Technical Guideline

QIAGEN

Technical guidelines for microRNA research

The study of microRNA requires specialized techniques because of their small size. Here's a guide on things to think about when isolating microRNA for your research.

MicroRNAs (miRNAs) are short RNA molecules that regulate gene expression and, in turn, protein biosynthesis. They do this primarily by base-pairing with complementary sequences in target mRNAs, typically within the 3' untranslated region (3' UTR). This interaction can result in mRNA degradation or inhibit translation directly. Alternations in miRNA expression have been linked to numerous oncological and other diseases, making them both promising biomarkers for clinical diagnostics (1) and potential candidates for therapeutic treatments (6).

Usually consisting of only 17–24 nucleotides, mature miRNAs are single-stranded RNA molecules derived from larger precursors (2). Due to their small size, specialized methods and techniques are required to study miRNAs and other classes of small RNAs. Therefore, when working with miRNAs, it is important to use RNA extraction kits, PCR assays and NGS library preparation kits that have been specifically developed with a focus on miRNAs.

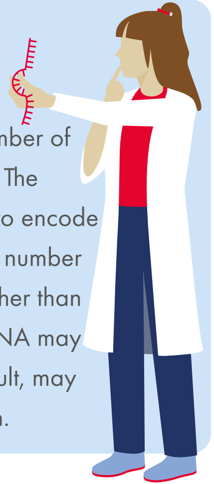
Milestones in miRNA research

Two papers by the Victor Ambros and Gary Ruvkun team were published back-to-back in Cell (3,4) in 1993, revealing for the first time the post-transcriptional regulation of the *lin-14* gene in *C. elegans* development by a non-coding RNA called *lin-4*. In 2002, Calin et al. conducted the first miRNA investigation in a human disease, discovering that peripheral blood samples from individuals with B-cell chronic lymphocytic leukemia had deleted *miR-15* and *miR-16* regions (5). Therefore, it took roughly ten years for the field of miRNA to advance from *C. elegans* to humans. In 2024, Ambros and Ruvkun were awarded the Nobel Prize for their discovery of microRNA's role in gene regulation.

Did you know?

miRNA vs mRNA

Compared to mRNAs and proteins, the number of miRNAs is often smaller in most organisms. The human genome is, for example, estimated to encode approximately 1900 (7) miRNAs while the number of mRNAs is typically considered to be higher than 89,000 (7). But keep in mind that one miRNA may regulate hundreds of mRNAs and, as a result, may have substantial effects on gene expression.



Purification of high quality miRNA

Isolating high-quality miRNA is essential for the success of any miRNA experiment and for accurately detecting and measuring miRNA expression levels. Over the years, miRNA isolation techniques have significantly improved, moving from labor-intensive methods, like phenol–chloroform extraction and ethanol precipitation, to faster, more reliable and higher-throughput techniques. The silica-based spin column method, with its quick "bind-wash-elute" workflow, enhances RNA yield and purity while minimizing hands-on time and contamination risk, ultimately improving the reliability and reproducibility of miRNA experiments.

Why do I need a specialized miRNA kit and can't just use any RNA kit for miRNA isolation?

Not all RNA isolation kits are designed to purify small RNAs. Early research often dismissed miRNAs as "junk" due to their small size and limited understanding of their function. As a result, most RNA isolation kits were developed to capture RNA molecules larger than 200 nucleotides, primarily targeting mRNA, which typically ranges from a few hundred to several thousand nucleotides. These kits, referred to as "total RNA isolation kits", do not actually isolate all forms of RNA, especially small RNAs like miRNA.

Therefore, it's crucial to remember that while many kits are optimized for "total RNA", they may not be suitable for isolating small RNAs. To avoid bias in recovering miRNAs versus larger RNAs, use kits like QIAGEN miRNeasy Advanced, designed to efficiently capture both miRNAs and RNA >200 nucleotides.

