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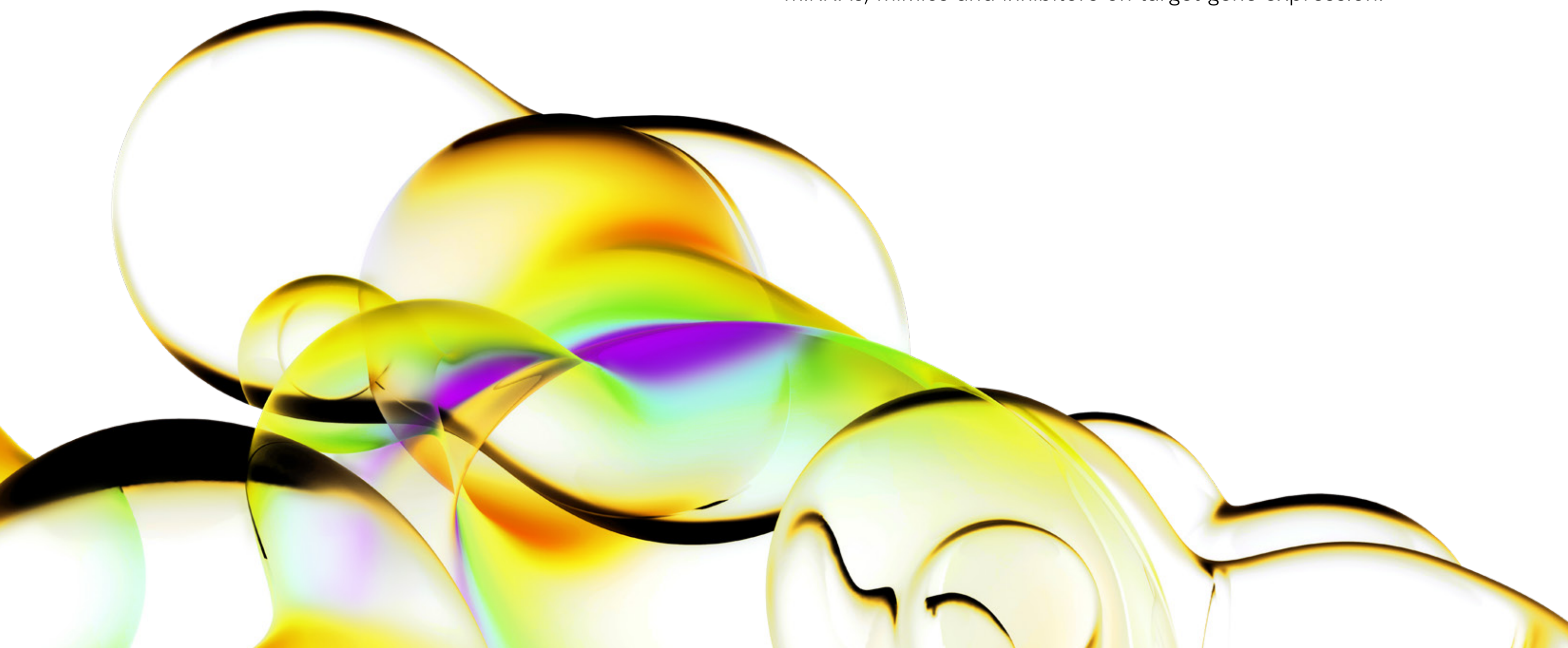
TECHNICAL NOTE

Dharmacon miRIDIAN mimics and inhibitors for microRNA functional analysis.

