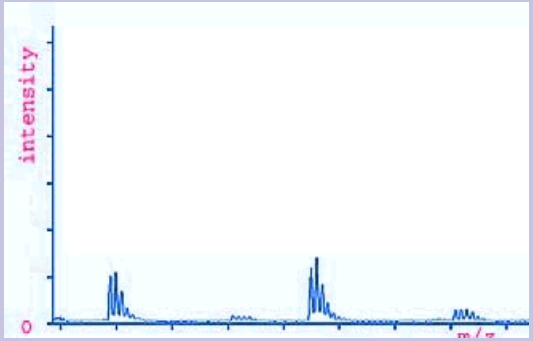
Target Validierung: Nicht-gelbasierte Proteomforschung zur systematischen und quantitativen Peptidanalyse

DPhG – Mainz, 6.Oktober 2005

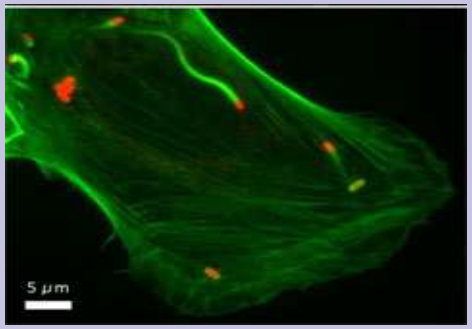
Lothar Jänsch



Organisation of Host-Pathogen Interaction Studies

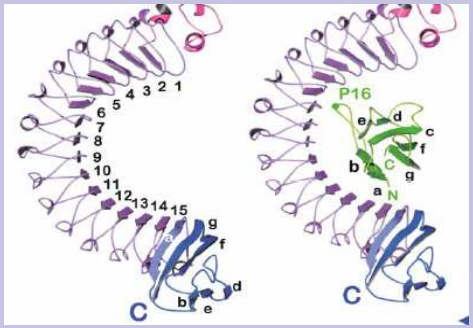
Cell Biology

Prof. Jürgen Wehland

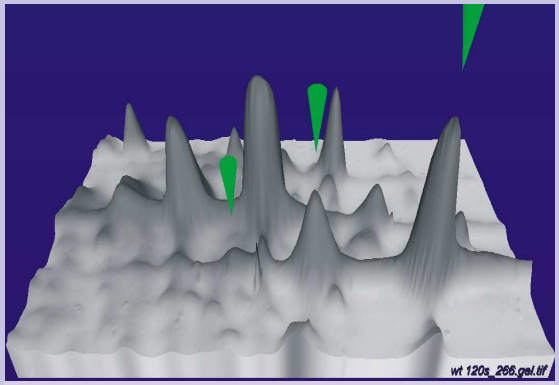


Structural Biology

Prof. Dirk Heinz



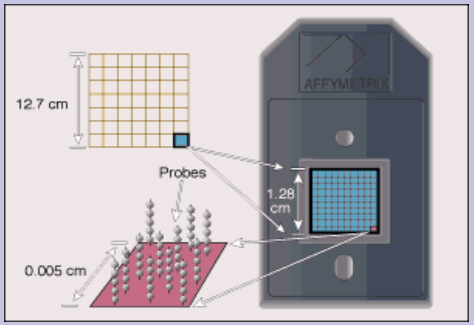
Proteome Research Group



Identi. & Characterisation of bacterial virulence factors

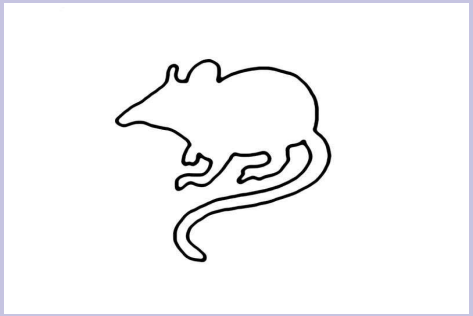
Affymetrix platform

Prof. Jan Buer

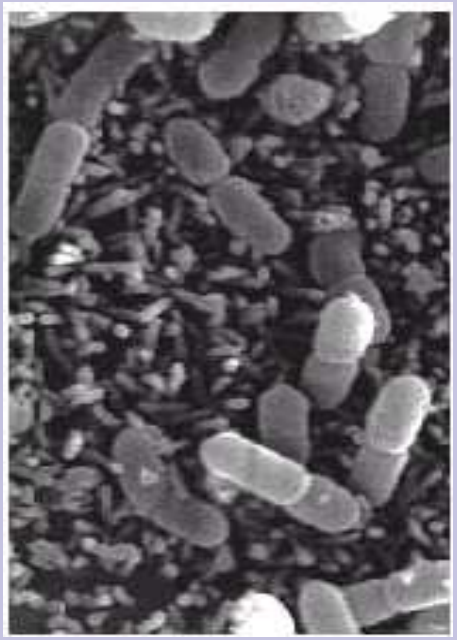


Mouse facility

Dr. Lengeling / Dr. Müller



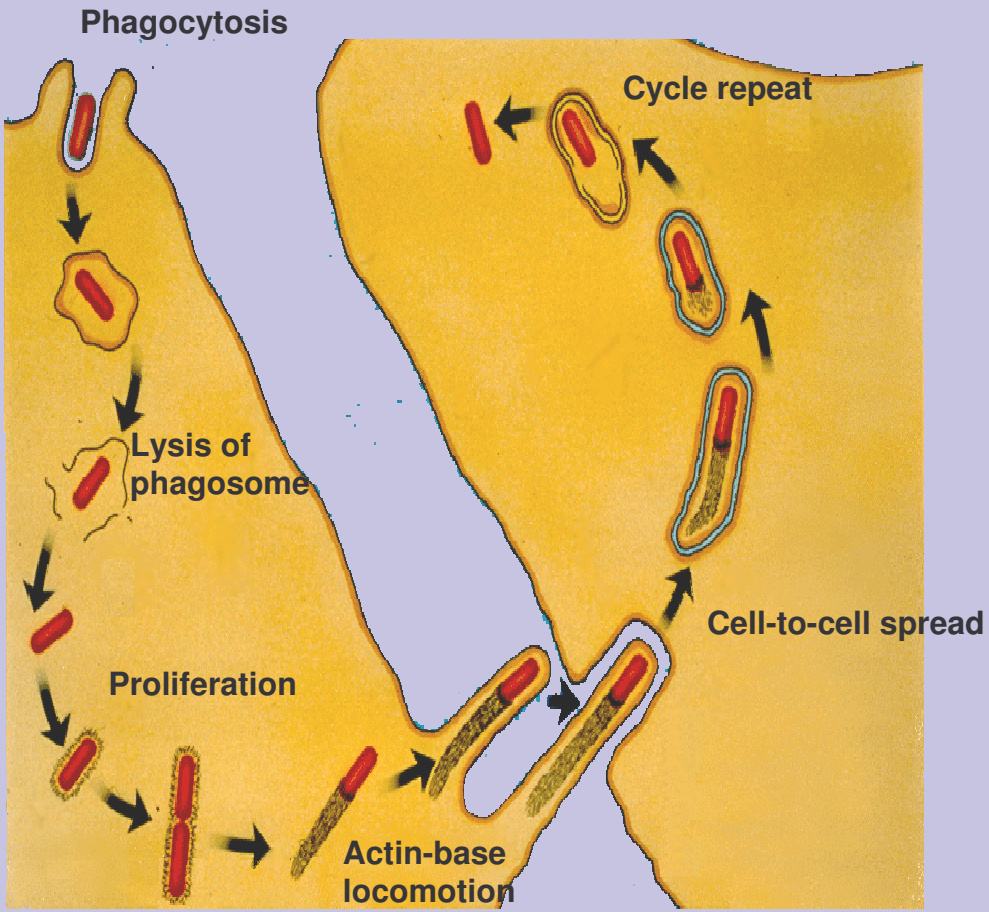
Listeria monocytogenes



- Gram-positive bacterium
- Facultative intracellular human pathogen
- Capacity to overcome three cellular barriers: intestinal-, brain-blood- and the placental barrier
- Genome published, *L. innocua & L. monocytogenes* (Glaser et al.,2001)

