APPLICATION NOTE

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Analysis of the Low-Abundance Plasma Biomarker Klotho in Less than Four Hours

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Key Words
SMART Digest ImmunoAffinity kit, immunoaffinity, peptides, trypsin digestion, transmembrane β-glucuronidase, UHPLC, Accucore C18, peptide mapping, LC-MS/MS, biomarker

Goal

To demonstrate the rapid quantitation of the low-level biomarker Klotho from plasma by LC-MS/MS. using the Thermo Scientific™ SMART Digest™ ImmunoAffinity (IA) kit, which combines the immunoaffinity capture and digestion process into a single well.

Introduction

Klotho, a transmembrane β-glucuronidase, is an important biomarker in aging research.[1-3] LC-MS/MS analysis provided single-run multiplexed quantitation of two specific peptides formed by the digestion of Klotho. The amount of the biomarker present was determined by quantitation of these signature peptides. An immunoaffinity step prior to digestion was used to purify the target biomarker for increased sensitivity and was able to purify all the isoforms of the biomarker.[4]

The SMART Digest IA kit is designed for biomarker quantitation. Since biomarker proteins are often present at low levels in complex biological matrices, it is often necessary to use immunoaffinity capture to reduce interferences and thus increase sensitivity. This step is often followed by protein digestion and subsequent quantitation of known unique signature peptides.

The SMART Digest IA kits are designed to remove issues often associated with sample preparation of proteins by delivering a process that is:

- Fast
- Simple
- Highly reproducible
- Sensitive
- Compatible with automation

The SMART Digest IA kits achieve this with their unique design, where the immunoaffinity reagents (either streptavidin, protein A, or protein G) and heat-activated, thermally stable trypsin are co-immobilized onto a single bead. Following the binding of a capture reagent to the bead and enrichment of the target, the enzyme is activated at elevated temperatures for accelerated

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digestion under protein denaturing conditions. This results in a fast, easy-to-implement, and sensitive workflow (Figure 1). Magnetic and non-magnetic versions of the beads are available.

