 25 °C, 28 °C, 31 °C, and 34 °C). Three repeats are shown in different rows. Higher absorbance stands for higher enzymatic activity. Reprinted with permission from reference 18. Copyright 2013 American Chemical Society.

This conversion from analog signal to digital signal using threshold chemistry enables the direct readout of a quantitative cystatin C assay in a reader-free manner, with high resolution (1.3X). This method can potentially be implemented into other clinical assays as well. The disadvantage of this chemistry lies in the fact that one assay is converted to multiple assays depending on the resolution requirement. However, microfluidics offers a solution, as it enables the parallel manipulation of many reactions, reducing the amount of sample used and the extra cost of integrating multiple assays.