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# Quantitative Bioanalysis of HT-KIT Vivo-Morpholino Using Hybridization LC-MS/MS

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## INTRODUCTION

Vivo-morpholinos are a type of conjugated phosphorodiamidate morpholino oligonucleotide (PMO), representing a significant advancement in genetic research and therapy (**Figure 1**). These molecules offer a robust tool for modulating gene expression by overcoming limitations of traditional gene knockdown techniques. The enhanced delivery moiety, specifically octa-guanidine dendrimer, improves cellular uptake and stability, allowing for precise modulation of genetic pathways. Vivo-morpholinos play a crucial role in developing targeted therapies and personalized medicine, and their accuracy and efficacy make them essential for studying gene functions, modeling diseases, and exploring new treatments in developmental biology and medical research.

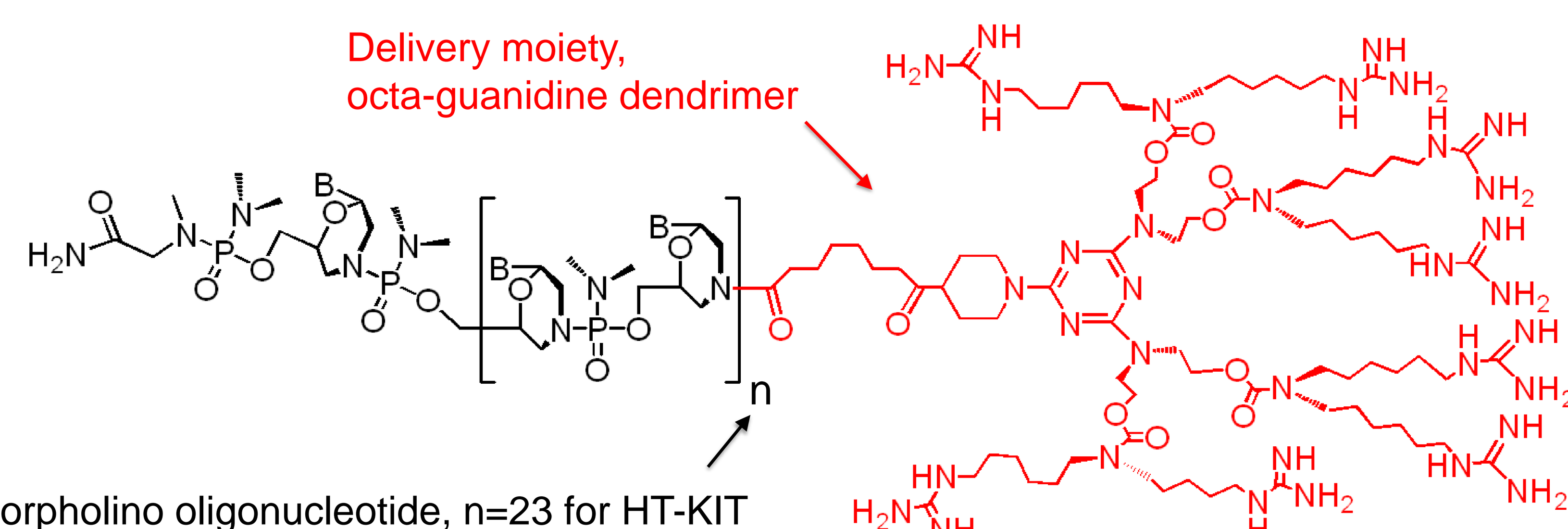
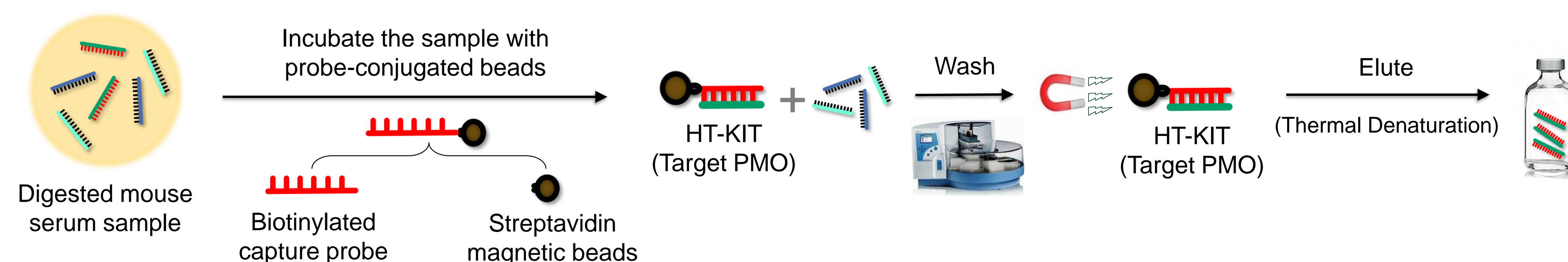



**Figure 1. Structure of a Vivo-morpholino**

To meet the increasing demand for evaluating the pharmacokinetics of these novel therapeutics, it is critical to develop sensitive and selective bioanalytical methods for determining vivo-morpholino concentrations in biological samples. However, there is limited data on the applicability of LC-MS for vivo-morpholino analysis, partly due to the lack of selectivity in traditional chemical extraction methods and compromised chromatographic resolution from strong electrostatic interactions between positively charged vivo-morpholinos and negatively charged silanol LC columns. In this research, we present a sensitive and selective analytical method that combines hybridization extraction with LC-MS/MS detection. This workflow was applied to the quantitation of HT-KIT, a novel antisense oligonucleotide under development for the treatment of cancers resulting from aberrant KIT signaling.

