

Newly Integrated Development of Aptamer and Artificial Cell Histamine Detection System

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Keywords: Aptamer, Artificial cell, Histamine detection, Histamine treatment

Abstract: In order to develop a more comprehensive and efficient bio-system for histamine detection and treatment, several gene pieces like CCD4, LacZ, and rDAO inserted into the artificial cell to construct the ability of histamine detection and degradation. The artificial cell had an obvious drawback that it could not detect the concentration of histamine. As for more sensitive and accurate detection, this work has improved the original system by adding aptamer. The advantages of aptamer-better ability of targeting-resulted in gaining the ability to detect the exact histamine concentration as well as the changes in histamine concentration. With aptamer added, this program succeeded in testing histamine concentration more sensitively than the forgone one on a slope about 0.147 (linear fitting) for the whole process.

1. Introduction

Aptamer^[1-5] has been regarded as a new technology for the past few years and it has been developed to use in many scientific fields. Aptamers have similar function as antibodies because they can precisely and strongly bind with different targets^[3,8,9,16]. This technology was firstly appreciated because of its ability of targeting, and improved its to cope with diseases, especially for cancer treatment^[3,7,10]. Additionally, aptamers are able to treat cancer in human organs like breast^[6] or some vulnerable organs with little negative side-effect because of their outstanding biocompatibility^[11,12,13,17]. However, it is known that aptamer has several limitations that negatively affect its functions when it is used in some conditions. Aptamers are short DNA sequences so that aptamers have weak signaling effect that is not likely to be detected and they are easily to be degraded^[14,15]. So, we imaged that aptamers could cooperate with other technologies, which could cover the drawbacks of aptamers. Artificial cell is case in point^[18,19,20]. Artificial cell also has plenty of advantages and functions. Many researches and studies have taken over its advantages and characteristics and represented that artificial cell could replace forgone carriers because they have better ability to carry and they are easily to be modified. Many works can utilize artificial cell to achieve excellent ideas by assembling variable sequences^[21]. So it is easy to think that they can have many usages in medicine. For example, artificial cells can produce proteins or enzymes through the opening riboswitch^[22-24] to deal with histamine. Histamine causes many responses in human body and plays a role in Vasorelaxing action and giving rise to different feelings^[25]. However, an unusual high level of histamine can cause many serious healthy problems^[26,27]. So, it is reasonable to have adopted artificial cells to deal with histamine^[22]. When these cells detect an unusually high level of histamine, the riboswitches in artificial cells will activate the whole sequence to produce enzymes or proteins that can reduce the concentration of histamine. The primary system succeeded in inducing response when the level of histamine is high, whereas they have no response when the level of histamine is low. But this detection is not accurate and sensitive enough. The system can not produce the relative amount of enzymes as the histamine concentration is changing. We believe that this problem can be perfectly solved by aptamers. As the figure 1. showing, we add an aptamer on the response sequence. When the histamine concentration is changing, the intensity of fluorescence and the amount of proteins are changing. This improvement has many advantages and benefits. To be more specific, with the ability to detect the change in concentration of histamine, people can know the exact concentration of histamine. It is useful whether in medicine or technology. We believe that this combination between artificial cell and

aptamer can be used for more than just detection of histamine, especially for future drug testing and material testing.

2. Results and Discussions Gene Amplification:

ccd4, the substance that oxidize Carotene into ionone, which produce a special smelling of violet to warn the patient that the histamine level in his or her body is increasing. So it was the first protein that we produced. We inserted the sequence of ccd4 into pET28-a Vector and transformed the plasmid into E. coli BL21. After PCR process, we examined the exact length of this sequence by electrophoresis. The results are in the expected range of 5000 bp (Fig. 2).

dao, a chemical that degrades histamine, can significantly reduce histamine levels in the mucous membranes of patients in the early stages of allergy, relieving symptoms and reducing pain. Therefore, the dao is a key element in our system. After inserting the sequence of rdao into pET28a Vector and converting it into BL21. The results of electrophoresis are about 5000 bp.

lacZ, could be recognized as a reporter gene, is mainly responsible for turn on the riboswitch after sensing the increase of histamine level. The expression of lacZ is also attributed to the expression of BL21. We successfully add the sequence to the whole system, and lead to the ability for detecting the histamine and inducing later functions.

ccd4, rdao, and lacZ were successfully gained or constructed as the following sequences in the table and replication in PCR process. PCR process replicated these DNA sequences systematically. Functional interest genes were collected and stored as the basis of our experiment.

