

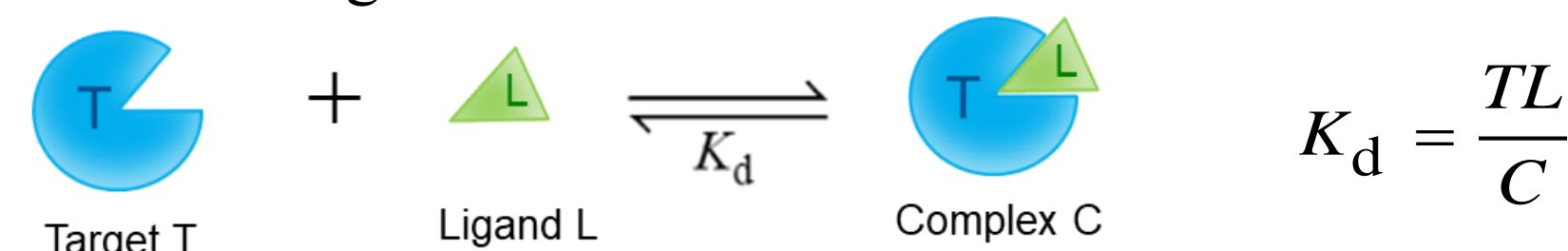
## 1. Abstract

The equilibrium dissociation constant ( $K_d$ ) characterizes stability of non-covalent molecular complexes. Determining  $K_d$  of complexes may be extremely inaccurate if the ratio between the concentration of the limiting component ( $L_0$ ) and the *a priori* unknown value of  $K_d$  exceeds an unknown threshold ratio. The only known approach to reveal this kind of inaccuracy in  $K_d$  requires building multiple experimental binding isotherms; it is resource intensive and, therefore, used very rarely. We introduced a single-isotherm approach for assessing  $K_d$  accuracy *via* determining the value of  $L_0/K_d$ , estimating the threshold ratio, and comparing  $L_0/K_d$  to the threshold ratio. In this proof-of-concept work, we present the theoretical basis and develop a step-by-step algorithm for our single-isotherm approach. We also demonstrate the experimental use of the developed algorithm.

## 2. Introduction

### Model:

Reversible binding reaction:

