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Review Article

Research progress in molecular biology related quantitative methods of MicroRNA

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Abstract: MicroRNAs (miRNAs) are small RNAs of 18-25 nucleotides in length that are widely distributed in eukaryotes and are produced by DNA transcription. As regulators of post-transcriptional gene expression, it plays an important role in the physiological processes of cells. As some miRNAs in the body are abnormally expressed at different and earlier stages of diseases, this phenomenon suggests that accurate, sensitive and specifical detection of them can be helpful for early and differential diagnosis. To expound the technological progress of miRNA detection, we reviewed all the related articles in PubMed database published before May 6, 2019, with the following keywords: "miRNA", "real-time fluorescent quantitative PCR", "electrochemical detection", "next-generation sequencing", "digital PCR technology". Original articles and reviews on the topics were selected. The present methods established for quantitative detection of miRNAs mainly relies on various probe design and labeling techniques, and the improvement of the sensitivity and specificity of detection is often through combination of microarray chips, real-time fluorescent quantitative PCR, high-throughput sequencing and other techniques. This paper combines the existing microRNA detection methods to provide a reference for researchers to choose the best detection method.

Keywords: miRNA, real-time fluorescent quantitative PCR, electrochemical detection, next-generation sequencing, digital PCR technology

Introduction

With the application of cutting-edge science and technology in the field of biological detection, more sensitive and accurate detection methods have been developed for testing microRNA (miRNA), these novel technologies have deepened our understanding of miRNAs [1]. Researchers have proved various regulatory roles of miRNAs in a variety of body fluids, including semen and saliva, and are associated with the pathogenesis of various diseases such as gynecological, obstetric and autoimmune diseases. The detection of miRNA expression profile can provide an rapid and objective basis for the diagnosis of relevant disease and early medication, so it can be used as a biomarker with high application value, and circulating miRNAs can be the best candidates for their rather stable form in serum [2]. However, some intrinsic properties of mature miRNA fragments make them hard to be detected. This leads to the following bottlenecks in the quantitative detection process [3, 4]: A) small size of sequence is an obstacle to design primers and probes; B) a high degree of similarity between family members, miRNA sequence of the same family only have 1-2 bases difference; C) low expression level in tissues and cells of different stages; D) miRNA precursors interfere with the specificity of detection. In order to overcome the above challenges, great efforts have been made to establish a variety of methods for quantitatively detecting miRNA during a long period of time, new motheds are faster, simpler, high-throughput and with high sensitivity and specificity [5]. This paper combines the existing methods of microRNA detection to pro-

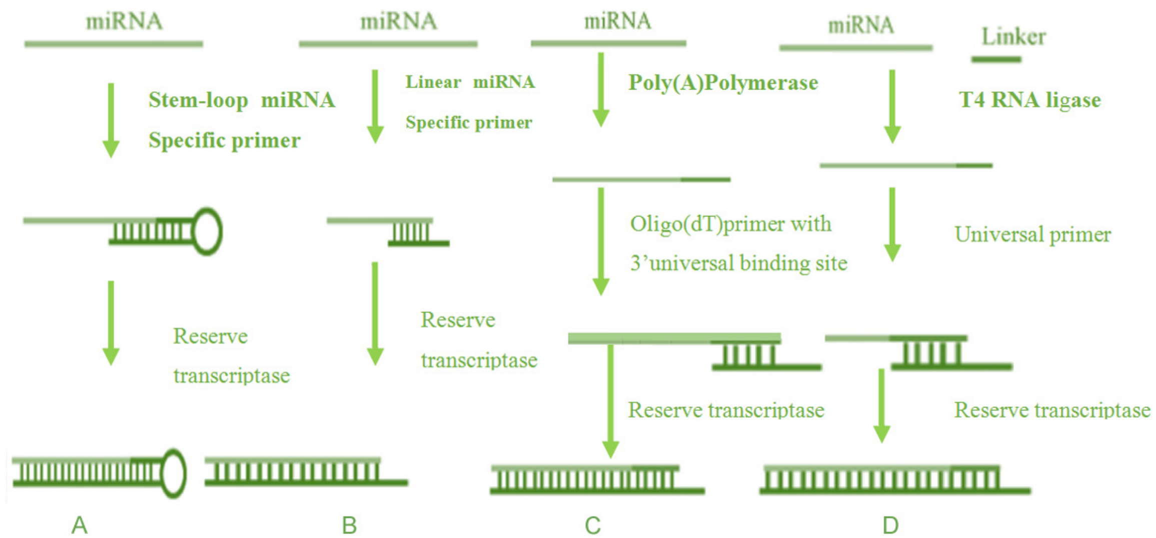

Figure 1. Zhu SR, Wu X, Chen YJ. Current progress in quantitative detection of microRNA [4]. qRT-PCR approaches for quantitative analysis of miRNA. Reverse transcription of individual mature miRNA using stem-loop (A) or linear (B) Primers and enzymatic tailing of the miRNA by using poly (A) polymerase (C) or T4RNA ligase (D).

vide a reference for researchers in making choice.