**(A) Hybridization Extraction**




**(B) LC-MS/MS Analysis**

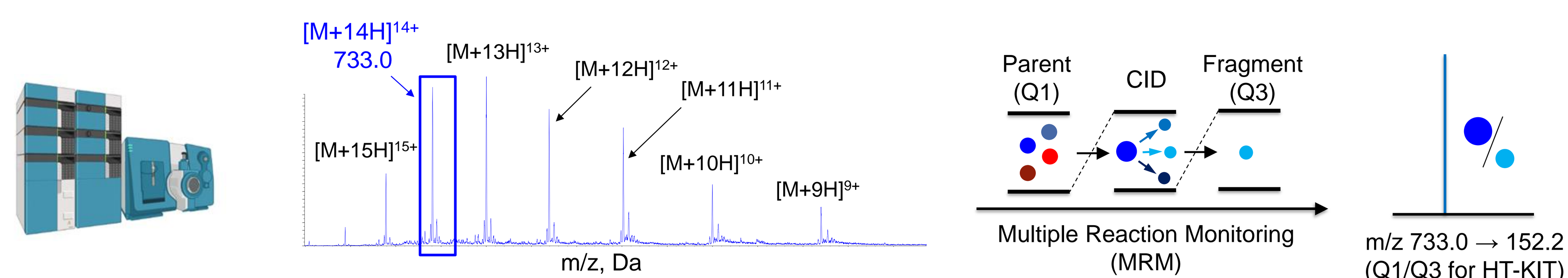



**Figure 2. Schematics of HT-KIT Hybridization Extraction (A) and LC-MS/MS Analysis (B).**

## METHODS

Mouse serum samples were fortified with HT-KIT, ranging from 1.00 to 250.00 ng/mL, and 25 µL aliquots were added to a 96-well plate. Proteinase K was used to digest proteins from the serum samples, ensuring complete HT-KIT release under mild buffer conditions for downstream hybridization. A full-length biotinylated capture probe, designed to complement the HT-KIT sequence, facilitated the formation of an HT-KIT-Probe-Bead complex. After multiple wash steps, HT-KIT was released from the capture probe via thermal denaturation. The internal standard, linear PMO, was added post-extraction and the resulting extracts were stored at 4ºC for LC-MS/MS analysis (**Figure 2**). Liquid chromatographic separation was performed on an XSelect Premier CSH C18 column (50 x 2.1 mm, 2.5 µm) with a mobile phase containing heptafluorobutyric acid (HFBA). HT-KIT and the linear PMO internal standard were detected using a SCIEX 6500+ QQQ operated in positive ESI/MRM mode.

**Table 1. Inter-Run Precision and Accuracy (3 Runs, 12 QC Replicates)**

| Parameters | QC LOQ 1.00 ng/mL | Low QC 3.00 ng/mL | Mid QC 125 ng/mL | High QC 187.5 ng/mL |
|---|---|---|---|---|
| % Nominal | 106.0 | 96.2 | 95.8 | 88.2 |
| % C.V. | 6.7 | 7.5 | 11.2 | 6.4 |

## RESULTS AND DISCUSSION

### LC-MS/MS Optimization

Ion-pairing is commonly used for LC separation of oligonucleotides, traditionally employing a combination of triethylamine (TEA) and hexafluoroisopropanol (HFIP). However, these conditions are unsuitable for PMOs, which are positively charged. Therefore, the mobile phase and analytical column must be optimized to minimize electrostatic interaction between positively charged PMOs and negatively charged silanol groups on LC columns. The Waters XSelect CSH C18 column, with its positively charged surface, was chosen to overcome issues such as peak shape asymmetry and poor loading capacity. HFBA was added to the mobile phase to improve the peak sharpness of HT-KIT and its separation from the linear PMO internal standard. To counteract HFBA's suppression of MS ionization and enhance sensitivity, ammonium bicarbonate was added post-column. Under optimized LC-MS conditions, baseline separation of HT-KIT and the internal standard was achieved with a lower limit of quantitation (LLOQ) of 1.00 ng/mL using a low sample volume (≤ 25µL).