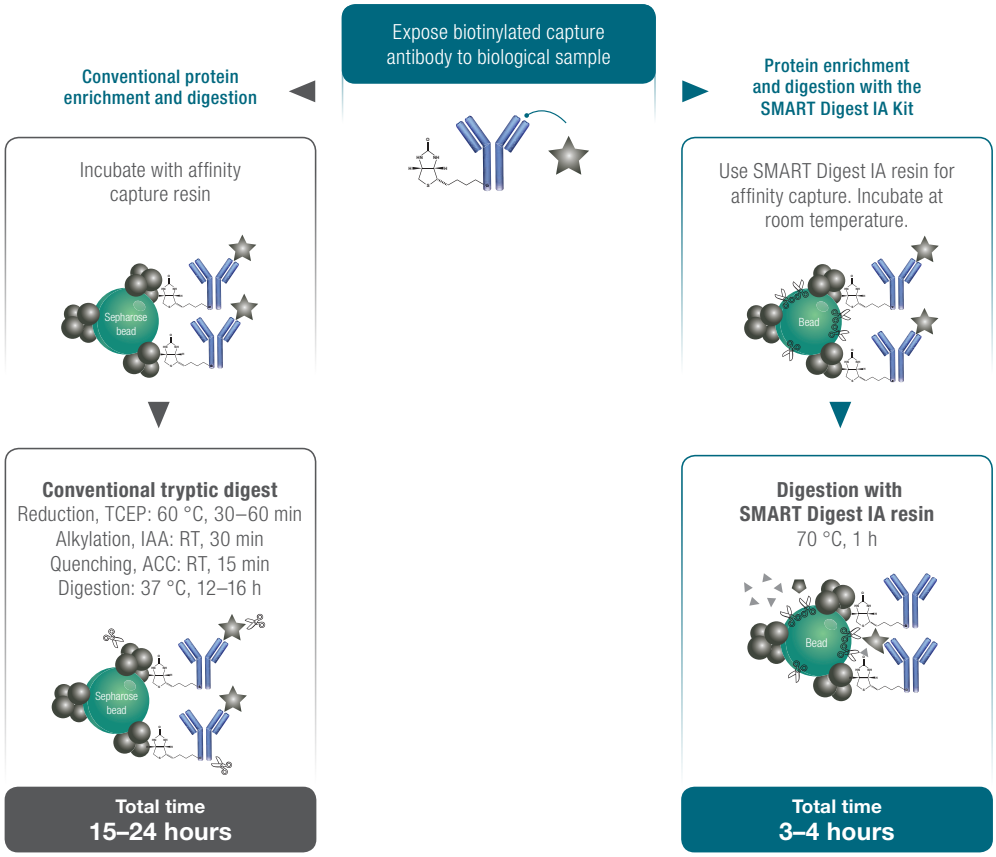


Figure 1. Comparison of SMART Digest IA kit workflow with a conventional workflow.

Experimental

Consumables

Affinity / Digestion

- SMART Digest IA Streptavidin Non-Magnetic Kit (P/N 60110-101)

Chemicals

- Deionized water, 18.2 MΩ/cm resistivity
- Fisher Scientific™ Optima™ Acetonitrile (ACN) (P/N A955-4)
- Fisher Scientific™ Formic Acid (FA) (P/N F/1900/PB08)
- Thermo Scientific™ Pierce™ Dimethylsulfoxide (DMSO), LC-MS Grade (P/N 85190)
- Thermo Scientific™ EZ-Link™ NHS-Biotin (P/N 20217)
- Human Klotho antibody from a reputable supplier
- Recombinant human Klotho (rhKlotho) from a reputable supplier
- Murine plasma from a reputable supplier

Sample Handling

- Thermo Scientific™ Pierce™ Microcentrifuge Tubes, 1.5 mL (P/N 69715)
- Thermo Scientific™ Mass Spec™ Certified 2 mL clear vial with blue bonded PTFE silicone cap (P/N MSCERT5000-341W)

Sample Handling Equipment

- Thermo Scientific™ MicroCL 17 microcentrifuge (P/N 75002449)
- Thermo Scientific™ LP Vortex Mixer (P/N 88880018)
- Heater/shaker equipped with heated block and lid

Antibody Pretreatment

Antibody biotinylation: 500 µL of 100 µg/mL Klotho antibody was biotinylated by the addition of 2.5 µL of 0.5 mg/mL NHS-biotin in DMSO. The solution was then incubated for 2 hours at room temperature (RT) on a heater/shaker set at ambient temperature and 1400 RPM before storage at 4 °C until subsequent use.

Standard Sample Preparation

rhKlotho was spiked into mouse plasma in varying concentrations ranging from 1.58 ng/mL to 500 ng/mL.

Immunoaffinity Purification Using the SMART Digest IA Kit

Purification: 500 µL of sample, 1 µg (10 µL) of biotinylated Klotho antibody, and 30 µL of SMART Digest IA resin were added to a 1.5 mL microcentrifuge tube, placed in a heater/shaker and incubated for two hours at room temperature and 1400 RPM. Following the capture step, 500 µL of supernatant was decanted, 500 µL of wash buffer was added to the samples before they were vortexed, and then centrifuged at 13,200 RPM (16,100 RCF) for 1 minute. This was repeated for a total of seven washes.

Digestion Using the SMART Digest IA Kit

After the final centrifugation, 460 µL of supernatant was removed, carefully with no removal of beads, by the pipetting step. 150 µL of SMART Digest buffer was added and samples were incubated at 70 °C and 1400 RPM for 90 minutes.

Separation Conditions

Instrumentation

Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system equipped with:

- SRD-3600 Solvent racks with degasser (P/N 5035.9230)
- DGP-3600RS Rapid Separation Pump (P/N 5040.0066)
- WPS-3000TRS Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
- TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)

Column

Thermo Scientific™ Accucore™ C18 column 2.1 mm × 50 mm, 2.6 µm (P/N 17126-052130)

LC Settings

Mobile Phase A	0.1% formic acid in 98% water and 2% acetonitrile
Mobile Phase B	0.1% formic acid in 5% water and 95% acetonitrile
Gradient	See Table 1
Flow Rate	0.5 mL/Min
Column Temperature	40 °C
Injection Details	5 µL

Table 1. LC gradient conditions.

