

## *In situ* Detection of MicroRNAs: The Art of MicroRNA Research in Human Diseases

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### Introduction

MicroRNAs (miRNAs) are a class of evolutionally conserved small non-coding RNAs that serve as a post-transcriptional regulator of gene expression [1]. Since the discovery of first microRNA lin-4 [2], more than 1200 miRNAs have been identified in human genome. More than 30% of the coding genes are under the regulation of miRNAs [3]. It is therefore not surprising that miRNAs play critical roles in almost every biological process, such as cell proliferation, differentiation, apoptosis, and cell fate determination. Today, it is known that deregulation of miRNAs is associated with various human diseases, including cancer, metabolic diseases, cardiovascular disease and neurodegenerative disorders [4-7]. Emerging evidence show that miRNAs will be useful as biomarkers and molecular targets for novel therapy in human disease. Therefore, accurate detection, analysis and quantification of miRNAs are essential in those applications in which miRNAs are used as diagnostic or therapeutic targets. This mini-review will discuss the current technology on miRNA detection and quantification.

### miRNA generation

Most of the miRNA genes are transcribed by RNA polymerase II or III to form primary miRNAs (pri-miRNAs) [8]. In the nucleus, the pri-miRNAs are cleaved by microprocessor complex containing the RNase III enzyme Drosha and the double-stranded RNA-binding domain (dsRBD) protein DGCR8/Pasha to produce the precursor miRNAs (pre-miRNAs) [9]. Pre-miRNAs are 70~100 nucleotides in length with a stem-loop structure. Pre-miRNAs are subsequently transported into the cytoplasm by exportin 5 [10], where pre-miRNA hairpins can be further cleaved by another RNase III enzyme named Dicer [11]. After the double-stranded duplex is separated, the mature miRNA molecules incorporate into RNA-induced silencing complex (RISC), subsequently induce posttranscriptional gene silencing by translational inhibition or target mRNAs degradation [11] (Figure 1).

### Common Methods to Detect and Quantify miRNA

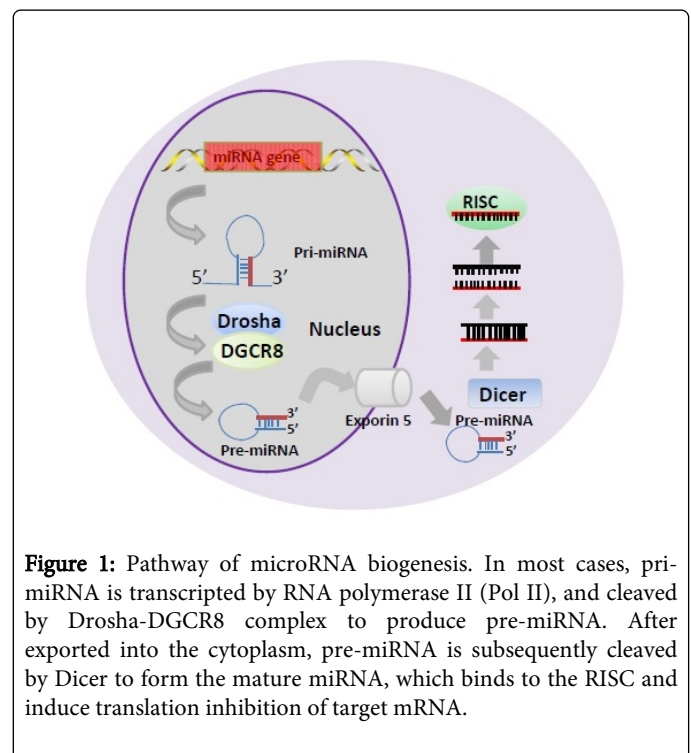
For miRNA detections, conventional methods include Northern blot analysis, qRT-PCR, and microarray (Table 1).

