Calculation of dissociation constant by electrophoresis

In this model, we attempted to use the basic electrophoresis approach to determine the dissociation constant of a given aptamer of amatoxin. Theoretically, by agarose gel electrophoresis of DNA, aptamer bound with target would experience conformational change, thus being distinguished from free aptamers. The fluorescence, which is the brightness of DNA bands, could quantify the concentration of aptamer-target complex and free aptamer.

By calculation of known binding constant of alpha amanitin and verify the feasibility, we are thus able to utilize the same model for the determination of dissociation constant of the aptamer against beta amanitin selected by our own SELEX process.To test the feasibility of the approach, we constructed the relationship between brightness of and total aptamer concentration derived from basic thermodynamic equation.

$$\frac{d[AT]}{dt} = Kon[Af][Tf] - Koff[AT] \qquad 1$$

Among these, [AT] is the concentration of aptamer-target complex, while [Af] is the concentration of free aptamer and [Tf] is the concentration of free target. Kon is the rate of association and Koff is the rate of dissociation, which are changing parameters in the equation following the relationship:

$$[Af] + [Tf] \underset{Koff}{\overset{Kon}{\rightleftharpoons}} [AT] \qquad 2$$

The ratio of Kon and Koff should be constant, which determines the affinity constants:

$$Kd = \frac{1}{Ka} = \frac{Koff}{Kon} \qquad 3$$

Since the concentration of free aptamer and free target are 2 unknown variables during the reaction, we need to rearrange the formula 1 using known variables, for example the total aptamer and total target concentration.

$$[Af] = [At] - [AT]$$
$$[Tf] = [Tt] - [AT] \qquad 4$$

At is the total concentration of aptamer and Tt is the total concentration of target. Thus, formula 1 can be expressed as:

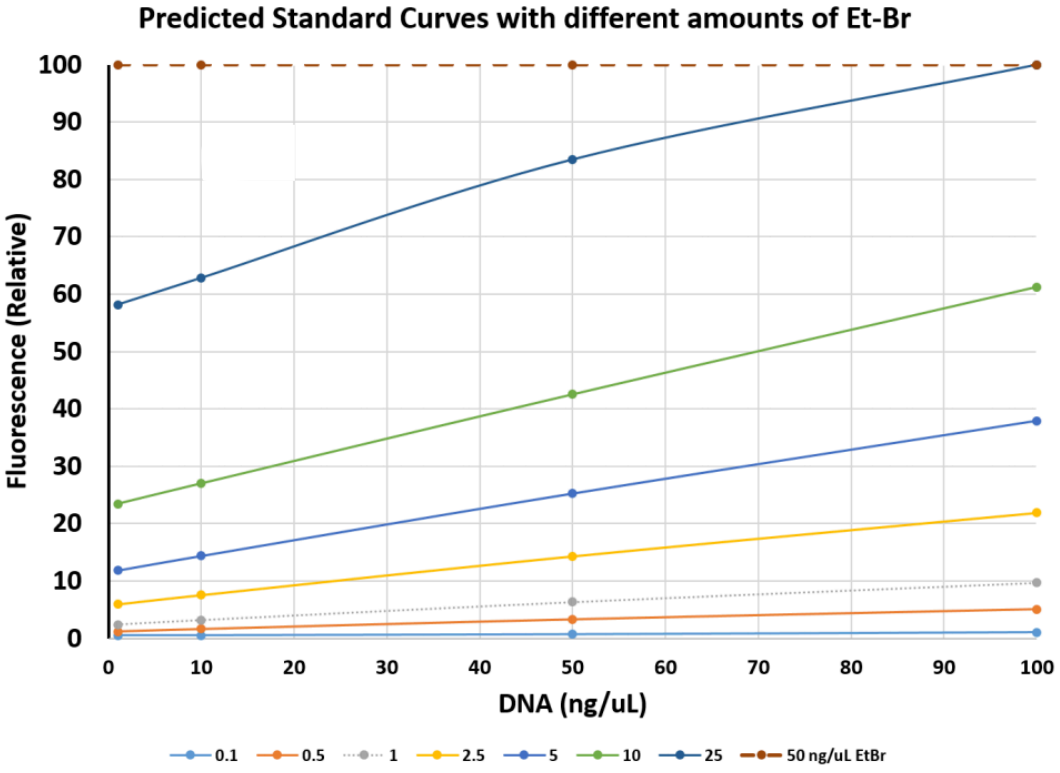
$$\frac{d[AT]}{dt} = Kon([At] - [AT])([Tt] - [AT]) - Koff[AT] \qquad 5$$

Now the equation is transformed into a solvable differential equation, the next step should be adapting the dependent variable we measured, brightness of DNA band in electrophoresis, into the equation.

