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Establishment of a protein thermal shift chip (PTSC) for COVID-19：exploring the future of protein chip in pharmacology

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In brief

Protein thermal shift chip can realize high-throughput protein target and drug screening by label free manner.

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ABSTRACT

The traditional protein chip based on a solid chip is impossible to provide drug and target screening in a label-free manner. Here, the concept of protein thermal stability chip (PTSC) was proposed based on fluorescence signal and has characteristics including low cost, high throughput, and label-free. The PTSC for COVID-19 contains twelve targets from SARS-CoV-2 and host. Series of quality control tests have been carried out for small molecule drugs, macromolecular antibody, and herbal medicine extracts. This chip can achieve a high-throughput screening of COVID-19 drugs, and provide a tool for screening the drug target of clinically effective drugs.

Keywords：Protein thermal stability chip, COVID-19, Drug target, Pharmacology

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1. Introduction

COVID-19 has been wreaking havoc on public health worldwide for more than two years, and there have been confirmed cases in every country and region in the world. The current consensus is that humans must live with the long-term existence of COVID-19. At present, the main methods of preventing the epidemic mainly rely on social distancing restrictions and immunization through vaccines[1]. Hundreds of vaccines have been investigated thus far, and several of them are already authorized by the World Health Organization, including inactivated vaccines, adenovirus vectored vaccines, recombinant protein vaccines, and mRNA vaccines[2].

Although the above strategies are effective, these measures to prevent the epidemic remain defective. Due to differences in the economies and the social and cultural backgrounds of different regions, social distancing limitations and travel restrictions are different in different regions of the world. In addition, there are already many kinds of mutant viruses, which appear new to mutate once every week on average. Due to differences in economic and social development, the vaccination inoculation speed was also different in different regions, leading to the risk of reduced vaccination protection rates [3]. Therefore, the final methods of terminating COVID-19 may depend on specific drugs[4] [5].

Drug screening and pharmacology research for COVID-19 is still being performed[6]. In the early days of COVID-19, researchers developed in vitro cell virus infection models based on multiple cell lines, such as the virus infection model developed by using cell lines[7]. Later, various models were developed and

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established with animals, such as rats, mice, and monkeys[8] [9]. On the other hand, some drugs derived from natural products have been demonstrated to inhibit viruses in in vitro models, but their drug targets remain unclear. A variety of compounds have been listed in the guidelines for the treatment of COVID-19 in China[10], which has been found to significantly inhibit SARS-CoV-2 replication[11]. Usually, the targets of small molecule drugs are screened by labelling with chemical probes. Protein targets are enriched and traced by probes and identified by methods including mass spectrometry, biochip, and other tools [12]. Traditional protein chips are based on glass substrates and rely on probe labelling to obtain detection signals. This greatly limits the speed of its application in drug and target screening. In pharmacology, new forms of protein chips need to appear.

The thermal stability of proteins is an important aspect of protein stability. The structure of protein will be destroyed in the process of heating. The temperature when half of the protein is in the unfolded state is defined as the protein melting temperature (Tm), which is usually related to conformational stability. The initial method to determine the Tm value of protein called typically different scanning calorimetry (DSC). Later, scientist used polarity sensitive dye 1-anilinonaphthalene-8-Sulfonic acid (ANS) to analyze the folding state of proteins, because proteins will gradually expose internal hydrophobic amino acids during the unfolding process, and then combine with hydrophobic dyes to stimulate fluorescence [13]. The discovery of hydrophobic residues bound to dyes makes it possible to automatically detect the thermal stability of proteins based on PCR

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instruments [14]. This method is called differential scanning fluorescence (DSF), DSF has a wide application in the field of protein science, including the optimization of stable buffer conditions, the optimization of crystallization parameters or ligands to determine the binding constants. By this technical principle, we propose a new form of protein chip: the protein thermal stability chip (PTSC), which use a small microscale and specific function protein cluster in liquid solvent to characterize the binding between protein and drug through thermal stability, has characteristics including a low cost, high throughput, label freedom, and fewer time requirements. Compared with the traditional protein chip, it does not need to label drugs, and can directly detect the interaction between drugs and proteins. At the same time, the conformation of proteins in the liquid phase can maintain better.

Take COVID-19 as an example, we put its main drug target on a PCR plate and constructed its PTSC, showed the application potential of PTSC. We optimized the reaction parameters, and implementation conditions, and automatically detected the thermal stability of COVID-19 drug target proteins, reflecting whether the drug molecule binds to the protein.

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2. Results and Discussion

2.1 Chip design

This chip was divided into two upper and lower regions, the drug experimental area and the blank control area, and each region contained twelve target proteins of COVID-19, as described in the methods section. Each target protein contains at least four independent repeats.

At present, as a prototype of PTSC, this chip is limited to twelve target proteins when 96-well plates are used with four multiple wells per protein. This platform strategy can be extended up to thousand protein targets at a time. To date, the types of PCR chips that can be queried have reached more than five thousand samples per plate (Smart Chip, Wafergen Biosystems). This number is far greater than the known number of human drug targets, can reach the level of proteome chip, and indicates that PTSC has a huge application space. Please refer to the supplementary materials for specific target information and methods.