Introduction

microRNAs (miRNAs) are naturally occurring short RNA molecules of about 22 nucleotide (nt) long that regulate gene expression by binding to target mRNA and suppressing its translation or initiating its degradation.[1] Since their initial description in *C. elegans* in 1993, almost four thousand miRNAs in over 40 species of animals, plants and viruses have been identified.[2] Subsequent functional and sequence analyses suggest that miRNAs play an important role in the regulation of many biological pathways. The identification of their role in cancer development, cancer characterization and potential cancer therapy has elevated interest in studying the function of these small regulatory molecules.[3,4]

This technical note discusses strategies for identifying and experimentally confirming miRNAs involved in the regulation of a single gene (Figure 1A) or a biological pathway of interest (Figure 1B). For single gene analysis, candidate miRNAs for the target gene are first computationally identified. For analysis of a pathway, an experimental screen to identify miRNAs involved in the regulation of the pathway is first performed (Section I). Candidate target genes for the miRNAs can then be computationally identified. Once identified, the authenticity of miRNA/target gene pairs can be confirmed by performing gain-of-function and loss-of-function experiments using miRNA mimics and inhibitors, respectively (Section II). These experiments are best performed in cells that express the appropriate level of the endogenous miRNA, which can be determined using the methods described in Section III. Finally, Section IV discusses the assays used for detecting the effect of miRNAs, mimics and inhibitors on target gene expression.



Identifying candidate miRNA/target gene pairs

miRNA-mediated gene regulation is achieved when an miRNA binds to an miRNA recognition element (MRE) in the 3′-untranslated region (UTR) of a target mRNA.[5] Unlike siRNA-mediated gene silencing where, in most cases, complete sequence complementarity between the siRNA and its mRNA target is necessary, miRNA-mediated gene regulation can be achieved through partial complementarity between the MRE and as few as six or seven nucleotides at the 5′-end of the miRNA, called the seed region (Figure 2A).[5] The short length of the seed region predicts that each miRNA would have multiple potential target genes in the genome. In fact, sequence analysis suggests that at least a third of all human genes may be regulated by miRNAs.[6,7] In addition, each target gene may be regulated by multiple miRNAs (Figure2B). It is proposed that the synergistic action of multiple miRNAs is important for the regulation of a target gene.[8]

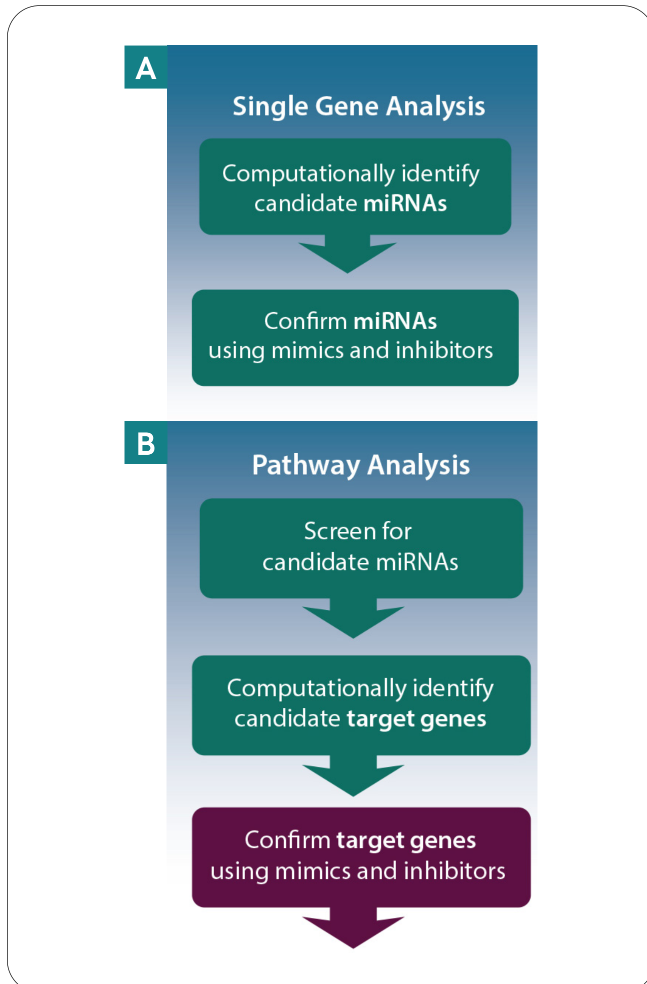


Figure 1: Strategies in miRNA functional analysis.