Nucleic acid amplification techniques

Real-time fluorescence quantitative PCR (qPCR)

Q-PCR is currently the most widely used detection technology based on polymerase chain reaction and application of fluorescent probe or primer. Two steps was involved in the process of detecting, which is firstly the synthesis of complementary DNA (cDNA) from a miRNA template, via reverse transcription, followed by quantitatively detecting the reverse-transcribed cDNA using normal real-time fluorescent PCR [6]. QPCR is a gold standard method of miRNA analysis that is difficult to be completely replaced in practical applications due to its many advantages (stability, good operability, good reproducibility, programmed design, and commercialization of reagents). The main obstacle to the detection of miRNA by qPCR is that the sequence is too short, usually consisting of about 20 bases, and it is difficult to design primers for reverse transcription and quantitative PCR by conventional means. Moreover, miRNA does not have A poly (A) tail like mRNA, so it cannot utilize this tail for enrichment and universal reverse transcription reaction [7]. In order to solve these problems, current miRNA detection methods based on RT-PCR mainly include the following designs (**Figure 1**).

The first way mimics the qPCR for quantitative analysis of mRNA, a poly (A) terminus is added to the short miRNA by a separate polymerase extension process prior to reverse transcription

The extension process is similar with the RNA tailing modification in vivo to some extent, which adds a poly (A/U) tail to the sequence of the target miRNA by RNA Poly (A)/Poly (U) polymerase. This is convenient for primer design and amplification. Shi et al. reverse-transcribed miRNAs with poly (A) tail added by the method described above, the primers used contain miRNA-specific forward primer and poly (T) adapter, then quantitative PCR reaction successfully performed [7]. This method allows multiple miRNAs to have a common tail sequence, conducive to the design of universal primers and improve experimental throughput. However, the introduction of the enzyme reaction makes the system more complicated, and, it's not applicable to some plant-derived miRNAs because the 2'-O-methyl-modifications of their 3' terminal ends prevent the terminal extension reaction.

The second way is to make a structural design of the primers

In order to increase the hybridization efficiency, melting temperature and specificity of reverse transcription, the primer is designed as a spe-

cial structure. Androvic et al. designed a two-tailed RT primer with partial bases hybridize to the miRNA at both the 3' and 5' ends, which is equivalent to extending the length of primer and increasing the stability of the hybridization [8]. Another highlight of this design is the introduction of a stem-loop structure between the 3' and 5' ends to increase the synergistic effect at both ends of the primer. This special structural primer designed for the reverse transcription increases the difficulty and complexity of the design process, while ensuring the specificity and sensitivity of the assay, but reduces the versatility of the design that means each miRNA requires a separate primer design [9]. Based on stem-loop reverse transcription and SYBR Green qPCR detection, Xue HH et al. established an improved method of qRT-PCR, which can not only directly judge the specificity of PCR reaction through melting curve, but also overcomes the shortcomings of low sensitivity and specificity of conventional SYBR Green qPCR by longer stem loop primer [10]. Others reported that the expression of miRNA-505 was rapidly, accurately and efficiently measured by a high efficient and specific stem-loop primer in the thalamus, pituitary, ovary, muscle and adipose tissue of two strains mice [11].

The third is the simplest design, the base-modified design

The principle of this design is to introduce Locked nucleicacids (LNA) into the primer for a reverse transcription or for PCR, the purpose of doing is to increase the melting temperature of the hybridization. It has been found that the hybridization stability can be greatly improved between the primers containing such artificial nucleotide-coupled bases and their complementary nucleic acid. Raymond et al. constructed a miRNA RT-qPCR assay by primers containing LNA to achieve efficient miRNA detection. [12] Qin ZF [13] also established a duplex locked nucleic acid real-time RT-PCR (Duplex LNA rRT-PCR) based on probes containing novel LNA for identifying virulent and attenuated strains of Newcastle disease virus (NDV). Two novel LNA probes were designed for the NDV gene cleavage site. The specificity of the method was verified by the detection of non-NDV positive samples. The sensitivity of the method was determined by detecting the paramyxovirus type I (APMV) and virulent (velogenic and mesogenic) NDV in different concentrations of virus solution. The result showed that the specificity for the detection was 100% (22/22). The sensitivity of the established Duplex LNA rRT-PCR to detect the virulent strain F48E9 and the attenuated strain LaSota was 10 EID50 and 0.1 EID50, respectively [13]. This method does not require complex primer design and does not require an enzymatic reaction, but introduces chemical modification, which increases the cost of the experiment [7, 12].

