

A Novel RT-qPCR Methodology for Quantitative Bioanalysis of Total and Conjugated siRNA in Antibody-Oligonucleotide Conjugates (AOCs)

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Jiaming Jiang, Hong Sun, Guohao Zhang, Hongmei Wang, Xiaoqi Wang, Zhiyu Li, Marc Gillard, Lili Xing, Yi Tao, Liang Shen

DMPK Department, WuXi AppTec, 31 Yiwei Rd. Waigaoqiao Free Trade Zone, Pudong New Area, Shanghai, China, 200131

INTRODUCTION

Background

Antibody-oligonucleotide conjugates (AOCs) represent an emerging class of targeted therapeutics designed to enhance the selective delivery of oligonucleotide payloads (especially siRNA) to specific tissues. Its complex structure, comprising of antibody, linker, and oligonucleotide, substantially complicates bioanalytical assessment. Comprehensive monitoring of AOC drug candidates requires quantification of multiple analytes, including conjugated and free antibody forms, as well as total and unconjugated siRNA payload. Such analyses are critical for understanding the pharmacokinetic (PK), toxicokinetic (TK), pharmacodynamic (PD), efficacy, and safety profiles of these therapeutics. While several bioanalytical techniques (ELISA, LC-MS/MS, and stem-loop RT-qPCR) have been employed for oligonucleotide measurement, existing methods for AOC quantification remain limited. Notably, RT-qPCR-based approaches exhibit exceptional sensitivity, with detection typically achievable from ng/mL down to mid-pg/mL concentrations.

This study presents a novel RT-qPCR methodology specifically developed for antibody-siRNA conjugate quantification, significantly advancing AOC research and development. The optimized qPCR protocol was rigorously validated for essential performance parameters, including linearity, sensitivity, amplification efficiency, and specificity.

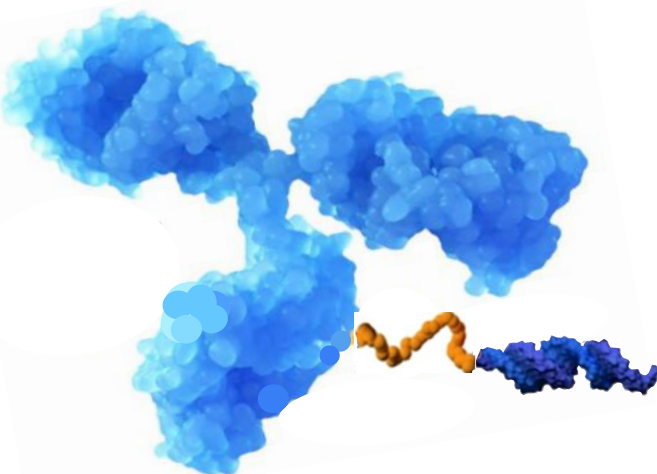


Figure 1. Structure of AOC

Novel aspect

Although numerous papers reported on detecting siRNA using the qPCR method, its application in the bioanalysis of antibody-oligonucleotide conjugates (AOCs) has been scarcely reported. For AOC therapeutics, the challenge in detecting total siRNA lies in the need to accurately quantify both the antibody-bound and unbound fractions simultaneously. However, detecting antibody-bound siRNA requires the prior separation and purification of the intact fraction to eliminate interference from incomplete fractions. This study introduces two validated RT-qPCR-based methods for accurately quantifying total and conjugated siRNA payloads in AOCs separately, addressing a critical gap in bioanalysis.

METHOD

1. Total siRNA Payload Analysis

Calibration standards and quality control (QC) samples were prepared in blank mouse plasma matrix. All samples (including standards, QCs, and test samples) underwent a 10-fold dilution with 0.25% Triton X-100 aqueous solution, followed by heat denaturation at 95°C for at least 10 minutes. The samples were subsequently centrifuged at 16,000 × g for 10 min at 4°C and the supernatant was collected. The total siRNA payload was subsequently reverse transcribed using a specific stem-loop RT primer and quantified by qPCR on a QuantStudio 7 Flex system.

2. Conjugated siRNA Payload Analysis

Calibration standards and QC samples were prepared following the same protocol as for total payload analysis. For conjugate isolation, 20-80 µL of protein G magnetic beads were added to each sample, followed by incubation at room temperature. Target AOC was washed on a KingFisher Flex system and eluted from the captured conjugates by heating at 95°C. The conjugated siRNA payload was subsequently reverse transcribed using a specific stem-loop RT primer and quantified by qPCR on a QuantStudio 7 Flex system.

RESULTS

1. Total siRNA Payload Analysis

In our mouse pharmacokinetic studies, we established a direct calibration curve using intact AOC as the reference standard. The assay demonstrated excellent linearity across a concentration range of 0.01-20 nM ($R^2 > 0.99$) and optimal amplification efficiency (90-110%). Given the potential coexistence of both free and conjugated siRNA in biological samples, it is essential to validate that AOC standards can accurately quantify free siRNA fractions. Bridging experiments confirmed that AOC standards provided accurate quantification of siRNA payloads, validating the method's reliability for total siRNA measurement. Notably, the assay exhibited equivalent detection capability for both antisense (AS) and sense strands (SS) of the siRNA duplex, allowing either strand to serve as a valid surrogate analyte for payload quantification.