### Northern blot analysis

Northern blot analysis is the earliest detecting method used to assess miRNA expression profiles [12]. To be noted, different forms of miRNA, including pri-miRNA, pre-miRNA and mature forms, all can be detected from total RNA without amplification. To date, the probe designing and labeling techniques have greatly improved for Northern blot analysis [13]. Although Northern blot analysis is recognized as a time- and sample-consuming method, it remains widely accepted as one of the reliable approaches for miRNAs determination.

### Quantitative PCR

Currently, qPCR detection of miRNAs is the most widely used method. This technology is based on the reverse transcription which converts miRNAs to cDNAs. Two methods are often used for miRNA reverse transcription, including stem-loop RT primers and poly (A) tail addition methods [14,15]. In general, qPCR method is deemed as the major approach for miRNAs quantification.



**Figure 1:** Pathway of microRNA biogenesis. In most cases, pri-miRNA is transcribed by RNA polymerase II (Pol II), and cleaved by Drosha-DGCR8 complex to produce pre-miRNA. After exported into the cytoplasm, pre-miRNA is subsequently cleaved by Dicer to form the mature miRNA, which binds to the RISC and induce translation inhibition of target mRNA.

miRNA detection method	Advantages	Disadvantages
Northern blotting	Detection without amplification, all forms of miRNAs can be detected.	low-throughput, consuming time and large amount of RNA, hard to detect miRNAs with low copy numbers
qRT-PCR	quick and easy to perform, relative low cost and small amounts of sample consumption	amplification needed, generation of non-specific amplification products
Microarray	high-throughput	amplification needed, non-specific hybridization, not accurate and need to be confirmed

**Table 1:** The advantages and disadvantages of common methods to detect and quantify miRNA

### miRNA microarray

Microarray technology is a high-throughput method to provide essential information for miRNAs screening [16]. It is a powerful tool for the genome-wide analysis of miRNA expression. To increase accuracy of miRNAs quantification, new methods in miRNA labeling and probe design have been developed and used in miRNA microarray [17]. However, more accurate quantification technique is still to be explored.

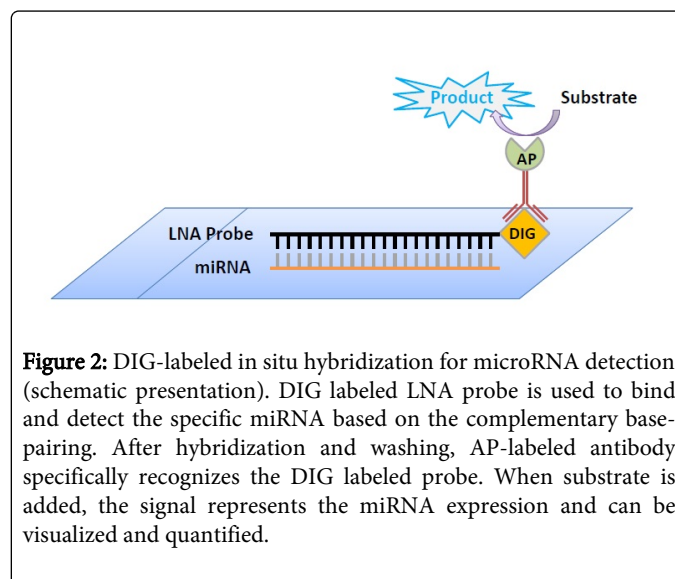
### *In situ* Detection of miRNA

The conventional methods of miRNA detection mentioned above are all indirect ways, in that the RNA from tissues or cells has to be first extracted before miRNA quantifications. As a result, these methods lose useful information about the spatial expression of miRNAs in tissue and cells. Evaluation of the spatial expression of miRNAs is especially crucial for those tissues with a number of different cell types. In 2004, *in situ* hybridization (ISH) technology was used for the first time in plants to assess miRNAs [18]. ISH technology is a direct and powerful technique to visualize the spatial localization of miRNAs at cellular level. Application of locked nucleic acid (LNA) probes in miRNA ISH has robustly improved the base-pairing specificity and stability of miRNA-mRNA complex [19]. Today, the ISH-based miRNA detection provides a reliable assessment of the physiologic function of miRNA at spatial location and the single-cell level [20]. For example, using fluorescence ISH system, the tumor-specific miRNAs miR-205 and miR-375 were respectively identified in two skin tumors, basal cell carcinoma (BCC) and Merkel cell carcinoma (MCC)[21]. Another published work showed that miR-21 is expressed in alveolar cells morphologically resembling alveolar type II cells in the normal lung and it is upregulated in cells surrounding fibrotic foci during lung fibrosis [22]. Therefore, *in situ* detection of miRNA not only demonstrates its expression, but also offers clues to possible functional roles.

