



A High-Sensitivity Stem-Loop RT-qPCR Method for RISC-Loaded siRNA Quantitation

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INTRODUCTION

- Small interfering RNA (siRNA), which is typically composed of 21-25 nucleotides, is a short, double-stranded RNA molecule that has become a pivotal tool for gene silencing via the RNA interference (RNAi) pathway. It guides the RNA-induced silencing complex (RISC) to a target messenger RNA (mRNA), enabling sequence-specific degradation and effectively silencing the target gene^[1]. Critically, only siRNA loaded into the RISC complex possesses pharmacological activity; free siRNA does not directly contribute to therapeutic efficacy. Therefore, quantification of total siRNA fails to indicate the amount of active RISC, leading to poor PK/PD correlation and unclear dosing rationale. Due to the extremely low abundance of RISC-complexes, a high-sensitivity method should be developed.
- By following the methods described in published papers, we found that detecting RISC-loaded siRNA is susceptible to interference from the large quantity of unbound siRNA due to the extremely low concentration of RISC-loaded siRNA in vivo, particularly during the early stages of administration. This study aims to establish and validate a highly selective and ultrasensitive bioanalytical method for quantifying RISC-loaded siRNA in target tissues. This approach will enable a more accurate assessment of drug PK/PD profiles and provide a scientific basis for dose optimization of siRNA therapeutics.