The type of sample also plays a key role in miRNA analysis success. Different samples, such as cell cultures, various tissue types, blood, urine, FFPE samples and plants can yield different quantities and qualities of RNA and miRNA. Each sample type presents unique challenges, so choosing the most appropriate isolation method is essential. Using tailored protocols and specialized kits help optimize extraction and maximize miRNA recovery while ensuring high-quality RNA.

"Total RNA" usually means RNA >200 nucleotides. Therefore, most total RNA kits are not suitable for miRNA isolation. Look for kits that have been specified for miRNA.

All "miRNA kits" are designed to isolate RNA >18 nucleotides – thus you will get miRNA in addition to larger RNAs, not just miRNA.

Do you need DNA and miRNA? The AllPrep® DNA/RNA/miRNA kit is designed for isolating miRNA/RNA and DNA in separate eluates.

Recommendations for miRNA isolation from various sample types

Sample collection and storage prior to miRNA isolation

After harvesting, RNA and miRNA become unstable due to down- or upregulation of certain genes and enzymatic degradation. They remain unprotected in the sample unless they are treated with stabilization solutions (such as AllProtect® or RNAProtect®), flash-frozen or homogenized with RNAse-inhibiting reagents. Our lysis buffers contain such reagents. Without immediate stabilization, the detected transcription profile may not accurately represent the actual pattern of gene expression.

Important: Process samples as soon as possible after collection. Delayed processing can lead to changes in RNA expression patterns due to cellular responses or RNA degradation.

Cells and tissues

Cells and tissues can be processed fresh or frozen. Keep the fresh tissue cold and process it quickly after dissection, or store it in RNAprotect or AllProtect. Fresh tissues/cells should be disrupted immediately in lysis buffer to inactivate RNases. The samples should not be left sitting in lysis solution without disruption.

Cultured cells are easier to work with since they are easy to disrupt. Cells grown in suspension are collected by centrifugation, washed and resuspended in lysis buffer. Disruption can be done just by vortexing or vigorous pipetting. Meanwhile, adherent cells can be lysed directly on the culture plate or flask by adding lysis buffer and scraping, then transferring to a tube for further disruption through vortexing or pipetting.

For tissue disruption methods, the range includes tissue grinders, mortar and pestle or bead-based techniques.

Difficult to lyse tissues

When working with difficult to lyse tissues, for example, tissues that are high in nucleases (pancreas) or fat (brain and adipose tissue), a more rigorous, phenol-based RNA isolation method using QIAzol® is recommended (e.g., miRNeasy kits).

For difficult-to-lyse tissues, such as muscles, disruption can be quite challenging. As only released miRNA can later be purified by the RNA isolation kit, efficient sample disruption is key to achieve good yields. Bead mills like TissueLyser III or TissueLyser LT or rotor–stator homogenizers like TissueRuptor® II can help optimize sample disruption.

Stainless-steel beads (3–7 mm in diameter) are ideal for animal tissues, but other disruption parameters should be determined empirically for each application.

Excessive mechanical disruption may cause shearing or RNA degradation due to heat. If longer bead beating is needed, put tubes on ice in between beating steps.

Small model organisms

Model organisms like *C. elegans, D. melanogaster* and *D. rerio* have proven to be convenient systems in miRNA research, and various transgenic models have been generated to learn more about miRNA function. All considerations that apply to tissue samples apply here as well.

Formalin-fixed paraffin embedded tissues

It has been demonstrated that there is a strong correlation between the extracted miRNA profiles from formalin-fixed paraffin embedded tissues (FFPE) and those from matched frozen reference samples. Compared to mRNA, miRNA seems to be remarkably stable and intact in FFPE tissues and much less affected by fragmentation (8). Nonetheless, it is important to work with kits that include efficient decrosslinking and efficiently recover small RNA, like RNeasy® FFPE kit (miRNA protocol).

For more general recommendations and information about FFPE samples, please see **QIAGEN FFPE Tech guide** or scan the QR code.

Samples with limited starting material

For limited samples like tissue biopsies or FACS-sorted cells, obtaining a high RNA concentration is essential, which requires eluting in small volumes. Specialized "micro kits," such as the miRNeasy Tissue/Cells Advanced Micro Kit, are designed to maximize yield from minimal sample material.

