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REVIEW PAPER

Circulating microRNA as a potential biomarker and its nanotechnology based detection methods: A literature review

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ABSTRACT

Micro ribonucleic acid (miRNA) is single-stranded RNAs that play a key role in gene regulation and development. The origin and precise activities of miRNAs have been uncovered in recent years, with an emphasis on their potential applications in the research field. Thus, miRNAs are promising diagnostic and prognostic biomarkers especially disease specific biomarker. Traditional methods for detection of miRNA lack sensitivity and specificity. Therefore, novel nanotechnology-based methods to detect microRNA have been developed. This literature review provides an overview of cutting edge nano approaches that have been used to identify distinct miRNA biomarkers. To date, no single or panel of miRNA marker has been developed for clinical application. Therefore, further research is needed on the detection of multiple miRNA biomarkers to diagnose various diseases at an early stage using nanotechnology.

Keywords: *Biomarker, Circulating miRNA, MicroRNA, Nanosensors*

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INTRODUCTION

Healthcare is an organized provision of diagnosis, treatment, prevention and management of diseases and improves the quality of patients. Worldwide, rural, and remote hospitals face barriers when accessing health services. Proteins and peptides are the known biomarkers with diagnostic, prognostic, and therapeutic uses [32]. Novel biomarkers are being researched especially molecular and genetic biomarkers [33]. Owing to their tissue- specific or cell-specific physiological and pathological roles, miRNA have emerged as potential biomarkers in recent years [34-37]. This review provides an outline on conventional and latest findings and prospective detection technologies for detect miRNAs.

MicroRNA (miRNAs) are small single stranded RNA found predominantly in eukaryotic cells that function as gene regulators, immune regulators, genomic protection, gene silencing and cellular functions [1]. The first miRNA is discovered in 1993 by Ambros was gene *lin-4* in Caenorhabditis elegans and Gary Ravkun identified the first miRNA target gene that provides a breakthrough in gene regulation [2]. The human genome contains approximately 2,600 mature miRNAs that are almost 22 nucleotides in length. Fig. 1 shows a simple explanation about miRNA biogenesis.

MiRNAs are processed from DNA sequences. The process of formation is divided into primary miRNAs (pri-miRNAs), precursor miRNAs (pre-miRNAs) and mature miRNAs. Majorly the miRNA binds to 3 prime un-translated regions (3' – UTR) which contain both binding sites for regulatory proteins and miRNAs it causing translation or degradation of transcripts and resulting in gene expression regulation of various mRNA. MiRNAs are released into extracellular vesicles (EVs) and help in cell-cell communication. Hence, alterations in miRNAs can serve as potential biomarkers for most diseases particularly in human cancer, cardiovascular diseases, and neurological diseases [3]. In particular, miRNA help in showing the status of internal tissues and organs and can be utilized for disease diagnosis, prognosis, and management of several diseases most importantly cancer. Due to chromosomal deformity, miRNA biogenesis flaws, and changes in transcriptional factors result in dysregulation of miRNA expression in diseases [4]. Majority of miRNA discharged in exosomes and remain abundant in bodily fluids like sweat, blood, urine and saliva and hence named as "circulating miRNAs" [5-8].

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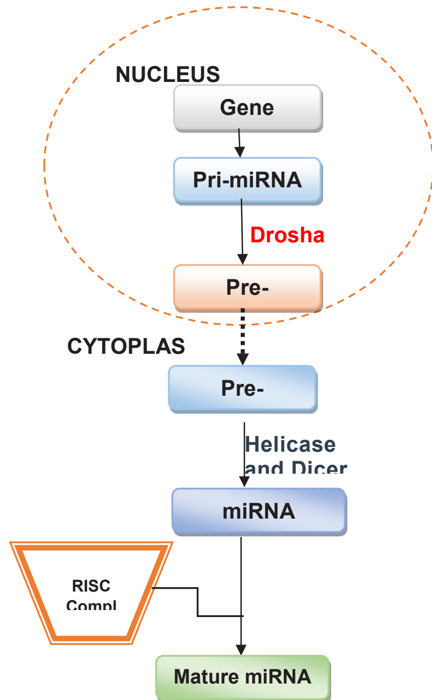

Fig. 1. Simple Representation of miRNA biogenesis

Gene perturbation or alteration in gene expression is the major mechanism involved in the diseased state, as determined by studying miRNA (posttranscriptional gene regulation). Hence, research on miRNAs has vastly increased in different disease states. Several research implies miRNAs are greatly altered in many diseases and hence, miRNAs are considered as potential biomarkers for diagnosis. Before proceeding with extraction and detection methods, the miRNA nomenclature [9] is summarized in Table 1.