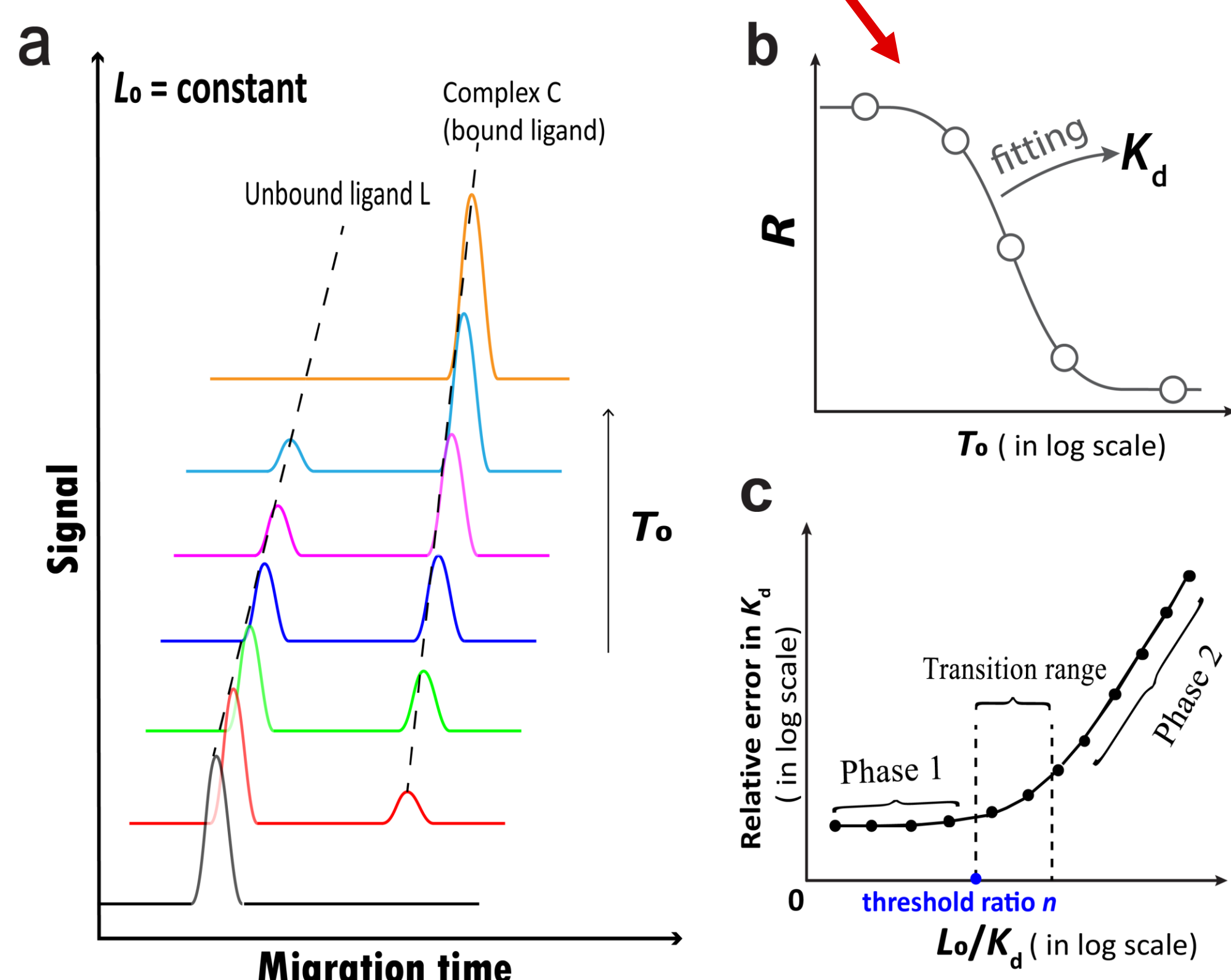


$T$ ,  $L$ , and  $C$  are equilibrium concentrations of the target, ligand, and complex, respectively.

### The classical approach of determining $K_d$ in equilibrium methods and the problem faced:<sup>1</sup>

- Calculate the fraction of unbound ligand  $R = L/L_0$  based on the measured signals.
- Build the binding isotherm of “ $R$  vs  $T_0$ ”.
- Fit the binding isotherm with theoretical dependence of  $R$  on  $T_0$  to determine  $K_d$ .

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



Measure signals from unbound and bound ligand at different total target concentrations ( $T_0$ ) by physically separating the ligand ( $L$ ) from the complex ( $C$ ).

**Problem:** Experimentally determined  $K_d$  can be significantly inaccurate if the ratio between the concentration of the limiting component ( $L_0$ ) and the *a priori* unknown value of  $K_d$  exceeds an unknown threshold value.

