PCR-based bioanalytical methods for short nucleic acid targets

TATAA BIOCENTER

PCR-based technologies are standard for bioanalytics, including PK, biodistribution, and biomarker profiling. However, short oligonucleotides such as siRNA and miRNA pose specific challenges. This guide discusses cutting-edge solutions for high-throughput PCR analysis of short targets.

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Introduction

PCR-based technologies, such as quantitative PCR (qPCR) and digital PCR (dPCR), surpass mass spectrometry in terms of specificity for detecting nucleic acids, speed, and simplicity of sample preparation, with significantly shorter data analysis times. PCR also offers high-throughput analysis by enabling the simultaneous detection of multiple samples or genes within a single experiment. This makes PCR a well-established tool for quantifying gene expression for biomarker analysis, target engagement and biodistribution, and DNA levels in fields like oncology and neurology for copy number variation. However, due to technical challenges, PCR traditionally has limitations when working with short targets. At TATAA Biocenter, we have developed Two-Tail PCR Technology to overcome these limitations, resulting in hundreds of validated assays using qPCR/dPCR for the detection of short nucleic acid drug products or biomarkers.

This guide covers setting up a high-throughput sample workflow for detecting short oligonucleotides such as siRNA, miRNA, and ASO using qPCR or dPCR. Such workflows streamline early-stage development efforts, allowing for efficient evaluation of efficacy, safety, and kinetics, indispensable in large-scale pharmacological studies and clinical trials, where the analysis of large cohorts are necessary.

- Extraction
- Assay development
- Assay validation
- High-throughput screening setup

Challenges

Extracting short oligonucleotides presents significant challenges, particularly because they are often present in very low abundance in biofluids or tissues. Consequently, extraction efficiency becomes crucial in the workflow. Designing PCR assays for short oligonucleotides requires unconventional methodologies, as standard primers used in qPCR and dPCR cannot effectively amplify these short RNA/DNA sequences. However, innovative primers have emerged as a solution to this problem, which we will explore further below.

Extraction

The accuracy of downstream PCR quantifications critically depends on the quality of the input RNA or DNA isolates. Not all short RNAs or DNAs are extracted equally; even the same type of molecule, such as siRNA, can vary in extraction efficiency depending on factors like sequence length, composition, modifications, and secondary structures. Numerous column-based and bead-based extraction methods are available for both manual and automated workflows, but each needs to be evaluated on a case-by-case basis. Extraction should always include controls and quality assessments to measure the efficiency, yield, integrity, and overall quality of the target isolate, whether short or long oligonucleotides, viral DNA/RNA, or other specific molecules. While certain extraction methods may work well for some matrices and oligonucleotide targets, there is no universal solution.

Extraction is optimized during the method development phase, accelerated by appropriate controls and spike-ins to understand the process and detect expected and unexpected events. Experienced scientists play a crucial role in ensuring the development moves forward efficiently, without technical surprises later on. At TATAA Biocenter, we have extensive RNA/DNA extraction expertise across diverse sample types, ranging from various tissues and biofluids to different fixation methods. Our facility is equipped with multiple instruments for automated extraction and optimized workflows, while we also perform manual extractions when it’s the best approach. Our deep experience enables us to identify the optimal solution for each project, minimizing the need for additional optimization and eliminating unexpected issues.

Platforms

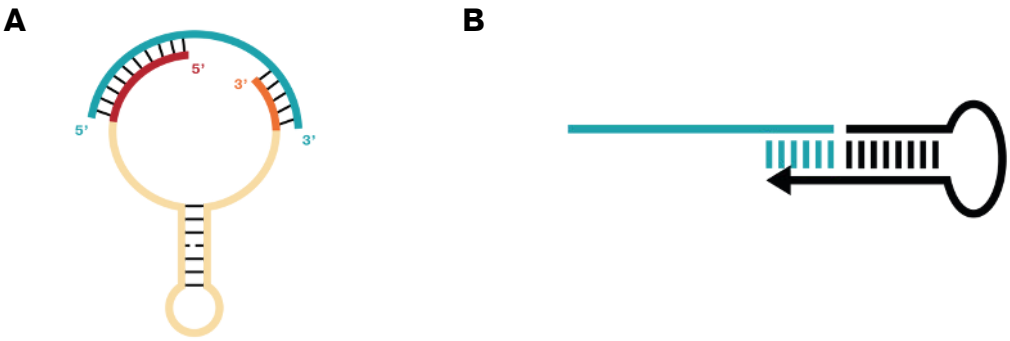
Quantifying short RNA can be achieved through two main approaches. Next-generation sequencing (NGS) methods offer comprehensive screenings, generating data on multiple targets simultaneously. Alternatively, targeted assays using modified primers for short oligonucleotides, facilitating high-throughput qPCR or dPCR analyses on numerous samples.

Next-generation sequencing (NGS)

Next-generation sequencing (NGS) enables comprehensive screening of short RNA molecules, much like standard transcriptomics but with a specific focus on short RNA species. Specialized library preparation protocols are employed, designed specifically for short RNA or even exclusively for miRNAs. This allows for the precise identification and quantification of small RNA molecules, offering the potential to discover novel RNA species. This technique is primarily utilized to investigate the regulatory roles of miRNAs and siRNAs in gene expression.

