# Waters<sup>™</sup>

#### Note d'application

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

Matthew E. Daly, Lisa Reid, Lee A. Gethings, Robert S. Plumb

Waters Corporation

Réservé à la recherche. Ne doit pas être employée en diagnostic clinique.

#### 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.<sup>1</sup> 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<sup>™</sup> 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 <sup>™</sup> C <sub>18</sub> (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 <sup>™</sup> 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

MRM of 5 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSNILLDER.3)         MRM of 3 mass pairs, Time 0.58 to 12.58, ES+ (PAK LLLDSYVK.2)         MRM of 4 mass pairs, Time 0.58 to 12.58, ES+ (PAK LLLDSYVK.2)         MRM of 4 mass pairs, Time 0.58 to 12.58, ES+ (MAP2K TDFEVLTK.2)         MRM of 4 mass pairs, Time 0.58 to 13.38, ES+ (MAP2K INPELN(+0.984016)(DK.2)         MRM of 5 mass pairs, Time 12.09 to 14.09, ES+ (PAK SYLDN(+0.984016)FIK.2)         MRM of 4 mass pairs, Time 12.09 to 14.09, ES+ (PAK SYLDN(+0.984016)FIK.2)         MRM of 4 mass pairs, Time 3.73 to 5.73, ES+ (PAK LLOTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLOTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLOTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLOTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLOTSNITK.2)         MRM of 4 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K N(+0.984016)TK.2)         MRM of 4 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K N(+0.984016)GSDFIPR.2)         MRM of 5 mass pairs, Time 9.24 to 11.24, ES+ (MAPK N(+0.984016)TLS2)         MRM of 5 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQC)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQC)         MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NLVLSDEDR.2)         MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NLVLSDEDR.2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)         MRM of 5 mass pair	
SR       Import       Import       Import       Import         SR       Import       Import       Import       Import         MRN of Smass pairs, Time 3.72 to 57.05 / 7.05 / 25.91 (MAPK ALDLEDK 2)       Import       Import         Import       Import       Import       Import       Import         Import       Import       Import       Import       Import       Import         Import       Import       Import       Import       Import       Import       Import         Import       Import       Import       Import       Impo	
Imple Der Peiz-E       Imple Der Pizz-E       Imple Der Pizz-E <t< th=""><th></th></t<>	
Image: 17.23       Image: 17.23 <t< th=""><th> </th></t<>	 
Information         Time           MRM of 4 mass pairs, Time 8.716 to 976, ES* (MAPK ALDLLDK2)         Time           MRM of 3 mass pairs, Time 8.21b to 10.52, ES* (MAPK ALDLLDK2)         MRM of 3 mass pairs, Time 4.11b e.11, ES* (MAPK ALDLLDK2)           MRM of 4 mass pairs, Time 4.22 to 6.22, ES* (MAPK ALDLLDK2)         MRM of 4 mass pairs, Time 4.22 to 6.22, ES* (MAP2 K NMSPER 2.)           MRM of 4 mass pairs, Time 6.21b e.81; ES* (MAP2 K NVFSNIU, VNI+0.984016)/LNNSR.3)         MRM of 4 mass pairs, Time 5.78 to 7.76, ES* (MAP2 K DVKPSNIU, VNI+0.984016)/LNNSR.3)           MRM of 4 mass pairs, Time 5.78 to 7.76, ES* (MAP2 K DVKPSNIU, VNI+0.984016)/LNNSR.3)         MRM of 3 mass pairs, Time 5.78 to 7.76, ES* (MAP2 K DVKPSNIU, VNI+0.984016)/LNSR.3)           MRM of 3 mass pairs, Time 6.56 to 10.55, ES* (MAP2 K DVKPSNIU, VNI+0.984016)/LLDER.3)         MRM of 3 mass pairs, Time 8.05 to 10.55, ES* (MAP2 K DVKPSNIU, VNI+0.984016)/LLDER.3)           MRM of 3 mass pairs, Time 8.05 to 10.55, ES* (MAP2 K DVKPSNIU, VNI+0.984016)/LLDER.3)         MRM of 4 mass pairs, Time 7.20 to 7.25, ES* (MAP2 K DVKPSNIU, VNI+0.984016)/LLDER.3)           MRM of 4 mass pairs, Time 10.20 to 14.05, ES* (MAP2 K DVKPSNIU, VNI+0.984016)/LLDER.3)         MRM of 4 mass pairs, Time 10.20 to 14.09, ES* (MAP2 K DVKPSNIU, VNI+0.984016)/LLDER.3)           MRM of 4 mass pairs, Time 10.20 to 14.09, ES* (MAP2 K DVKPSNUT, VNI+0.984016)/KL2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES* (PAK LLOTSNITK2)           MRM of 4 mass pairs, Time 3.72 to 5.72, ES* (PAK LLOTSNITK2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES* (PAK LLOTSNITK2)	
MRM of 4 mass pairs, Time 37/610 976, ESP (MAPK ALDLLDK2)         MRM of 3 mass pairs, Time 6.11 b0.22, ESP (MAPK ALDLLDR2)         MRM of 3 mass pairs, Time 6.11 b0.811, ESP (MAP2K KUDFGAAR2)         MRM of 4 mass pairs, Time 6.11 b0.811, ESP (MAP2K KUDFGAAR2)         MRM of 4 mass pairs, Time 6.11 b0.811, ESP (MAP2K KUDFGAAR2)         MRM of 4 mass pairs, Time 6.12 b1.2 ESP (MAP2K DVKPSNL/0.984016)LNNSR 3)         MRM of 4 mass pairs, Time 5.79 b7.78, ESP (MAP2K DVKPSNL/0.984016)MLVNTR3)         MRM of 4 mass pairs, Time 6.70 b7.78, ESP (MAP2K DVKPSNL/0.984016)TK3)         MRM of 3 mass pairs, Time 6.70 b7.78, ESP (MAP2K DVKPSNL/0.984016)TK3)         MRM of 3 mass pairs, Time 8.50 b10.55, ESP (MAP2K DVKPSNL/0.984016)TK3)         MRM of 3 mass pairs, Time 8.50 b10.55, ESP (MAP2K DVKPSNL/0.984016)TK3)         MRM of 4 mass pairs, Time 7.20 b7.2 ESP (MAP2K DVKPSNL/0.984016)K2)         MRM of 4 mass pairs, Time 7.20 b7.2 ESP (MAP2K DVKPSNL/0.984016)K2)         MRM of 4 mass pairs, Time 1.08 b10.105, ESP (MAP2K DVKPSNL/0.984016)K2)         MRM of 4 mass pairs, Time 3.70 b 5.73, ESP (PAK LLLDSYNK2)         MRM of 4 mass pairs, Time 3.72 b 5.72, ESP (PAK LLOTSNITX2)         MRM of 4 mass pairs, Time 3.72 b 5.72, ESP (PAK LLOTSNITX2)         MRM of 4 mass pairs, Time 3.72 b 5.72, ESP (PAK LLOTSNITX2)         MRM of 4 mass pairs, Time 3.72 b 5.72, ESP (PAK LLOTSNITX2)         MRM of 4 mass pairs, Time 6.72 b 6.72, ESP (PAK LLOTSNITX2)         MRM of 4 mass pairs, Time 6.72 b 6.	