Interest gene	Primers	Gene sequences
rdao	SEHS049	cctaggactgagctagccgtaaaTAAGCGCAACGCAATTAATGTA
	gbszp055	CCATCGGTGATGTCGGCGATATA
	038 pET28aAsR	CTATAGTGAGTCGTATTAAGATCTCGATCCTCTACGCCG
	pET28a R	catGGTATATCTCCTTCTTAAAGTTAAACAAAAT
	rDAO F	TAAGAAGGAGATATACCatgtgttagcg
	rDAO R	GGCTTTGTTAGCAGCCGGATCt
	pET28a F	GATCCGGCTGCTAACAAAGCCC
	SEHS051	cttcgcttatGATCCGGCTGCTAACAAAGCCC
	gbszp054	CCCAAGGGGTTATGCTAGTTATTGC

Interest gene	Primers	Gene sequences
lacZ	gbszp055	CCATCGGTGATGTCGGCGATATA
	038 pET28aAsR	CTATAGTGAGTCGTATTAAGATCTCGATCCTCTACGCCG
	039 T7AsF	TAATACGACTCACTATAGAAGGATCCAGTG
	SEHS019LacZ F	CCTGCTGGATCCAAACATGACCATGATTACGGATTCACTG
	LacZVF1	GACGGCAGTTATCTGGAAGA
	lacZVF2	GCAGAACAACCTTAAACGCCG
	lacZmut2R	GTGGCCTGATTCATTCCCAGCGACCAGA
	lacZmut1F	TGGGGAATGAATCAGGCCACGGCGC
	lacZmut1R	GCGACTGATCCACCCAGTCCCAGACGAA
	lacZmut2F	GGACTGGGTGGATCAGTCGCTGATTAAATAT
	042 lacZAsR	GGCTTTGTTAGCAGCCGGATCTTATTTTTGACACCAGACCAACTGG
	035 pET28a F	GATCCGGCTGCTAACAAAGCCC
		gbszp054
	Aptamer	AAGGATCCAGTGGGTTGAAGGAAAGTAACAGATCCTCCAGGATGC GACCCGGTTATCGAATTAAGGAGGTAATAAATGCAAGTCGACCTGC TGGATCCAAAC

Interest gene	Primers	Gene sequences
ccd4	gbszp055	CCATCGGTGATGTCGGCGATATA
	pET28a R	catGGTATATCTCCTTCTTAAAGTTAAACAAAAT
	CCD4 F	AAGAAGGAGATATACCatggataccctgagcagcag
	CCD4 R	gtgaagcttgctcagatcgtttcg
	pET28aCCD4 F	acgatctgaacaagcttcaccaccaccaccactga
	rDAO R	GGCTTTGTTAGCAGCCGGATCt

pET28a F	GATCCGGCTGCTAACAAAGCCC
gbszp054	CCCAAGGGGTTATGCTAGTTATTGC

3. Aptamer Utilization and Detection of Histamine

To detect the concentration of histamine precisely, aptamer played a role in response as the concentration of histamine changing. As showing in the table, the gene sequence of aptamer is colored in red. By constructing the sequence of J23106-aptamer-sfGFP, we successfully discovered that the intensity of fluorescence could change regularly with the change of histamine concentration, whereas the J23106-sfGFP, the original one without aptamer, lacks this significantly beneficial function (Fig. 3 a). With aptamer, we found that the intensity of fluorescence was directly proportional to the concentration of histamine, which was one of the most significant achievement in our experiment (Fig. 4 b). Additionally, the detection system is more sensitive even when the changes of histamine concentration are relatively low. As showing in the graph (Fig. 3 c), when the concentration of histamine changed from 0 μM to 60 μM , the intensity of fluorescence changed from about 345 to 395. This new function in the system served as more sensitive detector and replaced the forgone version. We constructed this new system and compared two systems (Fig. 3 d,e), and the error bars became smaller, that means we were making more accurate detector of the concentration of histamine.