Time (min)	A %	B %
0	98	2
1	98	2
6	50	50
6.1	10	90
7.5	10	90
7.51	98	2
9	98	2

MS Conditions

Instrumentation

Thermo Scientific™ Velos Pro™ Ion Trap Mass Spectrometer

MS Settings

Mode	Positive
Heater Temp	350 °C
Sheath Gas	60
Aux Gas	20
Spray Voltage	4 kV
Capillary temp	375 °C
S-Lens RF Level	55%

Data Processing

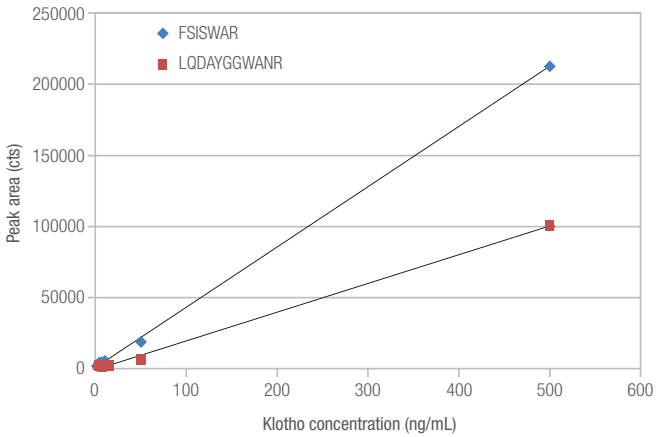
The Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System SR2 was used for data acquisition and analysis. MS instrument control was by Thermo Scientific™ Xcalibur™ software.

Results and Discussion

Using the SMART Digest IA Streptavidin kit and high flow LC-MS/MS, the method was able to achieve a LLOQ of 1.58 ng/mL in mouse plasma. CVs at the LLOQ were 6.3% and 12.5% without the use of isotopic standards.

Additionally, as seen in Figure 2, the data exhibited strong linearity across the dynamic range tested with excellent linearity at low levels of FISWAR marker.

A



B

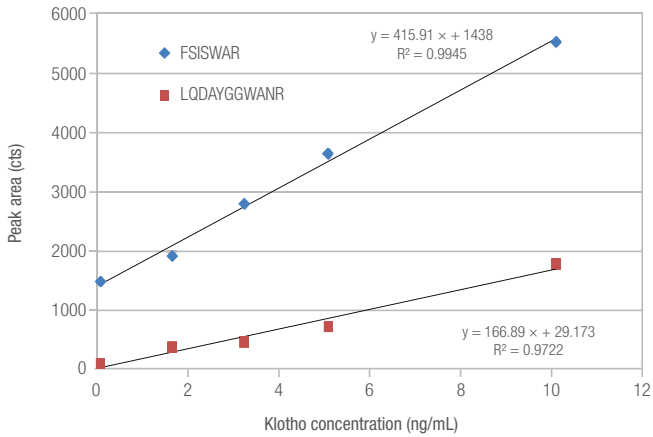


Figure 2. Calibration curves for the detection of Klotho from plasma using SMART Digest IA Streptavidin kit. Samples were run in triplicate. Figure 2A shows the full range of the calibration curve. Figure 2B shows the lower concentrations with linear fit.

The SMART Digest IA kit enabled the preparation of assay and calibration samples in 3 to 4 hours. The samples were analyzed using LC-MS/MS, with linearity coefficients of > 0.97 and CVs of < 17% for the two marker peptides being achieved across the calibration range.

Table 2. Determination of peptide at low, medium, and high levels of quantitation. A weighted fit was used (1/x where x is the analyte concentration).

Compound	Linearity Range (ng/mL)	R^2	CV (%) at LLOQ (n=3)	CV (%) at MLOQ (n=3)	CV (%) at HLOQ (n=3)
FSISWAR	1.58–500	0.9945	6.3	10.0	9.7
LQDAYGGWANR	1.58–500	0.9722	12.5	17.0	10.5

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Conclusions

- A simple, robust method for the quantification of Klotho was developed using the SMART Digest IA kit.
- The kit combines immunoaffinity enrichment and digestion in a single reactor to produce results in less than four hours.
- The full biological range of Klotho and its isoforms, as well as other low-abundance biomarkers, can be accessed using this generic strategy.

References

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