

# Fundamental Determinants of the Accuracy of Equilibrium Constants for Affinity Complexes

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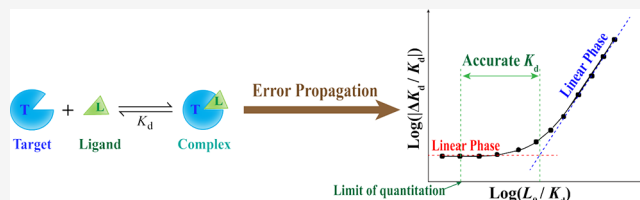
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**ABSTRACT:** The equilibrium constant of a chemical reaction is arguably the key thermodynamic parameter in chemistry; we naturally expect that equilibrium constants are determined accurately. The majority of equilibrium constants determined today are those of binding reactions that form affinity complexes, such as protein–protein, protein–DNA, and protein–small molecule. There is growing awareness that the determination of equilibrium constants for highly stable affinity complexes may be very inaccurate. However, fundamental (i.e., method-independent) determinants of accuracy are poorly understood. Here, we present a study that explicitly shows what the accuracy of equilibrium constants of affinity complexes depends on. This study reveals the critical importance of the choice of concentration of interacting components and creates a theoretical foundation for improving the accuracy of the equilibrium constants. The predicted influence of concentrations on accuracy was confirmed experimentally. The results of this fundamental study provide instructive guidance for experimentalists independently on the method they use.

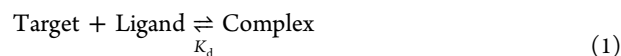


Equilibrium constants of chemical reactions are the cornerstone of chemical thermodynamics. We have seen them multiple times through the years of studies, starting with a freshmen-chemistry course. We learn how to calculate them using tabulated standard free energies of formation, typically presented with three to four significant figures. Accordingly, we naturally expect that equilibrium constants are accurately determined parameters. The real world, however, is very far from this utopia.

The vast majority of equilibrium constants that are published in the scientific literature today are those of binding reactions involving large biological molecules and forming highly stable affinity complexes.<sup>1–4</sup> These equilibrium constants cannot be calculated because standard free energies of formation of typical interactants, such as proteins, DNA, drug leads, etc., are unknown. Therefore, equilibrium constants of affinity complexes are determined experimentally using a large scope of methods.<sup>5–9</sup> Most of these methods are supported by commercial instruments in which a researcher essentially loads the solutions into the instrument, pushes a button, and reads the resulting value with multiple significant figures and a small standard deviation. While being enormously powerful and informative tools, user-friendly commercial instruments inadvertently create an illusion that the determination of equilibrium constants is a trivial technical step, which requires only following an instrument manual. Accordingly, equilibrium-constant values published in a single paper are typically determined with a single method and presumed to be accurate values. However, discrepancies between the values reported by different laboratories for the

same complex may reach orders of magnitude leading to misconceptions and wrong conclusions, e.g., about the potency of drug leads.<sup>10,11</sup> Great variations are often observed even for the values determined with the same method, suggesting that fundamental (method-independent) sources of inaccuracy play a critical role in inconsistencies of equilibrium constants for highly stable affinity complexes.<sup>12,13</sup> There is an understanding that the amount of inconsistent data grows rapidly due to the use of high-throughput methods.<sup>12,14,15</sup> The described “crisis of inaccuracy” in the field of thermodynamics of affinity complexes calls for a systematic search for sources of inaccuracy and approaches that could improve the accuracy. This work focuses on fundamental sources of inaccuracy of the equilibrium constants for affinity complexes.

In this study, we will use notations that are typically used in the field of affinity interactions. The interacting species is called a target and a ligand. Their binding with the formation of the target–ligand complex will be characterized by the equilibrium dissociation constant ( $K_d$ )



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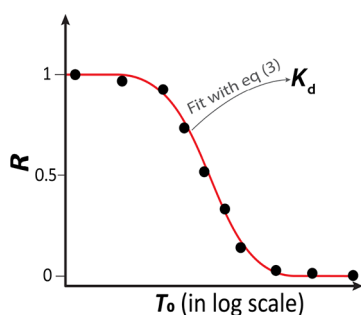
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$K_d$  is defined as

$$K_d = TL/C \quad (2)$$

where  $T$ ,  $L$ , and  $C$  are the equilibrium concentrations of the target, ligand, and complex, respectively, in the binding reaction (eq 1). Lower  $K_d$  values correspond to greater complex stability and are typically desired.<sup>16–18</sup> The values of  $K_d$  define therapeutic concentrations of drugs and analytical parameters of diagnostic methods.<sup>18–21</sup> Therefore, they are used not only for relative ranking of the ligands but also for assessment of their suitability for intended applications.<sup>19,20,22</sup> The most straightforward approach to  $K_d$  determination is an equilibrium (i.e., nonkinetic) method. It involves preparing a series of equilibrium mixtures of targets and ligands in which the total concentration of the ligand ( $L_0$ ) is the same, while the total concentration of the target ( $T_0$ ) varies. These mixtures are used to build a binding isotherm (dots in Figure 1), i.e., the experimental dependence of a fraction  $R$  of the



**Figure 1.** Example of determining  $K_d$  with a binding isotherm. A binding isotherm is the experimental dependence of the fraction of the unbound ligand ( $R$ ) on the total target concentration ( $T_0$ ), which is represented by black dots. The value of  $K_d$  is determined by the best fit (red line) of the isotherm with eq 3.

unbound ligand ( $R = L/L_0$ ) on  $T_0$  for constant  $L_0$ . The value of  $K_d$  is then typically found by fitting the binding isotherm with the theoretical dependence of  $R$  on  $T_0$ .<sup>23–25</sup>

$$R = -\frac{K_d + T_0 - L_0}{2L_0} + \sqrt{\left(\frac{K_d + T_0 - L_0}{2L_0}\right)^2 + \frac{K_d}{L_0}} \quad (3)$$

while varying  $K_d$  until the best fit (red line in Figure 1) is obtained. It is important to emphasize that eq 3 is obtained by solving a quadratic obtained from an expression for  $K_d$

$$K_d = \frac{T_0 - L_0(1 - R)}{(1/R - 1)} \quad (4)$$

which, in turn, is obtained from first principles: definitions of  $K_d$  (eq 2) and  $R$  as well as mass balance for the target and ligand ( $T_0 = T + C$  and  $L_0 = L + C$ ). In other words, eqs 3 and 4 are fundamental, i.e., method-independent, and obtained without assumptions, which would restrict the generality.

There are two fundamental requirements for  $K_d$  accuracy: (i) the binding reaction (eq 1) must approach the equilibrium, and (ii) the study must be done in a so-called binding regime, which is assured by satisfying a condition of  $L_0/K_d \ll 1$ .<sup>12,26–28</sup> According to a recent review by Jarmoskaite et al.,<sup>12</sup> of 100 publications dealing with  $K_d$  determination, fewer than 10 and 5% of publications reported

the satisfying of these two requirements. Failure to satisfy fundamental requirements may deem the vast majority of the published  $K_d$  values to be greatly inaccurate. The alarming level of researchers' ignoring the fundamental requirement is due to the lack of basic knowledge of how this can affect the accuracy of  $K_d$ . There are a few important contributions to the field, which assess  $K_d$  accuracy for specific methods,<sup>29–34</sup> e.g., isothermal titration calorimetry<sup>28,29</sup> and capillary electrophoresis.<sup>30–32</sup> However, there are no comprehensive studies that would explain on what errors of  $K_d$  are fundamentally dependent in the way that would further guide theorists and instruct experimenters. Accordingly, the field of experimental determination of  $K_d$  is largely an art in which researchers rely mostly on mnemonic rules and intuition in designing experiments and interpreting their results. The goal of our work was to initiate a graduate maturation of this field into a solid quantitative science through the understanding of what fundamental errors of  $K_d$  depend on and what it means for experimentalists.

## MATERIALS AND METHODS

**Chemicals, Materials, and Solutions.** All chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated. Fused silica capillaries with an inner diameter of 75  $\mu\text{m}$  and an outer diameter of 360  $\mu\text{m}$  were purchased from Molex Polymicro (Phoenix, AZ, USA) and used throughout this work. His-tagged recombinant *Thermus aquaticus* MutS protein (MW = 92.8 kDa) was purchased from Prospec (Ness-Ziona, Israel). An Alexa488-labeled MutS-binding aptamer was synthesized by Integrated DNA Technologies (Coralville, IA, USA) with the following sequence: 5'-Alexa488-CTT CTG CCC GCC TCC TTC CTG GTA AAG TCA TTA ATA GGT GTG GGG TGC CGG GCA TTT CGG AGA CGA GAT AGG CGG ACA CT-3'.<sup>35</sup> The aptamer stock solution was subjected to annealing by incubating at 90  $^{\circ}\text{C}$  for 2 min before cooling it to 20  $^{\circ}\text{C}$  at a rate of 0.5  $^{\circ}\text{C}/\text{s}$  prior to dilution and preparation of equilibrium mixtures. One single sample buffer (SB) was used for all experimental procedures: 50 mM Tris-Acetate at pH 8.2 was supplemented with 0.1% Tween 20 and 0.1% Tween 80 to reduce the adsorption of DNA and MutS protein to surfaces. All solutions were made using double-distilled water deionized with Milli-Q and filtered through a 0.22  $\mu\text{m}$  filter (Millipore, Nepean, ON, Canada); we termed it ddH<sub>2</sub>O.

**Poly(vinyl alcohol) (PVA) Coating.** To reduce sample adsorption, the inner wall of all capillaries used in capillary electrophoresis (CE) was coated with poly(vinyl alcohol) (PVA) using a previously described procedure.<sup>36</sup> PVA (5%, w/v) was prepared by dissolving the polymer in boiling ddH<sub>2</sub>O. Each time, two 130 cm long capillaries were coated simultaneously. The two uncoated fused silica capillaries were sequentially flushed with 0.1 M NaOH and ddH<sub>2</sub>O for 1 h under a pressure of 12 psi. The pretreated capillaries were then flushed with the PVA solution for 10 min at 15 psi and emptied using a 10 psi nitrogen gas flow for 10 min. PVA was immobilized on the capillary surface by drying overnight in an oven set at 140  $^{\circ}\text{C}$  and continuously flushed with nitrogen at a pressure of 5 psi. The detection window on the capillary was made by removing the outer polyimide layer with a fuming solution of H<sub>2</sub>SO<sub>4</sub>.

