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Nota de aplicación

# Endogenous Kinase Quantification Assay Combining Low-Flow LC and Tandem Quadrupole (QqQ) Mass Spectrometer

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*Para su uso en investigación únicamente. No se debe utilizar para procedimientos de diagnóstico.*

## Abstract

A bespoke targeted assay has been developed for 27 human endogenous kinase enzymes, chosen based upon biological significance and prevalence.

The assay has been designed to assess multiple unique targeted MS/MS transitions per enzyme class ensuring identification accuracy for each protein. The transitions include precursor and product masses experimentally confirmed for native peptides (using pure standards) and theoretically derived precursor and product masses for any peptides known to have the potential for phosphorylated residues. With the option of creating calibration curves using standards for every non-phosphorylated peptide there is the possibility of generating a semi -quantitative concentration value for each peptide, giving increased confidence in the experimental findings.

Method optimization and subsequent creation of calibration curves demonstrated 23 peptides with an LOD <100 pg on column, with a further 32 peptides with an LOD <1 ng on column. The LOD for the remaining 25 peptides ranged from 1.1 ng to 28.9 ng on column. A sample set consisting of cancer cell lines was then analysed as a feasibility experiment. This demonstrated the ability to distinguish between two cell lines (*i.e.*, with/without

mutations) using several of the peptide markers without prior sample enrichment at chromatographic flow rates consistent with routine analysis.

This targeted kinase assay has been designed to aid proteomics research, pharmaceutical drug discovery programs, research clinicians, and others within disease biomarker or tailored medicine research.

## Benefits

- Rapid, high throughput acquisitions
- Targeted protein analysis based upon multiple unique transitions
- Semi-quantification available through-the-use of quantification curves
- Available, easily tailored transitions allowing customers to modify targets as desired
- Data can be mined in MassLynx™, TargetLynx™ or imported into SkyLine

## Introduction

Kinase enzymes have been shown as significant biomarkers for different human diseases; in particular for certain cancers such as renal and liver cancer, with increased levels contributing to both favorable or unfavorable prognosis.[1] This makes them a useful target in translational medicine, potentially providing a prognostic biomarker for disease development of certain cancers. Two avenues of peptide targets can therefore be followed for this purpose; measurement of expression of total kinase proteoforms, or measurement of phosphorylated (activated) kinase proteoforms. The specific disease pathway may contribute to which method of quantitation may provide the most useful information regarding prognosis. However, due to the relatively low concentration of these kinases within the cytoplasm it may be difficult to achieve quantitation and as such, current methods employ prior enrichment steps to selectively enrich the target kinases, or operate at nanoflow scale chromatography which is not conducive to high-throughput analysis. We therefore present a comprehensive suite of peptide markers observed both experimentally and a theoretical subset that can be used for identification of specific kinase classes, and the application of these peptide markers to distinguish between two cancer cells lines at capillary scale chromatography with no prior enrichment.

## Experimental

A selection of 28 human kinase standards were purchased at a concentration of between 900 fmol/µL and 25.4 pmol/µL in UHQ Water. A total mix containing 1 µg of each standard was reduced with dithiothreitol (5 mM final concentration) in ammonium bicarbonate (50 mM final concentration, pH 7.8) containing 0.1% w/v RapiGest™ for 15 minutes at 60 °C, alkylated with iodoacetamide (15 mM final concentration) for 30 minutes and subject to digestion by sequencing grade trypsin at a 1:50 protease to protein ratio. Digestion was quenched via addition of formic acid to a final concentration of 0.1% v/v. A similar preparation protocol was also adopted for the cell line samples.

## LC Conditions

| | |
|---|---|
| LC system: | ACQUITY™ Premier FTN |
| Column(s): | ACQUITY Premier Peptide CSH™ $C_{18}$ (100 mm x 2.1 mm, 1.7 µm) |
| Column temperature: | 40˚C |
| Injection volume: | 5 µL (calibration curve) or 15 µL (cancer cell line samples) |
| Flow rate: | 0.5 mL/min |
| Mobile phase A: | Water + 0.1% formic acid |
| Mobile phase B: | Acetonitrile + 0.1% formic acid |
| Gradient: | Initial 5% B, 5% B 0–2 minutes, 5–30% B 2–25 minutes, 30%–60% B 25–27 minutes, 60% B 27–29 minutes, re-equilibrate initial conditions 29–35 minutes. |