MRM of 4 mass pairs, Time 77/619 7/6, ES* (MAPK ALDLLDK2)         MRM of 3 mass pairs, Time 6.11b 0.21, ES* (MAPK ALDLLDR2)         MRM of 3 mass pairs, Time 6.11b 0.21, ES* (MAPK ALDLLDR2)         MRM of 4 mass pairs, Time 6.11b 0.81, ES* (MAPK ALDLLDK2)         MRM of 4 mass pairs, Time 6.11b 0.81, ES* (MAPK ALDVFSNL/W)(+0.984016)SR 3)         MRM of 4 mass pairs, Time 6.12b 0.81, ES* (MAP2K DVKPSNL/0.984016)LNNR3)         MRM of 4 mass pairs, Time 6.79 b 0.79, ES* (MAP2K DVKPSNL/0.984016)MLVNTR3)         MRM of 4 mass pairs, Time 6.78 b 0.78, ES* (MAP2K DVKPSNL/0.984016)LLDER3)         MRM of 3 mass pairs, Time 6.50 b 0.56, ES* (MAP2K DVKPSNL/0.984016)LLDER3)         MRM of 3 mass pairs, Time 8.50 b 10.55, ES* (MAP2K DVKPSNL/0.984016)LLDER3)         MRM of 4 mass pairs, Time 7.12b 0.57, ES* (MAP2K DVKPSNL/0.984016)LLDER3)         MRM of 4 mass pairs, Time 7.05 b 12.58, ES* (MAP2K DVKPSNL/0.984016)K2)         MRM of 4 mass pairs, Time 1.30 b 13.38, ES* (MAP2K DVKPSNL/0.984016)K2)         MRM of 4 mass pairs, Time 9.19 b 11.19, ES* (MAP3K NIGSDFIPR2)         MRM of 4 mass pairs, Time 7.20 b 5.72, ES* (PAK LLOTSNITK2)         MRM of 4 mass pairs, Time 7.20 b 5.72, ES* (PAK LLOTSNITK2)         MRM of 4 mass pairs, Time 7.20 b 5.72, ES* (PAK LLOTSNITK2)         MRM of 4 mass pairs, Time 7.20 b 5.72, ES* (PAK LLOTSNITK2)         MRM of 4 mass pairs, Time 7.20 b 5.72, ES* (PAK LLOTSNITK2)         MRM of 4 mass pairs, Time 7.20 b 5.72, ES* (PAK LLOUSSDFIPR2)         MRM of 4	 
MRM of 3 mass pairs, Time 822 to 10.52, ES+ (MAPX KUDLDR2)         MRM of 3 mass pairs, Time 4.1 to 2.41, ES+ (MAPX KVNSPER2)         MRM of 4 mass pairs, Time 6.11 to 8.11, ES+ (MAPX KUDFGAAR2)         MRM of 4 mass pairs, Time 6.11 to 8.11, ES+ (MAPX KUDFGAAR2)         MRM of 4 mass pairs, Time 6.11 to 8.11, ES+ (MAPX KUDFGAAR2)         MRM of 4 mass pairs, Time 6.11 to 8.11, ES+ (MAPX KUDFGAAR2)         MRM of 4 mass pairs, Time 6.11 to 8.11, ES+ (MAPX KUDFGAAR2)         MRM of 4 mass pairs, Time 5.79 to 7.79, ES+ (MAPX KUDFGAR2)         MRM of 3 mass pairs, Time 5.79 to 7.79, ES+ (MAP2K DVKPSNILUDER3)         MRM of 3 mass pairs, Time 6.85 to 10.65, ES+ (MAP2K DVKPSNILLDER3)         MRM of 3 mass pairs, Time 10.86 to 10.56, ES+ (MAP2K DVKPSNILLDER3)         MRM of 4 mass pairs, Time 10.80 to 12.80, ES+ (MAP2K NUPELN(+0.984016)(LLDER3)         MRM of 4 mass pairs, Time 10.80 to 13.80, ES+ (MAP2K NUPELN(+0.984016)(CL2)         MRM of 4 mass pairs, Time 10.20 to 14.09, ES+ (PAK SVLDN(+0.984016)(KL2)         MRM of 4 mass pairs, Time 10.20 to 14.09, ES+ (PAK SVLDN(+0.984016)(KL2)         MRM of 3 mass pairs, Time 3.71 to 5.72, ES+ (PAK LL0TSNITK2)         MRM of 4 mass pairs, Time 9.12 to 1.17, ES+ (MAP3K NICSDFIPR2)         MRM of 4 mass pairs, Time 9.12 to 1.17, ES+ (MAP3K NICSDFIPR2)         MRM of 4 mass pairs, Time 9.12 to 1.17, ES+ (MAP3K NIC-0.984016)(TL2)         MRM of 4 mass pairs, Time 9.12 to 1.12, ES+ (MAPK NIC-0.984016)(SDEPIPR2)         MRM of 4 mass pairs	
MRM of 3 mass pairs, Time 0.41 to 2.41, ES+ (MAP2K SYMSPER 2)         MRM of 4 mass pairs, Time 4.21 to 2.2, ES+ (MAP2K DVKPSNL/V)(4.0 894016)SR.3)         MRM of 3 mass pairs, Time 6.12 to 8.11, ES+ (MAP2K DVKPSNL/V)(4.0 894016)ILVNSR.3)         MRM of 4 mass pairs, Time 6.12 to 8.12, ES+ (MAP2K DVKPSNL/V)(4.0 894016)ILVNTR.3)         MRM of 3 mass pairs, Time 5.79 to 7.78, ES+ (MAP2K DVKPSNL/V)(4.0 894016)ITR.3)         MRM of 3 mass pairs, Time 5.78 to 7.78, ES+ (MAP2K DVKPSNL/V)(4.0 894016)ITR.3)         MRM of 3 mass pairs, Time 8.65 to 10.55, ES+ (MAP2K DVKPSNL/V)(4.0 894016)ITR.3)         MRM of 3 mass pairs, Time 8.65 to 10.55, ES+ (MAP2K DVKPSNL/V)(4.0 894016)ILDER 3)         MRM of 4 mass pairs, Time 10.85 to 10.55, ES+ (MAP2K DVKPSNL/V)(4.0 894016)ILDER 3)         MRM of 4 mass pairs, Time 10.85 to 10.55, ES+ (MAP2K DVKPSNL/V)(4.0 894016)ICX 2)         MRM of 4 mass pairs, Time 10.85 to 10.55, ES+ (MAP2K NVFCPSNL/V)         MRM of 4 mass pairs, Time 10.85 to 10.55, ES+ (MAP2K NVFCPSNL/V)         MRM of 4 mass pairs, Time 10.810 to 12.82, ES+ (MAP2K NVFCPSNL/V)         MRM of 4 mass pairs, Time 10.810 to 13.84, ES+ (MAP2K NVFCPSNL/V)         MRM of 5 mass pairs, Time 10.810 to 13.84, ES+ (MAP2K NVFCPSNL/V)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLDTSNL/V)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLDTSNL/V)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLDTSNL/V)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLDTSNL/V)         MRM o	 _
MRN of 4 mass pairs, Time 4.22 to 6.22, ES+ (MAP3K IADFGAARR.2)         MRN of 3 mass pairs, Time 6.11 to 8.11, ES+ (MAP2K DVKPSNILUN(+0.984016)[KNSR.3)         MRN of 4 mass pairs, Time 6.21 to 8.21, ES+ (MAP2K IPEQILGK.2)         MRN of 4 mass pairs, Time 6.21 to 8.21, ES+ (MAP2K DVKPSNILUNOR.3)         MRN of 3 mass pairs, Time 6.79 to 7.78, ES+ (MAP2K DVKPSNILUN-0.984016)[ILVNTR.3)         MRN of 3 mass pairs, Time 6.60 to 8.66, ES+ (MAP3K LLGQGAFGR.2)         MRN of 5 mass pairs, Time 6.66 to 10.65, ES+ (MAP2K DVKPSNILUDER.3)         MRN of 4 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSNILLDER.3)         MRN of 4 mass pairs, Time 0.86 to 10.65, ES+ (MAP2K DVKPSNILLDER.3)         MRN of 4 mass pairs, Time 0.82 to 12.58, ES+ (PAKLLDSYVK.2)         MRN of 4 mass pairs, Time 0.28 LS = (MAP2K TDFEVLTK.2)         MRN of 4 mass pairs, Time 0.28 LS = (MAP3K KAPSYLYIHK.3)         MRN of 4 mass pairs, Time 1.13 to 13.38, ES+ (MAP3K SAYPSYLYIHK.3)         MRN of 4 mass pairs, Time 9.19 to 11.19, ES+ (MAP3K NIGSDFIPR.2)         MRN of 3 mass pairs, Time 9.19 to 11.19, ES+ (MAP3K NIGSDFIPR.2)         MRN of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAP3K NIC-0.984016)(TXC.2)         MRN of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAP3K NIC-0.984016)(SDFIPR.2)         MRN of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAP3K NIC-0.984016)(SDFIPR.2)         MRN of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAP3K NIC-0.984016)(SDFIPR.2)         MRN of 4 mass pairs, Time 6.21 to 8.72, ES+ (MAPK NIC-0.984016)(SDFIPR.2)<	
