Targeted Kinase Inhibitor Profiling Using a Hybrid Quadrupole-Orbitrap Mass Spectrometer

Scott Peterman[1], Ryan D. Bomgarden[2], Rosa Viner[3]
[1]Thermo Fisher Scientific, Cambridge, MA; [2]Thermo Fisher Scientific, Rockford, IL; [3]Thermo Fisher Scientific, San Jose, CA

Application Note 574

Key Words

Q Exactive, targeted peptide quantification, msxSIM, kinome profiling by MS, desthiobiotin nucleotide probes

Goal

To identify and quantify kinase inhibition by staurosporine using kinase active sites probes in combination with targeted, multiplexed SIM (msxSIM).

Introduction

Protein kinases are key enzymes involved in a wide array of complex cellular functions and pathways. Misregulation or mutation of protein kinases underlies numerous disease states, including tumorigenesis, making them ideal candidates for drug development. However, identifying specific kinase inhibitors is challenging due to the high degree of homology among subfamily members of the 500+ human kinases. In addition, overlapping kinase substrate specificity and crosstalk between cellular signaling pathways can confound attempts to identify kinase inhibitor targets *in vivo*.

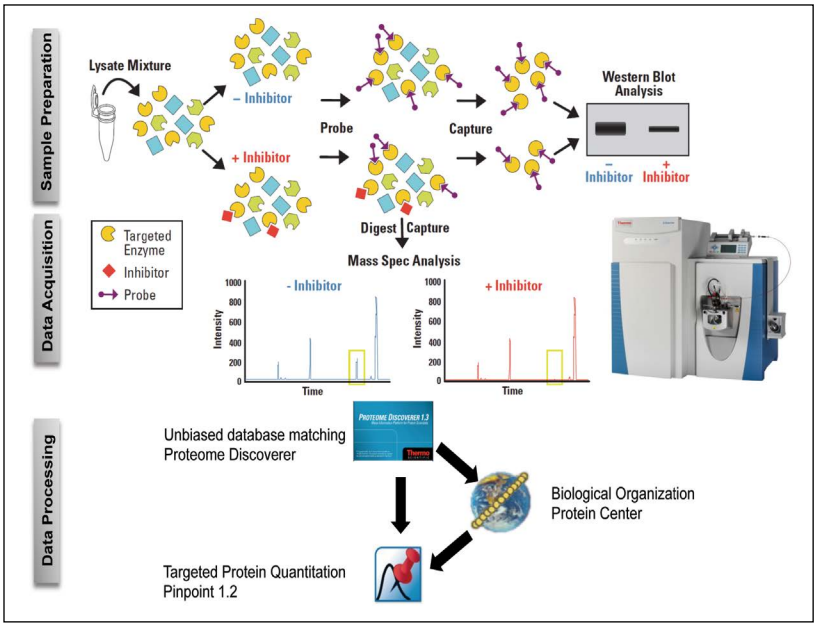


Figure 1. Integrated workflow of sample preparation/enrichment, data acquisition and data processing for global kinase identification and drug inhibition profiling

An emerging technology for identifying kinase inhibitor targets is based on chemical proteomic profiling of kinase inhibitor specificity and binding affinity. This technology combines mass spectrometry (MS)-based identification and quantitation with small molecule probe binding and enrichment to determine kinase active site occupancy during inhibition. One of these methods uses novel biotinylated ATP and/or ADP probes that irreversibly react with conserved lysine residues of kinase ATP binding sites.[1,2] Selective enrichment of active-site peptides from labeled kinase digests dramatically reduces background matrix and increases signal for MS analysis of low-abundance kinase peptides. Using this method, more than 400 different protein and lipid kinases from various mammalian tissues and cell lines have been identified and functionally assayed using targeted acquisition on an ion trap mass spectrometer.[1] The assays are available commercially from ActivX Biosciences as their KiNativ™ kinase profiling services.

Our approach incorporates desthiobiotin-ATP and -ADP probes with a hybrid quadrupole-Orbitrap™ mass spectrometer into an integrated workflow for global kinase identification and inhibition analysis (Figure 1). This workflow leverages the unique capabilities of the mass spectrometer for acquisition of MS and MS/MS spectra in unbiased or targeted modes with Orbitrap ion detection for high resolution and mass accuracy. A multiplexed data acquisition method was also employed to maximize instrument duty cycle and quantification of low-abundance kinase peptides through selected ion monitoring (SIM) on a nano-LC timescale. Overall, this approach resulted in significant improvements to kinase active-site peptide detection and relative quantitation for kinase inhibitor profiling.

Thermo SCIENTIFIC

Experimental

Sample Preparation

K562 leukemia cells (ATCC) were grown in RPMI media supplemented with 10% FBS. Cell lysates (1 mg) were desalted using Thermo Scientific™ 7K Zeba™ Spin Desalting Columns and labeled with 5 µM of Thermo Scientific ActivX™ Desthiobiotin-ATP or -ADP probes for 10 minutes as described previously.[3] For inhibitor profiling, cell lysates were pretreated with 0, 0.01, 0.3, 1, 3, and 10 µM of staurosporine before addition of the desthiobiotin nucleotide probes. Labeled proteins were reduced, alkylated, desalted, and digested with trypsin. Desthiobiotin-labeled peptides were captured using Thermo Scientific High-Capacity Streptavidin Agarose Resin for two hours, washed and eluted using 50% acetonitrile/0.4% TFA for MS analysis.