## MS Conditions

| | |
|---|---|
| MS System: | Xevo™ TQ-XS |
| Source: | ZSpray™ ESI |
| Ionization mode: | Positive ionization mode |
| Capillary voltage: | 0.5 kV |
| Sampling cone: | 35 |
| Source temperature: | 150 °C |
| Desolvation temperature: | 500 °C |
| Cone gas flow: | 150 L/hr |
| Desolvation flow: | 1000 L/hr |
| Collision energy: | Peptide specific between 13.5–29.8 V |
| Scan time: | Peptide specific |

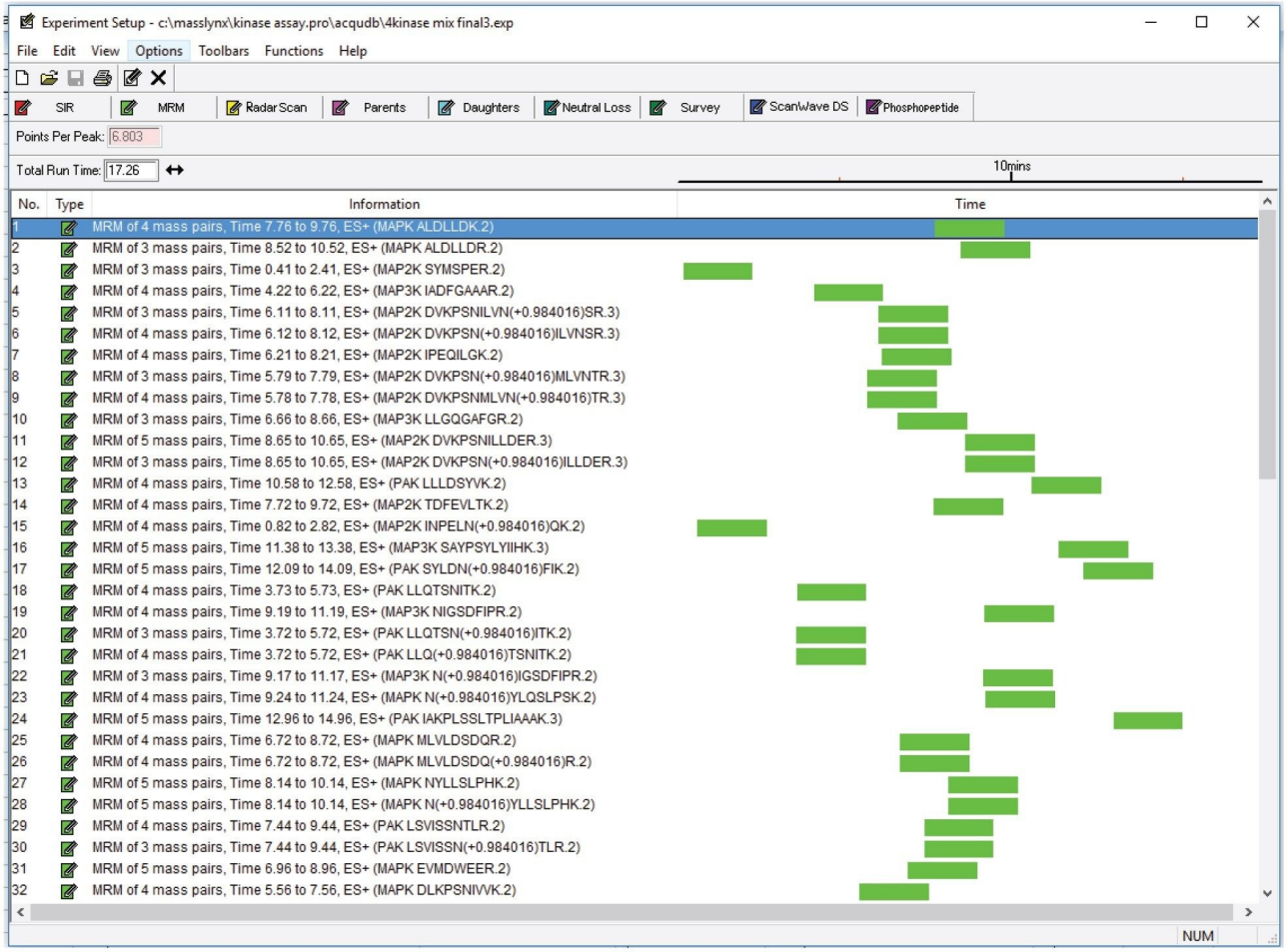


*Figure 1. Example MS experimental settings showing MS/MS transitions within the MassLynx experiment file.*