**Experimental Procedure.** All CE experiments were performed with a P/ACE MDQ apparatus (SCIEX, Concord,

Ontario, Canada) equipped with a laser-induced fluorescence (LIF) detection system. Fluorescence was excited with a blue line (488 nm) of a solid-state laser and detected at 520 nm. All capillaries were PVA-coated and had a length of 50 cm (approximately 40 cm from the capillary inlet to the detector). Prior to each run, the capillary was rinsed with running buffer at a pressure of 20 psi for 3 min.

All dilutions of sample components were made using SB. All equilibrium mixtures were prepared to final volumes of 40  $\mu\text{L}$  in SB and incubated at an ambient temperature of  $23 \pm 1$   $^{\circ}\text{C}$  for a minimum of 1 h prior to injection. A sample plug was injected into a capillary by pressure at 1.0 psi for 10 s (sample plug's length = 1.3 cm and volume = 59 nL) and then propagated at 0.9 psi for 30 s (propagation distance = 3.6 cm) with SB to pass the uncooled region of the capillary. The sample was then electrophoresed at 25 kV for 15 min with a negative electrode in the capillary inlet. The capillary coolant was set to 15  $^{\circ}\text{C}$ .

## RESULTS AND DISCUSSION

**Theoretical Problem.**  $K_{\text{d}}$  values are not measured; they are determined using eqs 3 or 4 from known values of  $T_0$ ,  $L_0$ , and  $R$  (in the case of eq 3, we also need to use nonlinear regression). Accordingly, a deviation of the determined  $K_{\text{d}}$  value ( $K_{\text{d,det}}$ ) from the true  $K_{\text{d}}$  value ( $K_{\text{d}}$ ) is a result of errors of  $T_0$ ,  $L_0$ , and  $R$ . So, the answer to the question of what the accuracy of  $K_{\text{d}}$  depends on is simple: it depends on the accuracy of  $T_0$ ,  $L_0$ , and  $R$ . However, there is a second important question: how does the error of  $K_{\text{d}}$  depend on the errors of  $T_0$ ,  $L_0$ , and  $R$ ? If the errors of  $T_0$ ,  $L_0$ , and  $R$  are not zero, which is always the case, then the error of  $K_{\text{d}}$  is the result of propagation of errors of  $T_0$ ,  $L_0$ , and  $R$ . Therefore, the answer to the second question is also conceptually simple: the dependence of the accuracy of  $K_{\text{d}}$  on the errors of  $T_0$ ,  $L_0$ , and  $R$  is defined by the rules of error propagation. Accordingly, we will apply error propagation rules to eq 4 to find how the deviation of  $K_{\text{d,det}}$  from  $K_{\text{d}}$  ( $\Delta K_{\text{d}} = K_{\text{d,det}} - K_{\text{d}}$ ) depends on deviations of  $T_0$ ,  $L_0$ , and  $R$  ( $\Delta T_0$ ,  $\Delta L_0$ , and  $\Delta R$ , respectively, defined similarly to  $\Delta K_{\text{d}}$ ) from their true values.

Note that deviations  $\Delta T_0$ ,  $\Delta L_0$ , and  $\Delta R$  are not random errors with a given error distribution; they can be both positive and negative.  $\Delta K_{\text{d}}$  is a systematic error, which defines the accuracy of  $K_{\text{d,det}}$ .  $\Delta K_{\text{d}}$  may be much greater than the random error of  $K_{\text{d,det}}$ . Furthermore,  $\Delta K_{\text{d}}$  is virtually impossible to determine since the true  $K_{\text{d}}$  value is fundamentally unknown owing to the absence of standard reference instruments for determination of  $K_{\text{d}}$  or standard reference  $K_{\text{d}}$  values.

**Propagation of Correlated Errors.** Since the sample preparation processes for both targets and ligands are similar and  $R$  values are determined from the signals of bound and unbound ligands, the error sources for  $T_0$ ,  $L_0$ , and  $R$  are linearly correlated in  $K_{\text{d}}$  determination experiments. Therefore, to investigate the error of  $K_{\text{d}}$  propagated from the errors of  $T_0$ ,  $L_0$ , and  $R$ , we can write the general dependence of  $\Delta K_{\text{d}}$  on  $\Delta T_0$ ,  $\Delta L_0$ , and  $\Delta R$  as

$$|\Delta K_{\text{d}}| = \left| \left( \frac{\partial K_{\text{d}}}{\partial T_0} \right) \Delta T_0 \right| + \left| \left( \frac{\partial K_{\text{d}}}{\partial L_0} \right) \Delta L_0 \right| + \left| \left( \frac{\partial K_{\text{d}}}{\partial R} \right) \Delta R \right| \quad (5)$$

By applying the error propagation rule (eq 5) to eq 4, and with a series of mathematical transformations (Note S2), we finally obtain

$$\left| \frac{\Delta K_{\text{d}}}{K_{\text{d}}} \right| = a + b \frac{L_0}{K_{\text{d}}},$$

For  $0 \leq \Delta R < 0.5$ :

$$a = t + \frac{r(1+r)}{0.5+r}, \quad b = \frac{0.5(t+l-r)}{1+r} + \frac{0.5r}{0.5+r}$$

For  $-0.5 < \Delta R < 0$ :

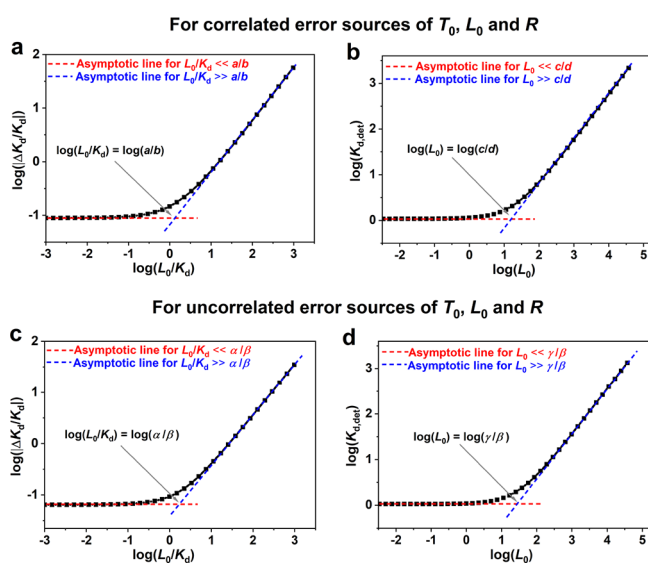
$$a = t + \frac{r(1-r)}{0.5-r}, \quad b = \frac{0.5(t+l-r)}{1-r} + \frac{0.5r}{0.5-r} \quad (6)$$

where  $t = |\Delta T_0/T_0|$ ,  $l = |\Delta L_0/L_0|$ , and  $r = |\Delta R/R|$ . Equation 6 reveals that, when the error sources for  $T_0$ ,  $L_0$ , and  $R$  are strongly correlated, the relative error of  $K_{\text{d}}$  is a linear function of  $L_0/K_{\text{d}}$  with an intercept dependent only on relative errors of  $T_0$  and  $R$  and slope-dependent on relative errors of all three variables:  $T_0$ ,  $L_0$ , and  $R$ . In the mathematical analysis (Note S2), we made a single simplifying assumption that the determined (from measured signals) value of  $R$  is equal to 0.5, which leads to the least erroneous  $K_{\text{d}}$ ,<sup>25</sup> thus, we are considering the best-case scenario and are finding the lower limit for the error of  $K_{\text{d}}$ .

Equation 6 explicitly shows the role of  $L_0/K_{\text{d}}$  in the relative error of  $K_{\text{d}}$ . The value of  $L_0/K_{\text{d}}$  is important only when the second term is greater than the first, i.e., when  $L_0/K_{\text{d}} > a/b$ . In other words, in contrast to a commonly used requirement of  $L_0/K_{\text{d}} \ll 1$ , it is unnecessary to decrease  $L_0/K_{\text{d}}$  much below  $a/b$ . Rather, using  $L_0/K_{\text{d}} \ll a/b$  will likely cause the opposite effect, namely, it will lead to an increase in  $|\Delta K_{\text{d}}/K_{\text{d}}|$  due to the increase in  $r$  when  $L_0$  is too low, causing an unacceptable signal-to-noise ratio ( $S/N$ ), which will be briefly discussed later. On the other hand, when  $L_0/K_{\text{d}} > a/b$ , the increase in  $L_0/K_{\text{d}}$  plays a crucial role in increasing the relative error of  $K_{\text{d}}$ ; an order of magnitude increase in  $L_0$  will lead to an order of magnitude increase in  $|\Delta K_{\text{d}}/K_{\text{d}}|$ . One should appreciate that if  $L_0/K_{\text{d}} \gg a/b$ , then  $K_{\text{d,det}}$  may differ from true  $K_{\text{d}}$  by orders of magnitude.

For a large range of  $L_0$  values, which is typically the case, it is more convenient to present the dependence of  $|\Delta K_{\text{d}}/K_{\text{d}}|$  on  $L_0/K_{\text{d}}$  on a double-log scale. A graph of  $\log(|\Delta K_{\text{d}}/K_{\text{d}}|)$  versus  $\log(L_0/K_{\text{d}})$  is triphasic: two asymptotically linear ranges flank a nonlinear transition range (Figure 2a and Figure S1a). For small values of  $L_0/K_{\text{d}}$ , i.e., for  $L_0/K_{\text{d}} \ll a/b$ , the dependence is a linear function with no dependence on  $L_0/K_{\text{d}}$ :  $\log(|\Delta K_{\text{d}}/K_{\text{d}}|) = \log(a) = \text{const}$ . For a large value of  $L_0/K_{\text{d}}$ , i.e., for  $L_0/K_{\text{d}} \gg a/b$ , the dependence is a linear function  $\log(|\Delta K_{\text{d}}/K_{\text{d}}|) = \log(b) + \log(L_0/K_{\text{d}})$  with an intercept with the ordinate equal to  $\log(b)$  and a slope equal to unity. The abscissa of the intersection of the two asymptotic lines is defined by  $\log(L_0/K_{\text{d}}) = \log(a/b)$ .