METHODS

Using established techniques, and with the goal of identifying research on circulating miRNAs as diagnostic biomarkers for the detection of various diseases, we underwent a literature search on PubMed Database for articles published between 1 January 2012 and 15 August 2022. The Mesh terms used are "circulating microRNAs" AND "nanotechnology", leading to 39 articles. The articles were selected if they satisfied the following requirements: (1) expression of circulating miRNA in human samples; (2) Used only blood samples, such as serum, plasma, and exosomes vesicles.

RESULTS

Conventional method

Body fluids are the common aim at the presence of biomarker because of minimally invasive way to detect disease and it has reasonable contents for the disease indication. At first, we review the conventional methods of miRNA detection helps to summarize the existing quantification and its drawback for the novel method. The most used methods are presented in Table 2.

Table 1. Nomenclature of MiRNA

Name	Meaning
mir	Precursor-miRNA
miR	Mature miRNA
Three letter prefix	Species name (eg. For human hsa-homo sapiens)
Additional suffix (number)	To distinguish between other miRNA
Additional Suffix (a or b)	Relating multiple miRNA by representing a letter in the suffix
Pri-miR	Primary transcript of miRNA
Pre-miR	Precursor form after processing
-3p	3' end of pre-miRNA into mature miRNA
-5p	5' end of pre-miRNA into mature miRNA

Table 2. Comparison of conventional methods of detection of miRNA biomarkers

Detection Method	Principle	Advantages	Disadvantages
Northern Blotting	RNA is isolated from the sample using gel electrophoresis Membrane transformation Hybridization with DNA or RNA probe Detection of miRNA	RNA size, amount and class can be detected Reuse of probes Quantitative method	Low throughput yield One gene analysis per time Time- consuming and expensive process Degradation of RNA due to harsh chemicals
Real time – Polymerase chain (RT-PCR)	RNA transformed to complementary DNA (cDNA) by reverse transcriptase cDNA is the PCR reaction template Quantification process	Highly sensitive and specific detection Fast quantification Quantification in different matrices No post amplification is necessary	Low-throughput method Degradation of RNA may occur during transcription
Microarrays	RNA sample preparation Labeling of oligo-nucleotide probe (cDNA probe) Hybridization process and washing to remove unbind molecules Detection and analysis	Easy to use Parallel-quantification method (thousands of gene from multiple samples) Non-requirement of large-scale DNA sequencing	Highly expensive experiment Large number of probe designs for low specific sequence Cross hybridization problems
In-situ hybridization	Tissue preparation Hybridization and washing Fluorescent detection Detection under autoradiography	Precise location nucleic acid within a cell Tissues can be preserved for future purpose	Low sensitivity in low volume nucleic acids

Northern blot

Northern blotting is a molecular biology technique used to detect RNA by gel electrophoresis technique using gel electrophoresis and membrane separation. Though northern blot identifies very specific and size of miRNA, it lacks its sensitivity and contamination affects the detection [10, 11]. To improve the sensitivity and yield efficient results with low volume sample, several research have done using altered locked nucleic acid (LNA) probes, combination of cross-linking with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) with LNA/DNA probe [13,14,17]. With these advancements, northern blotting lacks its sensitivity for detecting miRNA at low levels.

Real-time PCR (RT-PCR)

Quantitative is a molecular biology technique used to monitor and quantify the target nucleic acid, during the polymerase chain reaction (PCR) reaction. The basic steps involved in the techniques are reverse transcription and real time polymerase chain reaction. During the first step is to isolate the RNA using highly preserved methods like RNA isolation kits or TaqMan miRNA purification kit. The isolated mature miRNAs are extended using specific sequence primer and it is reverse transcribed. It helps to convert RNA to complementary DNA (cDNA). Later, in the second step, the reverse transcribed miRNAs are amplified and quantified by labeled hybridization probe (fluorescent) using data analysis tool [12]. The real time PCR had greater sensitivity than northern blotting but still it requires RNA template variability and degradation of RNA due to transcription [34].