## Results and Discussion

This targeted kinase assay was developed to allow the screening and semi-quantification of 27 individual kinase enzymes from blood products, cell lines or tissue homogenate. These kinase enzymes were chosen based upon their relevance to human biology (specifically cancer diagnosis and prognosis) and their availability as standards. The 27 kinase enzymes currently available within this assay with experimentally derived transitions are:

- PAK: 1, 2, 3, 4, 5 and 6

- MAPK: 1, 3, 7, 8, 9, 10, 11, 12, 13 and 14
- MAP2K: 1, 2, 3, 4, 5, 6 and 7
- MAP3K: 1, 2, 3, 8

Each kinase enzyme standard was purchased in a non-activated form and digested with trypsin following a typical proteomic workflow. Each digested kinase enzyme was then injected onto an ACQUITY™ M-Class coupled to a SELECT SERIES™ Cyclic™ IMS for untargeted screening for all peptides. The data acquired was imported into PLGS software for peptide identification, assessment, and fragmentation pattern information (Figure 2). Target peptides were assessed with regards to their uniqueness to a single kinase proteoform or unique to a class of kinases (*eg*. MAPK).

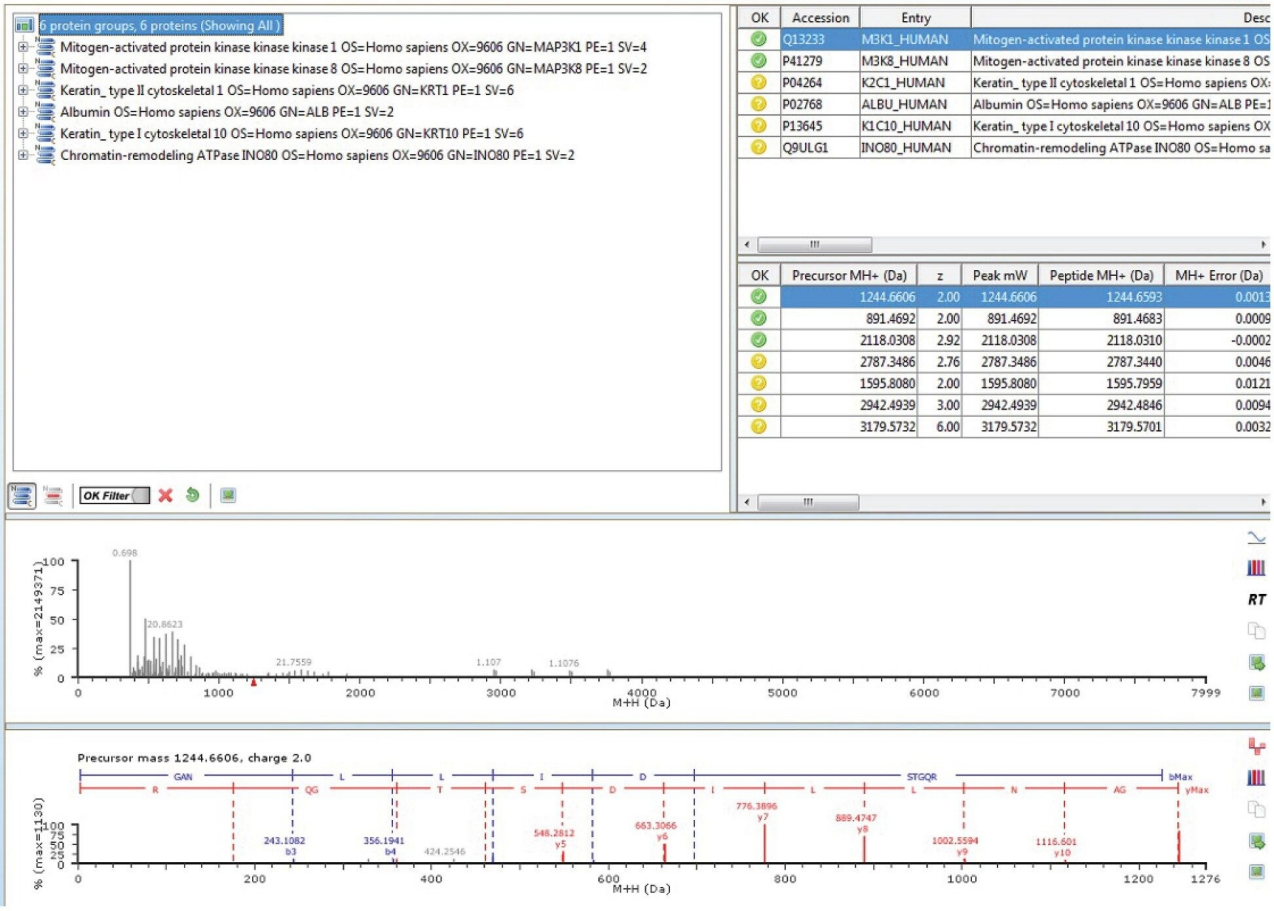


*Figure 2. Example PLGS view: showing an example single kinase enzyme digest peptide search (MAP3K1), of which seven individual peptides were found and identified, the fragmentation (b & y ions) found for the highlighted peptide (corresponding to (K)GANLLIDSTGQR(L)) can be seen in the bottom section of the view.*