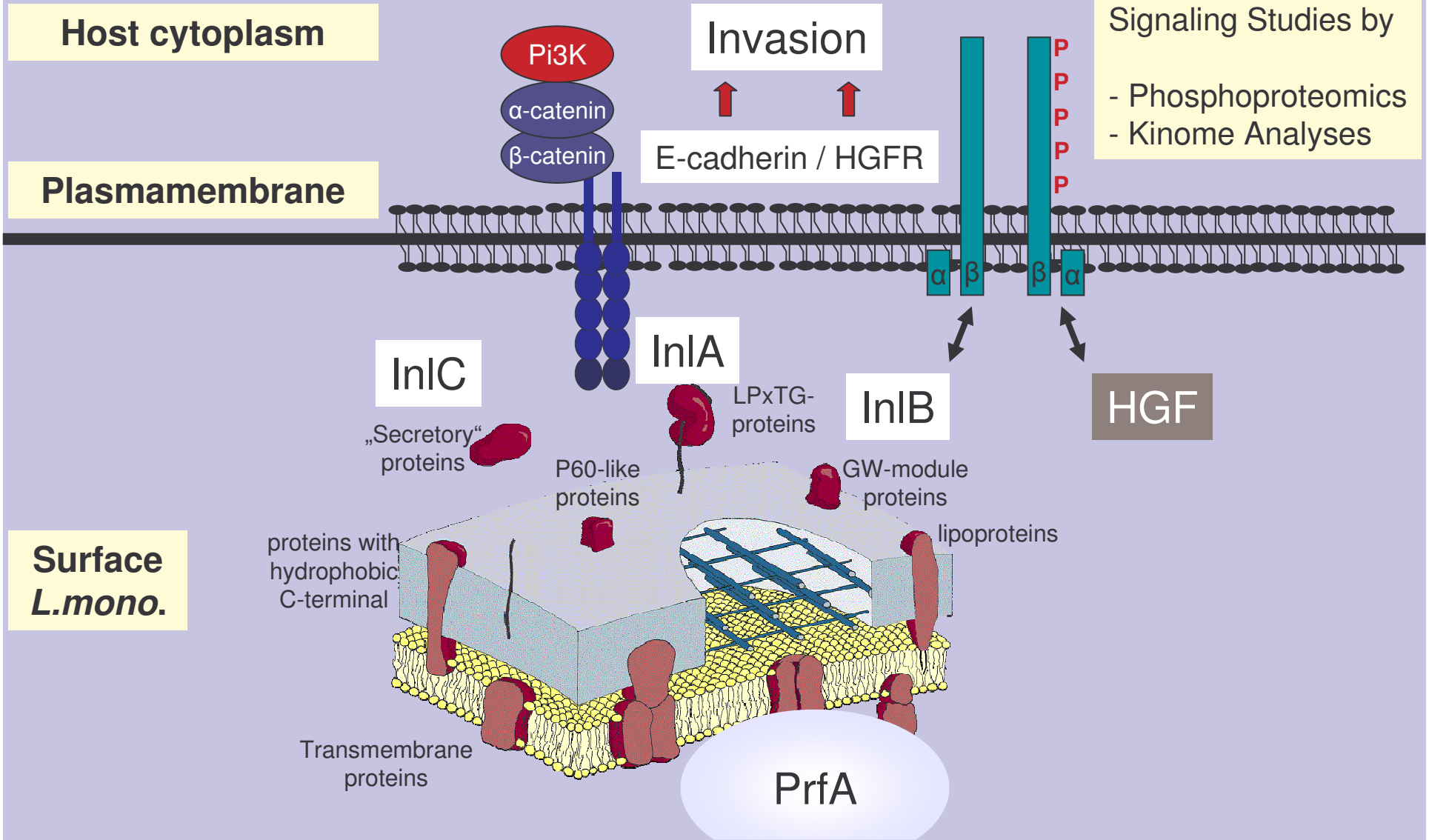
Picture taken from: J. A. Vazquez-Boland et al., *Clin Microbiol Rev* 14 (3):584-640, 2001

Host-Pathogen Interaction:
Listeria monocytogenes



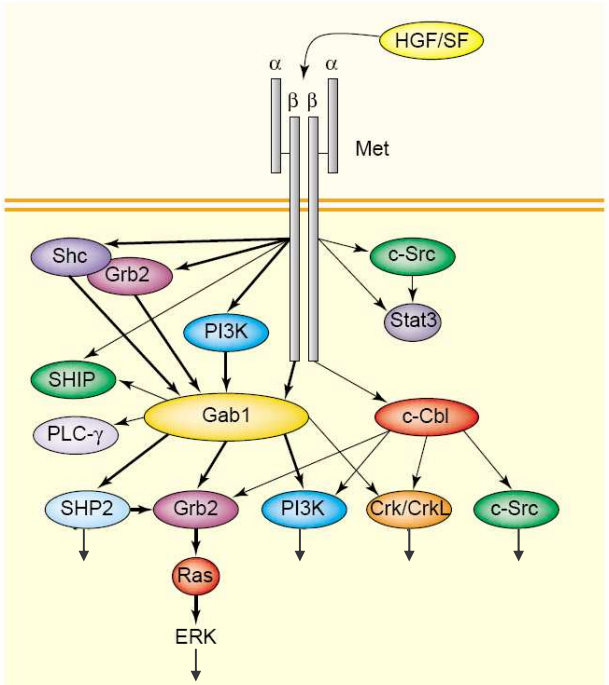
Internalin A, B	Cell entry
PlcA, Hly	Escape from Phagosomes
ActA	Intracellular motility
Mpl	Activation of PlcB
PlcB, Hly	Dissolution of double membrane

Host-Pathogen Interaction Studies



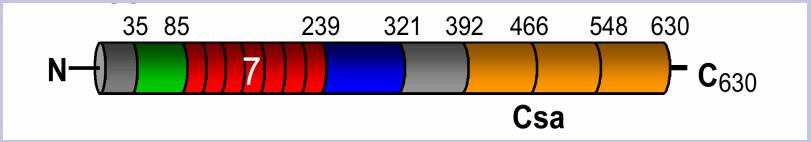
Comparison of HGF and InlB mediated host cell signalling

HGF:



cell growth, proliferation and motility

Internalin B:



Met

gC1q-R

glucosaminoglycans

Uptake of *Listeria monocytogenes* by non-professionel phagozytic cells

Rosario et al., Trends Cell Biol. 2003 Jun;13(6):328-35, modified

Signal transduction of the infected host upon interaction with pathogens

L. monocytogenes	InlB	cMet	Invasion
L. monocytogenes	InlA	E-Cadherin	Invasion
Y. enterolytica	Invasin	Integrin	Invasion
H. pylorii	CagA	Src	Persistence
P. aeruginosa	?	EGFR	Invasion
P. aeruginosa	ExoS	Ras/Raf	Cyto-toxicity

Further bacterial effectors / receptors have to be identified

or

We still have severe limitations in our knowledge about fundamental cellular signaling pathways

Aim & Strategy (started 2003)

Aim:

1) Define an experimental strategy for the functional analysis of receptor mediated signalling

2) Compare HGF and Internalin B induced epithelial cells to understand the level of „molecular mimicry“

Strategy:

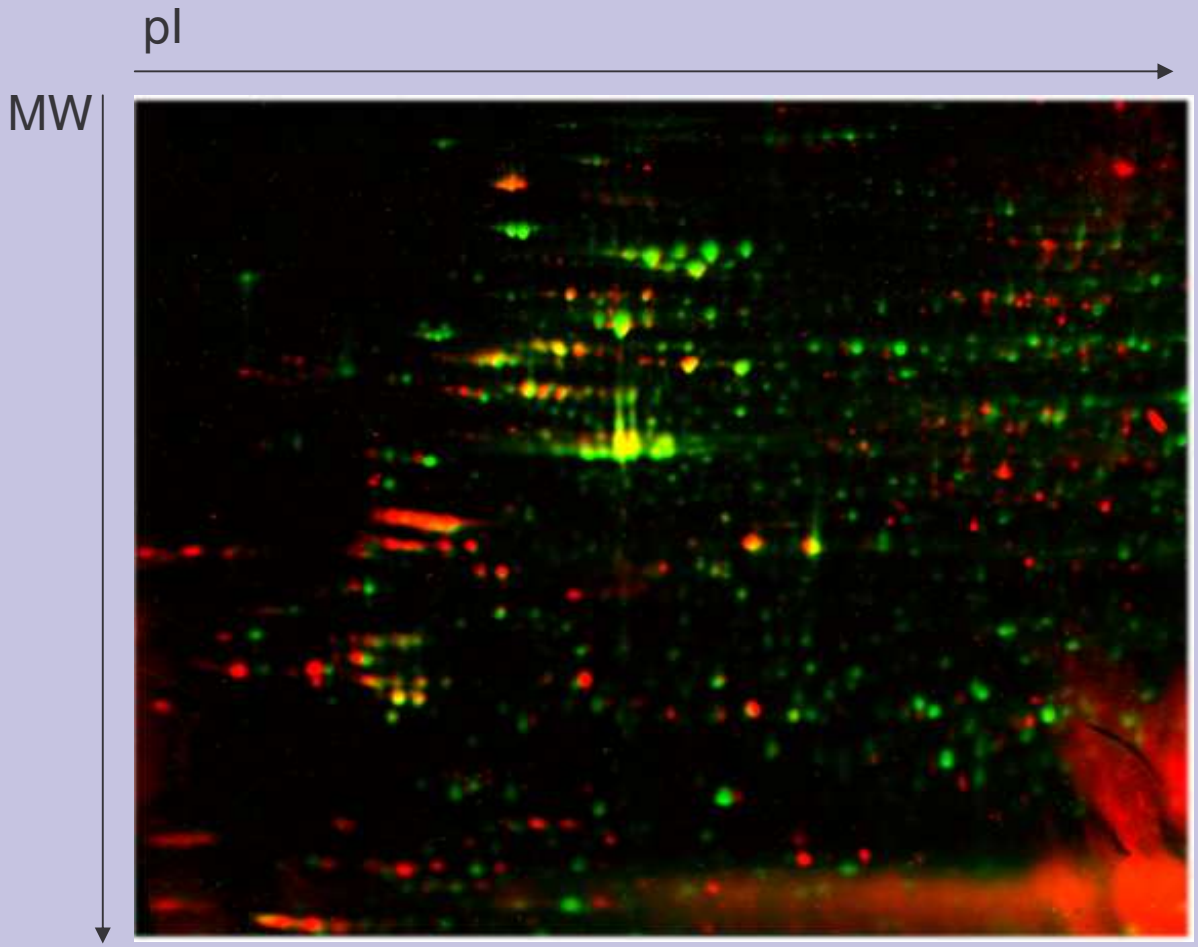
Cell lysis

Igepal, Phosphatase-/Protease-Inhibitors

„Proteomics“

Incubuation of 10^7 HeLa S3 cells

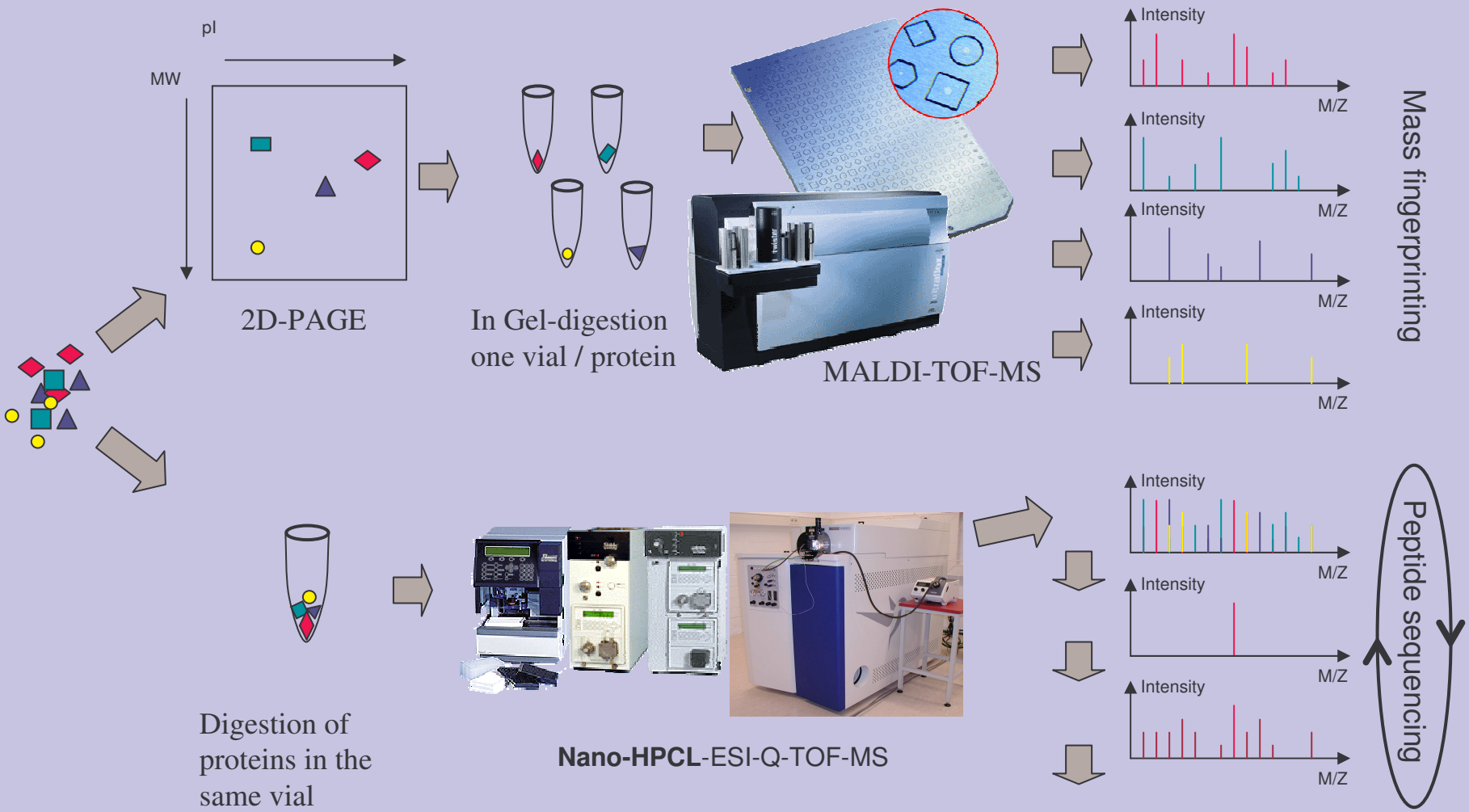
Host cell response analysis: Expression and Phosphoproteome analysis on traditional 2D-PAGE



