

# Quantitative Protein Profiling in Cell Signalling Networks

## TOF MS Profiling using the AB SCIEX TripleTOF™ 5600 System

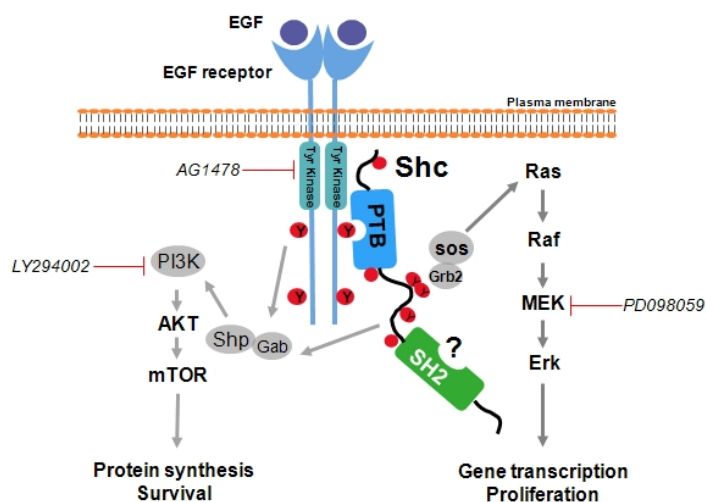
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Signal transduction is a key area of study in biology as it is the fundamental process by which communication occurs between cells. A stimulus at the cell surface is transmitted into the cell through receptors and results in a cascade of signals within the cell that produce a change in cell function. Understanding the components in these signal transduction cascades is widely studied using traditional techniques like Western blotting.

The Shc proteins are a family of adaptor proteins which are responsible for mediating signaling from activated cell surface receptors with ShcA being the most widely expressed in mammalian systems. The protein has two key binding domains, the PTB domain and the SH2 domain. The PTB domain binds to the intracellular portion of activated surface receptors through two phosphotyrosine recognition modules. This leads to recruitment of Grb2 and activation of the Ras/MAPK pathway and proliferation. There is also evidence for a Grb2-independent signaling from ShcA that leads to cytoskeletal reorganization and survival<sup>1</sup>.

The goal of this study is to understand how the protein ShcA coordinates the flow of molecular information downstream by determining the identities and abundances of the proteins



**Figure 1. Epidermal Growth Factor (EGF) Signaling.** The EGF signaling network is a dynamic and complex network of protein interactions that occur after a cell is stimulated with EGF. Proteins recruited to the complex upon signaling are key in understanding the resulting signal transduction.



recruited to the ShcA protein complex upon EGF stimulation of cells. After cell stimulation, the cells are lysed and the FLAG-tagged ShcA protein is immuno-precipitated under native conditions. The protein complex is preserved so all bound proteins are captured. By capturing the protein complex at various times after stimulation, the dynamics of this protein complex can also be studied. The proteins in the complex are analyzed using a combined qualitative / quantitative workflow (Profile workflow<sup>2</sup>) on the AB SCIEX TripleTOF™ 5600 system.

## Key Features of AB SCIEX TripleTOF™ 5600 System for the Profile Workflow

The key benefits of this qualitative and quantitative profiling are:

- Full scan high resolution TOF MS profiling with cycle time optimized to obtain 6-8 scans across LC eluting peaks
- High resolution and high mass accuracy TOF MS spectra allows for post-acquisition generation of extracted ion chromatograms (XICs) at >20,000 resolution (0.05 Da extraction window) for increased specificity
- Fast Information Dependant Acquisition (IDA) of high resolution, high mass accuracy MS/MS peptide spectra enables concurrent high confidence identification of many peptides and proteins<sup>3</sup>

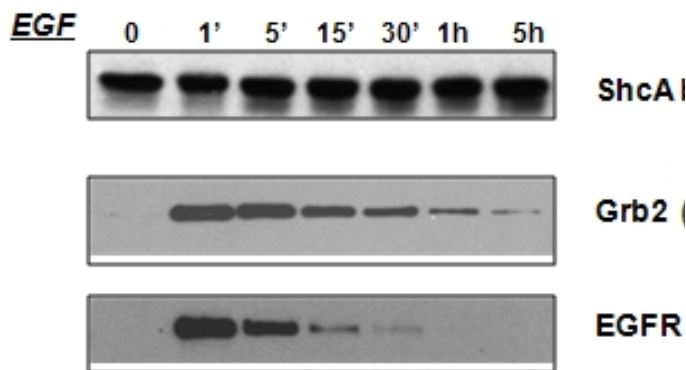
## Methods

**Sample Preparation:** ShcA and its interacting proteins were immuno-precipitated from Rat fibroblast cells using anti-FLAG™ antibody followed by on-bead tryptic digestion at 0, 1, 3, 5, 15, 30 and 60 minutes post exposure to EGF. Western blot analysis on a limited number of proteins was used to confirm the protein immuno-capture experiment was successful (Figure 2).

