Microfluidic Electrochemical Detection of Prostate Cancer using Telomerase Activity

by

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Abstract

Prostate cancer has become one of the leading causes of death and the most frequently diagnosed cancer in men, worldwide. It is highly treatable if detected early, but the current screening methods suffer from poor clinical specificity for prostate cancer, leading to unavoidable misdiagnosis and overtreatment. Elevated telomerase activity has been discovered as an indicator of a variety of cancers, including over 92% of prostate cancers. High clinical specificity of telomerase activity for prostate cancer compared to other prostate anomalies is an important concept promoted in this thesis. To overcome the quantification complexity of current telomerase activity detection techniques, we have designed and demonstrated the TAME assay (Telomerase Activity Measured Electrochemically): a microfluidic biosensor to detect and measure telomerase activity using an electrochemical technique known as E-DNA. Telomerase activity has been successfully correlated to a TAME parameter, TRAP (Telomeric Repeat Amplification Protocol) product concentration, using two E-DNA schemes: ‘signal-off’ and ‘signal-on’. In terms of the E-DNA signal change, telomerase activity and TRAP product concentrations we investigated are linearly correlated, which is promising for prostate cancer screening and detection. The signal-off scheme exhibits electrochemical signal suppression if telomerase activity is present with alternating cyclic voltammetry (ACV) at 50 Hz. The signal-on scheme shows the reverse effect with square wave voltammetry (SWV) at 150 Hz. ‘Signal-on’ and ‘signal-off’ are transferable by altering SWV’s frequency. The limit of detection of ‘signal-off’ and ‘signal-on’ on tested E-DNA chips using un-purified TRAP samples, originated from un-purified prostate cell extracts, are 55 nM and 10 nM. The TAME assay well-differentiates prostate cancer cells from healthy prostate epithelial cells based on telomerase activity expressions.

Due to the trend in medical devices towards miniaturization, portability, low power, and high integration capability, in addition to a microfluidic E-DNA chip, a polymerase chain reaction (PCR)
microfluidic chip is under development. The PCR chamber is 2.5 mm X 2.5 mm with the integration of micro-heaters and temperature sensors. PCR chips are designed to achieve heating uniformity, which has been evaluated by thermal imagining. In future, a fully integrated TAME chip including both E-DNA and PCR is anticipated.
Preface

This thesis work was completed in collaborations with Dr. Xiaolei Zuo from the University of California, Santa Barbara, USA and Dr. Jyh-Jian Chen from the National Pingtung University of Science and Technology, Taiwan. Dr. Zuo provided DNA sequences that were required for all E-DNA experiments. Dr. Chen proposed PCR chip designs. I performed all E-DNA related experiments. Moreover, I participated in the PCR chip design process, completed all PCR chip design drawings, fabricated PCR chips, and conducted preliminary validations of PCR chip designs.
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Chapter 1. Background

Prostate cancer has become the second leading cause of men’s death in the U.S. and the U.K., and the third leading cause in Canada. It is also the most commonly diagnosed cancer in men all over the world.[1] In 2009, one man in six will suffer from prostate cancer in his life with a higher likelihood in men with family history.[1] Ethnicity, age, and personal lifestyle are also common influences on men developing prostate cancer.[2] Particularly in Canada, every week, almost 500 men are diagnosed with prostate cancer, and 80 would likely die of prostate cancer; prostate cancer accounts 27 % of all new cancer cases in men, and about 11 % of all cancer deaths in men.[3]

During the past 10 years, prostate cancer mortality rates have declined in the U.S., the U.K., Canada, and Europe. Although the exact reasons are unclear, improvements in cancer treatments, well-organized health systems, and increased personal health attention have all positively contributed to this decline. Yet, in 2010, an estimated 32,000 men will still die from prostate cancer in the U.S. and 10,000 in the U.K. Furthermore, across Europe, prostate cancer accounts as much as 9.2 % of the total cancer deaths.[1]

If prostate cancer can be detected early, there is a high possibility that the cancer will be treatable.[4] Because prostate cancer develops very slowly, and its symptoms are not always obvious, early detection is very difficult.[5] Currently, prostate cancer detection largely relies on cancer screening. The common prostate cancer screening methods include the prostate specific antigen (PSA) blood test and digital rectal exam (DRE); transrectal ultrasound is another less frequently used method.[6] Biopsy is usually recommended when any screening method produces a positive result.[6] Although the PSA test and DRE are highly recommended by physicians as routine health examinations,
there is no proof, so far, that either individual test is related to the mortality reduction of prostate cancer.[2]

1.1 Current Detection Methods and Their Problems

The PSA test is the most common prostate cancer screening method worldwide. It examines the level of prostate specific antigen in blood. PSA is a protein produced by the prostate (molecular weight of 26,079 g/mole [7, 8]), which can be carried out from the prostate to the blood circulation.[9] When there is cancer forming in the prostate, the PSA level generally becomes elevated. Prostate cancer is suspected if the PSA concentration is higher than 4.0 ng/mL.[10] However, in addition to prostate cancer, many prostate anomalies can induce an elevated PSA level, including age, prostatitis, and ejaculation.[2] Moreover, the cut-off value of 4 ng/mL has been universally adopted in medical practice without “preliminary validation studies”. [2] In reality, the upper limit of the healthy PSA concentration range substantially varies from person to person, rather than the value of 4.0 ng/mL. Limited specificity, misdiagnosis, and overtreatment are therefore problems frequently encountered as a result of using the PSA test.

Much discussion has focused on the controversies of the PSA test. To date, the United States is the only country that officially recognizes the PSA test as a screening method. In Canada, using the PSA test as part of prostate cancer screening is not supported by government guidelines.[1]

DRE is a second, fast prostate cancer screening method. The prostate is a small organ located below the bladder and next to the rectum in men.[11] A healthy prostate is soft and mainly produces a fluid. When cancer is present, the prostate becomes hard. Thus, when a physician inserts a finger into the rectum, the physician tries to feel whether there are hard areas on the prostate, which could potentially be prostate cancer zones.[10] DRE is an ancient and common medical procedure for health examinations. It was the first prostate cancer screening method prior to the development of the PSA
test. Yet, DRE is invasive, uncomfortable, and its results are not always correct.[12] Many factors can potentially bias the examination, such as physician experience. DRE suffers from limited accuracy and limited specificity.

Transrectal ultrasound is the only other method for prostate cancer detection. Similar to DRE, transrectal ultrasound employs a probe, which produces sound waves, inserted from the rectum to generate images of the prostate. Any dark or dense areas can potentially be cancer zones.[6] As with DRE, transrectal ultrasound has suffered from low accuracy and low specificity.[12]

Biopsy is not a screening method, but a diagnosis for cancers. It is greatly recommended if the PSA test, DRE, or transrectal ultrasound produces positive results.[10] Though biopsy is invasive, painful, and uncomfortable, it conveys more certain messages about the cancer. After prostate tissues are collected, by observing the tissues in lab, the cancer can be confirmed and stage determination is possible. However, all medical and lab work can take up to one month to complete before letting patients know the results. This lag time is long and patients may miss the best treatment window. Patients’ quality of life is, therefore, substantially lowered and restricted. Biopsy’s sensitivity is also of concern to some researchers because it varies depending on the prostate size. After collecting tissue samples on various spots of the prostate, the cancer has a higher possibility to be detected from a small prostate than a big one.[2] Biopsy is, to date, still the best available cancer diagnosis.

Overall, low specificity, false negative results, and false positive results are main problems of the current prostate cancer screening methods (the PSA test and DRE). The current screening methods are therefore in urgent need of improvement: a more specific prostate cancer biomarker and a more reliable detection technique are necessary.

There have been other prostate cancer biomarkers explored in research, ranging from protein markers to tissue markers and urine markers.[13] They are all expected to be more specific than PSA,
but most of them are still in the validation stage. As one recent example, Getzenberg et al. claimed that EPCA-2 (Early Prostate Cancer Antigen 2) was a highly specific and highly sensitive biomarker to prostate cancer and its detection in blood could accurately confirm prostate cancer.[14] EPCA-2 is a serum protein that only appears in prostate cancer cells. They designed an assay for early prostate cancer detection. However, in 2008, the researchers and their university were sued due to their assay’s questionable validity and unstable performance.[15]

Improving current prostate cancer detection is a goal for many researchers, scientists, and engineers. Many obstacles exist and need to be overcome. The progress of developing a new generation of assays for prostate cancer detection has not been negatively affected by these difficulties. On the contrary, more pioneers are motivated and actively involved to achieve the aims of saving patients’ lives and improving their quality of life.

1.2 Elevated Telomerase Activity

Elevated telomerase activity has attracted many researchers’ attention as a promising biomarker for many cancers, including breast cancer and prostate cancer. The relation of elevated telomerase activity and a variety of cancers was discovered in the late 1970s and 1980s.[16] Higher telomerase activity has been correlated to a higher probability of the cancer, and potentially, a greater cancer severity.[17]

Telomeres are the ends of eukaryotic chromosomes helping to stabilize and protect the chromosomes.[18] Telomerase is an RNA reverse transcriptase with an internal template that can synthesize telomeric repeats at telomeres.[18] Telomerase activity is the ability of adding telomeric repeats, which are 6 bp repeats, (TTAGGG)$_n$ in vertebrates or (TTGGGG)$_n$ in ciliates.[19] Figure 1.1 presents telomerase structure and functions. When chromosomes or DNA strands are copied during cell divisions, there is always a loss of a few DNA sequences at one end of a newly copied DNA strand due to the mechanism of DNA replication. To prevent any loss of important DNA sequences at the end of
chromosomes, noncoding telomeric repeats are therefore sacrificed to protect the coding or regulatory sequences carried at the chromosome ends.[18]

Telomerase activity is only found in cells of the germline, unicellular eukaryotes, stem cells, and cancer cells.[20] For healthy adults, telomerase activity is usually not presented. However, in tumours, telomerase is activated and remains highly active for telomeric repeat addition, ensuring the continued fast growth and reproduction of cancer cells.

[Figure 1.1 The structure and functions of telomerase. Up right portion of the figure: the telomerase internal template synthesizes (TTGGG)\textsubscript{n} at the 3’ end of a ciliate’s chromosome. Left bottom portion of the figure: telomerase activity prevents DNA losses at the end of chromosomes [21]]

Figure 1.2 A demonstrates the relationship between telomerase activity and cancer development. In unstable genomes, a small portion of cells that do not have telomerase protection are able to proceed through the cell transformation process, becoming cancer cells with unusually high telomerase activity, while the majority of cells die from telomere attrition.[22] This phenomenon takes place not only in more than 92 % of prostate cancers and 90 % of breast cancers, but almost universally
across the cancer spectrums. It has been observed that high telomerase activity is present in 80 – 90 % of all cancers.[22, 23] Figure 1.2 B presents a model explaining the roles of telomerase activity in the process of cancer formation. Both checkpoint failures and telomerase dysfunction contribute to the cancer progression.[24]
Figure 1.2 Telomerase activity and cancer development. Part A: a schematic process flow of cancer development with high telomerase activity [22]. Part B: a model indicating the roles of telomerase at the process of cancer formation [24].
1.3 Telomerase Activity Detection

There are two generations of telomerase activity detection methods. The first generation is polymerase chain reaction (PCR) based methods, which include telomeric repeat amplification protocol (TRAP) and its variants. The second and more recent generation is the employment of modern techniques such as electrochemical and optical techniques. The continuous improvements in telomerase activity detection and measurement have maintained telomerase activity's importance and potential in medical practice.

1.3.1 The Traditional TRAP Assay

TRAP has been the gold standard for telomerase activity detection for decades. Almost all new developed telomerase activity detection techniques are first confirmed according to the TRAP results. The TRAP assay is a PCR process followed by gel electrophoresis (GE). The amplification principle of the traditional TRAP assay is shown in Figure 1.3, in which TS and RP are oligonucleotide forward primer strands and reverse primer strands, respectively. [25] If the telomerase extracted from cells is active, telomeric repeats will be added on the 3’ end of TS. A higher telomerase activity results in a larger number of TS having telomeric repeats, and therefore, leads to an increased number of TRAP product bands and their intensity on a gel image. Presented on a successful TRAP GE gel image is a series of TRAP product bands with 6 bp apart from each other. Theoretically, the amount of 6 bp-increment bands and their intensity indicate telomerase activity.

Although sensitive, the traditional TRAP assay is laborious, time consuming, and poorly quantifiable. [26] This is because there are many uncertainties while performing GE, including gel resolution and carry-over contaminations. Since TRAP products are usually small in length, between 36 to 200 bp, if the gel resolution is not optimum, it is difficult to clearly visualize the 6-bp increment between each product, and therefore, the detection of telomerase activity by GE is not always
convincing. In addition, gel imaging results are difficult to quantify. Thus, the traditional TRAP assay is only accounted as a relative measurement of telomerase activity.

Figure 1.3 The traditional TRAP assay [25]

1.3.2 TRAP Variants

To improve telomerase activity quantitation, recent work has attempted to alter the primer designs of the traditional TRAP assay. These primer modifications can be complicated, and their presence may inhibit or interfere with any downstream analysis of TRAP products.