Microarrays

Microarray method of miRNA detection is a hybridization method using nucleic acid between specific complementary probes with the target particle. The oligo-nucleotide probe design with amine modified is immobilized using the linker sequence on the glass slide through covalent bonding. Next, the miRNA from the sample is isolated and labeled with fluorescent dye and introduced to the glass or quartz slide for hybridization. Some of the labeling methods are guanine reagent, T4 RNA ligase, poly (A) polymerase (PAP) enzyme is used. The miRNA hybridizes with their specific complementary probe on glass slides.

After hybridization, fluorescence emission from the target-probe hybridization signal is obtained and further processed to detect the miRNA. The crucial step in microarray detection methods are probe design, sample preparation (quality of miRNA isolation) and proper labeling the miRNA. Some of the key advantages are robust, reliable method over decades of use, streamlined handling and lower cost [15]. Though microarray method is rapid and efficient method but still it was inadequate to detect too short miRNAs.

In situ hybridization

In situ hybridization is a useful method for determining the location and sequence of a gene, which is critical for understanding how genes are organized regulated, and function. Physiological as well as pathological processes are better understood with the help of determining the degree of expression and location of particular miRNAs in tissue slices, within individual cells. Sample tissues and cells are frequently treated to immobilize the target miRNA in position and enhance probe access for hybridization process. The pre-hybridization is obtained by labeling the probe with complementary DNA, RNA and locked nucleic acid (LNA). To increase the specificity of the binding process, solution parameters like temperature, salt can be changed i.e. optimal temperature should be maintained. The probe hybridization with target sequence is then quantified using fluorescence, or antigen-labeled bases [16].

Nanotechnology based mirna detection

Nanotechnology has changed our lives due its numerous benefits and uses. Different nanomaterials and techniques have been evolved and employed in biological applications such as imaging, diagnostics, and therapy. The limitations of the conventional method of miRNA detection are less sensitivity and specificity, large sample preparation, beyond economical, very time consuming, indirect quantification of miRNA and target amplification process [18]. To address this, nanotechnology-based miRNA detection has been developed to meet the above-mentioned criteria.

The term “biosensor” refers to effective and novel analytical equipment that includes a biological sensing element with huge variety of applications, consisting of drug delivery, diagnostics, biomedicine, environmental monitoring. The first biological sensor invented

through Clark and Lyons (1962) to quantify glucose in biological samples using an electrochemical detection. Biosensors serve as a foundation for technological development related to high-throughput and portable device which can be used by non-professionals. The few predominant technological techniques with various types of biosensor devices will be reviewed further.

Electrochemical mirna detection

Electrochemical biosensor is the measurement of electrical characteristics obtained from the change in biological events or biochemical events. In simply, electrochemical biosensors combine electro-analytical sensitivity with the biological components' bio-selectivity [22]. The electrochemical biosensor device has a biological recognition element (proteins, enzymes, DNA, RNA, antibody, cells, and tissues) that selectively interacts with target analyte and generates an electrical signal. The output signal is proportional to the analyte quantity.

Electrochemical miRNA biosensors work on the principle of precise hybridization of target miRNA with its complementary probe. The components that can create an electrochemical signal are usually labeled on the probe. The basic key fabrication steps of an electrochemical biosensor to detect miRNA involve: 1. A Complementary probe is immobilized on the electrode surface, 2. A miRNA target is hybridized with probe; and 3. Electrochemical analysis.

The unique properties of miRNA are very little amount to be observed in total RNA samples and highly similar pattern which greatly alter the specificity of the miRNA detection. Hence, nanomaterials or nano-particles incorporated with the biosensor minimizes the device size and improve the noble characteristics to biosensors, resulting in sensitive bio-analysis [19]. The different nano-architectures such as nanowires, nanoparticles, nanorods, nanoclusters, nanocomposites have been utilized with different nanomaterials to improve the efficiency of the nano-biosensor in terms of sensitivity and specificity with signal amplification. The phosphate groups in nucleic acid can be used as templates to direct the enzymatic deposition of nanoparticles and other nanostructures. By miRNA guided deposition of polyaniline nanowires or poly polymer film with polymerization using enzymes, Gao and *et al* created simple and sensitive electrical miRNA biosensor [20, 21].

