LABEL FREE MICRO-RNA BIOMARKER DETECTION IN SERUM SAMPLES FOR POTENTIAL DIAGNOSIS APPLICATION AT POINT-OF-CARE SETTINGS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Dr. Dharmakeerthi Nawarathna
Chair

Dr. Ivan T Lima Jr.

Dr. Sandeep Singhal

Approved:

07/28/2020
Date

Dr. Annie Tangpong
Department Chair

ABSTRACT

The number of new cancer cases is projected to rise to 23.6 million by 2030 according to the National Cancer Institute. Obesity & cardiovascular diseases are among the leading causes of death worldwide according to recent reports. Biomarkers— any molecules found within a human body that can be used to monitor an individual's health — have been shown to play a significant role in the detection of cancer, obesity, and cardiovascular diseases.

Recent studies have shown that in the diagnosis and screening of various human diseases, including cancer, obesity and cardiovascular diseases, circulating microRNAs (miRNAs) are important biomarkers. A crucial roadblock to using microRNA in screening applications is the lack of effective and low-cost microRNA detection. To address this issue, in this study, we have developed a viable method that combines the dielectrophoresis and electrical impedance. Results show this approach can measure very small concentrations of label-free microRNAs (1pM).

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CHAPTER 1. INTRODUCTION

1.1. Background Information

The most life-threatening disease hampering our world today is cancer [1]. The number of new cancer cases per year are expected to rise to 23.6 million by 2030 (National Cancer Institute, https://www.cancer.gov/about-cancer/understanding/statistics). The lung (16.7 percent of the total), prostate (15.0 percent), colorectum (10.0 percent), stomach (8.5 percent), and liver (7.5 percent) were the five most common cancer sites diagnosed in 2012 among men. Among women, the breast (25.2% of the total), colorectum (9.2%), lung (8.7%), cervix (7.9%), and stomach (4.8%) were the five most common incident sites for cancer [1]. Among women, there was a significantly greater prevalence of breast cancer (43.3 per 100 000) than any other cancer; the next highest incidence was colorectal cancer (14.3 per 100 000) [1]. Cancer mortality can be reduced if cases are detected and treated early (World Health Org, https://www.who.int/cancer/detection /en/). When the cancer is identified early, it becomes more likely to respond to effective treatment which results in greater probability of surviving. Various studies have showed that biomarkers play a significant role in diagnosis of cancer. The role of biomarkers is becoming increasingly significant for the effective diagnosis of cancer patients, because, more than 90% of the drugs which are supposed to treat cancer, are not even going to reach market due to the failure in clinical trials and due to not being able to demonstrate the benefits of the drug, which makes the process slower and the development of the cancer increases [2].

Obesity is a common condition in which a person carries an excessive, unhealthy amount of adipose tissue, more frequently called fat. Obesity is measured using Body Mass Index (BMI) obtained by dividing weight of a person in kilograms (kg) by the square of their height in meters (kg/m^2) (World Health Org, who.int/topics/obesity/en). Obesity has nearly tripled around the

world since 1975. About 1.9 billion people, aged 18 and older, were overweight in 2016. About 650 million of these were obese. In 2016 39 % of adults aged 18 years and older were overweight and 13% were obese. In addition, obesity is believed to raise the risk of developing a variety of diseases and disorders including cardiovascular disease and type 2 diabetes, sleep apnea, cancers, and depression [2, 3]. Biomarkers have been used to predict, & diagnose obesity-associated diseases such as type 2 diabetes. [4, 5].

Heart diseases are among the world's leading causes of mortality. Tobacco use continues to be the leading cause of preventable cardiovascular death in the US and internationally estimated to account for 7.2 million worldwide deaths in 2015 (World Health Org, https://www.who.int/healthinfo/global_burden_disease/GlobalHealthRisks_report_full.pdf). A recent study using a comparative risk assessment model reported that 45.4% of U.S. deaths from heart disease, stroke, and type 2 diabetes mellitus (DM) were due to poor dietary habits [6]. Physical inactivity, high blood pressure, & kidney diseases account for more cardiovascular disease deaths [6, 7]. By 2035, some form of cardiovascular disease is expected to occur in more than 130 million adults (45.1 per cent) in the US population [7]. Sudden cardiac death appears on 13.5 percent of death certificates (366 807 of 2 712 630) among the multiple causes of death, suggesting that one in every 7.4 people in the United States will die of sudden cardiac death [7]. Since some people survive sudden cardiac arrest, the risk of cardiac arrest is much greater for life [7]. In case of cardiovascular diseases, a biomarker may be able to determine the probability of a therapeutic response, degree of myocardial damage, seriousness of underlying cardiovascular disease, degree of left ventricular, the risk of potential recurrence, and the progression to failure of the heart [8].

Biomarkers are simply defined as biological markers, any molecules found inside a human body, which can be used to monitor the health of an individual [9]. A joint venture on chemical safety, the World Health Organization (WHO)-led International Program on Chemical Safety, and in cooperation with the United Nations and the International Labor Organization, has identified a biomarker as “any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease” [10].

1.2. Micro RNA

Micro RNA's (miRNAs) are small non-coding RNAs, 17-25 nucleotide long. Most miRNAs are transcribed into primary miRNAs (pri-miRNAs) from DNA sequences, and transformed into precursor miRNAs (pre-miRNAs) and mature miRNAs [11]. MiRNAs are essential to the normal development of animals and are involved in various biological processes [12]. miRNAs have emerged as major non-coding RNAs that regulate processes of the immune system, differentiation, tumorigenesis, cell death and neurodegeneration [13-22]. Many studies have found miRNAs in biological fluids, such as plasma and blood, saliva, breast milk and much more [13]. They were first discovered by Lee et al. in 1993 in *Caenorhabditis elegans* [14]. Circulating miRNAs were first used as serum biomarkers to examine patients with diffuse, large B-cell lymphoma [15]. miRNA dysregulation is involved in carcinogenesis and tumor progression. Several studies have since identified miRNAs as a possible biomarker in various human diseases [11-31]. Despite being unstable RNA molecules, miRNAs are in fact highly stable and detectable in serum and plasma [20].

In different biological processes, miRNAs are assigned to different families based on the function and location of miRNAs on the chromosomes. For example, the family *miR-34* controls cancer and apoptosis. It has been shown that the *miR-200* family inhibits the first stage of

metastasis, epithelial mesenchymal transformation (EMT), and the *let-7* family of microRNAs regulates cell death in human cancer cells [21]. *miR-21* has emerged as a major microRNA over-expressed in a variety of cancers including glioma, breast cancer, colorectal cancer, stomach / gastric cancer, hepatocellular cancer, pancreatic cancer, lung cancer, leukemia and prostate cancer. Studies using *miR-21* suggest that it post-transcriptionally downregulates tumor suppressor programmed cell death of target PDCD4 and tropomyosin 1 (TPM1) genes and stimulates invasion and metastasis in colorectal and breast cancers [21].

MiRNAs in malignant tissues are up- or down-regulated compared to normal tissue, and can be known as oncogenes or tumor suppressors, respectively. Since miRNAs have a very restricted tissue-specific expression, it is important to note that the apparent miRNA modulation in cancer tissues may be the manifestation of a different cell population in the tumor compared to the normal tissue [19-24]. A seminal study by Croce and colleagues showed that *miR-15a/16-1* cluster in chronic lymphocytic leukemia (CLL) is frequently deleted, involving these miRNAs as tumor suppressors [22]. From another study, *miR-155* or *miR-21* transgenic expression and *miR-15a/16-1* deletion is sufficient to initiate lymphomagenesis in mice. In contrast, systemic delivery of selected miRNAs including *let-7, miR-26a, miR-34a,* and *miR-143/145* inhibits by vivo tumor progression [23].

Recent studies have found micro-RNA to be a promising biomarker for cancer. Some studies have shown clearly that circulating miRNAs may derive from cancer tissues [21-28]. Numerous studies have shown that microRNAs are a promising biomarker in multiple types of cancer, including breast cancer; in one of the studies from Cortez et al., serum miRNA levels were highly correlated with breast tumor tissue types [25]. *miR*-21, *miR*-106*a*, and *miR*-155 were

significantly overexpressed in tumor specimens compared with in normal controls, whereas *miR*-126, *miR*-199*a*, and *miR*-335 were significantly under expressed [25].

Abue et al., conducted a study to analyze *miRNA-483-3p* and *miRNA-21* as biomarkers of blood plasma pancreatic ductal adenocarcinoma. The plasma samples were collected from 32 patients with pancreatic ductal adenocarcinoma [27], 12 patients with intraductal papillary mucinous neoplasm, and 30 individuals with healthy control [27]. The levels of these miRNA were assessed using real-time qRT-PCR, compared between groups, and clinically linked to each expression. In pancreatic ductal adenocarcinoma the plasma expression of both *miRNA-483-3p* and *miRNA-21* was found to be significantly higher than in healthy controls [27].

Review by Zhang et al., demonstrates few studies where the elevated levels of circulating *miR-122* was associated with patients with Hepatocellular carcinoma when compared to healthy individuals [28].

Recently, Kristensen M. and the group, assessed the miRNA expression in subcutaneous adipose tissue in 19 individuals with extreme obesity which included 10 women and 9 men before and after a weight loss intervention of 15 weeks. This action resulted in the up-regulation (*miR-29a-3p, miR-29a-5p*), and down-regulation (*miR-20b-5p*) of specific microRNA. And they concluded that weight loss influenced many miRNAs [29].

A review by Oses et al., identified four miRNAs overexpressed in obesity (*miR-222*, miR-142-3, *miR-140-5p*, and *miR-143*) and two miRNAs (*miR-122* and *miR-34a*) overexpressed in children with obesity and nonalcoholic fatty liver disease (NAFLD). They concluded that circulating miRNAs are promising biomarkers for the detection of obesity-associated diseases such as NAFLD and type 2 diabetes [4].

A recent study by Economou et al., found that micro-RNA has emerged as useful biomarkers in atherosclerosis and Coronary Artery Disease diagnosis, circulating *miR-133a, miR-208a* levels are up-regulated while *miR-126, miR-127, miR-92a, miR-155* levels are downregulated in patients with coronary artery disease compared to healthy subjects [30]. Another research done by Li et al., Concluded that plasma miRNA (*miR-22-5p, miR-150-3p*) could serve as candidate biomarkers for early diagnosis of Acute Myocardial Infraction [31].

1.3. Micro RNA Detection

Since the early miRNA study, northern blotting had been the normal and gold standard method [32]. Northern blotting can detect the relative molecular size of the miRNAs in total sample extract. But it also has many drawbacks, including semi-quantitative, poor throughput, slow, and times-consuming [33, 34].

Also, the gold standard when it comes to miRNA detection is the real-time quantitative reverse transcriptase polymerase chain reaction (real-time qRT-PCR) because of its wide dynamic range, and high sensitivity, it has become a popular technique for miRNA detection. However, real-time qRT-PCR is expensive, complex and has high demands on time and resources [33, 34].

The microarray is the method most commonly used to detect miRNAs quickly. This technique contributes to high throughput, and can simultaneously detect several miRNAs. The cost of microarray detection, however, is very high and also the sensitivity of analyzing miRNAs is not that good [33, 34].

Table 1.1: Comparison of different properties of most popular detection techniques used.

	Northern Blotting	Real-time qRT-PCR	Microarray
Sensitivity	LOW (>60%)	HIGH (>90%)	HIGH (>80%)
Limit of detection	LOW (~nM)	HIGH (~pM)	HIGH (~pM)
Cost	CHEAP (>$100)	HIGH (>$2000)	HIGH (>$1000)
Times consumption	HIGH (>24 hours)	HIGH (>7 hours)	HIGH (>2 hours)
Method	SIMPLE	COMPLEX	COMPLEX

The detection of miRNA is important for their use as a potential biomarker. In the past, studies showed that Northern Blotting was an effective method to detect miRNAs. However, because of poor sensitivity, high times requirements, and complexity, Northern Blotting may not be optimal for miRNA detection. Real-time Quantitative Reverse Transcriptase Polymerase Chain Reaction (real-time qRT-PCR) is another method used extensively for detection of miRNA, but due to its higher time and resource demand and the cost related with real-time qRT-PCR, it isn't a feasible method for the detection of miRNA. Similarly, microarray, another highly used method for the detection of miRNA, can analyze thousands of samples in a day; however, due to lower sensitivity, higher cost, and complexity, it is not the best method for detecting miRNA.