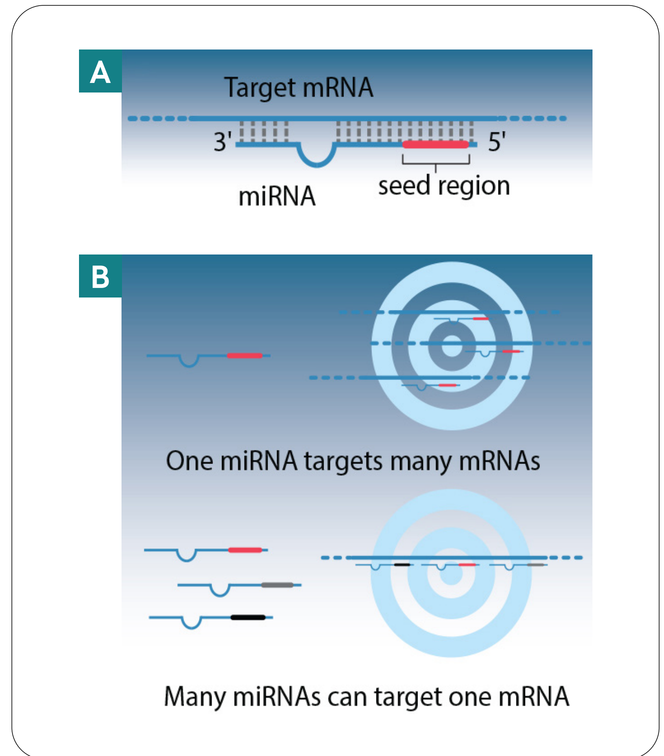


Figure 2: Short miRNA seed region targets many mRNAs. The seed region of an miRNA is six or seven nucleotides near its 5′-end (A). It allows binding of each miRNA to many mRNA targets (B, top) and binding of multiple miRNAs to one mRNA target (B, bottom).

The understanding of the relationship between the miRNA seed region and the MRE of its target gene enabled development of several bioinformatics tools to scan the 3′-UTR of mRNA sequences for the presence of MREs (Table 1). When a single gene is being studied, these tools can be used to predict candidate miRNAs for the gene. When a biological pathway that is comprised of multiple genes that are coordinately regulated is being studied, the miRNAs regulating the pathway need to first be experimentally identified before the tools can be used to identify candidate genes for the miRNAs.

Table 1: Online tools for computational analysis

Online tools for computational prediction of miRNA target genes
microrna.org[9]
targetscan.org[6]
pictar.mdc-berlin.de
ebi.ac.uk[10]

There are currently two screening approaches for identifying regulatory miRNAs for a specific biological pathway. The first approach uses miRNA microarray analysis, in which RNA samples containing endogenous miRNAs are hybridized to microarrays displaying probes for every identified miRNA in the species studied. This method can quickly and accurately identify miRNAs whose levels change with a specific phenotype compared to a control phenotype. For example, miRNAs that are present at higher levels in adipocytes compared to pre-adipocytes may be involved in the regulation of differentiation from pre-adipocyte to adipocyte.

In the second approach, individual miRNA mimics and inhibitors are transfected into cells to induce or inhibit a specific phenotype. miRNA mimics augment the function of endogenous miRNA for easier detection of a phenotypic change (Figure 3, Steps 5a and 6a). Conversely, miRNA inhibitors suppress the function of endogenous miRNAs, increase the expression of the target gene, and attenuate the presentation of the phenotype (Figure 3, Steps 5b and 6b). As there are currently several hundred miRNAs that have been characterized for each common experimental species, this method is most useful when the phenotypic assay is amenable to high throughput screening.