Using eq 6 and  $\Delta K_{\text{d}} = K_{\text{d,det}} - K_{\text{d}}$ , we can show that (for strongly correlated error sources) not only  $|\Delta K_{\text{d}}/K_{\text{d}}|$  depends linearly on  $L_0/K_{\text{d}}$ , but  $K_{\text{d,det}}$  is also a linear function of  $L_0$  (Note S3)



**Figure 2.** General trends in dependencies of  $\log(|\Delta K_d/K_d|)$  on  $\log(L_0/K_d)$  (a, c) and  $\log(K_{d,det})$  on  $\log(L_0)$  (b, d). In all panels, reasonable relative errors of  $L_0$ ,  $T_0$ , and  $R$  were used:  $|\Delta T_0/T_0| = |\Delta L_0/L_0| = 0.05$  and  $\Delta R/R = 0.02$  (the results corresponding to  $\Delta R/R = -0.02$  are shown in Figure S1a,c). In panels (b, d), we set  $K_d = 1$  (unitless) and  $\Delta K_d \geq 0$  (the results corresponding to  $\Delta K_d < 0$  are shown in Figure S1b,d). With these conditions, parameters  $a$ ,  $b$ ,  $c$ , and  $d$  were calculated to be 0.089, 0.058, 1.1, and 0.058, respectively;  $\alpha$ ,  $\beta$ , and  $\gamma$  were calculated to be 0.064, 0.036, and 1.1, respectively.

$$K_{d,det} = c + dL_0$$

$$\text{For } \Delta K_d \geq 0: c = (1 + a)K_d, d = b$$

$$\text{For } \Delta K_d < 0: c = (1 - a)K_d, d = -b \quad (7)$$

If presented in a double-log scale, then this dependence is expectedly triphasic (Figure 2b) and has features similar to the dependence of  $\log(|\Delta K_d/K_d|)$  on  $\log(L_0/K_d)$  (Figure 2a).

**Propagation of Uncorrelated Errors.** In some experimental cases, the error sources of  $L_0$ ,  $T_0$ , and  $R$  may be weekly correlated. For example, stock concentrations of the ligand and target may largely differ from the labeled values in the opposite direction. In these cases, the errors from the stock reagents are uncorrelated (independent of each other) and might be much larger than the errors caused by the instruments or methods used in sample preparation. In addition, using an uncalibrated instrument to detect ligand signals can cause the errors in determined  $R$  values to be uncorrelated to the errors of  $T_0$  and  $L_0$ . To study the error propagation with uncorrelated (orthogonal) error sources, we should write the dependence of  $\Delta K_d$  on  $\Delta T_0$ ,  $\Delta L_0$ , and  $\Delta R$  as

$$|\Delta K_d| = \sqrt{\left(\frac{\partial K_d}{\partial T_0}\right)^2 \Delta T_0^2 + \left(\frac{\partial K_d}{\partial L_0}\right)^2 \Delta L_0^2 + \left(\frac{\partial K_d}{\partial R}\right)^2 \Delta R^2} \quad (8)$$

Then, we apply the error propagation rule of eqs 8–4 and obtain (Note S4)

$$\left|\frac{\Delta K_d}{K_d}\right| = \sqrt{\alpha^2 + \lambda \frac{L_0}{K_d} + \beta^2 \left(\frac{L_0}{K_d}\right)^2}$$

For  $0 \leq \Delta R < 0.5$ :

$$\alpha = \sqrt{t^2 + \left(\frac{r + r^2}{0.5 + r}\right)^2}$$

$$\beta = \frac{0.5}{1 + r} \sqrt{t^2 + l^2 + \left(\frac{0.5r}{0.5 + r}\right)^2}$$

$$\lambda = \frac{t^2}{1 + r} + 2\left(\frac{0.5r}{0.5 + r}\right)^2$$

For  $-0.5 < \Delta R < 0$ :

$$\alpha = \sqrt{t^2 + \left(\frac{r - r^2}{r - 0.5}\right)^2}$$

$$\beta = \frac{0.5}{1 - r} \sqrt{t^2 + l^2 + \left(\frac{0.5r}{r - 0.5}\right)^2}$$

$$\lambda = \frac{t^2}{1 - r} + 2\left(\frac{0.5r}{r - 0.5}\right)^2 \quad (9)$$

Here, as explained in the previous section,  $t = |\Delta T_0/T_0|$ ,  $l = |\Delta L_0/L_0|$ , and  $r = |\Delta R/R|$  and we only investigate the case with the determined  $R$  being equal to 0.5 to simplify the study.

Although the forms of eqs 6 and 9 are very different, eq 9 shows a significant similarity to eq 6 regarding the triphasic dependence of  $|\Delta K_d/K_d|$  on  $L_0/K_d$ . For small  $L_0/K_d$  values, eq 9 approaches a linear function of  $|\Delta K_d/K_d| = \alpha = \text{const}$  that shows no dependence on  $L_0/K_d$ . For large  $L_0/K_d$  values, eq 9 approaches a linear function of  $|\Delta K_d/K_d| = \beta(L_0/K_d)$  that shows a high sensitivity of  $|\Delta K_d/K_d|$  to  $L_0/K_d$ . There is a nonlinear transition range between these two linear lines. Moreover, both eqs 6 and 9 suggest that the minimum  $|\Delta K_d/K_d|$  value depends on a parameter ( $a$  in eq 6 or  $\alpha$  in eq 9) that is defined only by  $t$  and  $r$ , while the sensitivity of  $|\Delta K_d/K_d|$  to  $L_0/K_d$  mainly depends on a parameter ( $b$  in eq 6 or  $\beta$  in eq 9) that is defined by  $t$ ,  $l$ , and  $r$ .

To show the dependence of  $|\Delta K_d/K_d|$  on  $L_0/K_d$  in a large range of  $L_0/K_d$  for uncorrelated error sources, we demonstrate “ $\log(|\Delta K_d/K_d|)$  versus  $\log(L_0/K_d)$ ” in Figure 2c. In the double-log scale (Figure 2c), the asymptotic lines for small and large  $L_0/K_d$  values are approximated with functions of  $\log(|\Delta K_d/K_d|) = \log(\alpha)$  and  $\log(|\Delta K_d/K_d|) = \log(\beta) + \log(L_0/K_d)$ , respectively. Figure 2c suggests that, to avoid a significant increase in  $r$  caused by an unacceptably low signal-to-noise ratio, it is unnecessary to decrease  $L_0/K_d$  much below a certain value  $\alpha/\beta$  that is defined by the abscissa of intersection of the two asymptotic lines (i.e.,  $\log(L_0/K_d) = \log(\alpha/\beta)$ ). According to the results in Figure 2 and Figure S1, with the same  $t$ ,  $l$ , and  $r$ , the value of  $\alpha/\beta$  is slightly greater than  $a/b$  because  $|\Delta K_d|$  obtained with eq 8 is smaller than that determined with eq 5.

Now, let us discuss how  $K_{d,det}$  depends on  $L_0$  when the error sources are uncorrelated. With eq 9 and the definition of  $\Delta K_d$  (i.e.,  $\Delta K_d = K_{d,det} - K_d$ ), we obtain (Note S5)



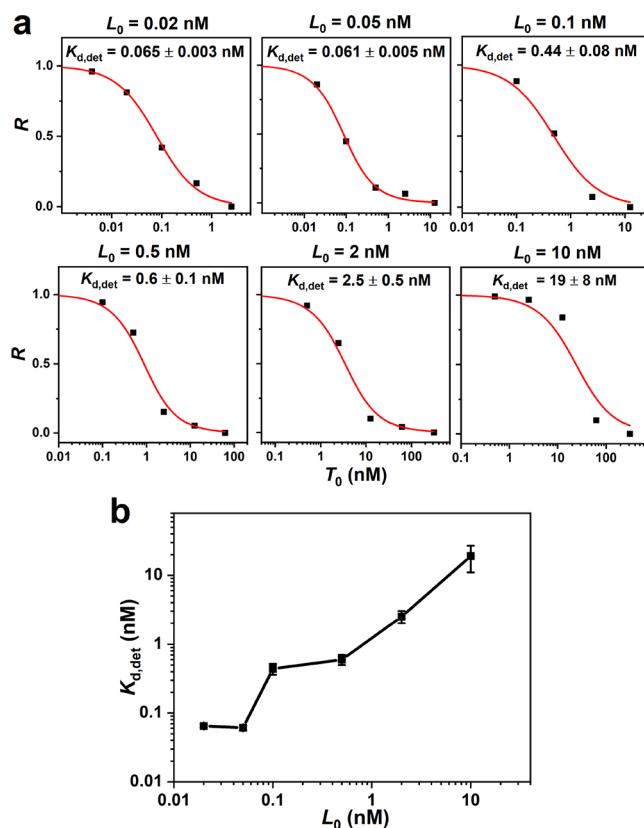
$$K_{d,\text{det}} = K_d + \sqrt{\alpha^2 K_d^2 + \lambda K_d L_0 + \beta^2 L_0^2} \text{ when } \Delta K_d \geq 0$$

$$= K_d - \sqrt{\alpha^2 K_d^2 + \lambda K_d L_0 + \beta^2 L_0^2} \text{ when } \Delta K_d < 0 \quad (10)$$

Equation 10 shows that the dependence of  $K_{d,\text{det}}$  on  $L_0$  is also triphasic. For small  $L_0$  values,  $K_{d,\text{det}}$  is insensitive to the change of  $L_0$  and eq 10 approaches a linear function of  $K_{d,\text{det}} = \gamma$  ( $\gamma = (1 + \alpha)K_d$  when  $\Delta K_d \geq 0$  and  $\gamma = (1 - \alpha)K_d$  when  $\Delta K_d < 0$ ) (Note S5). For large  $L_0$  values, eq 10 approaches another linear function of  $K_{d,\text{det}} = \beta L_0$  that shows high sensitivity of  $K_{d,\text{det}}$  to  $L_0$  (only applicable for  $\Delta K_d \geq 0$  since  $K_{d,\text{det}}$  has to be greater than zero) (Note S5). A nonlinear transition range exists between the two linear lines. Figure 2d shows an example of the dependence of  $K_{d,\text{det}}$  on  $L_0$  for uncorrelated error sources of  $T_0$ ,  $L_0$ , and  $R$  on a double-log scale, which has the expected triphasic feature.

**Experimental Validation.** The dependence shown in Figure 2b,d has two known variables,  $L_0$  and  $K_{d,\text{det}}$  and therefore, it can be tested experimentally, which we have undertaken in this work. Experimentally, we used a well-established solution-based method of nonequilibrium capillary electrophoresis of equilibrium mixtures (NECEEM) as the  $K_d$ -determination method and MutS-aptamer as the binding pair.<sup>25,37–39</sup> In NECEEM, the unbound ligand and complex (bound ligand) are separated by an electric field in a capillary due to their different charge-to-size ratios.<sup>37–39</sup> In six NECEEM experiments, the aptamer concentration ( $L_0$ ) was kept constant at 0.02, 0.05, 0.1, 0.5, 2, and 10 nM; the MutS concentration ( $T_0$ ) varied from 0 to 312.5 nM (all concentrations are given as nominal values with no significance to the number of digits). The lowest value of  $L_0$  (i.e., 0.02 nM) was chosen to be equal to the limit of quantitation (LOQ) (Figure S2) linked to  $S/N$  as discussed below. The influence of  $L_0$  on binding isotherms and the dependence of  $K_{d,\text{det}}$  on  $L_0$  are shown in Figure 3. Figure 3b shows that (in a double-log scale)  $K_{d,\text{det}}$  is insensitive to the change of  $L_0$  when  $L_0$  is at low concentrations (e.g.,  $\ll \sim 0.1$  nM), and  $K_{d,\text{det}}$  is sensitively dependent on  $L_0$  when  $L_0$  is at high concentrations. The results in Figure 3b are consistent with the theoretical predictions shown in Figure 2b,d, which confirm the validity of our theoretical analysis.