4. Purified Proteins and Rdao Treatment

After designing all the circuits, we purified the proteins expressed by them. Among them, the size of CCD4, rDAO and LacZ is exactly the same as that mentioned in the paper. We produced functional proteins and have tried to purified them. We successfully located where were the functional proteins in by observing the results of albumen glue. In the Figure. S1, we discovered that most proteins were found in rdao-cell lysates and supernatants. We have tried to purify the proteins in order to verify the effect of rDAO degradation of histamine obtained by us, we carried out the rDAO function examine experiments, and the result supported our expectation. Thus, rDAO could indeed degrade histamine. We inferred that this was due to the fact that the rDAO we had purified was not very pure, and they would be more intensive if we purified thoroughly.

5. Conclusion

In this subject, we successfully constructed and improved the cell-free system to complete the purpose for detecting and degrading histamine. The expression of CCD4 was one of the most important part of the subject, because it could involved into the whole cell-free system and played a role in detecting the concentration of histamine. Moreover, the aptamer, the most excellent part of this experiment, had vital function that it could help to detect the concentration of histamine by changing the intensity of fluorescence. The significant change in color detection functioned as CCD4 and J23106-aptamer-sfGFP along with the concentration of histamine changing reflected the advantage of our subject. This reaction accomplished the purpose in perception of the histamine concentration. rDAO was effectively incorporated with plasmid pET28a to form a gene circuit that was been expressed under particular circumstance. LacZ in the system was the starter of riboswitch. It had be supported that when the concentration of histamine increasing, the LacZ would lead riboswitch on. These materials were successfully concluded into cell-free system and played their roles systematically. Proteins were produced and purified to gain the final products of the gene circuit.

It is believed that our cell-free system assembles the ability of histamine detection and histamine degradation successfully, and the data got from the experiments supported the theories that the detection system with aptamer is able to inspect the concentration of histamine. More studies and work need to do besides the achievements in current stage. We believe that there is much more

potential advantages and applications of the system added aptamer, benefiting from its accuracy and efficiency in detection, especially in pharmaceutical science and medical science. To make the system working effectively, we hope to design a specific quantization table between histamine concentration and intensity of fluorescence to tell the accurate numerical value of histamine to users by conducting more precise experiments for many times. Additionally, more experiments should be done to build up a suitable cell-free system to actually have the ability to therapy hypersensitivity caused by excessive content of histamine by replaced the rDAO with hDAO. This therapeutic regimen is beneficial for the entire human race because it should has fewer side effect of drug.

The strategy mentioned in this essay have huge potential usages that are unexploited. There are many applications in several fields. It can be possible to solve out the true relationship that histamine has negative influences^[24-28], finding out results by detecting the change in concentration of histamine with our system. Additionally, Histamine also plays a key role in the regulation of gastric acid secretion^[29,30], so it will be important to measure the concentration of histamine for more proper regulation of secretion. With our cell-free system, it can help to detect the histamine concentration accurately by observing the intensity of fluorescence, which will be useful for doctors to offer appropriate treatment to patients with problems in secreting gastric acid^[29,30]. These are just some examples above to illustrate the effectiveness and potential advantages about the system. Moreover, it is easy to discover other values such as demonstrating quantitatively the effect of histamine on brain development^[31], testing the freshness of seafood by adopting the system as a test paper. According to all the applications mentioned above, we can believe there is large area that should be explored to utilize its potential and approach its capacity in function.

6. Method

6.1 The Laboratory Apparatus and Biological Reagents.

To complete the whole experiments and achieve the eventual purpose, the experiment required these laboratory apparatus: Electric Thermostatic Water Bath (DK) series, Electron-Heating Standing-temperature Cultivator (DH) series, TGreen Monitor (OSE-470M) Blue Light Transilluminators, Electrophoresis Apparatus EPS-300, Temperature-regulative Lab Shaker, 4 °C Refrigerator, -20 °C Freezer, Elaborate Pipettes, Vertical High Pressure Steam Sterilizer, Speed Refrigerated Centrifuge, ultrasonics Processor, Balance, Miniature Centrifuge, Thermodynamic Shakers, ELIASA, Vortex Mixers, Clean Bench, and PCR Machine. Additionally, the experiment conducted by using these materials: MCL Buffer P1, Buffer P2 (base and acid), PWT, DDH₂O, Primer R, Primer F, PFU mixture, Balance Buffer, PW, oePCR, GOL R, sensor F, Pfu Buffer, buffer HiFi2, primer 022, primer 023, Pet28a, Taq buffer, IPTG, Tris buffer, SDS Page Buffer.