For target-sample hybridization, all the current-detection method relies on times-dependent and unstable molecular diffusion which causes variation in the results [35, 36]. These issues affect both detection speed and detection limit [35]. Another issue is hemolysis—red blood cell lysis during the long pre-processing times. To minimize the hemolysis, the sample needs to be analyzed within 30 minutes after collection, but current pre-processing times is more than 4 h. One way to address this issue is to introduce a rapid miRNA analysis, ideally, at point-of-care settings [37, 38]. Therefore, in our study to minimize all the issues in current detection method (Times consumption, cost, complexity), we have purposed a novel technique for detection of miRNA at point-of-care settings, using Dielectrophoresis and Impedance measurement.

1.4. Dielectrophoresis

The term Dielectrophoresis (DEP) was first employed by Dr. Herbert A. Pohl, a scientist at the Anacosta Naval Research Laboratory (DC, USA). He defined DEP as a phenomenon seen in the relative motion of suspensions and media resulting from the polarizing forces produced by an inhomogeneous electric field [39].

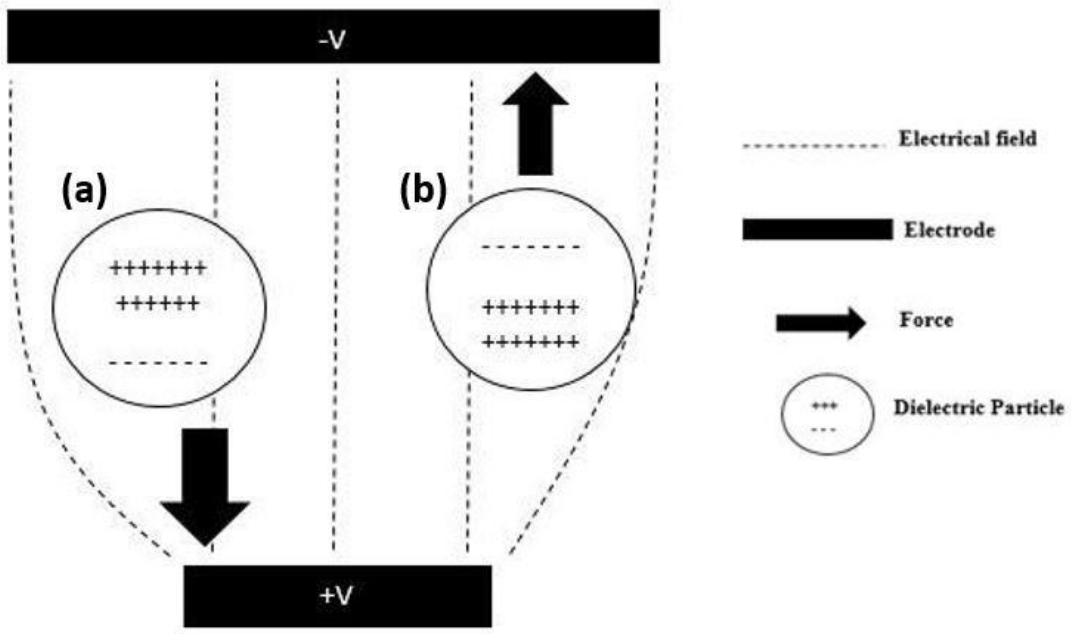

Figure 1.1: DEP particle separation which shows **(a)** p-DEP, the dielectric particle moves toward high gradient electric field; and **(b)** n-DEP, dielectric particle moves toward low electric field gradient.

DEP is the motion of a dielectric particle under the influence of a non-uniform electric field. Particulate motion in DEP is based on the polarizability difference between the particles and the surrounding medium. If the particles move towards the edge of the electrode, the area of high gradient of the electric field, then response is called positive DEP (p-DEP), as seen in the Figure 1.1 (a), while if the particles move away from the edge of the electrode, the response is called negative DEP (n-DEP), as seen in the Figure 1.1 (b) [39-41].

DEP force is generated when a non-uniform AC electric field is applied which manipulates particle motion by creating a gradient of polarizability between the particles and the suspended medium [39].

DEP force applied to the spherical particle of a radius r can be derived as:

$$F_{DEP} = 2\pi r^3 \varepsilon_m Re[K(\omega)]\nabla|E|^2$$

Where ω is the angular frequency of the applied field, , ε_m is the permittivity of the suspending medium, ∇ is the vector operator, E is the r.m.s value of the electric field, and Re[K(ω)] is the real part of Clausius-Mossotti factor (CM) [39-44]. According to this formula, DEP force is regulated by frequency-dependent CM factor. CM factor (K(ω))is expressed as $\frac{\varepsilon_p^* - \varepsilon_m^*}{\varepsilon_p^* + 2\varepsilon_m^*}$ where ε^*_p is the complex permittivity of particles and ε^*_m is the complex permittivity of medium [40, 41].

Previous studies performed using the Dielectrophoresis technique suggest that it is promising method for cell separation and concentration [41-44].

The dielectrophoretic response of prostate tumor initiating cells (TICs) in a microfluidic system using contactless dielectrophoresis (cDEP) was investigated in a study by salmanzadeh et al., It has been observed that the dielectrophoretic response of prostate TICs is markedly different from that for non-TICs, allowing them to be sorted using cDEP. This approach distinguishes the cells according to the difference between the membrane surface proteins and their electrical charge. The experiments have shown that at a particular frequency and voltage, PC-3, a Tumor Initiating Cell, displayed greater DEP force than other cells [41].

In another study, Lee et al, proposed a platform that integrates negative DEP (nDEP) force and gravitational forces to sort K-562 cells that are leukemia cells. Repeated nDEP barrier configuration has been shown to be an effective method of enhancing separation efficiency, achieving maximum separation efficiency and high throughput by the device they purposed [42].

Study by Cheng et al., present a high-purity and high-throughput DEP isolation of circulating tumor cells (CTCs) from the blood in a microfluidic chip with an antibody free approach. A 3D lateral dielectrophoretic (LDEP) configuration was used to isolate the lung cancer cells, AS2-GFP, from blood. The induced forces of LDEP on the blood cells and CTCs create

different velocities of LDEP that are common to the flow and balance the fluid viscosity at various equilibrium positions, resulting in CTC and blood cell separation [43].

Study by Velmanickam et al., purposes a new technique for *let-7b* miRNA detection using Dielectrophoresis and fluorescence imaging technique. This study proves that, let-7b miRNA were successfully concentrated in the hotspots of the T-Electrode, also manufactured by the group. The miRNA could then be successfully detected by fluorescence imaging [44].

1.5. Electrical Impedance

Electrical impedance is the amount of resistance a circuit experiences to a change in current or voltage. Where the voltage is V, Z is the electrical impedance, and the current is I.

$$Z = \frac{V}{I}$$

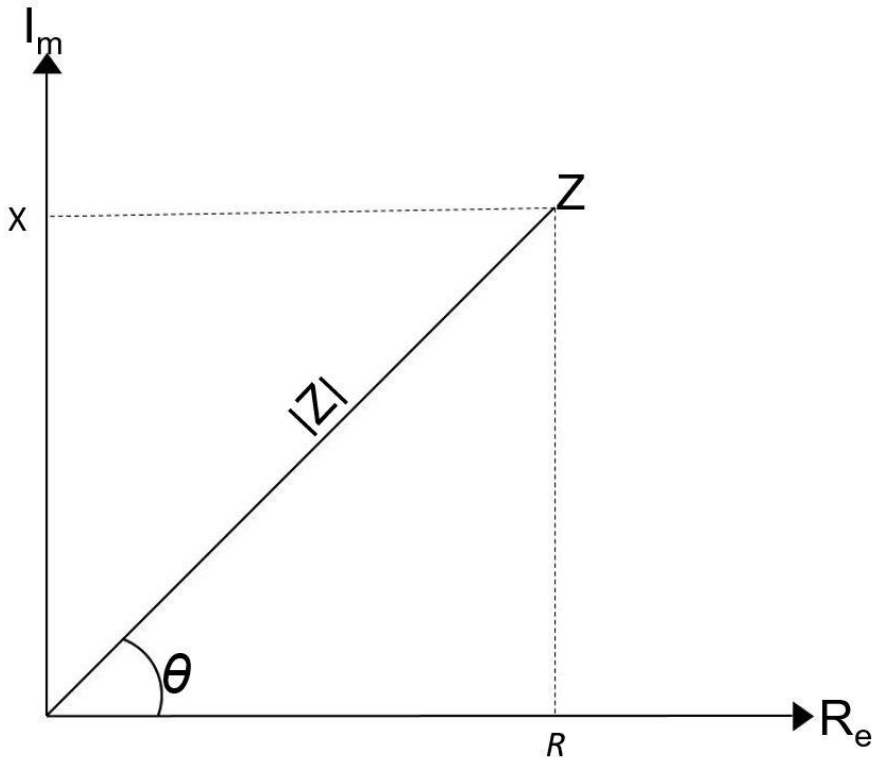

Figure 1.2: Graphical representation of complex impedance

A circuit impedance is the total effective resistance to current flow through a combination of the circuit elements (resistors, condensers, and inductors). There are two important characteristics of impedance they are, Impedance is a frequency function; and Impedance has to be represented as a complex number that is both real (R_e) and imaginary (I_m) parts need to be represented. Instead, to simplify issues, we state impedance as magnitude $|Z|$ (the total impedance) and angle of the phase θ [45].

From Figure 1.3, we can see that V_R, V_L, and V_C are the voltage across resistor, capacitor, and inductor. The voltages across all the three elements cannot be added, because V_L and V_C are considered to be imaginary parts; Here, R is the real part of the impedance, resistance and X is the imaginary part, reactance.

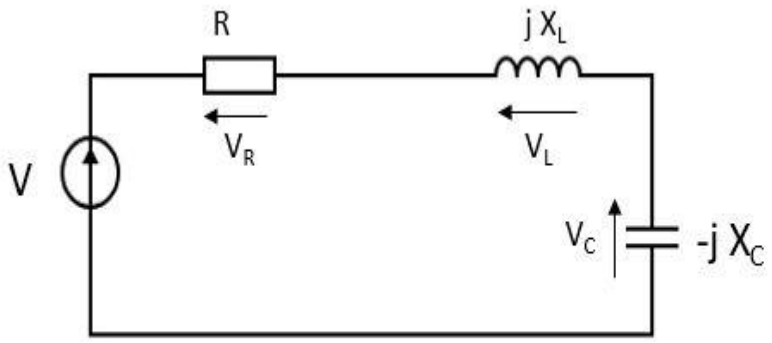

Figure 1.3: Circuit diagram showing how the components are connected

$$Z = R + j(X_L - X_C)$$

The graphical representation of complex impedance can be seen in Figure 1.2. Which constitutes of the real part of the impedance, the imaginary part, magnitude of the impedance, and the phase angle.

Now, the magnitude of Z is given as;

$$|Z| = \sqrt{R^2 + (X_L - X_C)^2}$$

The Phase angle (θ);

$$\tan\theta = \frac{X_L - X_C}{R}$$

Studies suggests that Electrical Impedance can be used for the detection of biomarker, and other microorganisms [46-49].

A research conducted by Lillehoj et al., proposed a microfluidic biosensor for rapid and multiplexed bacterial pathogens detection. Based on these experiments, antimicrobial peptide (AMP)-coated sensors displayed powerful preferential binding with negligible cross-binding to their corresponding targeted cells. For cell detection, an electrical impedance sensing scheme was used, in which S. Mutans and P. aeruginosa cells were distinguished at various concentration based on impedance measurements with lower detection limits and high throughput [46].

A research conducted by Stojadinovic et al., to determine feasibility and patient satisfaction with the electrical impedance screening (EIS) for early detection of breast cancer in young people resulted in, twenty-nine cancers identified among 1,103 women. They concluded that EIS seems promising to detect breast cancer early and to identify young women at elevated risk of developing the disease at screening [47].