In our lab Et-Br Nucleic Acid Gel Stain, 2.5X is utilized as DNA dye and according to previous modeling literature, brightness of band should be directly proportional to the concentration of DNA, with coefficient related to the relative size of DNA binding dye molecule and DNA molecule.

$$B = k[AT] \qquad 6$$

From the product instruction, the calibrated curve of DNA concentration and fluorescence representing brightness is shown below.



From the yellow line, at concentration of 2.5ng/mL, the coefficient k in equation above is calculated as the gradient of graph, k=0.16. Hence, k could become the constant of equation and thus we can substitute [AT] in equation 5 by B

$$\frac{1}{k}\frac{dB}{dt} = Kon([At] - \frac{B}{k})([Tt] - \frac{B}{k}) - \frac{KoffB}{k} \qquad 7$$

Where B is the brightness of band that represents the concentration of aptamer target complex, and the differentiation equation is about the rate of changing brightness, in which t represents the reaction time allowed for aptamer and target binding, 1h.

If we expand the equation and solve the differential equation by integration, we can obtain the following equations

$$\frac{1}{k}\frac{dB}{dt} = Kon[At][Tt] - Kon([At]+[Tt])\frac{B}{k} + Kon\frac{B^2}{k^2} - Koff\frac{B}{k} \quad 8$$

$$\int(\frac{Kon}{k}B^2 - \frac{Koff}{k}B - \frac{Kon([At]+[Tt])B}{k})dB = \int kKon[At][Tt]dt \quad 9$$

$$\frac{Kon}{3k}B^3 - (\frac{Koff + Kon[At]+[Tt]}{2k})B^2 = kKon[At][Tt]t + c \quad 10$$

In equation 10, the solved indefinite integration equation could be expressed with integral constant c. Since when the reaction time of aptamer and target is 0, there won't be any aptamer bound with target so that the brightness of band formed by aptamer-target complex should be 0 as well. Hence, c=0

$$\frac{Kon}{3k}B^3 - (\frac{Koff + Kon[At]+[Tt]}{2k})B^2 = kKon[At][Tt]t \quad 11$$

Here we can treat several terms as constant with relevant values and construct line graph of 2 variables. By substituting the experimental data of the 2 variables, we finally calculate the constant Kon and Koff that indicates the dissociation constant.

According the goal of our project, we aim to detect amatoxin presented in wild mushrooms, hence the rationale of dissociation constant calculation should be the situation that aptamer is in excess, thus depleting all amatoxin and detecting them. Therefore, we assume that target amatoxin is at certain concentration, 0.013μM, and the constant is found by varying the concentration of aptamer. The reaction time is assumed to be 1h according to relevant literature. K is 0.16 as calculated above.

$$t = 3600s, [Tt] = 0.013\boldsymbol{\mu M, k = 0.16} \quad 12$$

$$\frac{25Kon}{12}\boldsymbol{B}^3 - 3.125B^2(Koff + Kon[At] + 0.013) = 7.488Kon[At] \quad (13)$$