Biofluids (e.g. blood, plasma, serum), exosomes and other extracellular vesicles

We have developed dedicated workflows for profiling biofluid miRNAs, including the use of spike-ins and specialized sample QC methods.

More information on this can be found in our **Guidelines for Profiling Biofluid miRNAs** or by scanning the QR code.

Plants

Plants present unique challenges for miRNA isolation due to varying sample types like leaf tissue, starch-rich tubers or seeds, which contain different water levels and secondary metabolites. For most plant samples, homogenization with a 5 mm steel bead and miRNeasy purification is effective.

For more information, refer to **Supplementary Protocol: Isolation of total RNA, including small RNAs, from plant tissues** or scan the QR code.

Choosing the right path for miRNA purification: Total RNA including miRNA or miRNA-enriched fraction?

When purifying miRNA, you can choose between isolating total RNA (including miRNA) or focusing on miRNA-enriched fraction.

Purifying all RNA (>17 nucleotides) in one fraction offers a panoramic view by capturing not only miRNA but also various RNA types such as mRNA and long non-coding RNA (lncRNA). This broad spectrum enables comprehensive analyses, shedding light on diverse RNA regulatory mechanisms. In contrast, targeting miRNA specifically through enrichment techniques amplifies the "miRNA signal", which sometimes allows heightened sensitivity and specificity, making it easier to detect low abundant miRNAs. However, it also results in significant loss of information from your sample.

Keeping miRNA and longer RNA/mRNA in one fraction is usually the preferred choice since miRNA plays an important function as a regulator of gene expression. Most miRNA assays do not require enrichment and are designed to use total RNA, including miRNA. Researchers often prefer working with all RNA species due to

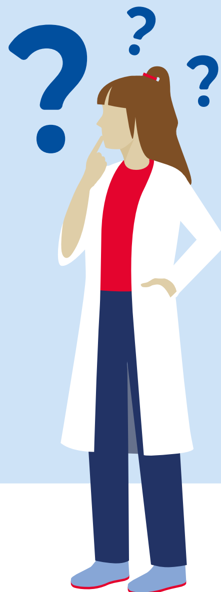
challenges in assessing RNA quality as typical standards like RIS or RIN values cannot be used. It has also been found out that enrichment can introduce bias in miRNA representation (13). Ultimately, the choice depends on your study's specific goals.

Why should you go phenol-free for your miRNA isolation?

Did you know?

Phenol is not only harmful to human health but also has the potential to bioaccumulate in living organisms, particularly in aquatic ecosystems (9).

Go phenol-free to protect the environment and your health and enjoy the easier handling of the Advanced kits.



The development of phenol-free methods for miRNA isolation marks a major improvement, enhancing safety, convenience and reducing environmental impact. Traditional phenol or phenol-based solutions, like Trizol, involve hazardous solvents, tedious phase separation workflows which can interfere with downstream applications. Phenol-free methods provide a safer, more reliable way to obtain high-quality miRNA samples.

If there is "Advanced" in a miRNA kit name, that typically indicates that it is free of phenol.

Odorless

- Work at the bench
- No hood required
- No special waste management necessary

Easier handling

- No phase separation required
- Faster procedure
- Less contamination

Less toxic

- Less exposure to health hazards
- Less harmful to your health
- Better for the environment

Do I need a DNase digestion step for my miRNA experiment?

A DNase digestion step is often used to remove genomic DNA (gDNA) contamination from RNA samples, which can interfere with downstream analyses like miRNA-Seq. The need for DNase digestion depends on the sensitivity of your downstream application to gDNA and whether your isolation method includes DNA removal, such as gDNA Eliminator columns. Our miRCURY® LNA® miRNA PCR Assays for detecting mature miRNA do not require further gDNA removal since they do not amplify DNA.