In Liju Yang's study a modern, simple and rapid impedance method to detect bacterial cells by using interdigitated microelectrodes to exploit the impedance properties of bacterial cell suspensions. This study produced a Limit of Detection that is comparable with many label-free immunosensors for pathogenic bacteria detection [48].

In Another analysis Jayasooriya et al., developed a micro-interdigitated electrode (IDE) to assess the label-free biomarker's electrical impedance. The detailed impedance data analysis carried out by the team can be used to identify the target biomolecules' physical process which is responsible for observed changes in the measured impedance. For this study they used biotin-

avidin system and one of the results they found was that as the solution's molarity increased, the impedance value decreased [49].

1.6. Data Analysis

Curve fitting is one of the most powerful and commonly used analytical methods. Curve fitting looks at the relationship between one or more predictors which are independent variables, and a response variable which are dependent variable, with the goal of determining the "best fit" model for the relationship.

The Levenberg- Marquardt method, was first introduced in 1944 by Kenneth Levenberg to provide solutions to problems called non-linear least square minimization [50]. It was a combination of the features of the steepest descent and the Newton's method. Donald Marquardt improved it in 1963 and incorporated in the update function the estimated local curvature information. This method of curve fitting is a combination of two other methods: gradient descent (Steepest descent) and the Gauss-Newton method. To find a solution, both the Gradient Descent methods and Gauss-Newton methods use a set of calculations (based on guessing x-value). The Gauss-Newton is a modification of Newton's system, which finds in the calculus x-intercepts [50].

Gradient descent starts with an initial guessing xo; and f(x) decreases after every iteration

$$x_{n+1} = x_n - \lambda \nabla f(x_n)$$

Here, ∇ represents gradient matrix, λ is the damping parameter. And $n \geq 0$.

Gauss-Newton also starts with an initial guessing x0; It uses first-order Taylor series approximation;

$$x_{n+1} = x_n \left(\nabla^2 f(x_n)\right)^{-1} \nabla f(x_n)$$

Then,

$$\nabla^2 f(x) = J(x)^T J(x)$$

Now,

$$\nabla f(x) = J(x)^T r(x)$$

Here, J is the Jacobian matrix. R is the residual vector, T is transportation;

$$x_{n+1} = x_n - (J(x)^T J(x))^{-1} J(x)^T r(x)$$

From this, The Levenberg-Marquardt algorithm is;

$$x_{n+1} = x_n - (H + \lambda I)^{-1} J(x)^T r(x)$$

Here, H is the Hessian matrix, I is the identity matrix.

In the above equation, small values of the damping parameter λ result in a Gauss-Newton update and large values of λ result in a gradient descent update.

1.7. Objective

This study was carried out to investigate

1. The main purpose of this research was to study let-7b miRNA and detection of Label-free let-7b miRNA in human serum sample.
2. To evaluate if the developed model could fit the curves better for the analysis of data.

1.8. Research Methodology Layout

This study was organized into five chapters.

1. Chapter 1: Introduction
2. Chapter 2: Literature Review
3. Chapter 3: Materials and Methodology
4. Chapter4: Results and Discussion
5. Chapter 5: Summary, and Conclusion

Chapter two, literature review, reviews studies that have enhanced the understanding on miRNA detection and history of detection methods. Chapter three, materials and methodology,

gives the details of the materials and equipment used in the study. It also explains the procedure adopted for the research and experiments in detail. Chapter four, results and discussion, presents the results obtained from the research and discussion on the obtained results. Chapter Five presents the summary and conclusion of this research.

CHAPTER 2. LITERATURE REVIEW

2.1. Introduction

This chapter presents a summary of past research on miRNAs, miRNA's role in various diseases, and miRNA detection. MiRNAs have been used as biomarkers for the diagnosis and detection of various diseases. This chapter will concentrate primarily on miRNA-based literature as a biomarker, and miRNA detection.

2.2. Background

MiRNA was first discovered in 1993 [14] and is an effective biomarker in the detection of various human diseases [13-20]. MiRNAs are vital to normal animal development and participate in various biological processes. Among others, they are present in biological fluids in human bodies such as blood, plasma, serum, urine, semen, breast milk [51, 52].

Cortez et al., study concluded that effective treatment for breast cancer depends on early detection of disease. Since aberrant miRNA expression is an early event in tumorigenesis, circulating miRNAs in breast cancer may represent non-invasive biomarkers [25]. Another study conducted by Daoud et al., found that distinct miRNA expression profiles can correlate with stages of malignant pancreatic disease and have potential as biomarkers [26]. A review by Zhang et al., summarizes the potential applications of circulating miRNAs as minimally invasive biomarkers for diagnosis and prognosis of Hepato-cellular carcinoma [28]. Other studies also indicate that miRNA is a possible biomarker for diseases related to obesity [4, 5] and cardiovascular diseases [30, 31].

2.3. Detection of miRNA Using Northern Blotting

2.3.1. Introduction

Northern blotting is a laboratory method for detecting different RNA molecules within an RNA mixture. Northern blotting can be used to analyze a sample of RNA from a given tissue or cell type to test the RNA expression of different genes. Northern blotting can detect the relative molecular size and relative abundance of the miRNAs. The basic concept of Northern Blotting is; digested RNA sample by endonuclease restriction is separated by agarose gel electrophoresis, which is then denatured and transferred to a nitrocellulose film or nylon membrane depending on its location in the gel, followed by reaction with the isotope or other marker-labeled samples. After washing the sample, miRNAs can be detected by autoradiography or other suitable techniques [33, 34].

2.3.2. Review of Studies That Used Northern Blotting

Small RNAs perform various functions within cells including regulating the expression of genes. In *Caenorhabditis elegans*, one class of regulatory RNA includes the small temporal geneslin-4 and let-7 RNA species. The lin-4 and let-7 RNAs are around 22 nucleotides (nt) in length, and are expressed stage-specifically by acting as antisense translational repressors, regulating important developmental transitions in worm larvae. Research carried out by Lee., (2001) to identify smaller lin-4 / let-7 class regulatory RNAs in *C. elegans*, used computer technology and cDNA cloning to select *C. elegans* genomic sequences which exhibited four lin-4 and let-7 characteristics namely expression of a mature RNA of 22 nucleotide in length; location in intergenic (non–protein-coding) sequences; high DNA sequence similarity between *C. elegans* and a related species, *Caenorhabditis briggsae*; and processing of the 22 nucleotide mature RNA from a stem-loop precursor transcript of about 65 nucleotide. Forty sequences in size

and structure similar to lin-4and let-7 were predicted by RNA folding program to form a stem-loop. Tests were carried out against Northern blots of total worm RNA complementing these sequences, and three of them detected small RNA transcripts [53].

In the Study performed by Calin et al., miRNAs are transcribed as short hairpin precursors (≈70nt) and processed by Dicer into active 21- to 22-nt RNAs, a ribonuclease that recognizes target mRNAs via base pairing interactions. The study says that miR15 and miR16 are located at chromosome 13q14, a region deleted from more than half of B cell chronic lymphocytic leukemia (B-CLL). Clear study of deletion and expression reveals that miR15 and miR16 are located within a loss area of 30 kb in CLL, and that both genes are deleted or down-regulated in the majority of CLL cases (68 percent). In order to determine the level of expression of miR15 and miR16 in normal tissues, Northern blot analysis was performed on a panel of normal tissues, including CD5+ B cells isolated from tonsils of normal individuals. Since B-CLL is characterized by progressive accumulation of CD5 + B lymphocytes, they used these cells as controls, and found widespread expression of both genes with the highest level in normal CD5 + lymphocytes, indicating that these genes play an important role in normal CD5 + B cell homeostasis. [22]

2.4. Detection of miRNA Using Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction

2.4.1. Introduction

Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (Real-time qRT-PCR) is a process by which the sum of the PCR product can be calculated in real time and is very useful for research in to the gene expression. It uses an increase in the intensity of the fluorescent signal produced by the dye or by the breakdown of the dye-labeled probe when the target sequence is amplified to detect nucleic acid either for their presence or absence, or for their

quantity. There are two real-time qRT-PCR detection methods, one based on sequence-specific probes such as the TaqMan probe; the other based on common non-sequence-specific double-stranded DNA-binding dye such as SYBR green. Real-time qRT-PCR is a very sensitive and effective analytical tool.

2.4.2. Review of Studies That Used Real-time qRT-PCR

Mohammadi-Yeganeh et al., developed a novel, cost-effective two-step RT-PCR (TaqMan real-time PCR) assay to detect miRNA expression. Two breast cancer cell lines, MCF-7 (tumorigenic, non-metastatic) and MDA-MB-231 (tumorigenic, metastatic) were used to detect miRNA expression. Micro RNA was extracted from the cancer cell lines; the samples were then reverse transcribed using expand reverse transcriptase for cDNA synthesis. Then, Real-time PCR was performed. In MCF-7, which is a non-metastatic cell line, 2 out of 20 miRNAs including miR-592 and miR-597 showed over expression. In MDA-MB-231, which is a more invasive breast cancer cell line, miR-31 was upregulated [54].

Lee et al., reported on a signature of microRNA expression associated with pancreatic cancer which has been identified by applying real-time PCR profiling of more than 200 microRNA precursors to specimens of human pancreatic adenocarcinoma, paired benign tissue, normal pancreas, chronic pancreatitis and nine cell lines of pancreatic cancer. miR-221, miR-376a and miR-301 were among the top differentially expressed miRNAs had increased expression in the tumor and cell lines compared to the normal pancreas and pancreatitis [55].

2.5. Detection of miRNA Using Microarray

2.5.1. Introduction

The microarray is the most commonly used method of detecting miRNAs easily and at high throughputs. In reality, microarray technology is focused on hybridization of nucleic acid

between the target molecules and their corresponding complementary probes. MicroRNA complimentary probes that normally have 5′ termini amine-modified are immobilized on glass slides via a covalent cross-linking between the amino groups and the SAM (monolayer self-assembly), creating a ready-to-use microRNA microarray. The isolated microRNAs are marked with fluorescent dye and then hybridized with microRNA microarray; each well's fluorescence intensity can be used to determine the level of expression of miRNAs.

2.5.2. Review of Studies That Used Microarray

A new Scanometric MicroRNA (Scano-miR) profiling array was created by Alhasan et al. using spherical nucleic acid (SNA)–gold nanoparticle. The assay used a universal linker probe to enzymatically ligate the isolated miRNAs to the miRNA microarray, followed by hybridisation. The universal SNA-functionalized gold nanoparticle conjugates (SNA – AuNPs) were subsequently hybridized after washing away unbound miRNA species to detect the captured miRNA targets. Depositing gold has amplified the intensities of the scattered light. The high-specificity and reproducibility light-scattering amplification protocol allowed the Scano-miR system to detect miRNA species of low abundance as low as 30 fM, which provides improved sensitivity for miRNA targets compared to molecular fluorophore-based detection systems. The platform demonstrates 98.8 percent accuracy when detecting deregulated miRNAs that are active in prostate cancer, demonstrating its possible usefulness in profiling and recognizing biomarkers for clinical and research. [56]

Ueno et al. Reported using ligase-assisted sandwich hybridization (LASH) which is a label-free and amplification-free miRNA microarray assay. The LASH coupled with a stem loop probe increased the sensitivity of miRNA to detect targets as low as 30 fM miRNA. In addition to that, the authors have also shown that miR-141 and miR-200a can be explicitly differentiated by

multicolor detection. Thus, they concluded that LASH-based microarray is a sensitive and rapid approach which can be applied to diagnosis of disease [57].

2.6. Detection of miRNA Using Dielectrophoresis

2.6.1. Introduction

Phol described Dielectrophoresis as "the movement of suspensoid particles relative to the solvent resulting from the polarizing forces created by an inhomogeneous electrical field" [39]. In the presence of electric fields all particles exhibit dielectrophoretic behavior.