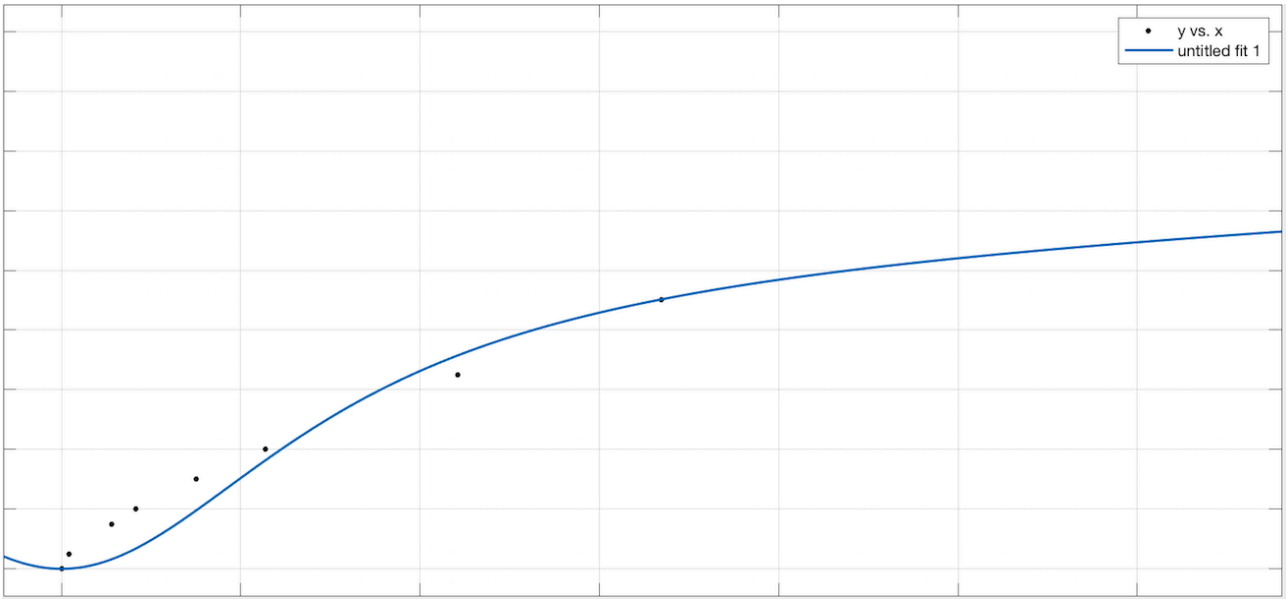
To estimate the dissociation constant, we first substitute the data of binding of existed aptamer of alpha amanitin and the toxin from recent literature regarding the varying concentration of aptamer-target complex and total aptamer concentration. Using k, we thus calculates th ebrightness of brand in our situation, hence calculating the proper range of Koff and Kon by fitting the data by curve with custom equation through MatLab.

B(brightness)	0	0.04	0.28	0.416	0.75	1.136	2.21	3.344	6.48
[AT] (concentration of complex)	0	0.25	1.76	2.60	4.69	7.1	13.8	20.9	40.5
[At](total aptamer concentration)	0	1.25	3.75	5.0	7.5	10.0	16.25	22.5	45

According to the data above, we optimize the function with parameter Kon and Koff by fitting the equation in form At=f(B), which is:

$$At = \frac{\frac{25\times Kon\times B^3}{12} - (\frac{13}{320} + \frac{25Koff}{8})\times B^2}{\frac{25\times Kon\times B^2}{8} + \frac{936\times Kon}{125}} \quad (14)$$

Hence, the curve with function as in formula 13 fitted by plotted points of data above is done by Matlab with optimized parameters(RMSE=3.687, SSE=95.16, R=0.9411)



The Koff calculated is -5.612 while Kon is much smaller in magnitude——0.2225. The dissociation constant can also be calculated following the thermodynamic formulae:

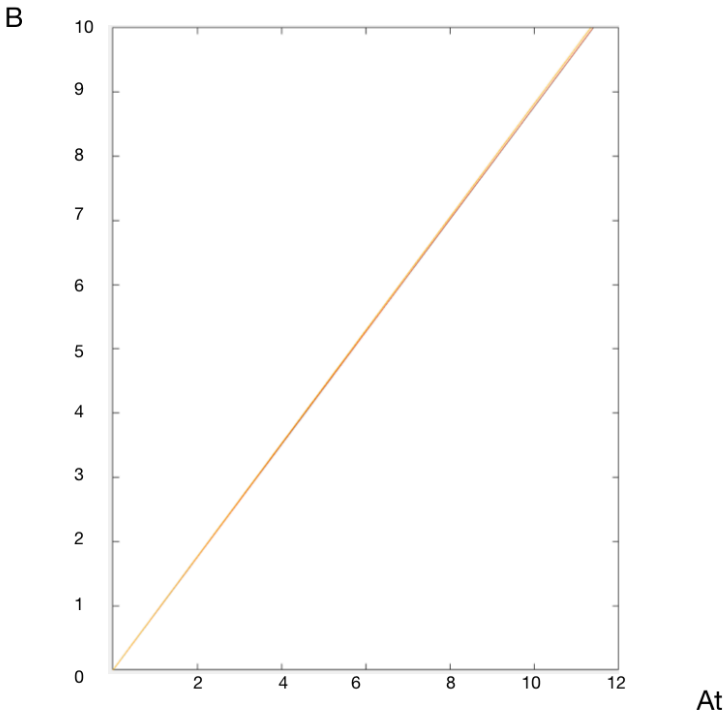
$$Kd = \frac{Koff}{Kon} = \frac{5.612}{0.2225} = 25.2 \quad (15)$$

