

A VALIDATED HYBRIDIZATION LC-MS/MS METHOD FOR QUANTITATIVE BIOANALYSIS OF HT-KIT, A VIVO-MORPHOLINO ANTISENSE OLIGONUCLEOTIDE

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PURPOSE

Vivo-morpholinos, a class of conjugated phosphorodiamidate morpholino oligonucleotides (PMOs), represent a significant advancement in genetic research and therapeutic development (**Figure 1**). By incorporating an octa-guanidine dendrimer, these molecules achieve enhanced cellular uptake and stability, enabling precise modulation of gene expression. They are increasingly applied in disease modeling, targeted therapies, and personalized medicine. To support pharmacokinetic evaluation, sensitive and selective bioanalytical methods are essential. However, conventional LC-MS approaches face limitations due to inefficient extraction and poor chromatographic resolution, largely driven by strong electrostatic interactions originating from cationic vivo-morpholinos.

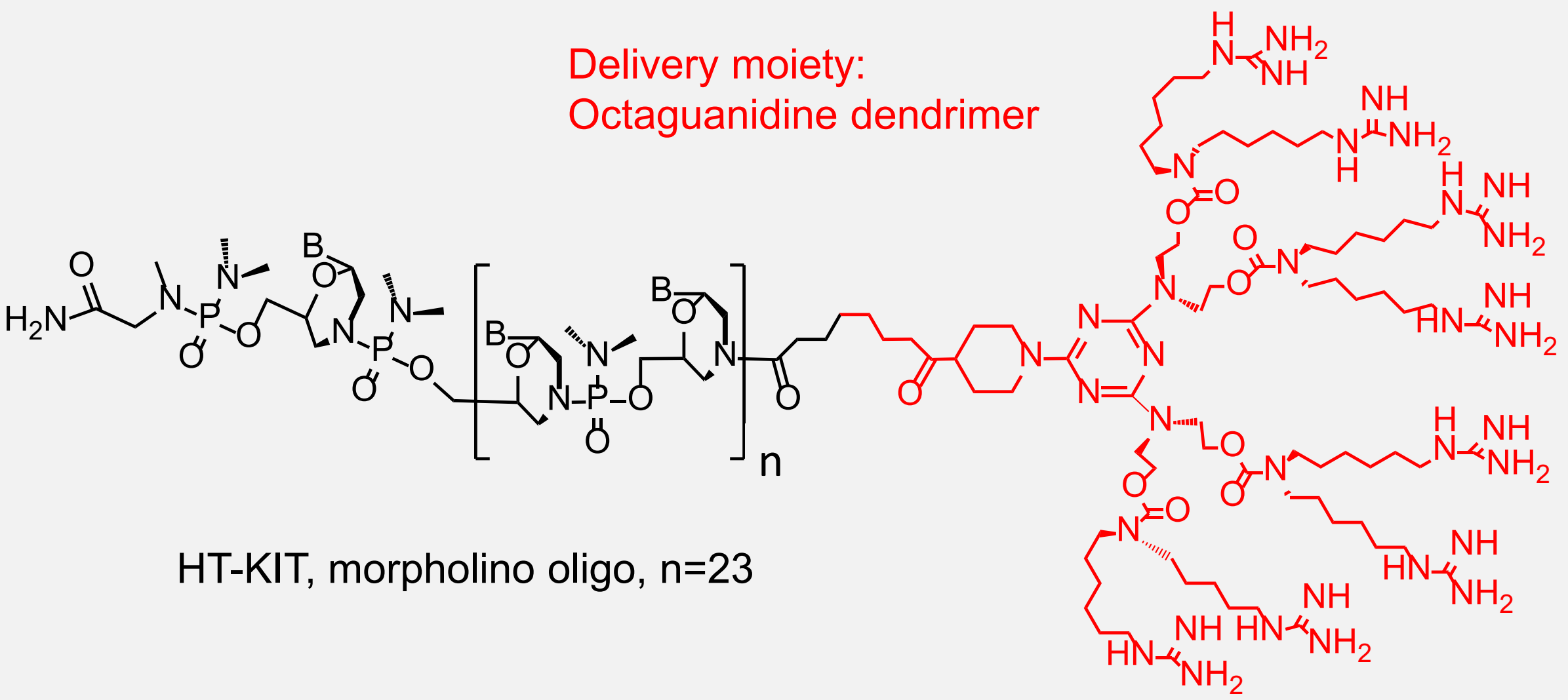


Figure 1. Structure of HT-KIT, Vivo-Morpholino

OBJECTIVE

To develop and validate a quantitative hybridization LC-MS/MS assay for HT-KIT, an octa-guanidine-conjugated vivo-morpholino, in mouse serum over an analytical range of 1.00 – 250 ng/mL.