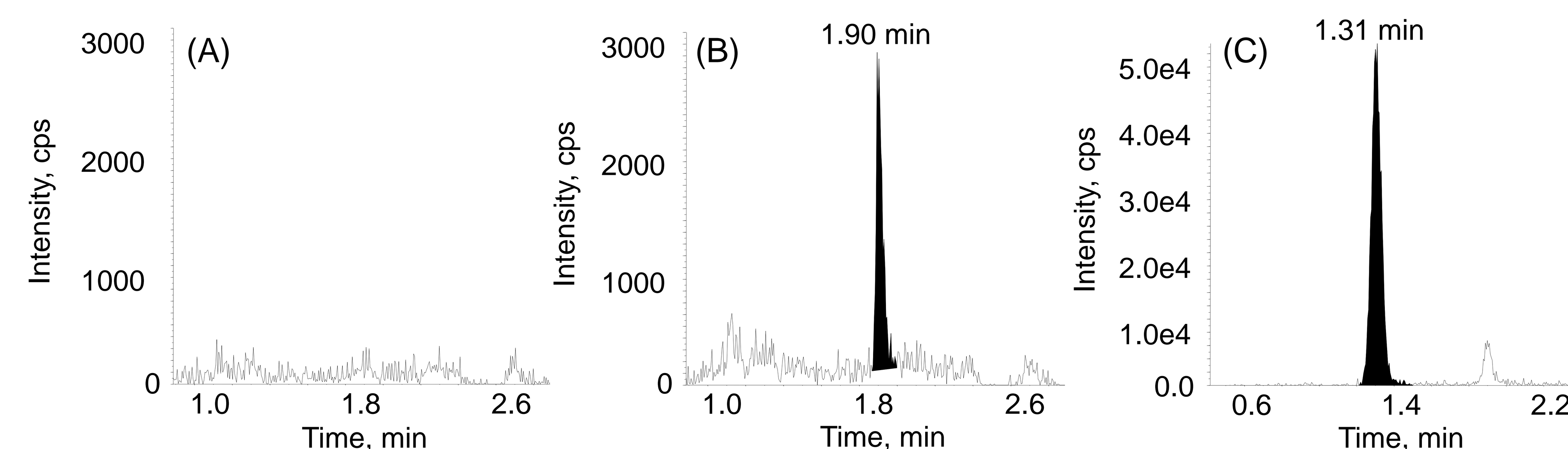



**Figure 3. Representative chromatograms of extracted Blank (A), Extracted LLOQ (1.00 ng/mL HT-KIT) (B), and Extracted IS (Linear PMO) (C).**

### Hybridization Optimization

In hybridization extraction, the formation of a stable analyte-probe-bead complex is crucial for effective sample clean-up and recovery. Unlike linear PMO, HT-KIT is conjugated with an octa-guanidine dendrimer, showing a different affinity to probe-conjugated magnetic beads. The effect of different lengths of complementary capture probes (15-, 20-, and 25-mer) on HT-KIT and internal standard extractability was investigated, with the full-length probe providing the best recovery. Different magnetic beads, including Dynabeads MyOne Streptavidin C1 and Cytiva Streptavidin Mag Sepharose beads, were tested. MyOne beads, with a larger surface area, showed severe non-specific binding (NSB) to the octa-guanidine moiety, resulting in reduced HT-KIT recovery. Conversely, NSB was negligible on Cytiva beads, achieving quantitative HT-KIT recovery.

### Method Qualification

The calibration curve was constructed with a range of 1.00 – 250 ng/mL established for HT-KIT using peak area ratios to the IS and applying a weighted ($1/x^2$) linear regression. Representative chromatograms (blank [BL], LLOQ, and Extracted IS) are shown in **Figure 3**. Precision and accuracy assessments were performed at four QC concentration levels (LLOQ, 3x LLOQ, geometric mean, and 75% of ULOQ), all within acceptance criteria (**Table 1**). The assay was specific and free of matrix effect, tested across 4 independent lots of mouse serum (**Table 2**).

**Table 2. Evaluation of Matrix Effect on the Quantitation of HT-KIT**

| Donors | Low QC: 1.50 ng/mL | | | High QC: 187.5 ng/mL | | |
|---|---|---|---|---|---|---|
| | Mean | %C.V. | %Bias | Mean | %C.V. | %Bias |
| Donor #1 | 1.56 | 3.6 | 3.7 | 165.9 | 1.1 | -11.5 |
| Donor #2 | 1.63 | 4.7 | 8.8 | 167.8 | 1.8 | -10.5 |
| Donor #3 | 1.51 | 9.0 | 0.6 | 171.0 | 2.9 | -8.8 |
| Donor #4 (hemolysed) | 1.49 | 4.0 | -1.0 | 165.6 | 3.8 | -11.7 |

## CONCLUSION

A sensitive and selective bioanalytical method for determining HT-KIT, a conjugated PMO, was successfully developed for an analytical range of 1.00 – 250 ng/mL from 25 µL mouse serum using hybridization LC-MS/MS. The method was linear, precise, and accurate, meeting all bioanalytical assessment criteria. The application of this hybridization LC-MS/MS method to the quantification of PMOs will accelerate the research and development of PMO therapeutics with improved efficacy, safety, and pharmacokinetic profiles.

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