The fluorescent TRAP assay is one example of a primer modification. It quantifies telomerase activity using fluorescence measurement instead of GE. This is achieved by employing an Amplifluor reverse primer. There is only fluorescence when double stranded TRAP (dsTRAP) products are present. The detailed amplification process is shown in Figure 1.4, in which TS is the primer strand and Amplifluor RP is the fluorescent reverse primer strand.
Figure 1.4 The fluorescent TRAP assay [27]

Most TRAP variants do not deviate from the traditional TRAP amplification principle, but rather seek to improve its quantitation. Table 1.1 summaries TRAP variants and comparison of their sensitivity, advantages, and disadvantages. [28] In the table, SPA stands for scintillation proximity assay; [29] HPA stands for hybridization protection assay; [30] ELISA stands for enzyme-linked immunosorbent assay; [31] ELIPA stands for enzymatic luminometric PP1 assay; [32] TRE stands for telomeric repeat elongation assay. [33]
<table>
<thead>
<tr>
<th>Name of the assay</th>
<th>Sensitivity [cells]</th>
<th>Advantages (compared to classical TRAP)</th>
<th>Additional requirements and disadvantages (compared to classical TRAP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAP</td>
<td>100</td>
<td>Information on telomerase product (amount, ladder appearance etc.) from sequencing gel</td>
<td>Laborious, high input of radioactivity, long exposure times</td>
</tr>
<tr>
<td>Direct assay</td>
<td>$= 10^6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRAP with fluorescent primers (F-TRAP)</td>
<td>100</td>
<td>Nonisotopic</td>
<td>Fluorescein-labeled primers needed</td>
</tr>
<tr>
<td>TRAP with fluorescent staining of products</td>
<td>50</td>
<td>Nonisotopic</td>
<td></td>
</tr>
<tr>
<td>TRAP-SPA</td>
<td>10</td>
<td>PAGE not necessary, high-throughput microplate format, easy quantitation</td>
<td>$[^3]$-TTP and biotinylated primer required</td>
</tr>
<tr>
<td>TRAP/HPA</td>
<td>10</td>
<td>PAGE not necessary, microplate format, easy quantitation, no radioactivity</td>
<td>AE-labeled probe required</td>
</tr>
<tr>
<td>TRAP with Amplifluor primers (TRAPezeXL)</td>
<td>50–100</td>
<td>PAGE not necessary, high-throughput format, fast and easy nonisotopic quantitation, differential labeling of product and control, available as a commercial kit</td>
<td>Price of the kit</td>
</tr>
<tr>
<td>TRAP-ELISA</td>
<td>10</td>
<td>Nonisotopic chromogenic detection without PAGE, available as commercial kits</td>
<td>Indirect detection, price of the kit</td>
</tr>
<tr>
<td>Real-time TRAP (using TRAPezeXL)</td>
<td>50</td>
<td>As in TRAPezeXL, plus direct recognition of false-negative and false-positive results, exact quantitation</td>
<td>Real-time PCR instrument required, price of the kit</td>
</tr>
<tr>
<td>In situ TRAP</td>
<td>$= 1$</td>
<td>Information on activity in intracellular and morphological context</td>
<td>Laborious procedure, low-throughput fluorescence microscope required</td>
</tr>
<tr>
<td>TMA/HPA</td>
<td>$= 1$</td>
<td>Isothermic amplification, elimination of PCR artifacts, detection without PAGE, high-throughput format</td>
<td>AE-labeled probe required</td>
</tr>
<tr>
<td>ELIPA</td>
<td>5</td>
<td>Linear dose–response — directly related to telomerase product, high-throughput format, elimination of PCR artifacts and PAGE, nonisotopic</td>
<td>Luciferase bioluminescence detection system and luminometer required</td>
</tr>
<tr>
<td>Optical biosensor</td>
<td>$= 10^3$</td>
<td>Nonisotopic detection, elimination of PCR artifacts and PAGE</td>
<td>Phosphothioate oligo substrate and fiber-optic apparatus required</td>
</tr>
<tr>
<td>TRE</td>
<td>$= 100$</td>
<td>Elimination of PCR-related artifacts and PAGE, high reproducibility, no interference of up to 1000-fold excess of background negative cells, information on reaction kinetics</td>
<td>BIACORE apparatus and biotinylated substrate primer required</td>
</tr>
</tbody>
</table>

Table 1.1 Comparisons of TRAP variants [28]
1.3.3 Biosensors

Bio-sensing underlies the modern generation of telomerase activity detection and measurement, including optical [34, 35], electrochemical [36, 37], magnetic [38], and nanowire conductive signal [39] techniques. Kulla and Katz have reviewed available telomerase activity biosensors that were published prior to 2008.[40] Bio-sensing definitely explores new territory for telomerase activity quantitation with a simple, efficient, and accurate fashion compared to the traditional methods.

In 2004, Xiao et al. published a telomerase detection scheme using catalytic beacons (Figure 1.5).[41] Part A and B are parts of a DNAzyme, and Part C is the primer that initiates telomerase extension activity. Parts A, B, and C make up a stem loop configuration, and the single stranded portion of this stem loop is completely complementary to telomeric repeat units. By adding dNTP and telomerase extracts from HeLa cells, telomerase extension occurs at the primer (Part C). Thus, the original stem loop is denatured, and the DNAzyme (Part A and B) is completely exposed in the testing solution. Next, in the presence of Hemin and H$_2$O$_2$, ABTS, a chemical compound, is oxidized by the DNAzyme with a color change, which could be measured by absorbance. Consequently, the telomerase activity is observed by ABTS oxidation. The detection limit is 500 HeLa cells.

![Figure 1.5 Analyzing telomerase activity by a functional DNA beacon that self-generates a DNAzyme [41]](image)

Figure 1.5 Analyzing telomerase activity by a functional DNA beacon that self-generates a DNAzyme [41]
Xiao has also participated in designing another telomerase activity detection platform involving chemiluminescence (Figure 1.6).[34] In Figure 1.6, Part (5) is a telomerase extension primer, which is immobilized on a gold (Au) surface. With the addition of telomerase extracts and dNTPs, the primer sequence is extended by telomerase becoming Part (6). A DNAzyme and a portion of single stranded sequence make up to Part (7), and this single stranded portion can quickly hybridize to Part (6) with the DNAzyme hanging in the testing solution. Under the help of Hemin and H$_2$O$_2$, luminol oxidation occurs, catalyzed by the DNAzyme, resulting in light emission. Hence, light intensity is the measurement parameter of telomerase activity.

**Figure 1.6 Telomerase activity analysis using DNAzyme labels and chemiluminescence as a detection signal [34]**

However, most of the existing biosensors for telomerase activity detection and measurement are not widely practiced, and there are several reasons. First, most of them have a complicated multi-step sensing process involving complex and proper folding or unfolding of certain elements, such as the...
DNAzyme in Figure 1.5, or require additional reagents during the sensing process, such as hemin and H$_2$O$_2$ have to be added at certain step in both Figure 1.5 and 1.6. Consequently, the overall performance of these sensors is largely restricted or limited by every element of the sensor. That is, telomerase activity is detected not only based on the telomeric repeat extension ability, but greatly influenced by the conditions of the sensor, such as whether there is enough hemin and H$_2$O$_2$ supply during sensing. If there is not enough, even the telomeric repeat extension was vivid, the sensor is not able to detect and measure telomerase activity accurately and precisely. Second, most of these sensors have difficulties to detect telomerase activity with complex samples, such as blood serum, soil extracts, and un-purified PCR products. It is very likely to have one or more elements that can interfere with the signaling process. Such as the DNAzyme labels in Figure 1.5. It is not surprising that there are additional DNAzymes in the complex blood serum producing signals along with the original desired sensor signal production, and therefore, telomerase activity is overestimated in this way. Hence, a more robust, straightforward, specific bio-sensing or signaling technique is required to detect and measure telomerase activity from complex samples.

1.4 Motivation

We would like to develop a biosensor for prostate cancer screening using telomerase activity measurements. Due to the unique correlation discovered between telomerase activity and many cancers, the elevated telomerase activity demonstrates a high specificity for prostate cancer compared to other prostate anomalies. This improved specificity is one of key benefits offered by our method, and promises to reduce false positive and false negative results of prostate cancer screening or detection. Being a bio-sensing technique to measure telomerase activity, our method should be more quantifiable, robust, and reliable than the existing TRAP related assays. We are further inspired, by previous telomerase activity biosensors, to design our sensor in a simple, straightforward, single-input-single-output fashion to achieve outstanding operation, time, and cost efficiencies. Therefore, we have
explored electrochemical and microfluidic technologies in addition to sensitive biomarkers to accomplish our goals. Finally, both patients’ needs and current medical device market’s demand have driven the development of this work.
Chapter 2. Principles of Electrochemical Detection

Electrochemistry is a well-established science focusing on reactions at the solution-electrode interface. It is a method with a range of techniques and potentially high selectivity for various detections.[42] It has gained popularity to detect and quantify DNA in recent years. All electrochemical measurements are performed in an electrochemical cell. The simplest electrochemical cell setup is a three-electrode cell, which is composed of a gold (Au) working electrode, a reference electrode, and a platinum (Pt) counter electrode (Figure 2.1). Common reference electrodes are Saturated Calomel Electrodes (SCE) and Silver/Silver Chloride Electrodes (Ag/AgCl).

![Three-electrode electrochemical cell](image)

**Figure 2.1 Three-electrode electrochemical cell [43]**

In electrochemistry, the counter electrode is used to polarize the working electrode and make sure the current does not flow to the reference electrode. Hence, once a potentiostat (indicated as Pot’stat in Figure 2.1) applies a voltage, electrons transferring between the working and the counter electrodes through the ionic electrolyte comprise a current.[43] An ammeter is used to detect the response of the working electrode under this condition. This electrochemical method is known as voltammetry: measurement of current as a function of potential.
A reference electrode is required because the working electrode’s potential is controlled against this standard. As no current passes through the reference electrode, it maintains a particular steady voltage.[43] Thus, the potential of the working electrode is measured refereeing to this stable reference voltage.

Most electrochemical based DNA detection techniques, summarized in Table 2.1, measure the electrochemical signal change before and after probe-target hybridization. In theory, if the target DNA mismatches with the probe DNA, an imperfect hybridization should not result in any electrochemical signal change compared to the signal produced by the probe only. In contrast, if the target is fully complementary base pairing to the probe, the amount of signal change is quantitative with the target concentration.
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Table 2.1 Summary of electrochemical-based DNA detection methods [44]

2.1 E-DNA Sensors

E-DNA sensors are defined as electrochemical sensors that detect nucleic acids and measure their concentrations based on the hybridization of target and electrode-bound probe oligonucleotides, which is normally presented by folding and unfolding of the probe.[45] It is a well-studied and well-established bio-sensing platform,[45-49] and one of the most extensively used electrochemical DNA detection schemes among those listed in Table 2.1. E-DNA’s advantages are its high specificity, simplicity, convenience, and effectiveness.[44] In addition, owing to its low cost, easy operation, and high integration capability, it has quickly become one of the most suitable candidates for point-of-care diagnoses from clinical samples, such as monitoring cocaine in blood serum.[47] Furthermore, the E-DNA method is very extensible. ‘Signal-off’ and ‘signal-on are two schemes that can be normally
performed by applying different voltammetry techniques, such as alternating cyclic voltammetry (ACV) and square wave voltammetry (SWV).

E-DNA sensors are suitable for complex samples because its signaling process is very selective and resistant to non-specific elements.[45] The E-DNA signals are the signal changes produced by the conformational change of the probes, either from folding to unfolding or a reverse process (shown in Figure 2.3 and 2.4). This conformational change process is very difficult to mimic by non-specific elements in the complex samples. Therefore, even many non-specific elements settle down on the SAM layer and they are electrochemically active (to produce some formats of signals), it is almost impossible to generate a signal change resembling E-DNA signals. Another reason is that the redox reporters, such as methylene blue (shown in Figure 2.3 and 2.4), employed in E-DNA sensors function at an almost unique electro-active range that is rarely seen from biomolecules, clinical samples, and environmental samples. Overall, E-DNA sensors are highly selective and robust. Figure 2.2 demonstrates the selectivity of E-DNA sensors showing minimal signals from various complex samples unless the target DNA is in presence. The signal suppression percentage is one of E-DNA signal parameters, which is explained in details in section 2.1.1.

![Signal suppression comparison](image)

**Figure 2.2** E-DNA sensors are highly selective even using complex samples.[45]
2.1.1 E-DNA Signal-Off Scheme

The simplest E-DNA scheme is shown in Figure 2.3.[44] The process is performed in a standard electrochemical cell. Stem-loop shaped DNA probes with methylene blue (MB), an electrochemical redox reporter, attached at one end are immobilized on the Au working electrode surface. MB’s proximity to the Au surface causes efficient electron transfer between them under the supply of a voltammetry. Once probe-target hybridization occurs, the probe undergoes a conformational change leading to an increase in MB-Au distance. As a result, the efficiency of electron transfer is largely reduced, as is the corresponding electrochemical signal, which is current in the technique of voltammetry.[46] Because the signal suppression is the indication of target concentrations, this scheme (Figure 2.3) is known as ‘signal-off’. The signal-off scheme is typically achieved using ACV at a frequency between 10 Hz and 50 Hz.

Figure 2.3 E-DNA signal-off detection scheme [44]
2.1.2 E-DNA Signal-On Scheme

A series of signal-on techniques were developed as a result of the overwhelming popularity of the signal-off approach. Instead of signal decrease, the electrochemical signal increases while the probe-target hybridization takes place. Therefore, the signal gain is quantitative with the target DNA concentration. The signal-on scheme also offers a lower limit of detection and theoretically infinite signal gain.[45] Figure 2.4 demonstrates three signal-on schemes.[45] All of them produce electrochemical signal gain during probe-target hybridization, due to the restoration of the proximity of the redox reporter (PC or MB indicated in Figure 2.4) and the Au working electrode surface. They are all achieved using ACV at a frequency between 10 Hz and 50 Hz.