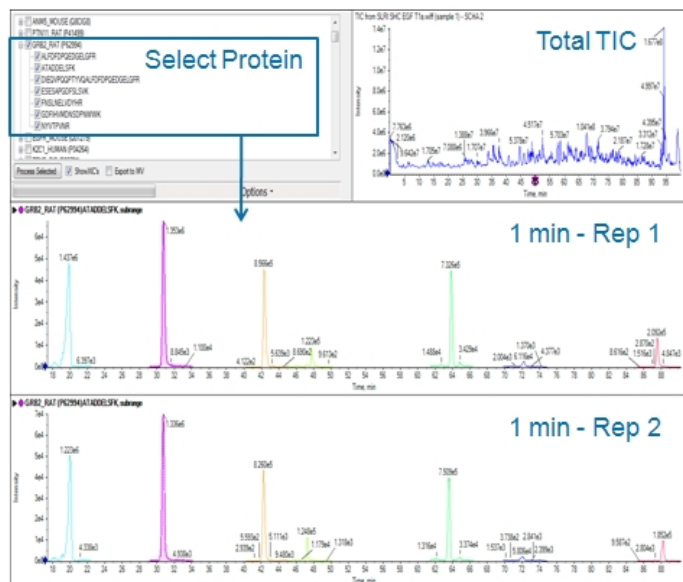
**Chromatography:** The sample was analyzed using the Eksigent nanoLC™-System combined with a homemade 10 cm 75 μM pulled emitter tip using 3.5 μM Zorbax C18 beads. A gradient of 2-30% acetonitrile (0.1% formic acid) over 90 minutes was used.

**Mass Spectrometry:** The acquisition method on the TripleTOF™ 5600 System consisted of a high resolution 250 ms TOF MS survey scan followed by 20 X 50 ms MS/MS at a cps trigger of 150. The resulting cycle time was <1.3 sec to ensure a minimum of 8 data points across the chromatographic peaks at half height for optimal quantification. Each sample was run in duplicate. The ShcA MRM assay was performed on a 4000 QTRAP® system using the *Scheduled MRM™* algorithm, with 214 MRM transitions for 3 or more peptides per protein with at least 2 MRM transitions per peptide. The acquisition method used a 5 minute detection window and a 3 second cycle time.

**Data Processing:** LC/MS/MS was processed using ProteinPilot™ Software 4.0, using Thorough searching and integrated false discovery rate analysis. TOF MS extracted ion chromatograms (XIC) were generated from all data files automatically using PeakView™ Software for all high confidence peptide identifications found in the search results. MS peak



**Figure 2. Quantitative Western Blot Analysis.** Proteins recruited to the Shc protein complex 0, 1, 5, 15, 30, 60 and 300 minutes after exposing the cells to EGF were quantified by Western blot. Amount of ShcA bait protein was measured with an anti-Shc antibody (top). The recruitment then release of the Grb2 protein (middle) and EGFR (bottom) was quantified using anti-Grb2 and anti-EGFR antibodies. ShcA bait protein can be used for normalization between LCMS injections.



**Figure 3. Automatic Generation of High Resolution TOF MS XIC for High Confidence Peptides.** TOF MS XICs for the masses of high confidence peptides identified using ProteinPilot™ software are extracted and integrated. Results can then be exported to MarkerView™ Software.

areas and identifications were imported into MarkerView™ software for visualization and statistical analysis. Principal Component Variable Grouping (PCVG) was used to identify protein cohorts that modulate in a synchronized manner.

## Protein Identification Results

The TripleTOF™ 5600 system data were submitted for protein database searching and a total of 145 proteins and 3003 unique peptides were identified at a 1% global false discovery rate using ProteinPilot™ Software. Mass accuracy calculations were carried out using the Descriptive Statistics Template and the outputs from the ProteinPilot Software searches. High mass accuracy was observed in the MS data for the precursor ions of the detected peptides, the RMS of the ppm error was 1.30.