Gold nanoparticles (AuNP) with the size of few hundreds of nanometers have attractive properties like optical, physical, chemical, and magnetic. The main characteristics are because of their excellent conductivity, catalytic properties and ease of functionalization and fabrication. In the past research, gold nanoparticles have gained attention in various fields like targeted cancer therapies, diagnosis, and prognosis [27]. The AuNPs are often tagged with bio-recognition elements to recognize targets. As a result, capturing AuNP on an electrode surface can produce significant shift in current. For effective miRNA sensing, AuNP should be used as labels to functionalize the capture probe or in combination as in composites like grapheme oxide (GO), reduced grapheme oxide (rGO), molybdenum sulfide (MoS_2), graphite like carbon nitride.

Gao et al. reported detection of miRNA expression using ultrasensitive assay that delivers electro catalytic nanoparticles tags such as ionized capped OsO2 nanoparticles to the indium tin oxide electrode for signal amplification. The limit of detection reached up to 200 picomoloar/litre [66]. Further Gao teamed with Peng and reported a impedimetry sensor with direct ligation by direct tagging of ruthenium oxide nanoparticles to target miRNAs which results in no mismatch hybridization [67].

Zhang and Chua reported a ultrasensitive detection of miRNA with silicon nanowires. The sensor incorporated peptide nucleic acid (PNA) as receptor to hybridize target miRNA without labeling. Hence, difference in resistance pre- and post hybridization was measured which directly proportional to miRNA concentration. The device sensitivity was observed in femptomolar [68]. Recently, carbon based material like graphene and its derivatives showed promising results in detecting biomarkers due to its superior stability, high electrical conductivity, high surface to volume ratio, tunable structure and low cost.

In a recent study by Huang and team developed a layered composite made of graphene oxide on top of graphene. The active probe for detecting target miRNA was placed on the oxide layer. The resistivity of the bottom graphene increases with increase in concentration of target miRNA-21 [69]. Further Torul and team developed a simple gold nanoparticles modified paper-based electrochemical sensor for detecting miRNA 155

and their limit of detection is around nanomolar. Further they observed good stability in sensor performance for about 60 days [70]. Torul and team advanced the study by incorporating reduced graphene oxide and molybdenum disulfide (MoS2) altered with gold nanoparticles (AuNP) on the paper based biosensor for the detection of miRNA 155 and miRNA 21. The results showed the greater sensitivity and simpler fabrication steps were observed with nanosheet modified paper sensor [71].

With advancement of MoS2 in bio applications, Dong and team developed a sandwich type biosensor incorporating flower like MoS2 – reduced graphene oxide. They observed increased active sites for depositing AuNPs with is nanocomposites which eventually shows promising results in detecting miRNA [72]. Bergveld developed a solid state device incorporating MOS transistor and a electrode ie., first FET biosensor to measure biological activities as a preliminary studies [73].). In field effect transistor (FET), the device has three electrodes, source, drain and gate. Among various method, FET based biosensor show great advantages due to high detection capabilities and sensitivity [39,42]. To increase the sensitivity of the gate surface, several nanostructures have been developed and the integration of nanostructures (nanowires, nanotube, nanoparticles, nanosheet) was a boon to medical diagnostics for real time diagnosis [74].

Electrochemical biosensors are briefly classified into five major classes depending on the kind of transducer and measuring technique. They are potentiometric, amperometric, voltammetric, impedimetric and conductometric. In amperometric method, the analyte is detected by measuring the current due to hybridization of target-probe happens. In voltammetric method, current is monitored due to change in potential. Goda et al. used potentiometric method to detect miRNA from exosomes by measuring the voltage between two electrodes [75]. The determination of electrolytic conductivity from the chemical reaction is measured in conductometric method. The effective impedance of an electrical component is measured using electrochemical impedance spectroscopy (EIS). Another research team reported the FET biosensor functionalized with phosphorodiamidate morpholino oligomers (PMO) and graphene quantum dots for ultrasensitive detection of exosomal miRNAs [76].