The confirmation of qPCR reaction system including several aspects: (A) Determination of primer concentration. The concentration of qPCR forward and reverse primers primers is concentrated between 0.1-1 μmol/L, especially at 0.1-0.2 μmol/L. Increasing the concentration of forward and reverse primers can improve the detection sensitivity, but it also leads to the increase of non-specific amplification; (B) the number of cycles. The number of qPCR cycles is concentrated between 35 x-45 x. The increase of the number of cycles will also lead to non-specific amplification. The results are generally believed when analyzing the data from cycles between 18-25 (C). What kind of qPCR detection is the most appropriate is still inconclusive, always has the same trend according to the detection specificity and the cost of experiment: specific probe method ≥ general probe method ≥ Sybr green dye method, comprehensive consideration should be given to the actual situation. Some literature compared different PCR methods. Sharbati-Tehrani S et al. [14] compared poly (A) tailing and stem-loop RT-PCR methods for miRNA detection in plants, suggesting that stem-loop RT-PCR detection is more accurate and trustworthy than polyA tailing due to methylation of plant miRNAs. Jung et al. [15] compared the specificity and sensitivity between universal probes and specific probes used for stem-loop RT-qPCR, the result was consistent, but using universal probes can greatly reduce the experimental cost. Varkonyi-Gasic et al. [16] showed that the stem-loop RT-PCR using the universal probe had better specificity than using the Sybr green dye. On the whole, poly (A) tailing method is cumbersome, but due to the introduction of universal poly (A) tail, reverse transcription can be performed using universal reverse transcription primers, which is suitable for miRNA expression profiling. The stem loop RT-PCR has high sensi-

tivity and is suitable for the specific detection of a few miRNAs [17]. If multiple miRNAs are to be detected simultaneously, the reverse transcription primers of these miRNAs can be mixed and reverse transcribed, but mixing may lead to non-specific amplification [18]. Therefore, it is best to determine the suitability of these primer combinations by pre-experiment before reverse transcription.

Digital PCR technology

Digital PCR (digital PCR) is a new technology that has been applied to the absolute quantification of miRNAs [19, 20]. It mainly composed of limited dilution PCR and fluorescence signal analysis. Limited dilution is the dispersion of DNA molecules into a large number of reaction chambers by manual dilution, microreaction valves or droplet generators. Each reaction chamber contains one or zero target molecules, then each reaction chamber is subjected to PCR amplification of the target molecule, and the fluorescence signal of each reaction chamber is analyzed after the end of amplification, and the fluorescence signal is read as 1, if no fluorescent signal is detected, it is interpreted as 0. The data obtained is analyzed by Poisson probability distribution function, according to the total number of reaction chambers, the number of cells containing the fluorescent signal and the dilution coefficient of the sample, the initial concentration in the sample can be obtained [21]. Since digital PCR is a terminal analysis method, if the target molecule is not well discretized (some reaction chambers contain multiple target nucleic acid molecules), the results obtained will be theoretically inaccurate (**Table 1**).

The method is completed in 4 steps, the first step is to mix the system evenly including the primers, probes or dyes (such as Taqman probe or EvaGreen dye) and the special digital PCR reaction solution. The second step is to use special instruments and reaction plates to prepare droplets, more than 10,000 droplets, each droplet corresponds to a single PCR reaction; the third step is to transfer the sample containing the droplet into the ordinary 96-well PCR plate and perform common PCR amplification; the fourth step is to achieve absolute quantification of genes by the fluorescent signal detection of PCR amplification products, using the special instruments [22]. The biggest feature of this technology is the improvement of instruments and reagents, which enables the absolute quantitative detection of trace samples, while the biggest disadvantage is that special instruments, reagents, and consumables must be used, which is expensive to operate. Currently on the market, commercially available instrument platforms for chip-type PCRs according sample dispersion mode are BioMark, QuantStudio3D and Starry sky 10k. Fluidigm's bio-mark HD system and THermo fisher's quants-todo3D system are representatives of chip and microdrop Chip, which uses microfluidic technology to evenly distribute samples into the independent reaction units that thousands to tens of thousands existing in the chip [23]. Starry sky10k system based on the counting principle of "Single-molecule polymerase chain reaction", through the chip-type dispersion system, the target sequence is fully separated in 10,000 micro-reactors, by identifying the proportion of positive and negative amplification results and special algorithm statistics, the copy number of the target nucleic acid can be directly obtained in an absolute quantitative manner.