2.6.2. Review of Studies That Used Dielectrophoresis

Nakano et al.'s study describes a microbead-based method that uses dielectrophoresis (DEP) for rapid detection of polymerase chain reaction (PCR) amplified DNA. For this study, Norovirus which is a common cause of gastroenteritis globally and has a segment of the norovirus GII genome which was used as target DNA. They amplified the GII norovirus through PCR in real-times. In the DEP microbead-based method the amplified norovirus GII was used. This study also compared the sensitivity and dynamic range of norovirus genome detection in real-times PCR and the microbread-based method. They noticed that target DNA could be reliably detected using real-times PCR at concentrations of 10–10^7 copies / reaction, with a lowered detection limit of 10 copies / reaction. Using the microbead DEP-based method, the biotinylated DNA amplified using real-times PCR was detected at each initial starting copy number as a change of conductance. They concluded that the detection limit for both methods was 10 copies / reaction, although real-times PCR had a greater dynamic range of 10–10^7 copies / reaction, than the DEP-based method which had the dynamic range of 10–10^5 copies / reaction. The DEP-based microbead method detected amplified DNA within 20 min. These results suggest that combined with conventional PCR, the microbead DEP method could offer an alternative to real-times PCRs [58].

The Velmanickam et al.'s study (iLuminate-miRNA, 2019) gives us an insight into cancer detection and the role of dielectrophoresis. For potential clinical applications at the point of care, they report a responsive, low-cost and timesly detection method for circulating microRNA (miRNA) called iLluminate-miRNA. The first step in the process was to extract miRNA (target and non-target) from the serum sample using a kit which was available commercially. The next stage was to selectively hybridize target miRNA with fluorophore-labeled supplementary DNA molecules at temperature of 95^0C for 5 minutes and subsequently cooling it down for 55 minutes at room temperature. The sample from the previous stage consisted of non-target miRNA, hybridized miRNA-DNA duplexes, and free complementary DNA that was transferred to an integrated array of gold-based microelectrodes. DEP force was used for the preferential concentration of miRNA-DNA molecules in hotspots.

The first step in the process was isolation of miRNA (target and non-target) from the serum sample using a commercially available kit. The next step was selectively hybridizing target miRNA with fluorophore labeled complementary DNA molecules (95^0C for 5 minutes and cool it down in room temperature for 55 minutes). The sample from Step B, composed of non-target miRNA, hybridized miRNA-DNA duplexes, and free complementary DNA, will be transferred to interdigitated array of microelectrodes manufactured in gold. DEP force can be used to selectively concentrate miRNA-DNA molecules in hotspots. Finally, a simple fluorescence image of the sample will be recorded. The image of the fluorescence intensity was determined and the molarity was determined by using the standard curve of known molarity versus the intensity of fluorescence. They also provided proof-of - concept data showing that the iLluminate-miRNA system was able to quantitatively and precisely detect Let-7b miRNA (highly expressed in human serum). This research demonstrated the efficacy of the iLluminate-miRNA method in measuring diluted Let-7b

miRNA (range: 0.0114 pM–12 nM) spiked into water or human serum. They also studied, single base pair mismatches using Let-7c, which differs by a single nucleotide to Let-7b. This effort yielded similar % recoveries for both Let-7b and Let-7c and could present certain drawbacks in measuring miRNA species with a high percentage of base pair similarity. Therefore, they purpose that the iLluminate-miRNA detection platform, that combines DEP and fluroscence imaging, would be effective at measuring unique miRNA species for early cancer detection [44].

2.7. Detection of miRNA Using Electrical Impedance

2.7.1. Introduction

Detection of miRNA as we know, is very crucial in determination of various diseases. Here, we discuss some literatures that focuses on using electrical platforms i.e. Impedance for detection of miRNAs.

2.7.2. Review of Studies That Used Electrical Impedance

Jolly et al.'s, research discusses a highly sensitive electrochemical platform for miRNA detection. As we are already aware, miRNAs play a very important role in human diseases, making them a promising biomarker. Since the amount of miRNAs is small in blood, miRNA requires an accurate and easy method of detection. They reported using peptide nucleic acid (PNA) as probes to be a simple and very sensitive dual mode electrochemical detection platform. PNA was used in this research because of various advantages such as neutral charge and high in stability. We also note that duplex with mismatches of PNA / miRNA is less stable than duplex with the same mismatches of DNA / miRNA. They used positively charged gold particles (AuNPs) to employ a powerful amplification strategy. With no redox markers, electrochemical impedance spectroscopy was used to monitor changes in the bilayer's dielectric properties through capacitance changes. The study focused on the miR-145 sequence and the application of the above detection strategy

through the use of electrochemical impedance spectroscopy. To reduce false positives, they developed a complementary detection technique using square wave voltammetry for validation of the signals on the same sensors. This was done by attaching thiolated ferrocene to the surface using PNA/miRNA duplex, by exploiting the availability of AuNPs, for the volumetric detection by the help of square wave voltammetry (SWV). A LOD of 0.37 fM with a wide dynamic range from 1 fM to 100 nM was achieved with the established dual detection system on the same sensor. This research concludes that the electrochemical framework built along with the development of microarray platforms can easily be extended to other miRNA / DNA detection [59].

Han et al., developed a simple, label-free and amplification-free electrochemical biosensor to detect miRNA using, for the first times, DNA origami nanostructure-supported DNA probes, with methylene blue (MB) serving as the redox hybridization indicator. They created cross-shaped DNA origami, containing ssDNA samples, by heating a mixture of M13mp18, which is a viral DNA, and staple strands which included ssDNA sample strands from 90oC to 15oC in a thermocycler for more than 12 hours. Then, DNA origami was purified to remove excess staple strands. The immobilization process of DNA origami of 1 nano Molar (nM) was carried out on the bare gold electrode in the immobilization buffer at room temperature for 4 hours. The modified electrode containing ssDNA probes was then incubated for 4 hours at room temperature with miRNA-21 in the hybridization buffer. In this study, they concluded that using cross-shaped DNA origami nanostructures that contain multiple single-stranded DNA probes at pre-selected locations on each DNA nanostructure may increase the accessibility and recognition efficiency of the samples. Electrochemical impedance spectroscopy and cyclic voltammetry methods have confirmed the successful immobilization of the DNA origami probes and their hybridization with targeted miRNA-21 molecules. To monitor the oxidation peak current of MB before and after

target hybridization, a differential pulse voltammetry technique was employed. This biosensor's linear detection range was 0.1 pm to 10.0 nM, with a lower detection limit of 79.8 fM. They also studied selectivity of the miRNA biosensor by observing the selection ability of single-base mismatched sequences. This technique showed great potential for sensitive, selective and label-free determination of miRNA for translational biomedical research and clinical applications due to the wider surface area and unparalleled customizability of DNA nanostructures [60].

2.8. Summary

Northern Blotting in conjunction with Polyacrylamide gels have been used in detection of miRNA by many researchers over the years. They combine Northern Blotting with Polyacrylamide gels to examine the expression of both the mature and precursor miRNAs, as it enables both quantitation of the levels of expression of the miRNA and determination of miRNA size. One major downside of this method is its poor sensitivity, especially when monitoring low-abundant miRNA expression. Consequently, for northern blotting, a large amount of total miRNA per sample is needed which is not feasible when the source of the cell or tissue is small. Therefore, because of poor sensitivity, times requirement, and abundance in sample a new and improve detection technique is important.

Another method which is widely used in miRNA detection is real-time qRT-PCR. The real-time qRT-PCR method is highly sensitive, but its use is limited by difficulty in primer design. The exact quantification of real-time qRT-PCR depends on the multi-step interconnection, and each step needs to be optimized. Several parameters such as RNA extraction, RNA integrity regulation, cDNA synthesis, priming design, amplicon detection and data normalization must be taken into account to obtain meaningful and repeatable results. Therefore, the step is complex and requires a lot of times to execute. Although high sensitivity is an important factor in detection technique but

complexity, cost and times consumption also plays a vital role in detection method and in developing new devices. Being expensive and having a high-time consumption real-time qRT-PCR isn't exactly feasible for many individuals.

Microarray can analyze thousands of samples in a day, but the cost related is very high. The microarray method also faces a multiple challenge like, difficulty in detecting too short miRNAs, or low copy number miRNA. The sensitivity of analyzing the miRNA is not so good. Microarray, although a newer technology in detection and monitoring of miRNAs it has a very high cost and is not simple which makes a place in detection techniques for a new and improve method of detecting miRNAs.

The study performed by Nakano et al., proved that Dielectrophoresis was indeed efficient method in the detection of DNA. Although, the dynamic range of the DEP-based method was lower than that of the real-time qRT-PCR method; the limit of detection was the same for the DEP-based method and the real-time PCR. The study done was important in understanding various details about real-time quantitative reverse transcriptase polymerase chain reaction and their method of microbead DEP-based method. While the real-time qRT-PCR is expensive, their method was comparatively less expensive. It also produced a Limit of detection which was similar to real-time qRT-PCR. This study proves that, DEP method could be effectively used and can be an important tool in the newer detection method for miRNA.

The study conducted by Velmanickam et al., Proved that Dielectrophoresis technique is indefinitely concentrating the micro RNA into the high electric field gradient region of the electrode. The study also compared both the iLluminate-miRNA method and the qRT-PCR method for Let-7b miRNA detection and found that percentage recoveries of spiked-in Let-7b miRNA to a 100% predicted recovery clearly showed that the iLluminate-miRNA method was more accurate

and accurate at all spiked-in Let-7b miRNA concentrations using both water and human serum as solvents. The iLluminate-miRNA method has resulted in smaller standard mean errors for Let-7b miRNA concentrations particularly in water compared with qRT-PCR. They concluded that the iLluminate-miRNA detection technique can successfully quantitate Let-7b miRNA molecules after purification from water or biological fluids in a cost-effective, fast, accurate and accurate manner that outperforms the current qRT-PCR gold standard method. The study done by Velmanickam et al., further proves that the method of Dielectrophoresis (DEP) is an efficient method and a cost-effective method for the detection of miRNA and could be used for further study in developing a new detection technique.

Furthermore, the study conducted by Jolly et al., and Han et al., concludes that Impedance technique can be successfully used for the detection of miRNA. Therefore, DEP and impedance-based detection method has more potential to be used for the detection of biomarkers because of its detection limit, cost effectiveness, and simple and quick method for the detection.

CHAPTER 3. MATERIALS AND METHODOLOGY

3.1. Introduction

This chapter provides the methods and details of the experimental process employed in the research. This study is divided into three major phases. In phase I, Dielectrophoresis technique was applied. In phase II, Electrical Impedance measurement can be seen carried out. The third and final phase of this research includes Data analysis.

3.2. Detection of miRNA

T-shaped interdigital electrodes, shown in Figure 3.1, were used in the execution of the experiments. Velmanickam et al., manufactured interdigital T-Electrodes using a low-resolution photolithography mask, and during photolithography, the photoresist film was over-exposed to UV light to produce rough-edged metal structures (or hotspots). Furthermore, using AutoCAD software, the electrode array and nanostructures were drawn to a scale and imported for stimulation into the COMSOL software. The hotspots had higher electric field gradient which was successful in concentrating let-7b miRNA in the T-Electrodes and the cost of this disposable device was estimated at around $60 [44].

