

Title	酸化物TFTを用いた病原体・ウイルスセンサの開発
Author(s)	呉, 維東
Citation	
Issue Date	2023-09
Type	Thesis or Dissertation
Text version	ETD
URL	http://hdl.handle.net/10119/18784
Rights	
Description	Supervisor: 高村 禪, 先端科学技術研究科, 博士

Doctoral Dissertation

**Development of pathogen and virus sensor employing
isothermal amplification and oxide TFT**

WU Weidong

Supervisor: Yuzuru Takamura

Graduate School of Advanced Science and Technology

Japan Advanced Institute of Science and Technology

Materials Science

September 2023

Content

Chapter 1: General Introduction	1
1.1 Introduction of infectious disease and its harmless.....	2
1.2 Infectious disease detection	4
1.2.1 Immunofluorescence	5
1.2.2 ELISA	6
1.2.3 Immune colloidal gold technology.....	7
1.3 Introduction of conventional nucleic acids testing (NAT) methods.....	8
1.4 Introduction of thin-film transistor (TFT) biosensor for nucleic acids detection.....	9
Chapter2. RPA-based Nucleic Acid Detection with PCM Temperature Control System.....	16
2.1 Nucleic acid isothermal amplification	17
2.2.1 LAMP	17
2.2.2 RCA.....	18
2.2.3 SDA	19
2.2.4 HDA	20
2.3 Recombinase Polymerase Amplification.....	20
2.3.1. Primer design	22
2.4 Phase change material.....	23
2.4.1 Solid-solid phase change materials	25
2.5 Objective	27
2.6 PDMS embedded PCM based temperature storage platform	27
2.7 RPA based nucleic acid detection with PDMS embedded PCM platform.....	28
2.8 Conclusions	31
2.9 Reference	31
Chapter 3. Factors of instability in oxide TFT biosensors and surface analysis	34
3.1 Introduction of instability in oxide TFT biosensor	35
3.2. Objective.....	35
3.3 Fabrication and modification of oxide TFT biosensor	35
3.4 Instability based on reference electrodes and measurement solution	38
3.5 Instability based on the water content.....	42
3.6 Target DNA sample detection and surface of TFT biosensor analysis	43
3.6 Polysilazane layer making and surface of TFT biosensor chip analysis	48
3.6.1 Make polysilazane layer on TFT biosensor surface	48
3.7 Prob DNA immobilization by linker (3-Glycidyloxypropyl)trimethoxysilane	50
3.8 Conclusion.....	51
3.9 Reference	52
Chapter 4. Exploration of nucleic acid detection that combined with isothermal amplification and TFT biosensors	54
4.1 Brief overview of experimental design.....	55
4.2 Objective.....	55
4.4 Results	58
4.4.1 RPA reaction system optimization on experimental outcomes.....	58
4.4.2 Leishmania HSP 70 detection by TFT biosensors	61

4.5 Discussion.....	63
4.5.1 Evaluating the impact of Proteinase K on experimental outcomes.....	63
4.5.2 Leishmania HSP 70 fragment detection.....	63
5. Conclusions	65
6. Reference.....	66
Chapter 5. Conclusion and Future works	70
5.1 Conclusions	71
5.2 Future works.....	72
Acknowledgments.....	73

Chapter 1: General Introduction

1.1 Introduction of infectious disease and its harmless

An infectious disease, also known as transmissible disease or communicable disease, is an illness resulting from an infection. Infectious diseases are usually caused by various pathogens such as bacteria, viruses, and parasites. It can be transmitted between humans, animals, and animals or between humans and animals.[1-3] Infectious diseases not only bring serious threats to people's lives and health but also have a major impact on economic and social life.

Infectious diseases have for centuries ranked with wars and famine as major challenges to human progress and survival. They remain among the leading causes of death and disability worldwide.[4] In history, there have been three major outbreaks of the Black Death, the most severe outbreak occurred in the Middle Ages 1346-1353, which took 75-200 million deaths of lives.[5] Smallpox is caused by the smallpox virus that spreads through the nose, saliva, and fluids in the pimples of carriers. It is first recorded as a pandemic in 1520, claiming at least 350 million lives in history. After nearly two centuries of struggle and hard work, smallpox has been eradicated by science in 1978.[6-7] The human immunodeficiency virus (HIV) is transmitted through bodily fluids, attacks the human immune system, and has a high mortality rate. The HIV virus has a long incubation period, and symptoms may not appear for many years after infection. Infected people normally don't necessarily know they have the virus, that's why it spreads so quickly. Currently, there is still no cure for Acquired Immune Deficiency Syndrome (AIDS). Globally, there are 37.7 million people were living with HIV, 1.5 million new cases, and 680,000 deaths due to AIDS-related illnesses, were reported yearly.[8] In recent years, the COVID-19 epidemic has become a severe and largest health crisis since the severe acute respiratory syndrome (SARS) outbreak in 2003. It is initially reported by China in late 2019. Now COVID-19 had spread to almost all countries in the world. COVID-19 was first identified in Wuhan as an emerging infectious disease. Its impact is yet to be determined, as observations and test results are changing rapidly, leading to high mortality rates. Among the common symptoms of the disease, cough, fever, shortness of breath and sometimes diarrhea can be mentioned.[9-10]

Fortunately, with the development of science and technology and the substantial improvement of productivity, great progress has been made in the mechanism of various infectious diseases, their

treatment, and prevention methods. In 1928, *Alexander Fleming* found penicillin in the plate culture dish of *Staphylococcus*. Subsequently, around 1940, Sir *Howard Walter Florey* and Professor *Ernst Boris Chain* proved that penicillin could treat bacterial infections and has a therapeutic effect through many experiments. In addition, they established a culture solution from *Penicillium* and the method of extracting penicillin.[11-12] The discoveries of these three scientists have brought penicillin into human life, saved thousands of lives, made humans enter a new era in the fight against diseases, and made great contributions to the improvement of human health. Over the next half century, people have used various traditional and genetic methods to carry out many transformations on penicillin-producing strains, constantly improving the culture medium and fermentation conditions, improving the fermentation equipment and related equipment, and controlling the fermentation process. The production level of penicillin has been continuously improved.[13] Especially in the past ten years, the synthetic route, and related metabolic pathways of penicillin in microorganisms have been comprehensively studied. In the production of strains, increasing metabolic regulation, controlling the fermentation process to use raw materials as much as possible, reducing the production of by-products, and improving the separation and purification technology have increased the fermentation titer by nearly 2,500 times. Since humans entered the 21st century, with the improvement of the medical level, humans have been able to understand the mechanism of most diseases and their treatment methods.[14]

However, even though humans have made great strides in the fight against infectious diseases, infectious diseases still threaten human health and life. Especially in today's highly interconnected world, infectious diseases will not only affect people's health and cause death, but the epidemics caused by infectious diseases will also severely limit and affect international cultural exchanges, business exchanges, and goods exchanges. Then affect the economic development of various countries and regions. Worse, epidemics may even cause serious social unrest in some countries and regions. As shown in Table 1, due to the impact of the Covid-19 epidemic, the economies of various organizations and countries around the world have been affected differently.

Table 1. Growth projections (real GDP, change of percent annually).[15]

	2019		2020		2021	
	WB	IMF	WB	IMF	WB	IMF
World output	2.9	2.4	-3.0	-5.2	5.8	4.2
Developed Economies	1.7	1.6	-6.1	-7.0	4.5	3.9
United States	2.3	2.3	-5.9	-6.1	4.7	4.0
Euro Area	1.2	1.2	-7.5	-9.1	4.7	4.5
Japan	0.7	0.7	-5.2	-6.1	3.0	2.5
Emerging Markets and Economies	3.7	3.5	-1.0	-2.5	6.6	4.6
East Asia and Pacific	5.5	5.9	1.0	0.5	8.5	6.6
Europe and Central Asia	2.1	2.2	-5.2	-4.7	4.2	3.6
Latin America and the Caribbean	0.1	0.8	-5.2	-7.2	3.4	2.8
Middle East and North Africa	1.2	-0.2	-2.8	-4.2	4.0	2.3
Sub-Saharan Africa	3.1	2.2	-1.6	-2.8	4.1	3.1

1.2 Infectious disease detection

Against such an important background, the detection of infectious diseases is particularly important to curb the spread of the epidemic.[14] In addition, global efforts to combat the emerging infectious diseases are largely based on post-outbreak control, drug and vaccine development, as well as awareness campaigns. However, due to lack of accessible diagnostics, time-consuming and delays in the detection of emerging pathogens have resulted in economic losses worldwide and high mortality rates. Normally infectious disease detection methods include culture, separation and identification, protein detection, biopsy, biochemical examination, X-ray examination, ultrasound examination, CT examination, nucleic acid testing and so on. However, precision instrument detection method including biochemical examination, X-ray examination, CT not only time

consuming but also expensive cost. early-stage diagnosis of the disease is important. It needs to develop the systems to monitor healthcare regularly. Therefore, develop a simple, sensitive and affordable point-of-care testing(POCT) has become the need of between diagnostics and treatment of deadly diseases prevailing in nowadays. Point-of-care testing for example medical diagnostic testing performed outside the clinical laboratory in close proximity to where the patient is receiving care are an excellent way to fight the epidemics faced by the world every now and then. Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Delivered to end user (ASSURED) need to be considered in the development of POCT. With the advancement of science and technology, point-of-care testing has become a very popular and important way to conduct infectious disease diagnosis. Point-of-care testing or in vitro diagnostic (IVD) tests that don't require the use of laboratory staff and facilities to provide the result. The analytical targets include proteins, nucleic acids, dissolved ions and gases, metabolites, human cells, drugs and microbes. Normally samples are blood, urine, saliva or other bodily fluids. Whether used "near the patient" in a hospital, or doctor's office, or at home to maintain health, manage disease or monitor treatment, or in the field to test the safety of water, food, or to comply with laws and regulations, these tests are widely accepted. Little or no pre-prepared samples and results in seconds to hours.[16] POCT of protein detection are shown below.

1.2.1 Immunofluorescence

According to the binding steps of the antigen-antibody reaction, immunofluorescence methods can be divided into the following three types: 1. direct method 2. indirect method 3. complement method.[17]

Direct method: The specific antibody labeled with fluorescein is directly combined with the corresponding antigen to check the corresponding antigen component.

The indirect method: First, the specific antibody is combined with the corresponding antigen, the unbound antibody is washed away. Then the fluorescein-labeled second antibody is combined with the specific antibody to form an antigen-specific antibody-fluorescein-labeled second antibody

complex. There are more fluorescent antibodies on this complex than the direct method, so this method is more sensitive than the direct method.

Complement method: The mixture of specific antibody and complement is used to react with the antigen on the specimen, the complement is bound to the antigen-antibody complex, and then the anti-complement fluorescent antibody is combined with it to form an antigen-antibody-complement-anti-complement fluorescent antibody complex. The part that emits fluorescence seen under a fluorescence microscope is the site where the antigen is located. Complement method has the advantage of strong sensitivity and is suitable for the labeling of specific antibodies from various species. It is the most used technical method in the detection of antibodies from various species.

1.2.2 ELISA

Enzyme linked immunosorbent assay (ELISA) is one of the most popular methods, which is also a commonly used biochemical analysis method. Different from immunofluorescence, ELISA can not only perform qualitative analysis, but also quantitative analysis.[17] It is firstly proposed by Eva Engvall and *Peter Perlmann* in 1971, it has become standardized for protein detection in nowadays.[18] The principle are:

1. The antigen or antibody is combined with the surface of a certain solid phase carrier to maintain its immune activity.

2. The antigen or antibody is combined with a certain enzyme to form an enzyme-labeled antigen or antibody, which not only retains the activity of the enzyme, but also retains its immune activity.

During the measurement process, the antibody or antigen in the sample to be tested and the enzyme-labeled antigen or antibody react with the antigen or antibody on the surface of the solid phase carrier according to different steps. The antigen-antibody complex formed on the solid phase carrier is separated from other substances by washing, and the amount of enzyme bound to the solid phase carrier is proportional to the amount of the tested substance in the sample. After adding the substrate of the enzyme reaction, the substrate is catalyzed by the enzyme into a colored product, and the amount of the product is directly related to the amount of the tested substance in the sample.

Therefore, qualitative or quantitative analysis can be performed depending on the depth of the color reaction. Due to the high catalytic efficiency of the enzyme, the reaction effect can be greatly amplified, and the assay method can achieve high sensitivity.

1.2.3 Immune colloidal gold technology

Immune colloidal gold technology is a new type of immunolabeling technology that uses colloidal gold as a tracer marker for antigen and antibody applications. Colloidal gold is polymerized into gold particles of specific size by chloroauric acid (HAuCl_4) under the action of reducing agents such as tannic acid, sodium citrate, ascorbic acid, and white phosphorus. Then become a stable colloidal state due to electrostatic action.[17] Its principle is to immobilize a known specific antigen or antibody on a certain zone on the Nitrocellulose membrane(NC membrane) as a T-band. After the sample is dripped in the sample area, the sample is surging on the NC membrane by means of capillary action, and the gold-labeled complex is formed. When it dissolves and reacts with the sample to form a complex, the complex continues to surge to the T-band of the NC membrane. The gold-labeled complex is captured by the T-band antigen or antibody, showing a red band. If there is no antigen or antibody to be tested in the sample, no binding occurs as well as no coloration occurs.[18]

However, although some of these detection reagents for antigens or antibodies have been marketed and used in infectious disease detection, there are still many defects such as long detection window period, cross-reactivity, and high false positive rate. Especially the long detection window period problem, most virus have an incubation period after infecting the host. After a virus infects a host cell, it needs to proliferate to a certain amount before the cell is ruptured and released. At this time, the antigen can be detected by means of protein detection, thereby judging the type of infectious disease. Therefore, it is not easy to detect infectious diseases from infected people during its incubation time.[19][20]

1.3 Introduction of conventional nucleic acids testing (NAT) methods

Nucleic acids testing technology or called nucleic acids detection is highly specific as well as sensitive to viral nucleic acids. It is based on the amplification of target regions of viral RNA or DNA, which can detect pathogens or viruses earlier than other screening methods, thereby shortening the window period of viral infection. Nucleic acids testing also addresses the benefits of serologically false reactive donations, which are important for donor notification and counseling.[21]

Nucleic acids detection plays an important role in food safety, environmental monitoring, criminal investigation, and infectious disease diagnosis. To overcome detection window period problem of infectious diseases, nucleic acids detection has become a popular detection method. In the early diagnosis of infectious diseases, the method of microbial culture was often used to verify the disease, which is not only time-consuming but also prone to misjudgment. Nowadays, polymerase chain reaction (PCR) based nucleic acids detection method has become an effective measure for infectious diseases detection. It is one kind of molecular biology technique that is used to amplify DNA fragments. It can be regarded as a special kind of DNA replication in vitro, and its biggest feature is that it can greatly increase the trace amount of DNA. PCR was invented by Kary Mullis in 1986 for the use of enzymes to amplify DNA in vitro.[20][22] The technology has been likened to a “molecular copier” because of its ability to recognize specific DNA sequences and synthesize large numbers of copies quickly and accurately.[23] The target DNA is supplied in small amount in the reaction and is copied by using short oligonucleotide primers which complementary to the ends of the template DNA.[24] Today it has revolutionized the field of molecular biology, especially DNA manipulation, diagnosis of infectious and genetic diseases, species investigation and criminal forensics. To date, various derivatization techniques based on the original PCR method have been invented. For example, real-time PCR, also known as quantitative PCR (qPCR), which combines PCR amplification and makes the detection become a single step. Now the qPCR assays mainly divide into two approaches: absolute quantification and relative quantification.[25]

Another technique known as reverse transcription polymerase chain reaction (RT-PCR). This technique uses RNA as the nucleic acid amplification template.[26] PCR involves repeated cycles

of heating and cooling of a reaction mixture containing DNA template, DNA polymerase, primers, and nucleotides. The PCR product will be visualized and analyzed by the using of agarose gel electrophoresis. Through the basis of size and charge, DNA products will be separated. Reverse transcription RT-PCR is used to amplify RNA targets. Through the enzyme that named reverse transcriptase, the RNA template is converted into complementary cDNA. After that, the cDNA serves later will be the template for exponential amplification using PCR. RT-PCR can be performed in one or two steps. In the one-step method, RT-PCR combines RT and PCR reactions in the same tube. This requires that only sequence-specific primers be used for the reaction. In the two-step method, during RT-PCR, the synthesized cDNA is transferred to a second tube for PCR. Oligonucleotides (dT), random hexamers, or gene-specific primers are required during this period. Oligo (dT) primers are often preferred because they hybridize to the 3' poly (A) tail in the mRNA transcript gene sequence, while random primers prime anything including ribosomal RNA.[27]

1.4 Introduction of thin-film transistor (TFT) biosensor for nucleic acids detection

To overcome conventional nucleic acids detection shortcoming, that requires laboratory setting, professional operation, over 2 hours cost.[28] TFT biosensor for nucleic acids detection is one another advanced and quick approach. The TFT is a special type of field-effect transistor (FET) where the transistor is thin relative to the plane of the device. It uses an electric field to control the flow of current in a semiconductor. TFT biosensor consist of three terminals: source electrode, drain electrode and gate electrode. TFT can control the flow of current by the application of a voltage to the gate, which in turn alters the conductivity between the source and drain electrode. It has the advantages of high input resistance ($10^7\sim 10^{15}\Omega$), low noise, low power consumption, large dynamic range, easy integration, no secondary breakdown phenomenon, and wide safe working area.

Over the past decade, the use of field-effect-based devices has become a fundamental structural element of a new generation of biosensors that enable label-free DNA analysis. Ion-sensitive TFTs are the basis for the development of entirely new methods for the specific detection and characterization of DNA because of their higher signal-to-noise ratios, fast measurement capabilities, and the possibility of being included in portable instruments. Reliable molecular characterization of

DNA as well as RNA is critical for disease diagnosis and for tracking changes in gene expression profiles. Therefore, TFT biosensors may become relevant tools for molecular diagnostics and point-of-care. It is now widely used in semiconductor chip industry.[28][29]

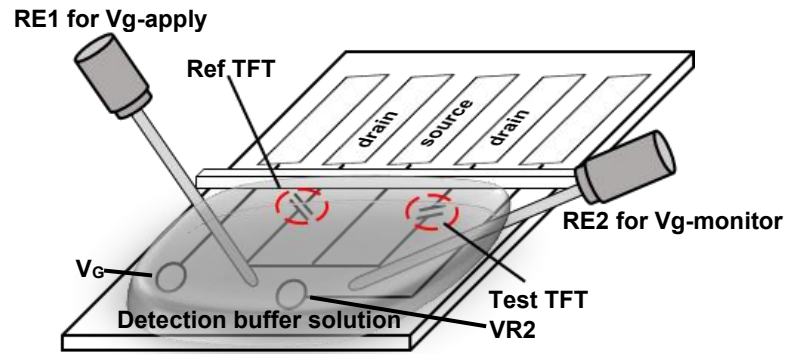


Figure 1 TFT biosensor structure

Because TFT working principle and excellent signal conversion ability, it can be used in voltage or current signal amplification and convert in biosensors. Figure 1 shows the TFT biosensor structure. All the TFT biosensors were made in our laboratory by photolithography technology. The biosensor generally has 2 drain electrodes and share with one same source electrode. The purpose of this design is to allow a single TFT biosensor perform two or more nucleic acid testing at the same time. It makes the TFT biosensor can not only improve the detection speed by one TFT biosensor but also can improve the detection accuracy. In addition, the TFT biosensor also has two artificial electrodes that made by Ag/AgCl paste. Ag/AgCl electrode VR2 is for recording voltage and current I_d changes. Ag/AgCl electrode V_G is the gate electrode of TFT biosensor, which is used to supply voltage for TFT biosensor.