LC-MS

All separations were performed using a Thermo Scientific EASY-nLC™ nano-HPLC system and a binary solvent system comprised of A) water containing 0.1% formic acid and B) acetonitrile containing 0.1% formic acid. A 150 x 0.075 mm capillary column packed with Magic™ C18 packing material was used with a 0.57% per minute gradient (5%–45%) flowing at 300 nL/min at room temperature. The samples were analyzed with a Thermo Scientific Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer in either data-dependent or targeted fashion. Details of the acquisition methods are summarized in Tables 1 and 2.

Table 1. Mass spectrometer parameter settings used for discovery experiments

Parameter	Setting
Source	Nano-ESI
Capillary temperature (˚C)	250
S-lens RF voltage	50%
Source voltage (kV)	2
Full-MS parameters	
Mass range (*m/z*)	380–1700
Resolution settings (FWHM at *m/z* 200)	140,000
AGC Target	1×10^6
Max injection time (ms)	250
Dynamic Exclusion™ duration (s)	70
Top n MS/MS	10
MS/MS parameters HCD	
Resolution settings (FWHM at *m/z* 200)	35,000
AGC Target	2×10^5
Max injection time (ms)	250
Isolation width (Da)	2
Intensity threshold	8×10^2 (0.1% underfill)
Collision energy (NCE)	27
Charge state screening	Enabled
Charge state rejection	On: 1+ and unassigned rejected
Peptide match	On
Exclude isotopes	On
Lock mass enabled	No
Lowest *m/z* acquired	10

Table 2. Mass spectrometer parameter settings used for targeted experiments

	Full	msxSIM
AGC Target Value	1×10^6	2×10^5
Max Injection Time (ms)	250	250
Resolution (FWHM at *m/z* 200)	140,000	140,000
Isolation Window (Da)	500–1300	4
# Multiplexed Precursors	-	4

Data Analysis

Thermo Scientific Proteome Discoverer™ software version 1.3 was used to search MS/MS spectra against the International Protein Index (IPI) human database using both SEQUEST® and Mascot® search engines. Carbamidomethyl (57.02 Da) was used for cysteine residue static modification. Desthiobiotin (196.12 Da) modification and oxidation were used for lysine and methionine residues, respectively. Database search results were imported into Thermo Scientific Pinpoint™ software version 1.2 for high-resolution, accurate-mass (HR/AM) MS-level quantitation. Data extraction was based on the four most-abundant isotopes per targeted peptide. The area under the curve (AUC) values were summed for the total AUC values reported. The relative AUC values for each of the isotopes were compared against the theoretical isotopic distribution for further confirmation and evaluation of potential background interference. The half-maximal inhibitory concentration (IC_{50}) values were determined by plotting AUC values for each peptide versus inhibitor concentration to generate a dose response curve of inhibitor binding (K_d) as described previously.[2]

Results & Discussion

Building a Kinase Active-Site Peptide Library

While protein kinase sequences and active sites are readily known from protein databases, it is still challenging to build a method for detection, verification, and quantification of kinase peptides. Our method focused on identifying and quantifying kinase active-site peptides since a large majority of these peptides are unique for their respective kinase. In addition, these peptides provide direct insight into kinase active-site inhibition for kinases that have multiple kinase domains.

To build a list of kinase active-site peptides, untreated cell lysate samples were labeled with the ActivX Desthiobiotin-ATP or -ADP probes for active-site peptide enrichment. An initial, unbiased Top10 data acquisition method (Table 1) was used to generate spectral libraries for database searching using Proteome Discoverer software. These search results were used to determine peptide sequences, desthiobiotin modification sites, and protein kinase family members. In addition, this experiment provided key data required for subsequent targeted acquisition methods including peptide retention times, precursor and product ion charge states, and HCD product ion distribution.

Peptide sequences were validated using Thermo Scientific ProteinCenter™ software for protein functional annotation. Figure 2 shows the proteins identified from peptides labeled with either desthiobiotin-ATP or -ADP probes. Both probes show high specificity for labeling known ATP binding proteins (77% and 83%, respectively) with protein kinases representing 13% of the total proteins identified using either probe. Although both probes enriched similar numbers of kinases with a modest degree of overlap, there was preferential binding of each probe for specific kinases as previously reported.[1]