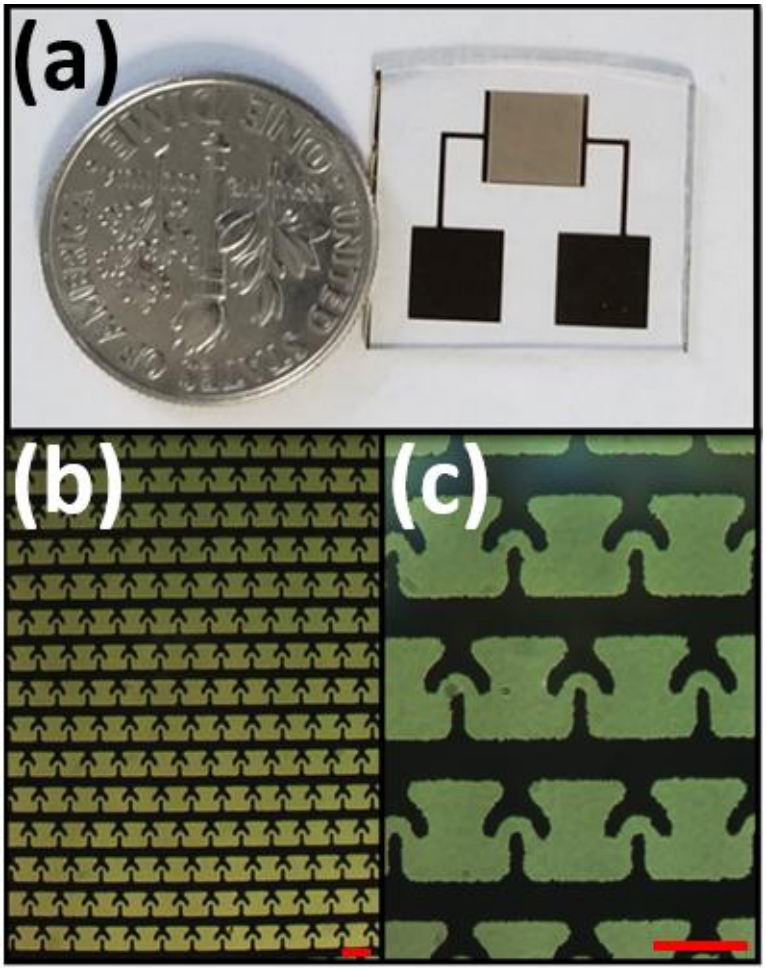

Figure 3.1: T-Electrodes (T-E) (a) The final version of the electrode that we used in experiments. (b&c) Close-up view of the T-E array showing how T-Es were designed. Scale bars indicate 50 μm.

The human let-7b miRNA (purchased from Integrated DNA Technologies), which is highly expressed in blood / serum and has been used as a biomarker for cancer, was used as proof of principle. The purchased miRNA sample was diluted with Tris-EDTA (TE) buffer, because TE buffer can solubilize miRNA without being degraded. First, the human albumin serum was serial diluted in Deionized (DI) water first 100 times and then 1000 times. For 100 times dilution, one part of serum was combined with 99 parts of DI water. The 1000 times dilution used; one part of 100 times diluted serum sample combined with 99 parts of DI water.

In both of the diluted serum samples, let-7b miRNA was spiked at different concentrations ranging from 10nM to 1pM. After miRNA was spiked at different concentration, 140 nM of a complimentary DNA probe was added to each sample and hybridized at 95^{o}C for 3 minutes, then left at room temperature for 10, 15, and 25 minutes to cool down [44]. We used a thermocouple to measure and record the sample temperature immediately after hybridization. This step measured the time taken for the sample to reach the room temperature of 21.5^{o}C. The typical cooling time used in previous study was 45 minutes [44], but we found that after 25 minutes of cooling down, the temperature of the sample reached the ideal room temperature of 21.5^{o}C.

Piranha solution was prepared in a 1:3 ratio of hydrogen peroxide to sulfuric acid. Piranha solution is used for cleaning substrates off any organic residues. Using piranha solution, the electrode was washed to remove any organic residues on the electrode that may have been present. The electrode was then washed using 70% ethanol. After the electrode was cleaned and dried, 10 μL of hybridized sample was pipetted over the electrode, the electrode that was connected to a function generator, (Figure 3.2(a); Tektronix, AFG 3021B, Beaverton, OR, USA) to enable the electrical connection. Electrical potential of 10 Vp-p and 1 MHz was applied to the electrode [44]. DEP then concentrated duplex molecules of miRNA-DNA near the electrode. Then, the samples' electrical impedance (0-100 kHz) was measured using Gamry's device (Figure 3.2(b); Gamry's Instrument, Reference 600). Figure 3.3 shows the overall steps involved in the process of miRNA detection.

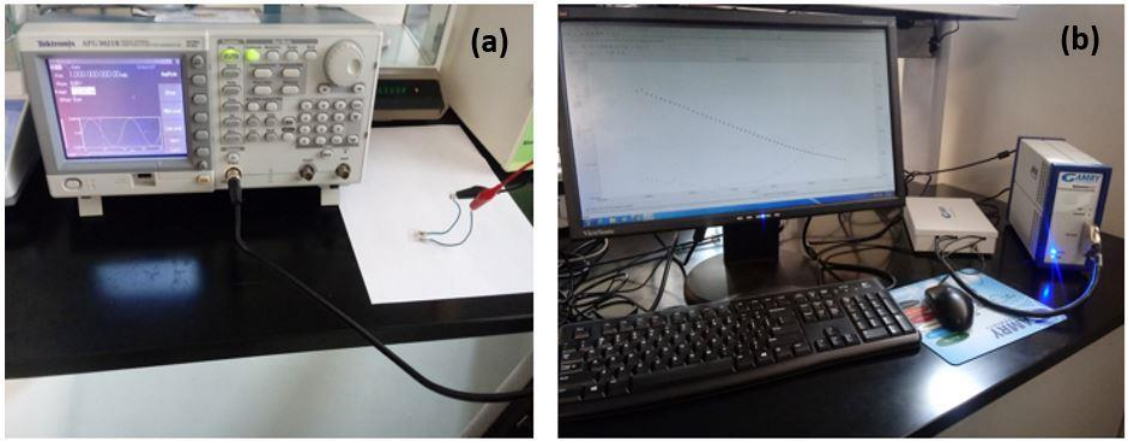

Figure 3.2: Experimental setup for the detection of let-7b miRNA **(a)** T-Electrode connected to a function generator, where DEP helps to concentrates let-7b miRNA in the electrode. **(b)** Electrode connected to Gamry's instrument where impedance is measured.

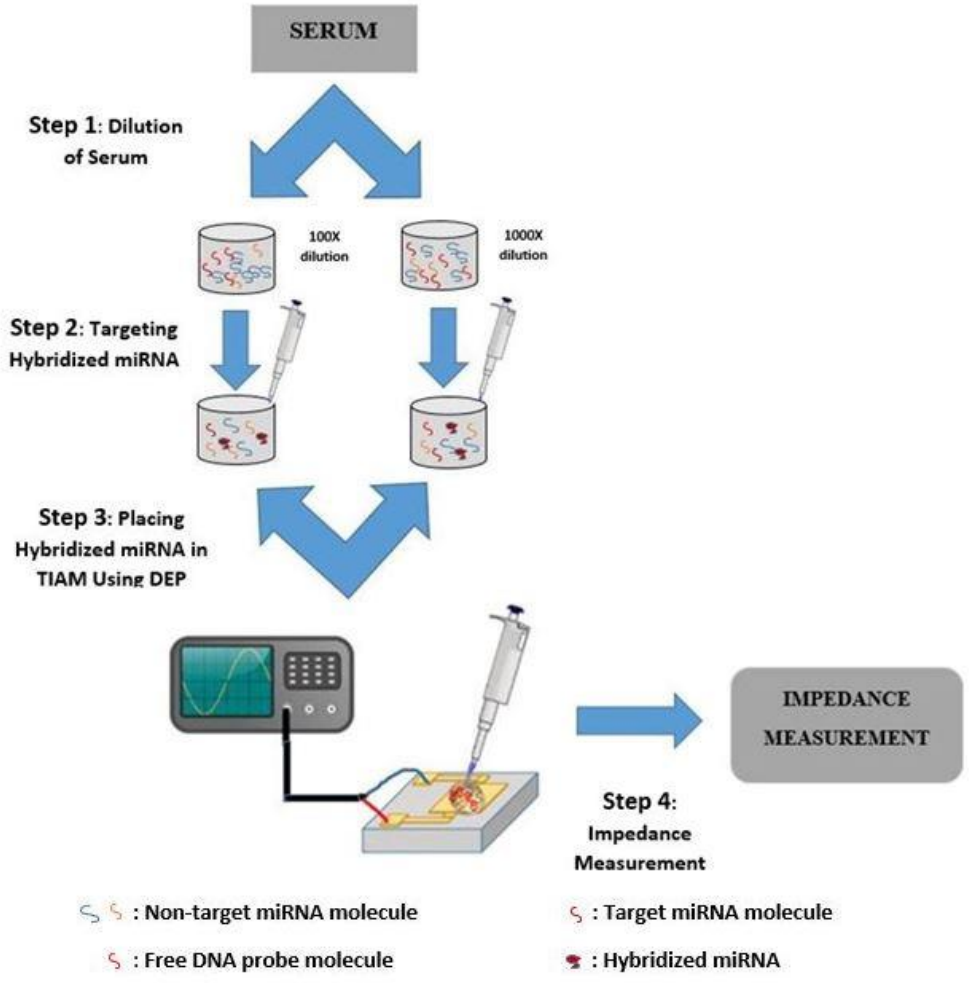

Figure 3.3: Diagram showing the steps involved in miRNA detection.

3.3. Data Analysis

The technique, or Data Fitting compares the obtained data from an electrical Impedance scan to a hypothetical resistor, capacitor, inductor, and other theoretical component model network. If the data matches the network (a "fit" of the data to the model), then the component network is considered a valid model for that data.

After obtaining impedance measurement for each individual miRNA molarities, by measuring the sample's electrical impedance. The obtained impedance was subjected to data fitting by using Gamry's Echem Analyst software. The Levenberg- Marquardt algorithm was used for fitting the curves.

Resistance, capacitance, and constant phase element was used in the developed model that can be seen in Figure 3.4. Gamry's Echem Analyst software was used to develop the model. Then, the previously obtained impedance spectra were fitted into the model. The change in Resistance, capacitance, and constant phase were recorded for each individual observation of let-7b miRNA. Also, to check if the model fitted the curve better, the change in the resistance, capacitance, and constant phase element was noted and the errors was calculated by Gamry's Echem Analyst software, which would be helpful in understanding the fitness of the model [61].

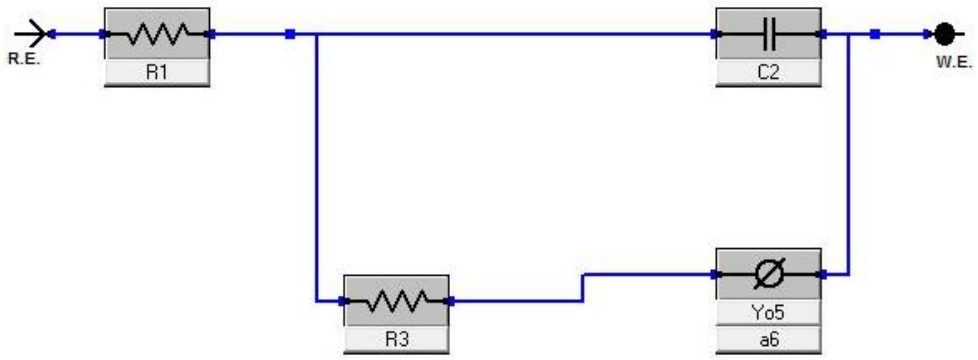

Figure 3.4: Model developed for curve fitting

Different Chemicals that have been used in the research, the names and usage of them are mentioned in the table below.

Table 3.1: Chemicals used in the research and their use.

Chemical	Used for
Molarities of miRNA+DNA (1nM, 100pM, 10pM, 1pM)	Conducting the experiment
Tris-EDTA (TE) buffer	Dilution of miRNA, and Complimentary Probe.
Deionized (DI) Water	Dilution of Serum sample
Diluted Serum Sample	Dilution and hybridization of different molarities of miRNA.
Hydrogen Peroxide	Preparation of Piranha solution.
Sulfuric Acid	Preparation of Piranha solution.
70% Alcohol	Cleaning the electrode.

CHAPTER 4. RESULT AND DISCUSSION

4.1. Introduction

This chapter focuses on the results obtained from the impedance measurement of the hybridized let-7b miRNA with its complementary DNA probe. Additionally, data analysis, which was analyzed by observing the error values from each equivalent circuit component by using the developed model.

