SureQuant™ AKT Pathway (Phospho) Absolute Quantitation Module

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

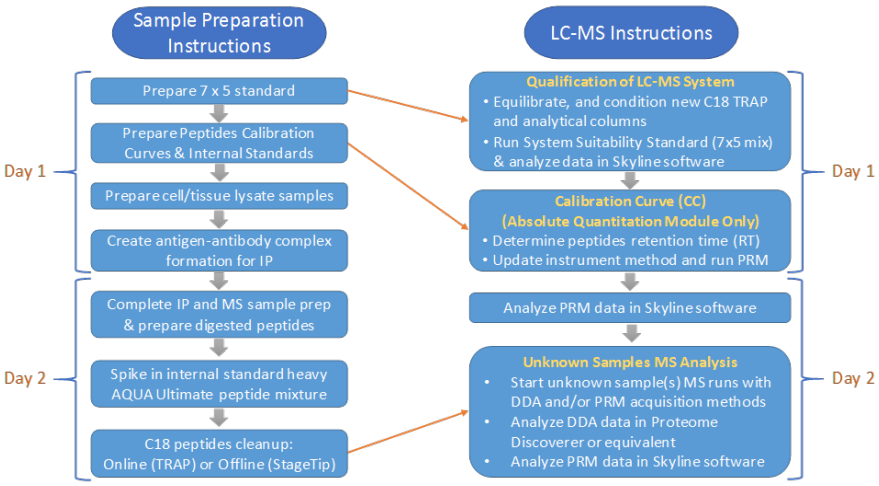
The Thermo Scientific™ SureQuant™ AKT Pathway (Phospho) Absolute Quantitation Module enables users to achieve absolute quantitation of 30 unique peptides from 12 AKT-mTOR signaling pathway target proteins using liquid chromatography (LC) and mass spectrometry (MS). To create each peptide calibration curve, a constant amount of an AQUA Ultimate HeavyPeptide is added to a corresponding unlabeled AQUA Ultimate LightPeptide of known quantity which spans a range of 200 fmol to 0.03 fmol on column. Thermo Scientific™ Pierce™ LC-MS/MS System Suitability Standard (7 x 5 Mix) enables the user to assess dynamic range of the nano or capillary flow LC-MS/MS systems before running the calibration curves and performing quantitative analysis of AKT-mTOR pathway target peptides from immuno-enriched, digested samples.

Contents

Kit Components	Storage
AKT Pathway AQUA Ultimate HeavyPeptides Mixture, 100 fmol/µL, 100 µL	-20°C
AKT Pathway AQUA Ultimate LightPeptides Mixture, 100 fmol/µL, 100 µL	
Pierce™ 6 Protein Digest Standard Equimolar, LC/MS Grade, 100 pmol, 1 vial	
Pierce™ LC-MS/MS System Suitability Standard (7 x 5 Mix), 0.5 pmol/µL, 25 µL	
Peptide Diluent, 5 mL	Room temperature
Low Protein Binding Collection Tubes, 0.6 mL, 50 each	

Note: Additional product documents must be downloaded from the website.

Procedure summary



Additional information

- Each AKT Pathway target peptide is unique and not modified at Serine, Threonine, or Tyrosine residues.
- Attachment A (web download) describes step-by-step how to qualify the LC-MS system using the 7 × 5 system suitability standard before running unknown samples. Attachment B (web download) describes data acquisition and data analysis for AKT Pathway target peptides.
- Use the Pierce™ LC-MS/MS System Suitability Standard (7 x 5 Mix) as a performance evaluation standard for both data-dependent acquisition (DDA) and parallel-reaction monitoring (PRM) modes of analysis with an LC-MS system.
- For ease of use and storage of AKT Pathway AQUA Ultimate HeavyPeptides (100 fmol/µL) and LightPeptides Mixtures (100 fmol/µL), prepare aliquots of the peptides in volumes of 15-20 µL using 0.6 mL low protein-binding tubes provided and store each aliquot at -20°C.
- All AQUA Ultimate HeavyPeptides are labeled with a heavy lysine ($^{13}C_6$ $^{15}N_2$, +8Da) or a heavy arginine ($^{13}C_6$ $^{15}N_4$, +10Da) at the C-terminus.
- Please refer to the attachments A and B (web download) for additional information about the data acquisition and data analysis of the system suitability standard, generation of the calibration curve, and calculations for absolute concentration of unknown samples using the Skyline software (at **skyline.ms**) and Excel™ template.
- Calibration curve solutions can be stored at -20°C until further use.