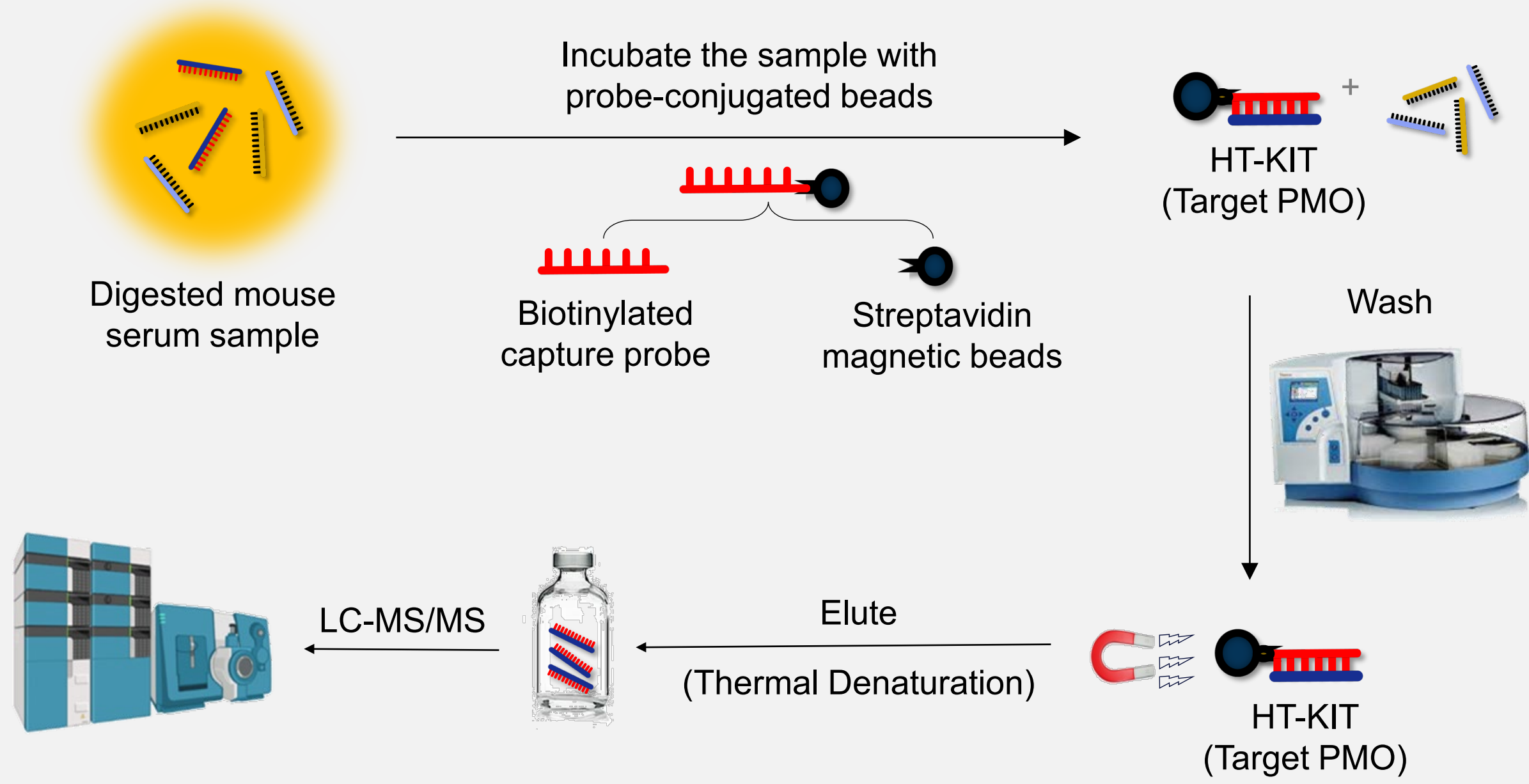


Figure 2. Schematic of HT-KIT Hybridization Extraction

METHODS

Sample Preparation

- 25 µL spiked mouse serum (1.00 – 250 ng/mL)
- Proteinase K digestion
- Hybridization extraction with a full-length biotinylated complementary probe pre-conjugated to streptavidin magnetic beads
- Automated extraction of HT-KIT on the KingFisher Flex magnetic particle processor (**Figure 2**)
- Linear PMO internal standard added post-extraction