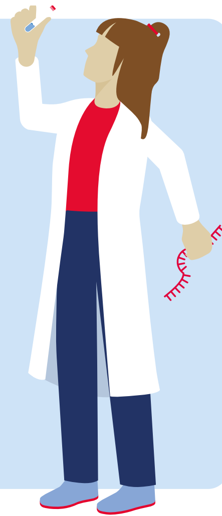
DNase selectively removes gDNA contamination from RNA samples without affecting RNA. However, over-digestion or improper handling can degrade RNA. To avoid this, strictly adhere to specified times and recommended temperatures.

While the miRNeasy Advanced kits remove most gDNA, trace amounts may still remain, especially with difficult samples like PBMCs that have challenging DNA to RNA ratio. Currently, no method guarantees complete DNA removal. For gDNA-sensitive applications, consider an on-column DNase digestion or digesting residual gDNA in the eluate, although this requires repurification via RNA cleanup.

gDNA eliminator columns are an efficient and convenient way to remove gDNA from your samples. Our Advanced kits typically include gDNA eliminator columns or plates.

How to check for DNA in your RNA sample?

Include a "minus-RT" control in your RT-PCR experiment. If a PCR product appears in your RNA sample lacking reverse transcription, it indicates DNA contamination. A "minus-template" control can help identify whether the contamination comes from the RNA extraction or RT-PCR reagents.



Automation – reproducible isolation of high quality miRNA

Automation decreases hands-on time and minimizes human error, resulting in more reliable experimental results. It also enables high-throughput miRNA isolation, allowing for the simultaneous processing of numerous samples. We provide a wide range of automation options for miRNA isolation.

Sample	Kit	Processing
96-well		
Cells and tissue	miRNeasy 96 (4 x 96)	Centrifugation, QIAvac/Centrifugation
	miRNeasy 96 Tissue/ Cells Advanced (4 x 96)	Centrifugation, QIAvac/Centrifugation
Kits for automation platforms		
Cells and tissue	EZ2® RNA/miRNA Tissue/ Cells (48)	Automated EZ2®
	QIAcube® HT RNA (5 x 96)	
	QIAzol® Lysis Reagent (200 mL)	Automated QIAcube® HT
	QIAsymphony® RNA (192)	Automated QIAsymphony®
Serum, plasma	miRNeasy 96 Advanced QIAcube HT (5 x 96)	Automated QIAcube® HT
Stabilized blood	QIAsymphony® PAXgene Blood RNA (96)	Automated QIAsymphony®

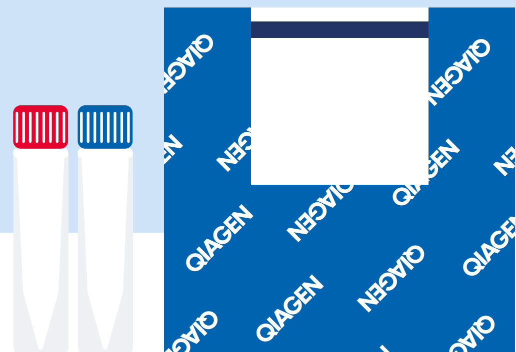
If you are interested in automating your miRNA purification protocol, reach out to our **Technical Support.** You can also scan the QR code.

Assessment of miRNA quality and quantification

Evaluating both the quality and quantity of isolated miRNA is crucial for ensuring reproducible and accurate miRNA profiling results. As most miRNA profiling techniques can utilize total RNA, including miRNA, as input, it is not always necessary to specifically evaluate the miRNA population. Instead, it is standard practice to assess the overall yield and extent of overall RNA integrity, for example, via capillary gel electrophoresis instruments such as the QIAxcel® Connect.

When assessing RNA extraction efficiency, you can spike-in a known amount of synthetic miRNAs that are not expressed in your biological sample early in the RNA isolation process. Measuring these spiked-in miRNAs in the recovered RNA serves as a control for extraction efficiency. However, ensure that the spike-ins do not interfere with your analysis methods and choose the appropriate spike-in solution accordingly.

QIAGEN offers specific spike-ins tailored for sequencing analysis to prevent unnecessary consumption of sequencing reads.



Assessing miRNA quality by PCR

We have developed a qPCR-based QC panel consisting of a set of synthetic RNA spike-ins and endogenous miRNAs for assessing quality of low-input RNA samples. The QC panel can be provided in both plate and individual assay tube formats for increased flexibility.