Microarray

It is a method of miRNA high-throughput analysis, which enables simultaneous quantitative determination of multiple miRNAs in a sample, and is widely used in differential analysis of miRNA expression profiles [24]. In this technique, high-density probes of known sequence are immobilized on a solid support, which can hybridized with certain miRNAs, thereby obtaining the expression profile of specific miRNAs base on detecting and analyzing the intensity and distribution of the signal after hybridization. High throughput is the biggest advantage of miRNA detection in microarray microarrays, but it is often accompanied by false positive results. Otherwise, it also have shortcomings in gene chip fabrication and detection costs, probe types and specificity [25]. So, it's often used for the preliminary screening of miRNAs [26], and it is difficult to be popularized in ordinary laboratories. The flow cytometry based on microsphere hybridization improves the deficiencies of microarray chips. A microsphere can be seen as a point on traditional microarray chip. The improvement is not only to increase the ability to capture the target miRNA to be tested, but also avoids cross-reactions in the

Table 1. Comparison of dPCR technology platform

System	Volume of reaction (uL)	Detection of throughput	Number of reaction units per sample	Elapsed time	Multiple ability (fluorescent channels)
QX200	20	96	220000	Droplets generated -2 min/8 Samples; PCR-2 h	2
RainDrop	25-50	8	5000000-10000000	Chip reading -1 min/chip, Droplets generated -30 min/8 Samples; PCR-3 hh	2
Naica	20	12	25000-30000	Droplet test -4 h/8 Samples; Droplets generated -10 min/12 Samples; PCR-2 hh	3
Quant Studio	14.5	24	20000	Droplet test -10 min/8 Samples; Sample loading -4 min/chip; PCR-2.5 h	2
TD-1	20-50	1-96	50000-100000	Droplet test -15 min/12 Samples; Droplets generated -4 min/8 Samples; PCR-2 hh	2

solid-state chip. In conclusion, although microarray chip realizes high-throughput detection, the technical support for the discovery of new miRNAs is weak and further improvements are needed.

New methods for microRNA detection

Next-generation sequencing technology

Since 2005, new generation technologies of high-throughput sequencing have made rapid progress. Up to now, these next-generation sequencing (NGS) technologies have been used to comprehensively analyze the differential expression of functional genes, miRNA expression profiles and genetic materials by transcriptome sequencing, small RNA sequencing, and degradation group sequencing. It helps multi-faceted analyzing of molecular and biological functions at genomic level, transcriptome level and protein level, which make them play an important role in the research of related fields [27, 28]. Among NGS technologies, two first to be introduced technologies are Roche's 454 pyrosequencing and Illumina (Solexa) sequencing. Both of them can qualitatively and quantitatively detect the gene expression level of miRNAs, not only can they measure the abundance of miRNA expression, but also can detect small changes in the length and sequence of known miRNAs, and are important tools for mining new miRNAs. Two sequencing methods differ in sequencing principle, data volume, data quality, and cost, but they are all based on sequencing by synthesis, and the sequencing steps are basically the same, mainly including: sample preparation, template preparation, sequencing and imaging, small RNA reads assembly and alignment, etc [29]. The 454 pyrosequencing technology performs PCR in microemulsion, that is, each fragment obtained after cleavage is separately bound to one microbead, and the only fragment bounded on microbead are independently amplified in oil droplets containing the PCR reaction system. The double-stranded DNA on the microbead is denatured into single-stranded DNA, then microbeads were added to a test plate in which micro-uppe holes are arranged and pyrophosphate-based sequencing of the fragments are performed on their surfaces. While the method of Solexa sequencing technology is using bridge PCR to amplify [30], sequencing with 3'-blocked reversible Terminator which ensures each time only one nucleotide can be added by polymerase, and specific wavelengths of fluorescence that represent specific nucleases are detected and recorded. The 454 sequencing technology has the advantage of being able to detect sequences of the longest length among second-generation sequencing, and is important in the search for new miRNAs and transcriptome sequencing. However, for pyrosequencing has no termination element to prevent continuous incorporation, the number of identical bases can only be inferred from the signal strength in the case of continuous identical bases such as AAAAA, resulting in insertion/deletion errors. Otherwise, this method relies on a series of enzymes for quantitative detection of miRNAs, so increases the false positives of detection. The advantage of Solexa squencing is that the throughput of sequencing is very high, and the Illumina Hiseq X platform is currently the highest. The main disadvantage is the relatively shorter read length due to the equal attenuation and shift of optical signal. In addition, the Solexa Genomic Analyzer incorporates one base per primer extension, and resulting in an increased probability of genetic mismatch. The accuracy of the Solexa sequencer is over 99% within a 50 bp read range, this feature makes it a novel detection method that goes hand in hand with the qRT-PCR method. In addition to these two methods, SOLID sequencing technology [31] introduced by ABI in 2007 is also one of the main sequencing platforms before 2012, with the highest accuracy (99.99%) in the second generation methods [32], it is the only method that does not use traditional polymerase for sequencing. The biggest difference from the previous method is that 16 oligonucleotide fragments can be distinguished by 4 different fluorophores. However, the experimental operation process of SOLID sequencing is far more complex than other methods, and its "double base Decoding" causes the disadvantage that it is difficult to be compatible with other sequencing software. Plus, the reading length of SOLID is limited and developed slowly after 2010, and SOLID system was eventually exited the competition of sequencing market after 2012. Detailed comparison of advantages and disadvantages of each sequencing technology platform is shown in **Table 2**.