MRN of 3 mass pairs, Time 6.11 to 8.11, ES+ (MAP2K DVKPSNI+0.984016)SR.3)         MRN of 4 mass pairs, Time 6.12 to 8.12, ES+ (MAP2K IPEQIL(4).984016)ILVNSR.3)         MRN of 4 mass pairs, Time 5.79 to 7.79, ES+ (MAP2K DVKPSNI+0.984016)ILVNTR.3)         MRN of 3 mass pairs, Time 5.78 to 7.78, ES+ (MAP2K DVKPSNILLDER.3)         MRN of 3 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSNILDER.3)         MRN of 3 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSNILDER.3)         MRN of 4 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSNILDER.3)         MRN of 4 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSNILDER.3)         MRN of 4 mass pairs, Time 1.058 to 12.58, ES+ (MAP2K DVKPSNILDER.3)         MRN of 4 mass pairs, Time 0.82 to 2.82, ES+ (MAP2K DVKPSNILDER.2)         MRN of 4 mass pairs, Time 1.958 to 12.58, ES+ (MAP2K DVKPSNILDER.2)         MRN of 4 mass pairs, Time 1.958 to 12.58, ES+ (MAP2K DVFEVTK2)         MRN of 4 mass pairs, Time 3.73 to 5.73, ES+ (MAP2K INPELN(+0.984016)GK.2)         MRN of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK 2)         MRN of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK 2)         MRN of 4 mass pairs, Time 9.19 to 11.19, ES+ (MAP3K NV(-0.984016)SINTK 2)         MRN of 4 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K NV(-0.984016)SINTK 2)         MRN of 4 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K NV(-0.984016)SINTK 2)         MRN of 4 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K NVLOSDOR)         MRN of 4 mass pairs, Time 7.24 to 8.72 (EA+ (LAVE)SSITTLP	
MRM of 4 mass pairs, Time 6.12 to 8.12, ES+ (MAP2K DVKPSN(+0.984016)/ILVNSR.3)         MRM of 4 mass pairs, Time 5.70 to 8.21, ES+ (MAP2K DVKPSN(+0.984016)/ILVNTR.3)         MRM of 3 mass pairs, Time 5.78 to 7.78, ES+ (MAP2K DVKPSNMLLVN(+0.984016)/TR.3)         MRM of 4 mass pairs, Time 6.66 to 8.66, ES+ (MAP2K DVKPSNMLLDER.3)         MRM of 5 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSNMLLDER.3)         MRM of 4 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSNMLDER.3)         MRM of 4 mass pairs, Time 0.85 to 12.58, ES+ (MAP2K DVKPSNMLDER.3)         MRM of 4 mass pairs, Time 10.58 to 12.58, ES+ (MAP2K TVFEVNL2)         MRM of 4 mass pairs, Time 10.58 to 12.68, ES+ (MAP2K TVFEVNL2)         MRM of 4 mass pairs, Time 10.58 to 12.68, ES+ (MAP2K TVFEVL1+K.2)         MRM of 4 mass pairs, Time 10.58 to 12.68, ES+ (MAP2K TVFEVL1+K.3)         MRM of 5 mass pairs, Time 10.59 to 11.40, ES+ (MAP3K SAVPSYLVIHK.3)         MRM of 4 mass pairs, Time 9.19 to 11.19, ES+ (MAP3K NICSOFIPR 2)         MRM of 4 mass pairs, Time 9.17 to 11.17, ES+ (PAK LLQTSNHK.2)         MRM of 3 mass pairs, Time 9.17 to 11.17, ES+ (PAK LLQTSNHK.2)         MRM of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAP3K N(+0.984016)/TX2)         MRM of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAPK N(+0.984016)/TX2)         MRM of 3 mass pairs, Time 9.24 to 11.24, ES+ (MAPK N(+0.984016)/TX2)         MRM of 5 mass pairs, Time 6.72 to 8.72, ES+ (MAPK N(+0.984016)/TX2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK N(+0.984016)/TX2) <td></td>	
MRM of 4 mass pairs, Time 5.21 to 8.21, ES+ (MAP2K IPEQILGK.2)         MRM of 3 mass pairs, Time 5.78 to 7.79, ES+ (MAP2K DVKPSNI(+0.984016)/IL.VNTR.3)         MRM of 3 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSNILLVN(+0.984016)/IL.DER.3)         MRM of 3 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSNILLDER.3)         MRM of 4 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSNILDER.3)         MRM of 4 mass pairs, Time 0.86 to 12.58, ES+ (MAP2K DVKPSNILDER.3)         MRM of 4 mass pairs, Time 0.82 to 2.82, ES+ (MAP2K DVKV2.2)         MRM of 4 mass pairs, Time 0.82 to 2.82, ES+ (MAP2K TDFEVLTK.2)         MRM of 5 mass pairs, Time 1.209 to 14.09, ES+ (PAK LLLDSYNUX.2)         MRM of 5 mass pairs, Time 1.209 to 14.09, ES+ (PAK SYLDN(+0.984016)/EX.2)         MRM of 5 mass pairs, Time 3.73 to 5.72, ES+ (PAK LLLOTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLLOTSNITK.2)         MRM of 3 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K NIGSDFIPR.2)         MRM of 4 mass pairs, Time 9.17 to 11.17, ES+ (MAP5K NIGSDFIPR.2)         MRM of 4 mass pairs, Time 9.17 to 11.17, ES+ (MAP5K NICSDFIPR.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (PAK LLOTSNICH)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAFK NL/0.984016)/TSNITK.2)         MRM of 4 mass pairs, Time 9.17 to 11.17, ES+ (MAP5K NICSDFIPR.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK NL/USDDQC+0.984016)/RLSDFIR.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK NL/USDDQC+0.984016)/RLSDFIR	
MRM of 3 mass pairs, Time 5.78 to 7.79, ES+ (MAP2K DVKPSN(+0.984016)MLVNTR.3)         MRM of 4 mass pairs, Time 5.78 to 7.78, ES+ (MAP2K DVKPSNLLVN(+0.984016)TR.3)         MRM of 5 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSNLLDER.3)         MRM of 6 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSNLLDER.3)         MRM of 4 mass pairs, Time 0.58 to 12.8, ES+ (PAK LLDSVVK2)         MRM of 4 mass pairs, Time 0.58 to 12.8, ES+ (MAP2K TVKPSNLLDER.3)         MRM of 4 mass pairs, Time 0.52 to 2.82, ES+ (MAP2K TVFEVLTK.2)         MRM of 4 mass pairs, Time 0.52 to 2.82, ES+ (MAP2K TVFEVLTK.2)         MRM of 4 mass pairs, Time 11.38 to 13.38, ES+ (MAP2K SAYPSYLYIIHK.3)         MRM of 4 mass pairs, Time 1.09 to 14.09, ES+ (PAK LLOTSNITK.2)         MRM of 4 mass pairs, Time 1.09 to 14.09, ES+ (PAK SAYPSYLYIIHK.3)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         MRM of 3 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         MRM of 3 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         MRM of 3 mass pairs, Time 9.24 to 11.24, ES+ (MAPSK NVC0.984016)/TK.2)         MRM of 3 mass pairs, Time 7.20 to 7.72, ES+ (PAK LLQTSNITK.2)         MRM of 3 mass pairs, Time 7.20 to 7.72, ES+ (PAK LLQTSNITK.2)         MRM of 4 mass pairs, Time 7.20 to 7.72, ES+ (MAPK NVC0.984016)/TLSLPK.2)         MRM of 3 mass pair	