Chromatography

- Shimadzu Nexera X2 UPLC
- Waters XSelect Premier CSH C18 column, 50 × 2.1 mm, 2.5 µm
- Mobile Phase A: 0.05% Heptafluorobutyric Acid (HFBA)
- Mobile Phase B: Acetonitrile
- Gradient Elution: (**Table 1**)
- Column Temperature at 60 °C
- Post-Column Addition (PCA): 100 mM Ammonium Bicarbonate

Table 1. Gradient Elution Profile

Time (minutes)	Flow (mL/min)	Mobile Phase B (%)	PCA Flow (mL/min)
0.00	0.600	20	0.200
2.00	0.600	32	0.200
2.25	0.600	85	0.200
2.50	0.600	100	0.200
2.51 – 4.25	0.850	Column gradient flush	0.000
4.26 – 6.00	0.600	Mobile phase re-equilibration	0.200

Mass Spectrometry

- SCIEX 6500+ Triple Quadrupole
- Electrospray Ionization in Positive Mode
- Multiple-Reaction Monitoring (MRM) (**Table 2**)

Table 2. Summary of LC-MRM Detection Conditions

Analyte	MRM Transition (m/z)	Dwell Time (ms)	Retention Time (min)
HT-KIT	733.0 > 152.1	200	1.98
Linear PMO (Internal Standard)	768.3 > 152.1	100	1.37