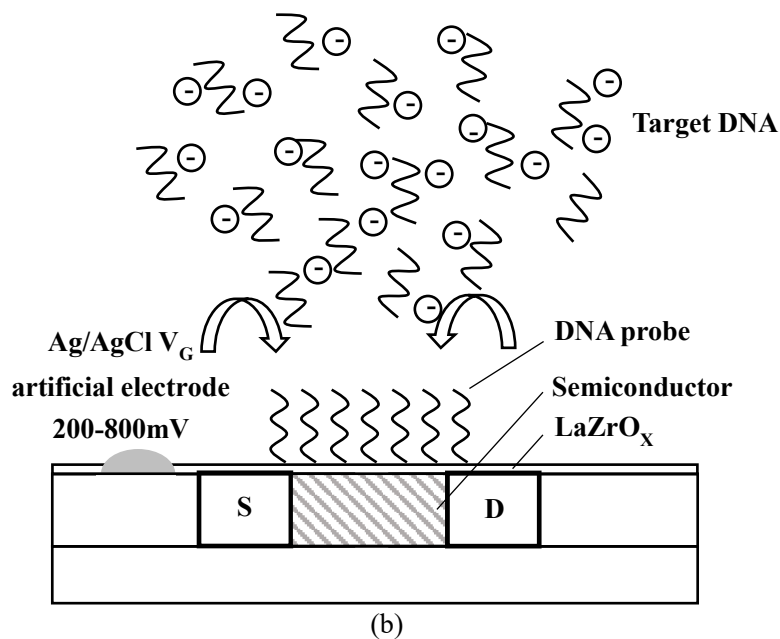
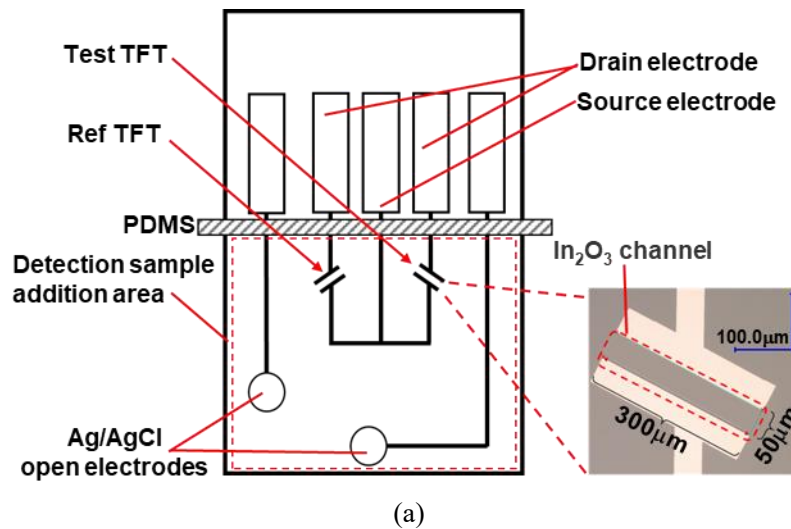


Figure 2: TFT biosensor working principle

Figure 2 shows the TFT biosensor working principle. First the DNA probe will be fixed on the surface of the TFT biosensor, then connect the source electrode, drain electrode and gate electrode of the TFT biosensor with semiconductor parameter analyzer. In some experiments, a standard reference electrode RE2 will be also set to monitor potential variation of detection sample as shown in Figure 1. Then add detection buffer solution onto the “detection sample addition area”, apply a voltage of 200mV between the source electrode and drain electrode, the passing current is recorded as I_d . At same time, a 200mV-800mV(in some case, it is 0mV-800mV) changing voltage V_G is applied to the Ag/AgCl gate. The gate and the TFT biosensor surface will form an electric field to attract electrons of TFT biosensor to its surface. As the number of electrons increases, the electrons

gathered in the semiconductor indium oxide (In_2O_3) channel will also increase. When the threshold value is reached, the currents I_d become able to flow from source electrode and drain electrode. At this point the current change graph VR2- I_d or RE2- I_d can be made. Then add the DNA sample to the “detection sample adding area”. If the sample contains the target nucleic acid, it will specifically bind to the DNA probe on the surface of the TFT biosensor. Since the nucleic acid is a negatively charged particle under the pH value of the buffer, this will lead to current I_d value decreases, so that whether the sample contains target nucleic acid can be judged and achieve the purpose of rapid nucleic acid detection.

1.6 Purpose and outlines

TFT biosensor has been recognized as a powerful technique for label-free, sensitive, real-time, and multifunctional biosensing, coupled with the characteristics, The TFT biosensor will help for nucleic acid testing with advantages of simple and rapid detection procedures, low sample consumption, and low cost.

This research is aimed to investigate the factors that makes the instability of TFT biosensor in measurements. Further finding the surface of TFT biosensor modification approach to improve its stability in measurements. Then combine the TFT biosensor with nucleic acid amplification to build more rapid and accurate nucleic acid detection system for POCT, making nucleic acids testing accessible for resource poor settings such as in developing countries and quick nucleic acids testing for infectious disease. These methods provide excellent specificity, sensitivity and simplicity to be used in the development of POCT devices for rapid and cost-effective health monitoring.

1.6 References

- [1]Aktar, M. A., Alam, M. M., & Al-Amin, A. Q. (2021). Global economic crisis, energy use, CO₂ emissions, and policy roadmap amid COVID-19. In *Sustainable Production and Consumption* (Vol. 26, pp. 770–781). Elsevier B.V. <https://doi.org/10.1016/j.spc.2020.12.029>
- [2]Albright, F. (1942). Communications to the editors. In *Journal of Clinical Endocrinology and*

- Metabolism* (Vol. 2, Issue 8, p. 537). <https://doi.org/10.1210/jcem-2-8-537>
- [3] Baynes J, D. MH. (n.d.). *Medical Biochemistry - John Baynes, Marek H. Dominiczak - Google book*.
- [4] Behbehani, A. M. (1983). The smallpox story: Life and death of an old disease. In *Microbiological Reviews* (Vol. 47, Issue 4, pp. 455–509). <https://doi.org/10.1128/membr.47.4.455-509.1983>
- [5] Buchholz, K. (2016). A breakthrough in enzyme technology to fight penicillin resistance— industrial application of penicillin amidase. *Applied Microbiology and Biotechnology*, 100(9), 3825–3839. <https://doi.org/10.1007/s00253-016-7399-6>
- [6] Daszak, P., Cunningham, A. A., & Hyatt, A. D. (2003). Infectious disease and amphibian population declines. In *Diversity and Distributions* (Vol. 9, Issue 2, pp. 141–150). <https://doi.org/10.1046/j.1472-4642.2003.00016.x>
- [7] David M. Morens, Gregory K. Folkers, & Anthony S. Fauci. (2004). The challenge of emerging and re-emerging infectious diseases. *Nature*, 430(July), 242–249.
- [8] Dodd, R. Y., Notari, E. P., & Stramer, S. L. (2002). Current prevalence and incidence of infectious disease markers and estimated window-period risk in the American Red Cross blood donor population. *Transfusion*, 42(8), 975–979. <https://doi.org/10.1046/j.1537-2995.2002.00174.x>
- [9] Duncan, C. J., & Scott, S. (2005). What caused the Black Death? In *Postgraduate Medical Journal* (Vol. 81, Issue 955, pp. 315–320). <https://doi.org/10.1136/pgmj.2004.024075>
- [10] Fardin, M. A. (2020). Covid-19 and anxiety: A review of psychological impacts of infectious disease outbreaks. In *Archives of Clinical Infectious Diseases* (Vol. 15, Issue COVID-19). Kowsar Medical Publishing Company. <https://doi.org/10.5812/archcid.102779>
- [11] Fraga, D., Meulia, T., & Fenster, S. (2008). Real-Time PCR. *Current Protocols Essential Laboratory Techniques*, 00(1), 10.3.1-10.3.34. <https://doi.org/10.1002/9780470089941.et1003s00>
- [12] Geddes, A. M. (2006). The history of smallpox. *Clinics in Dermatology*, 24(3), 152–157. <https://doi.org/10.1016/j.clindermatol.2005.11.009>
- [13] Glynn, S. A., Kleinman, S. H., Wright, D. J., & Busch, M. P. (2002). International application of the incidence rate/window period model. In *Transfusion* (Vol. 42, Issue 8, pp. 966–972). <https://doi.org/10.1046/j.1537-2995.2002.00200.x>

- [14]Gubala, V., Harris, L. F., Ricco, A. J., Tan, M. X., & Williams, D. E. (2012). Point of care diagnostics: Status and future. In *Analytical Chemistry* (Vol. 84, Issue 2, pp. 487–515). <https://doi.org/10.1021/ac2030199>
- [15]Hans, R., & Marwaha, N. (2014). Nucleic acid testing-benefits and constraints. In *Asian Journal of Transfusion Science* (Vol. 8, Issue 1, pp. 2–3). Wolters Kluwer -- Medknow Publications. <https://doi.org/10.4103/0973-6247.126679>
- [16]Hareru, H. E., Kaso, A. W., Ashuro, Z., & Mareg, M. (2022). Risky sexual practice and associated factors among people living with HIV/AIDS receiving antiretroviral therapy in Ethiopia: Systematic review and meta-analysis. *PLOS ONE*, *17*(4), e0266884. <https://doi.org/10.1371/journal.pone.0266884>
- [17]Im, K., Mareninov, S., Diaz, M. F. P., & Yong, W. H. (2019). An introduction to performing immunofluorescence staining. In *Methods in Molecular Biology* (Vol. 1897, pp. 299–311). Humana Press Inc. https://doi.org/10.1007/978-1-4939-8935-5_26
- [18]Jalali, M., Zaborowska, J., & Jalali, M. (2017). The Polymerase Chain Reaction: PCR, qPCR, and RT-PCR. In *Basic Science Methods for Clinical Researchers*. Elsevier Inc. <https://doi.org/10.1016/B978-0-12-803077-6.00001-1>
- [19]Keesing, F., Belden, L. K., Daszak, P., Dobson, A., Harvell, C. D., Holt, R. D., Hudson, P., Jolles, A., Jones, K. E., Mitchell, C. E., Myers, S. S., Bogich, T., & Ostfeld, R. S. (2010). Impacts of biodiversity on the emergence and transmission of infectious diseases. In *Nature* (Vol. 468, Issue 7324, pp. 647–652). Nature Publishing Group. <https://doi.org/10.1038/nature09575>
- [20]Ligon, B. L. (2004). Penicillin: Its Discovery and Early Development. *Seminars in Pediatric Infectious Diseases*, *15*(1), 52–57. <https://doi.org/10.1053/j.spid.2004.02.001>
- [21]Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., & Erlich, H. (1986). Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction. *Cold Spring Harbor Symposia on Quantitative Biology*, *51*(1), 263–273. <https://doi.org/10.1101/sqb.1986.051.01.032>
- [22]Oxner, E. S. (2020). Fet Technology and Application. In *Fet Technology and Application*. CRC Press. <https://doi.org/10.1201/9781003066149>
- [23]Pollard, A. J., & Bijker, E. M. (2021). A guide to vaccinology: from basic principles to new developments. In *Nature Reviews Immunology* (Vol. 21, Issue 2, pp. 83–100). Nature Publishing

Group. <https://doi.org/10.1038/s41577-020-00479-7>

[24]Promega. (2005). *PCR Amplification | An Introduction to PCR Methods | Promega*. Promega Corporation. <https://www.promega.jp/en/resources/guides/nucleic-acid-analysis/pcr-amplification/?cs=y>

[25]Sellars, M. J., Vuocolo, T., Leeton, L. A., Coman, G. J., Degnan, B. M., & Preston, N. P. (2007). Real-time RT-PCR quantification of Kuruma shrimp transcripts: A comparison of relative and absolute quantification procedures. *Journal of Biotechnology*, *129*(3), 391–399. <https://doi.org/10.1016/j.jbiotec.2007.01.029>

[26]Smith, K. F., Sax, D. F., Gaines, S. D., Guernier, V., & Guégan, J. F. (2007). Globalization of human infectious disease. *Ecology*, *88*(8), 1903–1910. <https://doi.org/10.1890/06-1052.1>

[27]Veigas, B., Fortunato, E., & Baptista, P. V. (2015). Field effect sensors for nucleic acid detection: Recent advances and future perspectives. In *Sensors (Switzerland)* (Vol. 15, Issue 5, pp. 10380–10398). Multidisciplinary Digital Publishing Institute. <https://doi.org/10.3390/s150510380>

[28]Wainwright, M. (1993). The mystery of the plate: Fleming's discovery and contribution to the early development of penicillin. *Journal of Medical Biography*, *1*(1), 59–65. <https://doi.org/10.1177/096777209300100113>

[29]Wang, C., Pan, R., Wan, X., Tan, Y., Xu, L., Ho, C. S., & Ho, R. C. (2020). Immediate psychological responses and associated factors during the initial stage of the 2019 coronavirus disease (COVID-19) epidemic among the general population in China. *International Journal of Environmental Research and Public Health*, *17*(5). <https://doi.org/10.3390/ijerph17051729>

**Chapter2. RPA-based Nucleic Acid Detection with PCM
Temperature Control System**

2.1 Nucleic acid isothermal amplification

Nucleic acid detection promises rapid, sensitive and specific diagnosis of infectious diseases. The next generation of diagnostic devices will interrogate the genetic determinants of such conditions at the point-of-care diagnosis, affording clinicians prompt reliable diagnosis from which to guide more effective treatment and epidemic prevention measures. The low abundance of nucleic acid targets and complex biochemical nature of in the majority of clinical samples and existing biosensor technology indicate that some form of nucleic acid amplification will be required to obtain clinically relevant sensitivities from the small samples used in POCT.[1]

In recent years, with the progress of society and science and the rapid development of molecular biology techniques, many nucleic acid testing-based infectious disease diagnostic methods have been established and widely used in infectious diseases detection, especially isothermal nucleic acid amplification technologies for POCT have been developed in nucleic acid detection. This technique can deal with protein assay and conventional PCR-based nucleic acid detection problems. In addition, because amplifying nucleic acid without a thermal cycle, it performs faster, more efficiently, and short time in nucleic acid detection. They can complete infectious diseases detection without special equipment, so it can support reducing the limitation for nucleic acid testing of POCT.[2-3]

2.2.1 LAMP

Loop-mediated isothermal amplification (LAMP) is an isothermal nucleic acid amplification technic that was published in the *Nucleic Acids Research* by Japanese scholar *Notomi et al* in 2000.[4] LAMP requires a set of four specially designed primers to recognize six distinct regions of the target and relies on auto cycling circulating strand displacing DNA synthesis with large fragments of *Bst* DNA polymerase under 60°C-65°C. Through the photometry, the amplification product can be detected. It can measure the turbidity caused by magnesium pyrophosphate precipitate in solution as a byproduct of amplification. Thus, the naked eye or via simple photometric detection approaches for small volumes can achieve the nucleic detection.[5-6]

Advantages of LAMP:

- (1) High amplification efficiency: it can effectively amplify 1-10 copies of the target gene within 1 hour, and the amplification efficiency is 10-100 times that of ordinary PCR.
- (2) The reaction time is short, the specificity is strong, and no special equipment is required.

Disadvantages of LAMP:

- (1) The requirements for primers are particularly high.
- (2) The amplified product cannot be used for cloning and sequencing, but can only be used for judgment.
- (3) Due to its strong sensitivity, it is particularly easy to form aerosols, causing false positives to affect the test results.

2.2.2 RCA

Rolling Circle Amplification (RCA) is one nucleic acid detection method combining target nucleic acid and signal amplification, which developed from the DNA amplification mechanism of self-replication in bacteriophage infection. This replication form can amplify cyclic single-stranded DNA at the same temperature with relatively infinite single-strand amplification. And this technique only requires room temperature. The template for RCA must be closed single-stranded cyclic DNA, so it has been applied to the study of single nucleotide polymorphism (SNP) and viral gene detection and gene expression profiles. Therefore, it is only limited to the single-stranded cyclic DNA such as viruses, plasmids, and circular chromosomes.[7]

Advantages of RCA:

- (1) High sensitivity: RCA has strong amplification ability, the efficiency of linear RCA can reach 10^5 times, and the efficiency of exponential RCA can reach 10^9 times, which has the potential to detect single copies.
- (2) High sequence specificity, which can distinguish different patterns of a single site.
- (3) The amplified product can be directly used for sequencing after phosphorylation.

(4) High-throughput: RCA can form a closed circular sequence on the target to ensure that the signal generated by RCA is concentrated at one point, to achieve in situ amplification and slide amplification.

Disadvantages of RCA:

(1) The complex probes are often close to 100 bp, and the synthesis cost is high.

(2) There is a background interference problem during signal detection.

2.2.3 SDA

Strand displacement amplification (SDA) was first proposed by American scholar Walker et al. in 1992. An isothermal DNA amplification technique in vitro based on enzymatic reaction. The basic system includes one restriction endonuclease and one enucleate. DNA polymerase with chain replacement activity, 2 pairs of primers, dNTP, and Calcium and magnesium ion and buffer systems. This amplification process can occur at 37°C~40°C. But the disadvantage is thermal denaturation step is required to open the double chain before isothermal amplification. And the products are not uniform, because there are some different single and double chain products that will be produced in the SDA cycle.[8]

SDA provides an amplification method that can be used for nucleic acid diagnostic analysis without temperature changes. Its significant advantage is high sensitivity and rapid amplification to obtain single-stranded DNA molecules. Compared with other DNA amplification techniques, SDA has the advantages of rapidity, high efficiency, and specificity, and does not require special equipment.

Disadvantages of SDA:

(1) In order to create a gap that can be used for amplification in SDA, non-standard nucleotides must be added to the reaction mixture, which not only increases the cost of the reaction, but also reduces the amplification efficiency.

(2) Both ends must have the recognition sequences of all endonucleases and their residues, so the SDA product is not suitable for direct cloning, which makes it no advantage in genetic engineering.

(3) SDA needs a heat denaturation to open before isothermal amplification. In the double-stranded step, because *exo-Klenow* has no thermal stability, it must be added to the system after the target DNA has been denatured, which is likely to cause contamination.

(4) The SDA product is not uniform, because some different single- and double-stranded products are always produced in the SDA cycle, which makes the electrophoresis detection of SDA amplification products inevitable tailing phenomenon.

(5) The current dominant method for SDA detection is the fluorescence polarization detection method, which requires a special instrument fluorescence spectrophotometer, which limits the wide application of SDA in clinical practice. In addition, SDA may not reflect the true content of target DNA in the sample due to the presence of unknown inhibitors in the sample. Competitive inhibition of low levels of target DNA by high levels of DNA in complex DNA is also common.

2.2.4 HDA

Helicase-dependent isothermal DNA amplification, HDA. It is an in vitro thermostatic gene amplification technique that simulates the replication mechanism of DNA in animals. It only requires 2 primers, and the design of the primers is simple. But the amplification reaction takes 75 min-90 min. It's not suitable for POCT of nucleic acid detection.[9]

HDA advantages:

(1) Simple and effective, easy to operate

(2) No expensive PCR instrument is needed, and it is more suitable for promotion and application in grassroots laboratories than bacteriology, immunology and PCR methods.

Disadvantages of HDA:

There are few researchers in HDA, and its development is not mature enough.

2.3 Recombinase Polymerase Amplification

In 2006, *Piepenburg* et al. developed the RPA technology using proteins involved in cellular

DNA synthesis, recombination and repair. The RPA process begins when the recombinase protein *UvsX* of the T4-like phage binds to the primer in the presence of ATP and a crowding agent and forms a recombinase-primer complex. This complex then interrogates the double-stranded DNA for homologous sequences and facilitates strand invasion of primers at the cognate site. The subsequently displaced DNA strands are stabilized by single-stranded binding proteins, a step that prevents the inserted primers from being ejected due to branch migration. Finally, the recombinase disintegrates, and a strand displacement DNA polymerase binds to the 3' end of the primer and elongates it in the presence of dNTPs. Eventually the cycle of this process repeats to achieve an exponential increase in nucleic acid amplification.