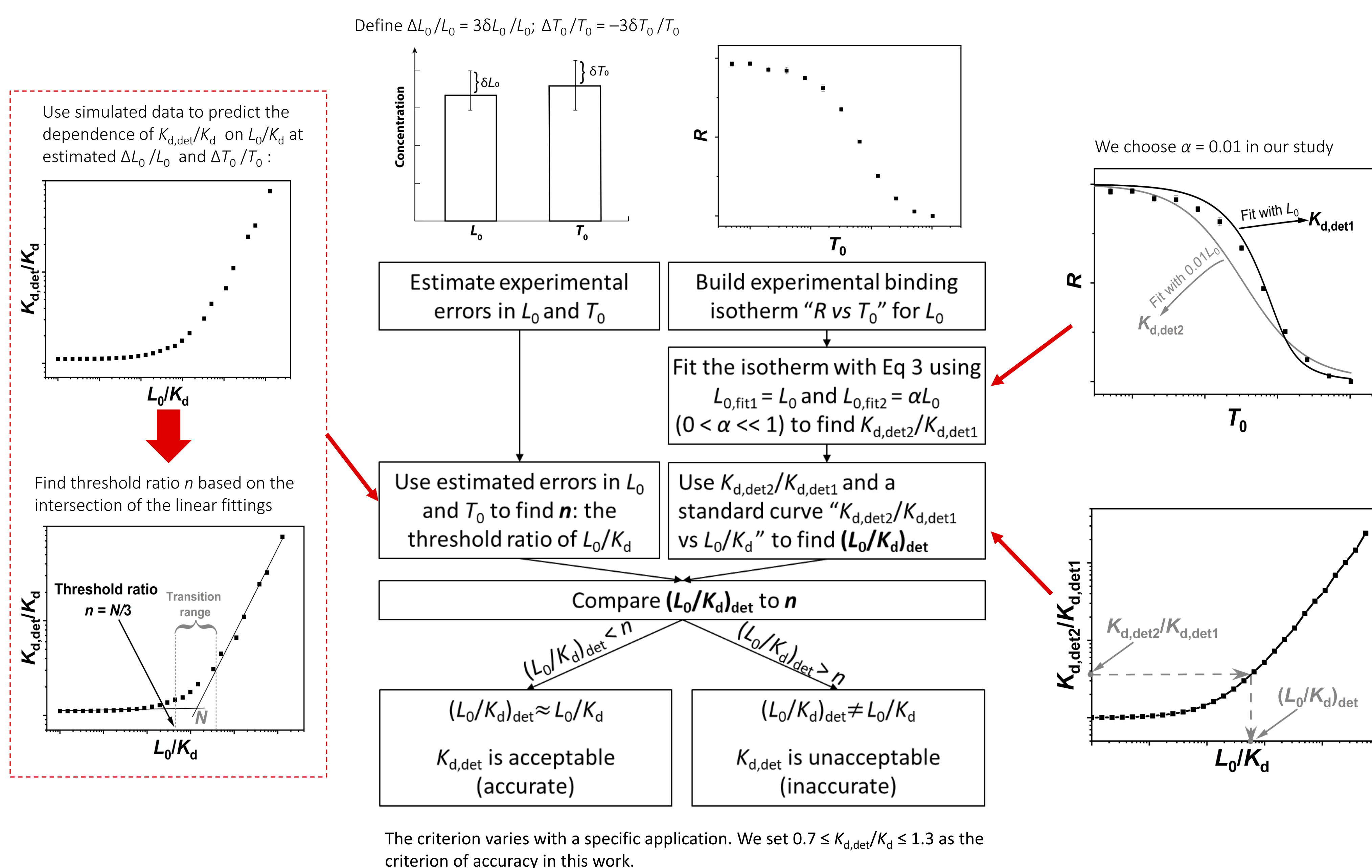
## 3. Previous solution

Experimentally obtain multiple binding isotherms within a range of  $L_0$  values. If determined  $K_d$  ( $K_{d,det}$ ) is found to be insensitive to changing  $L_0$ , then it is assumed that determined  $K_{d,det} \approx K_d$  (real  $K_d$  value).

**Disadvantage:** This approach drastically increases the amount of required experimental work and has been reported only in approximately 5% of publications concerned with the determination of  $K_d$ .<sup>2</sup>

## 4. Our Solution

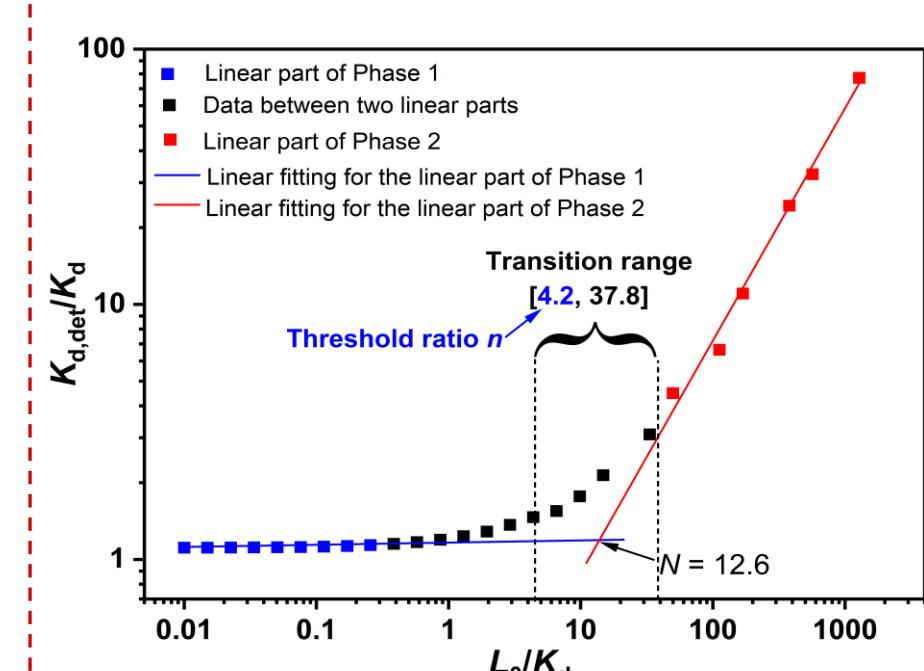
### $K_d$ -accuracy-assessment assay using a single binding isotherm



