

# In-Depth Qualitative Analysis of Complex Proteomic Samples Using High Quality MS/MS at Fast Acquisition Rates

## *Using the Explore Workflow on the AB SCIEX TripleTOF™ 5600 System*

A major challenge in proteomics research and biomarker discovery by mass spectrometry is the analysis of complex samples. As sample complexity increases, so does the need for powerful hardware with high sensitivity and large dynamic range capabilities. Equally important for a thorough analysis of complex samples is the ability to acquire the maximum number of MS/MS spectra without loss of spectral quality during an LC/MS/MS run.

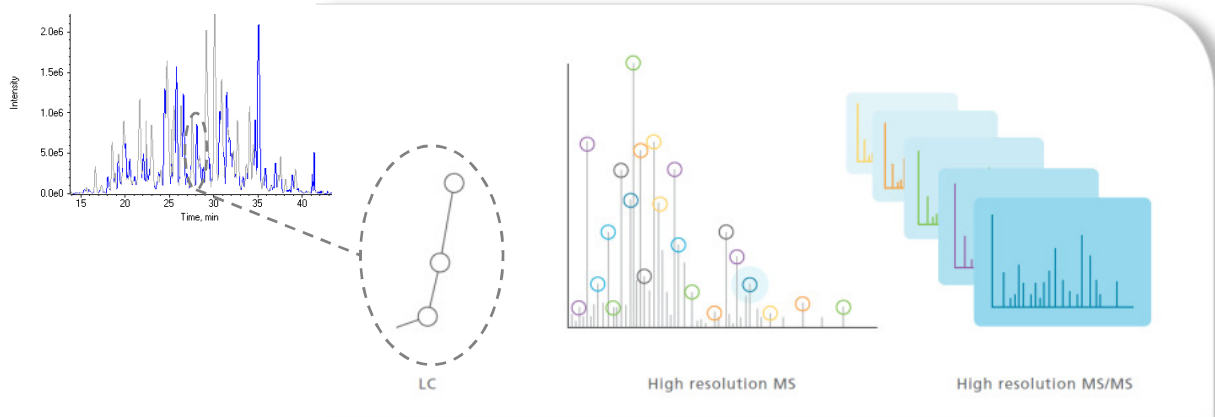
The AB SCIEX TripleTOF™ 5600 System has the speed and sensitivity to deliver comprehensive qualitative exploration, rapid profiling, and high-resolution quantification in complex matrices – *all on a single platform, all in a single run*. The system can acquire up to 50 MS/MS spectra in a second using powerful IDA (information-dependent acquisition) workflows. Even at the fastest acquisition rates, high-resolution and high mass accuracy is maintained in both MS and MS/MS mode, unlike other high mass accuracy, high resolution instruments.

While many different qualitative and quantitative workflows can be performed on the TripleTOF™ 5600 system, here we focus on the unique attributes of the whole system solution for depth of exploration of complex samples and for high fidelity results.



## Whole System Solution for the Analysis of Complex Proteomes

- The TripleTOF™ 5600 System can perform high speed acquisition of MS and MS/MS data while maintaining high resolution and high mass accuracy.
- The Eksigent nanoLC Ultra system with the cHiPLC™-nanoflex enables high quality separations with the highest reproducibility.
- Digging deeper requires that the database search algorithm is able to broadly detect many different PTMs. ProteinPilot™ Software with its unique Paragon™ Algorithm enables the simultaneous identification of hundreds of modifications, substitutions, and unexpected cleavages.