Materials required but not provided

- Pierce™ Formic Acid, LC-MS Grade (Product No. 28905)
- DMSO, Anhydrous (Product No. D12345)
- Pierce™ Water, LC-MS Grade (Product No. 51140)

Procedure

For instructions to download attachments and additional files, please visit **thermofisher.com/surequantdocs** and enter the code indicated with the flash drive to access instrument method and data analysis information.

Perform LC-MS/MS System Suitability Check

Note: Use recommended C18 analytical column (Product No. ES800) and on-line TRAP column (Product No. 164564) for the nanoLC-MS/MS analysis. See attachment A (web download) for the installation and conditioning of C18 columns.

1. Prepare 7 × 5 diluent (1% formic acid, 5% DMSO) for the 7 × 5 mixture.
   a. Add 10 µL of Formic Acid, LC-MS Grade, to a 1.5 mL low protein-binding tube or equivalent.
   b. Add 50 µL of DMSO, Anhydrous, and 940 µL of LC-MS-grade water.
   c. Vortex 10-20 seconds.
2. Thaw Pierce™ LC-MS/MS System Suitability Standard (7 x 5 Mix) for 5-10 minutes at room temperature.
3. Thoroughly vortex the solution for 2 minutes and quick spin by centrifugation.
4. Add 2 µL of 7 × 5 system suitability standard and 18 µL of 7 × 5 diluent prepared in step 1 to an autosampler vial.
5. Thoroughly mix this solution by repeatedly pipetting.
6. Transfer the autosampler vial to the nanoLC system and inject 4 µL of replicate samples.
7. Perform data-dependent acquisition (DDA) and subsequent parallel-reaction monitoring (PRM) modes of analysis using the relevant instrument method (web download). See attachment A for more details about the data acquisition and Skyline analysis.
8. Perform data analysis in Skyline using the DDA and PRM skyline documents. See attachment A for more details about the data analysis using Skyline software.
9. Assess dynamic range, linearity, and LLOQ for each peptide group using the standard curve generated in the Skyline software. See attachment A for more details on data analysis.

AKT Pathway Target Peptides Calibration Curves

Table 1 Calibration curve concentrations.

Calibration Curve No.	LightPeptides Concentration (fmol/µL)	HeavyPeptides Concentration	6-Protein Digest Mix Concentration
CC1	100	5 fmol/µL	500 fmol/µL
CC2	10		
CC3	1		
CC4	0.25		
CC5	0.0625		
CC6	0.0156		

1. Thaw the AKT Pathway AQUA Ultimate HeavyPeptide Mixture, AKT Pathway AQUA Ultimate LightPeptide Mixture, and Pierce™ 6 Protein Digest Standard for 15-20 minutes at room temperature.
2. **Thoroughly vortex AQUA Ultimate peptide mixtures for 2 minutes each.**
3. Prepare 5 fmol/µL AQUA Ultimate HeavyPeptide working solution*:
   a. Pipette 11 µL of AQUA Ultimate HeavyPeptide mixture into a 0.6 mL low protein-binding tube.
   b. Add 209 µL of peptide diluent provided with the kit.
   c. Thoroughly vortex solution for 2 minutes at the highest speed setting.

   * The HeavyPeptide mixture working solution is used as a constant-concentration reference for use in the calibration curve with 6-protein digest matrix as described in the following step:
4. Prepare AQUA Ultimate HeavyPeptide mixture with 6-protein digest matrix:
   a. Pipette 200 µL of 5 fmol/µL AQUA Ultimate HeavyPeptide mixture (prepared in step 3 above) to 6-protein digest standard vial.
   b. Pipette repeatedly to mix, washing the wall of the vial to completely collect the sample.