Compared with the PCR, although those isothermal amplification methods mentioned doesn't require a thermal cycle, some of them still require relatively high temperatures, or need complex primers, etc. Therefore, many of them are not easy to apply in POCT. Among the isothermal amplification technology, recombinase polymerase amplification (RPA), another isothermal amplification technique that can amplify DNA copies at 37°C-42°C within 10 min and less limitation in amplification, is more suitable for POCT of nucleic acid testing in vitro.[10-12] There are many papers already have reported the application of RPA, but most of them need electricity instrumented incubator or machine supporting. Some reports like *Lorraine Lillis* etc. tried to use chemical heater as heat source[12], but it can't provide stable temperature. *John P. Goertz* etc. applied phase change material to separate different reagents in order of reaction, and also applied PCM as thermal buffer area.[12-13] But the problem is it requires many materials and complex process to fabricate. In addition, the most important thing is the reaction temperature is unstable.

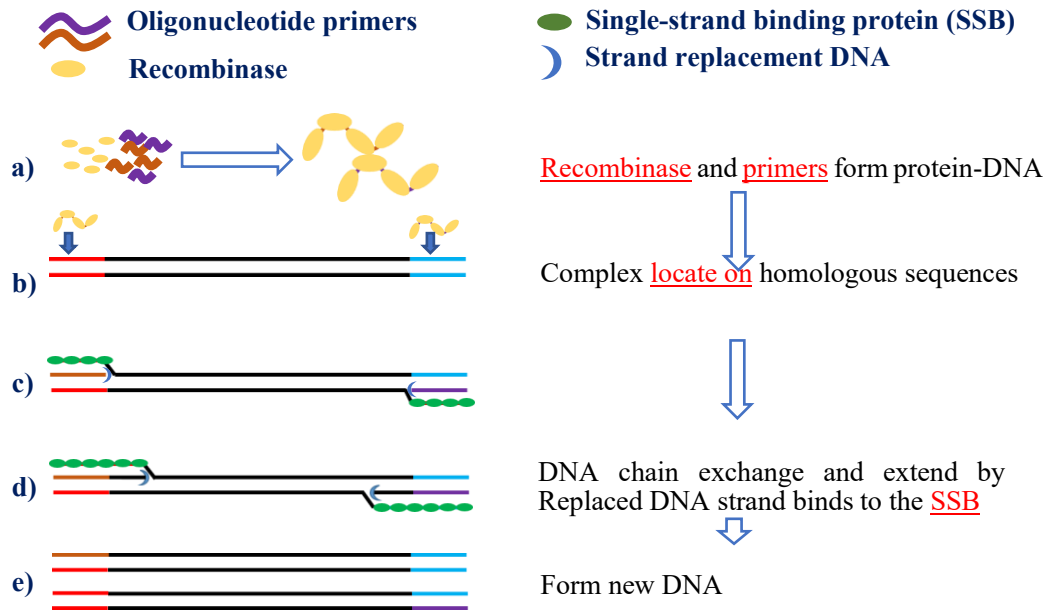


Figure 3 The RPA cycle

RPA technique relies on three enzymes: recombinase that can bind oligonucleotide primers, single-strand binding protein (SSB), and strand replacement DNA polymerase. Figure 3 shows the RPA reaction cycle. a) The protein-DNA complex formed by the combination of recombinase and primers. b) the protein-DNA complex can find homologous sequences in double-stranded DNA and locate on them. c) Once homologous sequences are located by primers, chain exchange reactions occur and DNA synthesis is initiated to exponentially amplify the target region on the template DNA. d) The replaced DNA strand binds to the SSB to prevent further replacement. e) Finally, new strands of DNA is formed. In this system, a synthetic event is initiated by two relative primers. The whole process is very fast, and detectable levels of amplified products can be obtained in less than 10 minutes.

2.3.1. Primer design

1. In general, RPA requires specially designed primers with a length of 30-35 bp. Longer primers such as 45 bp can also be used. In addition, RPA can also use common PCR primers to achieve efficient amplification. However, when designing primers, the length of the primers should not be too long, otherwise, dimers will be generated.

2. Second, primer design should avoid long G at the 5' end, while increasing cytidine is beneficial

to RPA, and guanine and cytidine at 3' tend to improve primer binding efficiency. GC content below 30% or above 70% is not recommended, and as with PCR primers, sequences that promote primer-primer interactions, secondary structure, or hairpins should not be used. Templates suitable for RPA amplification are between 100bp and 200 bp in length.

3. The primer selection process includes four steps: selection of target regions, design of primer candidates, experimental screening, and secondary and tertiary candidate screening if necessary. Self-avoiding molecular recognition (SAMR) oligonucleotides can also be used, in which the natural bases are replaced by A*, T*, G*, and C*, where A* is with T, T* with A, G* with C and C Pairs with G, but A* does not pair with T*, G* does not pair with C*, thus avoiding the formation of primer dimers.

2.4 Phase change material

With the rapid growth of the global population and economic development, non-renewable energy sources such as oil and natural gas are increasingly depleted, and the energy crisis is becoming more and more serious. However, in the process of energy exploitation and utilization, there is still no effective solution to the problem of low energy utilization. For example, in fuel vehicles, more than 50% of the energy in the fuel is dissipated into the air in the form of waste heat; in industrial production, a large amount of heat is dissipated in the form of waste heat. Energy is dissipated into the air in the form of heat, causing global warming while causing resource depletion. Therefore, the research and development of new energy storage materials to improve energy utilization has been an important research topic in the scientific community in recent years, which is expected to effectively alleviate the huge pressure caused by resource loss and environmental degradation. Phase-change materials (PCM) are a new type of green energy material. Although it cannot generate any form of energy by itself, it can use its phase-change thermal effect to convert the heat lost in the external environment into latent heat. It is stored in the form of energy, and the energy is released and utilized autonomously under suitable conditions, to achieve the purpose of improving the high utilization rate of energy. In addition, the use of phase change energy storage technology to collect and store the heat energy in sunlight, instead of the traditional fossil fuel

combustion for energy, can also provide a new way for the energy source of daily production and life.

At the same time, PCM can also control the ambient temperature near the phase transition point by absorbing or releasing a large amount of energy in the process of phase transition, while its own temperature fluctuates only slightly, so as to achieve the purpose of temperature control. Compared with the traditional thermal management method, this thermal management technology can efficiently remove heat from the heat source without any energy input, while ensuring that its own temperature has only a small fluctuation. With the advantages of high efficiency, energy saving and environmental protection, the thermal management technology of phase change materials has important development prospects in the fields of thermal management such as new energy vehicles and large energy storage batteries.

Phase change material is a green and recyclable energy storage material with extremely high latent heat of phase change, which can absorb or release a large amount of energy during the phase change process. From the perspective of thermodynamics, the principle of heat storage of phase change materials can be divided into two cases.

(1) The arrangement of molecules in the material changes: when the molecules are arranged in an orderly manner, the intermolecular vibration is slow and the internal energy is low; when the molecules are disordered, the intermolecular vibration is fast and the internal energy is high. When the molecular arrangement changes from an ordered arrangement to a disordered arrangement, the material on the macroscopic scale is endothermic; otherwise, it is exothermic. This kind of reaction is a physical reaction, and macroscopically manifests as the phenomenon of melting and solidification of materials, representing the solid-liquid reaction of organic phase change materials such as paraffin.

(2) Breaking and reorganization of bonds in materials: when bonds break in molecules, a large amount of energy needs to be provided to overcome the interaction force between atoms; on the contrary, when bonds are formed between atoms, This reduces the internal energy of the system and releases a lot of heat. This reaction is a chemical reaction, which means that inorganic phase change materials such as inorganic hydrates lose water and absorb water, and most of them are solid-

solid phase transitions.

There are many types of phase change materials. According to the material structure, phase change materials are divided into organic phase change materials, inorganic phase change materials and metal materials. Among them, the phase transition temperature of metal materials is generally above hundreds or even thousands of degrees Celsius, which is of little practical application significance. Generally, only organic phase change materials and inorganic phase change materials have large latent heat of phase change, and the parts with good thermal stability can be used for phase change energy storage. In addition, in actual production and life, the phase transition temperature is an important parameter for the selection of phase change materials. In the field of temperature control of phase change materials, the phase change temperature of the phase change material should be within the required optimum temperature range; in the field of heat storage of phase change materials, the phase change temperature should adapt to changes in the environment, while satisfying endothermic and exothermic requirements demand.

Phase change materials (PCM) is one kind of material that will change its physical state within a certain temperature range. When the physical state changes, the temperature of the material itself can be kept until phase transition process complete. Normally, PCM for latent heat storage is one of the most efficient ways.⁹ Therefore, it is being used in building construction material, goods transport, spaceflight, refrigeration and device cooling etc. To provide and control stable temperature for RPA-based nucleic acid testing, we used PCM as stable temperature providing media.

2.4.1 Solid-solid phase change materials

With the development of society and the improvement of people's health consciousness, POCT are being more and more important to us. Among them, nucleic acid testing is becoming more and more important in medical diagnostics. RPA, as a new DNA amplification method, the amplification and detection can be finished in 15 minutes under ideal condition. But the biggest problem is there are no stable temperature providing method without electricity instruments and low resource setting.

The phase change material could help to solve this problem.

According to its phase transformation process, PCM can be divided into solid-solid PCM, solid-liquid PCM, solid-gas PCM and liquid-gas PCM. At present, solid-liquid phase transformation materials are being widely used. However, solid-liquid PCM has the problems such as leakage, corrosion and volume change problems in application. To overcome these problems, solid-solid PCM were produced. The usual approach of produce solid-solid PCM is using carriers to lock the solid-liquid phase change materials. However, the essence of solid-solid phase change material in physical methods is still solid-liquid phase change material. Compared with physical methods, another method is chemical method. Chemical method refers to the chemical property change of carrier and organic phase change materials, usually contains polymerization and grafting, which results in the cross-linking of molecular chains. Chemical methods have completely changed the solid-liquid phase transition characteristics of organic phase change materials through intermolecular bonding, so it is a real solid-solid phase change material. This kind of solid-solid phase change material has stable performance, no supercooling, good mechanical properties and is easy to be processed into various shapes. However, it's less applied due to its high cost.

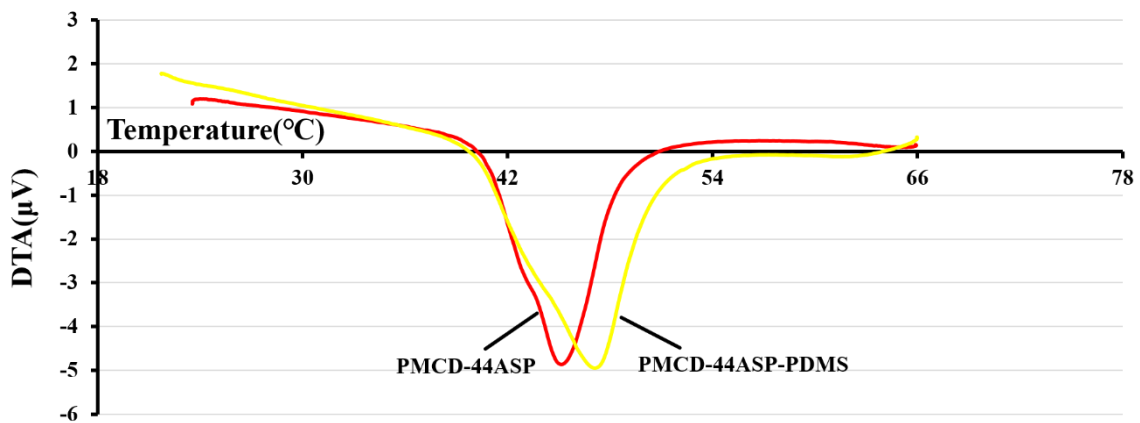


Figure 4 The DTA-temperature assay PMCD-44ASP and PMCD-44ASP-PDMS.

To balance the costs and simplify the preparation for solid-solid PCM. The approach that mix microcapsule PCM *PCMD-44ASP* powder with PDMS was found. *PCMD-44ASP* is one kind of microcapsule PCM, its center is Docosane ($C_{22}H_{46}$), the melting point is about 42°C-45°C, and it's cover by microcapsule wall. The Figure 4 shows the mixture has a high latent heat absorption around 40°C.

2.5 Objective

The aim of this this research is to combine PCM with RPA to make DNA detection can be used without electric instruments, enabling point-of-care testing for DNA detection and making DNA detection possible and facile in low-resource settings and in locations without electricity. With the PCM device, the detection of *Leishmania braziliensis* was demonstrated using the HSP70 gene as the target DNA. Polyacrylamide gel electrophoresis analysis was performed to confirm the RPA results. The success of HSP70 gene amplification also lays the foundation for the subsequent TFT detection

2.6 PDMS embedded PCM based temperature storage platform

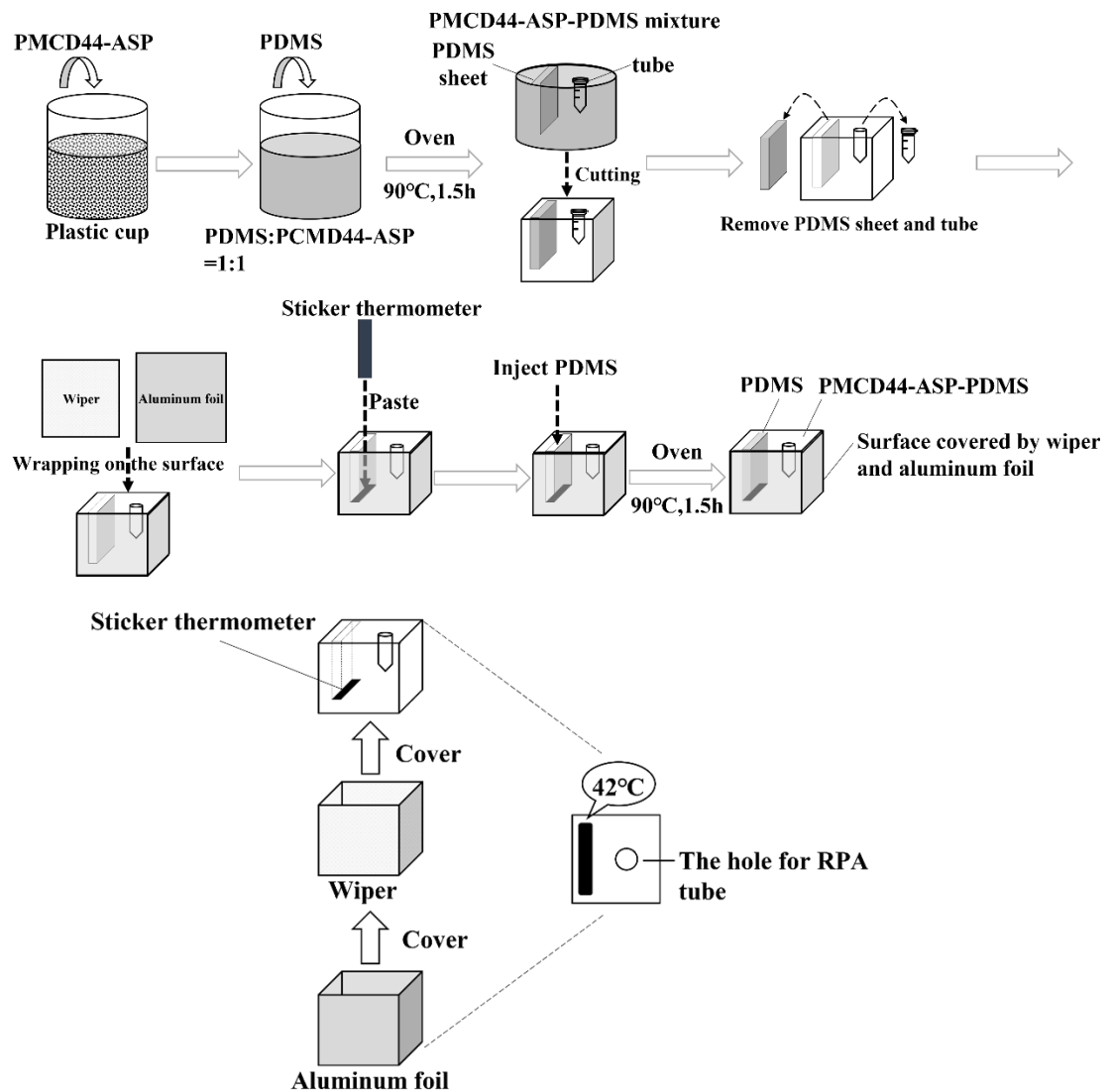


Figure 5 The space diagram and the real product of solid-solid PCM platform.

Figure 5 shows the structure of *PCMD-44ASP-PDMS* based solid-solid PCM platform. It mainly consists of 3 parts: *PCMD-44ASP-PDMS* mixture frame, sticker thermometer and RPA reaction area. To reduce heat exchange from environment and delay heat loss, thermal insulation material aluminum foil and wiper are used to cover the *PCMD-44ASP-PDMS* mixture frame. First use oven to heat the platform for a while, then cool it down at room temperature. When the sticker thermometer shows “40°C”, put the tube containing RPA sample into “RPA tube area”, and wait 10 min for RPA reaction.

The PCM device fabrication process shows in Figure 5. Firstly, using plastic cup to measure 5.5g *PCMD-44ASP* powder, mix with 11g *PDMS* and catalyst well. Then put the cup in vacuum pump to remove bubble about 40min. Use needle to wipe remainder bubble off as much as possible. Then put *PDMS* sheet and tube in *PCMD-44ASP* mixture. Put the plastic cup in oven to bake, 90°C 1.5h. After cooling down, take it out from plastic cup and cut the *PCMD-44ASP-PDMS* solid mixture into 3cm×3cm×1.5cm cube. In the same time, remove *PDMS* sheet and tube from solid mixture to reserve two holes, one for setting sticker thermometer and another for RPA tube. After that, wrap mixture in thermal insulation material, the purpose of this step is to reduce heat exchange from environment and delay heat loss. After pasting sticker thermometer onto the thermometer area, add *PDMS* to block and use vacuum pump to remove bubble again. Finally, put the PCM device in oven for 90°C 1.5h again. Then PCM device was fabricated. The physical map of PCM device was shown in Figure 6.

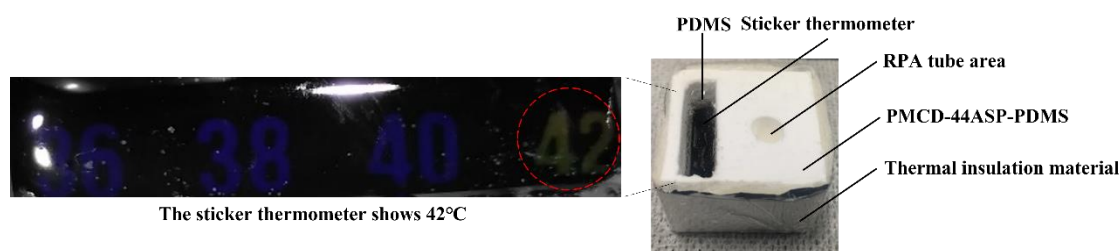


Figure 6 The physical map of PCM device

2.7 RPA based nucleic acid detection with *PDMS* embedded PCM platform

To investigate the temperature storage ability for RPA reaction. The Heat shock protein(HSP70) gene of *Leishmania braziliensis* was chose as target DNA for checking. Leishmaniasis is a sever

infectious diseases that caused by *Leishmania*. It can be transmitted via the bite of various phlebotomine sandfly species. HSP70 is conserved across prokaryotes and eukaryotes, one of the important characteristics of HSP in the biological world is their high conservatism in the process of evolution that can be used as identity for genus *Leishmania*. [14-15]

In the experiments, the RPA reaction is performed in thermo block rotator (thermos incubator) and PDMS embedded PCM platform at the same time. Then analyze the amplification results via 6% polyacrylamide gel electrophoresis. First according to the proportions of the manual, prepare positive and negative group of RPA sample, positive group contains template DNA, while negative group doesn't contain template DNA. To confirm the fluorescence whether from amplified DNA rather original DNA sample. One more negative control group sample, which doesn't contain primers but contain required reagent and template DNA, is also prepared. Then name the positive group, negative 1 group and negative 2 group as P, N1 and N2, respectively. After preparation, put the sample in thermos incubator and solid-solid PCM platform in the same time to amplify HSP70 gene of *Leishmania braziliensis*. Table 2 shows the solution composition. The details steps are as follows.