**Instructive Guidance for Experimentalists.** Although Figure 2 suggests that, with any type of experimental error sources (correlated or uncorrelated), we can always use an as-low-as-possible  $L_0$  to avoid the error of  $K_{d,\text{det}}$  being greatly magnified, the choice of the lowest experimentally suitable  $L_0$  is dictated by the limit of quantitation (LOQ) of an instrument used to measure the signal. The value of LOQ is, by definition, the analyte concentration (ligand concentration  $L_0$  in our case) for which  $S/N$  is equal to a certain value  $X \gg 1$ , which guarantees that the noise does not affect quantitation significantly. If the noise is independent of the signal and the value of LOQ is known, then  $S/N$  can be calculated for any given concentration of the ligand as  $S/N = XL_0/\text{LOQ}$  (Note S6). The minimum acceptable ligand concentration that satisfies this equality is  $L_0 = \text{LOQ}$ . Since  $R$  values are determined based on the measured signals  $S$ , using  $L_0 < \text{LOQ}$  is counterproductive as it will cause a significant increase in the relative error of  $R$  ( $r$ ), and, accordingly, in the relative error of  $K_{d,\text{det}}$ .



**Figure 3.** Influence of  $L_0$  on binding isotherms (a) and the dependence of  $K_{d,\text{det}}$  on  $L_0$  (b). In panel (a), all nonlinear regressions were conducted using OriginPro software with the iteration algorithm of Levenberg–Marquardt. See the Supporting Information for other details.

Based on the above analysis, to achieve good accuracy of  $K_{d,\text{det}}$  in an experiment, if  $\text{LOQ}/K_d < a/b$  (or  $\alpha/\beta$  for uncorrelated error sources), then  $L_0/K_d$  should satisfy

$$\frac{\text{LOQ}}{K_d} \leq \frac{L_0}{K_d} < \frac{a}{b} \left( \text{or } \frac{\alpha}{\beta} \right) \quad (11)$$

where  $a$ ,  $b$ ,  $\alpha$ , and  $\beta$  are defined by the absolute values of relative systematic errors of  $T_0$ ,  $L_0$ , and  $R$  (i.e.,  $t$ ,  $l$ , and  $r$ ) as shown in eqs 6 and 9. Because  $t$ ,  $l$ , and  $r$  are relatively difficult to determine, practically, the LOQ can be determined and used as the sole determinant of the ligand concentration to minimize the error of  $K_{d,\text{det}}$  in a  $K_d$ -determination experiment. It is important to emphasize that using  $L_0 = \text{LOQ}$  does not imply that  $L_0/K_d < a/b$  (or  $\alpha/\beta$ ) and thus does not guarantee the accuracy of  $K_d$ . Therefore, with the current state of affairs, the only way to confirm (indirectly) that  $L_0/K_d < a/b$  (or  $\alpha/\beta$ ) is to conduct experiments not only at  $L_0 = \text{LOQ}$  but also at  $L_0 > \text{LOQ}$  (e.g., the experiments corresponding to  $L_0 = 0.02$  and 0.05 nM shown in Figure 3). If  $K_d$  is not affected by the value of  $L_0$ , then one can assume that  $L_0/K_d < a/b$  (or  $\alpha/\beta$ ) and deem  $K_d$  accurate.

## CONCLUDING REMARKS

To conclude, in this study, we investigated the theoretical dependence of the relative deviation of  $K_{d,\text{det}}$  from  $K_d$  ( $|\Delta K_d/K_d|$ ) on the ratio of  $L_0/K_d$  with error propagations. Our analysis shows that with fixed correlated or uncorrelated

errors of  $T_0$ ,  $L_0$ , and  $R$ ,  $|\Delta K_d/K_d|$  always presents a triphasic dependence on  $L_0/K_d$  in a double-log scale: When  $L_0/K_d$  is small,  $|\Delta K_d/K_d|$  is insensitive to the change of  $L_0/K_d$  and approaches a constant that is defined by the errors of  $T_0$  and  $R$ . When  $L_0/K_d$  is large,  $|\Delta K_d/K_d|$  is sensitive to the change of  $L_0/K_d$ , i.e., an order of magnitude increase in  $L_0$  will lead to an order of magnitude increase in  $|\Delta K_d/K_d|$ . There is a nonlinear transition range between the two linear phases. We also theoretically demonstrated that the dependence of  $K_{d,\text{det}}$  on  $L_0$  should also show the triphasic features on a double-log scale, which was experimentally confirmed with NECEEM experiments using MutS-aptamer as the binding pair. The results of this work suggest that, without knowing the errors of  $T_0$ ,  $L_0$ , and  $R$ , experimentalists should use  $L_0 = \text{LOQ}$  (i.e., the smallest ligand concentration that does not sacrifice the accuracy of  $R$ ) in an equilibrium  $K_d$ -determination method and conduct an extra experiment with  $L_0 > \text{LOQ}$  to confirm the accuracy of the determined  $K_d$ . To emphasize, we foresee that our findings (e.g., eqs 6 and 9 and the triphasic dependence of  $|\Delta K_d/K_d|$  on  $L_0/K_d$  in Figure 2) can help to create an approach of assessing  $K_d$  accuracy from a single-binding isotherm if the ranges of relative errors in  $T_0$ ,  $L_0$ , and  $R$  can be estimated.

## ■ ASSOCIATED CONTENT

### SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.3c03557>.

Derivation of the theoretical dependence of the fraction of the unbound ligand on the total target concentration (Note S1); derivation of the dependence of relative systematic error of  $K_d$  on the  $L_0/K_d$  ratio for correlated error sources (Note S2); derivation of the theoretical dependence of determined  $K_d$  on  $L_0$  for correlated error sources (Note S3); derivation of the dependence of relative systematic error of  $K_d$  on the  $L_0/K_d$  ratio for uncorrelated error sources (Note S4); derivation of the theoretical dependence of determined  $K_d$  on  $L_0$  for uncorrelated error sources (Note S5); general trends in dependencies of  $\log(|\Delta K_d/K_d|)$  on  $\log(L_0/K_d)$  and  $\log(K_{d,\text{det}})$  on  $\log(L_0)$  (Figure S1); derivation of the relationship between the signal-to-noise ratio and the limit of quantitation (Note S6); determination of limit of quantitation (Figure S2); electropherograms of the NECEEM experiments (Figure S3); results of  $R$  values at different  $T_0$  for the NECEEM experiments with different  $L_0$  (Table S1) (PDF)

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## Notes

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## ■ ABBREVIATIONS

NECEEM, nonequilibrium capillary electrophoresis of equilibrium mixtures; LOQ, limit of quantitation

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## SUPPORTING INFORMATION

### Fundamental Determinants of the Accuracy of Equilibrium Constants for Affinity Complexes

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### Additional supplementary files

The following supplementary files can be found on Figshare:

DOI: 10.6084/m9.figshare.23624583

File name	Description/Experiment
Figures.zip	Figures in main text and supporting information (Origin and png files).



## Section S1: Derivation of equations

### Note S1: Derivation of the theoretical dependence of fraction of unbound ligand ( $R$ ) on total target concentration ( $T_0$ )

The definition of equilibrium dissociation constant  $K_d$  and mass balance for the target and ligand show:

$$K_d = \frac{TL}{C} \quad (S1)$$

$$T_0 = T + C \Rightarrow T = T_0 - C \quad (S2)$$

$$L_0 = L + C \Rightarrow C = L_0 - L \quad (S3)$$

where  $T$ ,  $L$ , and  $C$  are equilibrium concentrations of the target, ligand and complex, respectively;  $T_0$  and  $L_0$  are the total concentrations of target and ligand, respectively. The definition of fraction of unbound ligand  $R$  ( $0 \leq R \leq 1$ ) is:

$$R = \frac{L}{L_0} \Rightarrow L = RL_0 \quad (S4)$$

By inserting Eq S4 into Eq S3, we have:

$$C = L_0 - RL_0 = L_0(1 - R) \quad (S5)$$

By replacing  $C$  in Eq S2 with Eq S5, we obtain:

$$T = T_0 - L_0(1 - R) \quad (S6)$$

With inserting Eqs S4, S5 and S6 into Eq S1, we obtain:

$$\begin{aligned} K_d &= \frac{[T_0 - L_0(1 - R)]RL_0}{(1 - R)L_0} \\ &= \frac{[T_0 - L_0(1 - R)]R}{(1 - R)} \\ &= \frac{T_0 - L_0(1 - R)}{(1/R - 1)} \end{aligned} \quad (S7)$$

By rearranging Eq S7, we obtain a quadratic equation for  $R$ :

$$L_0R^2 + (K_d + T_0 - L_0)R - K_d = 0 \quad (S8)$$

With the quadratic formula, the expression of  $R$  is solved as

$$R = \frac{-(K_d + T_0 - L_0) \pm \sqrt{(K_d + T_0 - L_0)^2 + 4L_0K_d}}{2L_0} \quad (S9)$$

with two solutions. Now, let's discuss which solution should be picked to satisfy  $0 \leq R \leq 1$ .

When  $(K_d + T_0 - L_0) \geq 0$ , the only solution that satisfies  $R \geq 0$  is:

$$R = \frac{-(K_d + T_0 - L_0) + \sqrt{(K_d + T_0 - L_0)^2 + 4L_0K_d}}{2L_0} \quad (S10)$$

since the other solution leads to negative values for  $R$ .

When  $(K_d + T_0 - L_0) < 0$ ,

$$-(K_d + T_0 - L_0) = |K_d + T_0 - L_0| \quad (S11)$$

$$\sqrt{(K_d + T_0 - L_0)^2 + 4L_0K_d} \geq \sqrt{(K_d + T_0 - L_0)^2} = |K_d + T_0 - L_0|, \text{ since } L_0 \geq 0 \text{ and } K_d > 0 \quad (S12)$$

Therefore, the only solution that satisfies  $R \geq 0$  is also Eq S10:

$$R = \frac{-(K_d + T_0 - L_0) + \sqrt{(K_d + T_0 - L_0)^2 + 4L_0K_d}}{2L_0}$$

since the other solution leads to  $R < 0$ .