Optical based mirna detection

Optical-based methods are the currently under various research topics to detect miRNA. One such method is optical-fluorescence-based biosensors (fluorophores or quantum dots) that detect miRNA by using fluorescence labeled miRNA hybridized with immobilized probes which emits fluorescent signal when miRNA-probe hybridization occurs [23]. Furthermore, the intensity of the fluorescence per miRNA is used to quantify the capture miRNA [24]. Colorimetric-based miRNA detection utilizes metallic nanoparticles especially gold nanoparticles (AuNP) due to its optical properties (surface Plasmon resonance). Because of the coherent oscillation of AuNP surface electrons, AuNP of size 10-50 nm have red when it is distributed. The aggregation of metallic nanoparticles changes the gold nano-particle surface Plasmon from red to purple. The aggregation of nanoparticles is caused by inter-particle aggregation and non-cross-linking aggregation.

With detection technique based on inter-particle cross-linking aggregation of gold nanoparticles, Mirkin *et al* described an approach for miRNA targets with thiolated oligonucleotide modified gold nanoparticles (AuNP probe). They utilized two distinct probes and two different sets of AuNP-probe and aggregation occurred by the addition of linker which complementary to the two probes. Later they utilized single stranded DNA probe with the detection limit of femptomolar range [25]. With non-cross-linking aggregation of gold nanoparticles technique, Maeda *et al* developed the first biosensor to increase the sensitivity of the biosensor. Though many amplification techniques are used to increase the sensitivity, they are time consuming, high process complexity. Hence, a simple, cost-effective method or quantifying miRNA was developed based on inter-particle cross linking aggregation shows the higher sensitivity for low detection limits [26]. The summary of various biosensors for the detection of miRNA biomarker with their diseases are list in the Table 3.

DISCUSSION

Most biological cycle are regulated by miRNA. The levels of expression are influenced by a variety of human diseases. The miRNA are undeniable important regulators of several diseases like cardiovascular health to perform critical

Table 3. Summary of biosensor for the detection of various miRNA biomarker [28-31]

miRNA	Electrode	Technique	Linear range	Disease	Ref.
miR– 141	AuE	SWV	0.1 fM – 0.2 pM	Bladder cancer	[43]
miR-21	GCE/CNN/AuNP	SWV	10 fM – 1 nM	Gastric cancer	[44]
miR-155	GCE/Mo_2C/AuNP	DPV	0.1 fM – 1 nM	Lymphotic leukemia	[45]
hsa-miR-17-5p	AuE	LSV	100 aM – 0.1 nM	Breast cancer	[46]
CEA	Anti-CEA/PEDOT/Ag@BSA/rGO/CNT	CV and EIS	0.002-50 ng/mL	Colorectal cancer	[47]
p53	CdS NC/GO-AuNP	ECL	20-1000 fg/ml	Osteosarcoma	[48]
miR-155	MoS_2 FET	FET based sensor	0.1fM – 10 nM	Breast Cancer	[49]
miR-122	rGO/Au nanocomposite	SWV	10 µM – 10 pM	Liver Diseases	[50]
	Single labeled fluorescent probe	Fluorescence	2.5 – 500 nM		[51]
	Double-Strand Displacement Biosensor and Quencher-Free Fluorescence strategy	Fluorescence	5 – 1000 nM		[52]
	CdTe QDs – RLS sensor	RLS	0.16 – 4.8 nM		[53]
	Label-free resonance light scattering sensor	Resonance based light scattering (RLS)	200 pM – 20 nM		[54]
	rGO/Au nanocomposite modified electrode	DPV	10 pM – 10 µM		[55]
miR-32 miR-122	AuNP SPE/p19 protein	SWV	10 aM – 1µM	Liver Cancer	[56]
Let-7a	Str-AuNP biocomplex	DPV	100 fM – 1 nM	Tumor Suppressor	[57]
miR -182	Ferrocene–capped AuNP/Streptavidin conjugate	Amperometry	0.01 pM – 700 pM	Hepatocellular carcinoma	[58]
miR-21	LNA-hairpin/Bi-DNA/str-HRP/AuNP	Amperometry	0.01 pM – 700 pM		[59]
	Hemin-G-quadreplex/hairpin DNAzyme/hairpin/AuNP	EIS	5 pM – 5000 pM		[60]
	HRP/AuNP	Amperometry	1 fM – 100pM		[61]
	SPE/rGO-Au	DPV	1 µM – 1pM	Breast Cancer	[62]
	GCE/MWCNT	DPV	0.1fM – 5 pM		[63]
	NFG/AgNPs/PANI electrode	DPV	10 fM– 10 µM		[64]
miR-126, miR-208a, miR-21	Electrical Double Layer (EDL) gated AlGaN/GaN high electron mobility transistor (HEMT)	Electrical Measurement	1 fM	Cardiovascular Disease	[65]