RESULTS

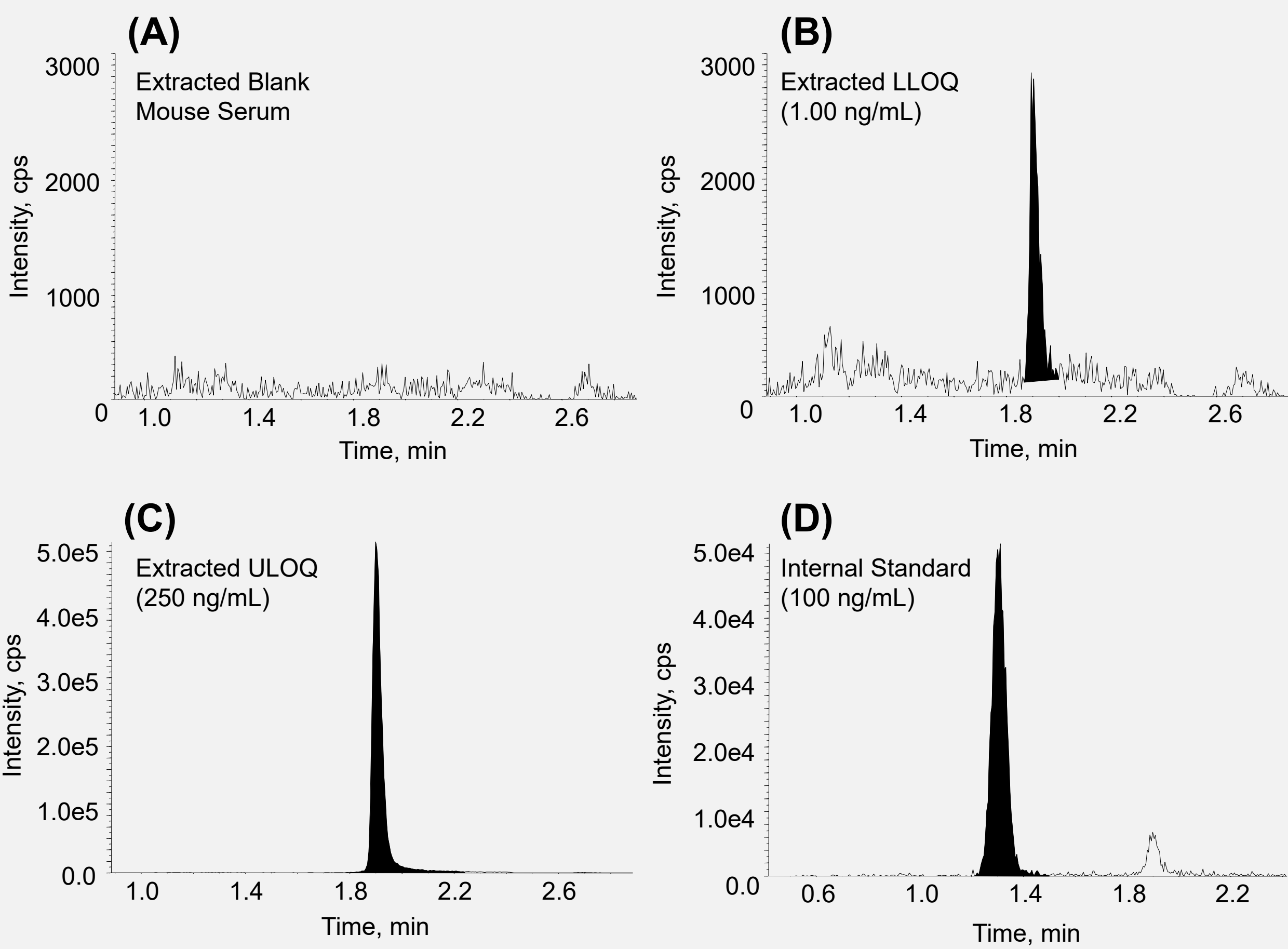


Figure 3. Representative chromatograms of extracted (A) Blank mouse serum, (B) LLOQ (1.00 ng/mL), (C) ULOQ (250.00 ng/mL), and (D) Internal standard

Table 3. Between-Run Precision and Accuracy

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	99.0	92.7	101.7	101.9
% C.V.	12.1	11.9	7.3	5.2

N = 3 batches, 6 QC replicates/concentration/batch

Table 4. Matrix Effect for HT-KIT in Mouse Serum

Mouse Serum Lot	Low QC (3.00 ng/mL)			High QC (187.5 ng/mL)		
	Mean	% Bias	% C.V.	Mean	% Bias	% C.V.
1	2.62	-12.7	3.4	205	9.3	3.6
2	2.65	-11.7	1.1	212	13.4	6.3
3	2.66	-11.3	1.1	197	5.6	11.0
4	2.66	-11.3	3.0	209	11.7	9.6
5	2.79	-7.0	15.1	205	9.6	6.3
6	2.68	-10.7	13.8	206	10.2	3.5
Hemolyzed	2.65	-11.7	4.2	214	14.5	2.8

Table 5. Stability of HT-KIT in Mouse Serum

Stability	Low QC (3.00 ng/mL)			High QC (187.5 ng/mL)		
	Mean	% Bias	% C.V.	Mean	% Bias	% C.V.
Long-Term (28 days at -20°C)	2.74	-8.7	6.2	183	-2.5	3.2
Long-Term (28 days at -80°C)	2.74	-8.7	5.1	186	-0.8	6.4
Short-Term (23.8 hours at Room Temperature)	2.70	-10.0	4.1	175	-6.9	2.8
Freeze-Thaw (4 Cycles)	2.52	-16.0	3.6	174	-7.0	2.9

Table 6. Summary of Method Validation Evaluations

Evaluation	Results
Specificity	No significant interference was observed in 6 screened blank matrix lots, including one hemolyzed matrix lot
Dilution integrity	500 ng/mL diluted 5-fold. Accuracy (%Bias): -5.8%, Precision (%CV): 7.7% 10.0 µg/mL diluted 100-fold. Accuracy (%Bias): -4.9%, Precision (%CV): 5.5%
Recovery	78.9% at Low QC, 89.5% at Mid QC, 98.0% at High QC
Re-Injection Reproducibility	Confirmed up to 189.7 hours at 4°C nominal

CONCLUSIONS

- A fully validated hybridization LC-MS/MS assay was developed for quantification of HT-KIT in mouse serum.
- The method requires only 25 µL of serum and enables accurate quantitation across a range of 1.00 – 250 ng/mL.
- Hybridization extraction with a full-length complementary capture probe provided high selectivity and sensitivity.
- The assay met all bioanalytical validation criteria and is well-suited for preclinical evaluations of vivo-morpholinos.