![Figure 2.4 Some signal-on E-DNA schemes](image-url)
2.1.3 Signal-On/Off Switching

Recently, White et al. [48] published an investigation presenting signal-on and/or signal-off E-DNA results from the same probe by simply altering voltammetry techniques and frequencies. The electrochemical techniques employed in this publication are ACV and SWV (Figure 2.5). The signal-off approach is achieved by applying ACV. In contrast, use of SWV results in both signal-off and signal-on measurements. In Figure 2.5, SWV was applied to both c) and d). By decreasing the frequency from 100 Hz to 5 Hz, the signal-off behaviour transitions to the signal-on behaviour. This paper has exquisitely illustrated the flexibility of the E-DNA technique.

The main reason why SWV is able to generate two opposite responses to one phenomenon is the unique current sampling method of SWV; it is also the result of combination of kinetics, sampling frequencies, and current responses. SWV samples current data at the end of every square pulse, as shown in Figure 2.5 a) as forward current and reverse current. The difference between both sampled signals is the current signal that is presented at each cycle. The collection of current signals from each cycle produces a “current response against potential” plot shown in Figure 2.5 c) and d). Rather than respect to potential, Figure 2.5 b) is generated by the collection of current signals from each cycle with correspondence to time. At different sampling time, the dominant signal is different from unbounded and bounded probes in terms of magnitude, which is mainly due to the effect of reaction kinetics. Compared to the reaction with a slower kinetic (bound probe in Figure 2.5 b), the reaction with the faster kinetic (unbound probe in Figure 2.5 b) produces a higher current or a lower current depending on if the current is sampled before or after the cross-over point. Figure 2.5 c) is the data produced by sampling before the cross-over point, which is a typical signal-off behaviour; d) is the data produced by sampling after the cross-over point, which is the signal-on behaviour. Overall, the switching between the signal-off and signal-on effects is the final effect of a combination of kinetics, sampling frequencies, and current responses with the voltammetry supply of SWV.
In Figure 2.5 b), the y axis is labeled as Normalized Current. Although White et al. did not state how the current was normalized and what was current normalized against in the paper, we assume the current is normalized against the electrode area or amount of immobilized probe DNA to eliminate the signal differences which could weaken the comparisons between different working electrodes and different experiments.

Figure 2.5 Square wave voltammetry’s frequency significantly affects the gain of E-DNA sensors [48]

2.1.4 Single Stranded DNA Generation

The E-DNA technique is only feasible for single-stranded DNA (ssDNA) measurements. This is due to the inherent requirement of the E-DNA technique: probe-target hybridization. Solid phase PCR, asymmetric
PCR, helper chemistry, primer modifications, lambda (λ) exonuclease digestion, and denature-freeze are available protocols for ssDNA generation from dsDNA samples.

Solid phase PCR and asymmetric PCR involve primer modifications. Although they are commonly used, their drawbacks are numerous. Solid phase PCR requires complicated chemistries and long processing time.[50, 51] Asymmetric PCR is straightforward, but requires precise PCR conditions, and proceeds only with a linear amplification rather than the exponential amplification of standard PCR.[49, 51]

Another method is to use “blocking or helper” oligonucleotides during the probe-target hybridization (Figure 2.6).[51] To implement this, a precise helper design and the knowledge of full product sequence are important. Helpers are complementary to the upstream and downstream sequences of the portion that would be hybridizing with the probe. This method is more applicable than solid phase PCR, primer modifications, and asymmetric PCR, due to its cost efficient, rapid, and simple implementation.

Figure 2.6 Use of "helper" oligonucleotides to facilitate the heterogeneous hybridization of targets [51]
Recently, λ exonuclease digestion has gained attention for use in generating ssDNA due to its simple application and fast reaction. λ exonuclease is an enzyme that digests the phosphorylated DNA strand among dsDNA as depicted in Figure 2.7. Owing to its simplicity, λ exonuclease digestion is the best candidate to achieve ssDNA production in a time and cost efficient manner.

![Diagram of PCR and λ exonuclease digestion](image)

**Figure 2.7 Using λ exonuclease to generate single stranded PCR products [52]**

The denature-freeze method for ssDNA generation is the least effective method among all available protocols, although it is fast and simple. dsDNA is heated up at 95°C for denaturation, and then quickly frozen in an ice bath. Ideally, ssDNA does not have enough time to form a perfect double helix, and hopefully, some of them are frozen at single stranded status.[49] The denature-freeze method has extremely low productivity, and is not recommended for any experimental use.

### 2.2 Microfluidic Diagnostic Platforms

Microfluidic electrochemical bio-sensing has become a fast growing research area. Microfluidics is a research focus that performs science in a miniature and well-defined space, such as a small chamber or a channel of dimensions less than 100 µm. Since its inception, many problems that are difficult to deal
with on benches, in pilot plants or industries have been resolved. Detecting rapid kinetics of reactions, reducing diffusion lengths, detecting activities of individual molecules, and characterizing surface interaction phenomenon are all typical unique tasks for microfluidic devices.[53]

The microfluidic platform offers many distinct advantages.[54] Because of its small size, there is less reagent usage, less power consumption, and therefore a fast science performance and a lower capital cost of experiments. As portability and miniaturization are greatly improved, microfluidic devices exhibit strong capability for automation and integration with other analytical tools. High throughput and ease of control are also offered by microfluidics, which deliver great hope to improve analytical sciences.

Microfluidics’ diverse characteristics have made it applicable to many disciplines. Chemical engineering is an example. Micro-reactors can carry out chemical processes with better controls and performances than the plant scale but with a lower production.[53] In addition, medical devices have taken advantages of microfluidics.[55] There are microfluidic medical sensors for blood pressure, temperature, and electrical impulse, and microfluidic action devices, such as fluid filters, separators, and pumps.

Nevertheless, microfluidics experiences difficulties that are absent from large scale work.[53, 54] Lack of mixing or fluid interactions can decrease the reaction efficiency substantially in micro-reactors. A huge surface area to volume ratio enhances non-specific adsorption and even causes inhibition of chemical and biological processes. Losses of working solution on device walls or by evaporation and energy through the device are fairly common. Although it is possible to counter, complicated and precise designs and time-consuming fabrications may be required. As examples, curved channel designs enhance mixing [56]; micro-pumps and micro-valves can transfer solutions in an automatic fashion on-chip [57].
A microfluidic device that can process a certain assay followed by an integrated analysis is categorized as “µ-TAS”, Micro-Total-Analysis Systems.[54] Such systems are certainly at the frontier of microfluidic research. It has extensively broadened the opportunities of integration between microfluidics with other practical analyses, such as capillary electrophoresis [58] and DNA microarray hybridization [59]. Furthermore, µ-TAS exhibits strong applicability to real life practices instead of being restricted in laboratory settings.

µ-TAS is particularly applicable for medical devices. It has been considered as a good platform for emergency and point-of-care diagnoses. As one example, Biosite Inc. has developed microfluidic chips for drug testing and cardiovascular disease testing. Inputting only a drop of blood, this chip enables to have results in 15 minutes.[60] The successful integration of µ-TAS and medical use has been a milestone in today’s technology development.

The E-DNA technique has been successfully achieved on a microfluidic chip.[47, 61, 62] One of recent work prepared by Ferguson et al. integrated ssDNA generation and the E-DNA technique using λ exonuclease digestion and presented them on-chip.[61]

### 2.3 Motivation

Combining elevated telomerase activity detection with bio-sensing technology, this project is further inspired by the E-DNA technique and microfluidics. We aim to present a microfluidic device for prostate cancer detection using telomerase activity measurement by E-DNA technique. Achieving an enhanced specificity, accuracy, operation efficiency, reliability, portability, and integration capability is the scope of this thesis project. In addition to patients’ needs, the trend of miniaturization in medical devices and the demand in point-of-care diagnoses have also significantly engaged the completing of this work.
Chapter 3. The TAME Assay

Our developed assay is named ‘TAME’, Telomerase Activity Measured Electrochemically. The TAME assay partially utilizes the traditional TRAP principle followed by single stranded product generation by λ exonuclease digestion. Then, sequence-specific electrochemical signals are collected before and after the ssTRAP hybridization to immobilized probes using the E-DNA technique. Detected signal change is quantitative with telomerase activity, and perhaps, cancer severity. The detailed TAME process is illustrated in Figure 3.1.

![Figure 3.1 The TAME assay for detection of telomerase activity. (A) Amplification of telomeric repeats with phosphorylated reverse primer followed by ssTRAP production using lambda exonuclease. (B) Stem loop DNA probes with redox reporters immobilized on a gold microfluidic surface. (C) Hybridization of probes and the ssTRAP products causing conformational change of the immobilized probes. (D) Detection of probe-TRAP hybridization by electrochemical techniques.](image)

Telomerase is extracted from a cell culture using published techniques.[25] The extracts are then uniformly mixed with other PCR components including the forward primer TS and the phosphorylated reverse primer CXP (Figure 3.1 A). Active telomerase adds telomeric repeats at the end
of TS. Subsequently, TS serves as a template for PCR amplification. The phosphorylated reverse primer is used for λ exonuclease digestion. With proper reaction conditions, λ exonuclease can selectively digest the phosphorylated strand, leaving only complementary ssTRAP products.

ssTRAP generation promises to improve downstream processing and novel analyses that are unable to perform on dsDNA, such as DNA microarrays. In addition, by digesting the phosphorylated strand, TAME allows the production of pure and unmodified ssTRAP samples for E-DNA quantification, which is unlike other TRAP quantification methods relying on the presence of biotin- or fluorophore-conjugated primer modifications. This pure TRAP output benefits the accuracy of TAME quantification and removes any incompatibility induced by these modifications in downstream analyses.

ssTRAP detection is performed on Au surfaces of a microfluidic chip. Stem-loop probes are first immobilized on the Au working electrodes using a self-assembled monolayer (SAM) (Figure 3.1 B). The probe sequence is self-complementary at its 5’- and 3’- ends and contains a thiol group, SH-, at one end and methylene blue, MB, on the other end. Besides acting as spacers between probe molecules, the SAM helps orient probes, exposing their sequence in electrolyte solutions. In the absence of complementary ssTRAP products, the MB group localizes near the Au electrodes as a redox reporter for electrochemical measurements. Once a voltammetry technique is applied, because of the proximity of the MB group to Au, efficient electron transfer occurs between them, resulting in a current response as a function of potential (Figure 3.1 D).

This probe-only signal is used as a standard; all ssTRAP measurement signals are compared against it. ssTRAP product injection into the microfluidic chamber on a E-DNA microfluidic chip results in hybridization and a corresponding stem-loop conformational change (Figure 3.1 C). This leads to a large reduction in electron transfer between the MB group and the Au working electrodes. ‘Signal-off’ and ‘signal-on’ measurements refer to two opposite responses to probe-ssTRAP hybridization (Figure 3.1 D).
Applying ACV at 50 Hz results in a significant peak current decrease compared to the standard (‘signal-off’ scheme). In contrast, providing SWV at 150 Hz results in a peak current signal increase (‘signal-on’ scheme). These procedures are consistent with recent work describing the electron transfer kinetics of the E-DNA system.[48]
Chapter 4. Experimental Setup

4.1 E-DNA Chip Design

The design criterion for the E-DNA chip is to perform electrochemical phenomena effectively and output signals to a computer successfully. These require the E-DNA chip to have two components: an electrochemical detection area and a connection lead for data acquisition. Electrochemical electrodes, contained within the detection chamber area, should include a reference electrode, a counter electrode, and at least one working electrode. Thus, once the chamber is filled with an electrolyte solution, it is supposed to act as a standard three-electrode electrochemical cell as shown in Figure 2.1.

There are many metals that can be used for fabricating electrodes. Based on literature review, Au has been selected as the working electrode material, which is standard in electrochemical setups.[63] In general, reference electrodes could be Saturated Calomel Electrodes (SCE), Silver/Silver Chloride Electrodes (Ag/AgCl); however, they are very hard to incorporate with microfluidic chips.[43, 63] Thus, a pseudo-reference electrode is the best approach for this chip. The pseudo-reference electrode is a metal film or wire that holds a relatively stable potential, but varies with every different electrolyte, which is because it is very sensitive to electrolyte components. The simplest pseudo-reference electrode is normally made with Pt. Normally, an internal standard of ferrocene is used in conjunction of pseudo-reference electrode to provide more understanding of the pseudo-reference potential, since there is no method that can calculate the reference potential.[64] In our experiments, ferrocene was not employed. However, in order to reduce the possibility of reference potential shift, all E-DNA experiments attempted to use a same working electrolyte. If the working electrolyte has to be different in terms of components, the 2nd electrolyte is diluted in the generally used working electrolyte to minimize the component differences so as to minimize the potential shift.
The most common counter electrode material is also Pt. This is owing to its inertness, so that the counter electrode does not interfere with the working electrode’s performance. If the electrolyte and the reaction is not corrosive, carbon, copper, and stainless steel are also commonly used electrode materials.[63]

Combining all design criteria, an E-DNA microfluidic chip was designed shown in Figure 4.1 A. All electrodes were thin films of 20 nm of Ti beneath a layer of 180 nm of Au or Pt. Figure 4.1 B is an image of a complete E-DNA chip with an open polydimethylsiloxane (PDMS) chamber. PDMS’s reversible bonding to glass surface without leaving residues has made the E-DNA chip reusable. Instead of a closed system, an open chamber decreases dead volume, reduces DNA loss on chamber walls or during fluid transfer, and minimizes the difficulties in probe immobilization. All electrochemical measurements of telomerase activity were performed on this chip.