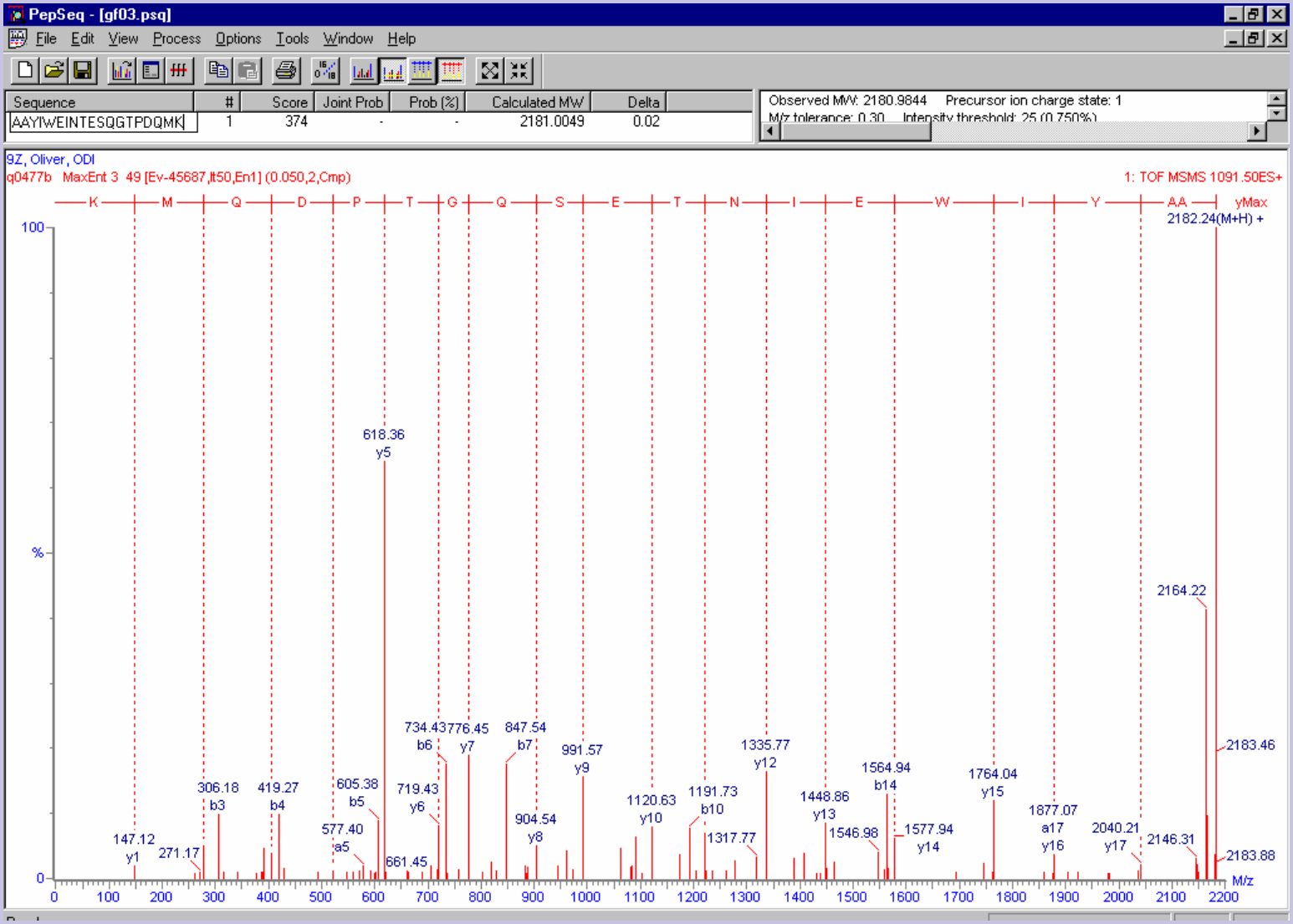
- Induction of HeLa S3 cells
- Lyse the cells after 30min (1% Igepal, Na_3V0_4, NaF)
- RuBPS-stain (green) for **expression analysis**
- ProQ-stain (red) for detection of **phosphorylated proteins**

Several differentially phosphorylated proteins can be detected

2D-PAGE vs. LC-MS/MS



Peptide-Sequencing



Frequency and suppressed ionization behavior of phosphorylated peptides in complex samples

HDEPLPANR

DFGR

LNNVRTSADCQWER

100 proteins generate about 40000 different typtic peptides

P

NH2-....JTGNVIPEERNGTDFGHSiPEYMHKGHDLHITRMFP....-COOH

TSADCQWER

LMNGFTWDEK

CDEPHLPASR

YREGVSPTQK

GWACVFLMASGTK

LC-MS/MS for the characterization of phosphorylated peptides

Prerequisites:

1. Specific enrichment of phosphorylated peptides.

2. Systematic access on kinases.

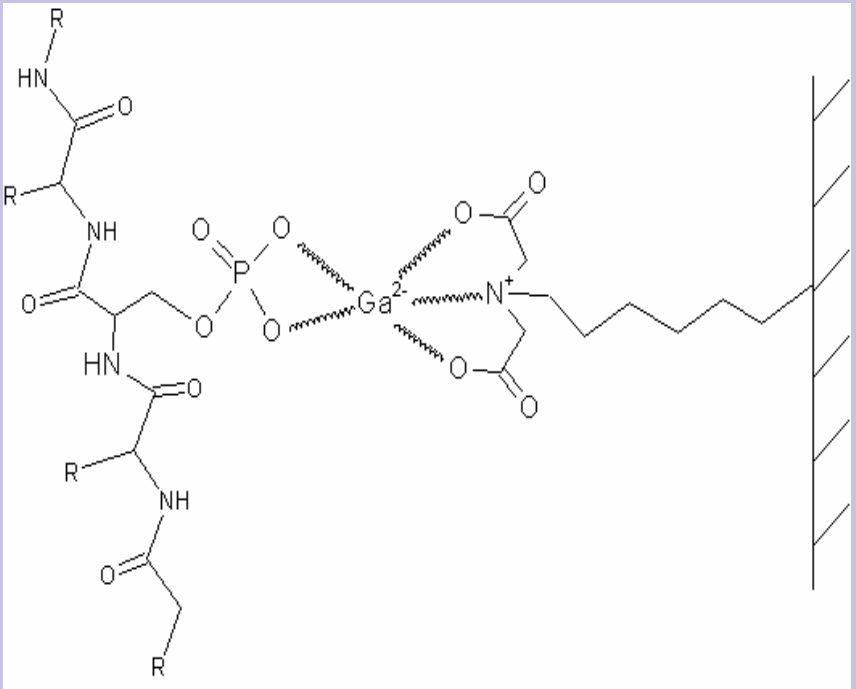
Preferable:

3. Relative and absolute quantification of single peptides.

1. Specific enrichment of phosphorylated peptides

(1.) IMAC
(Immobilized Metall Affinity Purification)

- Methylation of acidic amino acids
- Binding of phosphorylated peptides to immobilized Ga^{2+}
- Washing
- Elution
- Characterization by LC-MS/MS



Ficarro *et al.*, 2002: Systematic identification of phosphorylated peptides from *S. cereviseae.*

Systematic identification of phosphorylated peptides

1. Enrichment of phosphorylated peptides based on IMAC (Immobilized Metal Affinity Chromatographie, Ficarro et al., 2002)
2. Protein identification **and** phosphosite determination by LC-MS/MS.

Protein	ID	Phosphosite	Phosphobase	Scansite
TCOF1	gi|1587477	S139		Cdk5_Kin // Cdc2_Kin
		S287		
		S352		
		S812		
		S1133		
		S1134		
Src substrate cortactin	gi|2498954	T399		
		T401		
		S405		GSK3_Kin // Erk1_Kin
		S417		
		S418		
lamin A	gi|34228	S22	cdc2	Cdc2_Kin // Erk1_Kin
		S390		
		S392	cdc2	
		S403	pkc	
microtubule-associated protein 4	gi|107082	S280		
		T354		
		S358		
		T521		
nuclear ubiquitous casein kinase substrate	gi|12232387	S58		Casn_Kin2
		S61		Casn_Kin2
		S73		

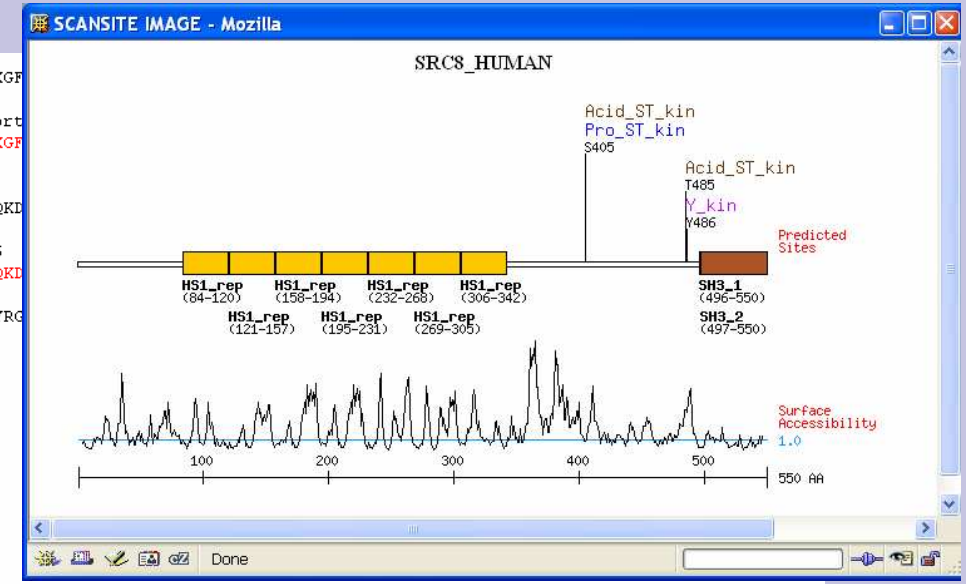
1D-LC-MS/MS (2004):

- Identification of 112 phosphorylated proteins
- 189 phosphosites
- known and unknown sites

Purification efficiency of phosphopeptide

> 94 %

Systematic evaluation of posttranslational modification: Phosphosite Report



FGGKFGVQMDRVDQSAVGFEYQGKTEKHASQKDYSSGFGGKYGVQADRVDKSAVGFDYQGKTEKHESQRDYSKGF
121 FGGKFGVQMDRVDQSAVGFEYQGKTEKHASQKD Cortactin 2
154 YSSGFGGKYGVQADRVDKSAVGFDYQGKTEKHESQRD Cort
191 YSKGF

DRQDKCALGWDHQEKLQLHESQKDYKTGFGGKFGVQSERQDSAAVGFDYKEKLAKHESQQDYSKGFGGKYGVQKD
241 DRQDKCALGWDHQEKLQLHESQKD Cortactin 5
265 YKTGFGGKFGVQSERQDSAAVGFDYKEKLAKHESQQD Cortactin 6
302 YSKGFGGKYGVQKD

KEQEDRRKAEAERAQRMAKERQEQEEARRKLEEQARAKTQTPPVSPAPQPTEERLPSSPVYEDAASFKAELSYRG
397 AKTQTPPVS (T401) HTP;HTP
397 AKTQTPPVSPAPQPTEER (S405) (T399)
398 KTQTPPVSPAPQPT (S405) Erk1_Kin
398 KTQTPPVSPAPQPT (S405) GSK3_Kin
399 TQTPPVSPAPQPTEER (S405) (T401)
399 TQTPPVSPAPQPTEER (S405) (T401)
399 TQTPPVSPAPQPTEER (T399) (T401)
399 TQTPPVSPAPQPTEER (S405) (T401)
399 TQTPPVSPAPQPTEER (T399) (T401)
399 TQTPPVSPAPQPTEER (S405) (T401)
399 TQTPPVSPAPQPTEER (T399) (S405)
399 TQTPPVSPAPQPTEER (S405) (T401)
399 TQTPPVSPAPQPTEER (S405) (T401)
399 TQTPPVSPAPQPTEER (S405) (T401)
401 T Phosphothreonine
401 TPPVSPAPQ (S405) MAP2K LTP;HTP
405 S Phosphoserine
414 RLPSSPVYE (S418) MAP2K LTP
415 LPSSPVYEDAASFK (S418)
415 LPSSPVYEDAASFK (S418)
415 LPSSPVYEDAASFK (S417)
415 LPSSPVYEDAASFK (S417)
415 LPSSPVYEDAASFK (S417)
415 LPSSPVYEDAASFK (S418)
415 LPSSPVYEDAASFK (S418)
417 SSPVYEDAA (Y421) Src HTP;LTP;LTP
418 S Phosphoserine
421 Y Phosphotyrosine

PEPTIDE MASCOT Close
TQTPPVSPAPQPTEER
Start: 399 End: 414
Rank: 1 Matched 1
mrCalc: 1935.84 delta -0.07
observed: 968.8907 charge 2
Experiment: RFI Diplomdaten IMAC Hela
DatFile: ../data/20050721/F047444.dat
Mascot

PEPTIDE Close
18 RLPSSPVYE
SwissProt ID: Q14247
Source (PubMed ID): 10537323
Kinase: MAP2K LTP
PhosphoELM (update Mar 2005)

ANNOTATION Close
SwissProt Annotation:
MOD_RES
Phosphotyrosine

PEPTIDES SCANSITE Close
YPAEDSTYDEYENDL
Start: 479 End: 493
Site: Y486
Motif Lck_Kin
Score: 0.4157 ZScore: -3.108
ScanSite Prediction