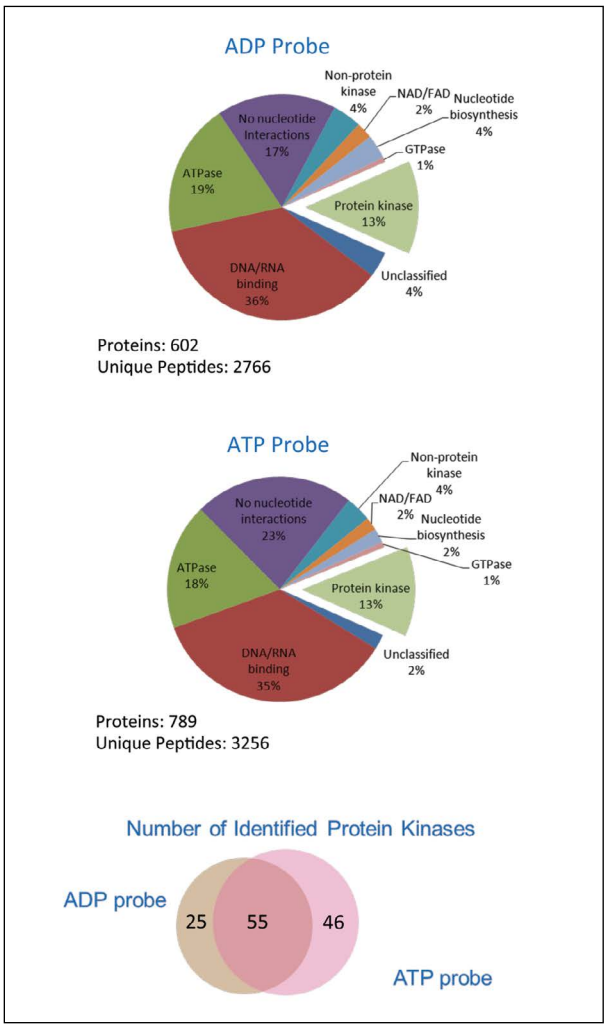


Figure 2. Proteins identified after desthiobiotin-ATP and -ADP probe labeling and enrichment categorized by protein function using Protein Center software. The Venn diagram shows the distribution of the resulting protein kinases identified using each probe.

The high resolution (up to 140,000) and mass accuracy of Orbitrap detection, as well as the ability to use the C-trap to collect/enrich the concentration of ions, facilitated kinase active-site peptide identification at the MS and MS/MS levels. Figure 3A shows an HR/AM MS spectrum at the retention time of the targeted active-site peptide **DIKAGNILLTEPGQVK** from Tao1/3. The complexity of the spectrum is typical even after active-site peptide enrichment and contains numerous highly charged peptides. The speed of the Q Exactive instrument for serial HR/AM MS and MS/MS acquisition, coupled with the C-trap's enrichment of the ion concentration in the Orbitrap mass analyzer, enabled the unbiased selection and sequencing of the +3 precursor despite it being the 14th most abundant peptide. Figure 3B shows the experimentally measured isotopic distribution and the corresponding theoretical isotopic distribution for the **DIKAGNILLTEPGQVK** +3 precursor charge state. Each isotope had less than 5 ppm mass error and less than 15% error compared to the theoretical isotope intensity distribution.

Figure 3C shows the HCD MS/MS spectra database search result matching 25 b- and y-type fragment ions. The average mass error for the fragment ions was less than 2 ppm across an order of magnitude range of measured product ion intensities. The ability to maintain a constant mass error across the entire mass spectrum is a particular advantage of Orbitrap detection, as it greatly increases peptide identification confidence. Ultimately, 126 kinase active-site peptides identified using Proteome Discoverer software were imported into Pinpoint software to evaluate relative abundance and generate an inclusion list for scheduled targeted acquisition (Figure 3D).