Once candidate pairs of miRNA/target genes are identified using the computational tools, functional analysis using miRNA mimics and inhibitors can be performed to experimentally confirm the pairs. As described in Section II, when a gene is a true target of an miRNA, its mimic will decrease the target gene expression level, while an inhibitor will increase the target gene expression level.

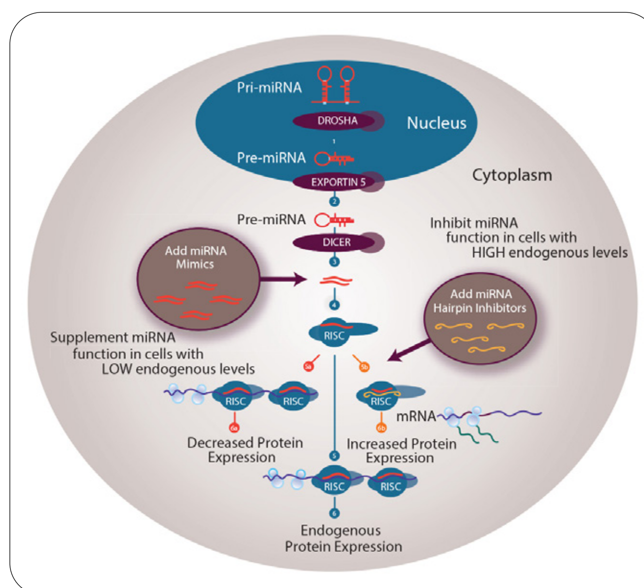


Figure 3: miRNA Mimics and Inhibitors simulate or attenuate the effect of endogenous miRNA on target gene expression. **(Steps 1-3)** describe the biogenesis of endogenous miRNA. When mature miRNA duplex loads into RISC, cleavage of the inactive strand of the miRNA results in the formation of programmed RISC **(Step 4)**. Programmed RISC binds to the 3'-UTR of a target mRNA **(Step 5)** and maintains low endogenous protein level **(Step 6)**. miRNA mimics supplement miRNA function **(Step 5a)** and further decreases protein level **(Step 6a)**. miRNA inhibitors compete with target mRNA for programmed RISC **(Step 5b)** and increase protein level **(Step 6b).**

Confirming miRNA targets using mimics and inhibitors

Unlike siRNA-mediated gene silencing where very high levels of mRNA reduction are reported, studies of miRNA function thus far show mRNA and protein level reduction of 50% or lower. The modest changes in mRNA and protein level present an experimental challenge when validating miRNA/target gene pairs. miRIDIAN Mimics and Hairpin Inhibitors are useful tools in these experiments as they either enhance or eliminate the effects of endogenous miRNA, respectively, and facilitate easier detection of the miRNA effects on a target gene.

Table 2 lists selected publications that report the use of mimics and inhibitors to confirm target genes for specific miRNAs. It also lists two important variables for performing these experiments: the concentration of the mimic or inhibitor, and the cells used. The optimal concentration of mimic and inhibitor will vary depending on the potency of the mimic or inhibitor used and the efficiency of mimic and inhibitor delivery into the cells. As with concentration, the effective assay time varies and should be empirically tested; 24 to 48 hours have been widely reported.

Table 2: Using Mimics and Inhibitors to confirm miRNA gene targets

miRNA	Cells	Mimic (nM)	Inhibitor (nM)
miR-21[11]	HeLa S3	40	120
miR-375[12]	MIN6	200	200
miR-196a[13]	HeLα	5 or 20 × 10^3	N/A
miR-143[14]	HeLα	N/A	20
miR-122a[15]	NIH/3T3	15–20	N/A
miR-125b[16]	PC-3, HeLα	N/A	160
miR-1, miR-124[17]	HeLa	100	N/A
miR-1, miR-133[18]	C2C12	200	200
miR-134[19]	Primary neruons	10^3	2 × 10^3

The choice of cells used for performing gain-of-function and loss-of-function experiments is important as each cell line has varying levels of endogenous miRNA. To clearly detect the effects of miRNA mimics and inhibitors in functional analysis studies, it is important to select a cell culture system that expresses the appropriate level of endogenous miRNA. Section III describes methods for assaying endogenous miRNA level.