Table.2 The composition of 3 RPA reaction system groups

	Positive control (μL)	Negative control 1 (without primers) (μL)	Negative control 2 (without template DNA) (μL)
Rehydration buffer	10	10	10
Template DNA	1.2	1.2	0
Forward primer	0.7	0	0.7
Reserve primer	0.7	0	0.7
Nuclease-free water	2.6	4	3.8
Magnesium acetate	0.8	0.8	0.8
Total	16	16	16

Take out the RPA pellet from the -20°C refrigerator and put it on ice box. Add 30 μL rehydration buffer, mixed by pipette. Divided into 3 tubes as positive control group (P), negative control 1 group

(N1, contain template DNA, no primers) and negative control 2 group (N2, contain primers, no template DNA), 10 μ L of rehydration buffer was dispensed to each tube. Then add 1.2 μ L template DNA, 0.7 μ L forward primer, 0.7 μ L reverse primer, 2.6 μ L nuclease free water in positive control. Add 1.2 μ L template DNA, 2.6 μ L nuclease free water to N1 tube. Add 0.7 μ L forward primer, 0.7 μ L reverse primer, 3.8 μ L fresh water to N2 tube. Finally, add 0.8 μ L magnesium acetate on the cap of each tube. After 10 min, put the tubes onto ice box to stop RPA reaction. Then add 3 μ L proteinase K into all tubes, incubate 37 $^{\circ}$ C for 10 min by thermo-block rotator. To ensure the effect of all RPA approach, 6% polyacrylamide gel electrophoresis were used in analyzing amplification effect. Polyacrylamide gel was made of polyacrylamide (19:1) 1.5mL, 5 \times TBE buffer 2mL, 10% APS 50 μ L, TEMED 10 μ L, nuclease-free water 6.5 mL. Then add proteinase K processed DNA sample of thermo-block rotator and PCM device into 2 lanes of polyacrylamide gel. After electrophoresis, stain polyacrylamide gel for ten minutes and using UV exposure. Finally, recode and analyze the electrophoresis intensity to compare DNA amplification ability in different heating approach.

Figure 7 shows the 6% polyacrylamide gel electrophoresis results. The DNA bind of solid-solid PCM platform shows same level with the bind in thermos incubator. It indicates solid-solid PCM platform can successfully amplify target DNA and has a ability for temperature storage.

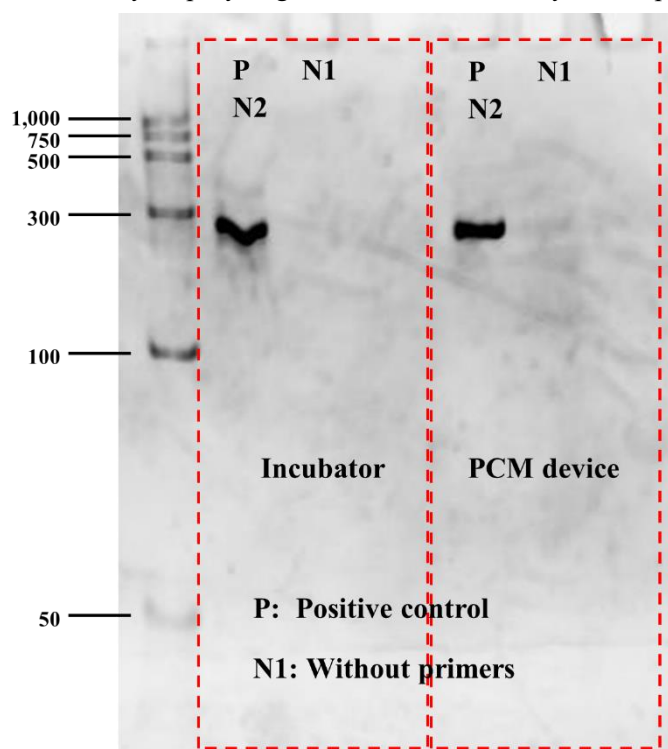


Figure 7 6% polyacrylamide gel electrophoresis results from P, N1 and N2

2.8 Conclusions

In initial scheme, 50 μ L RPA reaction was applying for DNA amplification, the divided it into two 25 μ L RPA reaction system, one for positive control (with template DNA), one for negative control (without template DNA). To confirm the fluorescence whether from amplified DNA rather original DNA sample. Another negative control sample that without primers but contain all other required reagent and template DNA was prepared. Therefore, to be able to show 3 control groups in the same time, 50 μ L RPA sample was divided into 3 groups, positive control (P), negative control 1 (N1, without primers) and negative control 2 (N2, without template) on the original basis (Table.2). Then the traditional method thermo-block rotator and PCM device for RPA were compared. From the Figure 7, the electrophoresis results of PCM device show PCM device has a good performance in amplifying DNA.

To reduce the time of reaching effective temperature control range for PCM device, oven was used to bake PCM device. Although using oven is impossible in low-resource setting and no electricity situation. However, it provides an idea that using such as water bath to heat PCM device to its stable temperature range. Once PCM device reach the stable temperature, it can hold that temperature for a long time, then it can be reused and provide stable temperature for RPA reaction, which makes DNA detection of POCT become possible and easier in low-resource setting areas and with no electricity situation.

2.9 Reference

- [1]Ali, M. M., Li, F., Zhang, Z., Zhang, K., Kang, D. K., Ankrum, J. A., Le, X. C., & Zhao, W. (2014). Rolling circle amplification: A versatile tool for chemical biology, materials science and medicine. *Chemical Society Reviews*, 43(10), 3324–3341. <https://doi.org/10.1039/c3cs60439j>
- [2]Augustine, R., Hasan, A., Das, S., Ahmed, R., Mori, Y., Notomi, T., Kevadiya, B. D., & Thakor, A. S. (2020). Loop-mediated isothermal amplification (Lamp): A rapid, sensitive, specific, and cost-effective point-of-care test for coronaviruses in the context of covid-19 pandemic. In *Biology* (Vol. 9, Issue 8, pp. 1–17). Multidisciplinary Digital Publishing Institute. <https://doi.org/10.3390/biology9080182>
- [3]Basu, N., Todgham, A. E., Ackerman, P. A., Bibeau, M. R., Nakano, K., Schulte, P. M., & Iwama,

- G. K. (2002). Heat shock protein genes and their functional significance in fish. *Gene*, 295(2), 173–183. [https://doi.org/10.1016/S0378-1119\(02\)00687-X](https://doi.org/10.1016/S0378-1119(02)00687-X)
- [4]Becherer, L., Borst, N., Bakheit, M., Frischmann, S., Zengerle, R., & Von Stetten, F. (2020). Loop-mediated isothermal amplification (LAMP)-review and classification of methods for sequence-specific detection. In *Analytical Methods* (Vol. 12, Issue 6, pp. 717–746). <https://doi.org/10.1039/c9ay02246e>
- [5]Craw, P., & Balachandran, W. (2012a). Isothermal nucleic acid amplification technologies for point-of-care diagnostics: A critical review. In *Lab on a Chip* (Vol. 12, Issue 14, pp. 2469–2486). The Royal Society of Chemistry. <https://doi.org/10.1039/c2lc40100b>
- [6]Craw, P., & Balachandran, W. (2012b). Isothermal nucleic acid amplification technologies for point-of-care diagnostics: A critical review. In *Lab on a Chip* (Vol. 12, Issue 14, pp. 2469–2486). The Royal Society of Chemistry. <https://doi.org/10.1039/c2lc40100b>
- [7]Euler, M., Wang, Y., Nentwich, O., Piepenburg, O., Hufert, F. T., & Weidmann, M. (2012). Recombinase polymerase amplification assay for rapid detection of Rift Valley fever virus. *Journal of Clinical Virology*, 54(4), 308–312. <https://doi.org/10.1016/j.jcv.2012.05.006>
- [8]Goertz, J. P., Colvin, K. M., Lippe, A. B., Daristotle, J. L., Kofinas, P., & White, I. M. (2018). Multistage Chemical Heating for Instrument-Free Biosensing. *ACS Applied Materials and Interfaces*, 10(39), 33043–33048. <https://doi.org/10.1021/acsami.8b11611>
- [9]Modak, S. S., Barber, C. A., Geva, E., Abrams, W. R., Malamud, D., & Ongagna, Y. S. Y. (2016). Rapid Point-of-Care Isothermal Amplification Assay for the Detection of Malaria without Nucleic Acid Purification. *Infectious Diseases: Research and Treatment*, 9, IDRT.S32162. <https://doi.org/10.4137/idrt.s32162>
- [10]Niemz, A., Ferguson, T. M., & Boyle, D. S. (2011). Point-of-care nucleic acid testing for infectious diseases. In *Trends in Biotechnology* (Vol. 29, Issue 5, pp. 240–250). Elsevier Current Trends. <https://doi.org/10.1016/j.tibtech.2011.01.007>
- [11]Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, 28(12), 63. <https://doi.org/10.1093/nar/28.12.e63>
- [12]Parida, M. M., Sannarangaiah, S., Dash, P. K., Rao, P. V. L., & Morita, K. (2008). Loop mediated

isothermal amplification (LAMP): A new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. In *Reviews in Medical Virology* (Vol. 18, Issue 6, pp. 407–421). Wiley InterScience. <https://doi.org/10.1002/rmv.593>

[13] Vincent, M., Xu, Y., & Kong, H. (2004). Helicase-dependent isothermal DNA amplification. *EMBO Reports*, 5(8), 795–800. <https://doi.org/10.1038/sj.embor.7400200>

[14] Walker, G. T., Little, M. C., Nadeau, J. G., & Shank, D. D. (1992). Isothermal in vitro amplification of DNA by a restriction enzyme/DNA polymerase system. *Proceedings of the National Academy of Sciences of the United States of America*, 89(1), 392–396. <https://doi.org/10.1073/pnas.89.1.392>

[15] Wang, X. J. (2011). Heat-shock protein 70 is associated with the entry of Marek's disease virus into fibroblast. *Acta Virologica*, 55(3), 189–194. https://doi.org/10.4149/av_2011_03_189

**Chapter 3. Factors of instability in oxide TFT biosensors and
surface analysis**

3.1 Introduction of instability in oxide TFT biosensor

V_G - I_d variation graphs of the TFT biosensor show instability in measurements. It is manifested in two or more V_G - I_d performing instability after replacing the measurement buffer. The instability of V_G - I_d in a TFT biosensor mainly comes from two aspects, inside of the TFT biosensor and measurement conditions. Depending on the detection conditions, its stability will also have a great impact.

3.2. Objective

In this chapter, it is expected to investigate the factors that affect the I_d variation instability of the TFT biosensor during the detection process by testing the TFT biosensors in different conditions, such as the measurement buffer concentration and so on. And the method of fluorescent labeling is used to analyze the surface of the TFT biosensor, expecting to find out whether the DNA hybridization on the chip surface is successful and find out the reason why the I_d variation of TFT biosensor shifted upward after DNA hybridization.

3.3 Fabrication and modification of oxide TFT biosensor

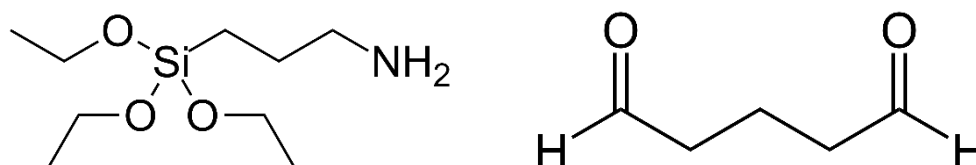
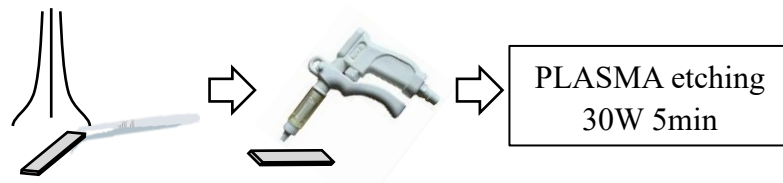


Figure 8 Structural formula of APTES (Left) and glutaraldehyde (Right)

To link the probe DNA on TFT biosensors, linkers reagents are required. The normal method is first reacting (3-Aminopropyl)triethoxysilane (APTES) with the hydroxyl(-OH) of the TFT biosensor surface to fix the APTES molecule on the chip surface. Then add glutaraldehyde on the chip surface to react with APTES to form the APTES-glutaraldehyde complex. Finally, glutaraldehyde reacts with probe DNA containing amino groups to complete the probe DNA immobilization.[1-3]

The TFT biosensors modification steps shows as follows:

Step1 Cleaning and increasing hydroxyl group



Use flowing deionized water(DIW) to wash TFT biosensors for around 10 seconds. Then dry the TFT biosensor with nitrogen gas. Process the biosensor by plasma ashing in 15 W, 300 seconds. Using plasma ashing not only can totally clean the surface of the TFT biosensor but also can slightly increase the hydroxyl group on the TFT biosensor.

Step2 making APTES layer

There are two main approaches that can generate an APTES layer on TFT biosensors: the wet APTES method and the vapor method.

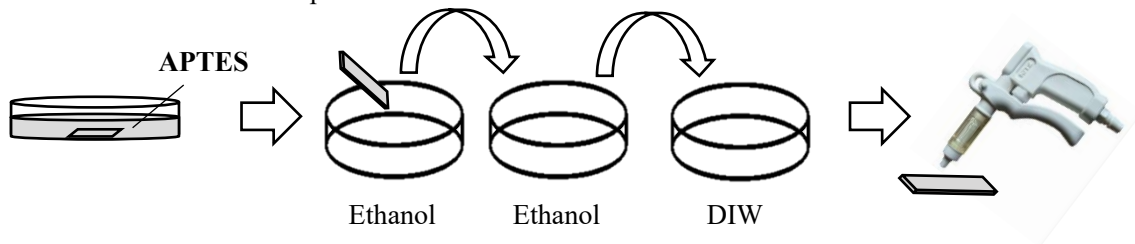


Figure 9 The wet APTES fabrication method for TFT biosensors.

Wet APTES method: First use 99.7% electronic industry ethanol to dilute the APTES to 1% solution. The composition is 100 μ L APTES with L 99.9 mL APTES. Second prepare one petri dish, use tweezers carefully transfer the TFT biosensor chips from the chip case into the petri dish, then add the 1% APTES to completely cover the chip, then cover the petri dish and react at room temperature for 30 min. Third prepare three additional petri dishes, add 99.7% electronic industry ethanol to the first two petri dishes, and add deionized water to the last petri dish. Transfer the 1% APTES-treated TFT biosensor chips to the three petri dishes in turn for rinsing to remove unreacted APTES solution. Finally, use nitrogen spray gun to dry the TFT biosensor. All steps were shown in Figure 9.

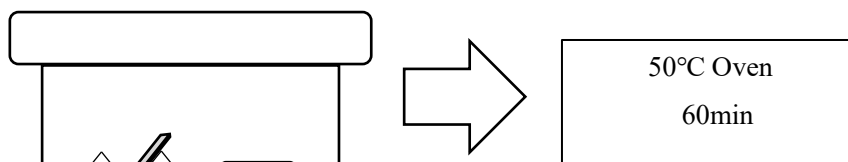


Figure 10 The vapor APTES fabrication method for TFT biosensor chips.

Vapor method: The vapor method requires generating vapor APTES to attach to the surface of TFT biosensors and react with hydroxyl groups of the chip surface to fix APTES molecule to the surface. To generate APTES vapor stably and uniformly, the method of heating APTES solution in an oven is adopted here. As shown in Figure 10, first prepare a W-shaped chip holder and a glass bottle which containing APTES solution. The shape of the holder can also be other, for example Photo 1. Then place the plasma-treated TFT biosensor chip on the holder and place the glass bottle containing APTES solution horizontally in the plastic box, the lid of the glass bottle need to be removed. Finally, place the plastic box in an oven at 50°C for 60 min so that the APTES steam could fully contact the TFT biosensors surface and fix on the surface.



Photo 1: The situation that the TFT biosensor chips are placed on the holder

Step3 After making the APTES layer for the TFT biosensors, the TFT biosensors are required to be baked to remove extra APTES on the surface. Place the TFT biosensors on a heater to bake at 110°C for 60 min.

Step4 The aldehyde group of glutaraldehyde can react with the amino group of APTES to attach to the TFT biosensors surface. In this study, the concentration of glutaraldehyde is not particularly important. Therefore, a 2.5% glutaraldehyde solution was chosen to make the glutaraldehyde layer here. Dilute the 25% glutaraldehyde solution to 2.5% by using 1 × phosphate buffered saline (PBS) solution at a 2.5% glutaraldehyde, the composition is 25% glutaraldehyde:1 × PBS=1:9. Then prepare one petri dish, and place TFT biosensors in the petri dish. Add the 2.5% glutaraldehyde

solution into the petri dish to react at room temperature for 60 min.



To remove extra unreacted glutaraldehyde solution, use deionized water to wash the TFT biosensor after the glutaraldehyde layer making step. Then use a nitrogen spray gun to dry the TFT biosensors. Finally, add amino group contained probe DNA sample to the In_2O_3 channel location of TFT biosensor chip, react at room temperature for overnight.

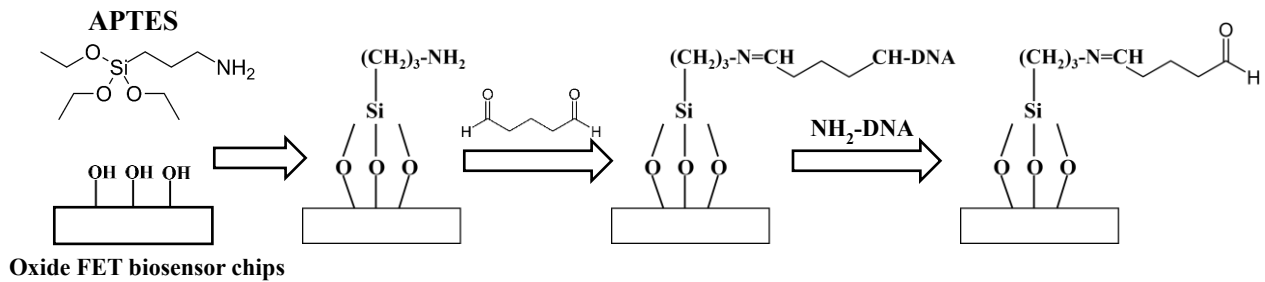


Figure 11 Probe DNA immobilization process and schematic.

Figure 11 shows probe DNA immobilization process and schematic. First, APTES molecule will react with the hydroxyl group on the chip surface to attach to the chip surface and form APTES layer. Then the amino group of APTES molecule will react with glutaraldehyde to attach to the TFT biosensors surface. Finally, glutaraldehyde reacts with the amino group contained probe DNA to achieve the goal that immobilizes probe DNA on the surface of TFT biosensor.

3.4 Instability based on reference electrodes and measurement solution

In sample detection, a shift of V_G -Id variation frequently occurs in two blank measurements even though the measurement conditions are totally same. This phenomenon greatly affects the nucleic acid detection results of TFT biosensors. Worse, it can even cause completely wrong detection judgments. To make V_G -Id variation of the TFT biosensor stable during detection, it is necessary to explore and discuss the unstable factors.