466 TEAVYESAE (Y470) Src LTP
478 HYPAEDSTYDEYEN (T485) Casn_Kin2
479 YPAEDSTYDEYEND (Y486) Lck_Kin
479 YPAEDSTYDEYEND (Y486) Fgr_Kin
479 YPAEDSTYDEYEND (Y486) Src_Kin

AEDSTYDEYENDLGYTAVALYDYQAAGDDEISFDPDDIITNIEMIDDGWWRGVCKGRYGLFPANYVELRQ
482 EDSTYDEYE (Y486) Src LTP
492 DLGYTAVALYDYQAAGDDEISFDPDDIITNIEMIDDGWWRGVCKGRYGLFPANYVELRQ SH3

2. Systematic access on kinases

Affinity Purification of Protein Kinases

Several small molecule kinase inhibitors exhibited a low substrate specificity

Immobilized inhibitor:	No. fished Kinases
Purvalanol	64
Pyridopyrimidine	61
Iressa	25
Imidoimidazol	23
Bisindolylmaleimides	11
Tarceva	10
SU 6668	9

A total of: 127 Protein Kinases (25% of the human kinome)

Mapping of Protein Kinases by affinity chroma-tography based on small kinase inhibitors

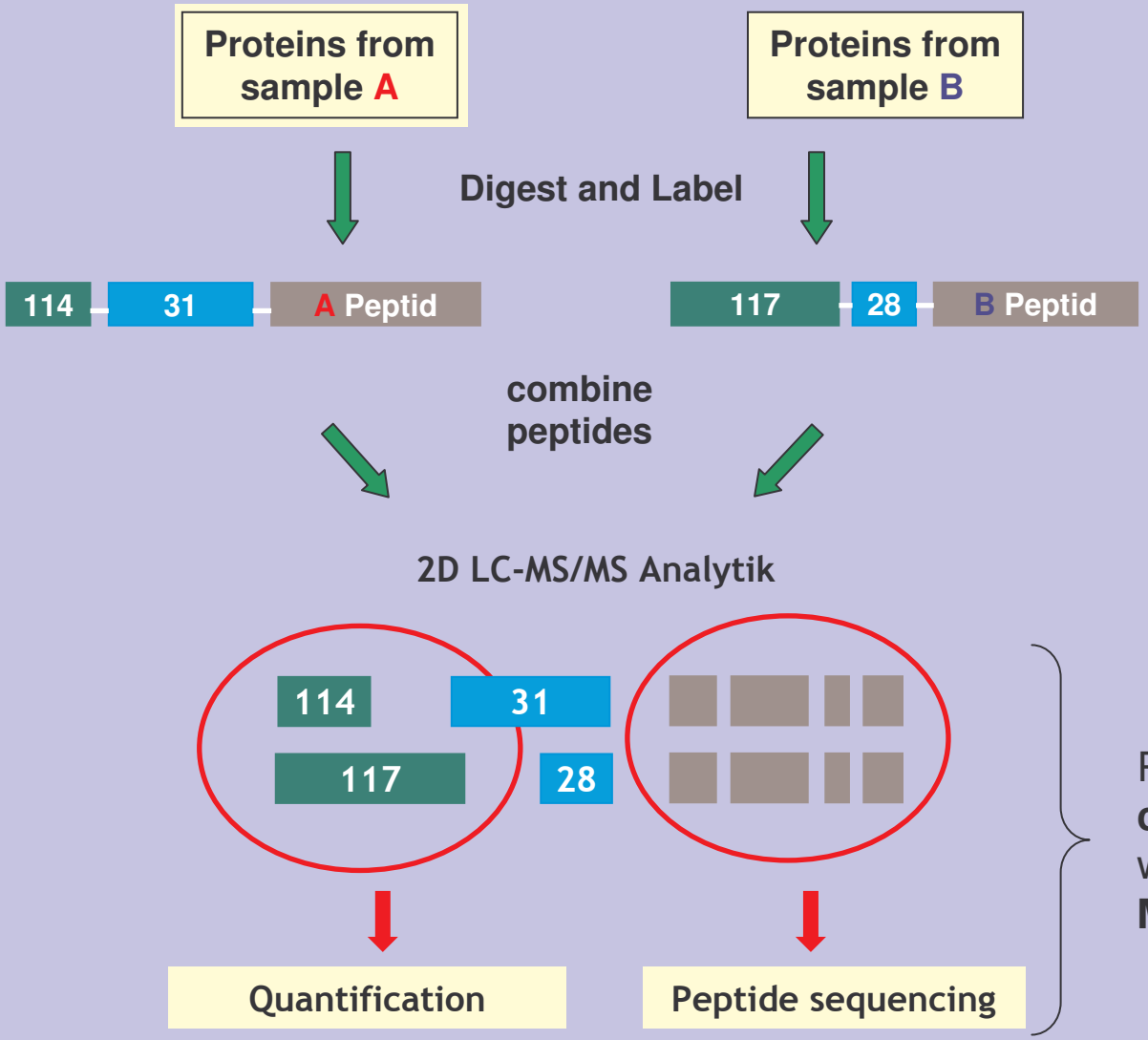
MATRIX SCIENCE Mascot Search Results

User : Mascot Daemon
Email :
Search title : FileName: <file_name>; FileText: <file_text>; Sample Group: <sample_group>; User: <user>
MS data file : \\Zibsrv34\common\Lc-ms_01\pkl_Dateien\Itraq Validierung\Z0883_1_1_1_1.pkl
Database : UniProt sprot (185639 sequences; 67143207 residues)
Taxonomy : Mammalia (mammals) (36615 sequences)
Timestamp : 2 Oct 2005 at 08:29:26 GMT
Significant hits:

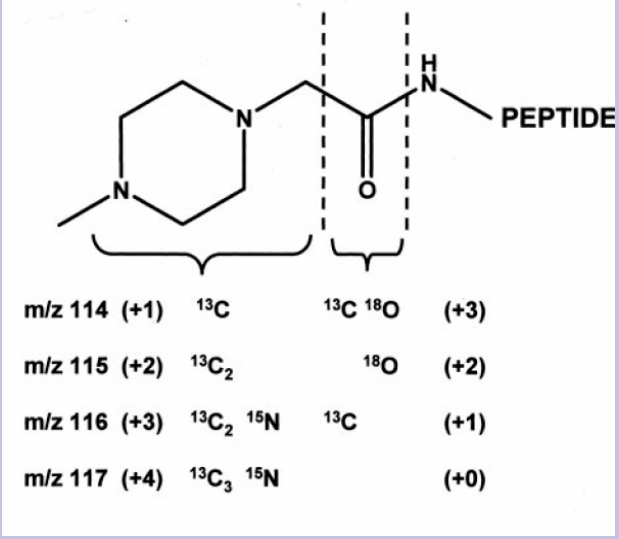
CSK_HUMAN	(P41240)	Tyrosine-protein kinase CSK (EC 2.7.1.112) (C-SRC kinase) (Protein-tyrosine kinase CYL)
CDK2_HUMAN	(P24941)	Cell division protein kinase 2 (EC 2.7.1.37) (p33 protein kinase)
CDK2_RAT	(Q63699)	Cell division protein kinase 2 (EC 2.7.1.37)
PDXK_HUMAN	(O00764)	Pyridoxal kinase (EC 2.7.1.35) (Pyridoxine kinase)
CDC2_HUMAN	(P06493)	Cell division control protein 2 homolog (EC 2.7.1.37) (p34 protein kinase) (Cyclin-depende
MK01_MOUSE	(P63085)	Mitogen-activated protein kinase 1 (EC 2.7.1.37) (Extracellular signal-regulated kinase 2)
MK03_HUMAN	(P27361)	Mitogen-activated protein kinase 3 (EC 2.7.1.37) (Extracellular signal-regulated kinase 1)
KS6A1_HUMAN	(Q15418)	Ribosomal protein S6 kinase alpha 1 (EC 2.7.1.37) (S6K-alpha 1) (90 kDa ribosomal protein
KS6A3_HUMAN	(P51812)	Ribosomal protein S6 kinase alpha 3 (EC 2.7.1.37) (S6K-alpha 3) (90 kDa ribosomal protein
CDK6_HUMAN	(Q00534)	Cell division protein kinase 6 (EC 2.7.1.37) (Serine/threonine-protein kinase PLSTIRE)
KC1A_HUMAN	(P48729)	Casein kinase I, alpha isoform (EC 2.7.1.-) (CKI-alpha) (CK1)
LCK_HUMAN	(P06239)	Proto-oncogene tyrosine-protein kinase LCK (EC 2.7.1.112) (P56-LCK) (LSK) (T cell-specific
KCC2G_HUMAN	(Q13555)	Calcium/calmodulin-dependent protein kinase type II gamma chain (EC 2.7.1.123) (CaM-kinase
CDK5_BOVIN	(Q02399)	Cell division protein kinase 5 (EC 2.7.1.37) (Tau protein kinase II catalytic subunit) (TP
AAKG1_HUMAN	(P54619)	5'-AMP-activated protein kinase, gamma-1 subunit (AMPK gamma-1 chain) (AMPKg)
AAKG1_BOVIN	(P58108)	5'-AMP-activated protein kinase, gamma-1 subunit (AMPK gamma-1 chain) (AMPKg)
KS6A2_HUMAN	(Q15349)	Ribosomal protein S6 kinase alpha 2 (EC 2.7.1.37) (S6K-alpha 2) (90 kDa ribosomal protein
MK09_HUMAN	(P45984)	Mitogen-activated protein kinase 9 (EC 2.7.1.37) (Stress-activated protein kinase JNK2) (c
KCC4_HUMAN	(Q16566)	Calcium/calmodulin-dependent protein kinase type IV (EC 2.7.1.123) (CAM kinase-GR) (CaMK I
PAK4_HUMAN	(O96013)	Serine/threonine-protein kinase PAK 4 (EC 2.7.1.37) (p21-activated kinase 4) (PAK-4)
KC1D_HUMAN	(P48730)	Casein kinase I, delta isoform (EC 2.7.1.-) (CKI-delta) (CKId)
M4K1_HUMAN	(Q92918)	Mitogen-activated protein kinase kinase kinase kinase 1 (EC 2.7.1.37) (MAPK/ERK kinase kin
MP2K2_HUMAN	(P36507)	Dual specificity mitogen-activated protein kinase kinase 2 (EC 2.7.1.-) (MAP kinase kinase
STK6_HUMAN	(O14965)	Serine/threonine-protein kinase 6 (EC 2.7.1.37) (Serine/threonine kinase 15) (Aurora/IPL1-
MP2K1_CRIGR	(Q63980)	Dual specificity mitogen-activated protein kinase kinase 1 (EC 2.7.1.-) (MAP kinase kinase
NEK9_HUMAN	(Q8TD19)	Serine/threonine-protein kinase Nek9 (EC 2.7.1.37) (NimA-related protein kinase 9) (Nercc1
GSK3B_HUMAN	(P49841)	Glycogen synthase kinase-3 beta (EC 2.7.1.37) (GSK-3 beta)
KC1G1_HUMAN	(Q9HCP0)	Casein kinase I, gamma 1 isoform (EC 2.7.1.-) (CKI-gamma 1)
AAPK1_HUMAN	(Q13131)	5'-AMP-activated protein kinase, catalytic alpha-1 chain (EC 2.7.1.-) (AMPK alpha-1 chain)
HCK_MACFA	(Q95M30)	Tyrosine-protein kinase HCK (EC 2.7.1.112) (p56-HCK) (Hemopoietic cell kinase)
MP2K2_MOUSE	(Q63932)	Dual specificity mitogen-activated protein kinase kinase 2 (EC 2.7.1.-) (MAP kinase kinase
CDK9_HUMAN	(P50750)	Cell division protein kinase 9 (EC 2.7.1.37) (Cyclin-dependent kinase 9) (Serine/threonine
AAKB1_HUMAN	(Q9Y478)	5'-AMP-activated protein kinase, beta-1 subunit (AMPK beta-1 chain) (AMPKb)
CDK7_HUMAN	(P50613)	Cell division protein kinase 7 (EC 2.7.1.37) (CDK-activating kinase) (CAK) (TFIIH basal tr
KPCT_HUMAN	(Q04759)	Protein kinase C, theta type (EC 2.7.1.-) (nPKC-theta)
SRC_MOUSE	(P05480)	Neuronal proto-oncogene tyrosine-protein kinase SRC (EC 2.7.1.112) (p60-SRC) (C-SRC)
GAK_HUMAN	(O14976)	Cyclin G-associated kinase (EC 2.7.1.-)
CCNB1_HUMAN	(P14635)	G2/mitotic-specific cyclin B1
SRC_HUMAN	(P12931)	Proto-oncogene tyrosine-protein kinase Src (EC 2.7.1.112) (p60-Src) (c-Src)
MAT1_HUMAN	(P51948)	CDK-activating kinase assembly factor MAT1 (RING finger protein MAT1) (Menage a trois) (CD