Since miRNA mimics decrease target gene expression, they are best used in cells that express a low level of the endogenous miRNA and correspondingly high target gene expression (Figure 4). If miRNA mimics are used in cells with high endogenous miRNA level and correspondingly low target gene expression, the effect of the mimics on the target gene may not be detectable. Instead, these cells are best used for studying the effects of inhibitors, which bind to endogenous miRNA and prevent it from repressing gene expression. The resulting increase in gene expression will be more pronounced and easily quantified in these cells compared to cells that highly express the target gene. An increase in gene expression in response to an miRNA inhibitor and a decrease in gene expression in response to an miRNA mimic provides evidence that the gene is regulated by the miRNA.

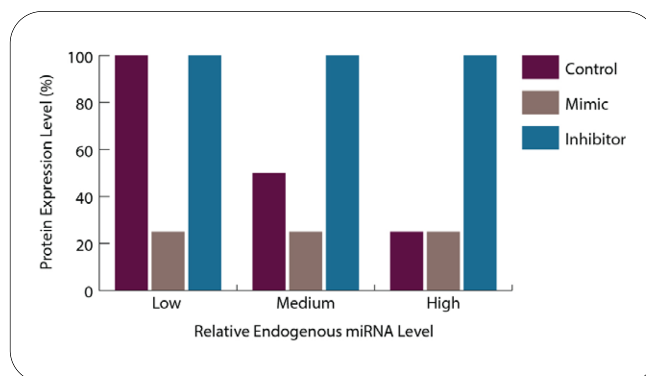


Figure 4: The effects of miRNA mimics and Inhibitors are best observed in cell lines expressing low or high endogenous miRNA levels, respectively. Protein level in cells expressing low, medium or high relative levels of endogenous miRNA are shown as purple bars. Protein level decreases in the presence of miRNA mimics (brown bars) and increases in the presence of miRNA inhibitors (blue bars).

Methods for assaying endogenous miRNA level

Endogenous levels of some miRNAs in a few cell lines are available in the public literature databases.[20] Levels of specific miRNAs may be assessed using northern analysis[21] or PCR-based assays.[22] As with mRNA analysis, these methods are appropriate for quantifying a small number of miRNAs. For a large number of miRNAs, microarray analysis is recommended as it allows accurate and simultaneous quantitation of relative levels of all miRNA with a known sequence in a specific cell line.[17,23]

An alternative to the direct assays described above is a reporter system, which provides the advantage of expressing a protein product that is easy to assay. The most commonly used reporter genes currently are luciferases (Photinus and Renilla) and green fluorescent protein (GFP). A reporter system is needed for each miRNA, as the recognition site of each miRNA is cloned into the 3′-UTR of the reporter cDNA (Figure 5).[21] The miRNA recognition site may be the antisense sequence of the miRNA or the MRE of a specific mRNA, when known. A reporter system specific for each miRNA produces low reporter expression in cells with a high endogenous miRNA level, and produces high reporter expression in cells with a low endogenous miRNA level.