To sum up, the theoretical dependence of  $R$  on  $T_0$  is Eq S10 or rearranged to:

$$R = -\frac{K_d + T_0 - L_0}{2L_0} + \sqrt{\left(\frac{K_d + T_0 - L_0}{2L_0}\right)^2 + \frac{K_d}{L_0}} \quad (\text{S13})$$

which is Eq 3 in the main text.

**Note S2: Derivation of the dependence of relative systematic error of  $K_d$  ( $|\Delta K_d/K_d|$ ) on  $L_0/K_d$  ratio for correlated (parallel) error sources**

Eq S7 shows:

$$K_d = \frac{T_0 - L_0(1-R)}{(1/R-1)}$$

When the error sources of  $T_0$ ,  $L_0$  and  $R$  are strongly correlated, the systematic error of  $K_d$  can be calculated with the error propagation rule:

$$|\Delta K_d| = \left| \left( \frac{\partial K_d}{\partial T_0} \right) \Delta T_0 \right| + \left| \left( \frac{\partial K_d}{\partial L_0} \right) \Delta L_0 \right| + \left| \left( \frac{\partial K_d}{\partial R} \right) \Delta R \right| \quad (\text{S14})$$

which gives,

$$\begin{aligned} |\Delta K_d| &= \left| \frac{R}{1-R} \Delta T_0 \right| + \left| -R \Delta L_0 \right| + \left| \left[ -L_0 + \frac{T_0}{(1-R)^2} \right] \Delta R \right| \\ &= \left| \frac{R}{1-R} \right| |\Delta T_0| + |R| |\Delta L_0| + \left| -L_0 + \frac{T_0}{(1-R)^2} \right| |\Delta R| \end{aligned} \quad (\text{S15})$$

Apparently, both  $R/(1-R)$  and  $R$  are greater than zero since  $R \in (0, 1)$  when we use Eq S7 to determine  $K_d$ . Now, to expand and rearrange Eq S15, let's determine the sign of " $-L_0 + T_0/(1-R)^2$ ":

From Eq S7, we obtain,

$$T_0 = (1/R-1)K_d + (1-R)L_0 \quad (\text{S16})$$

Then,

$$\begin{aligned} -L_0 + \frac{T_0}{(1-R)^2} &= \left[ \frac{1/R-1}{(1-R)^2} \right] K_d + \frac{(1-R)}{(1-R)^2} L_0 - L_0 \\ &= \left[ \frac{1-R}{R(1-R)^2} \right] K_d + \frac{1}{1-R} L_0 - L_0 \\ &= \frac{K_d}{R(1-R)} + \frac{R}{1-R} L_0 \end{aligned} \quad (\text{S17})$$

Eq S17 clearly shows that the term “ $-L_0 + T_0/(1 - R)^2$ ” is greater than zero since both  $K_d$  and  $L_0$  are greater than zero and  $R \in (0, 1)$ . Hence, we rewrite Eq S15 as:

$$|\Delta K_d| = \frac{R}{1-R} |\Delta T_0| + R |\Delta L_0| + \left[ -L_0 + \frac{T_0}{(1-R)^2} \right] |\Delta R| \quad (\text{S18})$$

To simplify the analysis, we only focus on the scenario of determined  $R$  equal 0.5, which is close to the least erroneous case.<sup>1</sup> Then, the actual  $R$  is:

$$R = 0.5 - \Delta R, \quad -0.5 < \Delta R < 0.5 \quad (\text{S19})$$

Insert Eq S19 into Eq S16, we can express  $T_0$  with  $L_0$  and  $\Delta R$ ,

$$T_0 = \frac{(0.5 + \Delta R)K_d}{0.5 - \Delta R} + (0.5 + \Delta R)L_0 \quad (\text{S20})$$

Now, divide  $K_d$  for both sides of Eq S18 to analyze the relative systematic error of  $K_d$ :

$$\left| \frac{\Delta K_d}{K_d} \right| = \frac{R}{1-R} \left| \frac{\Delta T_0}{K_d} \right| + R \left| \frac{\Delta L_0}{K_d} \right| + \left[ -\frac{L_0}{K_d} + \frac{1}{(1-R)^2} \frac{T_0}{K_d} \right] |\Delta R| \quad (\text{S21})$$

Or rewrite as:

$$\left| \frac{\Delta K_d}{K_d} \right| = \frac{R}{1-R} \left| \frac{\Delta T_0}{T_0} \right| \left( \frac{T_0}{K_d} \right) + R \left| \frac{\Delta L_0}{L_0} \right| \left( \frac{L_0}{K_d} \right) + \left[ -\frac{L_0}{K_d} + \frac{1}{(1-R)^2} \frac{T_0}{K_d} \right] |\Delta R| \quad (\text{S22})$$

By inserting Eq S19 and Eq S20 into Eq S22, we have:

$$\begin{aligned} \left| \frac{\Delta K_d}{K_d} \right| &= \frac{0.5 - \Delta R}{0.5 + \Delta R} \left| \frac{\Delta T_0}{T_0} \right| \left[ \frac{0.5 + \Delta R}{0.5 - \Delta R} + \left( \frac{L_0}{K_d} \right) (0.5 + \Delta R) \right] + (0.5 - \Delta R) \left| \frac{\Delta L_0}{L_0} \right| \left( \frac{L_0}{K_d} \right) \\ &+ \left[ -\frac{L_0}{K_d} + \frac{1}{(0.5 + \Delta R)^2} \left( \frac{0.5 + \Delta R}{0.5 - \Delta R} + \left( \frac{L_0}{K_d} \right) (0.5 + \Delta R) \right) \right] |\Delta R| \end{aligned} \quad (\text{S23})$$

If we set the absolute value of relative error of  $R$ ,  $|\Delta R/R| = r$  at  $R = 0.5 - \Delta R$  (Eq S19), then:

When  $0 \leq \Delta R < 0.5$ ,

$$\begin{aligned} \Delta R &= Rr = (0.5 - \Delta R)r \\ \Rightarrow \Delta R &= 0.5r - r\Delta R \\ \Rightarrow (1 + r)\Delta R &= 0.5r \\ \Rightarrow \Delta R &= \frac{0.5r}{1 + r} \end{aligned} \quad (\text{S24})$$

When  $-0.5 < \Delta R < 0$ ,

$$\begin{aligned} \Delta R &= -Rr = (\Delta R - 0.5)r \\ \Rightarrow \Delta R &= r\Delta R - 0.5r \\ \Rightarrow (r - 1)\Delta R &= 0.5r \\ \Rightarrow \Delta R &= \frac{0.5r}{r - 1} \end{aligned} \quad (\text{S25})$$

If we also set the absolute values of relative errors of  $T_0$  and  $L_0$  as  $|\Delta T_0/T_0| = t$  and  $|\Delta L_0/L_0| = l$ , respectively, Eq S23 is rearranged to (we omit some intermediate steps here):

When  $0 \leq \Delta R < 0.5$ ,

$$\begin{aligned} \left| \frac{\Delta K_d}{K_d} \right| &= \frac{0.5t}{0.5+r} \left[ \frac{0.5+r}{0.5} + \frac{0.5+r}{1+r} \frac{L_0}{K_d} \right] + \frac{0.5l}{1+r} \frac{L_0}{K_d} \\ &\quad + \left[ -\frac{L_0}{K_d} + \left( \frac{1+r}{0.5+r} \right)^2 \left( \frac{0.5+r}{0.5} + \frac{0.5+r}{1+r} \frac{L_0}{K_d} \right) \right] \frac{0.5r}{1+r} \end{aligned} \quad (S26)$$

$$\Rightarrow \left| \frac{\Delta K_d}{K_d} \right| = \left[ t + \frac{r(1+r)}{0.5+r} \right] + \left[ \frac{0.5(t+l-r)}{1+r} + \frac{0.5r}{0.5+r} \right] \frac{L_0}{K_d} \quad (S27)$$

When  $-0.5 < \Delta R < 0$ ,

$$\begin{aligned} \left| \frac{\Delta K_d}{K_d} \right| &= \frac{0.5t}{0.5-r} \left[ \frac{0.5-r}{0.5} + \frac{r-0.5}{r-1} \frac{L_0}{K_d} \right] + \frac{0.5l}{1-r} \frac{L_0}{K_d} \\ &\quad + \left[ -\frac{L_0}{K_d} + \left( \frac{r-1}{r-0.5} \right)^2 \left( \frac{0.5-r}{0.5} + \frac{r-0.5}{r-1} \frac{L_0}{K_d} \right) \right] \frac{0.5r}{1-r} \end{aligned} \quad (S28)$$

$$\Rightarrow \left| \frac{\Delta K_d}{K_d} \right| = \left[ t + \frac{r(1-r)}{0.5-r} \right] + \left[ \frac{0.5(t+l-r)}{1-r} + \frac{0.5r}{0.5-r} \right] \frac{L_0}{K_d} \quad (S29)$$

Or we can write Eq S27 and Eq S29 as:

$$\begin{aligned} \left| \frac{\Delta K_d}{K_d} \right| &= a + b \frac{L_0}{K_d}, \\ a &= t + \frac{r(1+r)}{0.5+r}, \quad b = \frac{0.5(t+l-r)}{1+r} + \frac{0.5r}{0.5+r} \quad \text{when } 0 \leq \Delta R < 0.5 \\ a &= t + \frac{r(1-r)}{0.5-r}, \quad b = \frac{0.5(t+l-r)}{1-r} + \frac{0.5r}{0.5-r} \quad \text{when } -0.5 < \Delta R < 0 \end{aligned} \quad (S30)$$

which is Eq 6 in the main text.

### Note S3: Derivation of the theoretical dependence of determined $K_d$ ( $K_{d,det}$ ) on $L_0$ for correlated (parallel) error sources

The definition of  $\Delta K_d$  is (with  $K_{d,det}$  representing the determined  $K_d$  value),

$$\Delta K_d = K_{d,det} - K_d \quad (S31)$$

By inserting Eq S31 into Eq S30, we get:

$$\left| \frac{K_{d,det} - K_d}{K_d} \right| = a + b \frac{L_0}{K_d} \quad (S32)$$

By multiplying  $K_d$  on both sides of Eq S32, we have:

$$\left| K_{d,det} - K_d \right| = aK_d + bL_0 \quad (S33)$$



When  $\Delta K_d \geq 0$ ,

$$\begin{aligned} K_{d,det} - K_d &= aK_d + bL_0 \\ \Rightarrow K_{d,det} &= (1+a)K_d + bL_0 \end{aligned} \quad (S34)$$

When  $\Delta K_d < 0$ ,

$$\begin{aligned} K_d - K_{d,det} &= aK_d + bL_0 \\ \Rightarrow K_{d,det} &= (1-a)K_d - bL_0 \end{aligned} \quad (S35)$$

Or we can combine Eqs S34 and S35 to be:

$$\begin{aligned} K_{d,det} &= c + dL_0, \quad c = (1+a)K_d, \quad d = b \quad \text{when } \Delta K_d \geq 0 \\ c &= (1-a)K_d, \quad d = -b \quad \text{when } \Delta K_d < 0 \end{aligned} \quad (S36)$$

Eq S36 (Eq 7 in the main text) suggests that the dependence of  $K_{d,det}$  on  $L_0$  is linear in the whole range of  $L_0$ .