6.2 Construct the Interest Genes and Build the Basis System

Take a 2 mL centrifuge tube with 2 mL of bacterial liquid and centrifuge at 12000 rpm for 1 min, then pour out the waste liquid. Repeat again and finally get the bacteria in 4 mL broth. 150 MCL Buffer P1 was added and vortex it. After that, 150 µL Buffer P2 (base) was added and gently mixed up and down for 8-10 times. Then Added 350 µL Buffer P2 (acid). Gently mixed it for 10 times and quickly mixed it for 10 times. The finished mixture was centrifuged at 12000 rpm for 7 min. The centrifuge was carried out on an adsorption column at 12000 rpm for 1min. PWT 300 µL was set for 2 min, then centrifuged it at 12000 rpm for 1 min, and the waste liquid was discarded. It was centrifuged at 12000 rpm again, idling for 3min, then put into a clean 1.5 mL centrifuge tube and dried it for 2 min. The DNA was dissolved in 50 µL of water solution, then left for 2 min and centrifuged at 12000 rpm for 2 min. The extracted DNA solution was mixed with buffer and injected into agarose gel to form bands under the action of electrophoresis. 12 µL DDH₂O, 1 µL Primer R, 1 µL Primer F, 1 µL Template DNA and 15 µL PFU Mix were successively added to the small centrifuge tube. Set the PCR machine at 95 °C for 5 min, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s. Repeat these steps for 30 times. Finally, set 72 °C for 5 min, and then cooled at 10 °C. The extracted DNA solution was mixed with buffer and injected into agarose gel to form

bands under the action of electrophoresis. Use adsorption column and collection tube. Add 450 mL Balance Buffer (salt), centrifuge it at 12000 rpm for 1 min, and discard the waste liquid. In order to add the sol solution at a ratio of 1:1 to 2, let it stand at 55 °C for 2 min, pour it into for adsorption, and centrifuge at 12000 rpm for 1 min. Pour the collected solution back to the adsorption column and let it stand the centrifugation was carried out at 12000 rpm for 1 min. The waste solution was discarded, and 600 mL Buffer PW (washing solution) was added, then let stand for 2 min, and centrifuged at 12000 rpm for 1 min. Repeat the washing process. The waste liquid was discarded and centrifuged at 12000 rpm for 3 min (idling). Discard the collection tube and replace with a 1.5 mL centrifuge tube, open the inner cover, dry at 55 °C for 5 min. 30 mL of water is added, centrifuged at 55 °C for 2 min and 12000 rpm for 2 min. After that, we should do Gel electrophoresis and concentration measurement. Then, create oePCR mixture. 11 mL water is added successively, 1 mL GOI R (primer), 1 mL Sensor F (primer), 1 mL Sensor, 1 mL water GOI and 15 mL 2 Pfu buffer. Then process PCR and Recycle the old glue. The procedure is the same as before.

6.3 Build Gibson Mix

Add a 2 L vector and 3 L DNA template at 50 °C for 1 h to a 5 L of 2 Gibson Buffer. Start Transformation process: DNA was adsorbed on the membrane surface with calcium ions, placed on ice for 30 min, and then placed in 42 °C for 80 s to expand the hole and facilitate the entry of DNA. Immediately ice bath for 5 min, the hole retracted. Liquid LB recovery for 1 h. Recovery process: 5 mL system and 30 mL sensitive state, ice bath for 30 min, then put in 42 °C heat shock 80 s, ice bath for 5 min, finally add 300 mL non-resistant LB, and store in 37 °C shaker for 200 rpm for 1 h. Take 150 mL and apply to LB plate. Colony PCR was used to test the bacteria. Make sure the T7-Sense-GoI is present and the sequence is consistent with the design. Construct PCR mix (30 µL *6) : add 78 µL water, 6 µL primer R (017), 6 µL primer F (018), and 90 µL 2* Taq buffer. Divide into 5 small centrifuge tubes, 30 µL each, and then pick up the bacteria and insert them into the µLtra-clean table. Gel TAE 50 mL, 0.6 g Agarose. Run glue, send sequencing. Preserve the bacterial solution at low temperature with 300 µL of glycerin (to prevent the bacteria from being frozen to death by ice crystals) and 300 µL of bacterial solution stored at -80 °C.