5. To prepare AQUA Ultimate LightPeptides calibration solutions, use 5 low protein-binding tubes (0.6 mL) and label as CC2, CC3, CC4, CC5 and CC6. CC1 solution is at the same concentration as the AQUA Ultimate LightPeptide mixture provided in the stock vial (100 fmol/µL).

   Note: The AQUA Ultimate LightPeptides calibration solutions (at **variant concentrations**) are combined with the AQUA Ultimate HeavyPeptide mixture plus 6-protein digest matrix at **constant concentrations**.

6. Prepare 0.6 mL tubes with appropriate volume of peptide diluent as shown in the second column in the table below. Take out 5 µL of CC1 (100 fmol/µL light mix) to make CC2 solution. Vortex for 60 seconds.

Calibration Curve (CC) No.	Peptide Diluent	CC Volume to Remove (From Column 1)
CC2	45 µL	5 µL CC1 (LightPeptide Mix)
CC3		5 µL CC2
CC4	30 µL	10 µL CC3
CC5		10 µL CC4
CC6		10 µL CC5

7. Remove 5 µL of CC2 to make CC3 and vortex for 60 seconds. Prepare remaining CC4 to CC6 solutions using 10 µL of CC solutions as shown in the table above (last column). Make sure to vortex 60 seconds every time to prepare each CC solution.
8. Label 6 autosampler vials for calibration curve solutions.
9. Using the same pipette tip, add 7.5 µL of the heavy peptides/6-protein matrix solution to each of the 6 appropriately-labeled autosampler vials.
10. Using a new pipette tip for each calibration solution, add 7.5 µL of each calibration solution containing AQUA LightPeptides (CC1, CC2, CC3, CC4, CC5 and CC6) to relevant labeled autosampler vials. Pipette 5 times to mix. Inject 4 µL on column for each CC solution.
11. To prepare AKT Pathway QC mix, remove 5 µL of CC2 LightPeptide solution and combine with 5 µL of HeavyPeptide working solution/6-protein digest mix using an autosampler vial.

Target peptides QC assessment (retention time correction) and standard curve analysis

1. Use the Xcalibur sequence to run AKT path QC sample followed by the calibration curve and absolute quantitation of unknown samples.
   a. After running AKT Pathway QC mix, correct retention time of each peptide using a Skyline DDA template. Export the new Isolation list with the corrected retention time. See attachment B (web download) for more details about Skyline DDA analysis and the isolation list.
   b. Import the updated inclusion list (.csv) file to PRM method. Run the calibration sequence starting with CC6, followed by increasing concentrations of calibration curve standards (CC5, CC4, CC3, CC2, and CC1, respectively). See attachment B for more details about Xcalibur sequence set-up and PRM data acquisition method.
2. Perform analysis of the calibration curve raw data using Skyline software. Use AKT Pathway PRM skyline template for the data analysis. See attachment B for more details about the AKT Pathway PRM data analysis using Skyline software.
3. Analyze data in Skyline to assess dynamic range, linearity, and LLOQ for each target peptide from the calibration curve. See attachment B for more details on Skyline data analysis.

AKT Pathway Target peptides absolute quantitation from IP-MS samples

1. Thoroughly vortex the AQUA Ultimate HeavyPeptide mixture for 2 minutes. If using frozen stock, thaw the AQUA Ultimat HeavyPeptide mixture for 15-20 minutes at room temperature before vortexing.
2. Prepare 2 fmol/µL AQUA Ultimate HeavyPeptide internal standard spiked-in solution*:
   a. Pipette 10 µL of AQUA Ultimate HeavyPeptide mixture into a 0.6 mL low protein-binding tube.
   b. Add 490 µL of peptide diluent.
   c. Thoroughly vortex solution for 2 minutes at highest speed setting.