The open PDMS chamber holds the volume of a solution up to 100 µL. All testing solutions during experiments were kept at or less than 100 µL with the open chamber. It is certainly possible to achieve E-DNA detection with much smaller volumes, such as less than 10 µL, which was demonstrated by experiments with a PDMS sealed chamber (results are shown in section 5.2.3). The performances with a bigger and a smaller volume of testing solutions are comparable, as are the open and sealed PDMS chambers.
4.2 Micro-Fabrication

Microfluidic chips are fabricated using techniques such as photolithography and etching. Since it deals with tiny features, microfluidic fabrication parameters are very stringent in order to achieve a desired feature or resolution. All processes and facilities introduced in this section are presented according to the Standard Operation Procedures (SOPs) stated by AMPEL Nanofabrication Facility at the University of British Columbia (UBC).

All micro fabrication completed in this thesis, mainly photolithography and thin film deposition, were practiced in a cleanroom following the SOPs. The cleanroom consists of a class 1000 lithography room and a class 10000 thin film room.[65] This means the particles in those two rooms are no more than 1000 /m$^3$ and 10000 /m$^3$. Generally, the photolithography process was performed in the class 1000 room, and the thin film deposition was performed in the class 10000 room.
4.2.1 Photolithography

Photolithography is a very common micro-fabrication technique. It includes photoresist coating, UV exposure, and developing (Figure 3.7).[66] This 3-step procedure can be repeated to fabricate multiple layers of designs on a same wafer. Wafer cleaning and baking are often critical during practical photolithography processes. Thus, in addition to the usual three steps, those steps are added as well.

In the cleanroom, a photoresist is spun on a cleaned wafer using a Laurell Spinner (Catalogue # WS-400-6NPP-LITE). Depending on wafer materials, an adhesive layer is optional prior to photoresist coating. Hexamethyldisilazane (HMDS) is normally sufficient for this purpose if needed, such as for glass wafers. UV exposure step is carried out with Canon Mask Aligner (PLA-501F). According to photoresist’s polarity, either positive, negative, or image reversal, different features can result after UV exposure (Figure 4.2).

![Figure 4.2 Photolithography process][66]
There is always a certain developer corresponding to a photoresist for the best and cleanest photoresist removal. This step removes all unwanted photoresist resulting in desired features on a wafer.

Baking is commonly necessary before and after UV exposure. Pre-bake, also called soft bake, can be performed on a hot plate with a certain set temperature. Evaporating the coating solvent and densifying the photoresist coating are the purposes of pre-baking.[66] Post-bake, also named hard bake, serves the almost same functions as the pre-bake does. However, the longer and the hotter the post-bake is, the harder the photoresist becomes to be removed in the developing step.

4.2.2 Thin Film Deposition
The thin film deposition process can be achieved by an evaporator (E-beam) in the cleanroom. E-beam operates under high vacuum, employing an electron beam to heat and melt the evaporating materials, which are normally located in individual carbon crucibles. Then, vapours of the melted material are deposited on substrates.[65] The thickness of the deposited film is measured by a programmable monitor. The evaporation process using E-beam is shown in Figure 4.3.[67] E-beam can be used for metal evaporation, such as Au and Pt.
4.2.3 Wafer Cleaning

Glass wafer cleaning is essential before starting any fabrication process. The piranha wash procedure is performed commonly to glass slides ensuring the surfaces are perfectly organic residue-free. Glasses are fully immersed in a sulphuric acid ($\text{H}_2\text{SO}_4$) and hydrogen peroxide ($\text{H}_2\text{O}_2$) mixed solution with 1:5 ratio.[65] The process is practiced at 120°C with various time lengths between 5 to 20 minutes. The piranha wash process is very hazardous; extra care of handling of this process is mandatory.

4.2.4 E-DNA Chip Fabrication Protocol

A detailed protocol and process flow of E-DNA chip fabrication is included in Appendix I. The E-DNA microfluidic chip was fabricated using the standard photolithography procedure. The process described below was performed twice for fabricating one E-DNA chip. The first round was for Au and the second was for Pt. Briefly, hexamethyldisilazane (HMDS) was spun on a borofloat glass wafer at 400 RPM for 45 seconds. Next, the photoresist of AZ 5214 (MicroChemicals, Branchburg, NJ, USA) was spun at 400 RPM for 6 seconds followed by 4000 RPM for 30 seconds. After being baked at 95°C for 90s, the glass was
exposed with UV (with an intensity of 6.90 ± 0.03 mW/cm²) through a mask for 130 seconds. Patterns were developed using AZ 400 Developer (MicroChemicals, Branchburg, NJ, USA) until clear features were visible. After rinsing with D.I. water, the wafer was evaporated with 20 nm of Ti as an adhesion layer followed by 180 nm of either Au or Pt. Lift-off using acetone followed by D.I. water rinsing resulted in the final patterned metal features. PDMS RTV 615 was purchased from GE Silicones (Waterford, NY, USA) and cast using standard techniques[68] to form a chamber seal. The E-DNA analysis volume was below 100 µL for all experiments.

4.3 Cell Culturing and TAME Protocol

Prostate cancer cell line LNCaP (Catalogue No. CRL-1740) and healthy prostate epithelia cell line RWPE-1 (Catalogue No. CRL-11609) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Handling, sub-culturing, and freezing procedures closely followed the instructions provided by ATCC. Dimethyl sulfoxide was ordered from Sigma (Oakville, ON), and all other culture media and supplements including RPMI-1640, Fetal Bovine Serum, Tripson TrypLE, Keratinocyte Serum Free Medium, Bovine Pituitary Extract, Human Recombinant Epidermal Growth Factor, Ca²⁺/Mg²⁺ Free Dulbecco’s Phosphate Buffered Saline were obtained from Invitrogen (Burlington, ON). Additional information about cell culturing, cell extraction, protein concentration determination, DNA concentration determination, and other TRAP preparations are included in Appendices.

TRAP TS and CXP primer sequences were ordered from Integrated DNA Technologies (Toronto, ON) with an analytical RP-HPLC and a standard desalting purification. TS sequence was 5’-AAT CCG TCG AGC AGA GTT-3’. CXP had a modification of 5’ phosphorylation and a sequence of 5’-CCC TTA CCC TTA CCC TTA CCC TAA-3’. The probe DNA for E-DNA measurements was ordered from Biosearch Technologies, Inc. (Novato, CA, USA) with a double HPLC purification. The sequence had two modifications: thiol C6 linker was added to the 5’ end and methylene blue was added to C7 at the 3’
end. The final sequence was 5′-/thiol C6 SS/GGC GAG GCT AAC CCT AAC CCC TCG CC/methylene blue/-3′.

Platinum Taq DNA Polymerase was obtained from Invitrogen (Burlington, ON), and the balance of the components including D.I. water (PCR grade water), lysis buffer, reaction mix, and dNTPs were provided in the TRAPEZE Telomerase Detection Kit obtained from Millipore (Catalogue No. S7700, Billerica, Massachusetts, USA). TRAP PCR mix was prepared with TS (100 ng/µL) and CXP (102 ng/µL) according to the proportions explained in the kit. However, CXP was not added until the start of thermal cycling; using only TS primer for extension at 30 °C for 30 minutes followed by 5 minutes at 95 °C prevented any digestion possibilities of CXP. 33 cycles of PCR amplification were performed with both primers with conditions of 1 minute at 95 °C followed by 1 minute at 60 °C and 2 minutes at 72 °C. The TRAP products were separated by GE at 120 V for 90 minutes. Precast 15% polyacrylamide gels were obtained from Dgel Sciences (Montreal, ON). Gel loading solution (Catalogue No. G7654) was ordered from Sigma (Oakville, ON). Staining dye was GelStar Nucleic Acid Gel Stain from Lonza (Burlington, ON).

λ exonuclease was obtained from New England BioLabs (Toronto, ON). The usage amount of λ varied depending on dsTRAP product concentration, but used a constant digestion protocol consisting of incubation at 37 °C for 15 minutes and 75 °C denaturisation for 10 minutes. Both dsTRAP and ssTRAP concentrations were measured by Quant-iT dsDNA Assay Kit (Catalogue No. Q33120) from Invitrogen (Burlington, ON). This kit shows the strongest signals with dsDNA, but it also detects ssDNA concentrations with a lower efficiency.

The experimental setup of performing E-DNA on a microfluidic chip is shown in Figure 4.4. The procedure of E-DNA probe immobilization on Au was described by Xiao et al.[46]. The E-DNA buffer used in this project was phosphate-buffered saline (PBS, 10 mM phosphate with 1.0 M NaCl and 1 mM Mg²⁺). All E-DNA signal measurements were collected through a 5 pin card-edge connector (CCESG-ND, Digi-Key, Thief River Falls, MN, USA) using an Electrochemical Analyzer Model 730 (CH Instrument, Austin,
TX, USA). A faraday cage (CH Instrument, Austin, TX, USA) was used to prevent electrical interferences. The signal-off detection experiments used TRAP PCR mix buffer as the electrolyte throughout data collection. Probe signals were acquired with the pure PCR mix. The signal-on detection experiments were conducted in the E-DNA buffer, which is phosphate-buffered saline (PBS, 10 mM phosphate with 1.0 M NaCl and 1 mM Mg²⁺)[46].

The E-DNA procedure was first validated using an artificial ssDNA target and a control stem-loop probe sequence. The control stem-loop probe had a sequence of 5' SH-AAT AAA ACG CCG ATC CA-MB 3’ (Biosearch Technologies, Inc., Novato, CA, USA). The artificial target to the control probe had a sequence of 5' -AAT AAA ACG CCG ATC CA- 3’ (Integrated DNA Technologies, Toronto, ON). Off-chip validation techniques followed those of Xiao et al.[46].

4.4 Experimental Setups

There are mainly two experimental setups for practicing TAME assay: off-chip and on-chip. Off-chip TAME experiments were carried out with three individual traditional gold rod working electrodes, one platinum counter electrode, and a silver/silver chloride reference electrode. The functioning area of each gold working electrode is approximately estimated as 2 mm². During experiments, all electrodes were placed in a glass cell containing 2 mL E-DNA buffer.

On-chip TAME experiments were performed with microfluidic E-DNA chips, instead of the traditional electrochemical cell setup with gold rod working electrodes and the silver/silver chloride reference electrode. Figure 4.4 is an image of the on-chip experimental setup. The individual available Au working electrode area is in the magnitude of µm², which is substantially smaller than the gold rod electrode area in the magnitude of mm². The open PDMS chamber holds a solution up to 100 µL. All testing solutions during experiments were kept at or less than 100 µL within the chamber.
The Au working electrode area exposed to the electrolyte in either off-chip or on-chip setup is the area available for probe immobilization. However, this physical exposed electrode area does not indicate the amount of functional probes. There is always a portion of probe DNA immobilized on the surface but cannot deliver any current signal with respect to the potential. The dead probes are commonly due to improper orientations. In order to understand probe coverage better, in this project, instead of estimate the coverage based on the available Au working electrode area, we measured the probe coverage using an electrochemical method. This method was exquisitely explained by Ricci et al. paper and its supporting material.[69]

Every experiment included 3 individual measurements at each of 3 working electrodes for a total of 9 measurements. Results presented in Chapter 5 are the average of these 9 measurements.

Figure 4.4 On-chip E-DNA experimental setup
Chapter 5. TAME Results and Discussions

5.1 Control Off-Chip E-DNA Experiments

The E-DNA technique was first performed off-chip using an artificial ssDNA target and a corresponding stem-loop probe. The artificial DNA target is a DNA sequence that was designed to hybridize with the probe DNA. Both detection schemes: ‘signal-off’ and ‘signal-on’ were confirmed by the off-chip experiments. This set of experiments validated the E-DNA performance in our laboratory.

5.1.1 Signal-Off Experiments

All signal-off experiments were performed with ACV at 50 Hz, unless stated alternatively, in a standard three-electrode electrochemical cell. Figure 5.1 clearly shows that the E-DNA technique successfully responds to different concentrations of target DNA in the signal-off scheme. A higher target DNA concentration corresponds to a larger peak current reduction compared to the probe-only signal. These results validated the designs of the probe DNA and artificial target DNA sequences. Similar results were observed when immobilizing 1.5 µL of 200 µM probe DNA and hybridizing it with 0.5, 1, and 1.5 µL of 200 µM target DNA.

![Figure 5.1 E-DNA signals when various volumes of 200 µM target DNA hybridize with 2 µL of 200 µM probe DNA](image-url)
Figure 5.2 summarizes the peak current signals before (diamond-shaped points) and after (square-shaped points) hybridization. The diamond-shaped points in Figure 5.2 are peak current data of 0.5, 1, 1.5, and 2 μL of 200 μM immobilized probe DNA. Then, 0.5, 1, 1.5, and 2 μL of 200 μM artificial target hybridized with 0.5, 1, 1.5, 2 μL of 200 μM probes, respectively; the hybridization peak current data are shown as square-shaped points. The amount of immobilized probes is expected to be quantitative with the probe peak current as a linear increase of peak current with the increasing in amount of immobilized probe DNA. The observed experimental data (diamond-shaped points) do not coincide with the expectation. There are two possible reasons. Once the probe volume is larger than 1 μL, the available working electrode surface area is almost fully saturated with probe DNA; therefore, the data for 1, 1.5, and 2 μL of 200 μM probe are not expressing substantial changes from each other. Another hypothesis is that the probe DNA did not form well on surfaces for the last three data points, which results in signal loss or undetectable signals from improper orientations of the probes on the electrode surfaces. Above two reasons could potentially violate the expectation contributing to the non-linearity.

All hybridization peak current data are smaller than the probe-only peak current data, which confirms the target and probe DNA sequence designs were correct, resulting in successful probe-target hybridizations. A substantial peak current reduction is one of the keys for performing a successful signal-off E-DNA experiment.
Figure 5.2 Peak current of various volumes of immobilized 200 µM probe DNA (shown in blue and diamond-shaped points) and peak current of hybridization by various volumes of 200 µM probe and target DNA (shown in red and square-shaped points).