To reduce the influence of ion species of measurement buffer, phosphate buffer solution (PB) with NaCl was used as the detection buffer in all sample measurement experiments

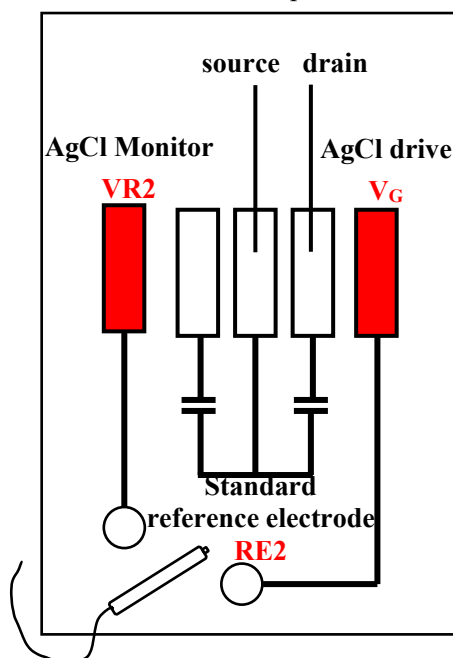
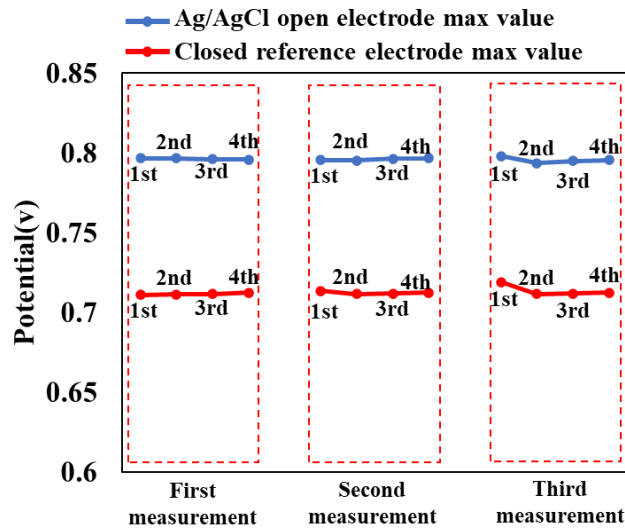
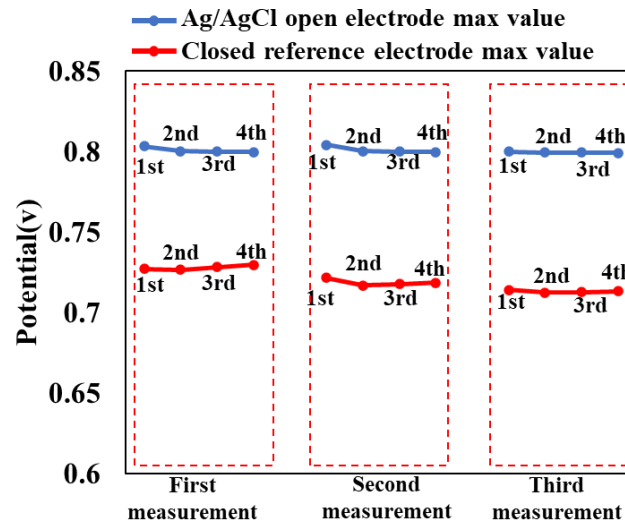


Figure 12 Schematic diagram of the chip used for the investigation

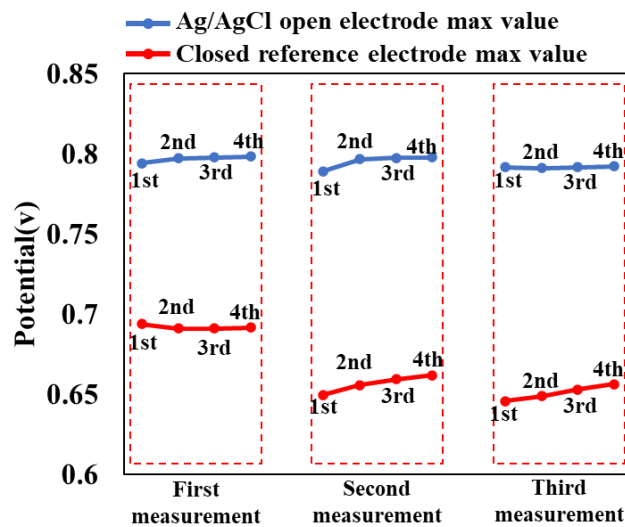
The influence of the artificial Ag/AgCl electrode was first investigated. As shown in Figure 12, two artificial Ag/AgCl electrodes are made on the surface of the TFT biosensor chip: the V_G electrode for supplying the voltage and the VR2 electrode for recording the voltage change of the detection solution. In addition, in order to accurately evaluate and judge whether VR2 can work correctly, the third carbon core type standard reference electrode RE2 is also used to record the variation of measurement voltage. In addition, a low-concentration measurement solution 1 mM PB+10 mM NaCl solution and a high-concentration measurement solution 1mM+100mM NaCl were prepared. Since the standard reference electrode is stored in 3M KCl solution before use, although the standard reference electrode will be cleaned with deionized water before use, due to its working principle and the W junction structure, there may be a small amount of KCl would flow into the measurement solution, causing the detected potential to change after change measurement solution. Therefore, to investigate its influence, another set of TFT biosensors was prepared. Before sample detection, the reference electrode was placed in the measurement solution and left to stand for about 5 minutes. During this process, the detection solution will be replaced with KCl in the W junction structure. In this way, the influence of KCl solution can be effectively avoided during the detection process.



(a) 1m MPB + 100mM NaCl, kept standard reference electrode into measurement buffer



(b) 1mM PB + 100mM NaCl, didn't keep standard reference electrode into measurement buffer



(c) 1m MPB + 10mM NaCl, didn't keep standard reference electrode into measurement buffer

Figure 13 The maximum potential value variation recorded by artificial Ag/AgCl electrode VR2

and standard reference electrode RE2

Figure 13 shows the maximum potential value variation in a low-concentration measurement solution of 1 mM PB+10 mM NaCl solution and a high-concentration measurement solution of 1 mM PB+100 mM NaCl, and the standard reference electrode of (a) group was previously kept in 1 mM PB+100 mM NaCl, and the standard reference electrode of (a) group was previously kept in 1 mM PB+100 mM NaCl before using. Comparing potential variation in the high concentration and low measurement solution, the results indicate high salt concentration can improve VR3 stability. The artificial Ag/AgCl electrode can correctly recode the potential variation with the voltage setting 0.2-0.8V, however, it's totally different from the potential value variation recorded by the standard reference electrode. Therefore, the use of V_G -Id or VR2-Id cannot correctly reflect the real potential-current variation in measurements. In addition, the maximum potential value variation in the group that kept the standard reference electrode in the measurement buffer shows more stability than the group that didn't keep the standard reference electrode in the measurement buffer.

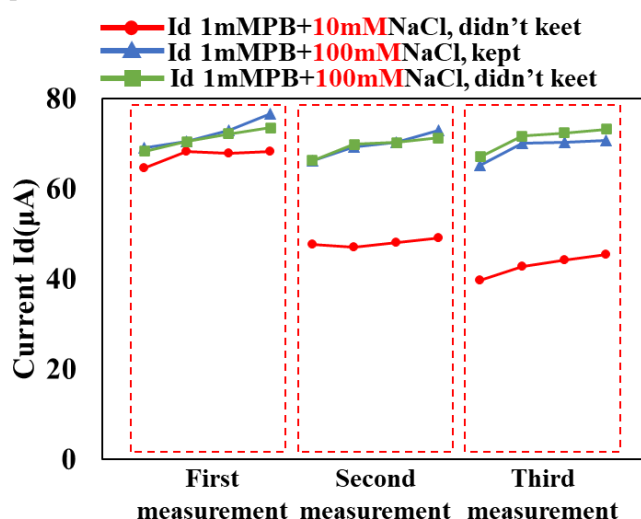


Figure 14 The Id variation when the $V_g=0.6V$

Figure 14 shows the current Id variation in different conditions. The current Id variation shows unstable even under stable voltage variation. Therefore, there are still other factors that will make the instability to TFT biosensor in measurements.

According to the Nernst equation, $E=E^0 - RT/nF * \log[\text{red}]/[\text{ox}]$, $25^\circ\text{C}=298.15\text{K}$. Therefore, the equation can be simplified to $E=E^0 - 0.05916/n * \ln[\alpha_{\text{red}}]/[\alpha_{\text{ox}}]$, according to $\text{AgCl} + e^- = \text{Ag} + \text{Cl}^-$, $n=1$. Finally the Nernst equation can be simplified to $E=E^0 - 0.059 * \lg(\alpha_{\text{Cl}^-})$. Therefore, the potential of measurement solution is relevant with Cl^- concentration.[4] If the standard reference electrode is not be

previously put into measurement solution, the high concentration KCl in the standard reference electrode will come out to change the Cl⁻ concentration of measurement solution. And according to lg (α_{Cl^-}) value calculation, the lower the concentration of the solution, the more easily the potential is affected. That's why the high concentration measurement solution can keep the potential stable.

3.5 Instability based on the water content

The water content (w), also known as natural water content or natural moisture content, is the ratio of the weight of water to the weight of the material.

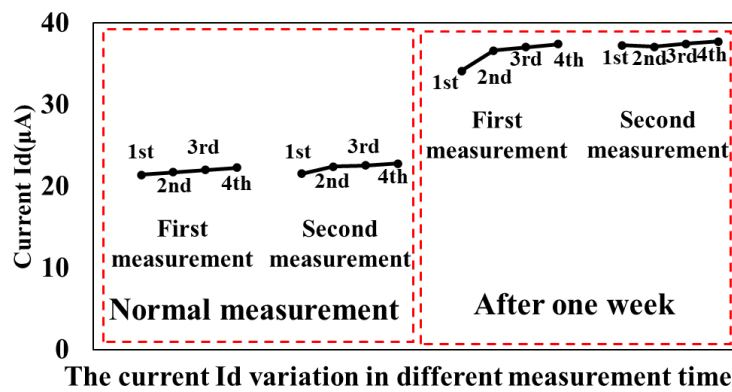


Figure 15 The current Id variation in different measurement time.

As shown in Figure 15, it is the current Id variation diagram obtained by the sample detection of the same TFT biosensors at different time points, and all the current Id values are the values when the reference electrode RE2=0.6V. Twice sample measurements were tested in two different periods. The current Id variation graph on the right side was measured again by the same method after one week. The conditions of the first group and the second group are almost same in each measurement, the only difference is that the measurement solution will be replaced in the second group. During the one week, the TFT biosensor was kept in a desiccator. Therefore, one reasonable reason is that humidity could affect measurement stability.

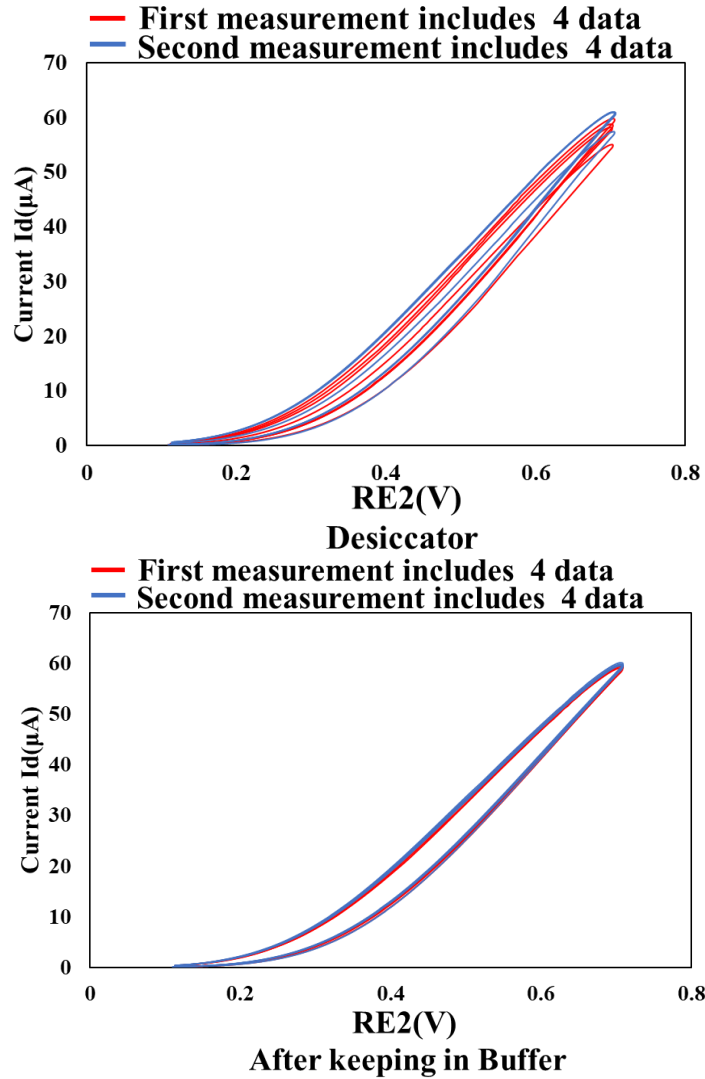


Figure 16 The I_d variation of two TFT biosensor chips that have different humidity.

To prove the reason that the humidity of TFT biosensor could affect the stability of measurement. Two new TFT biosensors were prepared to test. Firstly modify two TFT biosensors in the same conditions, then keep 1st TFT biosensor in desiccator one day, and keep 2nd TFT biosensor in the measurement buffer one day. Measure the two TFT biosensor chips and make RE2- I_d graph, the results show the TFT biosensor that kept in measurement buffer can improve the measurement stability.

3.6 Target DNA sample detection and surface of TFT biosensor analysis

To investigate the detection ability of the TFT biosensor chip, two different DNA probes were

immobilized on the surface of the chip. Then measure the Id variation before and after incubation.

The probe DNA and target DNA information are shown in table 3.

Table 3: The sequence of the probes and target DNA

Name	Sequence (5' to 3')
Probe A	[AmC6]-CCTATCGCTGCTACCGTGAA
Target A	[FITC]-TTCACGGTAGCAGCGATAGG
Probe B	[AmC6]-CATCTGACCTCTGTGCTGCT
Target B	[Cy5]-AGCAGCACAGAGGTCAGATG

The steps are as follows:

(1) At the first day, following the fabrication process to immobilize probe DNA probe on the surface of TFT biosensor.

(2) At the second, wash the TFT biosensors with deionized water t to remove excess DNA probe solution, then dry with a nitrogen spray gun.

(3) Wash the TFT biosensors 2-3 times with 0.1% sodium dodecyl sulfate (SDS) solution. Sodium dodecyl sulfate is detergent, which can effectively reduce the non-specific binding of the target DNA to TFT biosensor chip. Then rinse TFT biosensors with deionized water and dry with a nitrogen spray gun.

(4) Immerse the TFT biosensor in NaBH₄ solution. NaBH₄ can react with the excess or unreacted -NH₂ and other functional groups on the surface. This treatment can prevent the fluorescent molecules in the target DNA from reacting with the surface -NH₂ and other functional groups and cause erroneous results.

(5) Using deionized water to wash TFT biosensor. Then measure the RE2-Id variation by parameter analyzer.

(6) Use a pipette to add 1 μ M target DNA with fluorescein isothiocyanate (FITC) or DNA with Cyanines 5 (Cy5) solution on the surface of TFT biosensor chip for incubation one hour. After one hour, wash TFT biosensor chip with 1 x PBS buffer. Then measure the RE2-Id variation again. Finally, observe the fluorescence signal on TFT biosensor chips. About FITC, λ_{ex} =495 nm λ_{em} =519 nm, which shows green color.[5,6] While about Cy5, λ_{ex} =625;650 nm λ_{em} =670 nm, which shows far-red color.[7,8]

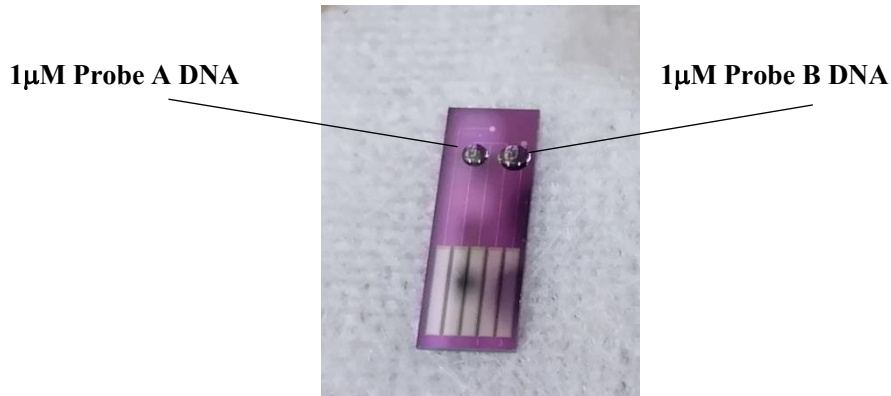


Photo 2: Diagram of when probe DNA is immobilized on the surface of TFT biosensor chip

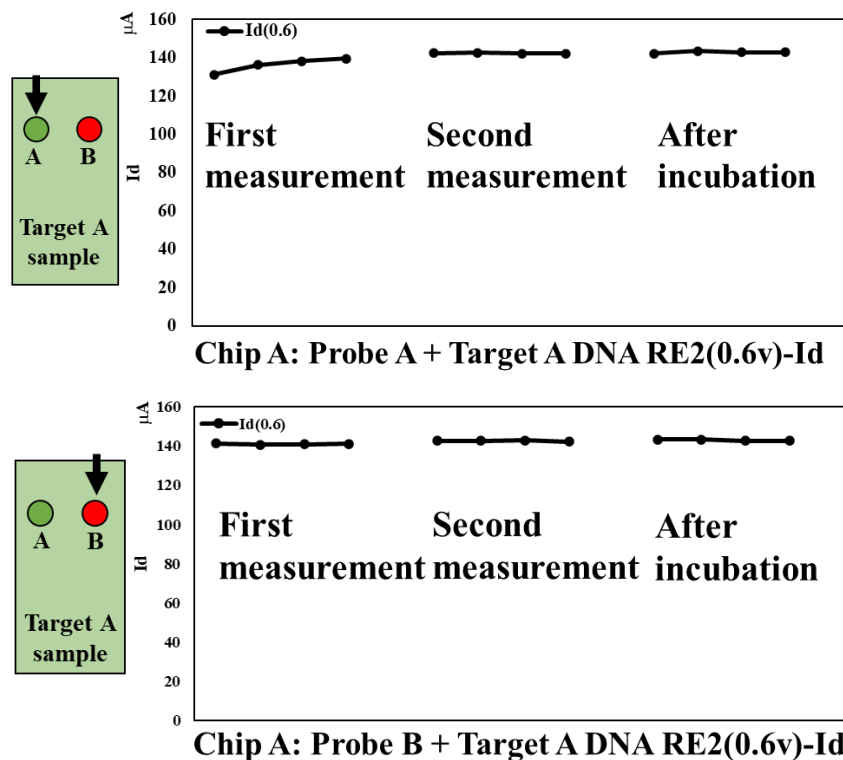


Figure 17 The RE2-Id variation in chip A

Probe A DNA and probe B DNA were immobilized on the two TFT channels of chip A, respectively. Then two blank measurements were performed on their 2 TFTs to obtain two RE2-Id variation graphs, denoted as “1st measurement” and “2nd measurement”. Then, the target A DNA solution was added to the TFT biosensors surface for one hour of incubation. After that, a blank measurement was performed on chip A to obtain the “After incubation” RE2-Id variation diagram.