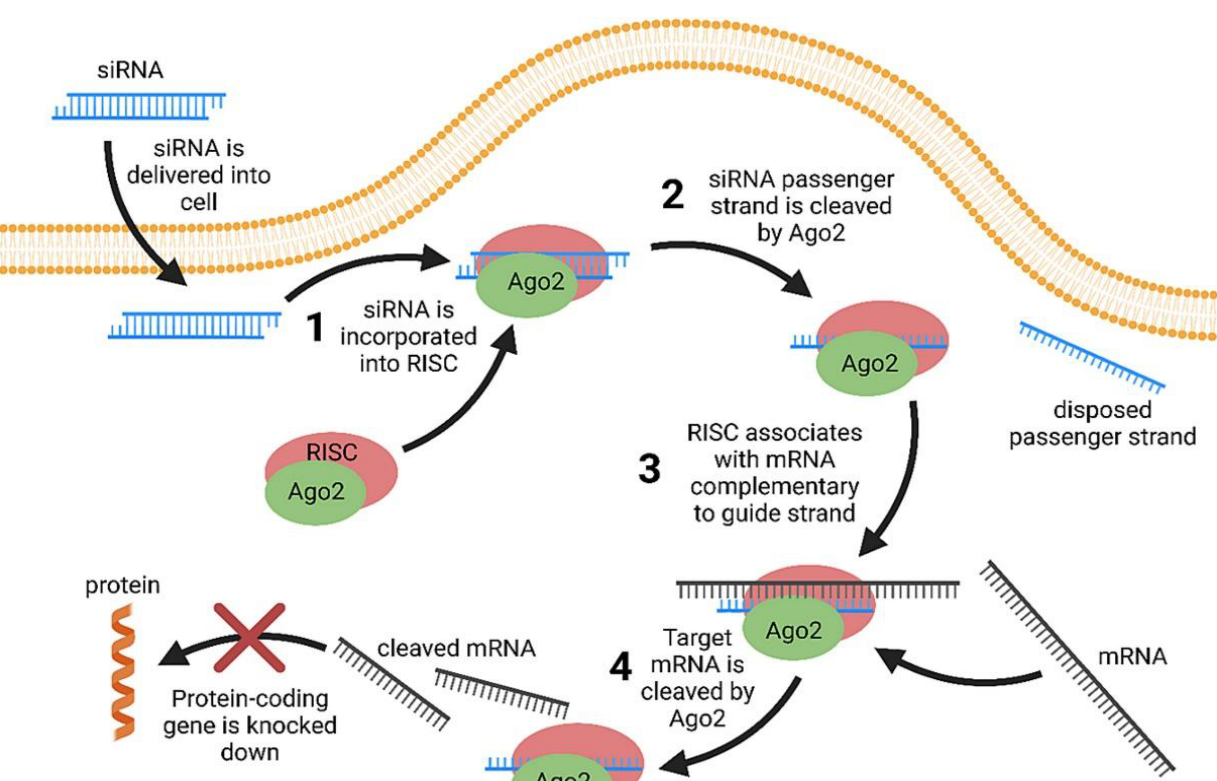


Figure 1. Mechanism of siRNA-based RNA interference (RNAi): 1) Exogenous siRNA delivered into the cell is incorporated into the RNA-Induced Silencing Complex (RISC), a multiprotein complex. 2) The argonaute-2 protein (Ago2) of RISC cleaves the siRNA passenger siRNA strand. 3) The remaining guide strand complexes with the target mRNA. 4) Ago2 cleaves the mRNA inhibiting its translation into protein.^[2]

METHOD

Sample preparation involved RNA immunoprecipitation (RIP) with Dynabeads (ThermoFisher Scientific), which are coupled to 60 µg anti-Ago2 antibody (or IgG as a negative control) per 10 mg beads. Precleared tissue homogenates were incubated with the antibody-coated beads at 37°C for 2 hours, the Dynabeads–Ab–Ag complexes were washed on a KingFisher Flex system, and Ago2-loaded siRNA was eluted via heating. The recovered Ago2-associated siRNA was then reverse transcribed using a specific stem-loop RT primer and quantified by qPCR^[3].

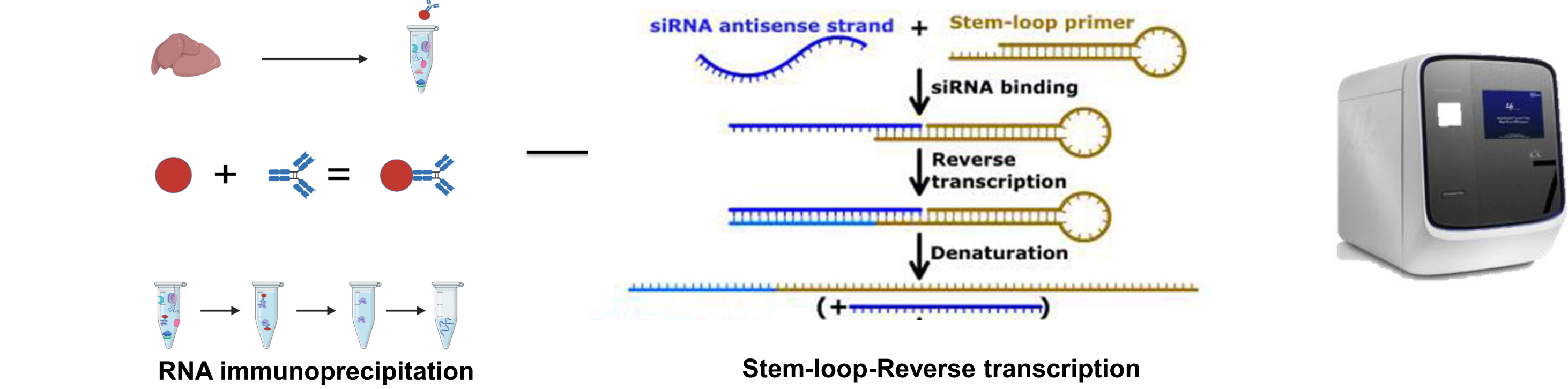


Figure 2. RNA immunoprecipitation & RT-qPCR detection

RESULTS

Specificity of immunocapture

The specificity of immunocapture for RISC-associated siRNA was evaluated by comparing the immunocapture via the antibody of Ago2 with a nonspecific IgG control. Ago2 IP samples contained substantially higher levels of the target siRNA than IgG controls across multiple biological replicates and tissue types, demonstrating selective enrichment of RISC species. Signal in IgG controls was near background, confirming minimal nonspecific binding of RISC to beads or other antibodies except for the antibody of Ago2. As an orthogonal positive control, endogenous miR21-an established Ago2 loaded microRNA-was markedly enriched in Ago2 IP fractions but not in IgG pulls, proving that the method specifically captures functional RISC complexes.