MRM of 3 mass pairs, Time 6.66 to 8.66, ES+ (MAP3K LLGQGAFGR.2)         MRM of 5 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSNILLDER.3)         MRM of 4 mass pairs, Time 10.58 to 12.68, ES+ (PAYLLDSYVK.2)         MRM of 4 mass pairs, Time 10.58 to 12.68, ES+ (PAYLLDSYVK.2)         MRM of 4 mass pairs, Time 10.58 to 12.68, ES+ (MAP2K TDFEVLTK.2)         MRM of 4 mass pairs, Time 10.58 to 12.68, ES+ (MAP2K NUPSNI(+0.984016)QK.2)         MRM of 5 mass pairs, Time 1.38 to 13.38, ES+ (MAP3K SAYPSYLJIIHK.3)         MRM of 4 mass pairs, Time 1.09 to 14.09, ES+ (PAK SVLDN(+0.984016)FIK.2)         MRM of 4 mass pairs, Time 3.73 to 5.73, ES+ (PAK LLQTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (MAP3K NIGSDFIPR.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQ(+0.984016)ITK.2)         MRM of 4 mass pairs, Time 9.19 to 11.17, ES+ (MAP3K NI         MRM of 4 mass pairs, Time 9.27 to 5.72, ES+ (PAK LLQ(+0.984016)ITK.2)         MRM of 4 mass pairs, Time 9.72 to 5.72, ES+ (PAK LLQ(+0.984016)ITK.2)         MRM of 4 mass pairs, Time 9.72 to 5.72, ES+ (MAPK NI<0.984016)ISCNER.2)	
MRM of 5 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSNILLDER.3)         MRM of 3 mass pairs, Time 10.58 to 12.58, ES+ (PAK LLLDSYVK2)         MRM of 4 mass pairs, Time 0.58 to 12.58, ES+ (PAK LLLDSYVK2)         MRM of 4 mass pairs, Time 0.58 to 12.58, ES+ (MAP2K TDFEVLTK2)         MRM of 4 mass pairs, Time 0.58 to 13.38, ES+ (MAP2K INPELN(+0.984016)(DK.2)         MRM of 5 mass pairs, Time 12.09 to 14.09, ES+ (PAK SYLDN(+0.984016)FIK.2)         MRM of 4 mass pairs, Time 13.73 to 5.73, ES+ (PAK LLOTSNITK2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLOTSNITK2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLOTSNITK2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLOTSNITK2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLOTSNITK2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLOTSNITK2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLOTSNITK2)         MRM of 4 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K N(+0.984016)TK2)         MRM of 5 mass pairs, Time 9.24 to 11.24, ES+ (MAPK N(+0.984016)TSNITK2)         MRM of 5 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ2)         MRM of 4 mass pairs, Time 6.74 to 8.72, ES+ (MAPK MLVLDSDQ2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)         MRM of 4 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISS	
MRM of 3 mass pairs, Time 8.65 to 10.65, ES+ (MAP2K DVKPSN(+0.984016)/LLDER.3)         MRM of 4 mass pairs, Time 10.58 to 12.58, ES+ (PAK LLLDSVVK.2)         MRM of 4 mass pairs, Time 0.72 to 9.72, ES+ (MAP2K TDFEV_TK.2)         MRM of 4 mass pairs, Time 0.82 to 2.82, ES+ (MAP2K NDFEV_TK.2)         MRM of 5 mass pairs, Time 11.38 to 13.38 ES+ (MAP2K SAVPSYLYIIHK.3)         MRM of 4 mass pairs, Time 1.209 to 14.09, ES+ (PAK SYLDN(+0.984016)FIK.2)         MRM of 4 mass pairs, Time 3.73 to 5.73, ES+ (PAK LLQTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQCTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQCTSNITK.2)         MRM of 4 mass pairs, Time 9.19 to 11.19, ES+ (MAP3K NIGSDFIPR.2)         MRM of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAP3K N(+0.984016))TCASLPSK.2)         MRM of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAPK N(+0.984016))TCASLPSK.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQR.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQR.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQR.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQR.2)         MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NLVLSDPHK.2)         MRM of 4 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NLVLSUSPHK.2)         MRM of 4 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)         MRM of 4 mass	
MRM of 4 mass pairs, Time 10.58 to 12.58, ES+ (PAK LLLDSYVK.2)         MRM of 4 mass pairs, Time 7.72 to 9.72, ES+ (MAP2K TDFEVLTK.2)         MRM of 4 mass pairs, Time 0.82 to 2.82, ES+ (MAP2K TDFEVLTK.2)         MRM of 5 mass pairs, Time 11.38 to 13.38, ES+ (MAP3K SAYPSYLYIIHK.3)         MRM of 4 mass pairs, Time 10.99 to 14.09, ES+ (PAK SYLDN(+0.984016)(FK.2)         MRM of 4 mass pairs, Time 3.73 to 5.73, ES+ (PAK LLQTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         MRM of 3 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQ(+0.984016))TK.2)         MRM of 3 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQ(+0.984016))TK.2)         MRM of 3 mass pairs, Time 9.19 to 11.19, ES+ (MAP3K N(+0.984016))TK.2)         MRM of 3 mass pairs, Time 9.24 to 11.24, ES+ (MAP3K N(+0.984016))TLQSLPSK.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (PAK LLQDSDQ(+0.984016))TLQSLPSK.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ(+0.984016)R2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ(+0.984016)R2)         MRM of 5 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ(+0.984016)R2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ(+0.984016)R2)         MRM of 4 mass pairs, Time 6.74 to 8.44, ES+ (PAK LSVISSNTLR.2)         MRM of 4 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTL2)         MRM of 4 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTL2)         MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVINDWEER.2)	
MRM of 4 mass pairs, Time 7.72 to 9.72, ES+ (MAP2K TDFEVLTK.2)         MRM of 4 mass pairs, Time 0.82 to 2.82, ES+ (MAP2K INPELN(+0.984016)QK.2)         MRM of 5 mass pairs, Time 1.38 to 13.38, ES+ (MAP2K SAYPSYLYIIHK.3)         MRM of 5 mass pairs, Time 1.209 to 14.09, ES+ (PAK SYLDN(+0.984016)FIK.2)         MRM of 4 mass pairs, Time 3.73 to 5.73, ES+ (PAK SYLDN(+0.984016)FIK.2)         MRM of 4 mass pairs, Time 9.19 to 11.10, ES+ (MAP3K NICSDFIPR.2)         MRM of 3 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSN(+0.984016)ITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSN(+0.984016)IGSDFIPR.2)         MRM of 4 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K N(+0.984016)IGSDFIPR.2)         MRM of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAPK N(+0.984016)IQSLPSK.2)         MRM of 4 mass pairs, Time 9.25 to 14.06, ES+ (PAK KLVLDSDQ.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ.2)         MRM of 4 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NLVLSLPHK.2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)         MRM of 3 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTL2)         MRM of 3 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTL2)         MRM of 3 mass pairs, Time 7.44 to 9.44, ES+ (MAPK EVMDWEER.2)         MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2) <td></td>	