In Figure 5.3, 2 µL of 200 µM probe peak current data exhibit small deviations, within 10% among 4 replicates (shown in diamond-shaped points). This suggests that the E-DNA immobilization method is fairly reproducible, robust, and reliable. Then, 0.5, 1, 1.5, and 2 µL of 200 µM target DNA hybridized with 2 µL of 200 µM probe, causing the hybridization peak current decreases as the target DNA concentration increases with an approximate $R^2$ value of 0.88 (shown in square-shaped points). A higher concentration of target DNA results in a larger number of probe-target hybridization, and therefore, a substantially larger peak current reduction. The same trend was also observed when hybridizing 1.5 µL of 200 µM probe DNA with 0.5, 1, and 1.5 µL of 200 µM target DNA individually. The result in this figure is consistent with the result presented in Figure 5.1.
We have conducted most of the E-DNA signal-off experiments using ACV at 50 Hz. We also investigated its performance at 1 Hz (Figure 5.4). Hybridizing 0.05, 0.1, and 0.2 µM artificial target DNA to 2 µL of 200 µM probe DNA, a linear correlation between the target DNA concentration and the signal decrease is held for both 1 Hz and 50 Hz. However, the signal decrease is much amplified with the higher frequency of 50 Hz than 1 Hz. Overall, different frequencies of ACV are unlikely to alter the trend of the E-DNA signal responses. This set of experiments was performed 3 times on 3 individual Au working electrodes, and the data used in Figure 5.4 is the average of 9 total measurements across 3 working electrodes. Because deviations are very minimal, they are not shown in the figure.
Figure 5.4 E-DNA signal-off experiments with different frequencies of ACV: the signal suppression increases as the target DNA concentration increases when same amount of probe is employed.

5.1.2 Signal-On Experiments

According to White et al.[48], SWV is more flexible than ACV in terms of current data sampling. By altering its frequency, the E-DNA performance can change from signal-off behaviour to signal-on behaviour. We have tested 1 Hz and 10 Hz using SWV (Figure 5.5 A and B) and both demonstrates ‘signal-on’ behaviour. The signal-on/off switch is clearly shown in Figure 5.6. In Figure 5.6, the signal-on behavior remains at 1 Hz and 5 Hz, but changes to signal-off at 60 Hz.
Figure 5.5 Signal-on behaviour with SWV 1 Hz and 10 Hz

Figure 5.6 Signal-on experiments conducted by Xiaolei Zuo (UCSB): signal-on and signal-off behaviours switching between 1 Hz to 60 Hz
Figure 5.7 presents the signal gain measured using SWV (shown as SQ in figure) at 1 Hz and 10 Hz as a function of target concentration, and further confirms that the signal-on scheme holds for SWV at 1 Hz and 10 Hz as shown in Figure 5.5 and 5.6. Although it is anticipated that a lower analysis frequency results in a relatively higher signal gain during probe-target hybridizations (from the observations of Figure 5.6), due to limited experimental data from using SWV at 1 Hz, the expected relationship was not observed compared to the results using SWV at 10 Hz. In addition, all data from 1 Hz and 10 Hz demonstrate that the signal gain decreases as a function of increasing target DNA concentration. This suggests that the signal-on approach has a lower limit of detection compared to the signal-off approach and is well suited for detecting small target concentrations. This set of experiments was performed 3 times on 3 individual Au working electrodes, and the data used in Figure 5.7 is the average of 9 total measurements across 3 working electrodes. Because deviations are very minimal, they are not shown in the figure.

![Graph showing DNA signal gain as a function of target concentration for different frequencies of SWV (SQ = SWV)](image)

**Figure 5.7 Signal-on experiments with different frequencies of SWV (SQ = SWV)**
5.2 Control On-Chip E-DNA Experiments

All on-chip E-DNA experiments were performed on a complete E-DNA microfluidic chip with a PDMS open chamber, unless stated alternatively. This set of experiments confirmed and validated the feasibility of performing E-DNA on a microfluidic chip.

5.2.1 Signal-Off Experiments

Figure 5.8 presents E-DNA experimental performance on our micro-fabricated E-DNA chip. The data shows that hybridizing 2 μM target DNA with 2 μM probes in this configuration resulted in 72 % signal reduction. 89 % of probes were regenerated by rinsing with D.I. water. A substantial signal decrease and a near-total signal recovery are the two requirements for a successful signal-off experiment. By fulfilling these requirements, this on-chip signal-off experiment is valid.

Compared to Figure 5.5, Figure 5.8 shows a potential shift between each time of E-DNA signal measurement. This shift is contributed by the Pt pseudo-reference electrode. Unlike the off-chip experimental setup using a standard Ag/AgCl reference electrode, Figure 5.8 plot was produced by on-chip Pt pseudo-reference electrode. As described earlier in section 4.1, this pseudo-reference electrode’s potential is strictly sensitive to electrolyte components; even the slightest change of component would result in a potential shift, which is what happened in Figure 5.8.
We have explored the influences of signal acquisition frequency and target concentration on signal-off performance on our microfluidic chips. Signal suppression data produced by hybridizing various artificial target concentrations with 2 µM probe at 50 Hz and 10 Hz using ACV are shown in Figure 5.9. The trend remains the same for both frequencies. The signal-off scheme experiences signal reduction saturation at approximate 78 % for 50 Hz and 63 % for 10 Hz. Thus, the signal-off scheme loses its detection ability corresponding to different target concentrations once the signal reduction saturation is reached. This set of experiments was performed 3 times on 3 individual Au working electrodes, and the data used in Figure 5.9 is the average of 9 total measurements across 3 working electrodes. Because deviations are very minimal, they are not shown in the figure.
5.2.2 Signal-On Experiments

The signal-on scheme was performed on-chip successfully with SWV 1 Hz and 10 Hz (Figure 5.10). The results shown in Figure 5.10 are consistent with the results obtained from off-chip with SWV 1 Hz and 10 Hz shown in Figure 5.5.
5.2.3 Signal-Off Experiments with a PDMS Sealed Chamber

In addition to performing experiments with an PDMS open chamber, we have tested using a PDMS sealed chamber (Figure 5.11). Our data demonstrate that the performance of using an open (Figure 5.8) and a sealed PDMS chamber is comparable. The sealed PDMS chamber has a substantially smaller working volume of 10 µL. Thus, it has the advantage of enabling the exploration of kinetics of the probe-target hybridization. However, the individual kinetics is not considered in the TAME assay, but the
The overall effect of the probe-target hybridization. 30 minutes of equilibration time for probe-target hybridization allows signals to change and stabilize on both the micro-scale (offered by the open chamber) and macro-scale (offered by the sealed chamber). Thus, the overall results from the small sealed PDMS chamber and the open PDMS chamber are comparable in terms of the E-DNA performance.

![Graph showing the relationship between potential and AC current for 2 μM Probe DNA and 2 μM Target Hybridization](image)

**Figure 5.11** On-chip signal-off experiment with a sealed PDMS chamber

### 5.2.4 Probe-Target Hybridization Equilibrium

Before collecting probe-target hybridization signals, 30 minutes of equilibrium time was allowed in every experiment. Figure 5.12 shows that the accumulated signal drop occurs during the first 5 minutes contributes a large portion of the overall signal drop for 25 minutes. Of course, a longer equilibrium time is correlated to a bigger signal drop, such as the data after 5 minutes of equilibrium shown in Figure 5.12; however, the change between each data after 5 minutes are relatively small comparing to the change during the first 5 minutes. Therefore, the signal of probe-target hybridization measured after 25 minutes would very likely include the majority of the signal change, which is expected to occur within the first 5 to 10 minutes. Consequently, we have chosen 30 minutes as a reasonable cut-off time for probe adjustment for hybridization. The cut-off equilibrium time could be a longer value, we decided to
use 30 minutes was based on the convenience level and overall experiment time of the TAME assay. Although this experiment was only performed once, this result has also been described by other work.[45, 46, 49, 69]

![Graph](image)

**Figure 5.12 Peak current responses to the probe-target hybridization along the equilibrium time**

### 5.2.5 Various SAM Preparations

We have investigated three methods of forming the probe-SAM layer using 6-mercapto-1-hexanol. The first method is to immobilize probe DNA for 1 hour followed by SAM for 2 hours. This method was mentioned to be the standard for E-DNA experiments by Xiao *et al.*[46], which is also the method for performing almost all experiments in this project. The second method attempted was immobilization of probe DNA for 1 hour followed by SAM layer formation overnight. The third method was immobilization of probe DNA overnight followed by SAM layer formation for 5 hours. All samples were incubated in E-DNA buffer for later E-DNA detection experiments.

Because the immobilization of probes and the SAM formation on the Au surface is a physical adsorption process, there is a molecular competition when there is more than one type of molecule
binding to the Au surface. Although probe DNA is first immobilized on the surface ahead of the addition of SAM molecules, the 6-carbon chained SAM molecules are still strongly competing with the immobilized DNA probes, and have chances to replace the original probe DNA on the surface if the SAM molecules have long enough immobilization time. That is, in order to get a good quantity of immobilized probe and stable probe signals, it is not desirable to allow SAM molecules to out-compete the DNA probes. In this sense, the first method described above is the best, which was observed and proven experimentally. Immobilizing probes with the SAM layer following such method produces a very proper layer of probe and SAM molecules on the electrode surface so that there are enough SAM molecules to space out probes without replacing the probes and help probe orientations without risking probe quantity. The E-DNA signals generated by the probes formed by such methods were observed as the most stable and most durable signals throughout this project. The shelf life of such probes can last as long as 10 days (shown in Figure 5.13).

It was observed that the second and the third methods resulted in unstable probes and unstable E-DNA signals, which are due to the imbalance amount of DNA probes and SAM molecules on the Au surfaces. The second method may have a strong competition between the SAM molecules and the probes, resulting in loose probes attached to the surfaces, and therefore many probes are replaced by SAM molecules. The third method may have too few SAM molecules as spacers for the probes, leading to a disordered and packed probe layer so that many probes are not alive for signaling. As a consequence, the most uniform probe-SAM layer is preferred for E-DNA experiments, and this is accomplished by DNA probe immobilization for 1 hour followed by the SAM layer for 2 hours.

5.2.6 Probe Stability

The probe-SAM layer was immobilized on the Au working electrodes of the E-DNA chip. DNA probes are scattered among the SAM layer. The stability of this layer therefore determines both the shelf life and
the reusability of the E-DNA chip. We measured E-DNA signal reproducibility as a function of time to estimate the SAM-probe stability. According to Figure 5.13, the probe-SAM layer can last for 11 days. There is a significant probe loss during the first 2 days following rinsing. Then, the amount of probe on the surface becomes very stable up to 10 days. Thus, if an E-DNA experiment was performed with a freshly prepared E-DNA chip, there may be probe loss following the first use. This causes poor probe regeneration so that the signal suppression occurs during the ‘signal-off’ approach is not clear whether it is due to the target-probe hybridization or the probe loss. A more stable probe-SAM layer is established for the subsequent E-DNA experiments after 2 days of incubation and good rinsing up to 10 days. Probes that were kept more than 10 days, such as from 10 to 12 days, suffer from serious degradation. The SAM-probe layer tends to lose the stability and soon degrades completely. Each data point in Figure 5.13 is the average of 3 replicate measurement values. Because deviations are very minimal, they are not shown in the figure.

![Probe-SAM Storage Time, Day vs. Current (ACV 50 Hz), I(A)](image)

**Figure 5.13** Probe-SAM layer stability and shelf-life data
5.2.7 SAM Length Impact on E-DNA Experiments

All probe DNA sequences were immobilized on Au electrodes among a layer of SAM using a standard method. If not stated alternatively, the SAM molecule used for probe immobilization was 6-mercapto-1-hexanol, which has 6-carbon chain from the Au surface. We have also attempted to use 11-mercapto-1-undecanol to form the SAM layer, forming a longer 11-carbon chain from the Au surface.

Compared to the 6-mercapto-1-hexanol, probes among the 11-carbon chain SAM layer require significantly longer probe-electrolyte equilibration time, up to 24 hours. Moreover, the E-DNA signals collected from the 11-carbon SAM layer are substantially smaller than the ones collected from the 6-carbon SAM layer. It is likely that the probes are buried in the 11-carbon SAM layer, causing steric hindrance and leading to the observed increased equilibration time and decreased signals. These impacts of 11-carbon SAM layer on E-DNA performance have been observed by others.[45, 70]

5.3 Performance of Various TRAP Assays

Telomerase activities from LNCaP and RWPE-1 cells were measured by performing various TRAP experiments. We observed that some types of commercial TRAP assays interfere with the E-DNA measurement performance, such as the fluorescent TRAP assay. We also observed the use of starting cell number for telomerase activity quantitation is biased. As a result, we designed and developed the TAME assay and employed dsTRAP product concentration following PCR and ssTRAP product concentration following λ exonuclease digestion as parameters for telomerase activity quantitation.

5.3.1 The Fluorescent TRAP Assay

We performed the fluorescent TRAP detection using a commercially available assay S7707 from Millipore. According to the S7707 manual, there is a clear association between the fluorescence value and the starting number of control cells for telomerase extraction. However, it is not observed in our experiments (Figure 5.14). There is no reproducible association between the two parameters by
repeated experiments. The reasons of lacking this association could be complicated; for example, the loss of telomerase activities during extraction or storage, and the nature of telomerase expression of LNCaP and RWPE-1 cells. Because any step involving telomerase handling could contribute to telomerase activity loss, comparing final telomerase activity quantitation to the initial number of starting cell accounts all uncertainties might occur during the process of telomerase handling. The uncertainties include the loss of telomerase activity physically by human error, inactivation or loss of activity by telomerase itself, and others.