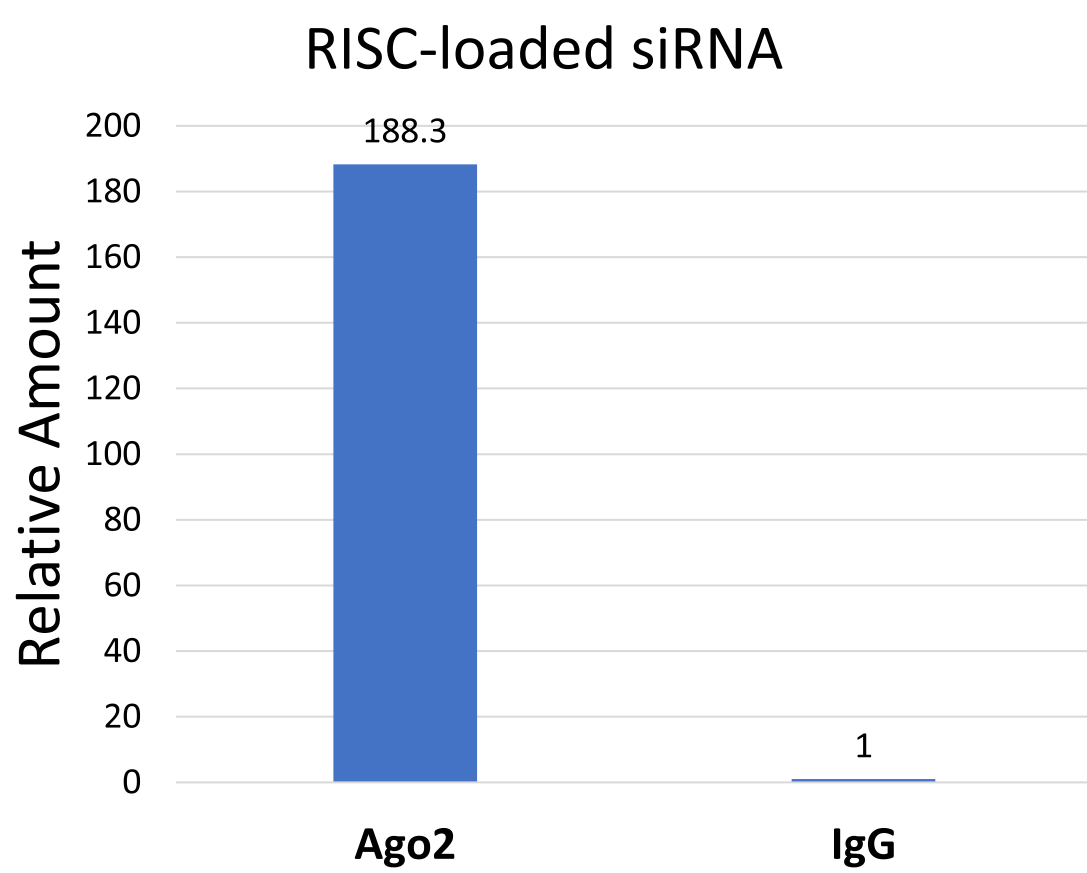


Figure 3. Enrichment of siRNA in RISC

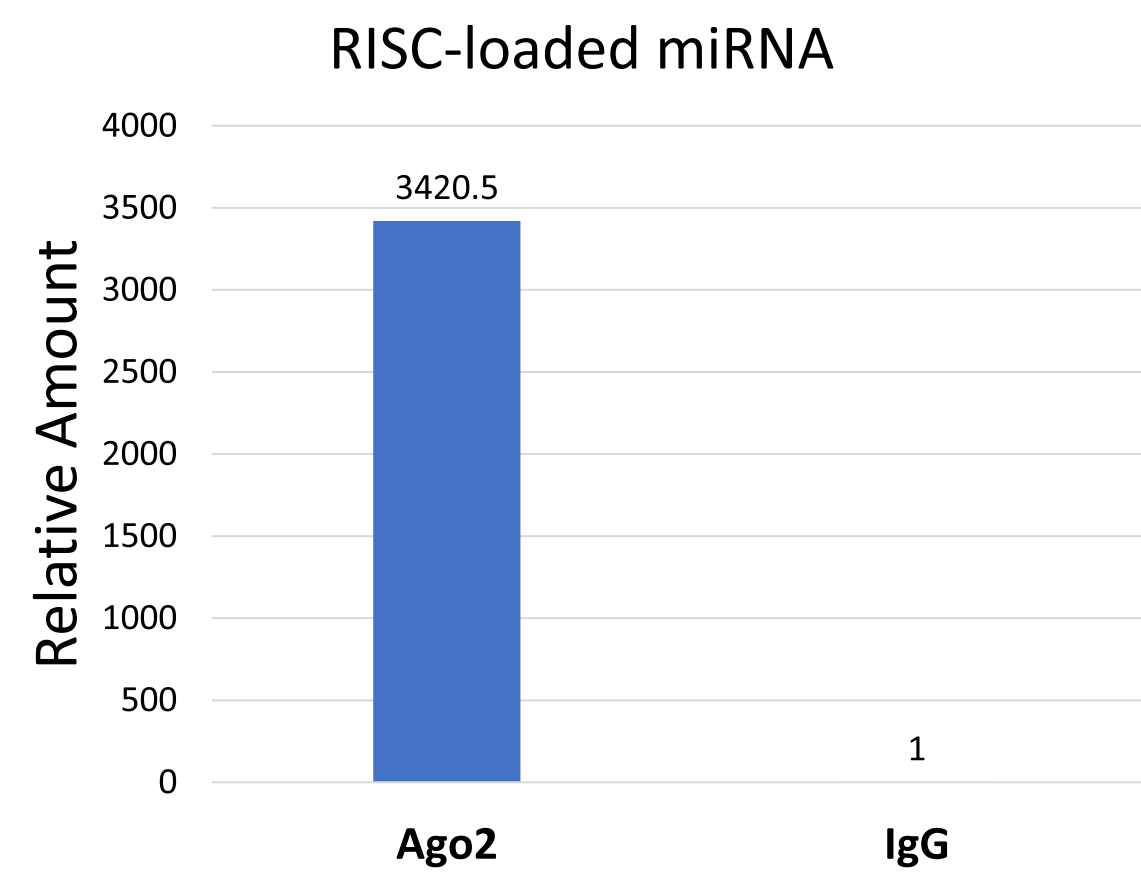


Figure 4. Enrichment of miRNA in RISC

Sensitivity and Linear Range

The stem-loop RT-qPCR assay demonstrated excellent analytical sensitivity and quantitative performance. The lower limit of quantification (LLOQ) was 0.72 pg/mL, with the upper limit of quantification (ULOQ) at 9,000 pg/mL, yielding a dynamic range spanning four orders of magnitude. The method's high sensitivity enables detection of trace amounts of RISC-loaded siRNA in tissues, including the late time points post dose when total tissue levels are quite low but pharmacologically relevant RISC fractions persist.