Detection and quantification of miRNAs

Several unique characteristics of miRNAs make accurate detection and quantification challenging. Their short length complicates annealing to traditional primers designed for reverse transcription and PCR, and unlike mRNAs, miRNAs lack a common sequence like a poly(A) tail for selective enrichment or universal primer binding site for reverse transcription. miRNAs are also a small fraction (~0.01%) (10) of the complete RNA mass, making selective detection difficult amidst other RNA species (see Figure 1).

Additionally, miRNAs show wide GC content variation, with over 20% having less than 40% GC, impacting unmodified primer T_m and causing detection failure of low GC% miRNA.

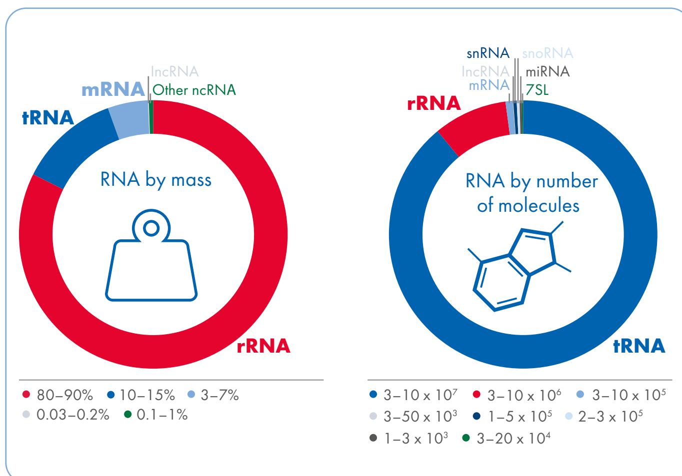


Figure 1. RNA distribution in a typical mammalian cell.

Furthermore, miRNAs within a family can exhibit minimal nucleotide differences, necessitating the ability to discriminate between forms with single nucleotide variations. Despite these challenges, methods like RNA-Seq, qPCR and dPCR remain effective for miRNA profiling when optimized tools and protocols are used.

The average copy number of miRNA species is estimated at around 500/cell, despite miRNAs comprising just ~0.01% of total RNA by weight. Due to their smaller molecular weight, miRNAs are abundant, but their concentration can still vary widely, spanning at least four orders of magnitude.

To explore this topic further, watch our webinar: **miRNA-Seq: Tools to interrogate a class of gene expression master regulators** or scan the QR code.

Uncover the hidden world of miRNAs with NGS

Unlike other techniques, miRNA sequencing is an open hypothesis approach that allows you to profile all miRNAs present in a sample. This includes novel miRNAs and isoforms that have not been previously annotated, enabling the discovery of new miRNAs.

Avoiding sequence bias using UMIs

Sequence bias in small RNA library construction often results from varying PCR amplification efficiencies between different miRNAs. The QIAseq® miRNA Library Preparation Kit minimizes this bias using Unique Molecular Indices (UMIs) of 12 nucleotides in length. During library preparation, RT primers incorporate UMIs, allowing nearly 17 million unique combinations to tag each RNA molecule, given that there are four nucleotide options at each of the 12 positions.

Traditional miRNA library sequencing often shows raw read counts affected by PCR bias, where the number of reads doesn't accurately reflect the true number of original RNA copies. This is illustrated in Figure 2, showing 6 reads for sample 1 and 3 reads for sample 2, resulting in a misleading 6:3 (2:1) ratio instead of the true 3:1 transcript ratio. Sequencing read quantification with UMIs corrects this bias, allowing the data to match the original RNA quantities. This approach allows for more accurate interpretation of gene expression changes and helps identify miRNA species of interest for further study.