4.2. Results & Discussion

We used a thermocouple to check how long it took for the sample (let-7b miRNA and its complimentary DNA spiked into 100 times diluted serum sample) that was hybridized at 95^{o}C for 3 minutes to reach the room temperature, which was 21.5^{o}C. From the results, we can see clearly that it took about 25 minutes for the sample to reach 21.5^{o}C from its initial temperature of 95^{o}C. We also found that it took 10 minutes for the sample to cool down to 22.5^{o}C, and 15 minutes to cool down to a temperature of 21.8^{o}C. In 2019, Velmanickam et al., hybridized the let-7b miRNA with its complimentary fluorophore labeled DNA probe for 95^{o}C, 5 minutes and kept in room temperature for 45 minutes, in our study we wanted to check if we could detect the target miRNA (let-7b) by minimizing the time taken for the sample (let-7b miRNA and its complimentary DNA spiked into diluted serum samples and hybridized) to rest in the room temperature. Therefore, we left the sample (let-7b miRNA and its complimentary DNA spiked into 100 times diluted serum sample) after hybridization to rest in the room temperature for 10, 15, and 25 minutes.

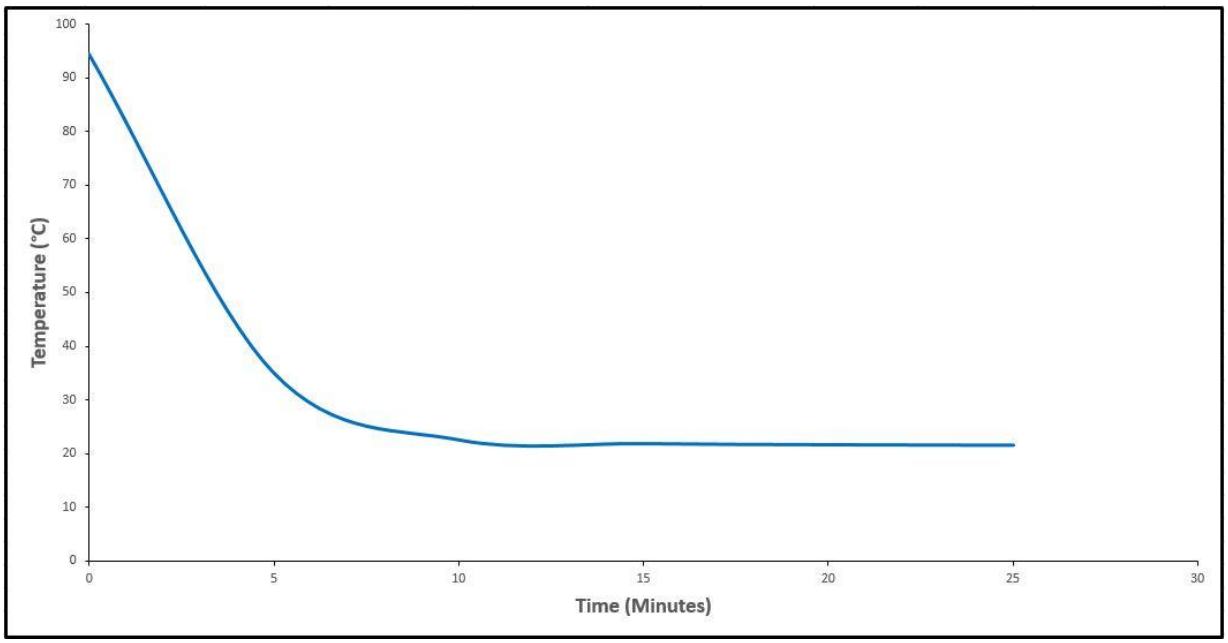

Figure 4.1: Change in temperature of let-7b miRNA with its complimentary DNA probe immediately after hybridization at 95^{o}C for 3 minutes with respect to time

The first evidence that our technique worked was when we could detect the serum sample which was 100 times diluted in DI water. From Figure 4.2, we can observe that after 10 minutes of cooling time, 1nM concentration of the sample (let-7b miRNA and its complimentary DNA spiked into 100 times diluted serum sample and hybridized) could not be properly distinguished from the 100 times diluted serum sample. We also know that as the concentration of molarity increases, the value of impedance must decrease. Therefore, we can say that 10 minutes of cooling time is not sufficient enough to detect let-7b miRNA using our technique.

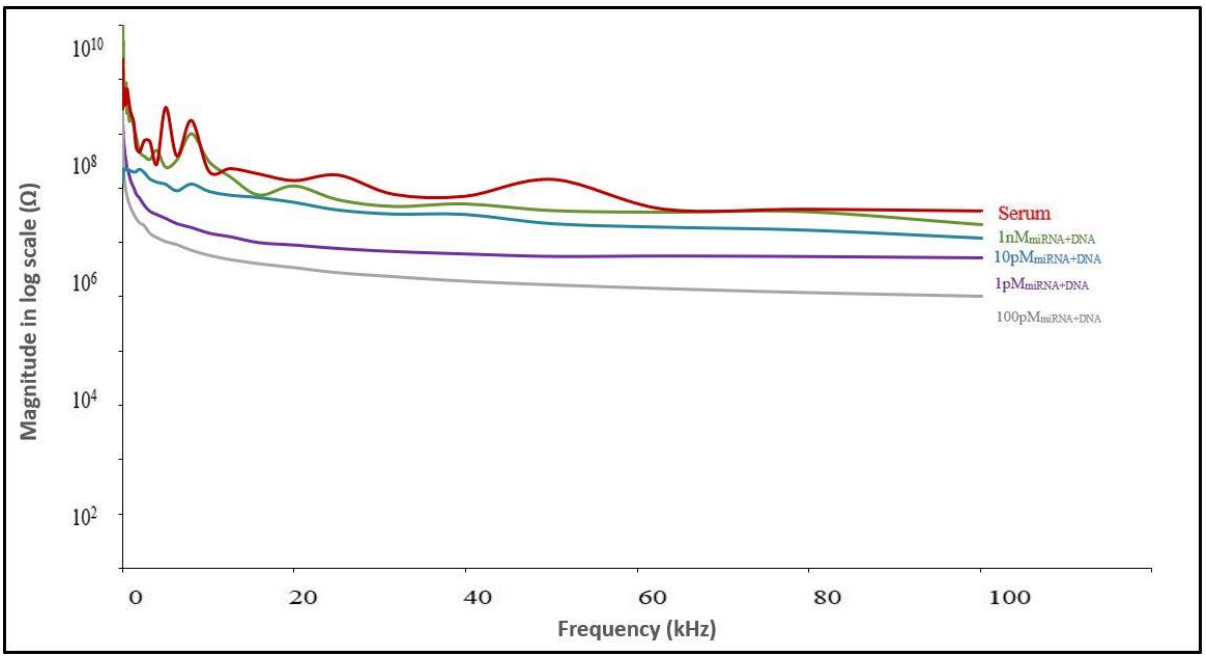

Figure 4.2: The obtained graph of the impedance measurement for different concentrations of 100 times diluted let-7b miRNA samples after 10 minutes of cooling time.

The second evidence in favor of our technique was when we could obtain independent observations for each individual molar concentration of sample (let-7b miRNA and its complimentary DNA spiked into 100 times diluted serum sample and hybridized) after 15 minutes of cooling time, as seen in Figure 4.3. While we could obtain independent observations for different molarities of the let-7b miRNA, impedance values of different molarities of sample (let-7b miRNA and its complimentary DNA spiked into 100 times diluted serum sample and hybridized) didn't decrease with increasing molarity. Therefore, from this result we can conclude that 15 minutes of cooling time is not sufficient either to detect let-7b miRNA using our technique.

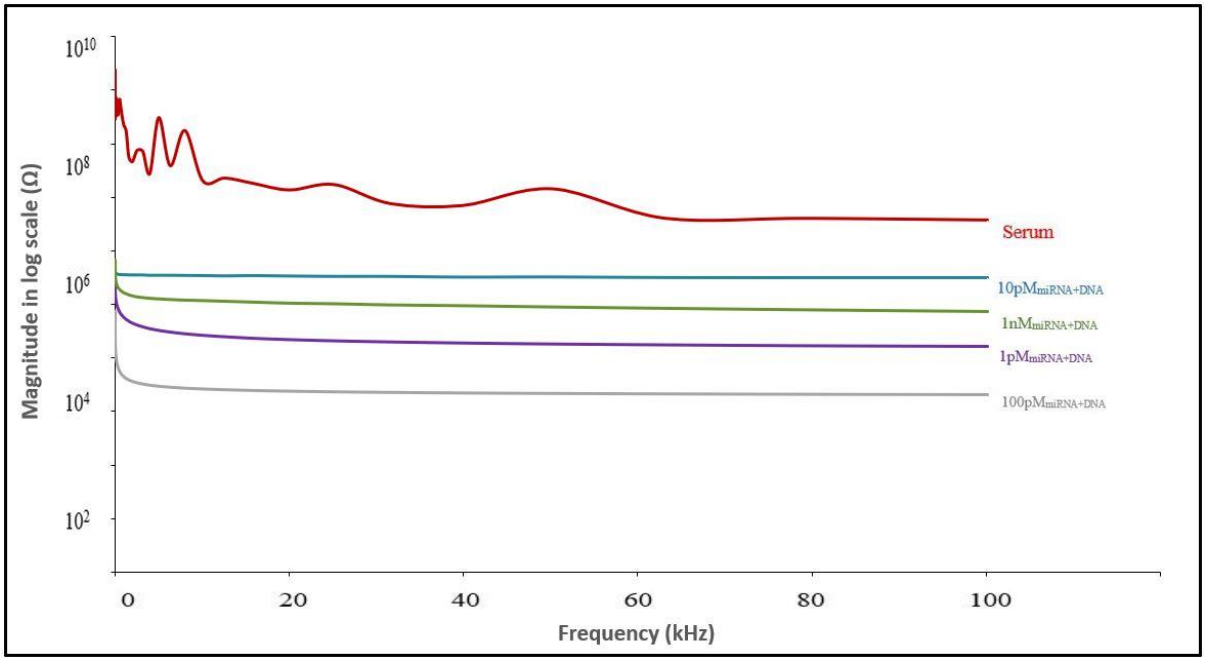

Figure 4.3 : The obtained graph of the impedance measurement for different concentrations of 100 times diluted let-7b miRNA samples after 15 minutes of cooling time.

We then left the hybridized let-7b miRNA with its complimentary DNA probe, which was spiked into 100 times diluted serum sample for 25 minutes of cooling, because it took 25 minutes for our sample to reach the room temperature.

As we can see from the results in Figure 4.4, we could obtain an independent observation for each molar concentration of sample (let-7b miRNA and its complimentary DNA spiked into 100 times diluted serum sample and hybridized). We can also see that as the molarity increased the value of the impedance decreased. We also found that we could measure up to 1pM concentration of sample (let-7b miRNA and its complimentary DNA spiked into 100 times diluted serum sample and hybridized) using our method. One of the important concerns was if we could detect up to 1pM of sample (let-7b miRNA hybridized with complimentary DNA probe) in a 1000 times diluted serum sample. We used the same process as we did before. We could successfully detect label-free let-7b miRNA up to 1pM concentration of sample (let-7b miRNA and its

complimentary DNA spiked into 1000 times diluted serum sample and hybridized) using our novel technique, which can be seen in Figure 4.5.

As we know, Conventional miRNA biosensors needs fluorescent labeling, which is complicated, time-consuming, laborious, and often times costly. A label-free detection technique allows miRNA to be detected directly without fluorescent labeling.