Theoretically, heating the PCR TRAP mix including the telomerase extracts at 85 °C for 15 minutes inactivates telomerase activity. However, Figure 5.1 suggests that simply heating may not be sufficient to deactivate telomerase in practice.

![Figure 5.14](image)

**Figure 5.14** Results with LNCaP and RWPE-1 cell lines from the modified TRAP using the kit S7707 (H represents samples that were heated at 85 °C for 15 minutes, RW represents RWPE-1 healthy prostate epithelial cells, other samples use LNCaP prostate cancer cells.)

### 5.3.2 The Traditional TRAP Assay

The traditional TAP assay was carried out using supplies from a commercial available kit S7700 from Millipore. The traditional TRAP assay was tested using LNCaP and RWPE-1 cell extracts to validate their
telomerase activities, since the traditional TRAP is expected to be the most sensitive assay for telomerase detection. GE is the only mean of telomerase activity detection coupling with the traditional TRAP assay.

In Figure 5.15, TRAP products spaced 6 bp apart are observed starting from 50 bp in LNCaP cell extract samples, which indicates high telomerase activities. The telomerase activities of the heated samples are not as high as the normal samples, because telomerase is sensitive to heat and inactivated by heating. In addition, D.I.water has an unexpected thick by-product band at 50 bp. Many common methods for eliminating PCR contaminations including re-ordering kit components, using filtered pipette tips, and autoclaving equipment, were tested, but the false products remained. Since the appearance of by-products was consistent among three S7700 kits with different lot numbers, it is likely that they were caused by certain element(s) provided by the kit.

Recently, Xiao et al.[71] published results addressing the by-product issue in negative controls present using S7700 supplies. Their investigation showed that by-products in negative controls rise with a threshold cycle value of 25.12 ± 0.12 in real-time PCR. Thus, it is likely that the by-product formation is unavoidable when running the traditional TRAP using the S7700 kit.

Contrary to what is stated in the S7700 kit manual, it is very hard to observe any association between the starting cell number for telomerase extracts and the TRAP production. This problem also occurred when using the S7707 kit.
5.3.3 The Modified TRAP Assay with ssTRAP Product Generation (Part of the TAME Assay)

In addition to GE, other parameters were also employed to evaluate telomerase activity. They were dsTRAP concentration following PCR and ssTRAP concentration following λ exonuclease digestion. As described above in section 2.1.4 and Chapter 3, the use of λ exonuclease requires a modified reverse primer, CXP. Figure 5.16 presents the GE results for the conventional TRAP assay conducted using a phosphorylated CXP primer. 6 bp telomeric repeat fragments are visible for each duplicate experiment with various starting cell numbers. The most concentrated product size is 44 bp and the shortest product size is 38 bp. Compared to the results from the kit S7700 (the traditional TRAP process), these products are smaller. That is, the use of CXP primer may favor shorter products across various template sizes.

However, noticeable differences in bands and band intensities of the duplicate experiments are observed in Figure 5.16, such as data from 3300 cells. These results are consistent with the phenomenon of lacking a correlation between starting cell number and TRAP product concentration discussed earlier. As another example of this phenomenon, Shu et al. has shown gel results after TRAP with various
numbers of starting LNCaP cells, but noticeable differences in band intensity were clearly present in duplicate experiments from 50 and 500 cells.[72] Consequently, using dsTRAP or ssTRAP product concentration replacing the starting cell number for telomerase extraction is very significant, because it eliminates uncertainties from the steps in the protocol before TRAP production.

![Image of gel results]

**Figure 5.16 Gel imagine results of the modified TRAP with CXP and TS primers**

Figure 5.17 presents the independence of TRAP product concentration measured using a fluorescent DNA detection kit (Invitrogen, Q33120) as a function of starting cell number. The data further demonstrates that no significant relationship was observed between TRAP product quantities and the starting cell number either in gel results (Figure 5.15 and 5.16) or TRAP product concentration (Figure 5.17). Figure 5.17 is representative data, and more data are presented in Appendix VIII. Although increases in extract template from 50 to 5000 LNCaP cells resulted in increases in detected telomerase activity, the correlation coefficient is too low to suggest any trend between them. This phenomenon can also be noted in Figure 5.14 when performing the TRAP using a fluorophore-conjugated reverse primer.
It was generally observed that the TRAP amplification produced a narrow product concentration range and product concentration saturation with telomerase extracts from 4000 cells and higher. The former could be due to the nature of telomerase expression of our LNCaP cell culture. The latter could perhaps be explained as the shortage of certain reaction component(s) and PCR inhibition. We have attempted to concentrate TRAP products following PCR using phenol chloroform extraction followed by ethanol precipitation to produce larger TRAP product concentrations for E-DNA detections. Due to the exceptionally small TRAP product sizes; however, the extraction yield was unfortunately too small to detect.

5.3.4 TRAP Product Improvement by BSA and DMSO

Investigation of methods to improve TRAP product quantity using LNCaP telomerase extracts was next performed. 0.8 µg/µL BSA and 5 % DMSO were added to the original TRAP PCR mix prior to PCR amplification. The results (Figure 5.18) show that the addition of BSA appeared to increase the TRAP product quantity. The addition of DMSO does not seem to be helpful for the quantity improvement of TRAP products, but it certainly provides the cleanest TRAP products, showing fewer smears on the gel. This investigation indicates that BSA out-competes DMSO to improve the TRAP PCR quantity. Further
experiments are preferred in order to confirm these findings, such as dsTRAP concentration measurement following the PCR.

![Image of TRAP products](image.png)

**Figure 5.18 TRAP product improvement using additives to the PCR mixture**

5.4 Telomerase Activity On-Chip Measurements

5.4.1 Signal-Off Experiments of TRAP Products with Fluorophore Conjugation

Figure 5.19 presents the E-DNA measurement of TRAP products with fluorophore conjugation. Although it was supposed to be a signal-off measurement, the measurement displayed ‘signal-on’ behavior. One possible explanation is that the fluorophore conjugation interferes with the E-DNA signals. There were also substantial potential shifts between each time of data collection. That is, MB is sensitive to certain element(s) in the buffer, and fluorophore conjugation may be one such element. It was therefore necessary to modify the experimental approach to avoid the use of fluorescent conjugation for quantification of the TRAP product with the E-DNA concept.
Figure 5.19 Signal-off E-DNA measurement of TRAP product with fluorophore conjugation

5.4.2 TAME Signal-Off Measurements

Unlike the fluorophore-conjugated data presented above, E-DNA signals are well correlated to unpurified TRAP products in terms of their concentrations. The signal-off detection of telomerase activity with ACV at 50 Hz was verified against dsTRAP concentration. Probe signals were acquired with a pure PCR mix. Then, the pure mix was replaced by the one containing ssTRAP products for probe-TRAP hybridization. Finally, the E-DNA substrate was rinsed and regenerated with the pure PCR mix. These three steps were performed for each telomerase activity measurement (Figure 5.20 A). A successful E-DNA signal-off measurement requires both a substantial peak current change following probe-TRAP hybridization and a near-total signal recovery after rinsing. A relatively linear relationship holds between dsTRAP concentration and the suppression percentage of peak current from probe-only to probe-TRAP hybridization (Figure 5.20 C). The minimum and maximum dsTRAP concentrations tested were 54.5 and 179 nM respectively. This relatively small range is restricted by the physiological TRAP production from the PCR amplification using LNCaP cell extracts, which is also illustrated in Figure 5.17. However, it is anticipated that the linear association between dsTRAP concentration and the percentage of peak current change is
consistent at higher target DNA concentrations until the approach of the signal reduction saturation, as shown in other published work.[47, 73]

Unlike having considerable peak current decrease with LNCaP telomerase extracts (Figure 5.20 A), undetectable signal change was present with RWPE-1 cell extracts (Figure 5.20 B). It is clear that healthy RWPE-1 prostate cells have little measurable telomerase activity. Moreover, neither D.I. water nor lysis buffer shows any observable signal suppression. Compared to the traditional TRAP assay, the signal-off scheme expresses excellent selectivity, detecting only TRAP products or telomerase activity and little response to negative controls. The limit of detection for the signal-off scheme is 55 nM. Yet, for the reasons discussed above, the signal-off approach will eventually undergo signal saturation, rendering the detection from high telomerase-activity samples impossible.[47, 73]
Figure 5.20 Signal-off on-chip E-DNA measurements of telomerase activity. (A) 64% signal decrease was observed from 3300 LNCaP cells, indicating elevated telomerase activity in cancer cells. (B) Negative control: no noticeable signal drop was observed from 20,000 RWPE-1 (normal prostate) cells. (C) E-DNA signal decrease as a function of dsTRAP production concentration.

5.4.3 TAME Signal-On Measurements

The signal-on detection scheme overcomes the saturation drawback from the signal-off experiments. The signal-on experiments were performed using SWV at 150 Hz. Telomerase detection was performed by diluting un-purified ssTRAP products 10-fold with the E-DNA buffer, which was the working
electrolyte for all measurements. This dilution allowed the investigation of the signal-on scheme for low telomerase activity measurements. The limit of detection for the signal-on measurements was 1.06 nM of ssTRAP or 1.23 nM of dsTRAP, 44-fold improvement compared to the signal-off measurements. The normalized percentage of current increase followed a positive linear correlation with ssTRAP concentration (Figure 5.21 C). On the other hand, only a minimal signal increase was observed from negative controls containing RWPE-1 telomerase extracts (Figure 5.21 B), D.I. water, or dsTRAP. Accordingly, signal-on measurements have an average background of 6.5 % on our E-DNA chip, which is absent when using the signal-off approach. The values used for Figure 5.21 C are presented without noise subtraction.

The signal-on detection should be capable of higher TRAP concentrations than the values tested in Figure 5.21 C. However, due to small TRAP production from LNCaP cell extracts and difficulties in short DNA sequence concentration, higher TRAP concentrations were not tested. We also tested 20-fold and 2.5-fold TRAP production dilution in the E-DNA buffer (data not shown); the most stable signals were achieved by the 10-fold dilution (Figure 5.21 C). Thus, this study mainly explored the limit of detection of the signal-on scheme with the 10-fold dilution. Clearly, the signal-on approach out-competes the signal-off approach in terms of the detection limit.

We observed a closer to complete probe recovery from the signal-on measurements than the signal-off measurements. This phenomenon might be due to the impacts on probes from different working electrolytes. The PCR mix containing Tween 20 [23] used in the signal-off scheme is a much more complex environment than the E-DNA buffer, which is simply a version of phosphate-buffered saline (PBS) solution [46]. Additionally, certain element(s) in the PCR mix might interfere with probe adsorption, thereby causing a small amount of probe loss during rinsing. Finally, in signal-on detections, diluting ssTRAP PCR buffer 10-fold in the E-DNA buffer substantially reduces the concentration of the inhibitory elements(s) so as to secure probe activity and overall probe stability.
Figure 5.21 Signal-on on-chip E-DNA measurements of telomerase activity. (A) 18.7 % signal increase was observed from 20,000 LNCaP (prostate cancer) cells. (B) Negative control showing 6 % signal increase from 20,000 RWPE-1 (normal prostate) cells. (C) E-DNA signal increase as a function of ssTRAP product concentration, measured after lambda exonuclease digestion.

5.4.4 TAME Signal-On/Off Switch

Importantly, our approach allows the alternation between signal-on and signal-off detections simply by altering the frequency from 150 Hz to 20 Hz with SWV, which is due to the unique method of data sampling when using SWV as explained earlier.[48] This transition is clearly seen in Figure 5.22. During
telomerase activity E-DNA measurements, signal gain transitions to signal drop at approximate 50 Hz. The linear relationship between signal drop or signal gain and TRAP concentration is still expected. Additionally, it is also possible to have much more aggressive signal gain with an applied frequency higher than 150 Hz, but the background noise will be amplified as well. Compared to the signal-off detection, the signal-on technique is more selective and expresses an exceptional advantage of detecting and measuring low telomerase activities.

Figure 5.22 Signal-off and signal-on alterations by applying SWV at various frequencies
Chapter 6. Microfluidic PCR

6.1 Motivation

Currently, TAME is only partially harnessing the advantages of convenience and ease of use from being in a microfluidic system. A fully integrated TAME µ-TAS device for prostate cancer detection requires coupling microfluidic PCR with microfluidic E-DNA to realize the maximum benefits of microfluidic analysis. Microfluidic PCR has the advantages of rapid operation, low cost, ease of control, fast thermal-cycling, less primer-dimer formation, good portability and integration with other analytical tools, and high throughput.

Performing PCR on a microfluidic chip has been presented by many researchers,[54, 58, 74] but it is still a very difficult task. Zhang et al. has given a thorough review of PCR microfluidics designs.[54] Due to its micro-chamber volume, many features, such as micro-heaters and micro-temperature sensors, are ideally integrated in this small area for direct heating and temperature sensing. Without a perfect sealing of the PCR chamber, bubble formation and PCR solution evaporation will occur. Micro-valves and micro-pumps are necessary to import and export the PCR solution. Major design criteria for micro-heaters are heating uniformity and fast cycling. Micro-temperature sensors function as feedback controls of heaters to adjust heating rates. Overall, the PCR chip design and fabrication have to be thorough and precise.

6.2 Low Power

One of the major design criteria for PCR chip is low power. To run a PCR chip, a voltage supply provided by a power supply must be in presence; here this voltage is 12 V. The PCR chip we have designed should be able to complete thermal cycling between 37 °C and 95 °C without difficulties with a small voltage supply of 12 V. Since the micro-heaters are designed to have resistance less than 200 ohms, the current
that drives the heating is only in the magnitude of mili-amperes. Therefore, from Ohm’s law, the power required to run a fully functional PCR chip is only a few Watts.