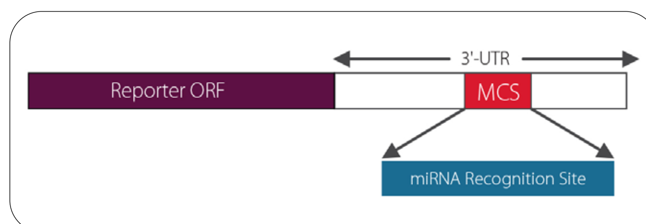


Figure 5: **miRNA reporter plasmid**
ORF: Open Reading Frame
UTR: Untranslated Region
MCS: Multicloning Site

Methods for assaying miRNA effects

As miRNAs modulate gene expression by both mRNA cleavage and translational attenuation, the effect of an miRNA and its mimic and inhibitor on a target gene should be assayed at both the mRNA and protein levels. Whether the miRNA results in translational arrest or mRNA cleavage most likely depends on the level of sequence complementarity between the miRNA and the MRE;[13] perfect or near-perfect sequence complementarity favors mRNA cleavage and degradation. The extent of mRNA level reduction and the time frame compared to the protein level reduction is currently being investigated.[24]

Methods for directly assaying protein level, shown in Table 3, use antibodies to quantify the amount of protein present in cell lysate (western analysis and ELISA) or in whole cells (immunocytochemistry). However, when an antibody is not available, proteins with well-established functions, such as enzymes and receptors, may be assayed by their activities. For example, the level of insulin may be measured by the metabolism of glucose, and the level of transferrin receptor may be measured by the cellular uptake of iron. Three common methods for assaying mRNA levels are northern analysis, RT-qPCR (reverse transcription quantitative real-time PCR) and branched DNA (bDNA).

Table 3: Methods for assaying protein and mRNA levels

Protein assays	mRNA assays
Western analysis	Northern analysis
ELISA	RT-qPCR
Immunocytochemistry	Branched DNA

When neither the protein nor mRNA level can be easily measured, the effect of miRNA function on a target gene can be measured using a reporter system as described in Section III. The candidate MRE is cloned into the 3′-UTR of the reporter plasmid. Binding of an miRNA or miRNA mimic to the MRE will result in reduced level of the reporter protein, while binding of an inhibitor to the miRNA will increase the reporter protein level.

When a biological pathway is being studied, phenotypic changes may be assayed as an indirect measure of miRNA effect on protein levels. Some phenotypic assays that are commonly used are cell proliferation, cell death, cell differentiation, and cell migration. There are many commercially available kits for assaying cell proliferation and cell death. Cell differentiation is usually measured by monitoring either morphological changes or the presence/absence of a specific marker protein. There are also well-established methods for measuring cell migration. In the field of infectious diseases, changes in viral infectivity or viral replication are easily measured as a function of target gene modulation.

While phenotypic assays reflect the ultimate desired biological effect, they are only indirect measures of regulatory changes affecting protein expression. They may not, by themselves, be sufficient for measuring miRNA function as a phenotypic effect may not directly correlate with the protein level. Some proteins may be functional over a wide range of intracellular concentration. In these situations, the small change that is generally expected from a miRNA may not be reflected in the phenotypic assay, and thus may not be a true measure of the miRNA function. Therefore, we recommend that a phenotypic assay be accompanied by a direct protein or mRNA level assay, when possible.

miRIDIAN microRNA mimics and inhibitors

miRIDIAN reagents are highly functional, chemically modified synthetic miRNA mimics and inhibitors for every human, mouse and rat miRNA present in the miRBase Sequence Database (microrna.sanger.ac.uk). miRIDIAN microRNA Mimics and Hairpin Inhibitors are available as single mimics or inhibitors to be used when confirming candidate miRNAs for a specific gene. They are also available as collections of mimics or inhibitors for every miRNA in each species, which are ideal for performing screening experiments to identify regulatory miRNAs for a specific biological pathway. To help assess the specificity of protein level or phenotypic changes observed in experiments using miRIDIAN microRNA Mimics and Hairpin Inhibitors, mimic and inhibitor negative controls are offered designed based on miRNA sequences from *C. elegans* miRNA. For details on package options and prices of miRIDIAN microRNA reagents, and to learn more about tools for miRNA research, please visit www.revvity.com.

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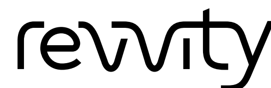

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