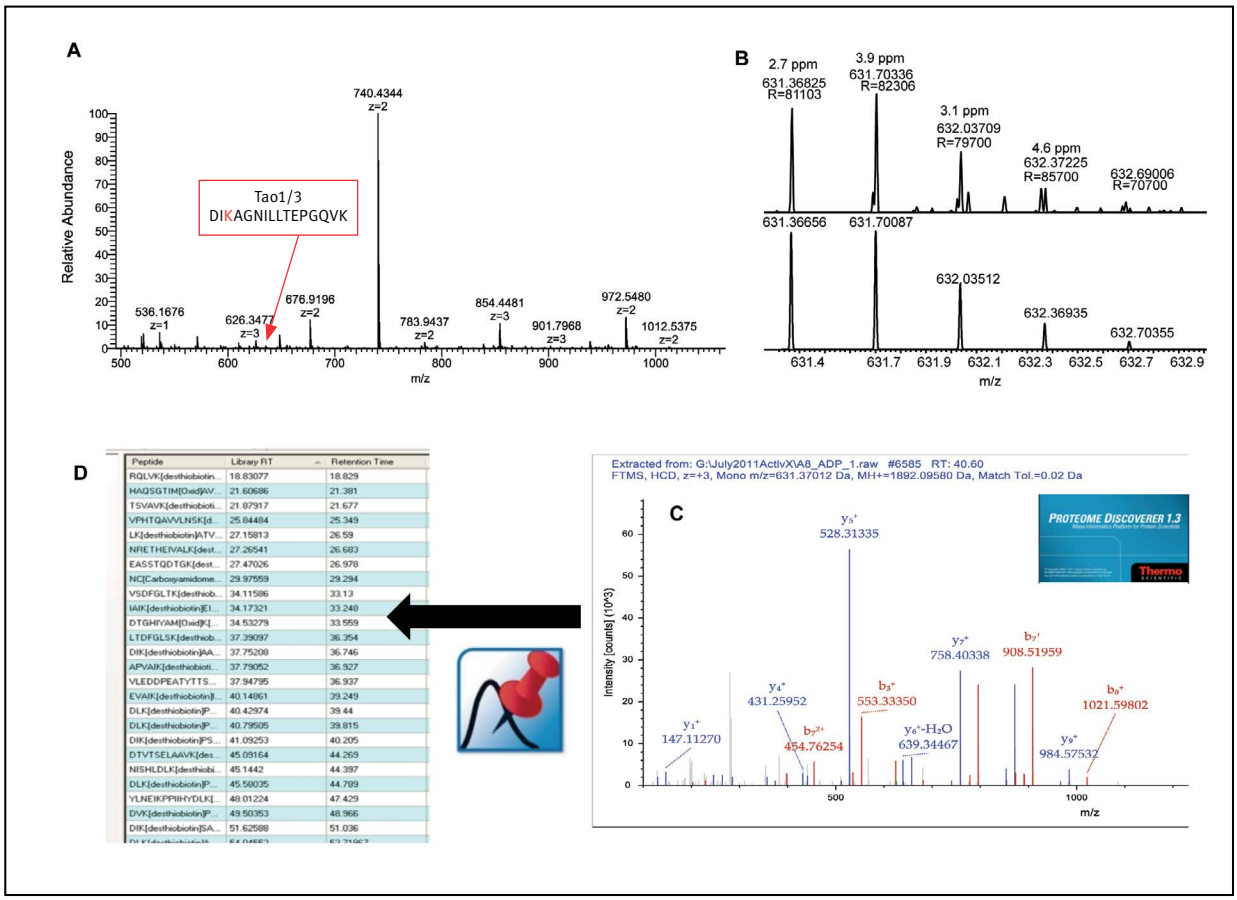


Figure 3. Processing strategy for building kinase active-site peptides spectral libraries and target lists. Example active-site peptide **DIKAGNILLTEPGQVK** from Tao1/3 kinase (A, B) was identified using Proteome Discoverer software from the HCD product ion spectrum (C) with Pinpoint software used to automate target-list method building (D).

Targeted Active-Site Peptide MS Method Development and Acquisition

Pinpoint analysis of kinase active-site peptide relative abundance in the enriched samples revealed a large dynamic range of peptide signals. More abundant active-site peptides were quantified using HR/AM full-scan MS and not included in the targeted experiment. Lower-abundance peptides were selected for a multiplexed SIM (msxSIM) acquisition to improve peptide ion signal intensity. A major advantage of the Q Exactive mass spectrometer is simultaneous C-trap filling during Orbitrap detection (Figure 4A and B).[4] This greatly reduces cycle times as selected ions can be trapped and detected in parallel. When targeted msxSIM events are scheduled, this benefit can be enhanced further by the trapping of multiple target ions in parallel. During Orbitrap detection, the C-trap is sequentially filled by ions defined by narrow mass ranges. Our experiment limited the multiplexing to a maximum of 4 mass windows but software capabilities enable up to 10 mass ranges to be collected and analyzed simultaneously. Cycling between full-scan MS and msxSIM is used to maintain target selectivity.

This approach of switching between full-scan MS and msxSIM was applied for targeted analysis of the ULK3 kinase active-site peptide **NISHLDLKPQNILLSSLEKPHLK** (Figure 4C). A narrow retention time region is displayed in centroid mode for which a total of 1.2 seconds was used to acquire data in both full-scan and SIM modes. The extracted ion chromatogram (XIC) plot from the full-scan mode shows a large number of peaks despite filtering using a mass tolerance of ±5 ppm. The highlighted region covers the expected elution time for the targeted active-site peptide, which was only 3% of the base peak intensity. The XIC profile for the SIM event shows that the scheduled, targeted data acquisition method clearly measures the peak of interest without interference.