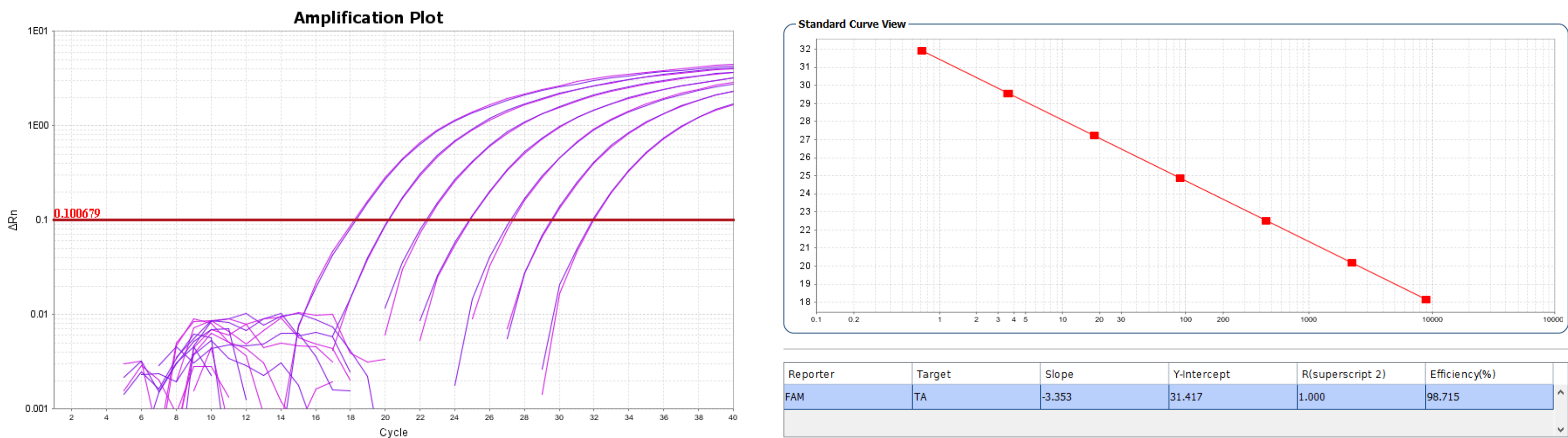


Figure 5. Amplification plot and standard curve

Dilution linearity

Because it is difficult to obtain standards of RISC-bound siRNA at known concentrations, samples diluted with blank tissue homogenate were used for the feasibility assessment of the method. The measured concentrations of the samples after dilution were proportional to the dilution factors, demonstrating dilution linearity and reliable quantitation of the method. Meanwhile, endogenous miR-21 levels remained essentially unchanged during the dilution, indicating that the dilution procedures did not introduce artifactual loss or enrichment of RISC-associated small RNAs and that the method is reliable.

Table.1. Relationship Between RISC Concentration and sample quantity

Sample name	CT value	ΔΔCT	Relative analyte Concentration	Relative theoretical concentration
10 mg-Ago2	24.8	4.56	23.3	25
2 mg-Ago2	27.2	2.16	4.5	5
0.4 mg-Ago2	29.4	1.00	1	1

Table.2. Ct values of endogenous miRNA-21 in samples with different dilution factors

Sample name	Dilution factor	CT value
10 mg-Ago2	1	23.4
2 mg-Ago2	5	23.3
0.4 mg-Ago2	25	22.8

PK-PD relationship

The method was applied to analyze rat liver tissue following the siRNA administration. Time-course analysis revealed a strong correlation between RISC-associated siRNA levels and pharmacodynamic outcomes: the extent and duration of target mRNA suppression corresponded closely with RISC-bound siRNA concentrations, and the recovery of mRNA expression coincided with the decay of RISC-loaded siRNA.

CONCLUSION

RNA immunoprecipitation (RIP) coupled with stem-loop RT-qPCR is a reliable and effective technique for quantifying RISC-loaded siRNA in pharmacokinetic studies. This method provides drug exposure data with greater pharmacodynamic relevance than total siRNA measurement, serving as a critical analytical tool to advance the development of siRNA therapeutics.

REFERENCES

[1]Prakash, P., Prerena, Hariprasad, M. G. (2025). International Journal of Advanced Research, 13(5), 538–543.
[2]Neary, M. T., Mulder, L. M., Kowalski, P. S., et. (2024). Journal of Controlled Release, 366, 812–833.
[3]Neugebauer, M., Grundmann, C. E., Lehnert, M., et. (2022). Pharmaceutics, 14(7), 1348.