Instead of a few Watts, the conventional bench top thermal cycler requires substantially higher power, in terms of a high voltage supply, such as 220 V, a high heater resistance, and a high current in the magnitude of amperes. In addition, the conventional thermal cycler normally utilizes a peltier cooler, which requires a significantly large amount of power to run. Overall, the power requirement for a conventional thermal cycler is very high. The PCR microfluidic chip particularly has advantages of low power so it is possible to be used at resource-inefficient environment, such as rural areas and emergencies.

6.3 Micro-Heaters: Joule Heating

Micro-heaters are usually fabricated from a thin film of metal, Pt, in this project, and function based on Joule heating, which transfers electric power to thermal power so as to elevate the surface temperature of the thin film.[75] Joule heating is induced by a passage of an electric current through a conductor. Joule heating postulates that the heat production is positively proportional to the product of the resistance and the square of current flow (Equation 1).

\[ Q \propto R \cdot I^2 \]  \hspace{1cm} (Equation 1)

\[ Q \propto V \cdot I \]  \hspace{1cm} (Equation 2)

\[ Q \propto \frac{V^2}{R} \]  \hspace{1cm} (Equation 3)

In above equations, \( Q \) is heat production, \( R \) is wire resistance, \( I \) is current flow, and \( V \) is voltage provided. A higher current or voltage supply is associated to a higher heat production.
The cause of Joule heating is the interactions of moving particles in current flow and atomic ions in the conductive material. Charged electrons that are accelerated by the electric field collide with atomic ions. Some of their kinetic energy is lost to atomic ions as heat causing the temperature rise of the conductive material.

6.4 Micro-Temperature Sensors: Resistance Vs. Temperature

Micro-temperature sensors are essential for the PCR chip as the thermal-cycling needs to be tightly controlled. The employment of a resistance temperature detector (RTD) is one of the methods of controlling temperature, others being use of an infrared camera or encapsulated thermochromic liquid crystals.[76] Except RTDs, the other two methods are both indirect, because RTDs can be programmed directly corresponding to micro-heaters, adjusting heating in a fast and accurate manner.[54]

The concept of how RTD works is based on the dependence between the resistance and the temperature of the RTD materials. The resistance of an RTD increases with a rising temperature.[58]

\[ R = \frac{\rho \cdot L}{A} \]  
(Equation 4)

\[ \rho = \frac{1}{e \cdot n \cdot \mu} \]  
(Equation 5)

\[ R = \frac{L}{A \cdot e \cdot n \cdot \mu} \]  
(Equation 6)

In equation 4, 5, and 6, \( R \) is resistance, \( \rho \) is resistivity, \( L \) is length, \( A \) is the cross-sectional area of the resistor, \( e \) is a constant of electron charge, \( n \) is electron density, and \( \mu \) is electron mobility.

For a particular resistor, such as a Pt thin film, \( A \), \( e \), and \( L \) are all constants. Therefore, Equation 6 can be simplified to Equation 7.

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Due to the characteristics of most metals including Pt, the value of $n \cdot \mu$ decreases with the increasing temperature. Thus, a positively correlated relationship between the rising temperature and the resistance is anticipated. However, it is not linear, as described by Callendar-Van Dusen equation (Equation 8). Practically, for a certain small temperature range, some materials, such as Pt, has a negligible value for the coefficient of B. From our experiments, the value of B for our fabricated Pt film is $-2.8E-6$. Therefore the relationship between the rising temperature and the resistance becomes linear, and the temperature value can be easily extrapolated from the resistance measurements of the thin metal.

$$R = R' (1 + A \cdot T + B \cdot T^2)$$ (Equation 8)

In Equation 8, $R$ is resistance, $R'$ is the resistance at the temperature of absolute zero, $A$ and $B$ are coefficients.

Among the most common RTD materials including platinum, nickel, and copper, platinum was selected. One of Pt advantages is a nearly linear positive coefficient, which means $B$ in the Callendar-Van Dusen equation is almost negligible. The material physical properties can withstand high temperature up to $800 \, ^\circ C$. Its chemical stability is also outstanding even under a high current. Finally, Pt is highly inert to oxidation, which is very important for a robust RTD. When the material is oxidized, its physical properties, especially resistance, change. Unknowingly using an oxidized RTD, the estimations of temperature measurements will be incorrect and invalid, and the RTD finally loses its function. Overall, therefore, Pt is the best candidate for fabricating RTDs.
6.5 Electromigration

Both micro-heaters and micro-temperature sensors must avoid electromigration. Electromigration is the result of “momentum transfer from the electrons to the ions that leads to the electrical failure of interconnects in relative short times, reducing the circuit lifetime to an unacceptable level”.[77] To simplify the above definition, under a high current, ions from the metal material, which are supposed to be stable without moving, actually flow with electrons. If metal ions are flowing from end A to end B, they are going to be accumulated on the downstream side end B, gradually losing the connection to the upstream side end A until the complete disconnection between end A and B occurs.

The micro-heaters and micro-RTDs were designed to resist the occurrence of electromigration. Accordingly, the current density of the metal film must be less than the electromigration limit, J, for a particular metal (Equation 9). The optimum dimensions of micro-heaters and micro-RTDs were obtained from trial and error calculations so as to achieve electromigration prevention.

\[
J < \frac{V}{R \cdot D \cdot W} \quad \text{(Equation 9)}
\]

In Equation 9, \(V\) is voltage supply, \(R\) is metal resistance, \(D\) is metal thickness, and \(W\) is metal width.

6.6 PCR Chip Designs

The volume of the PCR chamber on chip was designed to be 2.5 mm X 2.5 mm. The PCR chip designs were achieved with collaboration with Dr. Jyh-Jian Chen from the National Pingtung University of Science and Technology, Taiwan. 4 glass PCR chip designs are shown below as Figure 6.1, 6.2, 6.3, and 6.4. The one with the best uniform heating was selected as the final PCR chip design.
Figure 6.1 PCR chip design 1: heaters are in parallel and a RTD is around the heating area

Figure 6.2 PCR chip design 2: heaters are in series and one RTD is in the middle of heating area

Figure 6.3 PCR chip design 3: heaters are in series with dimension ratio of 1:2 and one RTD is in the middle of heating area

Figure 6.4 PCR chip design 4: heaters are in series with dimension ratio of 1:3 and one RTD is in the middle of heating area
6.7 PCR Chip Fabrication

A detailed PCR chip fabrication protocol and process are included in Appendix II. The fabrication procedure is the same as the one for the E-DNA chip using Pt as the thin film metal. A 200 µm SiO₂ layer was deposited by a plasma-enhanced chemical vapor deposition process over the PCR chamber to reduce any PCR inhibition and nonspecific binding. Figure 6.5 shows the layout of a PCR chip.

![Figure 6.5 PCR chip layout](image)

A plasma-enhanced chemical vapor deposition system (PECVD), located in the cleanroom, was employed to deposit SiO₂ and Si₃N₄. The working principle (Figure 6.6) is that the reacting gases, such as Di-ethyl silane (DES), NH₃, N₂, and N₂O, in the PECVD chamber are activated and catalyzed by the radio frequency (RF) or direct current (DC) generated by the electrode to form plasma for chemical reactions and then for thin film depositions.[67]
Figure 6.6 Schematic of PECVD [67]

The protocol of SiO$_2$ deposition using PECVD machine was performed under the pressure of 450 mTorr and the temperature of 190 °C. DES and N$_2$O were employed for SiO$_2$ deposition with flow rates of 4 and 71 standard cubic centimeters per minute, respectively. With above conditions, SiO$_2$ was deposited at the rate of 1 µm per second.

6.8 Preliminary PCR Chip Results

6.8.1 PCR Chip Calibration: Resistance Vs. Temperature

As expected, Pt has a nearly linear coefficient when tested from room temperature to 130 °C, as shown in Figure 6.7. The temperature between 20 °C and 130 °C contains the temperatures that are required for performing TAME. All 4 designs show a similar association between the resistance and temperature only with different starting resistance. The data of design 1 and 4 are presented in Figure 6.7.
6.8.2 Heating Uniformity

Both PCR design 3 and 4, heaters are in series with dimension ratios of 1:2 and 1:3, demonstrated the most uniform heating throughout the PCR chamber area. The heating uniformity tests were performed using an infrared camera Vario CAM HiRES IP54 from JENOPTIC Optical System. Inc. 5.2 V and 10 V of voltage supply were subsequently provided to every chip. Figure 6.8 A and B are thermal images of PCR design 4 (heaters are in series with a dimension ratio of 1:3) with the power supply of 5.2 V and 10 V. In both A and B, the temperature variation at points 1, 2, and 3 across (horizontally) the PCR chamber were (53.3, 55.9, 54.5 at 5.2 V and 85.5, 96, 90.5 at 10 V) within 6 °C. In contrast, the temperature profile across the PCR chip design 2 has a temperature variation as big as approximate 37 °C, shown in Figure 6.9.

The cooling rate performed on design 3 was faster than design 4. As the heater resistance of design 4 is bigger than design 3, more heat is sustained in heater design 4, and therefore more heat needs to dissipate while cooling down. Because the emissivity parameter for imaging was 1 and Pt has a
very low emissivity, 0.08, Pt heaters appeared to have lower temperatures than the glass area in thermal images; however, Pt heaters were perfectly functioning.

Figure 6.8 Thermal images of PCR chip design 4: heaters are in series with the dimension ratio of 1:3

Figure 6.9 Thermal image of PCR chip design 2: heaters are in series
Chapter 7. Conclusion

In conclusion, we have successfully demonstrated two microfluidic electrochemical techniques for prostate cancer detection: signal-off and signal-on E-DNA schemes. Both approaches demonstrate outstanding ability to detect and measure telomerase activity even in complicated un-purified samples. The signal-off platform is suitable for measuring moderate telomerase activities. In contrast, the signal-on approach demonstrates greater sensitivity, has high resolution for low telomerase activities and no limitation for high telomerase activity measurement in theory. Most importantly, the TAME method is much more specific than either the PSA test or DRE. Therefore, it should be possible to reject almost all possible false-negative and false-positive results. Overall, TAME is more reliable, easier to perform, and more quantifiable than current methods. These qualifications promise to make TAME a reliable prostate cancer screening method.

The next generation of medical devices require good portability, miniaturization, and sensitivity so as to improve operation efficiency and utilizations during emergency or at bedsides. Additionally, owing to the high correlation between elevated telomerase activity and most cancers, the TAME assay can potentially be useful to detect other cancers with enhanced efficiency. In general, the ability to detect enzyme activity using a low-power, modular electrochemical approach promises to speed diagnostic and basic science pursuits.

As miniaturization is a trend of both analytical tools and medical devices, implementing microfluidic PCR is significantly meaningful. In near future, an integrated all-in-one TAME chip including both PCR and E-DNA is anticipated. To this end, microfluidic PCR heaters and temperature sensors have been designed and tested. Preliminary data shows that Pt has a relatively linear correlation between our interested temperature range and resistance; this is particularly suitable for the functions of RTDs. Furthermore, two of heater designs: heaters are in series with dimension ratios of 1:3 and 1:2 exhibit
outstanding ability of uniform heating; therefore, those two designs will be employed for further experiments, and very likely, the final PCR on-chip demonstrations.
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Appendices

Appendix I. E-DNA Chip Fabrication Protocols and Process

<table>
<thead>
<tr>
<th>Steps</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. HMDS Coating</td>
<td>Spin at 4000 RPM for 45 seconds with acceleration of 400 RPM/s.</td>
</tr>
<tr>
<td>2. AZ 5214 photoresist coating (AZ 5214 is used as positive photoresist)*</td>
<td>Spin at 400 RPM for 6 seconds with acceleration of 300 RPM/s. Then, spin at 4000 RPM for 30 seconds with acceleration of 600 RPM/s.</td>
</tr>
<tr>
<td>3. Softbake</td>
<td>Bake on a hot plate at 95 °C for 90 seconds.</td>
</tr>
<tr>
<td>4. Exposure with a mask</td>
<td>Exposure with UV with an intensity of 6.90 ± 0.03 mW/cm² for 130 seconds.</td>
</tr>
<tr>
<td>5. Develop</td>
<td>Develop features for about 10 seconds using AZ 400 developer. This time length varies from every batch.</td>
</tr>
<tr>
<td>7. Evaporation</td>
<td>Evaporation of Ti/Au (20 nm / 120 nm) or Ti/Pt (20 nm / 120 nm) on the substrate.</td>
</tr>
<tr>
<td>8. Liftoff</td>
<td>Sonicate with acetone for maximum 10 minutes until the features are all shown.</td>
</tr>
</tbody>
</table>

* AZ 5214 can also be used as image reversal photoresist. The protocol is shown below