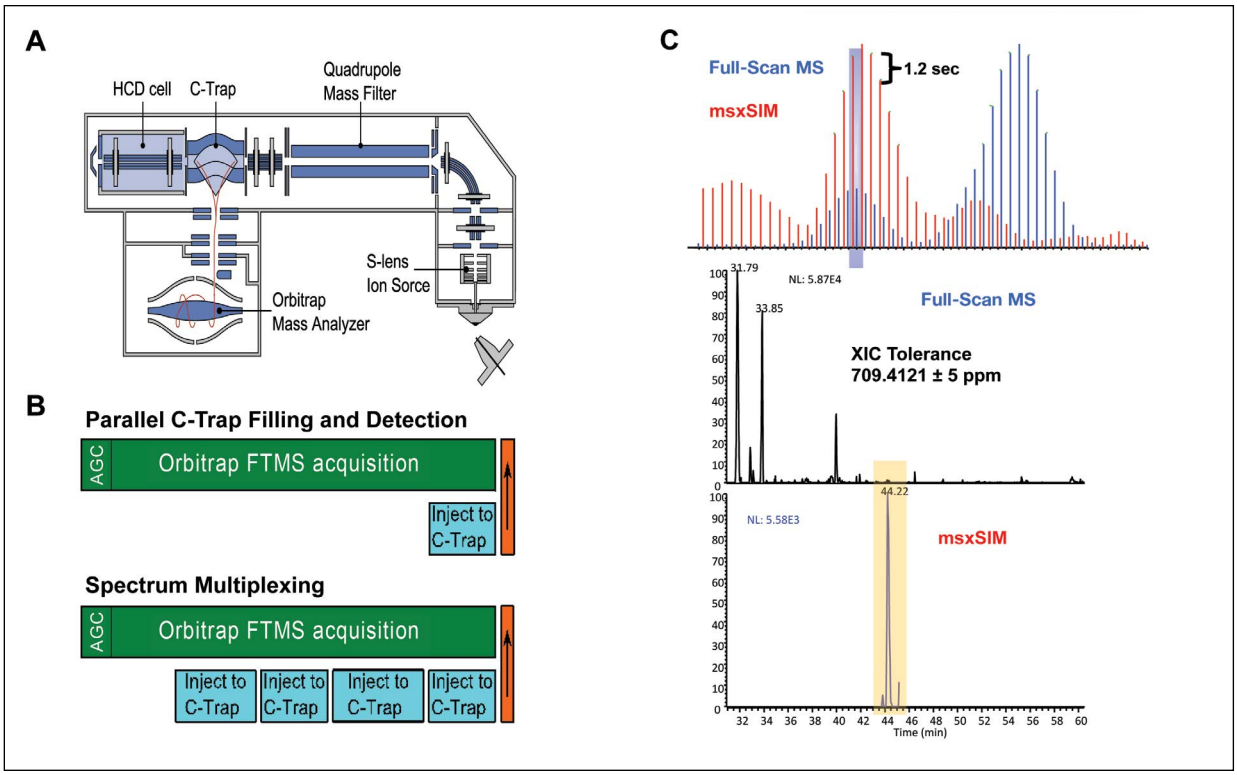


Figure 4. The Q Exactive mass spectrometer (A) is capable of multiple C-trap filling during Orbitrap detection (B) and producing a multiplexed spectrum (C).

Multiplexing the SIM event facilitated selective data acquisition for two additional active-site peptides that co-eluted in the same retention time window as the ULK3 active-site peptide (Figure 5A). Despite a large number of background ions in the full-scan MS scan, three target *m/z* values are easily acquired in the msxSIM window. Quadrupole mass filtering around the targeted *m/z* values enables greater accumulation of the target peptide precursor ions. Figure 5B shows the narrow mass region centered at the **NISHLDLKPQNILLSSLEKPHLK** +4 precursor *m/z* value for the full-scan MS, SIM, and theoretical isotopic distribution. Both full MS and SIM scans show a co-isolated matrix ion in the +3 charge state. However, the high resolution of the Orbitrap mass analyzer facilitates separation of the background signal from the targeted peptide even though the mass difference between the matrix ion and the A+2 target isotope is only 0.05 Da (76 ppm) at 20% relative intensity. The SIM event also provided greater fine structure compared to full-scan MS with increased signals for target peptide isotopes. Each peptide was confirmed in a separate targeted MS^2 experiment. Excellent retention-time correlation was observed between targeted msxSIM and targeted MS^2 experiments (data not shown).

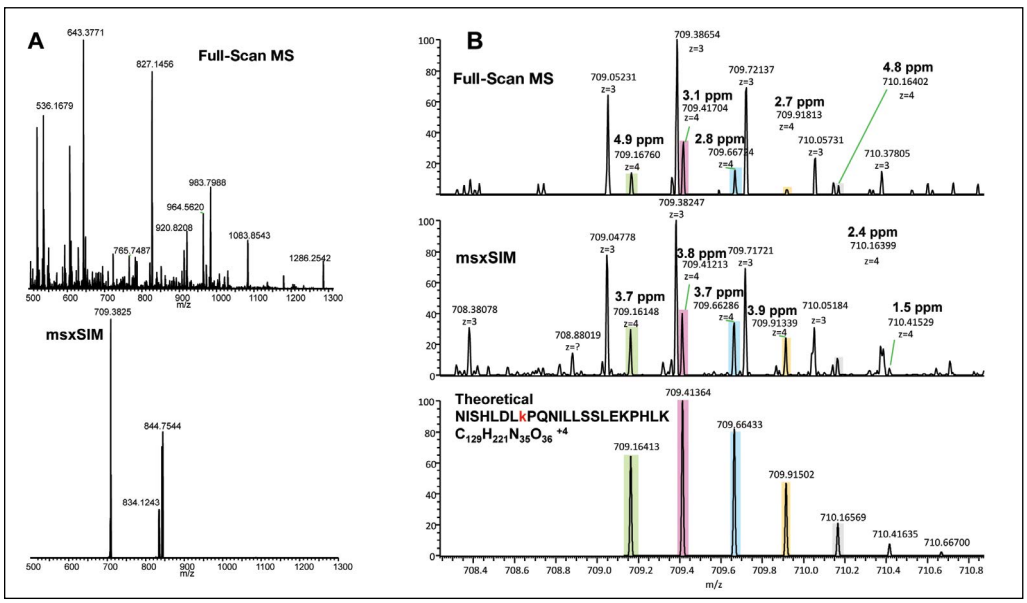


Figure 5. Comparative HR/AM mass spectra for the targeted active-site peptide **NISHLDLKPQNILLSSLEKPHLK** between full-scan MS and msxSIM at a determined retention time (A). Zoom in shows the +4 precursor charge state compared to the theoretical precursor isotopic distribution used for verification (B).

Post-acquisition data verification, integration, and relative quantitation using HR/AM MS data (full-scan and msxSIM data) was performed using Pinpoint software. Figure 6A shows the individual isotopic XICs (displayed in centroid mode) and the measured AUC values. A total of 1.8 seconds was needed to cycle from the msxSIM event to full-scan MS acquisition and back to msxSIM detection resulting in 12 scans acquired across the elution peak. User-defined sequence and modifications are used to calculate the theoretical *m/z* value for the four most abundant isotopes per charge state. An XIC extraction tolerance of ±5 ppm was used for full MS and msxSIM scans. Finally, an XIC is performed for each isotope independently, grouped, and then overlaid to identify and confirm target peptides versus retention time across all attributed precursor charge states and corresponding isotopes (Figure 6B).