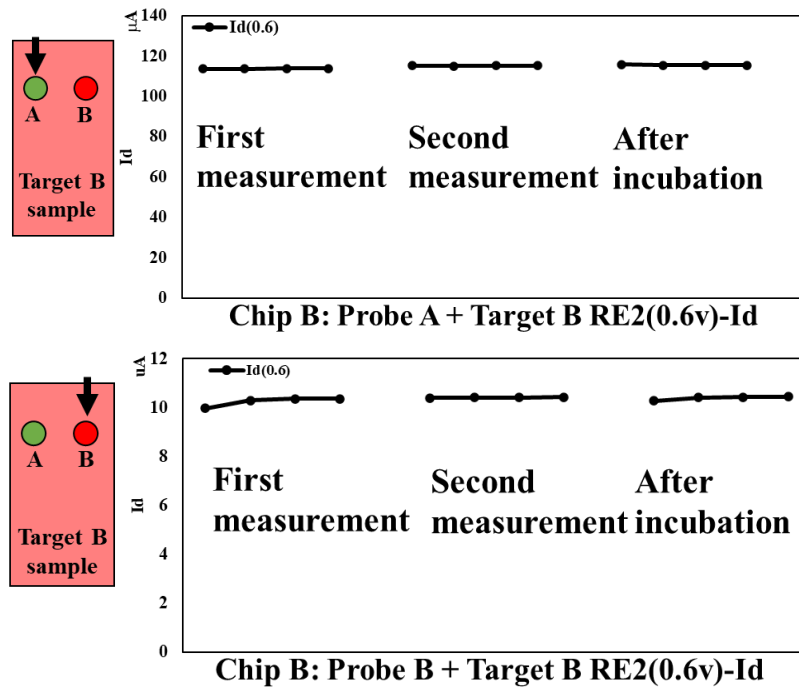


Figure 18 The RE2-Id variation in chip B

Then Use the same method to process chip B, Figure 18 shows its RE2-Id variation. According to the RE2-Id variation graphs in Figure 17 and Figure 18, it can be found that the current Id variation is still very stable and did not shift after twice blank detection step and incubation with the target DNA solution step. This is obviously inconsistent with the theory of TFT biosensor detection. In order to investigate the reason why the current did not shift, the chip surface was then observed using a fluorescence microscope to confirm the DNA hybridization situation.

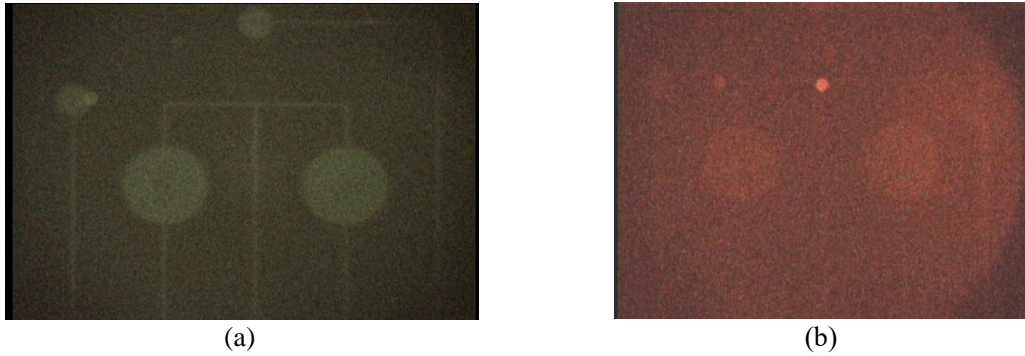


Figure 19 The fluorescence distribution graph. (a)Chip A: processed by target A DNA-FITC(b)Chip B: processed by target B DNA-Cy5 sample.

Figure 19 shows the fluorescence distribution situation. Judging from the photos, except for bottom electrode and wires, no fluorescence was observed in the photos. It can be inferred that there are two situations: the first is that the probe cannot be fixed on the TFT biosensors surface under the fixing method, and the second is that DNA hybridization failed. To confirm this conjecture, fluorescence analysis was performed using the silicon substrate. The silicon substrates were processed by the same method as the TFT biosensors.

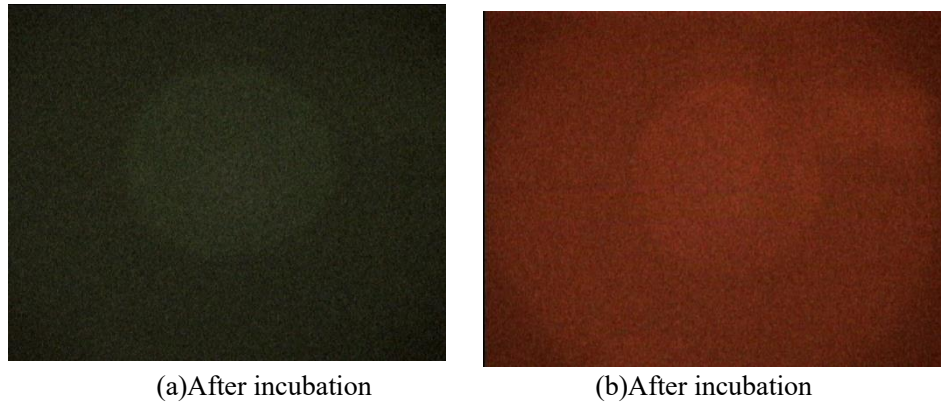


Figure 20 The fluorescence distribution graph after incubation in SiO_2 substrate.

Figure 20 shows the DNA hybridization is successful under existing process and incubation methods. This also shows that the probe DNA cannot be well immobilized on LaZrO_x layer surface.

3.6 Polysilazane layer making and surface of TFT biosensor chip analysis

3.6.1 Make polysilazane layer on TFT biosensor surface

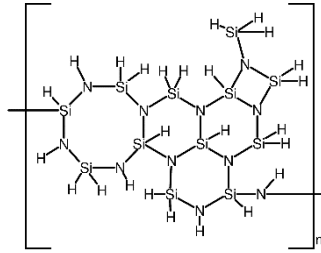
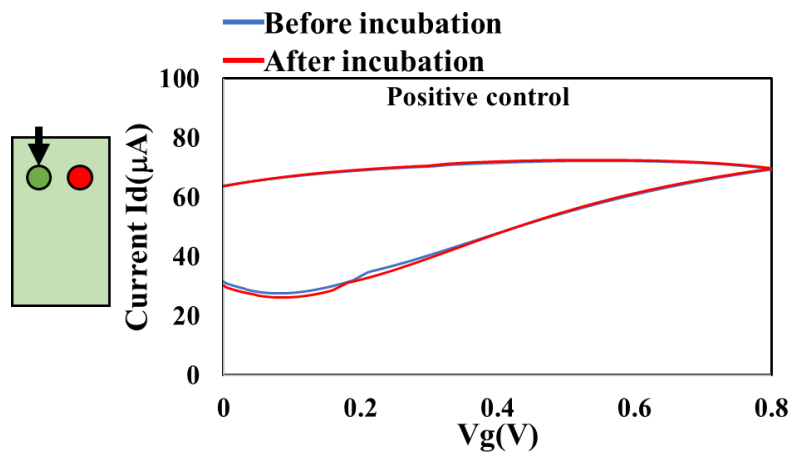


Figure 21 Structural formula of polysilazane

Using fluorescent DNA molecules to analyze the TFT surface, it was found that the probe DNA could not be immobilized on the surface of the TFT biosensor using wet APTES and vapor APTES methods.[9] In order to enhance DNA probe immobilization ability, the polysilazane solution was dropped on the TFT biosensor surface and then spin-coated.

The steps are as follows: First, the TFT biosensor was rinsed with deionized water to initially remove impurities on the TFT biosensor surface, and then plasma was used to further clean the TFT biosensor surface and increase the hydroxyl concentration on the TFT biosensor surface. Then, 2 drops of the polysilazane solution were added dropwise on the TFT biosensor surface, and the TFT biosensor was spin-coated at 3000 rpm for 30 seconds. The TFT biosensors were dried at 180°C for 30 s, 300°C for 5 min, and 450°C to allow the polysilazane to adhere to the TFT biosensor surface.

3.6.2 I_d measurement of polysilazane processed TFT biosensors



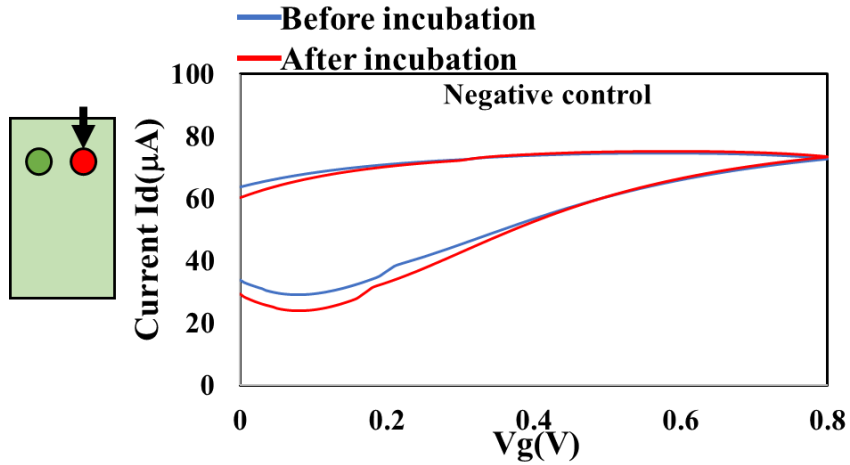


Figure 22 Vg-Id variation of positive control and negative control of polysilazane processed TFT biosensors

The Id variation results of polysilazane processed TFT biosensors were shown in Figure 22. The positive control shows there is no Id shift after DNA sample incubation. In the other hand, negative control shows there is slightly Id shift up occurs between 0 V to 0.4 V.

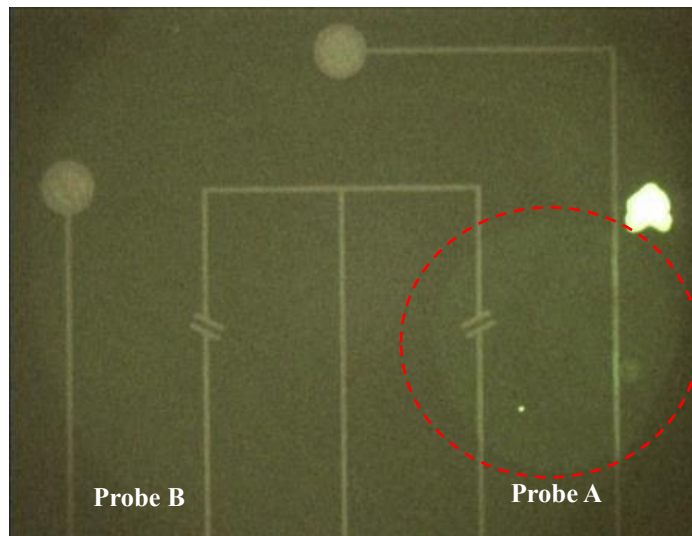


Figure 23 TFT biosensor surface fluorescence detection

The Id did not change as expected. To further investigate the cause, fluorescence detection was used for polysilazane processed TFT biosensor. As shown in Figure 23, there is a very clear fluorescence signal showed in positive control.

3.7 Prob DNA immobilization by linker (3-Glycidyloxypropyl)trimethoxysilane

Linker: (3-Glycidyloxypropyl)trimethoxysilane (GPTMS)

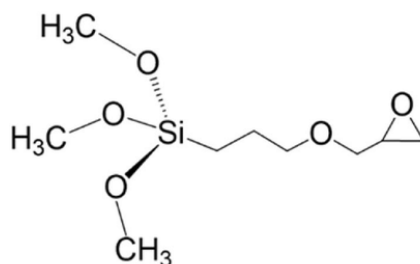


Figure 24 Structural formula of GPTMS

In sections 3.1 to 3.6, many experimental conditions had been used to formulate the plan for the stabilization of TFT biosensor I_d variation measurements. However, although the TFT biosensor I_d measurement tends to be stable, the I_d variation after DNA hybridization did not shift. Through TFT biosensor surface fluorescence detection experiments, using APTES as a linker can correctly hybridize DNA have been figured out. Therefore, it is possible that APTES as a linker affects the detection sensitivity of the TFT biosensors. In this section, APTES was replaced to GPTMS to deal with the problem that I_d couldn't shift after DNA hybridization. GPTMS can also immobilize DNA probes on the surface of the TFT biosensor. The reaction principle is similar with APTES and Glutaraldehyde, the methyl $-CH_3$ of GPTMS molecular will first react with hydroxyl to link on the surface of the TFT biosensor, then its terminal functional groups will react with amino groups of DNA probe to finally achieve the purpose of immobilizing the probe on the surface of TFT biosensors.[10] Different from APTES and Glutaraldehyde, GPTMS only needs one step, which can greatly reduce DNA probe immobilization time.

In this approach, firstly clean can increase hydroxyl group by deionized water and plasma ashing. Then use 1% GPTMS aqueous solution to process TFT chips for 30 min and immobilize DNA probe on the surface of TFT biosensors. After that, use ethanolamine to process TFT biosensors. In this experiment, all TFT biosensors didn't have the $LaZrO_x$ layer.

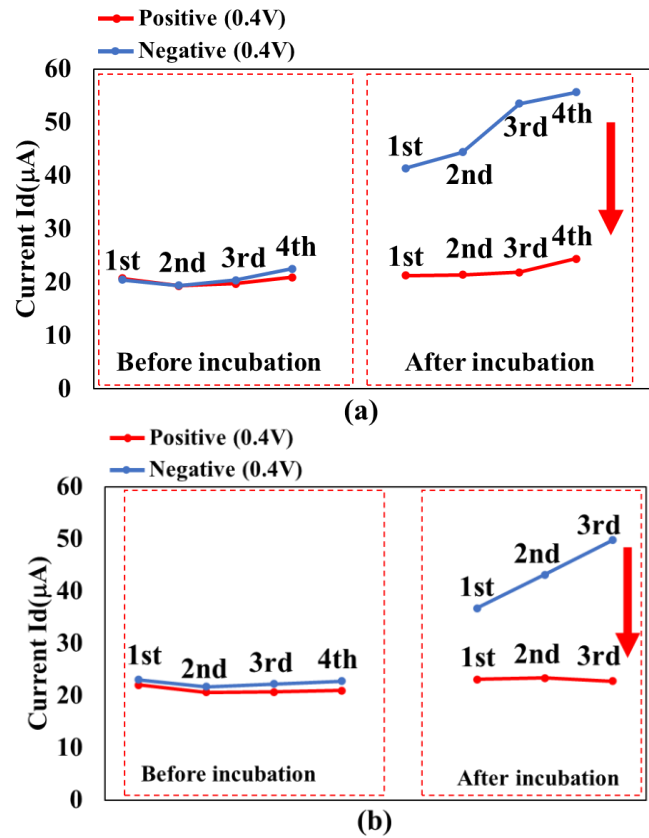


Figure 25 The I_d variation of chip(a) and chip(b)

Same measure approaches were used in GPTMS processed TFT biosensors which without $LaZrO_x$. One TFT biosensor immobilized with DNA probe A as positive and probe B as negative control. Then use 1 mM PB + 30 mM NaCl to measure the I_d variation before and after DNA sample incubation. The I_d variation of two TFT biosensors which modified in same condition were shown in Figure 25. Figure 25 indicates both positive control and negative control will shift up after incubation, however, it obviously the I_d shift magnitude of negative control is huge. Even though the I_d shift direction was not as expected, it can be clearly judged that the solution contains the target DNA. It also proved that GPTMS is a better DNA probe immobilization scheme with no $LaZrO_x$ TFT biosensor.

3.8 Conclusion

Different buffer concentration does affect the I_d variation stability, high salt concentration can improve RE2 stability. In some conditions, V_G and artificial Ag/AgCl electrode(open electrode) can

not reflect the real potential variation of the measurement buffer in I_d measurement. In this section, we also proved that previously put standard reference electrode in measurement buffer can improve I_d measurement stability. In addition, high water content of TFT biosensor can also improve the I_d measurement stability. After that, DNA sample was detected by TFT biosensor, however, the I_d didn't shift after incubation. The target DNA with fluorescent molecules FITC and CY5 was hybridized on the surface of the TFT biosensor, however, there were no fluorescent signals were observed, which indicated that DNA hybridization on the TFT biosensor surface might have failed. That may be a reason why the I_d did not show shift after DNA hybridization. Instead, we treated the TFT biosensor surface with polysilazane, expecting that the DNA probes would be correctly immobilized on the chip surface in the way we understand and expect. From the results of fluorescent labeling, DNA hybridization successfully occurred on the TFT biosensor surface, however, strangely there was no I_d shift occurred. Finally, we tried to use the linker GPTMS to immobilize DNA probes. The I_d measurements results shows the positive control and negative control can be clearly distinguished, which indicated the GPTMS processed TFT biosensors that without LaZrO_x can successfully detect the target DNA sample.

3.9 Reference

- [1] Adam, T., & Hashim, U. (2015). Highly sensitive silicon nanowire biosensor with novel liquid gate control for detection of specific single-stranded DNA molecules. *Biosensors and Bioelectronics*, 67, 656–661. <https://doi.org/10.1016/J.BIOS.2014.10.005>
- [2] Cheng, Y., Wang, N., Ren, Z., & Xu, C. (2022). Development of fluorescence-based nucleic acid blot hybridization method using Cy5.5 labeled DNA probes. *Journal of Microbiological Methods*, 197, 106479. <https://doi.org/10.1016/J.MIMET.2022.106479>
- [3] Gunda, N. S. K., Singh, M., Norman, L., Kaur, K., & Mitra, S. K. (2014). Optimization and characterization of biomolecule immobilization on silicon substrates using (3-aminopropyl)triethoxysilane (APTES) and glutaraldehyde linker. *Applied Surface Science*, 305, 522–530. <https://doi.org/10.1016/J.APSUSC.2014.03.130>
- [4] Feiner, A. S. (1994). The Nernst equation. *Journal of Chemical Education*, 71(6), 493–494.

<https://doi.org/10.1021/ED071P493>

[5] Huy, T. Q., Hanh, N. T. H., Van Chung, P., Anh, D. D., Nga, P. T., & Tuan, M. A. (2011). Characterization of immobilization methods of antiviral antibodies in serum for electrochemical biosensors. *Applied Surface Science*, 257(16), 7090–7095.

<https://doi.org/10.1016/J.APSUSC.2011.03.051>

[6] Imhof, A., Megens, M., Engelberts, J. J., De Lang, D. T. N., Sprik, R., & Vos, W. L. (1999). Spectroscopy of fluorescein (FITC) dyed colloidal silica spheres. *Journal of Physical Chemistry B*, 103(9), 1408–1415. <https://doi.org/10.1021>

[7] Van Hal, N. L. W., Vorst, O., Van Houwelingen, A. M. M. L., Kok, E. J., Peijnenburg, A., Aharoni, A., Van Tunen, A. J., & Keijer, J. (2000). The application of DNA microarrays in gene expression analysis. *Journal of Biotechnology*, 78(3), 271–280. [https://doi.org/10.1016/S0168-1656\(00\)00204-2](https://doi.org/10.1016/S0168-1656(00)00204-2)

[8] Zhu, Z., Chao, J., Yu, H., & Waggoner, A. S. (1994). Directly labeled DNA probes using fluorescent nucleotides with different length linkers. *Nucleic Acids Research*, 22(16), 3418–3422. <https://doi.org/10.1093/NAR/22.16.3418>

[9] Stueger, H., Fuerpass, G., Mitterfellner, T., & Baumgartner, J. (2010). Synthesis and reactivity of NH₂ derivatives of dodecamethylcyclohexasilane. *Organometallics*, 29(3), 618–623. https://doi.org/10.1021/OM9010017/SUPPL_FILE/OM9010017_SI_001.CIF

[10] Li, F., Chen, W., & Zhang, S. (2008). Development of DNA electrochemical biosensor based on covalent immobilization of probe DNA by direct coupling of sol–gel and self-assembly technologies. *Biosensors and Bioelectronics*, 24(4), 781–786. <https://doi.org/10.1016/J.BIOS.2008.06.052>

Chapter 4. Exploration of nucleic acid detection that combined with isothermal amplification and TFT biosensors

4.1 Brief overview of experimental design

Chapter 2 highlights the exceptional amplification efficiency of RPA, the entire process of nucleic acid amplification takes approximately 10 mins, demonstrating significant potential in nucleic acid detection. It is poised to supplant conventional PCR-based amplification methods in nucleic acid detection. The core principle of nucleic acid amplification detection lies in rapidly amplifying trace amounts of nucleic acid to boost the detection signal. However, this isothermal amplification method isn't devoid of application shortcomings. For example PCR, the amplification results from isothermal nucleic acid amplification require comparative analysis through gel electrophoresis. This procedure necessitates the use of additional reagents such as loading buffers, dyes, and PCR markers. Moreover, electrophoresis is time-consuming, which isn't favorable for POCT. By merging the isothermal nucleic acid amplification method with the TFT biosensor, the speed of nucleic acid detection can be significantly accelerated. This combination will provide a substantial contribution to the nucleic acids detection.