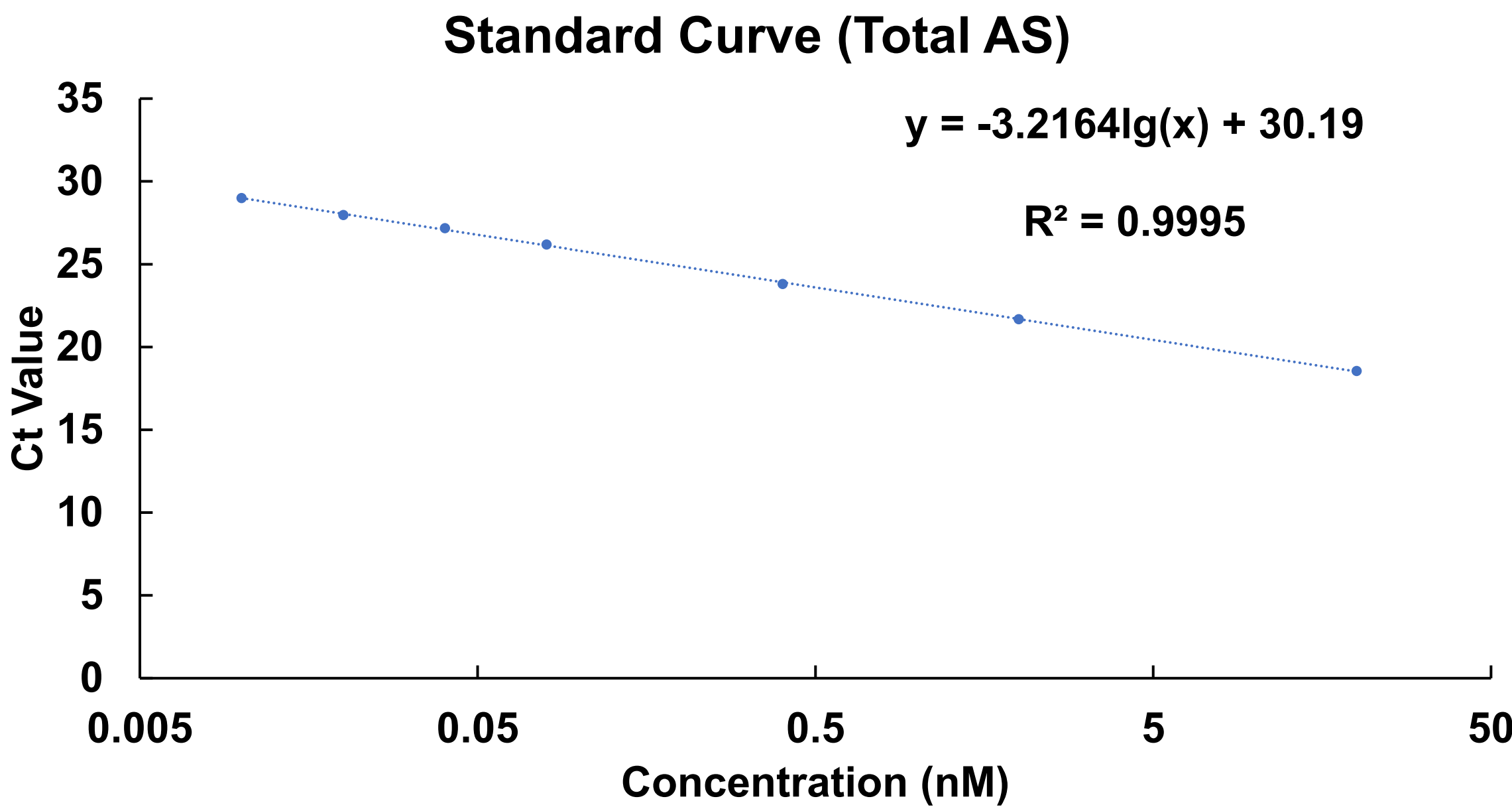


Figure 2. Linear regression of standard curve for plasma

Table 1. Bridging study (accuracy of AOC-conjugated AS strand detection using siRNA as standards)

Sample Name	Theoretical concentration (nM)	Mean Back-Calculated Quantity (nM)	%CV	%Bias
AOC_AS_HQC	2.50	2.41	17.61	-3.60
AOC_AS_MQC	0.200	0.195	11.07	-2.43
AOC_AS_LQC	0.0300	0.0312	8.57	4.12

2. Conjugated siRNA Payload Analysis

For the quantification of conjugated siRNA, our optimized method achieved high capture efficiency through magnetic bead isolation, enabling sensitive detection with an LLOQ of 0.05 nM. Following immunocapture, the antibody-conjugated siRNA was efficiently released via thermal denaturation, after which both antisense and sense strands were accurately quantified using our validated stem-loop RT-qPCR assay. The method's ability to specifically quantify conjugated siRNA while differentiating it from free siRNA represents a significant advancement for pharmacokinetic studies of AOCs, addressing a critical bioanalytical challenge in the development of these new modalities.