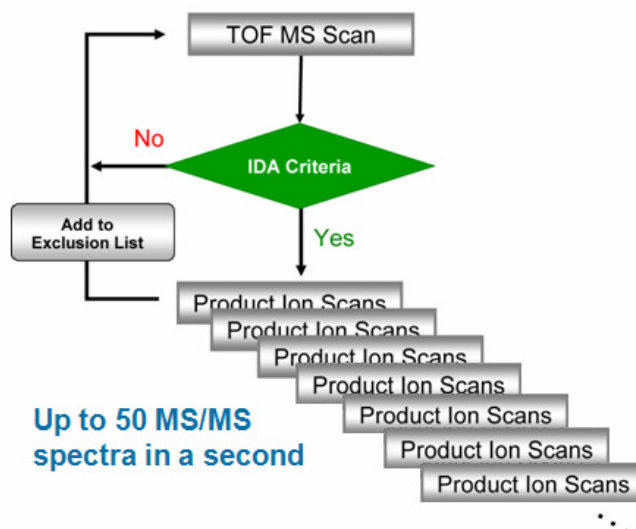
**Figure 1. Explore Workflow for Comprehensive Protein Identifications.** In the Explore workflow, high resolution TOF MS spectra are acquired and precursors are selected and sent for MS/MS acquisition. The acquisition method is designed such that maximal time is spent in MS/MS mode to obtain the most peptide identifications and therefore deep proteome coverage. Requiring as little as 20 ms to obtain a high quality MS/MS spectrum, very fast acquisition rates are achieved.

## Methods

**Sample Preparation:** Yeast cell lysate was denatured using RapiGest, then reduced with tris-(2-carboxyethyl)phosphine and alkylated with iodoacetamide. An overnight digestion with trypsin was performed at 37 °C. RapiGest was cleaved with 0.5% trifluoroacetic acid and sample was centrifuged. A final concentration of 400 ng/mL was obtained.

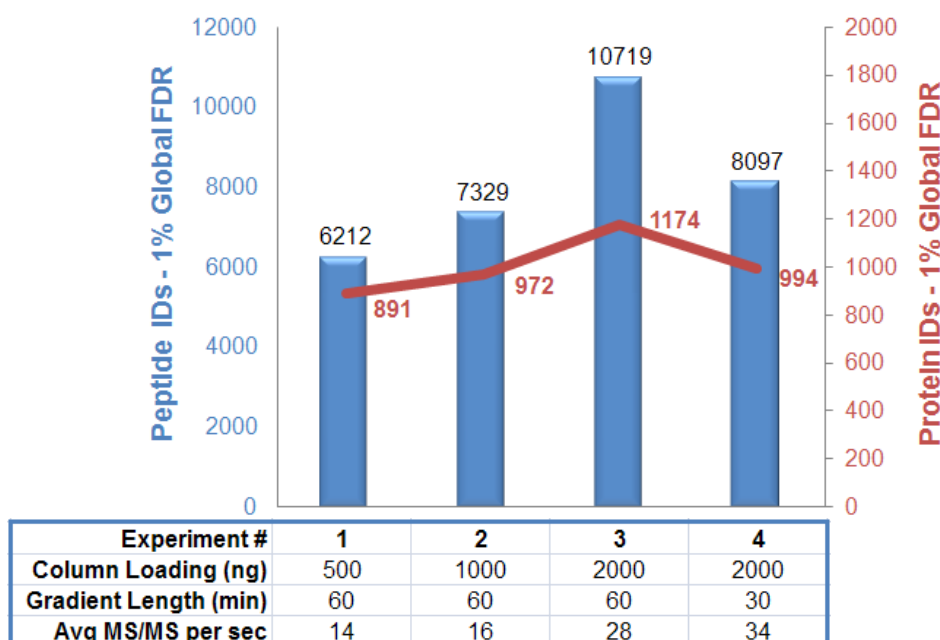
**Chromatography:** The sample was analyzed using the Eksigent nanoLC-Ultra™ 2D System combined with the cHiPLC™-Nanoflex system in Trap-Elute mode. The cell lysate was loaded on the cHiPLC trap (200 μm x 500 μm ChromXP C18-CL 3 μm 300 Å) and washed for 10 mins at 4 μL/min. Then, an elution gradient of 5-35% acetonitrile (0.1% formic acid) in either a 30 or 60 min gradient was used on a nano cHiPLC column (75 μm x 15 cm ChromXP C18-CL 3 μm 300 Å).