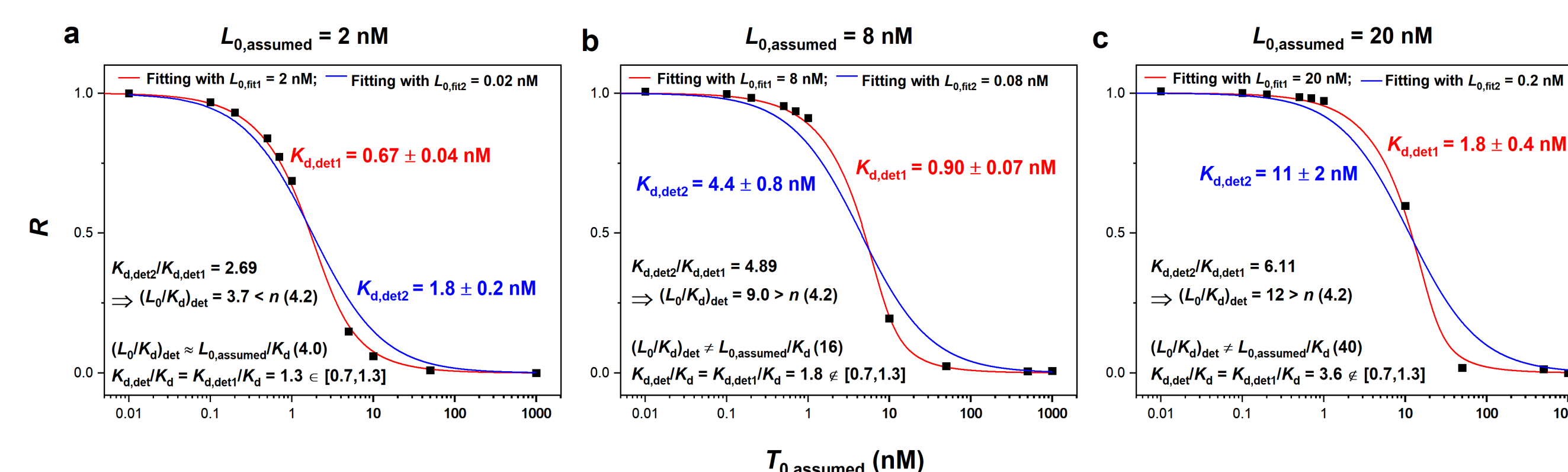
**Advantages:** This assay does not require additional binding isotherms. It will equip researchers with a “no-cost” analytical tool for assessing the accuracy of  $K_d$  values determined in equilibrium experiments.

## 5. Validate $K_d$ -accuracy-assessment assay with virtual experiments

Assume  $\Delta L_0/L_0 = 0.1$  and  $\Delta T_0/T_0 = -0.1$ . Determine the threshold ratio with the approach introduced in the assay