As the values of [Tt] and [At] are in unit μM, the dissociation constant calculated should be 25.2 μM, which is similar and slightly above the value calculated in literature.

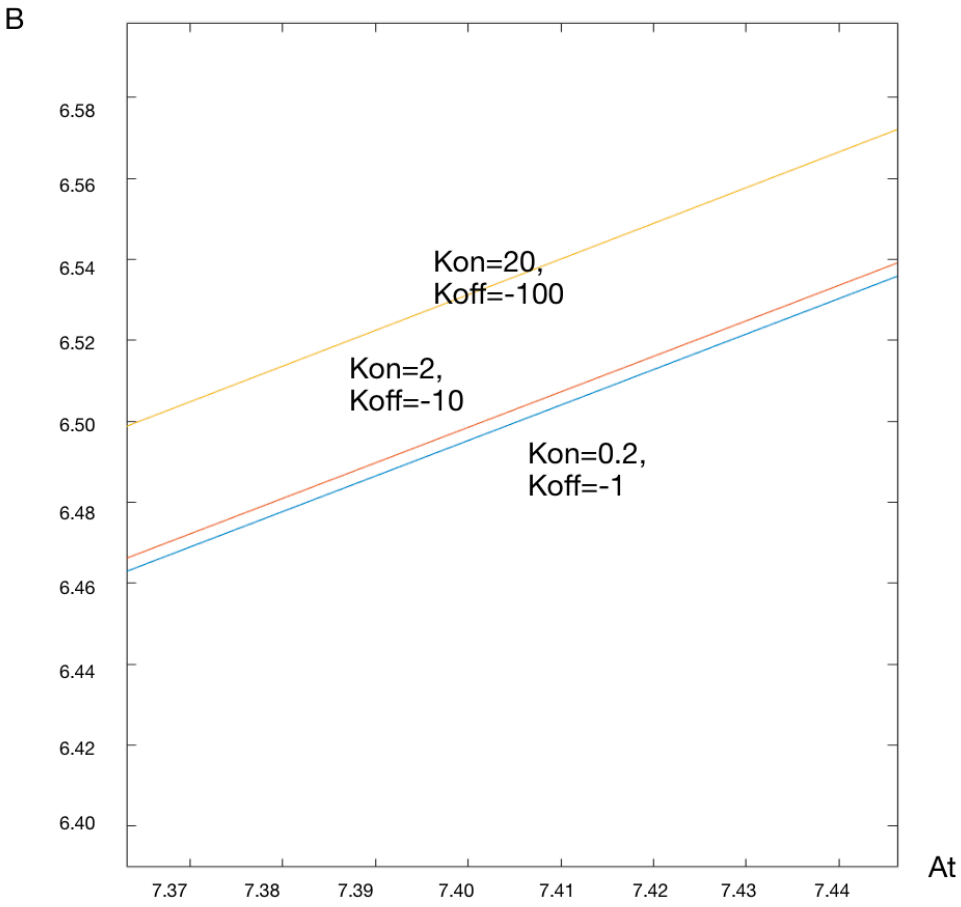
Then, we roughly making the ratio of Koff and Kon consistent with the value in literature, 5 μM, and further vary the relative value of Kon and Koff and look for the change in graph of total aptamer concentration against brightness of the DNA ban d indicting bound DNA concentration. The assumption embedded should be the linear relationship of Kon and Koff, to ximplify the whole system. According to the value of Kon and Koff calculated by curve fitting toolbox, the range of Kon is determined as (0,20) while the range of Koff is (-100,0). The values are selected in geometric sequence.