Peptide targets were selected based on criteria such as minimum sequence length and were taken forward for further method development using the Tandem Quadrupole Mass Spectrometer. Peptides were then investigated to ensure each was observed, charge state and fragmentation ions were evaluated and selected for maximum signal intensity. Figure 3 shows a typical peptide signal, as a combined TIC (typical view when running the assay) also as the XIC for the three transitions included to generate the TIC.

For each peptide between three and eight transitions were included within the method. The assay contains a total of 47 non-redundant individual experimentally derived peptides, with a total of 80 targets including post-

translational modifications. Due to the standards available for purchase being in a non-active form, typically phosphorylated residues (when activated) were observed in a non-phosphorylated state. Of the 80 peptides included within the assay 26 can be potentially phosphorylated (one or more residue) based upon literature searching. Theoretical transitions for phosphorylated versions of the peptides are also included within the experiment file.

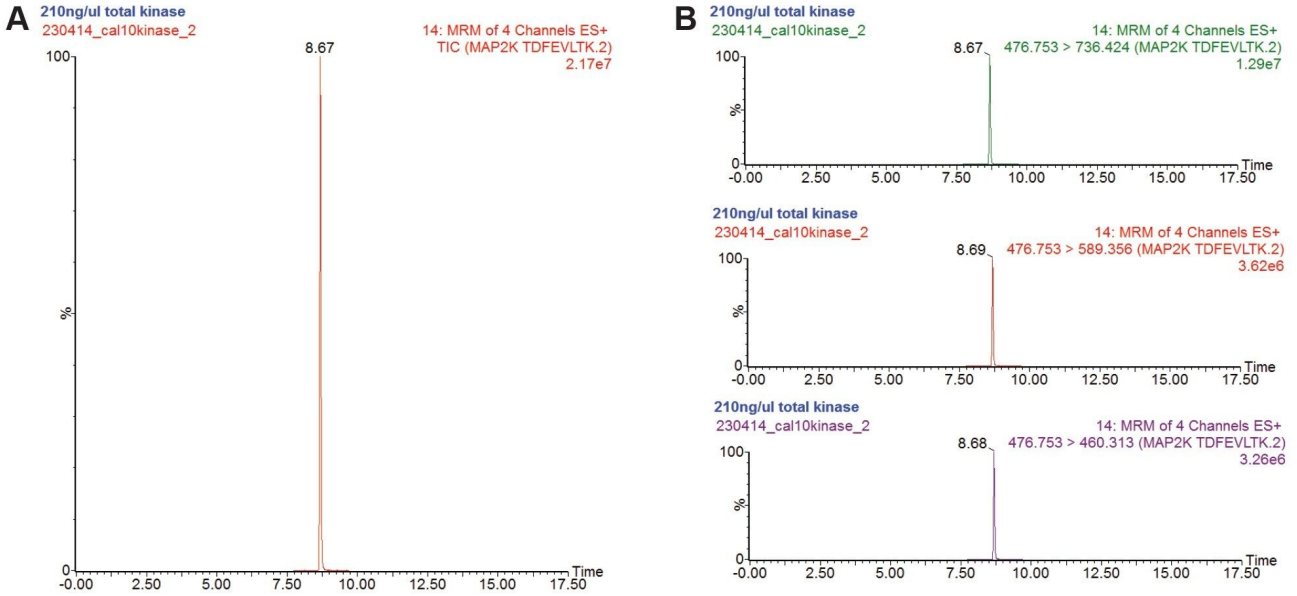


*Figure 3. Example Masslynx views: (A) TIC produced when all signals are combined (three selected transitions) to confirm the presence of MAP2K7 within the sample. (B) XIC of the three individual transitions.*