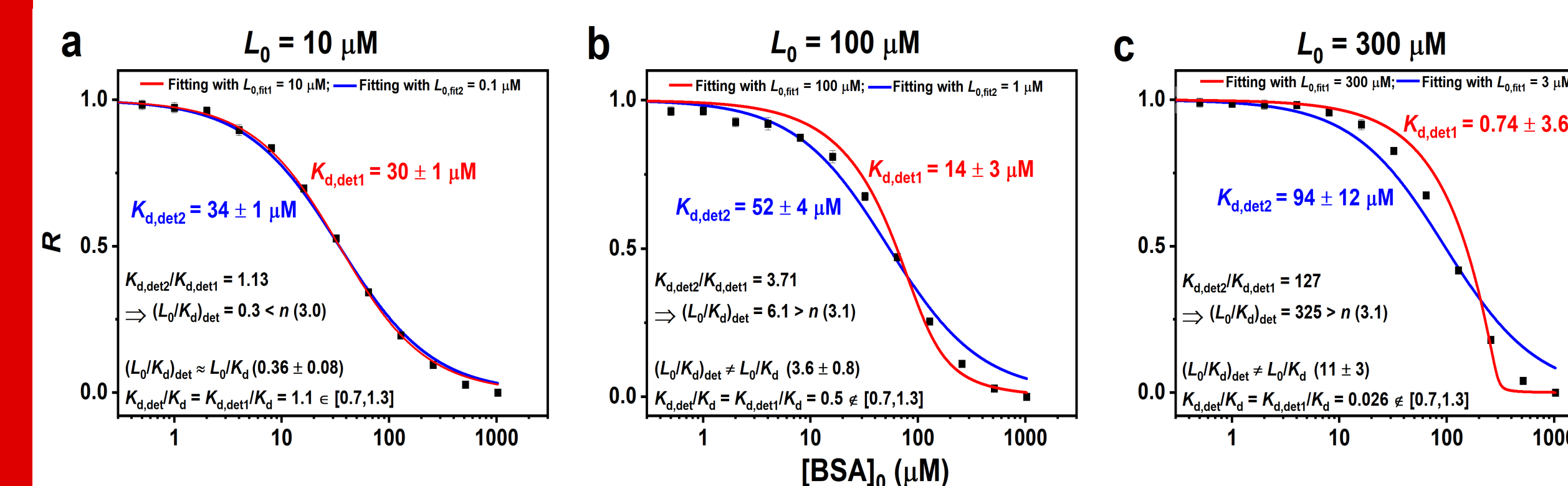


The results of virtual experiments conducted with COMSOL Multi-physics software fully validated the  $K_d$ -accuracy-assessment assay.



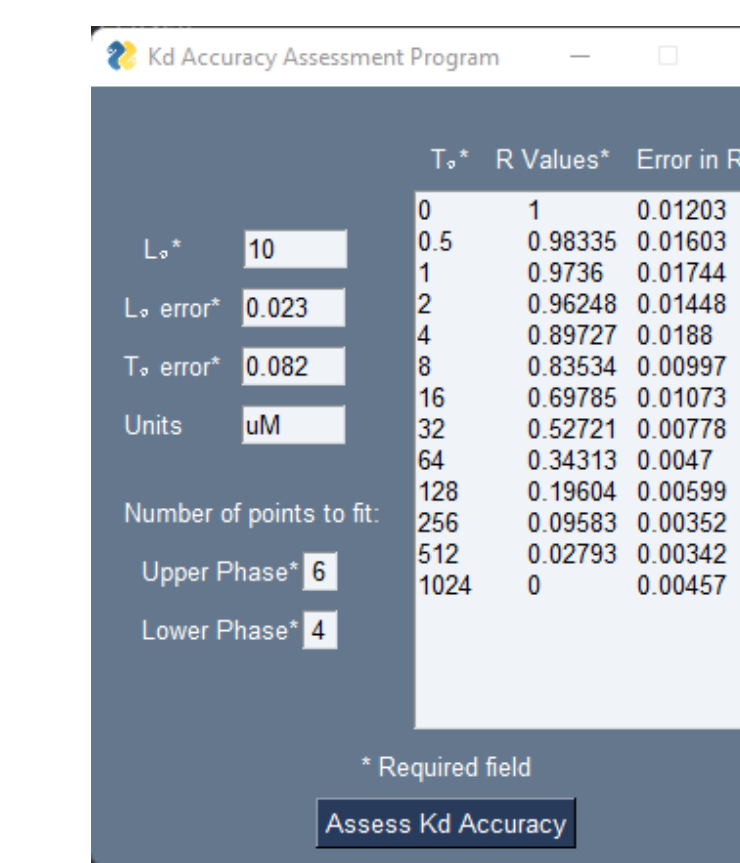
## 6. Validate $K_d$ -accuracy-assessment assay with real experiments

- $K_d$ -determination method:** Accurate Constant via Transition Incomplete Separation (ACTIS)<sup>3</sup>
- Molecular pair:** BSA–fluorescein
- Reference  $K_d$  value:**  $28 \pm 6 \mu\text{M}$



The results of real also confirmed the validation of the  $K_d$ -accuracy-assessment assay.

## 7. Python program



One click

$K_{d,det}$  is acceptable

$K_{d,det}$  is unacceptable

## 8. Conclusion

- In this proof-of-principle study, we introduced an approach for assessing the accuracy of a determined  $K_d$  value from a single experimental binding isotherm, which was named as  $K_d$ -accuracy-assessment assay.
- $K_d$ -accuracy-assessment assay can assess the accuracy of determined  $K_d$  by solving two problems directly: (i) determining the threshold value for  $L_0/K_d$  under estimated errors in  $L_0$  and  $T_0$  and (ii) finding out if  $L_0/K_d$  is smaller than the threshold.
- The assay was validated with both virtual and real experiments.
- Using the algorithm of  $K_d$ -accuracy-assessment assay, we developed an Python program that can assess the accuracy of determined  $K_d$  in a few seconds.

### Acknowledgement

Thanks for Jessica Latimer (orcid.org/0000-0001-7961-6286) for writing the Python script for the  $K_d$ -accuracy-assessment assay.

### Reference

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