MRM of 4 mass pairs, Time 0.82 to 2.82, ES+ (MAP2K INPELN(+0.984016)QK.2)         MRM of 5 mass pairs, Time 11.38 to 13.38, ES+ (MAP3K SAYPSYLYIIHK.3)         MRM of 5 mass pairs, Time 12.09 to 14.09, ES+ (PAK SYLDN(+0.984016)FIK.2)         MRM of 4 mass pairs, Time 3.73 to 5.73, ES+ (PAK SYLDN(+0.984016)FIK.2)         MRM of 4 mass pairs, Time 9.19 to 11.19, ES+ (PAK SYLDN(+0.984016)FIK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSN(+0.984016)/TK.2)         MRM of 4 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K NI<0.984016)/GSDFIPR.2)	
MRM of 5 mass pairs, Time 11.38 to 13.38, ES+ (MAP3K SAYPSYLYIIHK.3)         MRM of 5 mass pairs, Time 12.09 to 14.09, ES+ (PAK SYLDN(+0.984016)FIK.2)         MRM of 4 mass pairs, Time 3.73 to 5.73, ES+ (PAK LLQTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         MRM of 4 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K N(+0.984016))TSNITK.2)         MRM of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAPK N(+0.984016))TQSLPSN.2)         MRM of 5 mass pairs, Time 6.72 to 8.72, ES+ (PAK LLQCSLPSL2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ2)         MRM of 5 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ2)         MRM of 4 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NVLSLPHK.2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)         MRM of 4 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLP.2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLP.2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLP.2)         MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2)	
65       MRM of 5 mass pairs, Time 11.38 to 13.38, ES+ (MAP3K SAYPSYLYIIHK.3)         7       MRM of 5 mass pairs, Time 12.09 to 14.09, ES+ (PAK SYLDN(+0.984016)FIK.2)         8       MRM of 4 mass pairs, Time 3.73 to 5.73, ES+ (PAK SYLDN(+0.984016)FIK.2)         9       MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLOTSNITK.2)         9       MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLOTSNITK.2)         10       MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLOTSNITK.2)         11       MRM of 3 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K NI<0.984016)/TSNITK.2)	
8       MRM of 4 mass pairs, Time 3.73 to 5.73, ES+ (PAK LLQTSNITK.2)         9       MRM of 4 mass pairs, Time 9.19 to 11.19, ES+ (MAP3K NIGSDFIPR.2)         0       MRM of 3 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         0       MRM of 3 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         0       MRM of 3 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSNITK.2)         0       MRM of 4 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K NI(-0.984016)/TLQSLPSK.2)         0       MRM of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAPK NI(+0.984016)/TLQSLPSK.2)         1       MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ(+) 0.984016)/R.2)         1       MRM of 5 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ(+) 0.984016)/R.2)         1       MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ(+) 0.984016)/R.2)         1       MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ(+) 0.984016)/R.2)         1       MRM of 4 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NVLLSLPHK.2)         1       MRM of 4 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)         1       MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLP.2)         1       MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2)	
B       MRM of 4 mass pairs, Time 9.19 to 11.19, ES+ (MAP3K NIGSDFIPR.2)         MRM of 3 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQ(+0.984016)ITK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQ(+0.984016)ITK.2)         MRM of 3 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K N(+0.984016)IGSDFIPR.2)         MRM of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAPK N(+0.984016)IGSDFIPR.2)         MRM of 4 mass pairs, Time 9.29 to 14.08, ES+ (PAK LLQ(+0.984016)YLQSLPSK.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK N(+LQSLPSK.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ.2)         MRM of 5 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ.2)         MRM of 4 mass pairs, Time 6.74 to 9.44, ES+ (MAPK MLVLDSDQ.2)         MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NVLLSLPHK.2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)         MRM of 3 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTL2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTL2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTL2)         MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2)	
MRM of 3 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQTSN(+0.984016)/TK.2)         MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQ(+0.984016)/SNITK.2)         MRM of 4 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K N(+0.984016)/IGSDFIPR.2)         MRM of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAPK N(+0.984016)/ISQSLPSK.2)         MRM of 4 mass pairs, Time 1.29 to 14.96, ES+ (PAK LLQ(+0.984016)/ISQSLPSK.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVDSDQR.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ(+0.984016)/R.2)         MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK MLVLDSDQ(+0.984016)/R.2)         MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NVLLSLPHK.2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)         MRM of 3 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLP.2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLP.2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLP.2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLP.2)         MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2)	