High resolution and mass accuracy are also used to maintain target ion selectively for both full-scan MS and msxSIM MS analysis. Pinpoint software can determine potential background interference per isotopic XIC. Figure 6C shows the isotopic distribution overlap between experimental and theoretical values for the +4 and +5 precursor charge states of the **NISHLDLKPQNILLSSLEKPHLK** peptide. The dot-product correlation coefficients for the charge states are 0.95 and 0.98, respectively. The consistent peak shape and relative isotopic distribution increase confidence in the measured signal being attributed to the target peptide and not to the matrix background. Figure 6D shows the consolidated histogram for the +4 charge state for all biological conditions and technical replicates. The calculated correlation coefficient was greater than 0.95 for all files, indicating the externally calibrated mass accuracy was maintained for the duration of the sample analysis.

Kinase Inhibitor Profiling and IC_{50} Determination

Using the combined full-scan MS and targeted msxSIM methods described, relative abundance of kinase expression can be readily assessed for multiple samples or treatment conditions. Kinase inhibitor profiling is a powerful application that can be assessed by measuring changes in kinase active-site peptide relative abundance before and after drug treatment. Inhibitor binding affinities can also be determined by titrating inhibitor concentrations to determine IC_{50} values from dose-response curves. Targeted msxSIM acquisition greatly aids this type of analysis as inhibited kinases peptide signals will be decreased as inhibitor concentrations increase.

Figure 7 shows the relative quantitation of three co-eluting active-site peptides measured using the msxSIM method from samples treated with increasing amounts staurosporine, a broad-specificity kinase inhibitor. Figure 7A shows the overlaid XIC plots for each targeted peptide per sample with two technical replicates. The GCK kinase (**DTVTSELLAAVKIVK**) and ULK3 kinase (**NISHLDLKPQNILLSSLEKPHLK**) both show significant inhibition at < 0.1 µM staurosporine while PKR kinase (**DLKPSNIFLVDTK**) shows modest inhibition to staurosporine treatment. Relative peptide signal intensity was used to plot dose-response curves and calculate IC_{50} values for each kinase (Figure 7B). For staurosporine kinase inhibition profiling, comprehensive data is also available from several alternative assays and is very similar to values obtained in the present study.[5]