For second generation sequencing platforms, they have two shortcomings. One, the read length is relatively short. Second, the second generation sequencing technology is based on PCR, so it is difficult to guarantee that amplified molecules does not have deviate from the templates in number, which has a greater impact on gene expression analysis. So, the third and fourth generation sequencing technology came into being, they do not require PCR amplification, and have the characteristics of higher throughput, longer reading, higher accuracy, shorter sequencing time and lower cost. They mainly include true single molecular sequencing (tSMS), single molecule real-time sequencing (SMAT), fluorescence resonance energy transfer (FRET), and the United Kingdom company's nanopore single molecule sequencing (Technology) [33]. The representatives are single molecule real-time sequencing of Pacific BioSciences and Oxford Nanopore. The third generation technologies are mainly used for whole genome sequencing, targeted gene sequencing and mRNA full-length sequencing, etc [34]. At present, the second generation sequencing system is still the best choice for miRNA sequencing. (see **Table 2** for the advantages and disadvantages of each system).

Electrochemical detection based on enzymatic signal amplification

Enzymes have catalytic activity that allows a single hybrid molecule to be converted into a large number of monitorable molecules. Therefore, enzymes can be used as labels for bioanalysis, this feature promote increasingly applications of enzyme-based electrochemical methods implemented in miRNA detection and analysis [35]. In 2012, Wang Cui combined the two amplification techniques of circular strand-displacement polymerasereaction (CSDPR) and hybridization chain reaction (HCR) to successfully construct an electrochemical sensor for ultrasensitive detection of DNA. The hybridization between hairpin-like capture probe and the target DNA results in a conformational change in the capture probe and exposes the stem sequence. Then, the exposed stem sequence hybridizes to the initiation strand and initiates CSDPR. At the same time, the initiation chain can also initiates an HCR reaction in the presence of two biotin-labeled hairpin probes, following the alkaline phosphatase-labeled streptavidin to be captured for electrochemical detection. This method has been successfully applied in the quantitative analysis of p53 gene mutations [36]. Lin et al. [37] established the third-generation E-DNA sensor with further improved sensitivity suitable for detecting miRNAs, in which the thermodynamic stability of the stem-loop structure DNA well regulated by DNA tetrahedron to reduce the background signal and enhance the reaction specificity, and then stronger electrocatalytic signal by attached enzymes can be effectively captured to amplify the detection. This approach has significant advantages in distinguishing the members of highly conserved miRNA family. Deng et al. [38] hybridized the target miRNAs to DNAzyme-capped capture probes (DZ-CPs) bound on special magnetic beads, and then cut it with a specific nuclease, removed the unreacted DZ-CPs and magnetic beads by a magnet. The rest DNA molecules in the solution can be used as catalysts to promote color reaction of 3,3',5,5'-benzidine in the presence of hydrogen peroxide, and then high sensitivity detection achieved by the colorimetric method. Although the method of enzyme signal amplification meets the requirements of high sensitivity and high specificity for miRNA assay, there are still defects such as the difficulty of designing sequences for template amplification and high background signals. Therefore, the enzyme-assisted electrochemical detection of miRNAs still needs to be greatly improved [39].