6.4 Express Rdao on a Vector and Use Final Proteins to Show Results:

To Dissolve rDAO, we need to centrifuge (5000 g) for 5 min. Add 100 µL water (special, from refrigerator). Incubate it at room temperature for 10 min. Then the mixture was briefly centrifuged under less than 4000 g for a little time, until the liquor is spin on the bottom. Store the plasmid. To be continued, prepare 1 µL buffer HiFi2 (for plasmid, HiFi1 for genome) 1 µL rDAO, 1 µL primer 022, 1µL primer 023 and 12 µL water at 95 °C for 5 min, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min and 30 s, and 72 °C for 5 min, then kept at 10 °C. Then the step moved to set up PET28a: 15 µL buffer HiFi2, with 1 µL PET28a, and 1 µL primer 021, 1 µL primer 024 and 12 µL H₂O at 95 °C for 5 min, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 5 min and 10 s, and 55 °C for 7 min, finally, at 10 °C, the mixture was rested. Use these to Electrophoresis. After that 3 µL DNA loading buffer, 10 µL marker, 30 µL sample of each hole. Gel recycling and Gibson Assembly: 5 µL Gibson mix, 3 µL rDAO, 2 µL PET28a. at 50 °C for 1 h. (rDAO3-PET28a1). start the Transformation: add 50 µL 5a and 5 µL mixture and pour it on the LB medium with K antibiotic. Prepare 9 conical flasks. 50 mL non-anti-LB, 0.5 mL (1:100) bacterial solution (rDAO BL21), 50 µL K anti-LB (1:1000). IPTG induction: Add 5 µL, 15 µL, 25 µL, 50 µL 1M IPTG to each of the eight bottles (add two bottles of each, the remaining one for control). IPTG concentration gradient: 0.1, 0.3, 0.5, 1 at 20 °C shaking table 16 h to 20 h. The protein induced by the original bacterial solution was loaded into a 50 mL centrifuge tube, and a small amount of water/Tris Buffer was added to make the two with the same mass (balancing in a large centrifuge with an error less than 0.5). After centrifugation, the supernatant was discarded and 15 mL Tris Buffer was added to the precipitate. The ultrasonic process was to put each tube of bacteria into the µLtrasonic instrument for 15 min vibration (stop 1 min for each 5 min vibration to prevent the µLtrasonic instrument from overheating), and clean the

column with a drop bottle after the vibration. 40 μL of the original bacterial liquid was taken and put into the eighth tube, then 50 μL of each tube was taken, centrifuged at 12000 rpm for 5 min, and 40 μL of supernatant was added into the eighth tube. Add 10 μL SDS Page Buffer to each. (Both the supernatant and the original bacteria may contain proteins, and the purification is denatured/non-denatured according to the electrophoresis situation). The electrophoresis process required 100 V, 75 min. Carefully crack the small glass with a thin sheet and rinse. Cut off the concentrated glue with the blade and cut both ends of the separating glue. Rinse in the box with water and let the separator slide into the water. Pour out the water, add the dye solution, shake and dye for 20 min. Follow the glue and observe the bands.

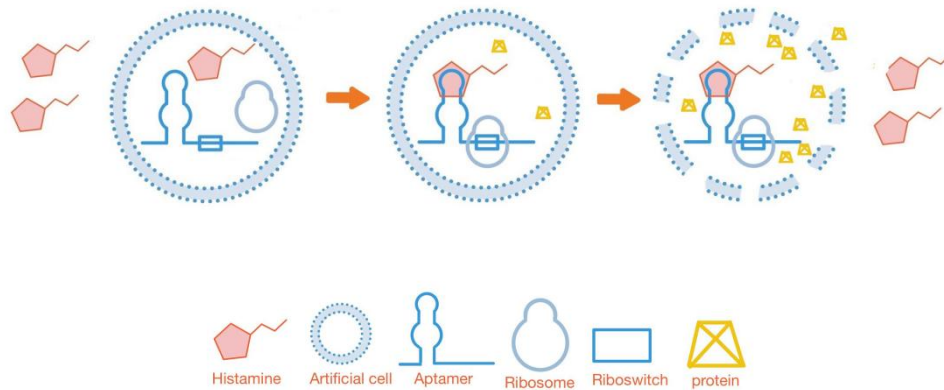


Figure 1 Schematic Image

This figure shows the functions of our cell free systems and the whole process of detection of histamine and production of proteins. As the histamine binds to the aptamer, the whole process begins, leading to the opening of riboswitch. Ribosome produces proteins while the cell free system gets broken so that the proteins can emit out of the artificial cell.