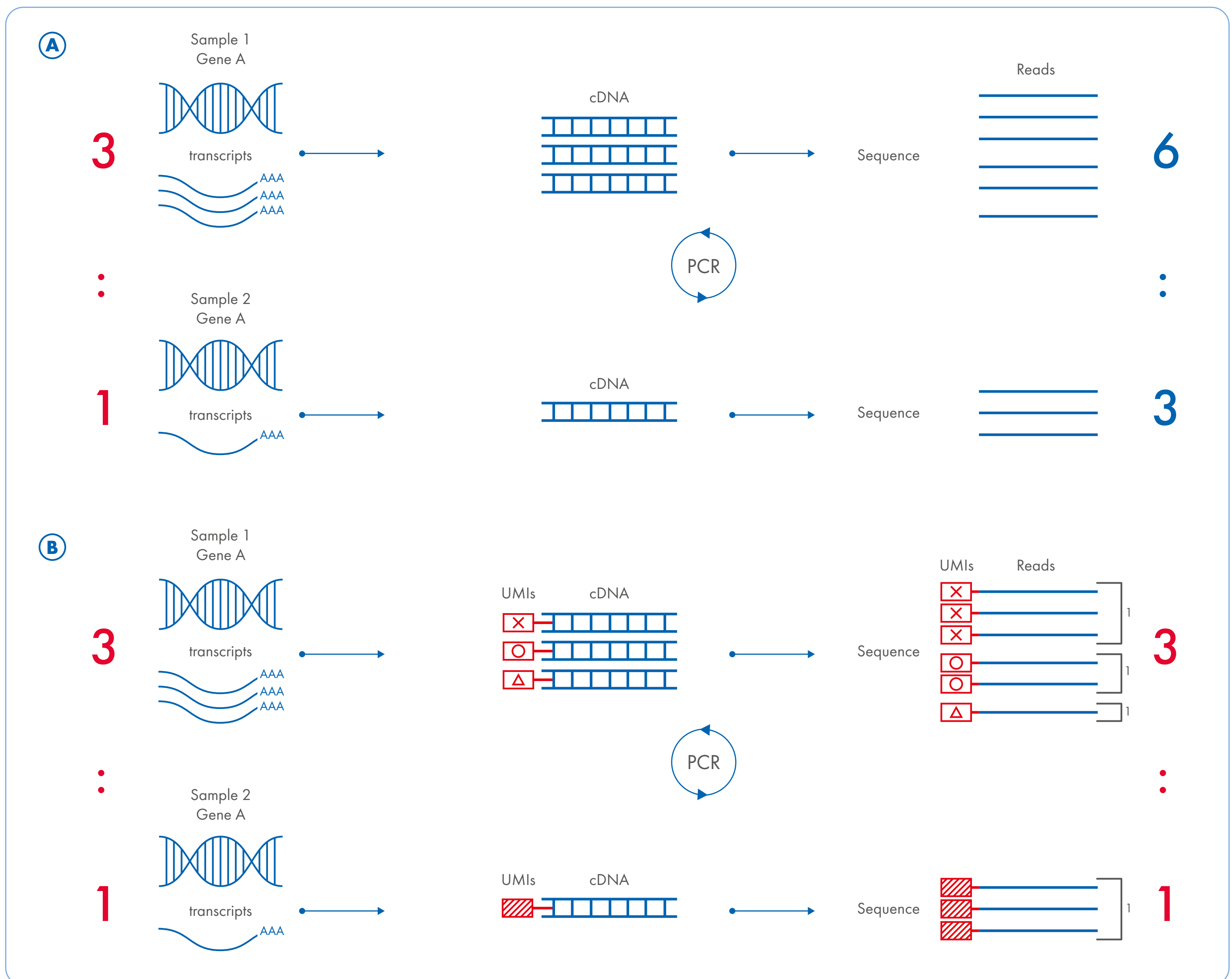


Figure 2. PCR bias correction using UMIs.
A. Without UMIs, the number of reads does not accurately represent the number of copies of the original RNA. **B.** With UMIs, the data accurately reflect the original transcript abundance.

miRNA quantification using LNA PCR

Locked nucleic acids (LNA) are high-affinity RNA analogs with a "locked" ribose ring that is the ideal conformation for Watson–Crick binding, enhancing their binding affinity to complementary strands (Figure 3). This provides superior sensitivity and specificity, making LNA-enhanced oligos ideal for detecting and differentiating miRNA targets. QIAGEN miRCURY LNA miRNA PCR Assays use LNAs to enable uniform detection of any miRNA, even AT-rich targets, and allow for single nucleotide discrimination.