Kon	-100	-5	-0.25
Koff	20	1	0.05
Kd	5	5	5

It is discovered that although Kon and Koff vary to a large extent in the proper range, the graphs with same dissociation constant, which is the ratio of Koff and Kon are about the same



When the dissociation constant remains constant, there's an almost linear relationship between the total aptamer added(x-axis) and brightness of band representing aptamer-target complex(y-axis), with a slight curvature.The difference in graph with distinctive Kon and Koff value as in table above is subtle, which can only be detected when the graph is magnified

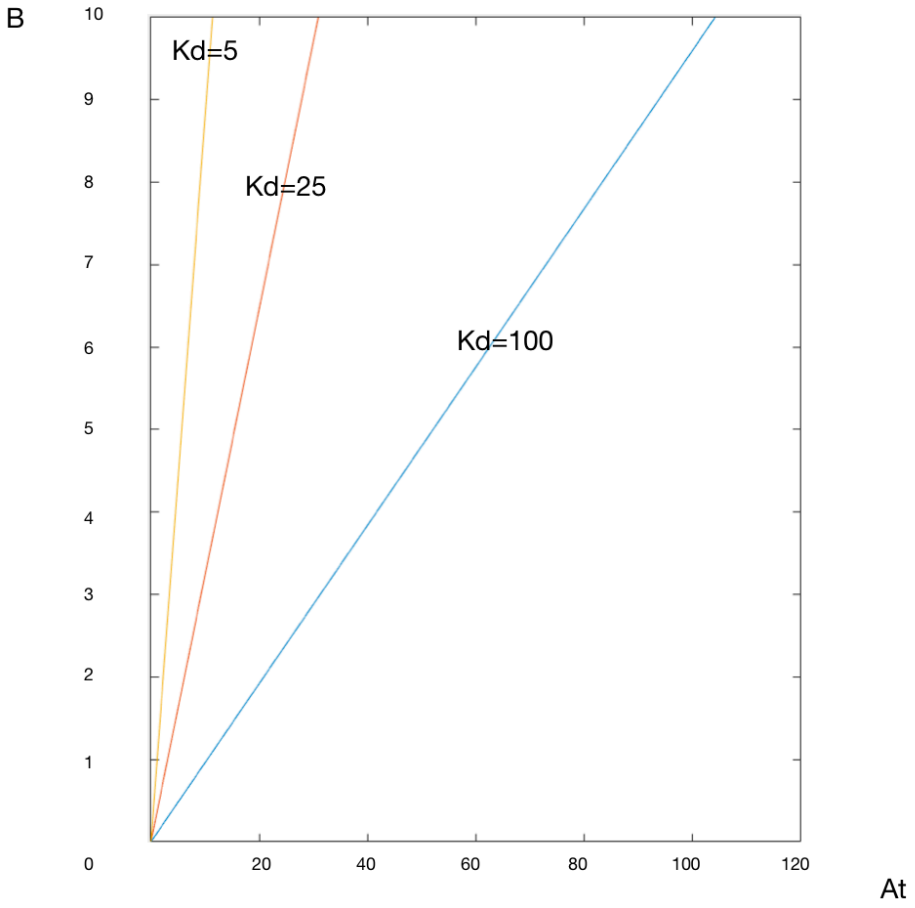


From the graph, when Kon and Koff is the lowest(yellow line), the graph is slightly bove the other 2, indicating higher proportion of aptaemr-target complex form under same concentration of aptamer and toxin added. Overall, this model is robust upon the specific values of Kon and Koff, which doesn't significantly affect the percentage of aptamer and toxin bound. This robustness correctly fit the real situation and physical meaning of Kon and Koff, verifying the reliability of the model.

Then, another aspect of the model should also be tested, that is, the change of graph and proportion of bound aptamer when the dissociation constant is varying. From the graph with varying Kon and Koff, we proved that the specific value of Kon and Koff is not a significant factor changing the shape of graph. Hence, by fixing Kon at 1, we vary the value of Koff in range (-100,0), thus changing the dissociation constant from 5μM(in literature) to 100μM(hypothesized)

Kon	-100	-25	-5
Koff	1	1	1
Kd	100	25	5

By adjusting the dissociation constant, the graph with 3 distinctive dissociation constant values are significantly different.



As expected, when the dissociation constant is highest(Kd=100), the graph is significantly lower than the other 2, indicating that more aptamer should be added initially to form similar amount of aptamer-complex with similar brightness of band. Hence, the proportion of bound aptamer is lower when dissociation constant increases, which is consistent to the definition of dissociation constant.