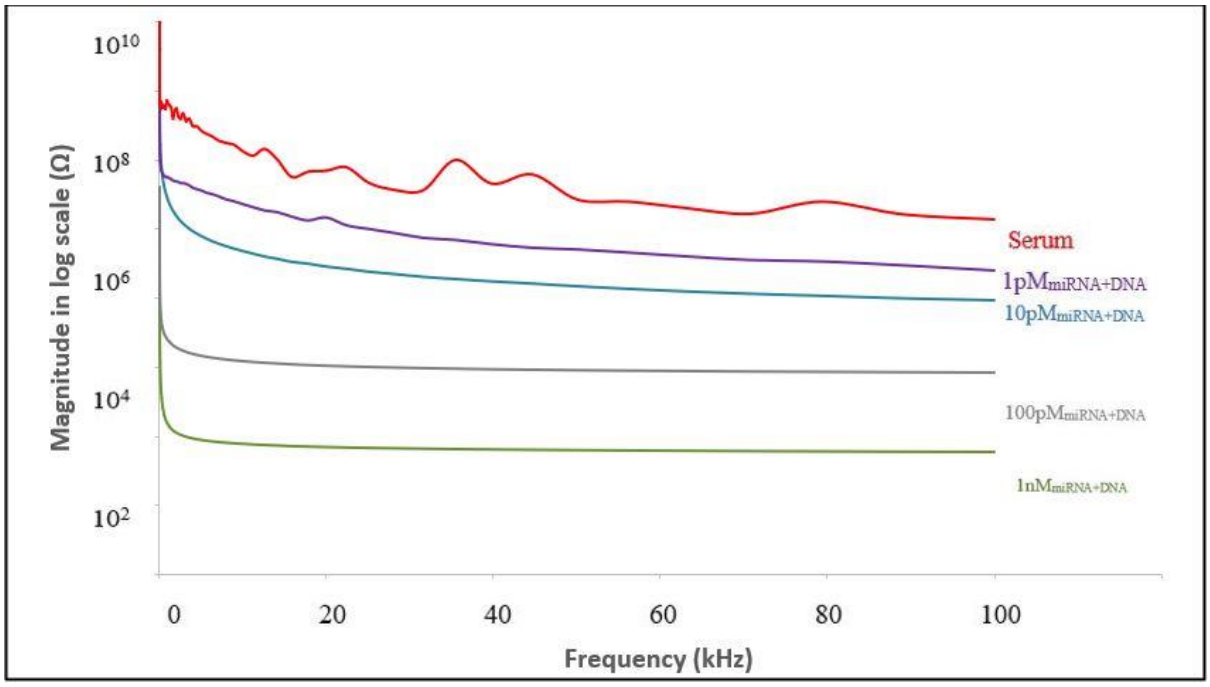

Figure 4.4 : The obtained graph of the impedance measurement for different concentrations of 100 times diluted let-7b miRNA samples after 25 minutes of cooling time.

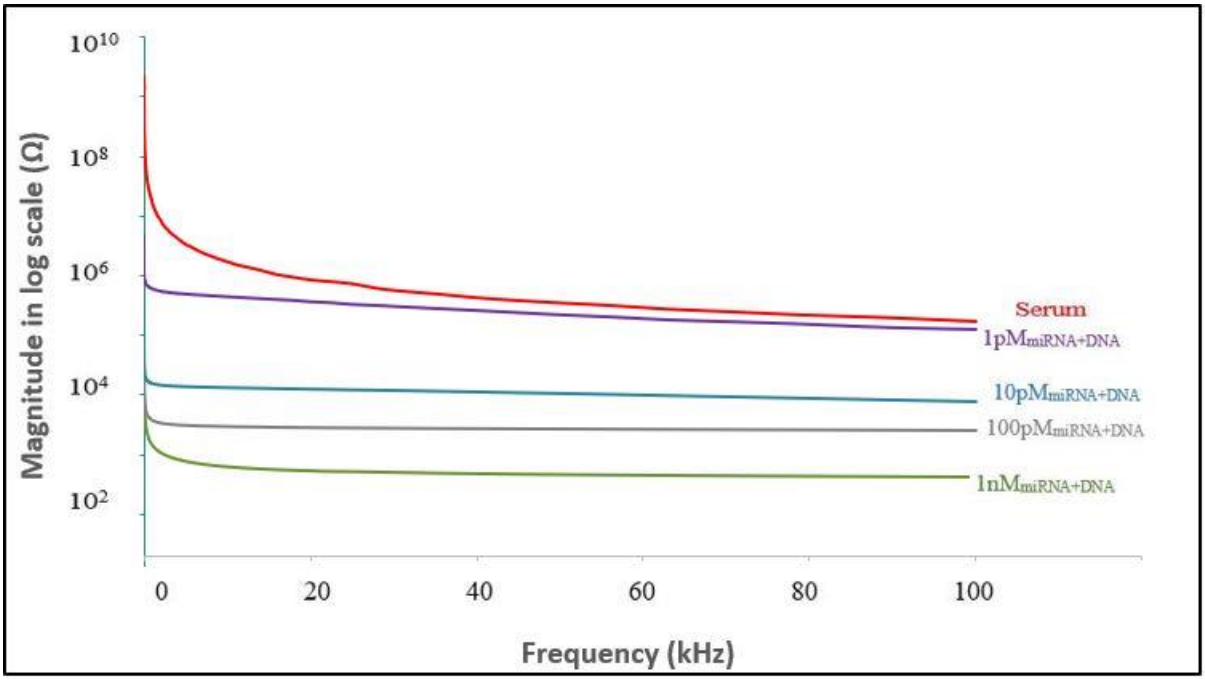

Figure 4.5: The obtained graph of the impedance measurement for different concentrations of 1000 times diluted let-7b miRNA samples after 25 minutes of cooling time.

The standard error that is shown in Figure 4.6 – Figure 4.13, is calculated by the maximum likelihood estimation statistical method in the Gamry Echem Analyst software. This outputs one error value per element in the model. An Information Matrix is built by Inverting the Hessian Matrix (a second order derivative matrix). Next the variance-covariance matrix is calculated, and the standard errors are the square roots of the diagonal terms in the variance-covariance matrix. Variance refers to the spread of a data around the mean value, while a covariance refers to the measure of relationship between two random variables.

The model used to fit the data serves an equation that has frequency as the independent variable and impedance and phase as the dependent variable. The model is made to fit over a range of frequencies.