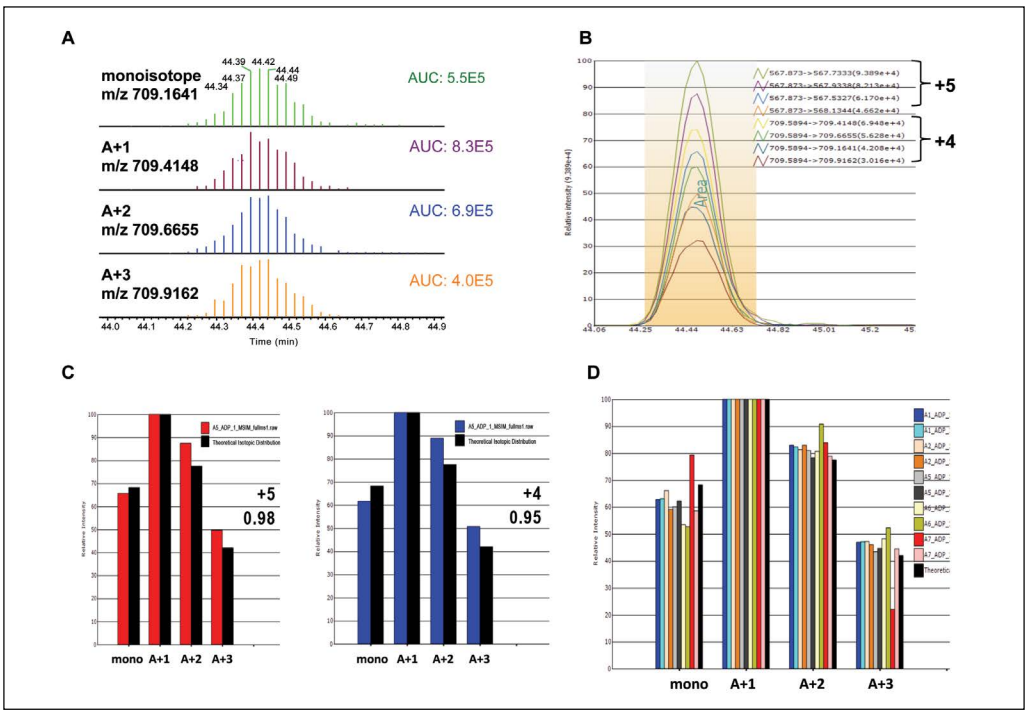
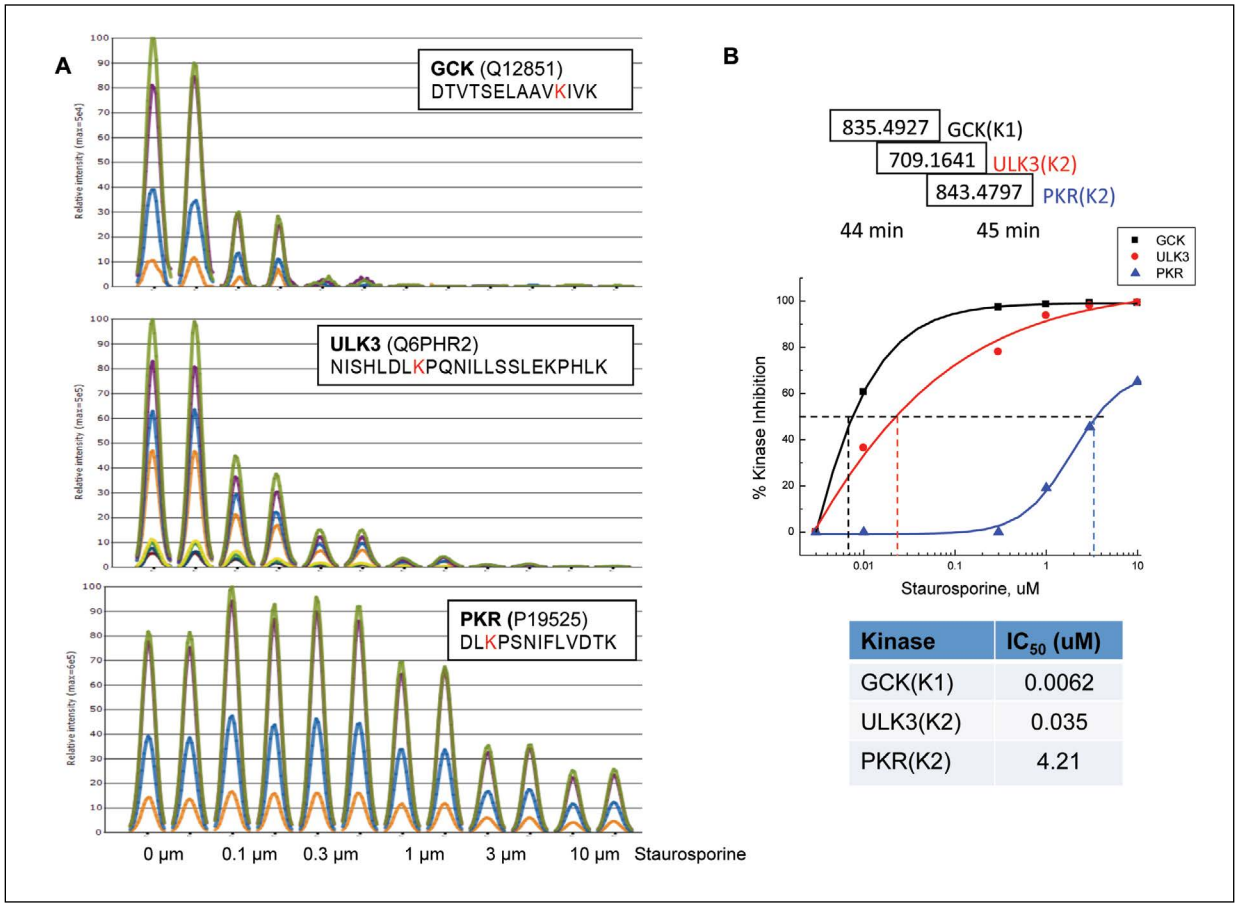


Figure 6. Data processing strategy using Pinpoint software for HR/AM MS and msxSIM data where (A) shows the individual isotopic XIC plots in centroid display mode, (B) shows an overlaid XIC plot for retention-time determination, (C) shows the isotopic distribution overlap between experimental and theoretical profiles for each charge state, and (D) shows the resulting isotopic distribution for all resulting RAW files acquired in the study.



Kinase	IC_{50} (uM)
GCK(K1)	0.0062
ULK3(K2)	0.035
PKR(K2)	4.21

Figure 7. Targeted msxSIM analysis for low-level co-eluting active-site peptides. Comparative overlaid XIC plot at each titrated staurosporine level (A). Dose-response curves of the measured peptide signal inhibition as a function of titrated staurosporine concentration with calculated IC_{50} values for each active-site peptide (B).

Table 3 lists all kinases quantified in this study after staurosporine treatment. By comparing relative intensity of untreated to staurosporine-treated (10 µM) samples, kinases were grouped by their percent inhibition. This analysis clearly shows which kinases bind to staurosporine and can be used to identify kinase subfamilies that share common inhibition profiles.