**Mass Spectrometry:** The TripleTOF™ 5600 System has high sensitivity which enables very fast MS/MS acquisition rates, as low as 20 ms accumulation time for MS/MS in Information Dependent Acquisition (IDA) mode. To fully leverage the instrument speed and obtain the best depth of coverage, the IDA workflow has been optimized such that software overhead is minimized (Figure 2). The IDA method consisted of a high resolution TOF MS survey scan followed by up to 50 MS/MS in a second with a minimum accumulation time of 20 msec.



**Figure 2. Information Dependent Workflow (IDA).** Acquisition of MS/MS spectra at high speeds requires the minimization of software overhead. The maximum number of precursors and the minimum accumulation time is specified by the user. During acquisition, the precursor list is automatically generated and the accumulation time will be dynamically distributed across detected precursors per cycle.

**Data Processing:** All data was processed using ProteinPilot™ Software 4.0 with integrated false discovery rate (FDR) analysis. Further data analysis was performed using the accompanying ProteinPilot Descriptive Statistics Template.



**Figure 3. Protein and Peptide Identifications from Yeast Cell Lysate.** Single injections of yeast were run using varied gradient lengths and varied loading amounts. As more protein is loaded on column, more proteins and peptides are detected.

## Fast MS/MS Acquisition Yields Greatest Proteome Coverage

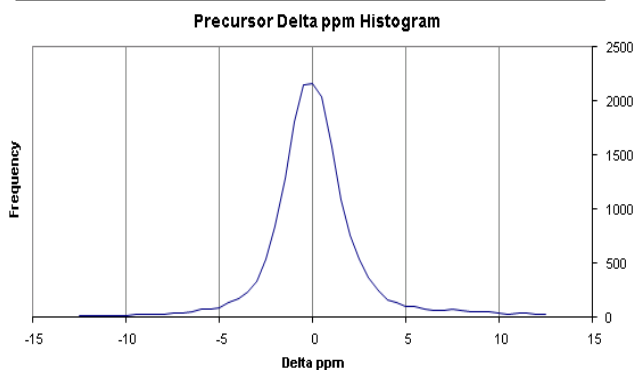
At any given moment in a gradient when analyzing a complex proteome, many peptides are co-eluting. The speed of acquisition and the quality of the MS/MS spectra will determine the depth of coverage obtained on the sample and the confidence of the results obtained.

The TripleTOF™ 5600 System has high sensitivity which enables very fast MS/MS acquisition rates, as low as 20 ms accumulation time for MS/MS in IDA mode. At this acquisition rate, MS/MS spectra at >15,000 resolution are acquired with high mass accuracy. This speed and quality, in combination with the dynamic IDA strategy (Figure 2) provides the flexibility and dynamic range needed when analyzing a broad range of samples from diverse proteomes.

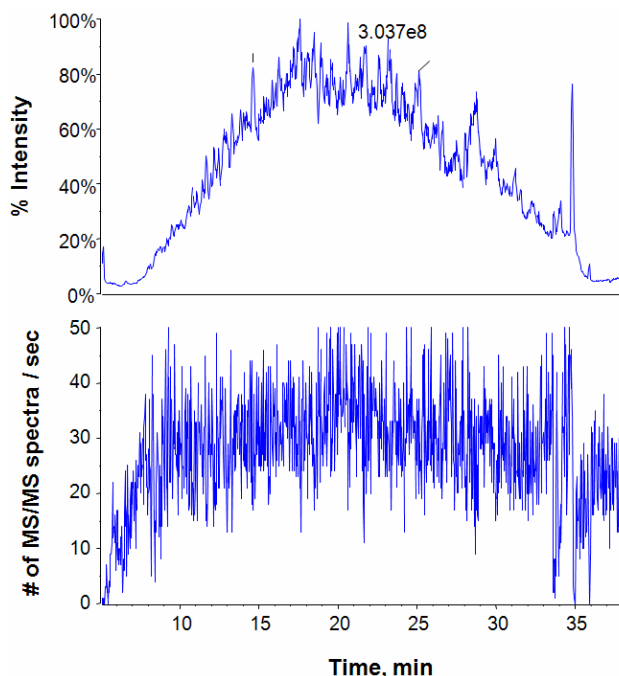
In this experiment using a complex yeast cell lysate digest, the utility of speed was explored. The acquisition methods were created using a maximum of 50 MS/MS in a second and minimum accumulation times of 20 msec. By increasing the sample loading across injections, there is an increased analysis burden. On the TripleTOF 5600 system, IDA dynamically adjusts the number of precursors selected per cycle based on the precursors available. This allows for acquisition to be accelerated in precursor rich regions of the gradient and slowed down when fewer precursors are eluting.