<table>
<thead>
<tr>
<th>Steps</th>
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<td>1. HMDS Coating</td>
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</tr>
<tr>
<td>2. AZ 5214 photoresist coating (AZ 5214 is used as image reversal photoresist)*</td>
<td>Spin at 400 RPM for 6 seconds with acceleration of 300 RPM/s. Then, spin at 4000 RPM for 30 seconds with acceleration of 600 RPM/s.</td>
</tr>
<tr>
<td>3. Softbake</td>
<td>Bake on a hot plate at 95 °C for 90 seconds.</td>
</tr>
<tr>
<td>4. Exposure with a mask</td>
<td>Exposure with UV with an intensity of 6.90 ± 0.03 mW/cm² for 130 seconds.</td>
</tr>
<tr>
<td>5. Postbake</td>
<td>Bake on a hot plate at 90 °C for 90 seconds. This may vary for different sizes of substrate. This condition is optimized to 4” borofloat glass substrate.</td>
</tr>
<tr>
<td>6. Flood exposure</td>
<td>Expose under UV with an intensity of 6.90 ± 0.03 mW/cm² for 90 seconds without any mask.</td>
</tr>
<tr>
<td>7. Develop</td>
<td>Develop features for about 10 seconds. This time length varies from every batch.</td>
</tr>
<tr>
<td>9. Evaporation</td>
<td>Evaporation of Ti/Au (20 nm / 120 nm) or Ti/Pt (20 nm / 120 nm) on the substrate.</td>
</tr>
<tr>
<td>10. Liftoff</td>
<td>Sonicate with acetone for maximum 10 minutes until the features are all shown.</td>
</tr>
</tbody>
</table>
A borofloat glass substrate

1. Spin AZ positive photoresist

2. UV exposure using a mask for Pt

3. Develop

4. Pt evaporation

5. Lift off \(\rightarrow\) Pt electrodes

6. Pt counter and reference electrodes

7. Spin AZ positive photoresist

8. UV exposure using a mask for Au

9. Develop

10. Au evaporation

11. Lift off \(\rightarrow\) E-DNA Chip
Appendix II. PCR Chip Fabrication Protocols and Process

<table>
<thead>
<tr>
<th>Steps</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
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<td>1. HMDS Coating</td>
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</tr>
<tr>
<td>3. Softbake</td>
<td>Bake on a hot plate at 95 °C for 90 seconds.</td>
</tr>
<tr>
<td>4. Exposure with a mask</td>
<td>Exposure with UV with an intensity of 6.90 ± 0.03 mW/cm² for 130 seconds.</td>
</tr>
<tr>
<td>5. Develop</td>
<td>Develop features for about 10 seconds using AZ 400 developer. This time length varies from every batch.</td>
</tr>
<tr>
<td>7. Evaporation</td>
<td>Evaporation of Ti/Pt (20 nm / 120 nm) on the substrate.</td>
</tr>
<tr>
<td>8. Liftoff</td>
<td>Sonicate with acetone for maximum 10 minutes until the features are all shown.</td>
</tr>
<tr>
<td>9. SiO₂ deposition for 450 nm</td>
<td>Before deposit SiO₂, the electrode leads (to the card-edge connector portion) need to be covered by Kepton tape, which can stand up to 250 °C. Then, the deposition is accomplished by using PECVD machine with following parameters: Pressure: 450 mTorr Temperature: 190 °C Gas: DES and N₂O Flowrates: DES = 4 standard cubic centimeters per minute, N₂O = 71 standard cubic centimeters per minute, Deposition rate: 1 μm per second.</td>
</tr>
</tbody>
</table>
A borofloat glass substrate

1. Spin AZ positive photoresist

2. UV exposure using a mask for Pt

3. Develop

4. Pt evaporation

5. Lift off \(\rightarrow\) Pt features

6. SiO$_2$ Deposition \(\rightarrow\) a thick layer
Appendix III. Protocols for LNCaP Cell Culturing

Main materials and equipment used for sub-culturing and freezing LNCaP cells included the basal medium of RPMI-1640 (Invitrogen, Burlington, ON), Fetal Bovine Serum (Invitrogen, Burlington, ON), Dimethyl sulfoxide (Sigma, Oakville, ON), Trypsin trypLE (Invitrogen, Burlington, ON), cell culture flasks (T-75 flasks, canted neck, vented cap, Sarstedt, Burlington, ON), conical tubes (5mL, 15mL, 50mL, PP, sterile, Sarstedt, Burlington, ON), an automated cell counter (Cedex, Innovative Directions, Pinole, CA, USA), a centrifuge, and an incubator.

The procedures of dealing with LNCaP cells are presented as follows. The handling procedure for frozen cells is from steps 1 to 8. The sub-culturing procedure is from steps 9 to 16. The freezing procedure is from steps 17 to 23.

1. Make the complete growth medium by adding FBS to RPMI-1640 to 10%.

2. Thaw the frozen vial of LNCaP cells in a 37°C water bath.

3. Transfer the content from the vial to a 50 mL conical tube and add 9 mL of the compete culture medium.

4. Centrifuge the 10 mL LNCaP solution for 4 min with 1000 RPM.

5. Remove the supernatant from the LNCaP solution, which includes all dead cells and DMSO.

6. Add 5 mL complete medium to the LNCaP pellet and gently pipette in order to break the cell pellet for a uniform cell scattering.

7. Add 15 mL complete medium into a T-75 flask, and pre-warm it in a 37°C incubator with 5% CO₂ atmosphere.

8. Transfer the uniform cell scattered solution (5 mL) to the pre-warmed flask, and incubate the culture in an incubator.

9. Once the cell confluency reaches 90%, the culture is ready for sub-culture.
10. Remove all old medium solution from the culture flask, and wash the flask with the basal medium in order to obtain only viable cells attached at the flask.

11. Add 5 mL Trypsin solution to the old culture flask and incubate at 37°C for 4 min.

12. Transfer the LNCaP-Trypsin solution into a conical tube, wash the old culture flasks with 5 mL complete medium and transfer the washing solution into the same conical tube.

13. Centrifuge the conical tube at 1000 RPM for 7 min.

14. Remove the supernatant, and add 11 mL complete medium plus gentle pipetting to break the cell pellet.

15. Take 1 mL for cell counting.

16. Transfer the rest 10 mL to 2 pre-warmed T-75 culture flasks, and store the new culture flasks in the incubator.

17. By the time there is enough cells depending on needs, LNCaP cells is ready to be frozen.

18. Make the cryoprotectant medium by adding DMSO to the complete medium to 5%.

19. Repeat the steps 10 to 13.

20. Remove the supernatant, and add 11 mL cryoprotectant medium plus gentle pipetting to break the cell pellet.

21. Take 1 mL for cell counting.

22. Take 1 mL to 10 individual vials.

23. Freeze all 10 vials to -80°C freezer overnight and -150°C freezer for longer time storage.
Appendix IV. Protocols for RWPE-1 Cell Culturing

Main materials and equipment used for sub-culturing and freezing RWPE-1 cells included a basal medium of Keratinocyte Serum Free Medium (Invitrogen, Burlington, ON), Bovine Pituitary Extract (Invitrogen, Burlington, ON), Human Recombinant Epidermal Growth Factor (Invitrogen, Burlington, ON), Ca$^{2+}$/Mg$^{2+}$ free Dulbecco’s phosphate buffered saline (D-PBS, Invitrogen, Burlington, ON), Fetal Bovine Serum (Invitrogen, Burlington, ON), Dimethyl sulfoxide (Sigma, Oakville, ON), Trypsin trypLE (Invitrogen, Burlington, ON), cell culture flasks (T-75 flasks, canted neck, vented cap, Sarstedt, Burlington, ON), conical tubes (5mL, 15mL, 50mL, PP, sterile, Sarstedt, Burlington, ON), an automated cell counter (Cedex, Innovative Directions, Pinole, CA, USA), a centrifuge, and an incubator.

The procedures of dealing with RWPE-1 cells are similar to LNCaP. However RWPE-1 cells are more sensitive to certain elements and require more careful media preparations. The handling procedure for frozen cells is from steps 1 to 8. The sub-culturing procedure is from steps 9 to 18. The freezing procedure is from steps 19 to 25.

1. Make the complete growth medium by adding 0.05 mg/mL BPE and 5 ng/mL EGF in K-SFM.
2. Thaw the frozen vial of RWPE-1 cells in a 37°C water bath.
3. Transfer the content from the vial to a 50mL conical tube and add 9 mL of complete culture medium.
4. Centrifuge the 10 mL RWPE-1 solution for 4 min with 1000 RPM.
5. Remove the supernatant from the RWPE-1 solution, which includes all dead cells and DMSO.
6. Add 5 mL complete medium to the RWPE-1 pellet and gently pipette in order to break the cell pellet for a uniform cell scattering.
7. Add 15 mL complete medium into a T-75 flask, and pre-warm it in a 37°C incubator with 5 % CO$_2$ atmosphere.
8. Transfer the uniform cell scattered solution (5 mL) to the pre-warmed flask, and incubate the culture in an incubator.

9. Once the cell confluency reaches 90 %, the culture is ready for sub-culture.

10. Remove all old medium solution from the culture flask.

11. Wash the flask with Ca\(^{2+}\)/Mg\(^{2+}\) D-PBS in order to obtain only viable cells attached at the flask.

12. Add 5 mL Trypsin solution to the old culture flask and incubate it at 37°C for 8 min.

13. Transfer the RWPE-1/Trypsin solution into a conical tube.

14. Wash the old culture flask with 5 mL washing solution (2 % FBS in Ca\(^{2+}\)/Mg\(^{2+}\) D-PBS) and transfer the washing solution into the same conical tube.

15. Centrifuge the conical tube at 1000 RPM for 7 min.

16. Remove the supernatant, and add 11 mL complete medium plus gentle pipetting to break the cell pellet.

17. Take 1 mL for cell counting.

18. Transfer the rest 10 mL to 2 pre-warmed T-75 culture flasks, and store new culture flasks in an incubator.

19. By the time there are enough cells depending on needs, RWPE-1 cells are ready to be frozen.

20. Make cryoprotectant medium by adding DMSO and FBS to the complete medium to be both 10 %.

21. Repeat steps 10 to 15.

22. Remove the supernatant, and add 11 mL cryoprotectant medium plus gentle pipetting to break the cell pellet.

23. Take 1 mL for cell counting.

24. Take 1 mL to 10 individual vials.

25. Freeze all 10 vials to -80°C freezer overnight and -150°C freezer for longer time storage.
Appendix V. Protocols for Cell Extraction

Cell extraction was performed to obtain telomerase from LNCaP and RWPE-1 cells. However, in addition to telomerase, cell extracts also include DNAs and all other proteins. Since telomerase is RNA reverse transcriptase, it is very sensitive to RNase. That is, endogenous or exogenous RNases will destroy telomerase activity. Thus, all solutions used for dealing with telomerase have to be RNAse free. RNase inhibitor (Invitrogen, Burlington, ON), lysis buffer (S7700, Millipore, Billerica, Massachusetts, USA), phosphate buffered saline (Invitrogen, Burlington, ON), and a centrifuge were materials and equipment required to perform cell extraction.

Cell extraction was performed on both LNCaP and RWPE-1 cells. The procedure has following steps[25]:

1. Make the lysis medium: add RNase inhibitor to the lysis buffer with a concentration of 200 units/mL. The lysis buffer needs to be prepared according to the cell number, which is 200 µL/10⁶ cells.
2. Suspend the frozen cell pellet (was stored in -180°C freezer) with the lysis medium.
3. Store the cell/lysis medium tubes on ice for 30 minutes.
4. Pre-cool down the centrifuge to 4°C.
5. Centrifuge the cell/lysis medium tubes for 20 min with 12000 Xg.
6. Recover as much supernatant as possible to new tubes, the supernatant solution is cell extracts.
Appendix VI. Protocols for Protein Concentration Determination

As mentioned in Appendix III, cell extraction includes proteins. If the protein concentration is too high, it will interfere with the TRAP performance, and may even inhibit the TRAP process. Therefore, protein concentration determination of cell extracts is necessary. Once protein concentration is detected exceeding 750 ng/µL[25], the cell extract needs to be diluted with the lysis medium mentioned in Appendix III.

Materials and equipment used for this step were Coomassie (Sigma, Oakville, ON) Protein Assay kit (Sigma, Oakville, ON), micro-well plates (96 well plate, flat transparent bottom, black polyethylene material, Corning), a Microplate Reader for spectrophotometer (Tecan), and bovine serum albumin (Sigma, Oakville, ON).

Protein concentration determination procedure includes following steps[78]:

1. Make series dilution of BSA as standards: 2000 µg/mL, 1500 µg/mL, 1000 µg/mL, 750 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 25 µg/mL, and 0 µg/mL.
2. Add 5 µL of each standard and LNCaP and RWPE-1 cell extracts to a micro-well plate.
3. Add 250 µL of Kit reagent solution into each well and mix them by gentle pipetting.
4. Incubate the plate for 15 minutes at room temperature.
5. Use the Microplate Reader to measure absorbance at 595 nm.
6. Plot the standard curve, and calculate the protein concentration of LNCaP and RWPE-1 cell extracts according to the standard.
Appendix VII. Protocols for DNA Concentration Determination

Quant-iT dsDNA Assay Kit with High Sensitivity (Invitrogen, Q33120, Burlington, ON) was employed for dsTRAP product concentration determination. Materials and equipment required for performing TRAP product concentration determination were reagents, buffers, and standards are provided by the kit (Invitrogen Q33120), a micro-well plate (96 well plate, flat transparent bottom, black polyethylene material, Corning), and a Microplate Reader for Spectrophotometer (Tecan).

The procedure of concentration determination included[79]:

1. Equilibrate the assay components to room temperature.
2. Make the working solution by diluting the HS reagent 1:200 in the HS buffer.
3. Load 200 µL of working solution in each micro-well plate well.
4. Add 5 µL of each HS Standards to individual well and mix well.
5. Add 5 µL of TRAP product to individual well and mix well.
6. Incubate the mixture solution at room temperature for 10 minutes.
7. Measure fluorescence using Microplate Reader (Tecan) at Excitation/Emission = 485nm/530nm.
8. Use the standard curve to determine TRAP product concentration.
### Appendix VIII. Starting Number of Cell Vs. TRAP Product Concentration

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<td>TRAP Product Concentration, (ng/μL)</td>
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