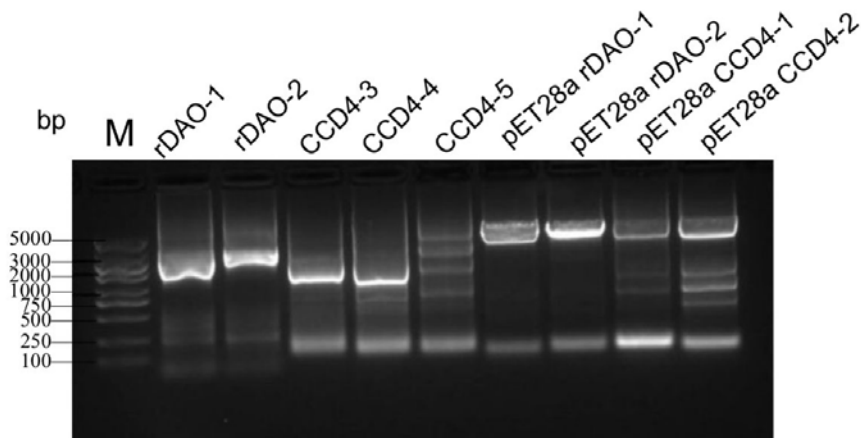


Figure 2 Results of electrophoresis.

From left to right, the first two are the rDAO-1 and rDAO-2 genes that are in the proper range between 2000 bp and 3000 bp. The third and the fourth are CCD4 genes that is about 1500 bp. The last four are that pET28a assemblies with rDAO and CCD4, and these are approximately 5000 bp.

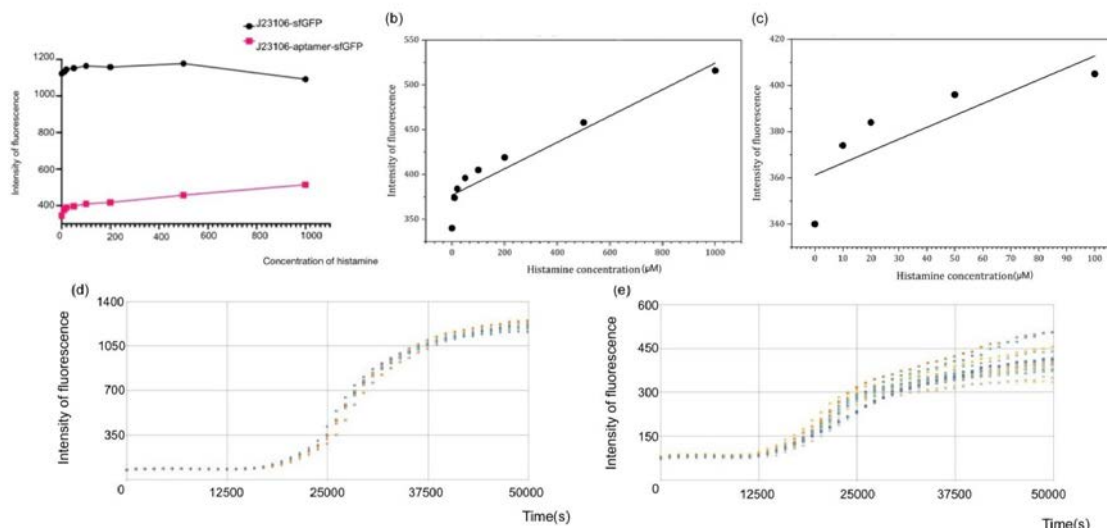


Fig.3 Results about Detection of Histamine by Evaluating the Intensity of Fluorescence

(a) is the contrary between two systems whether there is aptamer or not. The red one reflects the system with aptamer that can detect the changes in histamine but the system without aptamer which is demonstrated by the black line cannot. (b) and (c) are the relation between the histamine concentration and the intensity of fluorescence. (d) and (e) are graphs that demonstrate the relation between the time and the intensity of fluorescence.

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[32] Figure S1. is results of albumen glue of rdao-cell lysate, rdao-cell supernatant-1, flow through, wash, elute-1, elute-2, elute-3, elute-4, and rdao-cell supernatant-2 (from left to right).