Creation of calibration curves using only purified standards does not sufficiently mimic the complexity of "real-life" samples and does not consider the effect this has on peptide response through competitive ionisation. Therefore, to mimic a "real-life" sample, the total kinase standard mix was serially diluted with MassPREP™ E. coli digest standard (p/n: 186003196 <https://www.waters.com/nextgen/global/shop/standards--reagents/186003196-massprep-e-coli-digest-standard.html> ) ranging from 10.5 pg to 210 ng of total kinase on column. Calibration curves were created and the limit of detection for each peptide was calculated as blank + 3 x SD, with the peptide GANPLAIDLLGR representing the MAPK class providing the lowest LOD of 35.38 pg (29 fmol) on column. The calibration for this peptide remained linear over at least 3.5 orders of magnitude. Overall, 23 peptides had an LOD <100 pg on column, with a further 32 peptides with an LOD <1 ng on column. The LOD

for the remaining 25 peptides ranged from 1.1 ng to 28.9 ng on column for the PAK peptide marker IGEGSTGIVCIATVR.

Relating calculated limit of detections to physiological levels is difficult due to the heterogeneity between patient samples and their involvement in a complex cascade of networks. Further, changes in disease state may be revealed by not only an increase or decrease in active protein kinases (*i.e.*, phosphorylated), but also an increase or decrease in the total abundance.[2,3] Therefore, to assess the suitability of the peptide markers for use in a targeted assay, cancer cell lines were lysed and enzymatically digested with trypsin in the presence of phosphatase inhibitors. No other sample enrichment was completed.

Analysis of the cell lines was undertaken using chromatographic conditions as above and demonstrated that four of the peptide markers could differentiate between the cell lines analysed based on intensity alone (Figure 4). Therefore, the utility of this assay and associated peptide markers for relative quantitation of some protein kinases present at levels consistent with cancer cell lines is demonstrated. It is possible that with further sample enrichment, other peptides markers could be detected, and with phosphopeptide-specific enrichment it may also be possible to provide a measure of relative activation/phosphorylation.

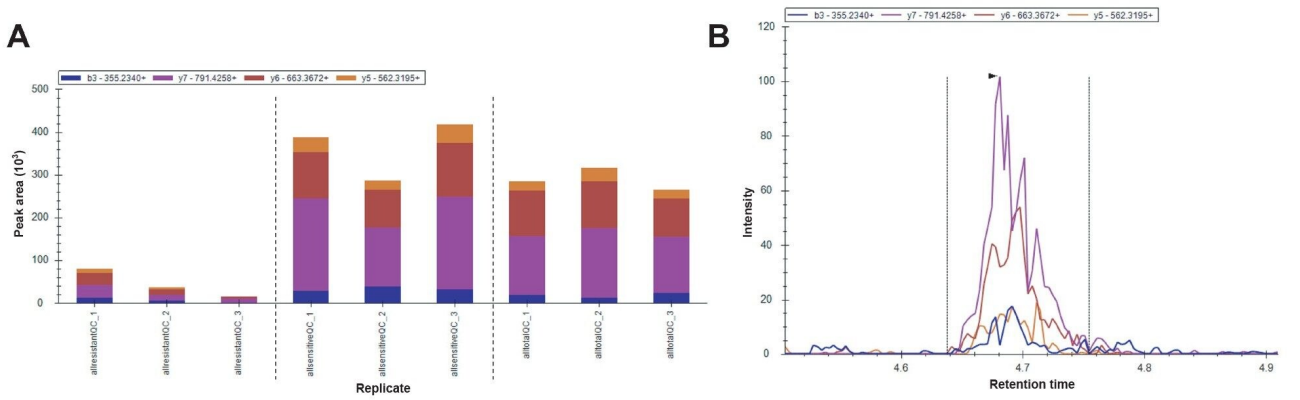


*Figure 4. (A) Ion ratios for the PAK marker peptide LLQTSNITK demonstrating the difference in peak area between non-mutated cell line (low abundance), mutated cell line (high abundance) and a mix of the two (intermediate abundance). (B) Extracted ion chromatograms from analysis of the mutated cell line of each of the four fragment ions monitored for the peptide eluting at the expected retention time of 4.7 minutes.*

## Conclusion

The combination of the Xevo TQ-XS and robust peptide markers have allowed the relative quantitation of protein kinases at physiological levels without sample enrichment and at high chromatographic flow rates, opening the possibility for routine analysis. Four of the peptide markers defined could differentiate between two cancer lines based on the abundance of those markers alone, with two of the markers being within the top ten most sensitive with an LOD >50 pg on column. The peptide markers defined in this application note can be used to not only reveal differences in total kinase abundance but may also be used to give a measure of phosphorylation.

## References


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