Compliant PCR assays

A compliant PCR assay requires thorough validation to ensure it meets the acceptance criteria for robustness, accuracy, precision, sensitivity, and specificity. Techniques to elongate short oligonucleotides are necessary for high-throughput PCR assays targeting biodistribution, kinetics, or biomarker analysis. This can be achieved by adding a poly-A tail with an oligo dT primer or by using an elongation method with a stem-loop primer approach. At TATAA, we have developed a technology that utilizes a Two-Tailed RT-PCR primer. Similar to the stem-loop primer, this primer extends short RNA during cDNA



The short oligonucleotides are elongated before qPCR/dPCR using either (A) the Two-Tailed Primer, which binds to the short RNA/DNA with two interaction sites to improve specificity, or (B) the stem-loop RT primer, which binds to the short RNA and elongates it by including the primer in the cDNA.

synthesis, enabling quantification using standard qPCR or dPCR methods. However, unlike the stem-loop primer, our Two-Tailed primer features two binding sites for the short RNA, significantly enhancing specificity. The qPCR/dPCR assay is performed with target-specific

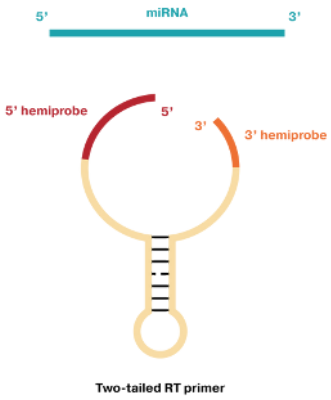
forward and reverse primers after the Two-Tail RT-PCR step. This differs from the stem-loop primer method, where the reverse primer targets the primer sequence rather than the target RNA. In addition, the Two-Tailed PCR technology offers greater flexibility in selecting primer binding sites, unlike the stem-loop primer, which binds exclusively to the 3' end of short RNA with no flexibility. This allows the Two-Tailed primers to be adjusted for optimal binding and PCR performance. With the stem-loop primer, the binding either works or it doesn't, which may not meet the stringent requirements of a validated assay.

Two-Tailed Primer Assays

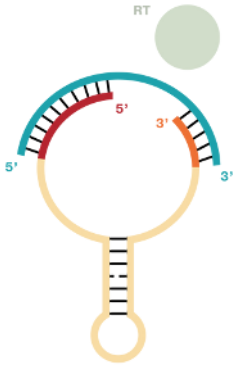
The Two-Tailed Primer was published in 2017 and has since been utilized in various applications. These include the detection of short oligonucleotides and other scenarios where high specificity is crucial, such as distinguishing between single base pair differences in the target. Two-Tailed assays can be conducted either individually (singleplex) or with multiple targets simultaneously (multiplex).

HOW IT WORKS

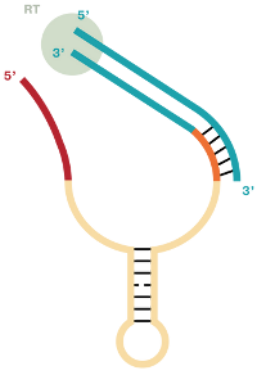
Watch our Two-Tail video



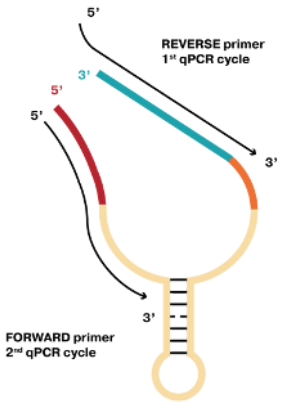
Two-tailed RT primer with two hemiprobes connected by a hairpin folding sequence.



The hemiprobes bind coope- ratively, one at each end of the target miRNA, forming a stable complex.



Reverse transcriptase binds the 3´-end of the hybridized Two-tailed RT primer and elongates it to form tailed cDNA.



The cDNA is amplified by qPCR using two target-specific primers.

Androvic, P. et. al, Two-tailed RT-qPCR: A novel method for highly accurate miRNA quantification. Nucleic Acids Res. 2017, 45, e144.
Androvic, P. et al, Two-tailed RT- qPCR panel for quality control of circulating microRNA studies. Scientific Reports – Nature 2019, 9:4255.

Quality beyond the data

Assay validation is essential for generating reproducible and reliable data. At TATAA, we offer an infrastructure specifically designed to deliver high-quality results. We are accredited by SWEDAC for GLP, compliant with GCLP, and hold ISO/IEC 17025 accreditation, ensuring adherence to the highest standards.

Comprehensive quality management system (QMS):
Ensures consistent adherence to quality standards and procedures.

Laboratory information management system (LIMS): Streamlines data management, tracking, and reporting.

Backup power generator:
Provides uninterrupted power supply to maintain lab operations and safeguard samples/data.

Data quality control (QC):
Rigorous data validation and verification processes to minimize errors and ensure accuracy.

Data quality assurance (QA):
Systematic monitoring and evaluation of data generation, analysis, and reporting.

Secure data storage and backup:
Robust data protection measures with multiple backup levels to prevent data loss.

Skilled and qualified personnel:
Well-trained and experienced staff ensuring high-quality services and adherence to GCLP standards.

Regular audits and inspections:
Ongoing internal and external assessments to maintain compliance and identify areas for improvement.

Continuous improvement process:
Commitment to refining processes and procedures to enhance the quality of services offered.

Transparent communication:
Open and proactive communication with clients, providing updates and addressing concerns throughout the project.

High-throughput workflow

We frequently engage in scalable projects, using specialized instrumentation tailored to specific applications. These range from niched projects like highly multiplexed digital PCR assays to high-throughput automated extraction and liquid handling robots and walk-away instruments for qPCR capable of analyzing hundreds of samples per hour, twenty-four hours a day, seven days a week.

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