MiRNA analysis by ligation-based identification

The principle of the ligation-based miRNA identification

It mainly includes the following aspects: Firstly, both two DNA probes are designed to hybridize with target miRNAs, and then ligases was added after hybridization to connect the perfectly matched two probes into a complete probe, which finally initiates subsequent signal generation. The representative of such methods used in miRNA detection is the rolling ring amplification technology (RCA) [7], it also known as rolling circle replication [40].

miRNA detection by RCA-based methods

Jonstrup et al. [38]. first used RCA technology for miRNA analysis in 2006. In this case, a padlock probe was used as the template for a poly-

Table 2. Comparison of the various NGS technologies

Platform	Technology	Amount of data generated in a single run (GB)	Average reading length (bp)	Advantages and disadvantages
Illumina genome analyzer	Synthetic sequencing	Up to 900 (Illumina HiSeq X is currently the highest-throughput instrument available)	Up to 300	Advantages: Small number of samples required; Data error is small; The process is simple Disadvantages: The error rate accumulates with the read length
Roche454 genome sequencer	Parallel pyrophosphate synthesis sequencing	Up to 0.7	Up to 1000	Advantages: The reading length of the sequence is the longest in Second-Generation Sequencing, which is mainly used for genome and transcriptome sequencing of new species Disadvantages: The continuous incorporation of homopolymer produces errors. Reagent prices are relatively high
AB Life technologies' SOLiD system	Magnetic bead based parallel clone connected DNA sequencing	Up to 320	Up to 75	Advantages: The highest accuracy; It is suitable for small fragment RNA study Disadvantages: Sequence reads are relatively short
Ion Torrent	Detection of pH change by ion sensitive Ion sensitive field effect transistor (ISFET)	Up to 15	Up to 600	Advantages: Incorporation of nucleic acid bases can be determined directly; DNA synthesis under natural conditions (modified bases are not required) Disadvantages: The step-by-step elution process can lead to the accumulation of errors; There are potential difficulties in reading highly repetitive and homopoly sequences
Pacific Biosciences Sequel	Synthetic sequencing Fluorescence/Optics	Up to 7	Up to 350,000	Advantages: High average reading length, lower than the sequencing time of the first generation; No amplification Disadvantages: DNA polymerases cannot be added to sequencing array efficiently; The chance of accuracy reaching the standard at one time is low; DNA polymerases are degraded in the array
Nanopore PromethION (Beta)	Electrical current	Up to 4000	Hundreds to thousands of kb	Advantages: Nanopores can be produced at a cost; No fluorescent labeling or optical means are required Disadvantages: Severed nucleotides may be misdirected; It is difficult to produce devices with multiple parallel holes