To investigate the detection capability of oxide TFT biosensors for RPA products, the heat shock protein (*HSP70*) gene of *Leishmania* was selected as the target DNA for validation. The entire experiment consists of two parts. In experiment 1, the effect of RPA reaction system optimization on experimental outcomes was explored. Three RPA conditions were examined: untreated RPA sample, a proteinase K-added sample, and purified samples. The Id-Vg curves of the TFT biosensor were measured before and after incubation with the RPA products, with each group being measured three times. In experiment 2, following the optimization of RPA reaction conditions, we utilized the TFT biosensor to detect RPA products at different template DNA concentrations, ranging from 0 copies/ μL and 10^1 copies/ μL - 10^6 copies/ μL , in order to determine the detection limit of the TFT biosensor for *Leishmania HSP70* RPA products.

4.2 Objective

This work is underpinned by the belief that by integrating the isothermal nucleic acid amplification method with the TFT biosensor, we can significantly expedite the nucleic acid detection process. To investigate the effects of RPA reaction system optimization on experimental outcomes. This

objective entails the evaluation of three different RPA conditions: untreated RPA samples, proteinase K-added samples, and purified samples. It also involves analyzing the Id-V_g curves of the TFT biosensor before and after incubation with the RPA products.

To ascertain the detection limit of the TFT biosensor for Leishmania HSP70 RPA products. To achieve this, we plan to use the TFT biosensor to detect RPA products at varying template DNA concentrations, ranging from 0 copies/ μL to 10^1 copies/ μL - 10^6 copies/ μL , post the optimization of RPA reaction conditions.

4.3 RPA products of Leishmania detection with TFT biosensors.

Leishmaniasis is a serious infectious disease with effects on the human skin and visceral organs. The main clinical features are long-term irregular fever, splenomegaly, anemia, emaciation, a decreased white blood cell count, and increased serum globulin. [1][2] Without proper treatment, certain types of leishmaniasis can lead to death within 1-2 years after infection. Leishmaniasis mainly occurs in Mediterranean countries as well as tropical and subtropical regions, with cutaneous leishmaniasis being the most common form. At least 20 species of parasites from the genus Leishmania can be transmitted via the bite of various phlebotomine sandfly species. It is important to note that, although some types such as mucocutaneous leishmaniasis and visceral leishmaniasis may cause severe health issues and death, cutaneous leishmaniasis is typically not life-threatening and can heal on its own in many cases. [3][4]

HSP70 is conserved across prokaryotes and eukaryotes, one of the important characteristics of HSP in the biological world is their high conservatism in the process of evolution that can be used as the identity for the genus Leishmania. Therefore, the HSP70 is suitable for confirming the presence of the genus Leishmania. [5]

We designed primers and probe, the sequence information was shown in Table 1. The DNA probes are pre-immobilized on the surface of the TFTs, specifically on the “Ref TFT” and “Test TFT”. The source electrode, two drain electrodes of the TFTs are then connected to a Semiconductor Parameter Analyzer (Keysight 4156C). A detection buffer solution is added to the “Detection sample addition area”. A reference electrode (RE1) is inserted in the solution for applying gate voltage (V_g-apply). Another standard reference electrode (RE2) is also put in the solution to monitor the actual potential (V_g-monitor)

applying the solution. Then, drain voltage V_g of 200mV is applied between the source and drain electrodes. Concurrently, a variable voltage ranging from 0-800mV is applied to the gate electrode RE1, denoted as V_g . The resultant currents flowing the drains of Ref TFT and Test TFT are recorded as I_d . The I_d - V_g curves are plotted using I_d and V_g -monitor instead of V_g -apply. In this dissertations, V_G stands for Ag/AgCl open electrode, V_g stands for standard electrode.

The I_d - V_g curve measurement is performed before and after DNA sample in-troduction. If the sample contains the target nucleic acid, it will bind specifically to the DNA probe on the surface of the TFT biosensor.

Table 4. The sequence information of the *Leishmania HSP 70* gene, primers and probe immobilized on surface of the biosensor.

Name	Sequence
<i>Leishmania</i> Heat shock protein 70 gene	5'-CATATCACCATCACCAACGACAAGGGCCGACTGAGCAAGGACGAG ATCGAGCGCATGGTGAACGATGCGTCGAAGTACGAGCAGGCCGACAA GATGCAGCGGAGCGCGTGGAGGGCGAAGAACGGCCTGGAGAACTAC GCGTACTCGATGAAGAACACGGTCTCCGACACGAACGTGTCCGGCAA GCTGGAGGAGAGCGACAGGTCCGCGCTGAACTCGCGATCGACGCGG CGCTGGAGTGGCTGAACAGCAACCAGGAGGCGTCGAAGGAAGAGTAC GAGCA-3'
Forward primer	LB-hsp70-sp-Fwd 5'-TACACAGCAC[CCC]CATATCACCATCACCAACG-3'
Reverse primer	LB-hsp70-sp-Rev 5'-TGCTCGTACTCTTCCTTCG-3'
Probe	Anti-capture-NH ₂ 5'-GTGCTGTGTAATTTTT[AmC7]-3'

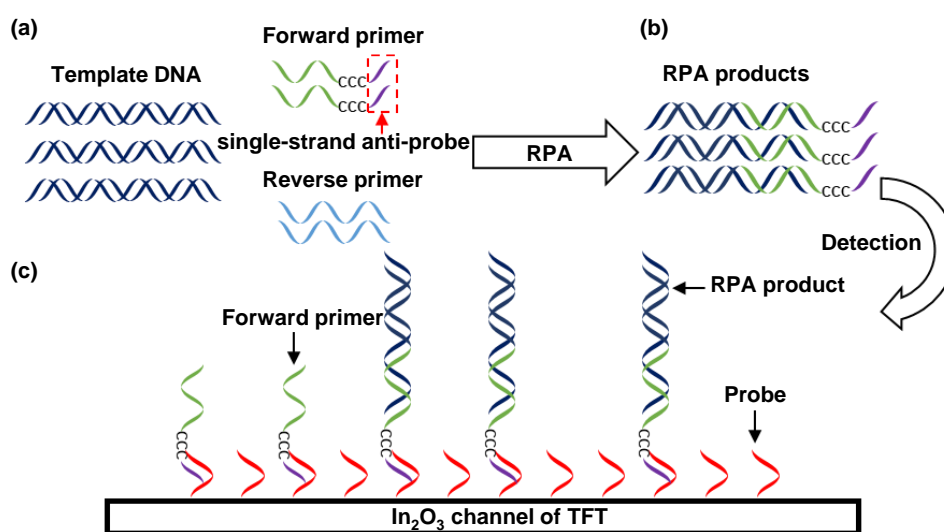


Figure 26. The process of nucleic acid detection using TFT biosensor in combination with RPA. From (a) to (b), template DNA is amplified by RPA. (c) The process of sample detection.

Figure 26 shows the process of nucleic acid detection. In order to enhance the hybridization between RPA amplicon and probe on TFT, a special designed forward primer is used in this study. The Forward primer in (a) contains not only the sequence that specifically binds to the template DNA, but also a 3-carbon block, namely CCC, and a single-strand anti-probe section. The 3-carbon block is used to prevent the polymerase from further extending the DNA chain, whereas the single-strand anti-probe remains as single strand still in amplicon. Therefore, it is expected to bind to the probe on the TFT effectively compared to the all double strand amplicon produced by conventional RPA, or PCR etc. The sample with the template DNA are mixed with these primers and RPA reagents, then RPA is undergone and the trace target nucleic acid fragments in sample are amplified in large quantities (b). The amplified products carry the single-strand anti-probe segment from the forward primer. After RPA, the sample is put on the TFT biosensor to let the amplicon hybridize to the probe on TFT (c). The amplicon can bind effectively to the probe on the TFT because they have single-strand binding section. The negative change of bound amplicon DNA shift the Id-Vg curve of TFT in right-down direction. From the shift, we can detect the hybridization of the DNA. Notably, some of the unreacted forward primers may also bind to the surface of TFT biosensor. This effect is discussed in the result and discussion section. In this study, we used a *Leishmania HSP70* fragment as the target detection nucleic acid. Its sequence and primer sequence are shown in Table 4.

4.4 Results

4.4.1 RPA reaction system optimization on experimental outcomes

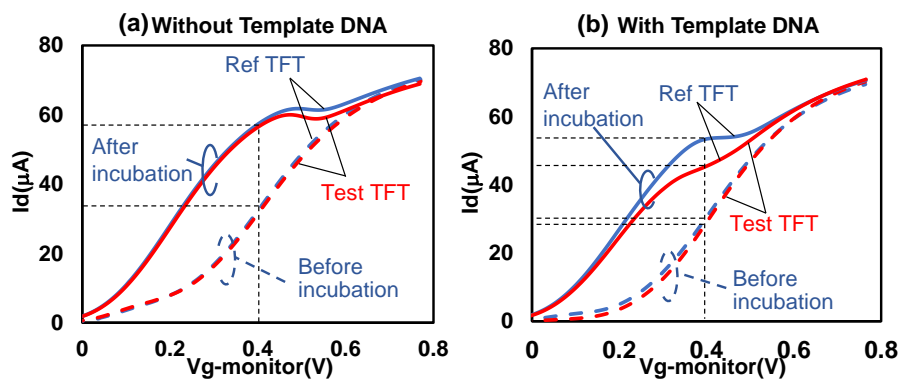


Figure 27. The Id variation of (a) purification RPA products without template DNA and (b) purification RPA products with template DNA.

RPA products with template DNA.

Figure 27 depicts the typical changes in Id-Vg curve on the TFT biosensor before and after the incubation with RPA products. Here, the curves are the 3rd measurements of protenase-K-added and purified sample. The difference between (a) and (b) is that in (a), the RPA reaction system did not contain template DNA (replaced with same amount of ultra-pure water), while in (b), the RPA reaction system contained template DNA. Both (a) and (b) show that the Id value increases after incubation. However, in (a), there is no significant change in the Id values of both “Ref TFT” and “Test TFT” before and after incubation. In contrast, in (b), after incubation with purified RPA samples, the Id value of “Test TFT” decreases significantly compared to “RF TFT” especially at Vg near 0.4V.

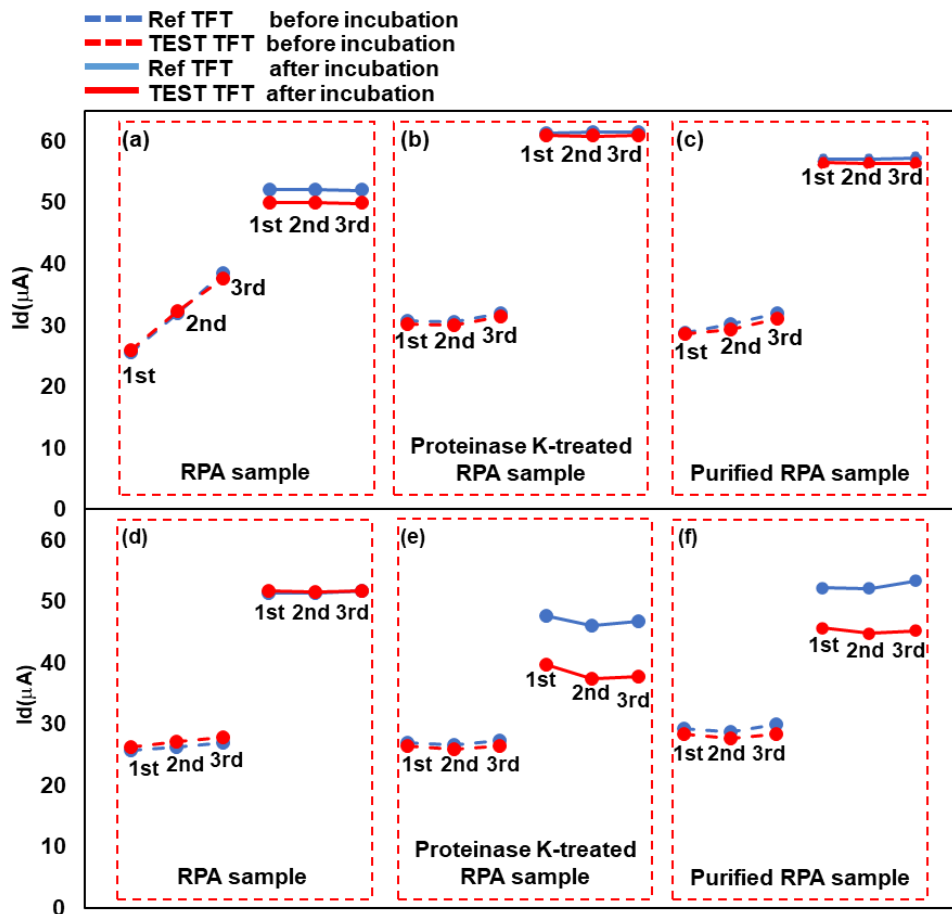


Figure 28. Change in current before and after sample addition. The three values on the left are pre-incubation readings, whereas those on the right are post-incubation readings. The detected RPA samples

were (a, d) untreated RPA-amplified products, (b, e) proteinase K-treated RPA products, and (c, f) purified RPA products. In each graph, “Ref TFT” denotes the reference TFT, which will not bind with the target DNA in the sample, whereas “Test TFT” refers to the Test TFT, capable of binding with the target DNA segment. In (a), (b), and (c), ultrapure water instead of the template DNA was added to the RPA reaction solutions. In (d), (e), and (f), template DNA was added to all RPA solutions.

Table 5. RPA sample processing conditions.

	a	b	c	d	e	f
Template DNA	-	-	-	+	+	+
Proteinase K	-	+	-	-	+	-
Purification	-	-	+	-	-	+

Figure 28 summarized the I_d change at $V_g = 0.4$ V in various conditions of (a) – (g) shown in Table 2. Three times measurements were taken in each before and after incubation for the all conditions. The three values on the left are the before incubation readings, while those on the right are after incubation readings. From left to right, the detected RPA samples were: Untreated RPA amplified products, Proteinase K-treated RPA product, and purified RPA products, respectively. In each graph, “Ref TFT” denotes the reference TFT, which will not bind with the target DNA in the sample, while “Test TFT” refers to the Test TFT, capable of binding with the target DNA segment. In the group (a) (b) (c), no template DNA was added to the RPA reaction solutions; instead, ultrapure water was used. In contrast, in the group (e) (f) (g), template DNA was added to all RPA solutions. According to the results, in the group (a) (b) (c), regardless of the type of RPA sample, there was no discernable change in I_d between “Ref TFT” and “Test TFT”. Contrarily, in the group (e) (f) (g), for both of (e) the Proteinase K-treated

RPA sample and (f) the purified RPA sample, there was a noticeable decrease in Id in “Test TFT” while no Id decrease was observed in (e) no treated RPA product. The magnitude of change in (e) and (f) is roughly equivalent. Hence, it was observed that the TFT biosensor could not differentiate whether the RPA sample contained the target amplified DNA fragment. This implies that the TFT biosensor detects RPA product preferentially while the TFT less responds to the forward primer. This may be due to the difference of the length of DNA.

4.4.2 *Leishmania HSP 70* detection by TFT biosensors

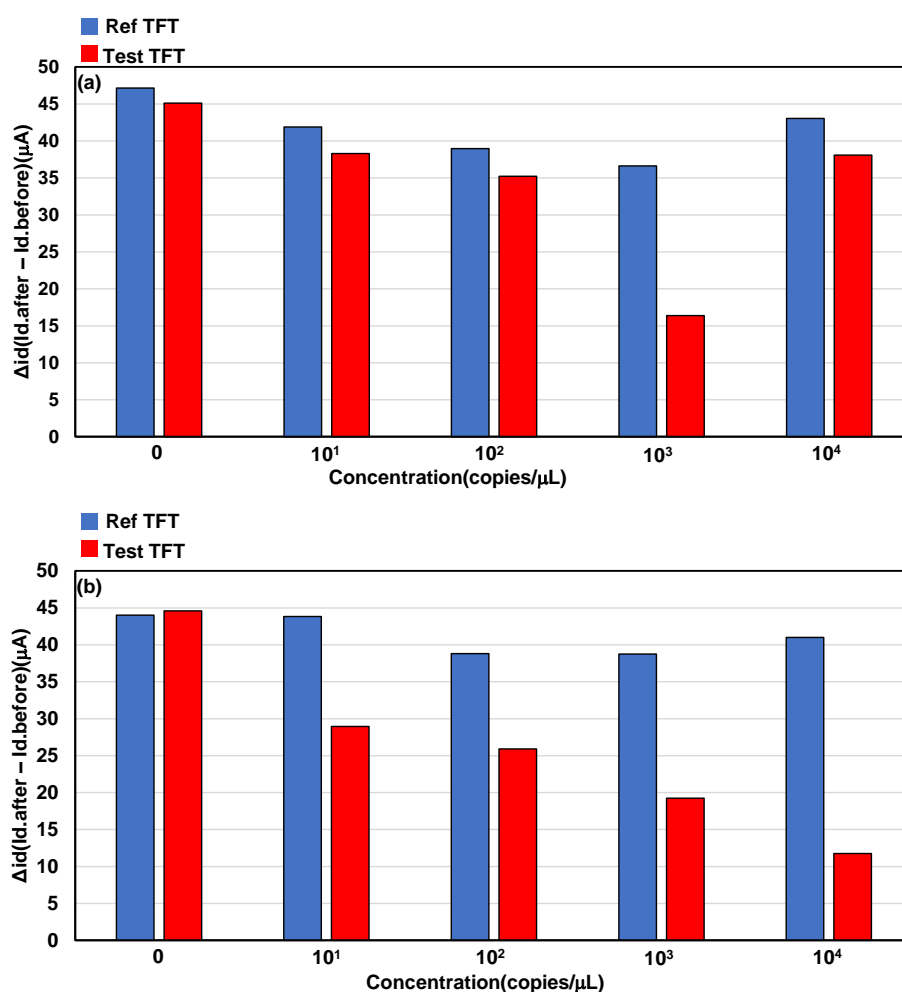


Figure 29. The change in current values for the positive group and the negative group at the 0.4V

position before and after incubation. (a) represents RPA conditions at 42°C for 20 minutes.

(b) represents RPA conditions at 42°C for 20 minutes, with the addition of 1% SDS after amplification samples were treated with proteinase K.

Figure 29(a) presents the results obtained from detecting RPA samples of varying concentrations, which ranging from 10^1 copies/ μL to 10^4 copies/ μL , using TFT biosensors, along with a blank control group. At a concentration of 10^3 copies/ μL , a significant discrepancy was observed in the current values between the positive and negative groups. Figure 29(b) illustrates the results obtained from assessing TFT biosensors using RPA amplification at concentrations spanning from 10^1 copies/ μL to 10^4 copies/ μL , complemented by a blank control group, under identical RPA reaction conditions (42°C for 20 minutes). The primary distinction lies in the application of Proteinase K treatment. Following the RPA reaction, 3 μL of Proteinase K and 3 μL of 1% SDS were incorporated into the reaction system and subsequently incubated at 37°C for 10 minutes. The results revealed a more pronounced decrease in the current of the positive sample post-SDS treatment in comparison to the previous group.