Therefore, the entire model is verified and could be utilized with substitution of our own experimental data later on.

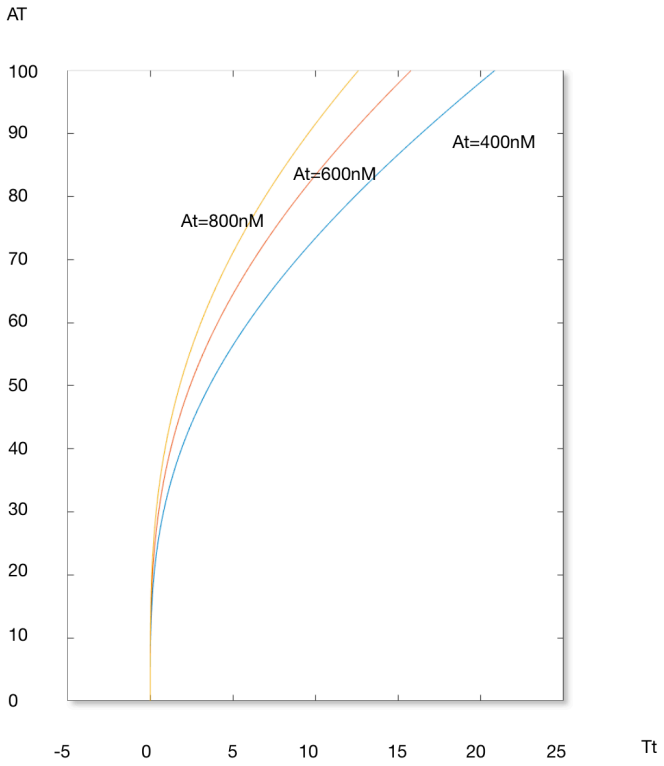
Based on the previous curve fitting, we can deduce suitable Kon and Koff value according to the model or previous literature, thus calculating Kd. This model of dissociation constant is significant, not only due to the basic calculation of Kd, but also further utilization in the competitive test we developed in our project. Combining the model and previous findings, we now assume Kon=5.0 and Koff=0.5 to achieve this, for simplicity.

$$\frac{125}{12} * B^3 - \frac{0.5 + 5 * [At] + [Tt]}{0.32} * B^2 = 2880 * [At] * [Tt]$$

To further explore this relationship, we conducted an experiment of concentration gradient to identify the dynamic range of aptamer toxin binding. The sensitive range of aptmamer, [At], to is identified to the range of 400nM-800nM, in which there's a significant difference between 0(absence of free toxin) and 1(excess free toxin) in the fluorescence of final DNA product. If more than 800nM, then excessive aptamer would bond with immobilized toxin anyway, making non difference. If lower than 400nM, there won't be sufficient aptamer bound to immobilized toxin even without the competition from free toxins. Hence, we expect the concentration of toxin and bound toxin would vary with a narrow dynamic range within this range of aptamer concentration, with each aptamer concentration respectively. Now we aimed to convert back the brightness of band to [AT], bound aptamer toxin complex, by the relationship B=k[AT]

$$\left(\frac{16}{375}\right)*AT^3 - \left(\frac{1+10*[At]+2*[Tt]}{25}\right)*[AT]^2 = 2880*[At]*[Tt] \qquad 17$$

Taking the range of AT from 0 where there's no toxin binding the aptamer at all and 100 which implies an idealistic concentration of toxin binds all aptamer in certain fixed concentration. From the optimized concentrations of aptamer verified by our experiment, we take distinc values of 400nM, 600nM and 800nM for demonstrating the trend of target concentration and complex formation through graphs. Here it is noticeable that "0" in AT indicates a high concentration of amplified aptamer, thus a bright red test line. Vice versa, "100" in AT means a total inhibition of amplification, indicating an absent of color at test line.



From the graph sketched above, we can find the limit of detection of the competitive in each distinctive aptamer concentration value. Within the range identified by experiments, the LOD presented by aptamer concentration of 800nM is the lowest shown by a narrower dynamic range to reach the maximum capacity of aptamer, compared to the other 2. The KD could also be deduced by finding the concentration of target at AT=50, in which the value varies according to the concentration of aptamer, signifying the importance of identify the most suitable and sensitive aptamer concentration fro detection, 800nM.