**Note S4: Derivation of the dependence of relative systematic error of  $K_d$  ( $|\Delta K_d/K_d|$ ) on  $L_0/K_d$  ratio for uncorrelated (orthogonal) error sources**

When the error sources of  $T_0$ ,  $L_0$  and  $R$  are uncorrelated, the systematic error of  $K_d$  can be calculated with the error propagation rule:

$$|\Delta K_d| = \sqrt{\left(\frac{\partial K_d}{\partial T_0}\right)^2 \Delta T_0^2 + \left(\frac{\partial K_d}{\partial L_0}\right)^2 \Delta L_0^2 + \left(\frac{\partial K_d}{\partial R}\right)^2 \Delta R^2} \quad (S37)$$

By applying Eq S37 to Eq S7 and square both sides, we have,

$$\Delta K_d^2 = \left(\frac{R}{1-R}\right)^2 \Delta T_0^2 + R^2 \Delta L_0^2 + \left[-L_0 + \frac{T_0}{(1-R)^2}\right]^2 \Delta R^2 \quad (S38)$$

By replacing  $T_0$  with Eq S16, we obtain:

$$\Delta K_d^2 = \left(\frac{R}{1-R}\right)^2 \Delta T_0^2 + R^2 \Delta L_0^2 + \left[\left(\frac{1}{1-R}\right)\left(\frac{K_d}{R} + RL_0\right)\right]^2 \Delta R^2 \quad (S39)$$

Now, divide  $K_d^2$  for both sides of Eq S39 to analyze the relative systematic error of  $K_d$ :

$$\begin{aligned}
\left(\frac{\Delta K_d}{K_d}\right)^2 &= \left(\frac{R}{1-R}\right)^2 \left(\frac{\Delta T_0}{K_d}\right)^2 + R^2 \left(\frac{\Delta L_0}{K_d}\right)^2 + \left[\left(\frac{1}{1-R}\right)\left(\frac{1}{R} + R\frac{L_0}{K_d}\right)\right]^2 \Delta R^2 \\
&= \left(\frac{R}{1-R}\right)^2 \left(\frac{\Delta T_0}{T_0}\right)^2 \left(\frac{T_0}{K_d}\right)^2 + R^2 \left(\frac{\Delta L_0}{L_0}\right)^2 \left(\frac{L_0}{K_d}\right)^2 + \left[\left(\frac{1}{1-R}\right)\left(\frac{1}{R} + R\frac{L_0}{K_d}\right)\right]^2 \Delta R^2 \\
&= \left(\frac{R}{1-R}\right)^2 \left(\frac{\Delta T_0}{T_0}\right)^2 \left[\frac{(1/R-1)K_d + (1-R)L_0}{K_d}\right]^2 + R^2 \left(\frac{\Delta L_0}{L_0}\right)^2 \left(\frac{L_0}{K_d}\right)^2 + \left[\left(\frac{1}{1-R}\right)\left(\frac{1}{R} + R\frac{L_0}{K_d}\right)\right]^2 \Delta R^2 \\
&= \left(1 + R\frac{L_0}{K_d}\right)^2 \left(\frac{\Delta T_0}{T_0}\right)^2 + R^2 \left(\frac{\Delta L_0}{L_0}\right)^2 \left(\frac{L_0}{K_d}\right)^2 + \left[\left(\frac{1}{1-R}\right)\left(\frac{1}{R} + R\frac{L_0}{K_d}\right)\right]^2 \Delta R^2
\end{aligned} \tag{S40}$$

After setting  $|\Delta T_0/T_0| = t$  and  $|\Delta L_0/L_0| = l$ , Eq S40 can be expanded and rearranged to be:

$$\left(\frac{\Delta K_d}{K_d}\right)^2 = \left[t^2 + \left(\frac{\Delta R}{R(1-R)}\right)^2\right] + \left[2Rt^2 + 2\left(\frac{\Delta R}{1-R}\right)^2\right] \frac{L_0}{K_d} + \left[R^2t^2 + R^2l^2 + \left(\frac{R\Delta R}{1-R}\right)^2\right] \left(\frac{L_0}{K_d}\right)^2 \tag{S41}$$

When  $0 \leq \Delta R < 0.5$ , by inserting Eqs S19 and S24 into Eq S41, we obtain:

$$\left(\frac{\Delta K_d}{K_d}\right)^2 = \left[t^2 + \left(\frac{r(1+r)}{0.5+r}\right)^2\right] + \left[\frac{t^2}{1+r} + 2\left(\frac{0.5r}{0.5+r}\right)^2\right] \frac{L_0}{K_d} + \left[\left(\frac{0.5}{1+r}\right)^2 \left(t^2 + l^2 + \left(\frac{0.5r}{0.5+r}\right)^2\right)\right] \left(\frac{L_0}{K_d}\right)^2 \tag{S42}$$

or

$$\left|\frac{\Delta K_d}{K_d}\right| = \sqrt{\left[t^2 + \left(\frac{r(1+r)}{0.5+r}\right)^2\right] + \left[\frac{t^2}{1+r} + 2\left(\frac{0.5r}{0.5+r}\right)^2\right] \frac{L_0}{K_d} + \left[\left(\frac{0.5}{1+r}\right)^2 \left(t^2 + l^2 + \left(\frac{0.5r}{0.5+r}\right)^2\right)\right] \left(\frac{L_0}{K_d}\right)^2} \tag{S43}$$

When  $-0.5 < \Delta R < 0$ , by inserting Eqs S19 and S25 into Eq S41, we obtain:

$$\left(\frac{\Delta K_d}{K_d}\right)^2 = \left[t^2 + \left(\frac{r(1-r)}{r-0.5}\right)^2\right] + \left[\frac{t^2}{1-r} + 2\left(\frac{0.5r}{r-0.5}\right)^2\right] \frac{L_0}{K_d} + \left[\left(\frac{0.5}{1-r}\right)^2 \left(t^2 + l^2 + \left(\frac{0.5r}{r-0.5}\right)^2\right)\right] \left(\frac{L_0}{K_d}\right)^2 \tag{S44}$$

or

$$\left|\frac{\Delta K_d}{K_d}\right| = \sqrt{\left[t^2 + \left(\frac{r(1-r)}{r-0.5}\right)^2\right] + \left[\frac{t^2}{1-r} + 2\left(\frac{0.5r}{r-0.5}\right)^2\right] \frac{L_0}{K_d} + \left[\left(\frac{0.5}{1-r}\right)^2 \left(t^2 + l^2 + \left(\frac{0.5r}{r-0.5}\right)^2\right)\right] \left(\frac{L_0}{K_d}\right)^2} \tag{S45}$$

We can combine Eqs S43 and S45 as:

$$\begin{aligned}
\left| \frac{\Delta K_d}{K_d} \right| &= \sqrt{\alpha^2 + \lambda \frac{L_0}{K_d} + \beta^2 \left( \frac{L_0}{K_d} \right)^2}, \\
\alpha &= \sqrt{t^2 + \left( \frac{r+r^2}{0.5+r} \right)^2}, \quad \beta = \frac{0.5}{1+r} \sqrt{t^2 + l^2 + \left( \frac{0.5r}{0.5+r} \right)^2}, \quad \lambda = \frac{t^2}{1+r} + 2 \left( \frac{0.5r}{0.5+r} \right)^2 \quad \text{when } 0 \leq \Delta R < 0.5 \\
\alpha &= \sqrt{t^2 + \left( \frac{r-r^2}{r-0.5} \right)^2}, \quad \beta = \frac{0.5}{1-r} \sqrt{t^2 + l^2 + \left( \frac{0.5r}{r-0.5} \right)^2}, \quad \lambda = \frac{t^2}{1-r} + 2 \left( \frac{0.5r}{r-0.5} \right)^2 \quad \text{when } -0.5 < \Delta R < 0
\end{aligned} \tag{S46}$$

**Note S5: Derivation of the theoretical dependence of determined  $K_d$  ( $K_{d,\text{det}}$ ) on  $L_0$  for uncorrelated (orthogonal) error sources**

By inserting Eq S31 into Eq S46, we get:

$$\left| \frac{K_{d,\text{det}} - K_d}{K_d} \right| = \sqrt{\alpha^2 + \lambda \frac{L_0}{K_d} + \beta^2 \left( \frac{L_0}{K_d} \right)^2} \tag{S47}$$

By multiplying  $K_d$  on both sides of Eq S47, we have:

$$|K_{d,\text{det}} - K_d| = \sqrt{\alpha^2 K_d^2 + \lambda K_d L_0 + \beta^2 L_0^2} \tag{S48}$$

When  $\Delta K_d \geq 0$ ,

$$\begin{aligned}
K_{d,\text{det}} - K_d &= \sqrt{\alpha^2 K_d^2 + \lambda K_d L_0 + \beta^2 L_0^2} \\
\Rightarrow K_{d,\text{det}} &= K_d + \sqrt{\alpha^2 K_d^2 + \lambda K_d L_0 + \beta^2 L_0^2}
\end{aligned} \tag{S49}$$

From Eq S49, we can infer that, at low  $L_0$  range,  $K_{d,\text{det}}$  approaches a constant:

$$K_{d,\text{det}} = K_d + \alpha K_d = (1 + \alpha) K_d \tag{S50}$$

Or we can write,

$$K_{d,\text{det}} = \gamma, \quad \gamma = (1 + \alpha) K_d \tag{S51}$$

At high  $L_0$  range, the dependency of  $K_{d,\text{det}}$  on  $L_0$  approaches a linear equation:

$$K_{d,\text{det}} = \beta L_0 \tag{S52}$$

When  $\Delta K_d < 0$ ,

$$\begin{aligned}
K_d - K_{d,\text{det}} &= \sqrt{\alpha^2 K_d^2 + \lambda K_d L_0 + \beta^2 L_0^2} \\
\Rightarrow K_{d,\text{det}} &= K_d - \sqrt{\alpha^2 K_d^2 + \lambda K_d L_0 + \beta^2 L_0^2}
\end{aligned} \tag{S53}$$