Kinase	% Inhibition	Kinase	% Inhibition	Kinase	% Inhibition	Kinase	% Inhibition
ABL1/2	75%	EphB4	88.00%	MARK2	100.00%	PLK1	0.00%
ACK	100.00%	Erk1/2	0.00%	**MARK3**	100.00%	PKN2	100.00%
AMPKa1/2	100.00%	FAK1	100.00%	MASTL	0.00%	PRPK	0.00%
ATR	50.00%	**FER**	100.00%	MET	0.00%	PRP4	40.00%
ATM	0.00%	**GCK**	100.00%	**MLKL**	0.00%	**PRKDC**	0.00%
ARAF	0.00%	GCN2	75.00%	MLK3	100.00%	PRKCI	0.00%
BAZ1B	100.00%	JAK1	100.00%	**MST3**	93.00%	ROCK1/2	100.00%
BRAF	0.00%	IKKa	0.00%	MST4	100.00%	RSK1	95.00%
CaMK1a	75%	IKKb	0.00%	**NDR1/2**	95.00%	**RSK1/2/3**	100.00%
CaMK2g	100.00%	ILK	10.00%	NEK1	20.00%	**RSK2(1)**	100.00%
CAMKK2	100.00%	IRE1	90.00%	NEK3	0.00%	RSK2(2)	0.00%
CASK	100.00%	**IRAK4**	100.00%	NEK4	0.00%	**SLK**	100.00%
CDC2	50.00%	KHS1/2	100.00%	NEK7	0.00%	SMG1	20.00%
CDK5	100.00%	LATS1	100.00%	NEK8	0.00%	SRPK1/2	50.00%
CDK6	65.00%	LKB1	100.00%	NEK9	40.00%	STLK6	0.00%
CDK7	100.00%	LOK	100.00%	OSR1	30.00%	SRC	100.00%
CDK8/11	100.00%	LYN	100.00%	p70S6Kb	0.00%	TAO1/3	100.00%
CDK9	0.00%	MAP2K1/2	90.00%	p70S6K	15.00%	TAO2	100.00%
CDK10	100.00%	**MAP2K3**	100.00%	PAN3	0.00%	**TBK1**	100.00%
CDK12	0.00%	**MAP2K4**	95.00%	PDK1	85.00%	TEC	100.00%
CDK13	0.00%	**MAP2K6**	100.00%	PHKg2	100.00%	TP53RK	100.00%
CHK1	100.00%	MAP3K1	80.00%	PKCi	80.00%	**TLK1/2**	100.00%
CHK2	97.00%	MAP3K2	50.00%	PITSLRE	0.00%	**ULK3**	100.00%
CSK	85.00%	MAP3K4	0.00%	PKD2	100.00%	Wnk1/2/4	0.00%
EF2K	0.00%	**MAP3K5**	100.00%	PKD3	100.00%	YES	100.00%
EGFR	30.00%	MARK1/2	100.00%	**PKR**	30.00%	ZAK	0.00%
EphA2	88.00%						

% Inhibition > 90% | 60%-89% | 30%-59% | <30%

Table 3. Identified kinases (active-site peptides) and corresponding relative quantitation determined using HR/AM MS data after inhibitor treatment. Kinase peptides measured using targeted multiplexed SIM are shown in bold.

Conclusion

Thermo Scientific ActivX Desthiobiotin-ATP and -ADP probe technology combined with Q Exactive LC-MS analysis creates a powerful workflow enabling global kinase identification and drug-inhibition profiling. These novel active-site probes specifically captured ATPase subfamily members and improved kinase MS detection for inhibitor selectivity profiling and binding affinity assessment. Unique data acquisition methods incorporating HR/AM full-scan MS and multiplexed SIM events were used to build spectral libraries and target kinase active-site peptides. Multiplexed SIM events were also used to maximize cycle times for co-eluting active-site peptides which increased signals 7-fold over background. This increased dynamic range enabled low-level peptide quantitation necessary for IC_{50} determination. Integrated software further facilitated automated method building and data processing to reduce analysis time. This workflow was successfully used to identify and quantify inhibition of over 126 kinases in a 60 minute gradient using the same instrument platform.

Acknowledgment

The authors would like to thank Dr. M. Patricelli of ActivX Biosciences for his valuable insights and discussions.

References

1. Patricelli, M.P., *et al. Chem Biol*, **2011**, *18*(6), 699-710.
2. Patricelli, M.P., *et al. Biochemistry*, **2007**, *46*, 350-358.
3. Bomgarden, R., *et al.* Thermo Scientific Application Note 515, **2011**.
4. Michalski, A., *et al. Mol Cell Proteomics*, **2011**, *10*(9), M111.011015
5. Karaman, M.W., *et al. Nature Biotech*, **2008**, *26*, 127-132.

www.thermofisher.com

© 2016 Thermo Fisher Scientific Inc. All rights reserved. KiNativ is a trademark of ActivX Biosciences, Inc. Magic is a trademark of Bruker-Michrom. SEQUEST is a registered trademark of the University of Washington. Mascot is a registered trademark of Matrix Science Ltd. All other trademarks are the property of Thermo Fisher Scientific and its subsidiaries. This information is presented as an example of the capabilities of Thermo Fisher Scientific Inc. products. It is not intended to encourage use of these products in any manners that might infringe the intellectual property rights of others. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details.

Africa-Other +27 11 570 1840
Australia +61 3 9757 4300
Austria +43 1 333 50 34 0
Belgium +32 53 73 42 41
Canada +1 800 530 8447
China +86 10 8419 3588
Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland/Norway/Sweden +46 8 556 468 00
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9434
Italy +39 02 950 591
Japan +81 45 453 9100
Latin America +1 561 688 8700
Middle East +43 1 333 50 34 0
Netherlands +31 76 579 55 55
New Zealand +64 9 980 6700
Russia/CIS +43 1 333 50 34 0
South Africa +27 11 570 1840
Spain +34 914 845 965
Switzerland +41 61 716 77 00
UK +44 1442 233555
USA +1 800 532 4752

AN63662_E 06/16S

Thermo SCIENTIFIC
Part of Thermo Fisher Scientific