2.2 Chip development and production

First, we tested the reaction buffer, which was water, PBS buffer (pH=7.4, 0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl), PBS buffer with disaccharide (1% trehalose; disaccharide was added to increase protein stability), PBS buffer with 1% trehalose and 0.1% bacteriostatic agent (5-chloro-2-methyl-4-isothiazolin-3-one2-methyl-4-isothiazloin-3-one, 3:1), and PBS buffer with 0.02% NaN_3. After testing, the PBS buffer had the same Tm value as that of water, but the fluorescence signal obtained by PBS was stronger. After adding

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bacteriostatic agent, the Tm value of the protein was greatly changed compared with that of the PBS buffer. The existence of some bacteriostatic agents affects the stability of some enzyme proteins and reduces the Tm value. Careful consideration should be given when using bacteriostatic agents in the reaction buffer. PBS, as the basic buffer used in this experiment, can result in less interference. It is recommended to use PBS for short-term storage (Figure 1).

According to the conventional targets of SARS-CoV-2, we designed and developed this chip, and its target and function are shown in figure 2A. We screened the protein concentration used in the chip, as shown in figure 2B, using Nfkb1-p50 as a representative target. Finally, we determined 0.045 μg/μL as the ideal protein concentration for the chip. We conducted a quality control test for all proteins on the chip, and the standard curves of all proteins on the chip were measured, as shown in figure 2C-L. Different proteins exhibited completely different patterns on the dissolution curve. Different Tm values are related to different properties of proteins, such as structural conformation, number of subunits, and number of disulfide bonds. The same protein showed a single peak pattern, indicating the purity of the protein and the repeatability of this experiment. This indicates that the chip can be used for applications in the small molecule drug screening field.

2.3 Testing the application of the chip

The application of this chip mainly involves three aspects. The first aspect is the screening of small molecule antiviral drugs. We tested small molecules with known targets. Previously, we found that the small molecule drug chlorogenic acid (CLA) can

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combine with its active protein targets, chlorogenic acid can efficiently combine with annexin A2 protein, change its original conformation, and then change its thermal stability [15]. In figure 3A, the red curve represents the thermal stability curve of annexin a2 protein without CLA, while the green curve represents the thermal stability curve when annexin a2 was combined with CLA. The thermal stability of the annexin a2 protein changed after CLA binding. After three independent repeated experiments, a *t test* showed that the change in the Tm value after CLA binding was significantly different.

Second, the chip can also be used to detect macromolecular drugs and judge whether binding occurs. The interaction of antigen or antibody also changes the thermal stability of each component. In this case, we found that the binding between the nucleocapsid protein and its antibody was successfully detected, as shown in figure 3B. The thermal stability curve of the nucleocapsid protein is shown (red curve), and the binding of a specific antibody with it increases the thermal stability. The blue curve represents the thermal stability curve of nucleocapsid protein under the action of antibody; because its antibody is also a protein, there is another thermal stability curve for the antibody (green curve).

This study proposes the following new concept: a protein thermal stability chip based on a PCR chip. We chose twelve potential targets for COVID-19 treatment, screened the chip buffer, and optimized the protein concentration. This protein chip is the first device with a high-throughput detection for the protein stability of COVID-19. Its potential applications are reflected in the following aspects. First, the

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protein thermal stability principle is used to achieve a low-cost and high-throughput screening of COVID-19 drugs (small molecule, macromolecule, complex chemical herb medicine), which is of great significance for the global epidemic situation. On the other hand, this chip can also be used for pharmacological research and provides a tool for screening the drug target of complex herbal drugs.

Third, it can also be used for pharmacological research to identify unknown targets of small molecules. One of the most remarkable features of this chip is that it can efficiently identify protein targets of complex chemical composition systems. In the literature, many herbs have been proven to be effective in clinical anti-COVID-19 practice. The China Food and Drug Administration (CFDA) approved three compound drugs for the clinical treatment of COVID-19. *Pinellia ternata* is one component in the Traditional Chinese. *Pinellia ternata* is widely used in the clinical treatment of COVID-19 in China, but its molecular mechanism is unclear [16]. We tested the potential application of our chip with the aqueous extract of *Pinellia ternata*. The results showed that *Pinellia ternata* contains chemical components that can strengthen the thermal stability of the SARS-CoV-2 receptor ACE-2. As a control, the water extract of *Pinellia ternata* did not change the thermal stability of another virus receptor (AXL) protein (Figure 4).