Figure 3. Schematic presentation of LNA.

Absolute quantification of miRNAs with high accuracy and precision using digital PCR

The QIAcuity® dPCR System, using miRCURY LNA miRNA PCR Assays, enables reliable detection and quantification of miRNAs with high sensitivity, even for low-expressed targets. The LNA-enhanced assays provide increaseed specificity, ensuring accurate detection and quantification. The QIAcuity dPCR System also demonstrates increased tolerance to inhibitors, allowing effective quantification of miRNA even in challenging samples.

To learn more watch our webinar: **Absolute quantification of miRNAs and CNVs with high accuracy and precision using digital PCR** or scan the QR code.

Broad dynamic range allows quantification of low- and high-expressed miRNAs

The QIAcuity dPCR System enables miRNA quantification of up to 200,000 copies per reaction with high accuracy and precision. Precise thresholding is important for absolute quantification of miRNAs.

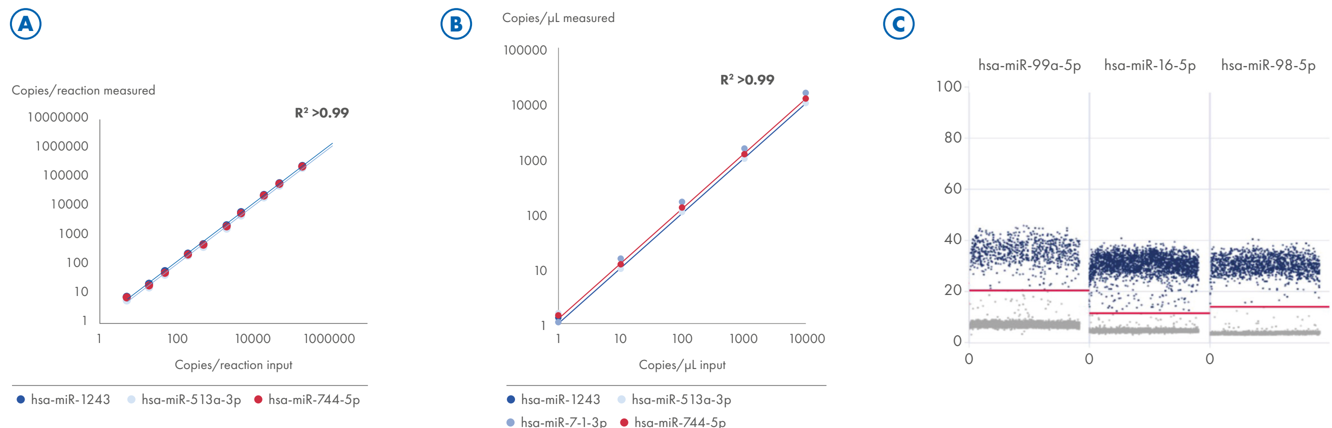


Figure 4. QIAcuity dPCR dynamic range on 26k and 8.5k nanoplates. A Serial dilutions of synthetic cDNAs were performed from 5 copies/reaction to 200,000 copies/reaction on 26k nanoplates and **B** from 12 copies/reaction up to 120,000 copies/reaction on 8.5k nanoplates. **C** 1D-scatterplot of 3 human miRNAs with a high signal-to-noise ratio. miRCURY LNA miRNA PCR assays together with the QIAcuity EG MM were used for miRNA quantification.

Study of miRNA function with LNA-enhanced tools

Understanding the complex world of miRNA and its network of interactions is crucial for validating miRNAs as potential biomarkers. Through RNA interference and the RISC complex, miRNAs regulate numerous mRNA targets. We provide tools to explore miRNA roles using a variety of approaches (Figure 5).