MRM of 4 mass pairs, Time 3.72 to 5.72, ES+ (PAK LLQ(+0.984016)TSNITK.2)         MRM of 3 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K N(+0.984016)[GSDFIPR.2)         MRM of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAPK N(+0.984016)[CQSLPSK.2)         MRM of 5 mass pairs, Time 12.96 to 14.96, ES+ (PAK LLQ(+0.984016)[CQSLPSK.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK NLVLDSDQR.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ(+0.984016)R.2)         MRM of 5 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ(+0.984016)R.2)         MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NLVLDSDQ(+0.984016)R.2)         MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NLVLDSDQ (+0.984016)R.2)         MRM of 4 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTL+2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTL+2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTL+2)         MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2)	
22       MRM of 3 mass pairs, Time 9.17 to 11.17, ES+ (MAP3K N(+0.984016))GSDFIPR.2)         38       MRM of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAPK N(+0.984016))YLQSLPSK.2)         49       MRM of 5 mass pairs, Time 12.96 to 14.96, ES+ (PAK IAKPLSSLTPLIAAAK.3)         50       MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ.2)         51       MRM of 5 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ.2)         52       MRM of 5 mass pairs, Time 8.72 to 8.72, ES+ (MAPK MLVLDSDQ.2)         53       MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NLVLSDPLK.2)         54       MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NLVLSUPHK.2)         55       MRM of 4 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NLVLSUPHK.2)         56       MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)         57       MRM of 4 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLP.2)         58       MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLP.2)         59       MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2)	
33       MRM of 4 mass pairs, Time 9.24 to 11.24, ES+ (MAPK N(+0.984016)YLQSLPSK.2)         44       MRM of 5 mass pairs, Time 12.96 to 14.96, ES+ (PAK IAKPLSSLTPLIAAAK.3)         55       MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQR.2)         56       MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ(+0.984016)R.2)         57       MRM of 5 mass pairs, Time 8.72 to 8.72, ES+ (MAPK MLVLDSDQ(+0.984016)R.2)         58       MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NVLLSLPHK.2)         59       MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NVLLSLPHK.2)         50       MRM of 3 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)         50       MRM of 3 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSN(+0.984016)TLR.2)         51       MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2)	
MRM of 5 mass pairs, Time 12.96 to 14.96, ES+ (PAK IAKPLSSLTPLIAAAK.3)           MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQR.2)           MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQL(+0.984016)R.2)           MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NVLLSLPHK.2)           MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (MAPK NVLLSLPHK.2)           MRM of 3 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)           MRM of 3 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)           MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2)	
MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQR.2)         MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ(+0.984016)R.2)         MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NVLSLPHK.2)         MRM of 5 mass pairs, Time 7.44 to 10.14, ES+ (MAPK NVLSLPHK.2)         MRM of 4 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)         MRM of 3 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLP.2)         MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2)	
MRM of 4 mass pairs, Time 6.72 to 8.72, ES+ (MAPK MLVLDSDQ(+0.984016)R.2)         MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NVLLSLPHK2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (MAPK NVLSLPHK2)         MRM of 4 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)         MRM of 5 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)         MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2)	
7         MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NYLLSLPHK.2)           8         MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK NYLLSLPHK.2)           9         MRM of 4 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)           9         MRM of 3 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)           1         MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2)	
3         MRM of 5 mass pairs, Time 8.14 to 10.14, ES+ (MAPK N(+0.984016)YLLSLPHK.2)           3         MRM of 4 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)           4         MRM of 3 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSN(+0.984016)TLR.2)           5         MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2)	
9         MRM of 4 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)           9         MRM of 3 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSN(+0.984016)TLR.2)           1         MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2)	
B         MRM of 4 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSNTLR.2)           MRM of 3 mass pairs, Time 7.44 to 9.44, ES+ (PAK LSVISSN(+0.984016)TLR.2)           MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2)	
MRM of 5 mass pairs, Time 6.96 to 8.96, ES+ (MAPK EVMDWEER.2)	
2 MRM of 4 mass pairs, Time 5.56 to 7.56, ES+ (MAPK DLKPSNIVVK.2)	