ACE-2 is the main receptor of SARS-CoV-2 in the respiratory system, and ACE2 expression is extremely low in various human tissues, especially in the respiratory tract[17]. The AXL specifically interacts with the N-terminal domain of SARS-CoV-2 Spike protein. This finding suggests that the role of *Pinellia ternata* may partly be to

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block the invasion of viruses in the respiratory system, but not in other tissues that are dependent on the AXL receptor [18]. This suggests that *Pinellia ternata* may have small molecules that specifically bind to ACE-2 receptors. This indicates the direction for further drug discovery and pharmacological research. We can test the components from *Pinellia ternata* to determine which small molecules play a role. If combined with high-throughput automatic equipment, such as robots, this method will have a profound impact on the discovery of natural drugs. This is also the direction of our future research efforts.

This chip is a method for preliminary drug and its target screening. The advantage of this method is high-throughput and rapid drug screening, which cannot be realized by isothermal titration calorimetry (ITC), surface plasmon resonance (SPR) and other technologies. This method also has disadvantages, and the drugs after preliminary screening still need to be verified by a variety of methods. We suggest that this chip be combined with ITC and other technologies to achieve the aim of rapid and accurate screening of drugs and targets.

DECLARATION OF COMPETING INTERESTS

The authors declare no conflicts of interest.

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Graphical abstract

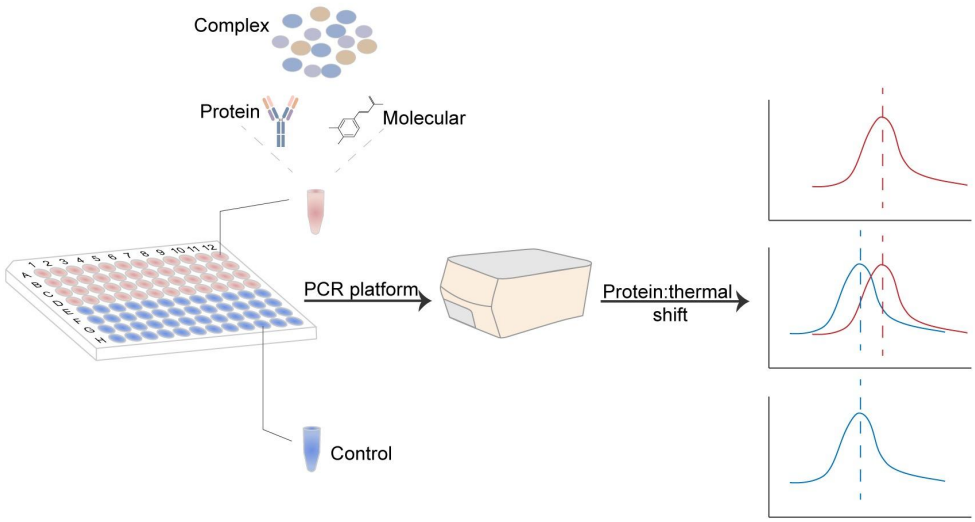

Diagram of the chip workflow. In this project, a protein thermal chip for COVID-19 target drug screening based on a conventional PCR instrument and protein thermal stability was constructed. The chip has characteristics including being a molecular labelling-free and simple process. It can be used for the initial screening and pharmacological study of antiviral drugs.

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Figure Legends

Figure 1 | Screening of reaction buffer system.

Pure water, pH = 7.4, 0.01 M PBS buffer and pH = 7.4 PBS buffer (0.01 M) with 1% trehalose was tested using 3CLpro protein. The results show that pH = 7.4 M PBS buffer can improve the reaction signal relative to that of the water system and does not affect the Tm value. Buffer 1: water; 2: PBS buffer (pH=7.4, 0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl), 3: PBS buffer with 1% trehalose, 4: PBS buffer with 1% trehalose and 0.1% bacteriostatic agent (5-chloro-2-methyl-4-isothiazolin-3-one2-methyl-4-isothiazloin-3-one, 3:1), and 5: PBS buffer with 0.02% NaN_3.

Figure 2 | Quality control test for all proteins on the chip.

(A) The twelve target proteins selected in this chip are partly from the antiviral targets of the host (human) and partly from SARS-CoV-2. (B) NFkB1-p50 was used as an example to explore the protein concentration (0.045-0.36 μg/μL). (C-L) The thermal stability curve of the protein selected in this chip without adding drugs. Independent repetitions were performed on the curve of each protein.

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Figure 3 | Application test on small molecules and macromolecules.

(A) Chlorogenic acid is a small molecule that has been reported to bind to annexin a2. We tested the thermal stability before and after the combination. Chlorogenic acid binding to annexin resulted in an increase in the thermal stability (P<0.05). (B) Similarly, we tested the thermal stability of the N protein in SARS-CoV-2 and the anti-N protein antibody before and after binding. The stability of the N protein clearly increases after antibody binding (P<0.05). Independent repetitions were performed on the curve of each protein.

Figure 4 | Application test on a complex chemical system.

(A) The extract of *Pinellia ternata* contains chemical components that can strengthen the thermal stability of the SARS-CoV-2 receptor ACE-2. (B) As a control, the extract of *Pinellia ternata* did not change the thermal stability of another virus receptor (AXL) protein.