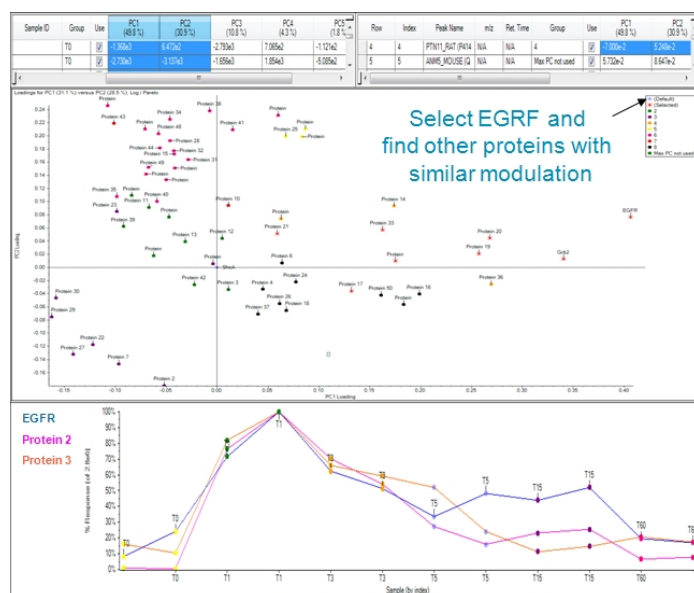
The ProteinPilot Software protein identification results were imported into PeakView™ Software for automatic processing. All identified proteins were selected and extracted ion chromatograms (XIC) were automatically generated from the TOF MS data for the high confidence peptides (Figure 3). The same peptide XICs were generated for the duplicate analyses across all the time points. Because of the high mass accuracy and resolution of the TOF MS data, narrow XIC windows of 0.01 Da were used for the extraction, to obtain higher specificity and higher quantitative quality. High reproducibility in TOF MS data was observed between analytical replicates.

## Statistical Analysis of Time Course Data

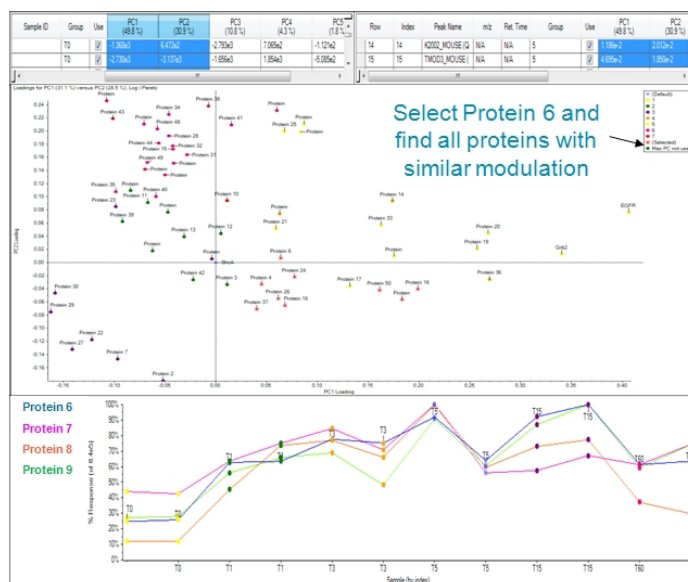
After extraction and integration of all the TOF MS data for the proteins / peptides across all the LC/MS/MS runs, the data are imported into MarkerView™ Software for statistical analysis and visualization. First, principal component analysis (PCA) is performed to compare the data across the multiple samples, which reveals groupings. Then, principle component variable grouping (PCVG) analysis is performed on the PCA loadings values to find the peptides that share a common pattern<sup>4</sup> (Figure 4).

When using a label free approach such as this, using a normalization strategy can increase the accuracy of quantification. In this case, the bait protein was used to normalize the TOF MS areas.

The EGFR receptor was quantified in the sample and shows a distinctive time course. Immediately after stimulation, EGFR protein is complexed to ShcA protein, but then ShcA quickly dissociated from the receptor. There are a number of other proteins that show this same time course of association with ShcA, two of which are shown in Figure 4. These were found by looking at other members in the same variable group as EGFR. A similar analysis was done using another set of proteins which are recruited more slowly and show a more sustained interaction (Figure 5).



**Figure 4. Statistical Analysis and Visualization of EGFR and Other Rapidly Recruited Proteins.** EGFR and other proteins in the same PCVG group were selected and the quantitation data were visualized. These proteins are rapidly recruited into the protein complex then rapidly released.