From Eq S53, we can infer that, at low  $L_0$  range,  $K_{d,\text{det}}$  approaches a constant:

$$K_{d,\text{det}} = K_d - \alpha K_d = (1 - \alpha) K_d \tag{S54}$$

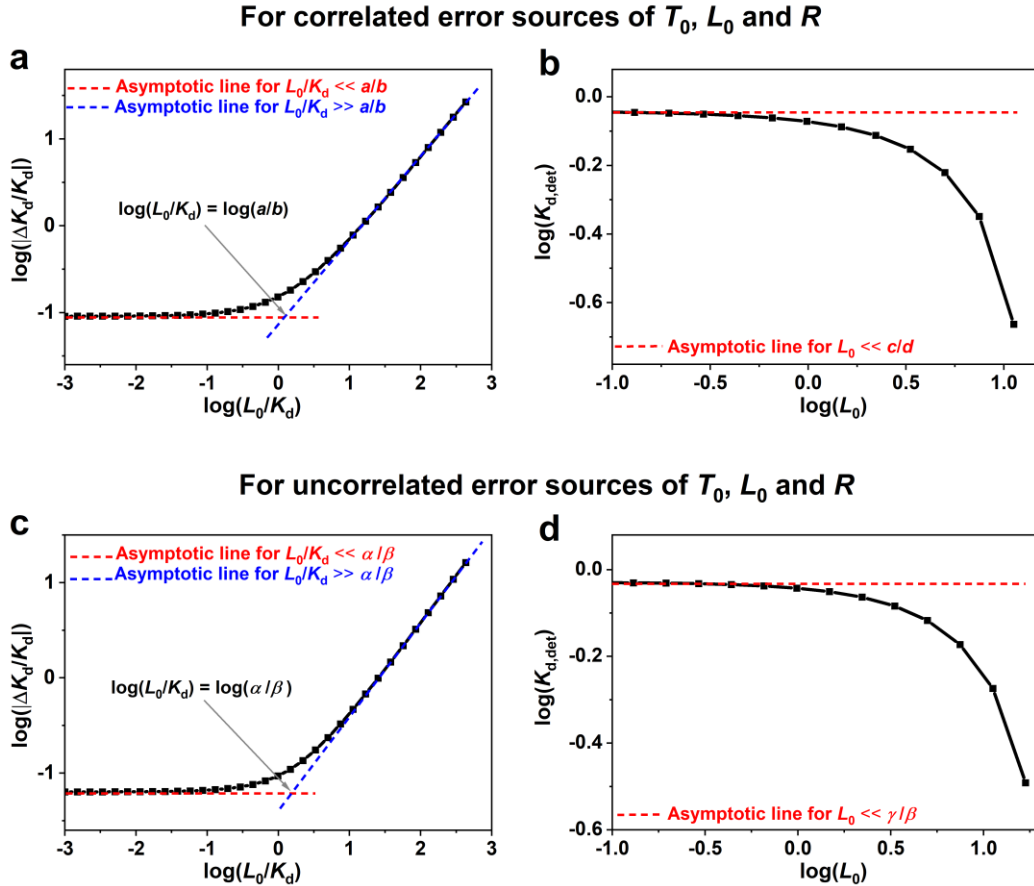
Or we use the same parameter  $\gamma$  to express Eq S54 as,

$$K_{d,\text{det}} = \gamma, \gamma = (1 - \alpha)K_d \quad (\text{S55})$$

The dependency of  $K_{d,\text{det}}$  on  $L_0$  at high  $L_0$  range is unavailable because  $K_{d,\text{det}}$  is required to be greater than zero.

**Figure S1: General trends in dependencies of  $\log(|\Delta K_d/K_d|)$  on  $\log(L_0/K_d)$  and  $\log(K_{d,\text{det}})$  on  $\log(L_0)$**

Panels (a) and (c) show the general trends in dependency of  $\log(|\Delta K_d/K_d|)$  on  $\log(L_0/K_d)$ ; panels (b) and (d) show the general trends in dependency of  $\log(K_{d,\text{det}})$  on  $\log(L_0)$ . In all panels, reasonable relative errors of  $L_0$ ,  $T_0$  and  $R$  were used:  $|\Delta T_0/T_0| = |\Delta L_0/L_0| = 0.05$  and  $\Delta R/R = -0.02$ . In panels (b) and (d), we set  $K_d = 1$  (unitless) and  $\Delta K_d < 0$ . With these conditions, the parameters  $a$ ,  $b$ ,  $c$  and  $d$  were calculated to be 0.011, 0.040, 0.99 and  $-0.040$ , respectively;  $\alpha$ ,  $\beta$  and  $\gamma$  were calculated to be 0.064, 0.037 and 0.94, respectively.



With Eqs S30, S36, S46 and S51, we produced representative graphs to show the dependence of  $|\Delta K_d/K_d|$  on  $L_0/K_d$  and the dependence of  $K_{d,\text{det}}$  on  $L_0$  in double-log scale. The graphs corresponding to  $0 \leq \Delta R < 0.5$  and  $\Delta K_d \geq 0$  are shown in Figure 2 in the main text, and the graphs corresponding to  $-0.5 < \Delta R < 0$  and  $\Delta K_d < 0$  are shown in Figure S1. Figures 2a, 2c (in main text) and Figures S1a, S1c show the same general trends in dependency of  $\log(|\Delta K_d/K_d|)$  on  $\log(L_0/K_d)$ . Although Figures S1b and S1d cannot show the part at high  $L_0$  range (where  $K_{d,\text{det}}$  equals physically unfeasible negative values), the results in Figure S1b and S1d suggest a dependency of  $K_{d,\text{det}}$  on  $L_0$  that is similar to the trend shown in Figures 2b and 2d (in main text):



$K_{d,det}$  is insensitive to the change of  $L_0$  when  $L_0$  is at low concentrations, while the sensitivity of  $K_{d,det}$  to  $L_0$  increases with the increase of  $L_0$ .

**Note S6: Derivation of the relationship between signal to noise ratio ( $S/N$ ) and the limit of quantitation (LOQ)**

The limit of quantitation (LOQ) of an instrument is defined as the analyte concentration (ligand concentration in our case) for which the signal to noise ratio ( $S/N$ ) is equal to a certain value  $X \gg 1$  (e.g.,  $X = 10$ ) which guarantees that the noise does not affect quantitation significantly, which can be expressed as:

$$\frac{S_{LOQ}}{N} = X \Rightarrow S_{LOQ} = XN \quad (S56)$$

where  $S_{LOQ}$  is the signal measured at LOQ. For one instrument and a specific ligand, since the measured signal  $S$  is proportional to the ligand concentration, we can assume:

$$S_{LOQ} = k \cdot LOQ \Rightarrow k = \frac{S_{LOQ}}{LOQ} = \frac{XN}{LOQ} \quad (S57)$$

where factor  $k$  is the ratio of measured signal to ligand concentration. Now, if the noise  $N$  is independent of the signal  $S$ , the signal to noise ratio for any total ligand concentration  $L_0$  can be calculated as:

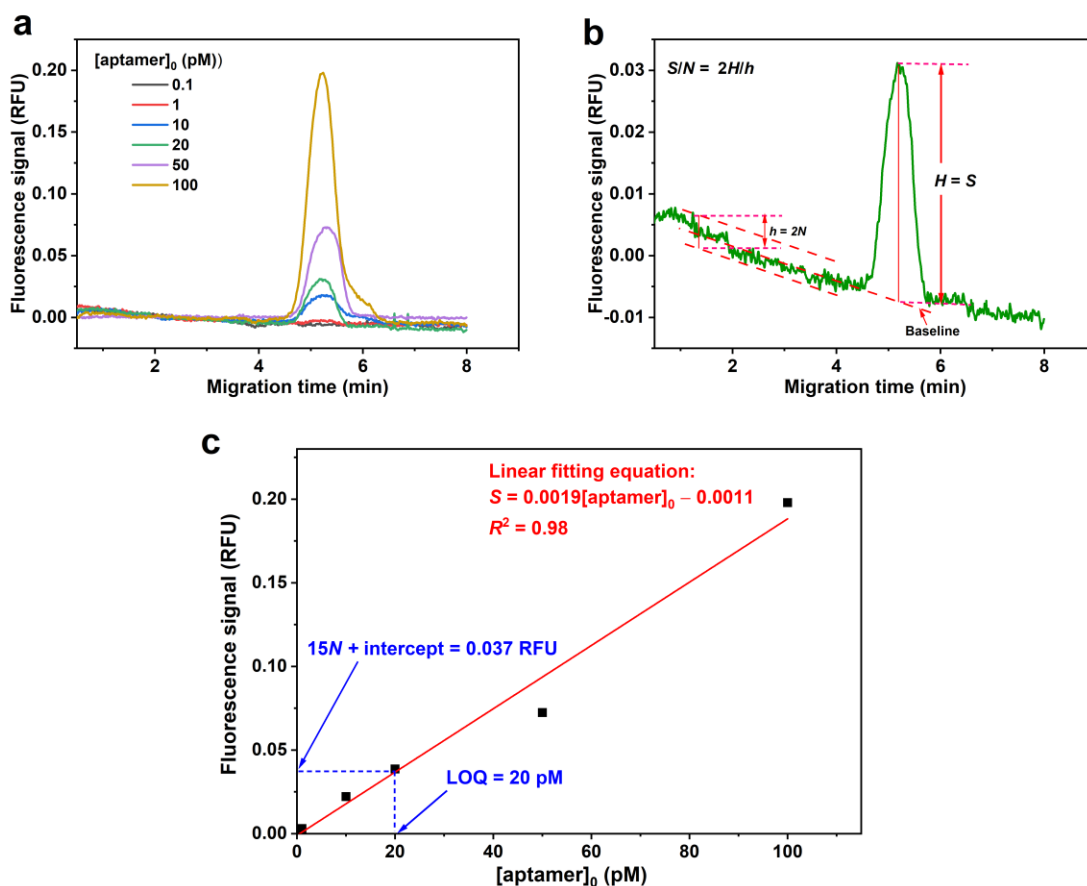
$$\begin{aligned} \frac{S}{N} &= \frac{k \cdot L_0}{N} = \frac{XN}{LOQ} \cdot \frac{L_0}{N} \\ \Rightarrow \frac{S}{N} &= X \frac{L_0}{LOQ} \end{aligned} \quad (S58)$$

The minimum acceptable ligand concentration which satisfies Eq S58 is  $L_{0,min} = LOQ$ .