### Mass Error Summary Statistics Table

	<i>Std. Deviation</i>	<i>RMS</i>	<i>Average Error</i>
Delta m/z error	0.00126	0.00126	0.00001
Delta ppm error	1.72	1.72	-0.03
Delta Sqrt m/z error	2.49E-05	2.49E-05	2.62E-08



**Figure 5. Mass Accuracy.** (Top) Table of important mass accuracy values and (bottom) plot of the histogram of the delta m/z values seen for all detected peptides from Experiment 3. This information can be obtained using the ProteinPilot Descriptive Statistics Template that is provided with ProteinPilot™ Software 4.0. Similar RMS error was observed in all yeast runs.



**Figure 4. Visualization of the MS/MS Acquisition Speed.** (Top) the total ion chromatogram (TIC) of Experiment 4 (2000 ng sample with 30 min gradient) shows how peptide rich the chromatogram is. In the bottom graph, the number of MS/MS obtained in each cycle is computed across the gradient.

As seen in Figure 3, the average number of MS/MS acquired in each experiment dramatically increases as sample load increases or LC gradient time decreases due to the dynamic nature of IDA. Shown in Figure 4 is an example of the acquisition rate across the whole LC gradient for experiment 4. In this scenario where the entire sample is being forced to elute in half the time by using a much shorter gradient, the maximum speed of 50 MS/MS in a second was reached several times during the gradient with the average number of spectra acquired at 34 MS/MS spectra in a second. This indicates that the complexity of the sample at any one moment in time demands these faster acquisition rates in order to adequately observe all detected precursors.

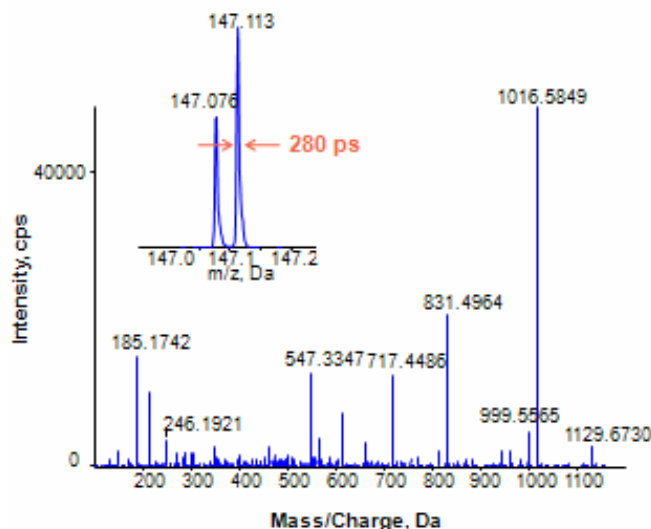
### MS Mass Accuracy

Accurate detection of the precursor mass is an important aspect of the fidelity of the database searching. Because of the inherent stability of the instrument, the AB SCIEX TripleTOF™ 5600 System can maintain an RMS error of <2 ppm over extended acquisition times. The mass accuracy for experiment 3 is shown in Figure 5, where the RMS error for the whole acquisition, including the many low abundant peptides is 1.72 ppm.

## High Quality MS/MS Data

The TripleTOF™ 5600 system has two operational modes for MS/MS acquisition, high sensitivity mode (~15,000 resolution) and high resolution mode (~30,000 resolution). The unique performance attributes of this instrument allow maintaining the resolution down to very low masses. As shown in Figure 6, this enables resolution of immonium ions and low  $m/z$  fragment ions. High resolution and therefore mass accuracy of the sequence ions are also advantageous for specificity when performing database searching for peptide / protein identification, especially when using tag-based algorithms such as the Paragon™ Algorithm in ProteinPilot™ Software. The TripleTOF™ 5600 System is typically run in high sensitivity mode for MS/MS acquisition when analyzing proteomic complex mixtures to further facilitate the high speed acquisition.