   *The HeavyPeptide internal standard spiked-in solution is used as an internal standard, in which IP-MS samples should be reconstituted as described in the following step.
3. Add 20 µL of AQUA Ultimate HeavyPeptide internal standard spiked-in solution (2 fmol/µL) to each digested and speedvac-dried immunoenriched sample. Store remaining AQUA Ultimate HeavyPeptide internal standard spiked-in solution at -20°C.
4. Vortex each sample for 60 seconds and quickly centrifuge.
5. Remove 12 µL of digested samples with spiked-in AQUA Ultimate HeavyPeptides to a new autosampler vial and place it in autosampler. Inject 5 µL on column for the internal standards spiked-in unknown sample. Store remaining sample at -20°C.
6. Perform data-dependent acquisition (DDA) and subsequent parallel-reaction monitoring (PRM) modes of data acquisition using the relevant LC-MS instrument acquisition method provided with the web download documents for the AKT Pathway. See attachment B for more details about the AKT Pathway DDA and PRM data acquisition methods.

   Note: DDA and PRM LC-MS acquisition methods were optimized using the ES800 C18 column, Easy-nLC, Dionex™ RSLC nano system, and Q Exactive™- and Fusion™-series MS instruments.
7. Perform DDA data analysis using Thermo Scientific™ Proteome Discoverer™ Software or similar.
8. Perform PRM data analysis in Skyline using the AKT Pathway PRM skyline documents provided with the web download. See attachment B for more details about the AKT Pathway PRM data analysis using Skyline software.
9. Export the analyzed data from Skyline and refer to attachment B for more details on Excel™ data analysis to calculate the concentration of each target peptide per IP from unknown samples.

Troubleshooting

Observation	Possible cause	Recommended action
Hydrophilic peaks not detected.	Starting percentage of organic mobile phase (%B) is too high.	Decrease starting mobile phase %B to <3%.
	High organic (>5% acetonitrile) in sample loading solvent or in sample diluent.	Reduce acetonitrile to <3% in sample loading solvent and/or in sample solvent.
Hydrophobic peaks are not detected or severely decreased in intensity.	C18 TRAP and/or analytical column was compromised.	Evaluate column with 7 × 5 system suitability standard mixture. Replace with new column if necessary.
	Hydrophobic peptides are aggregated or retained on column and/or in tubes.	Ensure that low protein-binding tubes are used to prepare peptides, and that tubes are vortexed at least 2 minutes.
Not all target peptides are identified in the Positive Control Lysate.	Some peptide loss may occur with different C18 sample clean up methods.	Use on-line C18 Trap column (Product No. 164564) or C18 Spin Tips (Product No. 84850).
Peaks are resolved late in the gradient and/or the gradient shifted to the right.	Flow sensor calibration issue.	Recalibrate flow sensor module.
	Dead volume in nanoLC system.	Purge nano pumps (A + B) and flush air nanoLC system.
Peaks are too broad.	Gradient was too shallow.	Increase slope of gradient.
Peaks overlap.	Gradient was too steep.	Reduce slope of gradient.
Peptide masses are incorrect.	Mass spectrometer needed calibration.	Calibrate mass spectrometer.
Some peaks are not detectable.	C18 TRAP and/or analytical column is compromised.	Evaluate column with 7 × 5 system suitability standard mixture. Replace with new column if necessary.
	Mass spectrometer front end is dirty.	Run calibration mix to evaluate MS performance and/or clean the front end.
Variable peak intensities.	Injector had bubbles.	Clean injector with 50% methanol.
	Gradient was too steep.	Reduce slope of gradient.

Related products

Product	Cat. No.
EASY-Spray™ LC Analytical Column, 75 µm × 150 µm, 3 µm	ES800
Acclaim™ PepMap™ 100 C18 Trap Column, nanoViper™	164564
Pierce™ 0.1% Formic Acid (v/v) in Water, LC-MS Grade	85171
Pierce™ 0.1% Formic Acid (v/v) in Acetonitrile, LC-MS Grade	85175
Pierce™ Acetonitrile (ACN), LC-MS Grade	51101
Pierce™ Water, LC-MS Grade	51140
Pierce™ Trifluoroacetic Acid (TFA)	28904
Pierce™ LTQ Velos ESI Positive Ion Calibration Solution	88323

Limited product warranty

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