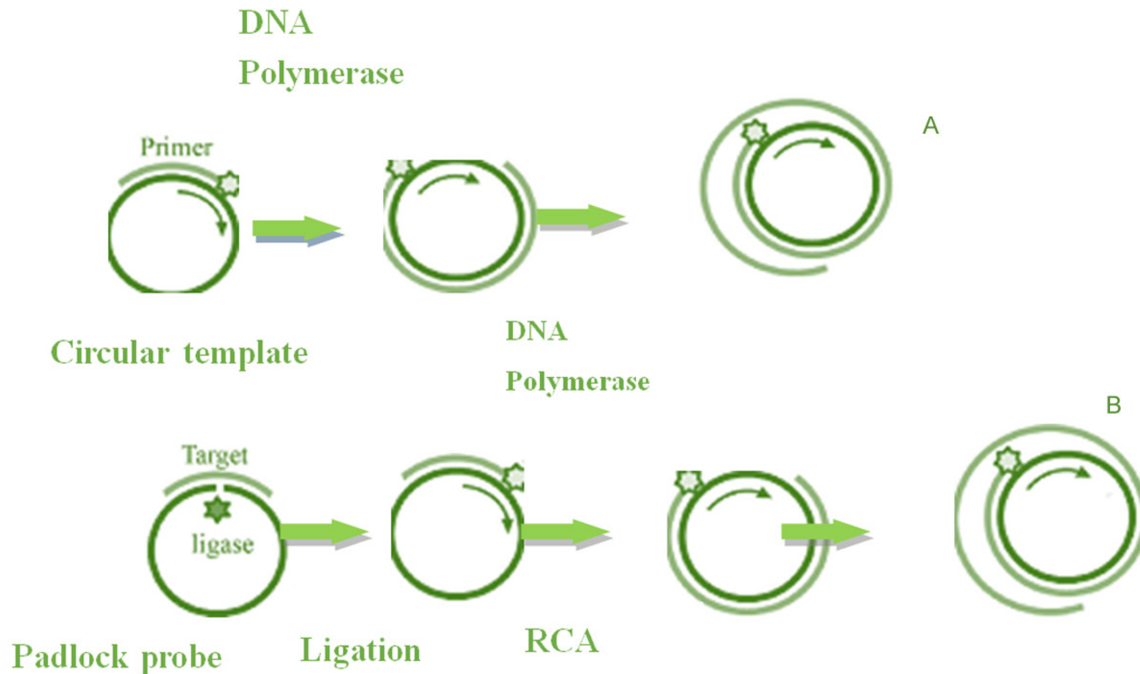

Figure 2. Rolling circle amplification (RCA) (A) and Padlock-RCA (B) [39]. Jonstrup SP, Koch J, Kjems J. A microRNA detection system based on padlock probes and rolling circle amplification.

merase, namely the primer-mediated extension polymerization was based on the special DNA polymerase phi29 (**Figure 2**). The characteristics of the RCA reaction is that the reaction product can theoretically extend infinitely, so it is a very sensitive analytical technique for signal amplification at constant temperature. Specifically, This technology uses the target miRNA as a ring-forming template for a semi-annular padlock probe, that is, under the action of miRNA, the padlock-type probes can be ligated into loops by RNA ligases with the target miRNA as the template, and the ligated loop as a template in turn is amplified with the target miRNA as a primer, products are identified by Northern blot. Cheng et al. [41] also established a method for miRNA detection based on RCA in 2009. Unlike the former, this method introduces a hyperbranched RCA amplification technique, which is to add a second primer based on the original amplification. The sequence of the second primer is identical to the partial sequence of the circular RCA products, so that the products of the RCA once again as a template can be further amplified by hybridization with the second primer, to achieve the purpose of further improving the sensitivity. Furthermore, the way of signal generation is also improved, the fluorescent dye SYBR Green I is embedded in the nucleic acid to enhance the fluorescence intensity.

The second type of method for miRNA identification by ligation

First of all is the ligation-mediated PCR reaction. In 2011, it was reported that a size-coded ligation-mediated PCR was established [42]. It's based on miRNA ligation to generate PCR templates of different lengths. After PCR, different bands are obtained by electrophoresis of products, which can achieve highly sensitive and specific multi-miRNA analysis, but this method cannot effectively quantify miRNA. The research team of Li et al. established a method for quantitative analysis of miRNAs based on such way. Two short probes containing upstream and downstream primers bind to the target miRNA and are linked into a PCR template, and then the quantitative PCR is performed [43]. In addition, the research team still improved the method by implementing single-copy detection based on digital PCR and multiple miRNA analysis. The high sensitivity of ligation-mediated miRNA detection is usually due to the sensitive monitoring technology coupled. The detection limit of the RCA-based method can reach 10 fM, and the method based on

quantitative PCR can reach 0.2 fM, and it can reach 20 aM based on Digital PCR. In addition, since the ligation reaction is highly demanding for base matching, this method has extremely high sensitivity and is enough for distinguishing single base mismatches. Comparing to RCA technology, PCR technology increases the throughput of some experimental parts, but has highly demanding of an instrument for variable temperature reaction. RCA technology enables a constant temperature reaction that reduces the precision of the instrument and can be developed for on-site analysis (**Table 3**).

Technology of nanoparticle signal amplification

In recent years, there are many methods established for detecting miRNAs through using nanoparticles to construct biosensors, which is a research hotspot that has been widely concerned due to its unique photoelectric property, size effect, and surface effect. Its classification is also diverse, according to methods of downstream signal detection, can be divided into optical signals, electrochemical signals, mechanics, thermals and so on. There are some recent studies reported below. Vance S A et al. uses surface plasmon resonance imaging (SPRI) technology and surface polymerization for miRNA detection. The principle is as follows. Firstly, a linker sequence is specifically bound to the target miRNA, then the complex is hybridized with a single-stranded capture probe containing LNA residues (on the surface of a gold-coated chip) and a gold Nanoparticle (AgNC)-labeled signal probe (composed of about 30 bases T). Following the sandwich complex formed, the result was obtained via SPRI technology [44].