function. The review clearly showed numerous studies were observed for cancer detection and cardiovascular detection [35, 36, 38]. But no specific miRNA biomarkers are recognized for the diseases. Several difficulties need to be rectified to identify the biomarker or combination of biomarkers which are specific to diagnosis and prognosis of various health problems. Likewise, different combination of detection methods and technologies were discussed with their limit of detection. This clearly shows the sensitivity of the electrode configuration for the biomarkers based on their level in the sample. The technological improvement evidently shows very sensitive electrode (atto-molar concentration) can be designed or fabricated with different hybridization techniques for ultra-sensitive detection.

This review gives a broad overview of recent developments and state-of-art research on different types of nanomaterials that have been used to functionalize the FET to make ultra-sensitive electrochemical biosensor for miRNA detection. Likewise, label-free and amplification free, no sample preparation, very less sample volume with ultrasensitive detection of analyte especially miRNA can be achieved through optical technology especially surface plasmon resonance (SPR). Graphene-based nanosensors have demonstrated exceptional superiorities in electrochemical biosensors because of its superior conductivity and biocompatibility, as well as variety of other intriguing features. Additionally, transition metal dichalcogenides especially MoS2 shows promising growth in biosensor. Based on graphene and MoS2 properties, they would be novel nanohybrid for miRNA detection and further studies on nanohybrid should be required.

From the table, proposed miRNA biomarker has benefits and limitations in terms of disease specific biomarker. Some of the limitations observed from this review are lack of sample size, prospective investigations, multi-disease biomarker and specific and non-specific geographical population studies. The study of cardiovascular miRNA biomarkers includes subjects with cardiovascular issues and healthy one. Thus miRNA specific to both cardiovascular and other disease had never been studied or included in the study. Hence correlation of miRNA biomarkers with different disease should be studied to improve the specificity of the any sensor. Hence disease specific miRNA along with biomarkers should be included in a panel for effective. The continued development of these approaches offers hope for next generation lab-on-a chip devices that can achieve the technology's goal such as point-of-care clinical analysis.

CONCLUSION

The review addressed the biogenesis of miRNA, the isolation of miRNA from the sample and various detection methods available in the research. The intriguing realm of miRNA related studies and clinical application is enormously evolving. Based on the review, the following knowledge is gained.

(i) The miRNA potential has a great potential for disease diagnosis and management. Still the transfer of research to commercial product requires nanotechnology and technical

advancement, sensitive, repetitive, and reliable results.

(ii) The high performance, ultra-sensitive, easy to manufacture, cost-effective and highly specific detection of miRNA biomarker for various diseases can be carefully designed with utmost importance to characterization of the electrode and proper, chemical-free hybridization with stable performance and no prior preprocessing of sample.

(iii) Graphene and MoS2 nanomaterials and its nanohybrid shows promising results for nanosensors to detect miRNA from biological fluids.

(iv) To increase sensitivity with respect to disease specific miRNA, further research should emphasis on “fingerprint miRNA” for each disease.

FUTURE PERSPECTIVE

Latest studies and research shows that miRNA play a crucial role in early diagnosis and therapy management of diseases. Further in future, it will be a huge boom in implementing the nano-biosensor for the detection of miRNA for early detection of diseases with less turnaround time and can be effectively implemented in point-of-care devices [41, 42]. Hence, a sensor based on nanoparticles modified electrode that are cheap, ultrasensitive, commercially accessible and easily operable with significant influence on early disease diagnosis especially acute myocardial infarction by utilizing the biological fluids such as blood. Rapid point-of-care (POC) diagnostics are an important method for accomplishing these objectives and allow for reliable evidence-based diagnosis and triage choices especially in rural, low-resource settings areas. These tests offer key information about the miRNA cardiac marker with more simply and rapidly, rapidly allowing staff to identify patients and improve health outcomes.

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