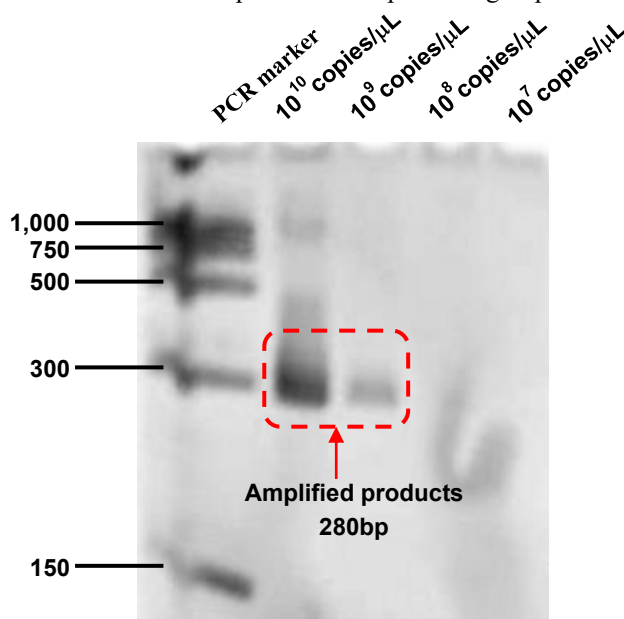


Figure 30. The 6% polyacrylamide gel electrophoresis of RPA product at the initial template DNA concentration ranging from 10^{10} copies/ μL to 10^7 copies/ μL .

Figure 30 presents the results of 6% polyacrylamide gel electrophoresis for the RPA product at the initial template DNA concentration ranging from 10^{10} copies/ μL to 10^7 copies/ μL . The results demonstrate that when the concentration of template DNA falls below 10^9 copies/ml, the amplified product cannot be detected using gel electrophoresis.

4.5 Discussion

4.5.1 Evaluating the impact of Proteinase K on experimental outcomes.

In Figure 28, there are 6 kinds of RPA samples were prepared with the TFT biosensors for *Leishmania* HSP70 gene detection. In Figure 28 (e) the Proteinase K-treated RPA sample and (f) the purified RPA sample, there was a noticeable decrease in I_d in “Test TFT” while no I_d decrease was observed in (e) no treated RPA product. One of the potential reason for this is the effect of single-stranded binding protein (SSB) in RPA reagents. In RPA, SSB protein binds to the single-stranded DNA during the amplification process, protecting it from degradation and preventing unwanted secondary structures from forming, thus promoting the amplification of DNA. SSB protein interacts with single-stranded DNA to form a more stable protein-nucleic acid complex than DNA-DNA, protecting the single-stranded DNA and increasing the efficiency and accuracy of amplification. However, due to the presence of SSB, the amplified DNA may cannot bind well to the probes on the chip surface. Proteinase K is expected to degrade the SSB protein in RPA samples. This is the reason why the proteinase-K treated sample in Figure 28 (e) and (f) show a clear decrease in I_d while non-treated sample in Figure 28 (d) doesn't exhibit such phenomenon.

4.5.2 *Leishmania* HSP 70 fragment detection

In this experiment, nucleic acid detection has been successfully achieved by combining an oxide

TFT biosensor with RPA. According to the results shown in Figure 29, the combination of the RPA and TFT biosensor was able to detect *Leishmania* HSP70 with a concentration of at least 10^1 copies/ μ L. Therefore, the results of the experiment demonstrate a high sensitive detection with 20 min isothermal amplification.

In Figure 29, we investigated the effect of 1% SDS addition at post-amplification treatment. By comparing reaction conditions of 42°C at 20 minutes and 42°C at 20 minutes with 1% SDS process as shown in Figures 7(a) and (b) respectively, we found that the addition of SDS significantly improved the detection capability of the chip. This is also considered be caused by SBB. SDS may denatured the SSB more completely together with proteinase K, and improved the binding between RPA amplicon and probe DNA on TFT.

In this study, we innovatively used RPA amplification products containing specific nucleotide sequences for DNA detection. The primer was designed with the 5'-TACACAGCAC[CCC]-3' sequence, where [CCC] is a three-carbon structure that can block DNA replication from extending beyond the primer's extra sequence. Therefore, the amplified product contains a 5'-TACACAGCAC-3' fragment of the amplified nucleic acid, which can specifically bind to the surface of the chip containing the 5'-GTGCTGTGTA-3' sequence. Thus, the detection of the HSP70 gene fragment can be converted into the detection of 5'-TACACAGCAC-3' and 5'-GTGCTGTGTA-3' sequences. This approach not only eliminates the need for probe design but also increases the versatility of TFT chips for nucleic acid detection, which can be applied to the detection of other nucleic acid fragments.

In Figures 27 to 29, the all Id value increased by an amount after incubation . It is speculated by the non-specific binding of some substances in RPA reagents. The increase can be cancel out by comparing

the I_d of Ref TFT and Test TFT. In the case of low-concentration template DNA, the amplification efficiency is low, resulting in less consumption of the primer. Thus, most of the bound molecules on the chip surface are forward primers rather than RPA products in low and zero concentration cases. Even though, the larger decrease in I_d were observed at higher concentration of template cases in Figure 29. This indicate that the sensitivity of TFT for primer is much less than that for RPA amplicon. Actuary, the primer length is short, about 20 bases in comparison with the HSP70 nucleic acid fragments as long as 280bp. Furthermore, due to the length of the HSP70 fragments, they may tend to adopt a flopped state on the chip surface.

5. Conclusions

In this research, we successfully designed and fabricated a TFT biosensor based on inorganic material and glass substrate. The TFT biosensor we developed in this study can realize rapid nucleic acid detection by monitoring the current changes before and after sample hybridization. This innovative approach provides a novel method for the rapid diagnosis of infectious diseases. Furthermore, the use of inorganic material and glass substrate allows for these biosensors to be produced rapidly and cost-effectively via printed circuits. This type of TFT biosensors not only enables rapid detection of nucleic acids but also boasts the advantages of being reusable and easy to operate due to moisture tolerance. Notably, the probe we used has a wide range of applicability, and it does not require the design of new probes for different detection targets. This "design once, use multiple times" strategy significantly enhances our research efficiency and design investment. Furthermore, this label-free probe eliminates the need for additional instruments to observe probe results, such as fluorescence inspection. By

combining the TFT biosensor with isothermal amplification technology RPA, we were able to detect the *Leishmania* HSP70 fragment, achieving an impressive limit of detection of 10^1 copies/mL. Moreover, it is not affected by non-target amplification products, and compared to traditional gel electrophoresis detection and fluorescence detection, this TFT biosensor can significantly improve the detection capability for target nucleic acids.

However, despite the significant progress we have made, our work is far from over. We noted that although our TFT biosensor can detect trace levels of nucleic acids in combination with the RPA method, the TFT biosensor's current performance still needs further optimization. We acknowledge this as an area that requires our continuous effort and exploration. We will seek new methods and strategies to further enhance the stability and performance of the TFT biosensor.

6. Reference

1. Smith, K.F.; Sax, D.F.; Gaines, S.D.; Guernier, V.; Guégan, J.F. Globalization of human infectious disease. *Ecology* 2007, 88, 1903–1910. DOI:10.1890/06-1052.1.
2. Fardin, M.A. Covid-19 and anxiety: A review of psychological impacts of infectious disease outbreaks. *Arch Clin Infect Dis* 2020, 15. DOI:10.5812/archcid.102779.
3. Keesing, F.; Belden, L.K.; Daszak, P.; Dobson, A.; Harvell, C.D.; Holt, R.D.; Hudson, P.; Jolles, A.; Jones, K.E.; Mitchell, C.E.; et al. Impacts of biodiversity on the emergence and transmission of infectious diseases. *Nature* 2010, 468, 647–652. DOI:10.1038/nature09575.
4. Duan, R., Zuo, X., Wang, S., Quan, X., Chen, D., Chen, Z., Jiang, L., Fan, C., & Xia, F. Lab in a tube: Ultrasensitive detection of MicroRNAs at the single-cell level and in breast cancer patients using quadratic isothermal amplification. *Journal of the American Chemical Society*, 2013, 135(12), 4604–4607. DOI:10.1021/JA311313B
5. Oladepo, S. A., Nzila, A., Aminu, A., & Sankaran, S. Non-enzymatic detection of miR-21 in cancer cells using a homogeneous mix-and-read smart probe assay. *Analytical Biochemistry*, 2022,

645, 114601. DOI:10.1016/J.AB.2022.114601

6. Min, X., Zhuang, Y., Zhang, Z., Jia, Y., Hakeem, A., Zheng, F., Cheng, Y., Tang, B. Z., Lou, X., & Xia, F. (2015). Lab in a Tube: Sensitive Detection of MicroRNAs in Urine Samples from Bladder Cancer Patients Using a Single-Label DNA Probe with AIEgens. *ACS Applied Materials and Interfaces*, 2015, 7(30), 16813–16818. DOI:10.1021/acsami.5b04821
7. Wang, C.; Pan, R.; Wan, X.; Tan, Y.; Xu, L.; Ho, C.S.; Ho, R.C. Immediate psychological responses and associated factors during the initial stage of the 2019 coronavirus disease (COVID-19) epidemic among the general population in China. *Int J Environ Res Public Health* 2020, 17. DOI:10.3390/ijerph17051729.
8. Veigas, B.; Fortunato, E.; Baptista, P.V. Field effect sensors for nucleic acid detection: Recent advances and future perspectives. *Sensors (Basel)* 2015, 15, 10380–10398. DOI:10.3390/s150510380.
9. Krsihna, B.V.; Ahmadsaidulu, S.; Teja, S.S.T.; Jayanthi, D.; Navaneetha, A.; Reddy, P.R.; Prakash, M.D. Design and development of graphene FET biosensor for the detection of SARS-CoV-2. *Silicon* 2022, 14, 5913–5921. DOI:10.1007/s12633-021-01372-1.
10. Wang, S.; Hossain, M.Z.; Shinozuka, K.; Shimizu, N.; Kitada, S.; Suzuki, T.; Ichige, R.; Kuwana, A.; Kobayashi, H. Graphene field-effect transistor biosensor for detection of biotin with ultrahigh sensitivity and specificity. *Biosens Bioelectron* 2020, 165, 112363. DOI:10.1016/j.bios.2020.112363.
11. Park, J.; Nguyen, H.H.; Woubit, A.; Kim, M. Applications of field-effect transistor (FET)-type biosensors. *Appl Sci Converg Technol* 2014, 23, 61–71. DOI:10.5757/ASCT.2014.23.2.61.
12. Xu, S.; Zhang, L.; Wang, B.; Ruoff, R.S. Chemical vapor deposition of graphene on thin-metal films. *Cell Reports Physical Science* 2021, 2. DOI:10.1016/j.xcrp.2021.100372.
13. Sun, C.; Wang, X.; Auwalu, M.A.; Cheng, S.; Hu, W. Organic thin film transistors-based biosensors. *EcoMat* 2021, 3. DOI:10.1002/eom2.12094.
14. Yamashita, Y. Organic semiconductors for organic field-effect transistors. *Sci Technol Adv Mater* 2009, 10, 024313. DOI:10.1088/1468-6996/10/2/024313.
15. Li, J.; Tsukada, H.; Miyasako, T.; Tue, P.T.; Akiyama, K.; Nakazawa, H.; Takamura, Y.; Mitani, T.; Shimoda, T. High-transconductance indium oxide transistors with a lanthanum-zirconium gate

- oxide characteristic of an electrolyte. *J Appl Phys* 2020, 127. DOI:10.1063/1.5119210.
16. Oxner, E.S. *Fet Technology and Application*. Fet Technology and Application; CRC Press, 2020. DOI:10.1201/9781003066149.
 17. Lobato, I.M.; O’Sullivan, C.K. Recombinase polymerase amplification: Basics, applications and recent advances. *Trends Analyt Chem* 2018, 98, 19–35. DOI:10.1016/j.trac.2017.10.015.
 18. Tan, M.; Liao, C.; Liang, L.; Yi, X.; Zhou, Z.; Wei, G. Recent advances in recombinase polymerase amplification: Principle, advantages, disadvantages and applications. *Front Cell Infect Microbiol* 2022, 12, 1019071. DOI:10.3389/fcimb.2022.1019071.
 19. Zheng, T.; Li, X.; Si, Y.; Wang, M.; Zhou, Y.; Yang, Y.; Liang, N.; Ying, B.; Wu, P. Specific lateral flow detection of isothermal nucleic acid amplicons for accurate point-of-care testing. *Biosens Bioelectron* 2023, 222, 114989. DOI:10.1016/j.bios.2022.114989.
 20. Fraga, J.; Montalvo, A.M.; De Doncker, S.; Dujardin, J.C.; Van der Auwera, G. Phylogeny of *Leishmania* species based on the heat-shock protein 70 gene. *Infect Genet Evol* 2010, 10, 238–245. DOI:10.1016/j.meegid.2009.11.007.
 21. da Graça, G.C.; Volpini, A.C.; Romero, G.A.; Oliveira Neto, M.P.; Hueb, M.; Porrozzi, R.; Boité, M.C.; Cupolillo, E. Development and validation of PCR-based assays for diagnosis of American cutaneous leishmaniasis and identification of the parasite species. *Mem Inst Oswaldo Cruz* 2012, 107, 664–674. DOI:10.1590/s0074-02762012000500014.
 22. da Silva, L.A.; de Sousa, C.dos S.; da Graça, G.C.; Porrozzi, R.; Cupolillo, E. Sequence analysis and PCR-RFLP profiling of the hsp70 gene as a valuable tool for identifying *Leishmania* species associated with human leishmaniasis in Brazil. *Infect Genet Evol* 2010, 10, 77–83. DOI:10.1016/j.meegid.2009.11.001.
 23. Gelanew, T.; Hurissa, Z.; Diro, E.; Kassahun, A.; Kuhls, K.; Schönian, G.; Hailu, A. Case report: Disseminated cutaneous leishmaniasis resembling post-kala-azar dermal leishmaniasis caused by *Leishmania donovani* in three patients co-infected with visceral leishmaniasis and human immunodeficiency virus/acquired immunodeficiency syndrome in Ethiopia. *Am J Trop Med Hyg* 2011, 84, 906–912. DOI:10.4269/ajtmh.2011.11-0055.
 24. Gedda, M.R.; Singh, B.; Kumar, D.; Singh, A.K.; Madhukar, P.; Upadhyay, S.; Singh, O.P.; Sundar, S. Post kala-azar dermal leishmaniasis: A threat to elimination program. *PLOS Negl Trop*

Dis 2020, 14, e0008221. DOI:10.1371/journal.pntd.0008221.

25. Leishmaniasis. Available online: <https://www.who.int/news-room/fact-sheets/detail/leishmaniasis>.

26. Leishmaniasis – PAHO/WHO. Available online: <https://www.paho.org/en/topics/leishmaniasis>; Pan-American Health Organization.

27. Rathore, H., Biyani, R., Kato, H., Takamura, Y., & Biyani, M. (2019). Palm-size and one-inch gel electrophoretic device for reliable and field-applicable analysis of recombinase polymerase amplification. *Analytical Methods*, 11(39), 4969–4976. DOI:10.1039/C9AY01476D

28. Piepenburg O, Williams CH, Stemple DL, Armes NA. DNA detection using recombination proteins. *PLoS Biol.* 2006 Jul;4(7):e204. DOI:10.1371/journal.pbio.0040204.

29. Daher, R. K., Stewart, G., Boissinot, M., & Bergeron, M. G. Recombinase Polymerase Amplification for Diagnostic Applications. *Clinical Chemistry*, 2016, 62(7), 947–958. DOI:10.1373/CLINCHEM.2015.245829

Chapter 5. Conclusion and Future works

5.1 Conclusions

Chapter 1 focuses on introducing several major infectious disease crises in human history, followed by the discovery and use of penicillin. With the progress and development of modern science, enabling human to actively face the crisis of infectious diseases. This chapter also introduces the rapid detection methods for infectious diseases, among which the conventional nucleic acid detection method PCR is becoming more and more important in the detection for infectious diseases. However, due to some limitations, it cannot be easily used in point-of-care testing. In addition, FET biosensor chips make rapid nucleic acid detection possible due to their excellent properties.

In chapter 2, some known nucleic acid isothermal amplification methods are mainly introduced, such as LAMP, RCA, SDA, HAD and so on. And this chapter 2 briefly introduced the research topics of the next monk period, the phase change material platform based on RPA nucleic acid isothermal amplification. The construction of the phase change material platform used below is briefly introduced, as well as the method and process of using the phase change material platform to provide a suitable reaction temperature for RPA. Finally, polyacrylamide gel electrophoresis was used to analyze the RPA amplicon using the traditional incubator and the phase change material platform. According to the electrophoresis results, the nucleic acid can be successfully amplified using the phase change material platform. The method can replace PCR nucleic acid detection to a certain extent in terms of speed and accuracy.

In chapter 3, the method of immobilizing the probe DNA to the surface of the FET biochip is first introduced. Then, the factors that cause the current to be unstable on the FET biochip are introduced. The first point is that the high-concentration buffer can stabilize the current better than the low-concentration buffer. The second point is that the standard reference electrode needs to be placed in the test before the test. The high-concentration KCl solution at the end of the standard electrode is replaced in the buffer. The third point is that the water content of the chip will affect the current stability. The current of the chip with high water content is more stable than that of the chip after drying. Then use the optimal detection conditions, that is, use a relatively high concentration of detection buffer, place the reference electrode in the buffer before detection, soak the chip in the

buffer before detection, etc., but according to the current change diagram, on the chip After adding the target nucleic acid sample and incubating, the current is very stable without offset. In this case, it can be deduced that there are two possibilities. One is that the sensitivity of the chip is not good, and the other is that the DNA hybridization fails. Since the target nucleic acid molecule used has fluorescent molecules, the surface can be fluorescently analyzed by a polarized light microscope. According to the results, there were no obvious fluorescent molecules on the chip surface. Finally, GPTMS, as another linker, has been used for DNA probe immobilization. The Id variation measurements indicate GPTMS treated TFT biosensor chip can clear distinguish the positive control and negative control.

In chapter 4, the prospect of combining RPA technology and TFT biosensors are described. And show the Id variation of the chip before and after adding the RPA products to the TFT biosensor chip. According to the Id variation measurement results, TFT biosensor chips that without LaZrOx can successfully detect the target RPA products. It shows that the combination of the RPA technique and TFT biosensor chip is feasible, which provides the possibility for the POCT of DNA detection. Oxide TFT biosensor can successfully detect RPA products with template DNA. The following conclusions were obtained 1. Without proteinase K process, RPA products can not be detected. 2. The detection effect of proteinase K-treated RPA sample is same with purified RPA sample. 3. So far, our TFT biosensor can detect the concentration of 101 copies/ μ L for *Leishmania* HSP70. 4. 1%SDS processed RPA sample can improve the detection ability of TFT biosensors. 5. Oxide TFT biosensor can selectively and specifically detect *Leishmania*.

5.2 Future works

In future works, the investigation of the factors affecting the Id variation will still be the primary content of the works. Secondly, improve the TFT biosensor chip modification method and design, then strive to apply and promote the TFT biosensors in the actual nucleic acid detection. Finally, try to combine the technology of Crispr-cas technology with TFT biosensor to explore its possibility in DNA detection.

Acknowledgments

Throughout the writing of this thesis, I have received a great deal of support and assistance.

I would firstly like to express my sincere gratitude to Prof. Yuzuru Takamura, who accepted me into his laboratory and provides an excellent platform for me to have the opportunity to learn knowledge and related experimental skills in the field of biosensors. I would like to acknowledge Asst. Prof. Daisuke Hirose, whose continued guidance and support really taught me a lot in experiments as well as life attitude. I would also like to thank Prof. Manish Biyani for his advice in the biotechnology field which helped me to overcome many troubles in my research. I would also like to express my thanks to all the members of Takamura laboratory who have helped me in my research with their important advice and for all the fun we did together outside the laboratory.

I would like to sincerely thank the China Scholarship Council for its strong support of my scientific research and daily life.

In addition, I would also like to express my gratitude to JAIST for providing this excellent chance and platform to me.

I would like to thank Prof. Takahiro Honsaka to support and help me to complete my minor research project in his laboratory.

Finally, I would like to thank my family and friends for their wise counsel and sympathetic ear. I could not have continued my Ph. D course without their encouragement.

Weidong WU
JAIST, September 2023