3. PK analysis of AOC in rat plasma

Following intravenous administration of the AOC compound to rats, we applied this validated method to comprehensively characterize the pharmacokinetic profiles of both total and conjugated siRNA in plasma. The analysis revealed favorable concentration-time curves for both total and conjugated siRNA.

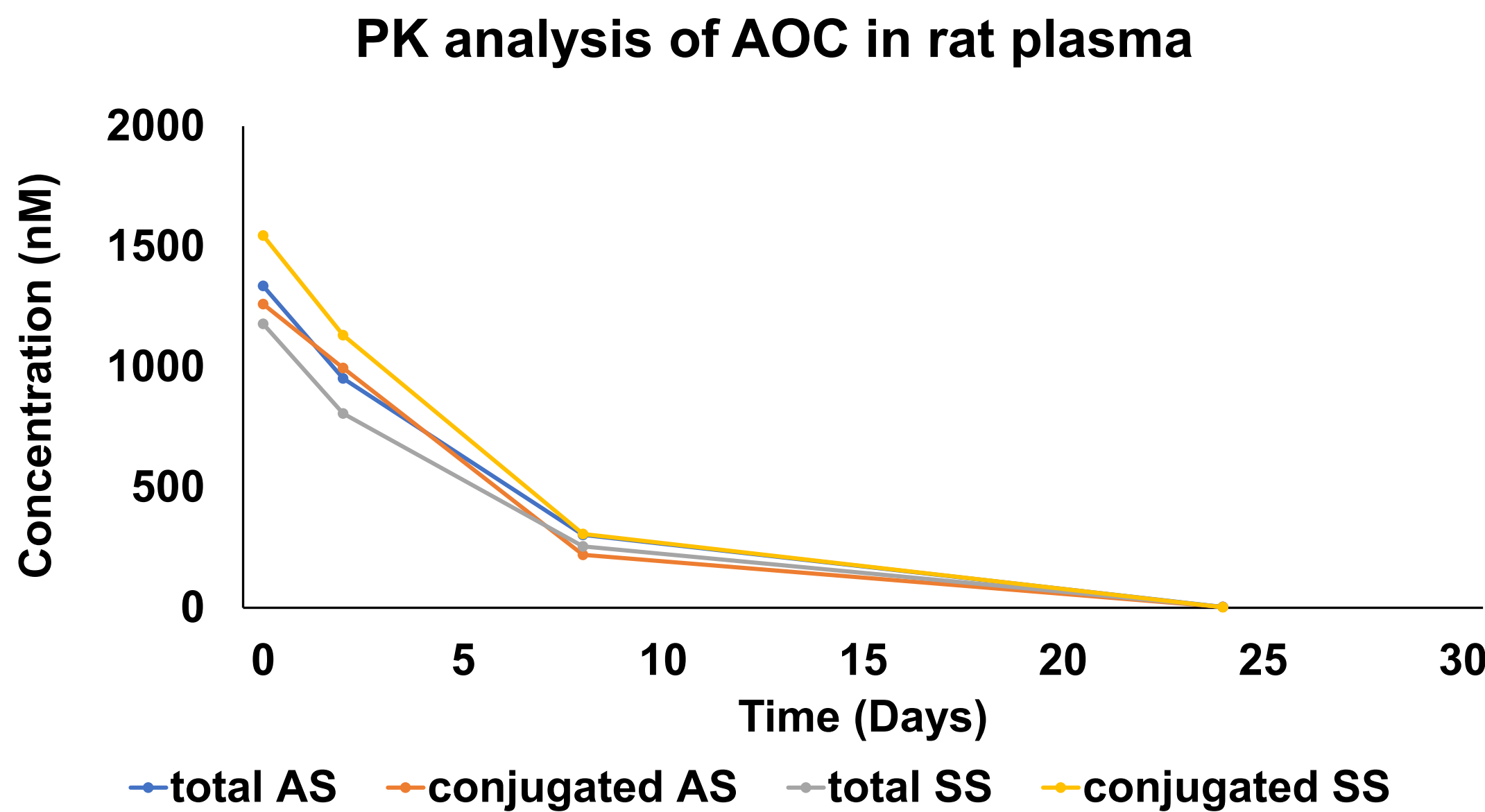


Figure 3. Concentration-time curves for both total and conjugated siRNA

CONCLUSION

This study establishes a highly sensitive qPCR-based method for precise quantification of oligonucleotide payloads in AOCs. The optimized assay achieves exceptional sensitivity (LLOQ: 0.01 nM) for both total and conjugated siRNA analysis while requiring minimal sample volumes (~3 µL plasma), and features strand-specific detection capability for both antisense and sense strands. These methodological advances address critical needs in the growing field of targeted oligonucleotide delivery, offering researchers a reliable tool for comprehensive pharmacokinetic assessment and payload quantification across various conjugate platforms.