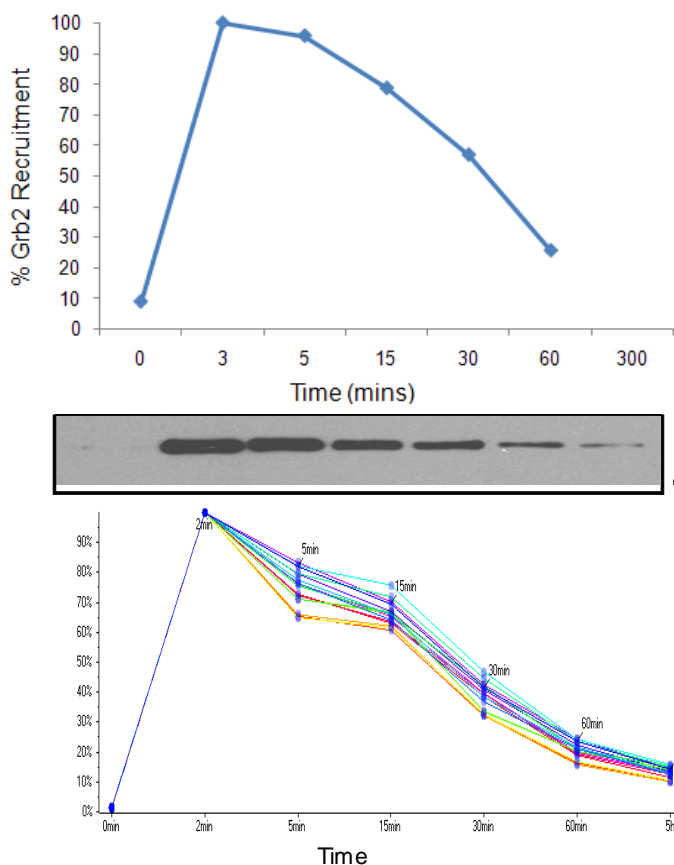


**Figure 5. Statistical Analysis and Visualization of Protein 6 and Other Slowly Recruited Proteins.** Protein 6 and other proteins in the same PCVG group were selected and the quantitation data were visualized. These proteins are slowly recruited into the protein complex and sustained.

Novel binding proteins were also identified which had not been seen in previous discovery experiments. Using the XIC areas, direct quantification of these was possible across the stimulation time course. Direct information about their presence and also their kinetics of interaction was obtained. Peptides observed for these novel binding proteins were then transferred via MRMPilot™ Software and included into the MRM assay for confirmation of their interaction profile.

## Validation of Quantitative Results

To confirm the quality of the quantitative results using this TOF MS approach on the TripleTOF™ 5600 system, results were compared with two other quantitative technologies. Quantification of Grb2 protein shows a rapid increase in binding to ShcA after stimulation followed by a rapid slow dissociation (Figure 6, top). This result was confirmed by Western Blot analysis, where strong anti-Grb2 binding is observed initially followed by a steady decline (figure 6, middle). Finally, the results were compared to those obtained by Multiple Reaction Monitoring (MRM) on the 4000 QTRAP® System. An MRM assay was developed to detect peptides from SchA and its interaction partners using MRMPilot™ software. This assay was used to analyze the time course samples and the extracted data for each of the proteins of interest showed very similar time course profiles (Figure 6, bottom) to the automated TOF XIC method.



**Figure 6. Validation of Quantitative Results.** The variation in Grb2 complexed with ShcA as measured by TOF MS quantitation with the TripleTOF™ 5600 system (top) is confirmed both by Western blot analysis (middle) and MRM analysis on the 4000 QTRAP® system (bottom).

## Conclusions

- TOF MS quantification using the AB SCIEX TripleTOF™ 5600 System provides a routine and automated strategy for high quality protein quantification from medium complexity proteomic samples.
- Sufficient sensitivity, dynamic range and precision were obtained for observing biological trends as confirmed by comparison to MRM and Western blot quantification results.
- MarkerView Software provides powerful statistical analysis and visualization tools to distil complex data sets into valuable biological information.
- The Profile mode on the TripleTOF™ 5600 system provides an easy transition to MRM analysis for high throughput MRM quantification using QTRAP® systems because the instruments share the same ion source and atmospheric pressure interface as well as the same LINAC® collision cell.

## References

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3. In-Depth Qualitative Analysis of Complex Proteomic Samples Using High Quality MS/MS at Fast Acquisition Rates - Explore Workflow on the AB SCIEX TripleTOF™ 5600 System– AB SCIEX Technical Note 0450210-01.
4. MarkerView™ Software 1.2.1 for Metabolomic and Biomarker Profiling Analysis – AB SCIEX Technical Note 0970210-01

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