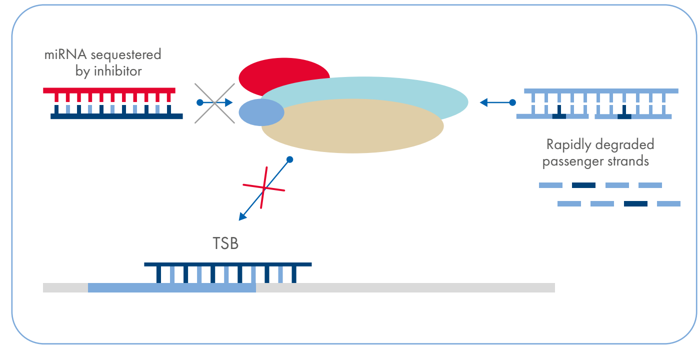
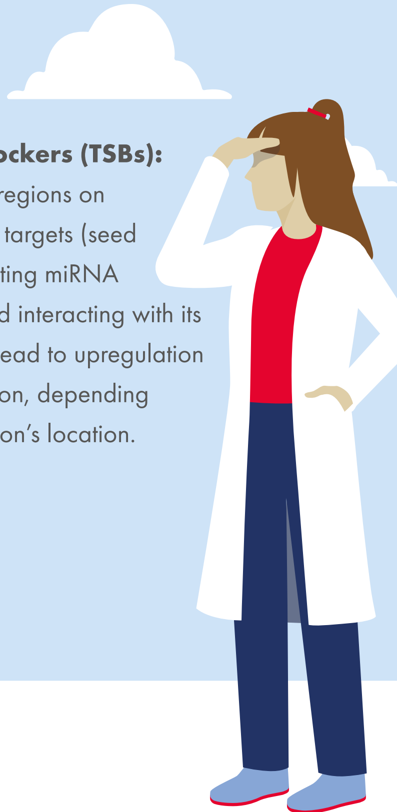


Figure 5. Mode of action of LNA-enhanced miRNA analysis tools.

These miRNA functional tools can be used in both in vitro and in vivo experiments for developing RNA therapeutics

- **miRNA inhibitors (antagomiRs):** Used to suppress miRNA activity, helping to determine the role of miRNAs in cellular processes and pathological pathways, and for identifying and validatingmiRNA targets.
- **miRNA mimics (agomiRs):** Simulate mature miRNAs to mimic their activities in functional analysis studies, such as miRNA pull-down assays.
- **Target site blockers (TSBs):** Bind to specific regions on mRNA/lncRNA targets (seed regions), preventing miRNA from binding and interacting with its target. This can lead to upregulation or downregulation, depending on the seed region's location.



Localization of miRNA

Utilizing LNA technology, highly sensitive and specific in situ hybridization probes have been developed for cellular and sub-cellular miRNA localization studies, determining spatial miRNA expression. Due to the short length of miRNA, it is only possible to generate such probes with the use of LNA.

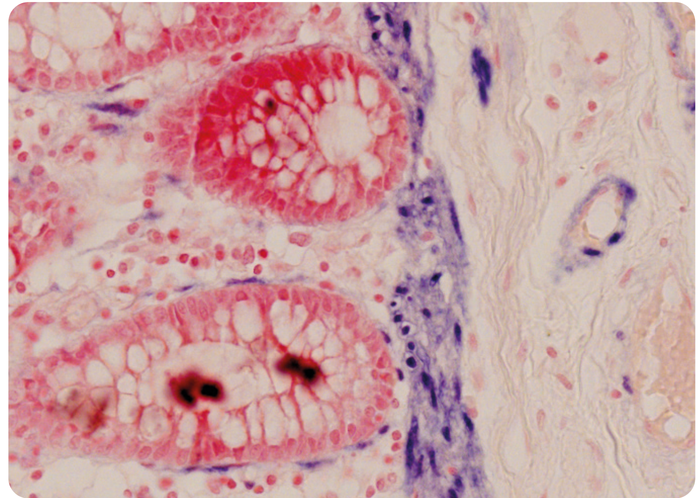


Figure 6. miR-145 detection in human colon.

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