### Current Methods to Detect miRNA *In situ*

A variety of miRNA ISH protocols have been published, for both tissue sections and cultured cells [23-25]. Fixation using paraformaldehyde is needed in cultured cells and cryosections in order to prevent the loss of miRNA during hybridization. For the paraffin embedded-, formalin-fixed tissue, microRNA ISH requires several additional steps. These steps, such as deparaffination and predigestion, are used to enhance the binding affinity/specificity and optimize permeability before hybridization. The same steps are performed after pretreatment, including hybridization, washing, blocking and detection.

For current miRNA ISH, LNA™ probes have been the most accepted, because that the use of LNA™ efficiently increases the specificity in target detection [26]. In addition, appropriate labeling method for LNA-probes is important given that a variety of labeling choices are available for ISH detection. The techniques for probe modifications include the labels with fluorescence, biotin, digoxigenin (DIG), enzymes such as alkaline phosphatase (AP) and horseradish peroxidase (HRP), as well as radio isotopes [27-31]. The fluorescence-labeled probe is especially a useful technique known as fluorescent *in situ* hybridization (FISH). To be noted, fluorescence-based probe is not sensitive enough to detect the miRNAs with low copies. In the presence of substrate, the enzyme which is conjugated to the probes catalyzes substrate into colored products, which can be detected and quantified. Biotin-labeled system has been used for nucleic acid detection, given that it is stable and highly sensitive. However, endogenous biotin in tissues can cause high levels of background after hybridization. Currently, DIG label is the most popular, convenient, and effective one for miRNA ISH. Compared with the biotin one, DIG system has much lower background. AP conjugated DIG antibody is often used to bind to the LNATM probe (Figure 2). Although the radioactively labeled probes are less used today, they remain the most sensitive method which can be used to quantify miRNA ISH [32]. As mentioned above, LNA-based miRNA ISH is an essential technique which provides a unique method to locate and quantify miRNA expressions in tissue and cells. Traditional methods, such as Northern blot analysis, qPCR and microarray, fail to locate the miRNAs within tissue or cells.



**Figure 2:** DIG-labeled *in situ* hybridization for microRNA detection (schematic presentation). DIG labeled LNA probe is used to bind and detect the specific miRNA based on the complementary base-pairing. After hybridization and washing, AP-labeled antibody specifically recognizes the DIG labeled probe. When substrate is added, the signal represents the miRNA expression and can be visualized and quantified.

However, miRNA ISH also has its own disadvantages. Strong background and absence of detecting signal are the major problems with miRNA ISH. An optimized miRNA ISH protocol with good signal-to-noise ratio is crucial. This optimizing process is often time-consuming, given that the concentration of buffer, the duration and temperature of hybridization all have to be adjusted for each probe in order to acquire the most accurate data.

In summary, ISH-based miRNA detection is a powerful tool to detect and characterize miRNA location/expression in tissue and cells. It provides a direct method of analyzing spatial profile in formalin-fixed paraffin embedded (FFPE) tissue and small biopsied specimens (cryosections). Along with the increasing knowledge of miRNAs, the histological analysis of their expression and location has been used in the development of diagnostic and therapeutic tools for human diseases, such as bladder cancer [33], thyroid nodules[25], Alzheimer's disease[34], and lung fibrosis[22]. miRNA ISH improves our understanding on this small molecule and its important role as biomarkers or therapeutic targets.

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