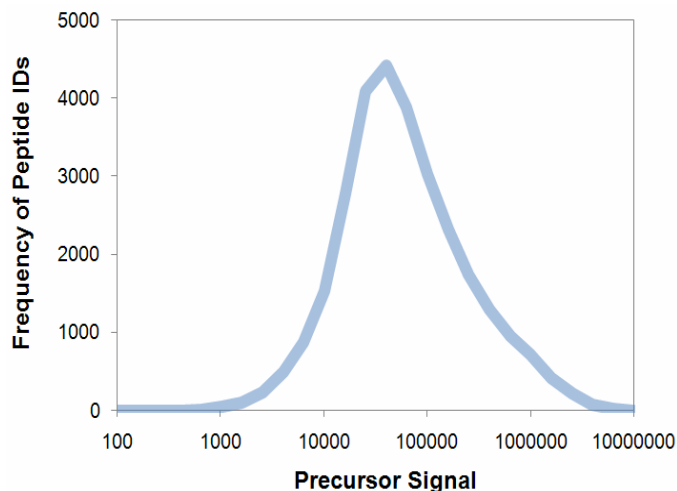
Methods for protein identification are easily created using the template methods provided in the Method Wizard. Pre-determined defaults designed for optimal acquisition are used. For example, selecting the method template for “Prot Complex IDA” will automatically set key acquisition parameters for protein ID from complex mixtures, such as faster MS/MS acquisition.



**Figure 6. High Resolution MS/MS Spectra.** Low mass  $y_1$  ions of lysine and glutamic acid can be resolved easily with MS/MS resolution of >25000 at low mass. Highly resolved peaks at low mass are only 280 psec in width, however the 25 psec acquisition rate of the 40 GHz TDC detector provides ample sampling and good peak shape.

## Dynamic Range of Detected Peptide Signal

The MS intensity of every detected peptide is determined in ProteinPilot™ Software 4.0 and can be used in many detailed analyses. Figure 7 shows a plot of the numbers of peptides identified across a range of MS precursor signals as provided by the ProteinPilot Descriptive Statistics Template and demonstrates the broad dynamic range of signal that can be interrogated in a single acquisition.



**Figure 7. Dynamic Range of Detected Peptide Signals.** Using the MS peak intensities of each detected peptide, the signal range of identifications can be plotted. Shown is the data for experiment 3, where 4 orders of magnitude in precursor signal are successfully identified in a single acquisition.

## Key Features of the TripleTOF™ 5600 System for Protein Identification

- High mass accuracy, high sensitivity and high resolution in both MS and MS/MS mode
- High acquisition rates of up to 50 MS/MS in a second in IDA mode with no loss in resolution or mass accuracy
- High specificity during database searching using tighter mass tolerances for higher confidence in results
- Good dynamic range for depth of coverage

## Conclusions

The TripleTOF™ 5600 System establishes a new standard for acquisition speed, while still maintaining high data quality in both MS and MS/MS mode to deeply interrogate a proteomic sample. The yeast experiment demonstrated that there is an ever increasing need for faster acquisition as the sample complexity increases. As shown, higher loading and shorter gradients force the system to acquire data faster, reaching maximum capacity of 50 ms/ms in a second in heavily co-eluting areas of the gradient. Very high numbers of high confidence peptide and protein identifications are obtained as a result of the high numbers of high quality MS/MS spectra acquired.

Mass accuracies of sub 2 ppm are easily achievable using the TripleTOF 5600 system and ProteinPilot Software 4.0. Coupled together with high fidelity LC separations using the Eksigent nanoLC Ultra with cHiPLC™-nanoflex, the TripleTOF™ 5600 System provides a powerful solution for proteomics.

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