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<sup>™</sup> M-Class coupled to a SELECT SERIES<sup>™</sup> Cyclic<sup>™</sup> 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).

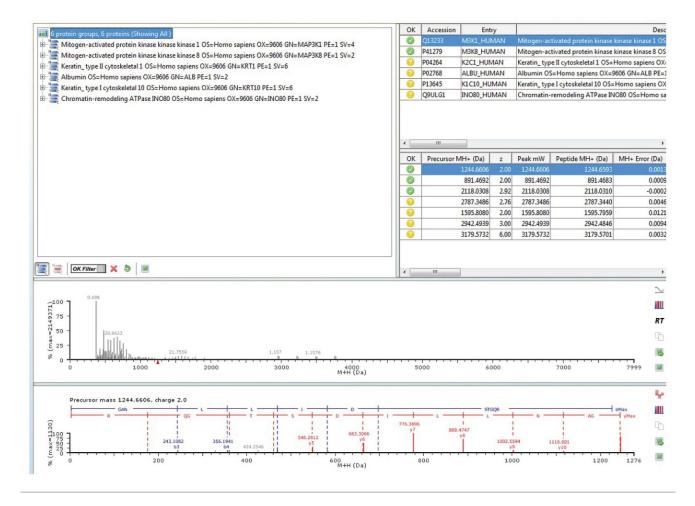


Figure 2. Example PLGS view: showing an example single kinase enzyme digest peptide search (MAP3K1), of which seven individual peptides were found and identified, the fragmentation (b & y ions) found for the highlighted peptide (corresponding to (K)GANLLIDSTGQR(L)) can be seen in the bottom section of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