Obtaining readings based on the good conductivity of nanomaterials which enables the electrons to move freely between the surface of electrodes and biomolecules, this technology has obtained ideal results [27]. Yin et al. immobilized biotin-labeled single-stranded signal DNA and Reporter LNA on gold nanoparticles to form a DNA-AgNCs-LNA bio bar codes, while the hairpin probe on the electrode can specifically hybridize with the target miRNA (miR-21) and expose binding site for Reporter LNA in the bio bar codes, thus formed complex can be ultra-sensitively detected by the enzymatic amplification signal when the added streptavidin-labeled horseradish catalase bind to the biotin in the bio bar codes [45]. Dong et al. applied a molecular beacon (MB) coupled with Hg^{2+} to bind target miRNA under the help of assistant probe, bonding results in release of Hg^{2+} ions that can make the fluorescence of AgNCs quenching and the quenching is monitored for miRNA quantification, furthermore, with the aid of endonuclease the assistant probe can be regenerated and reused. This technology not only avoids complicated temperature control and labeling, and greatly improved miRNA detection with a linear range from 10 pM to 1 fM [46]. In 2018, Zhao et al. prepared nanoparticles and the electrochemical sensor of dual signal amplification for detection of miRNA. The DNA probe modified with CdS quantum dots on a magnetic bead, and the double-stranded structure of the probe is formed by hybridizing to a target miRNA and can be cut by double-strand specific nuclease (DSN). After enzyme cleavage, the miRNA free into solution for next hybridization and shear and obtain exponential amplification of the signal through multiple cycles, and the signal molecule CdS quantum dots can release a large amount of Cd^{2+} after dissolution, which can further amplify the response signal. Result signal is detected by anodic stripping voltammetry (DPASV), and the obtained electrochemical signal is linear with the concentration of the target miRNA. The miRNA in the cell extract was detected with a linear range of 0.001-10000 pM L^{-1} and a detection limit as low as 0.48 fM L^{-1} [47]. Nanoparticle technology enables miRNA detection to have high sensitivity, high throughput, and high specificity without the need for sequence amplification and reverse transcription, can enables instant detection. However, nanoparticles are susceptible to external solution pH or ionic strength. In addition, probe density also affects the efficiency of specific hybridization. Therefore, when selecting nanotechnology to detect miRNAs, we should try to avoid the above problems (**Table 3**) [48, 49].

Prospect

MiRNAs have become a hot spot in the field of biomedicine for their important roles in the pathogenesis of diseases. The present methods established for quantitative detection of miRNAs mainly relies on various probe design and labeling techniques, and the improvement

Table 3. Comparison of technical characteristics of miRNA detection methods

Type	Method	Sensitivity detection	Specificity of	throughput	Application characteristics
Traditional method	RT-PCR	high	middle	low	Accurate, suitable for extract detection, widely used, but the kit is more expensive
	Microarray	low	low	high	Large-scale screening of expression level, difficult to quantify, suitable for complex systems, high testing costs, high false positive
New method	Electrochemical detection based on enzymatic signal amplification	high	high	low	Low cost
	MiRNA analysis by ligation-based identification	high	high	low	Complex design, combined with a variety of signal conversion and amplification technology
	Nano gold labeling	high	high	high	Nanoparticles are susceptible to environmental effects
	Second generation sequencing	high	high	high	The testing cost is high and the application is limited

of the sensitivity and specificity of detection is often through combination of microarray chips, real-time fluorescent quantitative PCR, high-throughput sequencing and other techniques. For the reliability and intuitiveness of RT-qPCR analysis, a growing number of researchers have chosen this method to verify the expression level of miRNAs. Otherwise, as the cost of next-generation sequencing technology decreasing, its usage in related research is also increasing. However, each method has non-negligible defects. Therefore, developing a more economical, simple, rapid, sensitive, and high-throughput method for miRNA analysis can promote the progress of miRNA research and clinical application, especially for circulating miRNA in complex systems (such as serum), this may become the main direction of future research.

At the same time, the cost of NGS technology keeps decreasing, and the detection technology of miRNA high-throughput database construction by using sequencing analyzer develops rapidly. In recent years, the concept of target mRNAs for regulating gene expression or miRNA for disease detection has been established, which has become a hot spot for future research and development. Compared with traditional miRNA detective methods, With the advantages of high throughput, high quality, high accuracy and repeatability, the new generation sequencing technology, RCA and other detection methods are widely used in the prediction of new miRNA, screening of differentially expressed functional genes, prediction of miRNA target genes and detection of miRNA isomers.

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