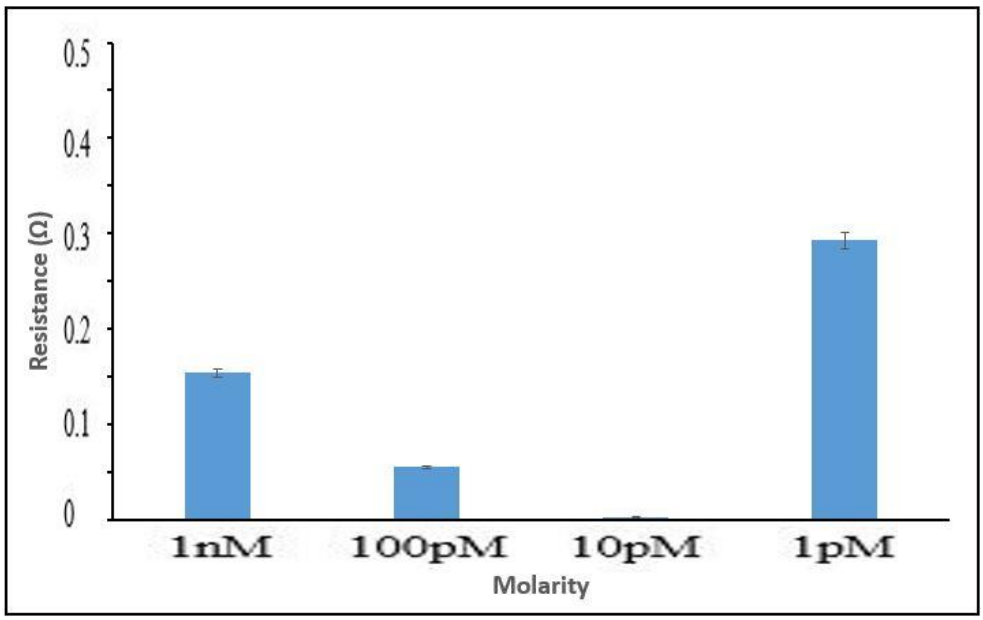

Figure 4.6: Errors value of R1 for 100 times diluted sample

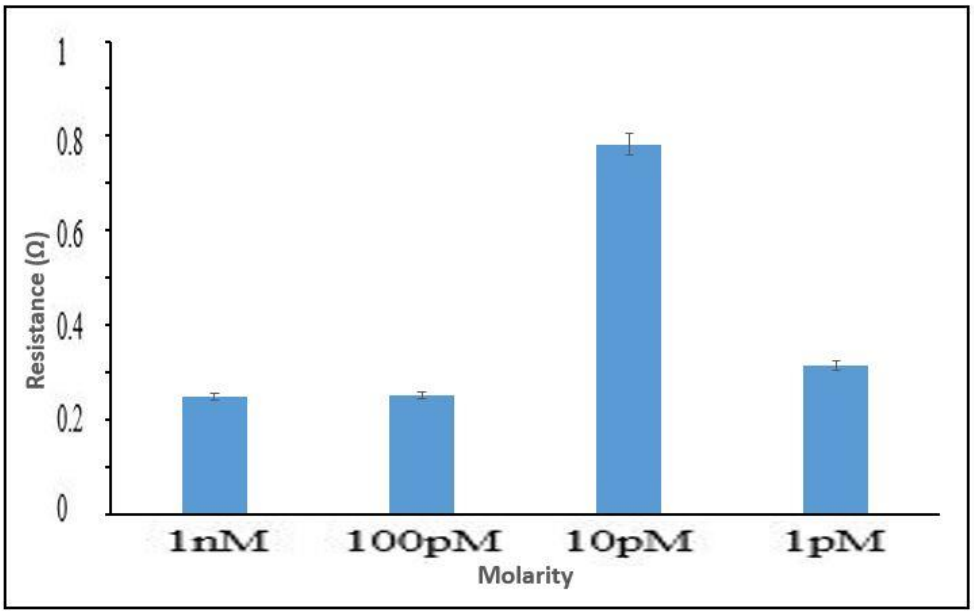

Figure 4.7: Errors value of R1 for 1000 times diluted sample

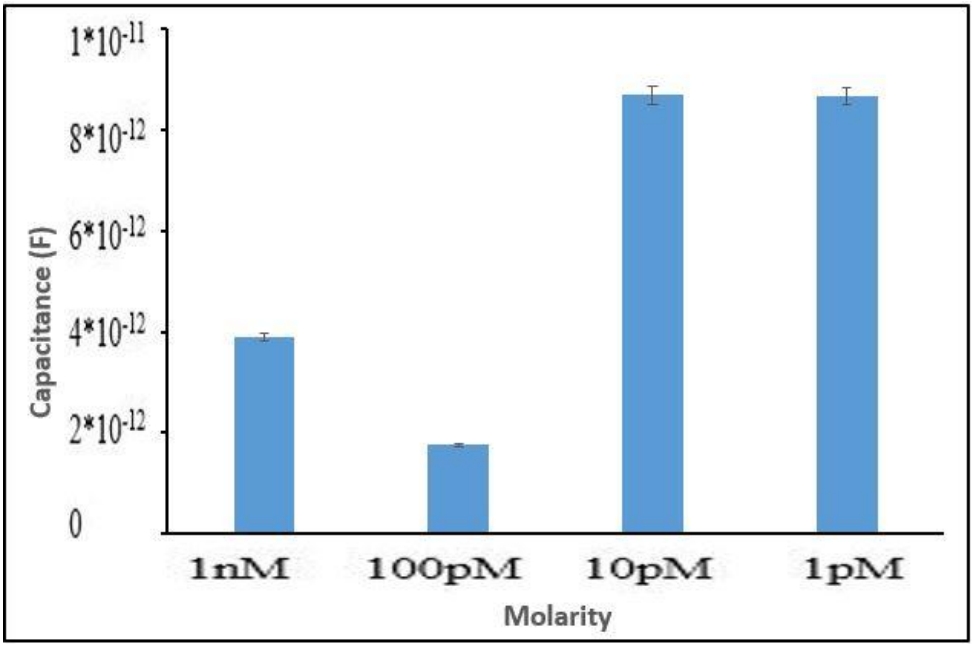

Figure 4.8: Errors value of C for 100 times diluted sample

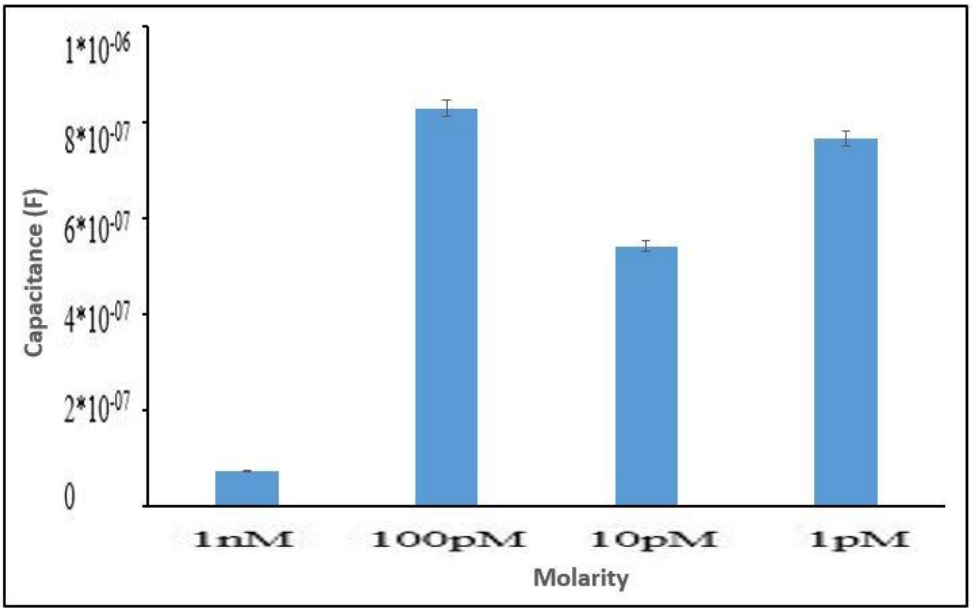

Figure 4.9: Errors value of C for 1000 times diluted sample

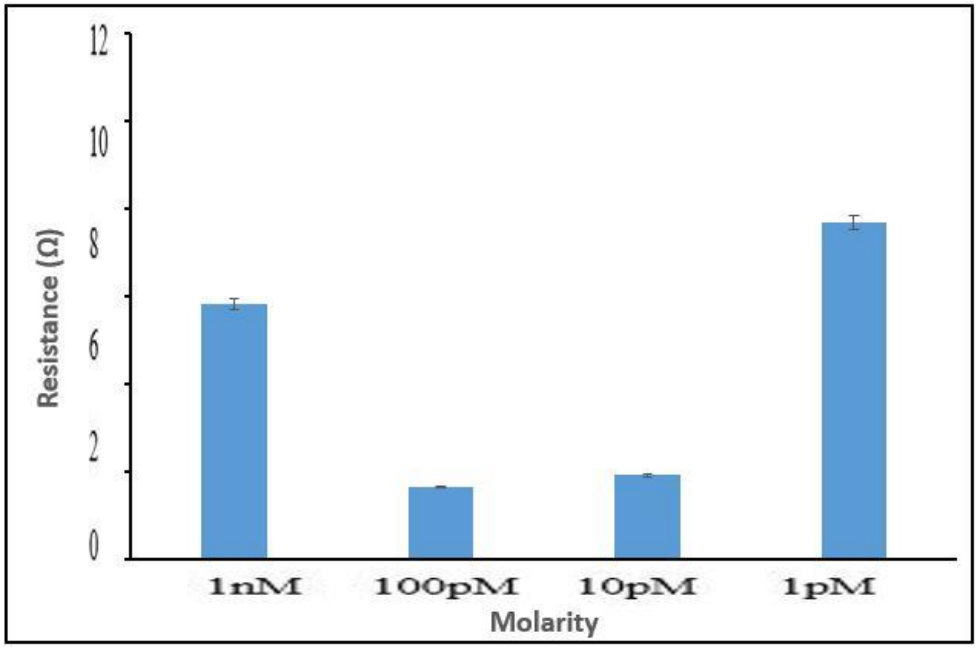

Figure 4.10: Errors value of R3 for 100 times diluted sample

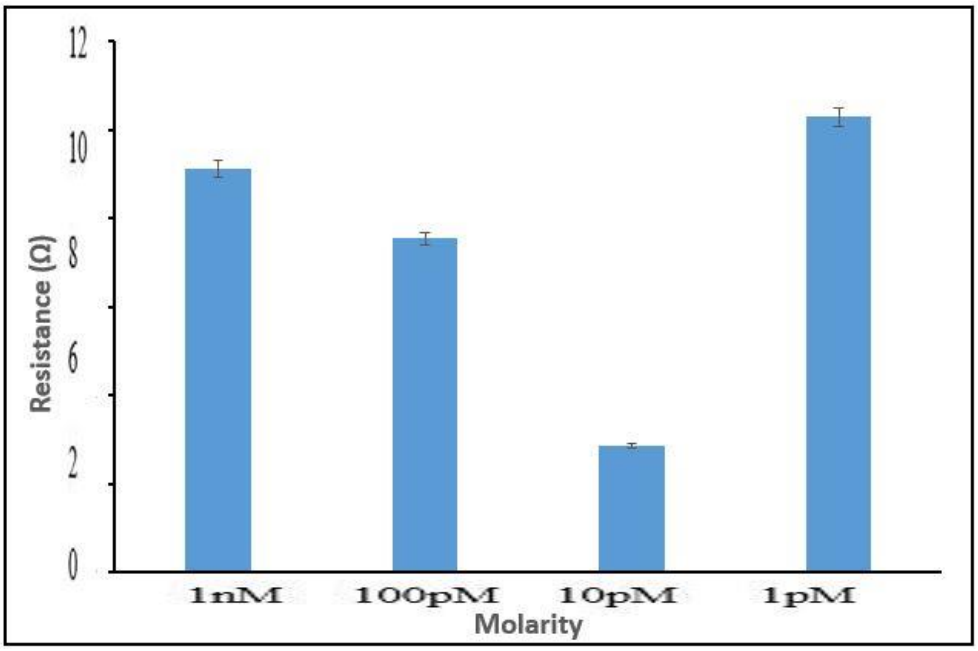

Figure 4.11: Errors value of R3 for 1000 times diluted sample

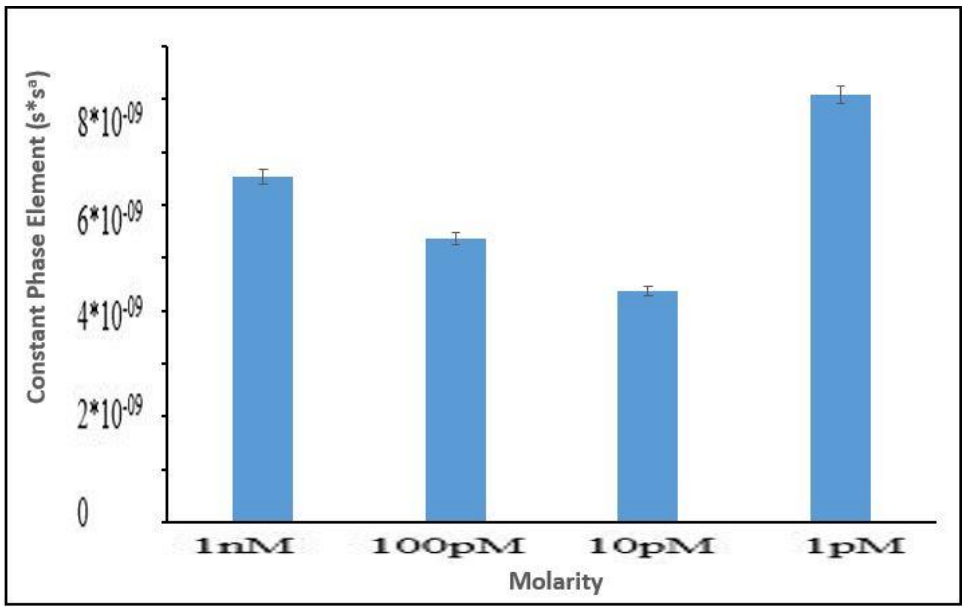

Figure 4.12: Errors value of constant phase element for 100 times diluted sample

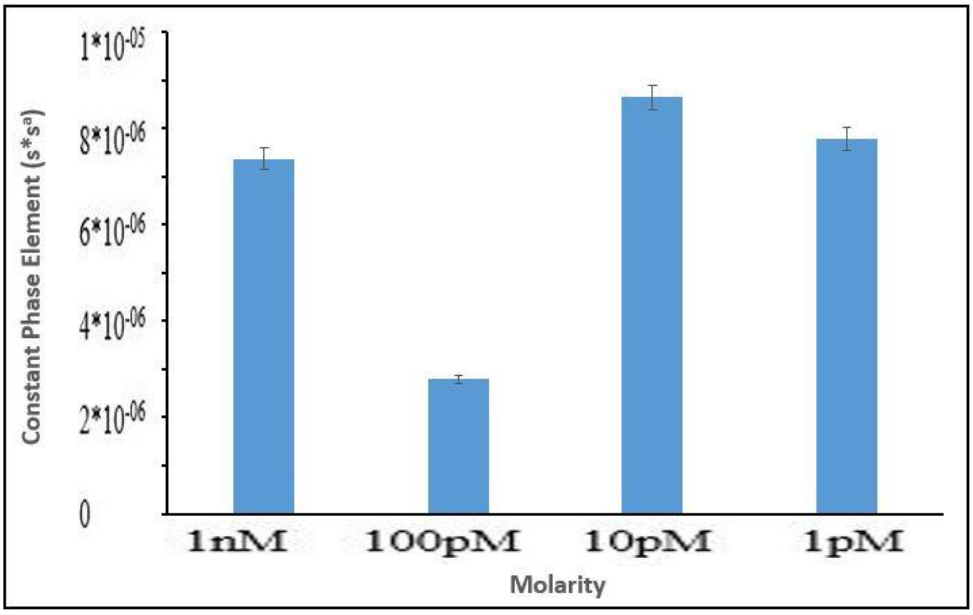

Figure 4.13: Errors value of constant phase element for 1000 times diluted sample

Data Analysis was done to check if the developed model (Figure 3.4) could fit the obtained data. The developed model was fitted into curves obtained from the electrical impedance measurement. By the errors obtained from the 100 times diluted serum sample by using the model, we could say that the model definitely had less errors for each circuit component. As we know, the lesser the error values, the better the fit for the data. Therefore, from the error values obtained

from R1 (Figure 4.6), C (Figure 4.8), R3 (Figure 4.10), and constant phase element (Figure 4.12) we can see that the error values were smaller, which means the model that we developed fit the data of our research. Similarly, from the error values obtained from R1 (Figure 4.7), C (Figure 4.9), R3 (Figure 4.11), and constant phase element (Figure 4.13) using 1000 times diluted serum sample by the developed model we could say that the model had less error values and was a good fit for our data. We also checked goodness of fit for all of the fitted data and found out that the goodness of fit value was smaller too, which further proved that our model was a good fit for the data.

CHAPTER 5. SUMMARY AND CONCLUSION

5.1. Summary

Micro RNA (miRNAs) have attracted growing interest among the scientific community since their first detection in 1993. miRNA, found abundantly in body fluids of humans, is considered to be a potential biomarker which can be used for the detection of various diseases including cancer such as pancreatic cancer, breast cancer, colorectal cancer, etc.

The main purpose of current research was to study let-7b miRNA and detection of Label-free let-7b miRNA in human serum samples. This research focused on the two main principles of dielectrophoresis, and electrical impedance. The let 7-b miRNA was focused and concentrated on the high electric field gradient area of the T-E electrode. Then, electrical impedance obtained independent observations of individual molarities of hybridized let-7b miRNA with its complimentary DNA probe which was spiked in the diluted serum.

The next portion focused on data analysis using curve-fitting technique. For data analysis a model was developed using Gamry's Echem Analyst Software. The model consisted of resistance, capacitance, and a constant phase element. The data fitting was conducted using Gamry's Echem Analyst tool, and the errors of each equivalent circuit component were measured.

This was a proof-of-principle concept, which was performed to check if let-7b miRNA could be detected using our novel technique.

5.2. Conclusion

The data obtained from Figure 4.4 and 4.5 indicates that miRNA can be detected successfully using our novel technique that combined dielectrophoresis and electrical impedance.

Our technique experimentally demonstrated that dielectrophoresis effectively concentrated and targeted biomarkers (let-7b miRNA) in the high electric field gradient area of the electrode,

which was followed by electrical impedance to measure the different molarities of biomarker (let-7b miRNA). The limit of detection was calculated and found to be 2pM for 1000 times diluted serum sample, and 1.5pM for 100 times diluted serum sample.

The developed model fit the obtained curve because the developed model had a lower error values for each equivalent circuit component.

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