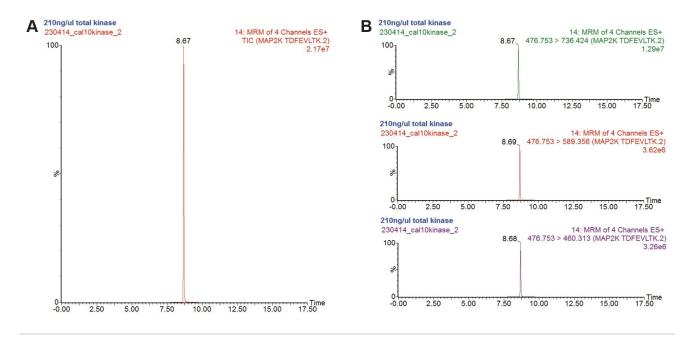


Figure 3. Example Masslynx views: (A) TIC produced when all signals are combined (three selected transitions) to confirm the presence of MAP2K7 within the sample. (B) XIC of the three individual transitions.

Creation of calibration curves using only purified standards does not sufficiently mimic the complexity of "reallife" 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<sup>™</sup> 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.<sup>2,3</sup> 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.

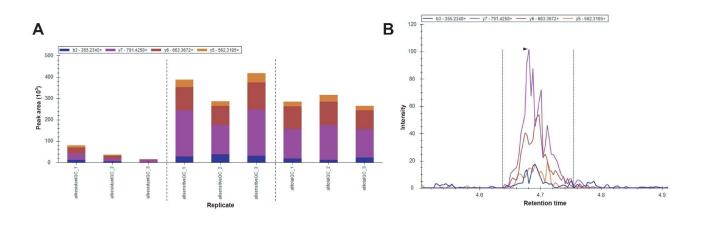


Figure 4. (A) Ion ratios for the PAK marker peptide LLQTSNITK demonstrating the difference in peak area between non-mutated cell line (low abundance), mutated cell line (high abundance) and a mix of the two (intermediate abundance). (B) Extracted ion chromatograms from analysis of the mutated cell line 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

- Bhullar, K.S., Lagarón, N.O., McGowan, E.M. *et al.* Kinase-targeted Cancer Therapies: Progress, Challenges and Future Directions. *Mol Cancer* 17, 48 (2018). https://doi.org/10.1186/s12943-018-0804-2 < https://doi.org/10.1186/s12943-018-0804-2>
- Duong-Ly KC, Peterson JR. The Human Kinome and Kinase Inhibition. *Curr Protoc Pharmacol.* 2013 Mar;Chapter 2:Unit2.9. doi: 10.1002/0471141755.ph0209s60 <a href="https://pubmed.ncbi.nlm.nih.gov/23456613/">https://pubmed.ncbi.nlm.nih.gov/23456613/></a>. PMID: 23456613; PMCID: PMC4128285.
- Lu Z, Hunter T. Degradation of Activated Protein Kinases by Ubiquitination. Annu Rev Biochem.
   2009;78:435–75. doi: 10.1146/annurev.biochem.013008.092711 <a href="https://pubmed.ncbi.nlm.nih.gov/19489726">https://pubmed.ncbi.nlm.nih.gov/19489726</a>>.
   PMID: 19489726; PMCID: PMC2776765.
- 4. Pillay N, Tighe A, Nelson L, Littler S, Coulson-Gilmer C, Bah N, Golder A, Bakker B, Spierings DCJ, James DI, Smith KM, Jordan AM, Morgan RD, Ogilvie DJ, Foijer F, Jackson DA, Taylor SS. DNA Replication Vulnerabilities Render Ovarian Cancer Cells Sensitive to Poly(ADP-Ribose) Glycohydrolase Inhibitors. *Cancer Cell*. 2019 Mar 18;35(3):519–533.e8. doi: 10.1016/j.ccell.2019.02.004 < https://pubmed.ncbi.nlm.nih.gov/30889383/>. PMID: 30889383; PMCID: PMC6428690.

## Featured Products

ACQUITY UPLC I-Class PLUS System <https://www.waters.com/134613317>

ACQUITY Premier System <

https://www.waters.com/nextgen/global/products/chromatography/chromatography-systems/acquity-premier-system.html>

ACQUITY UPLC M-Class Chromatography System <

https://www.waters.com/nextgen/global/products/chromatography/chromatography-systems/acquity-uplc-mclass-system.html>

Xevo TQ-XS Triple Quadrupole Mass Spectrometry <a href="https://www.waters.com/134889751">https://www.waters.com/134889751</a>

SELECT SERIES Cyclic IMS <https://www.waters.com/waters/global/SELECT-SERIES-Cyclic-IMS-ion-mobilitymass-spectrometer/nav.htm?cid=135021297>

MassLynx MS Software <https://www.waters.com/513662>

TargetLynx <https://www.waters.com/513791>

720008020, September 2023

 $\wedge$ 

© 2023 Waters Corporation. All Rights Reserved.

Conditions d'utilisation Confidentialité Marques Carrières Cookies Préférences de cookies