## Section S2: Experiments

### Figure S2: Determination of limit of quantitation (LOQ)

**a)** Electropherograms of the measurements for samples of MutS-binding aptamer with concentrations of 0.1, 1, 10, 20, 50, and 100 pM. The instrumental setup and experimental procedure are described in “Materials and Methods” section in the main text. **b)** An example ( $[\text{aptamer}]_0 = 20 \text{ pM}$ ) of determining signal and noise for an electropherogram: the intensity difference between the highest noise peak and the lowest noise peak was determined as the noise height  $h$ , which equals 2 times noise  $N$ ; the midline of the noise peaks was determined as the baseline; the intensity difference between the highest signal of the signal peak and the baseline was determined as signal height  $H$ , which equal the signal  $S$ . **c)** Determining LOQ based on the linear plotting of “signal versus  $[\text{aptamer}]_0$ ”.

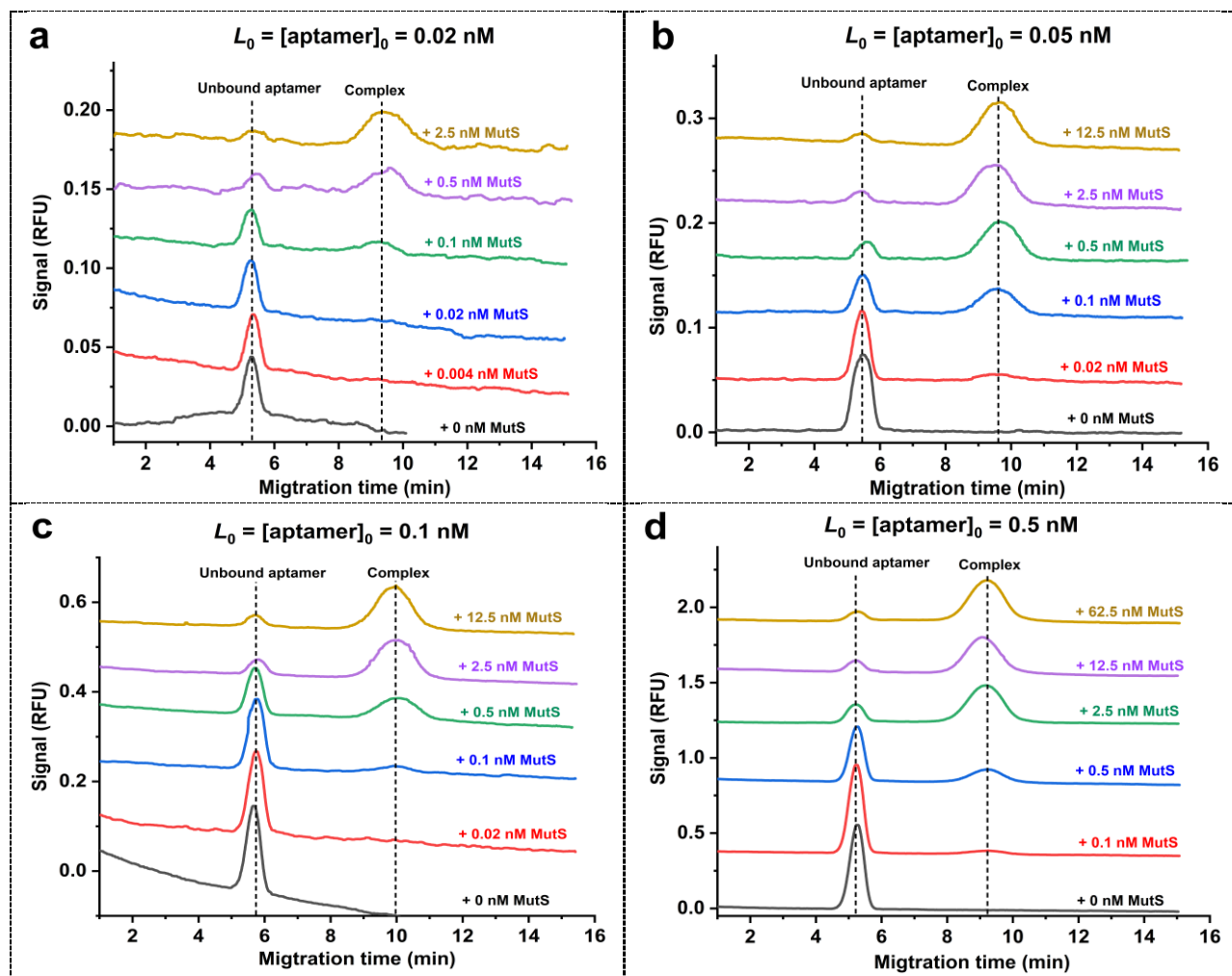


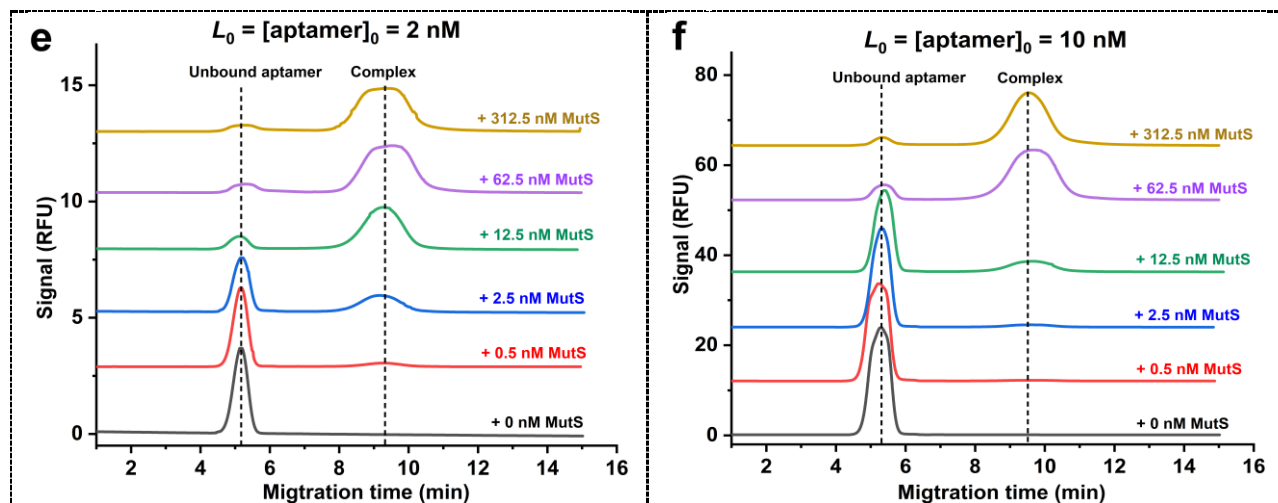
To determine the LOQ of our laser-induced fluorescence (LIF) detection system for the fluorescently labeled MutS-binding aptamer, we measured the aptamer samples with concentrations ( $L_0$  or  $[\text{aptamer}]_0$ ) of 0.1, 1, 10, 20, 50, and 100 pM. In the measurements, the instrumental setup and experimental procedure were identical to that used in the NECEEM (nonequilibrium capillary electrophoresis of equilibrium mixtures) experiments as explained in “Materials and Methods” section in the main text. The obtained electropherograms are shown in Figure S2a. Based on the electropherograms, the signals of aptamer at each concentration were determined to be 0.0020, 0.0031, 0.022, 0.039, 0.072, and 0.20 RFU, respectively. By analyzing all six electropherograms, the average noise was determined to be 0.0025 RFU. One example of determining signal ( $S$ ), noise ( $N$ ), and  $S/N$  is demonstrated in Figure S2b. Then, the results of “signal versus

[aptamer]<sub>0</sub>” were plotted (Figure S2c), and a linear fitting was applied to the data points. Conventionally, the analyte concentration corresponding to signal to noise ratio ( $S/N$ ) of 10 (i.e.,  $X = 10$  in Eq S56) is defined as LOQ.<sup>2-4</sup> In this work, considering the signal of unbound aptamer decreases with the increase of protein concentration, we chose the [aptamer]<sub>0</sub> corresponding to  $S/N = 15$  as LOQ. For different applications, one can choose different values for  $X$  in Eq S57 to achieve a desired accuracy and precision of measured signals at LOQ. By inserting the signal equal 15 times of average noise plus the intercept of the fitting line (for correcting the signal offset caused by background signal) into Figure S2c, LOQ for this work was determined to be 20 pM (i.e., 0.02 nM).

### Figure S3: Electropherograms of the NECEEM experiments

Panels a–f show the electropherograms of the NECEEM experiments with  $L_0$  ([aptamer]<sub>0</sub>) of 0.02, 0.05, 0.1, 0.5, 2, and 10 nM, respectively. The instrumental setup and experimental procedure are explained in “Materials and Methods” section in the main text.





Based on the electropherograms shown in Figure S3, the areas of the peaks for unbound aptamer and complex were measured with NECEEM Area Analysis Program (NAAP) that can be downloaded from <https://www.yorku.ca/skrylov/resources.html>. In each set of NECEEM experiments, since the corresponding highest MutS concentration was much greater than the expected  $K_d$  value range of  $\leq 0.1$  nM, we presumed that the highest MutS concentration in each set of NECEEM experiments saturated the binding to the aptamer, and the remaining unbound aptamer was the impurity (*i.e.*, non-binders) in the aptamer sample. Therefore, from each unbound aptamer area, we subtracted the unbound aptamer area in corresponding “aptamer + highest MutS concentration” electropherogram to obtain the actual unbound aptamer area. Then, the determined areas were corrected by dividing them by their migration times.<sup>1</sup> Based on the corrected areas, the actual fractions of unbound aptamer ( $R$  values) at different  $T_0$  ( $[MutS]_0$ ) were calculated, and the results are shown in Table S1. The binding isotherms for the NECEEM experiments are shown in Figure 3a in the main text.

**Table S1: Results of  $R$  values at different  $T_0$  ( $[MutS]_0$ ) for the NECEEM experiments with different  $L_0$  ( $[aptamer]_0$ )**

$T_0$ (nM)	$R$ ( $L_0 = 0.02$ nM)	$R$ ( $L_0 = 0.05$ nM)	$R$ ( $L_0 = 0.1$ nM)	$R$ ( $L_0 = 0.5$ nM)	$R$ ( $L_0 = 2$ nM)	$R$ ( $L_0 = 10$ nM)
0	1.0	1.0	1.0	1.0	1.0	1.0
0.004	0.96	N/A	N/A	N/A	N/A	N/A
0.02	0.81	0.86	1.0	N/A	N/A	N/A
0.1	0.42	0.45	0.89	0.94	N/A	N/A
0.5	0.17	0.11	0.52	0.73	0.92	0.99
2.5	0.0	0.066	0.073	0.15	0.65	0.97
12.5	N/A	0.0	0.0	0.053	0.10	0.84
62.5	N/A	N/A	N/A	0.0	0.041	0.098
312.5	N/A	N/A	N/A	N/A	0.0	0.0



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