3. Relative and absolute quantification of single peptides.

Quantification of Peptides by iTRAQ™

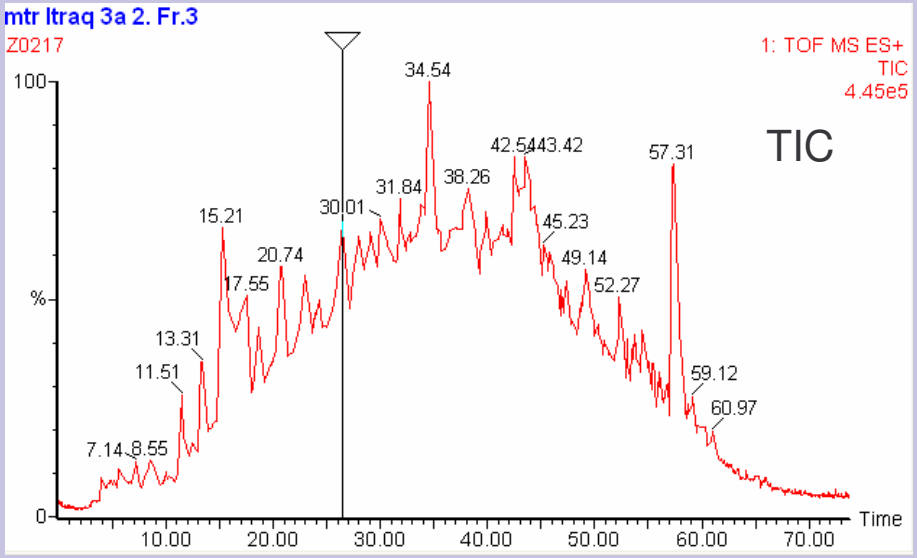


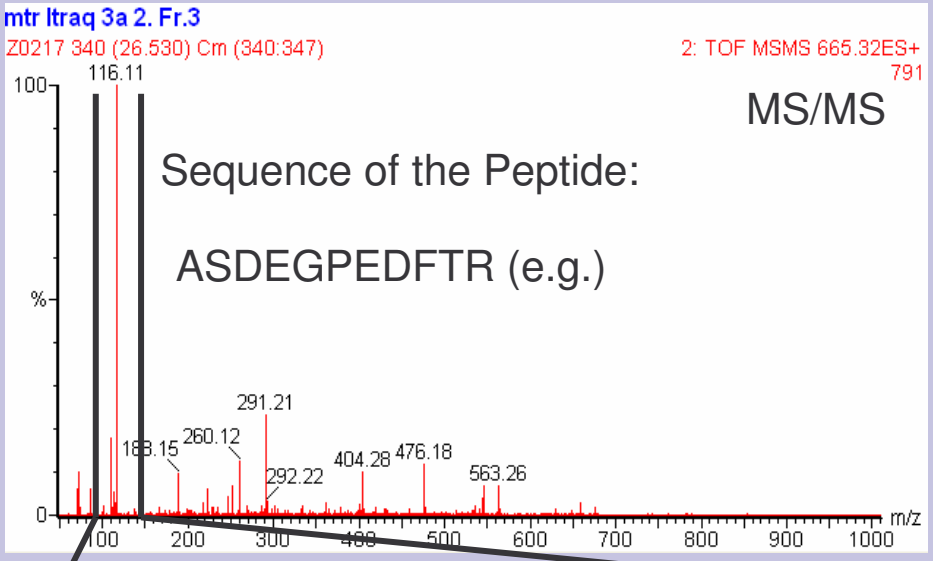
(taken from Ross *et al.*, MCP, 2004)

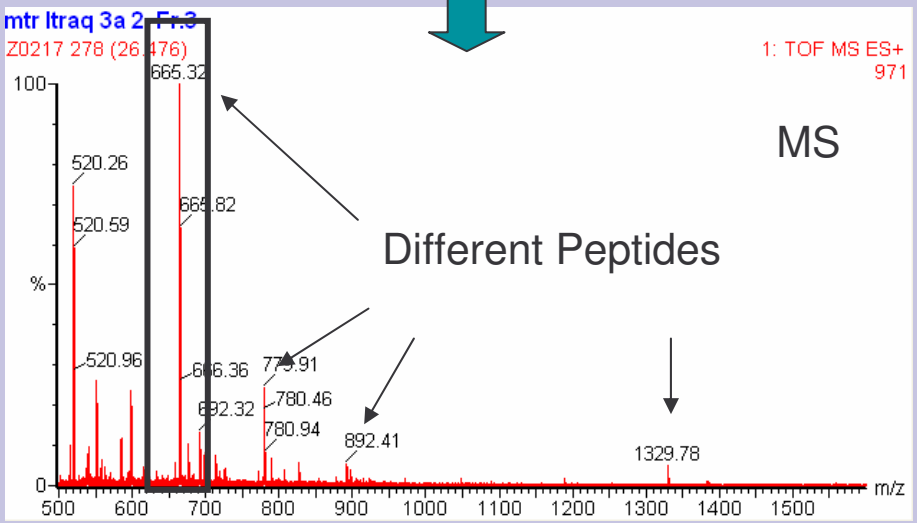


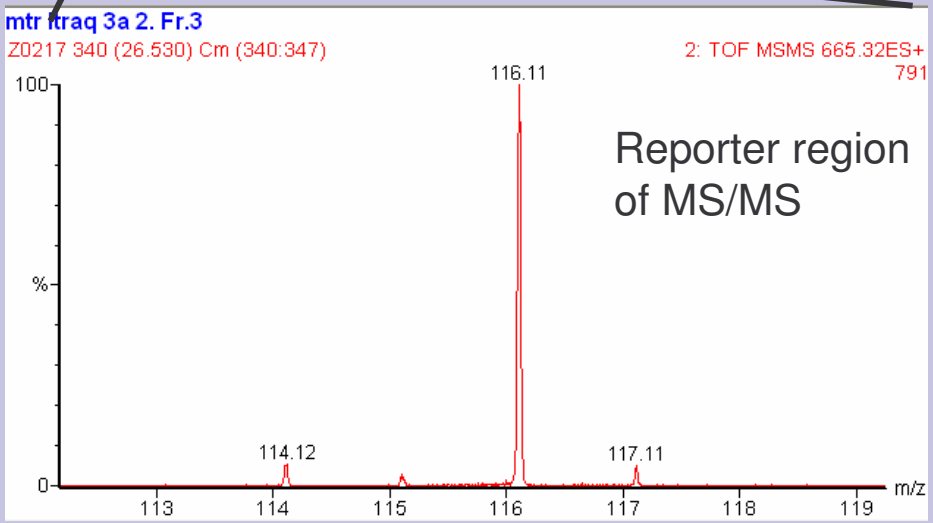
Peptides with the **same sequence co-elute** at the same time and were Analyses in the same **MS/MS experiment**

Quantification of Peptides by iTRAQ™

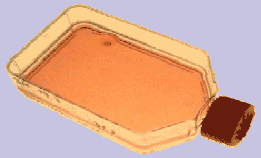








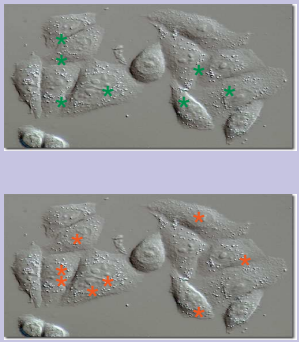
Relative quantification of phosphorylated peptides



HeLa cells

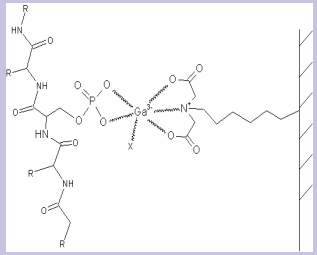
+HGF

+InlB

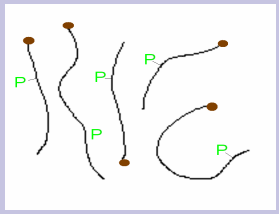


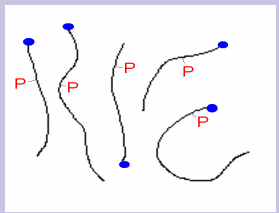
Induced phosphorylations

- -Cell-Lysis
- -Protein extraction
- -Proteolysis
- -Methylation



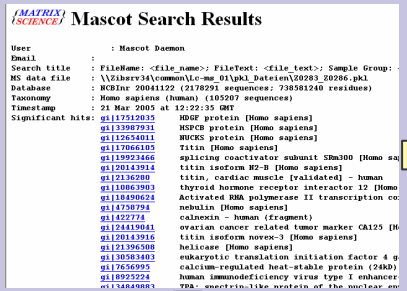
Immobilized **M**etal **A**ffinity **C**hromatography (**IMAC**)

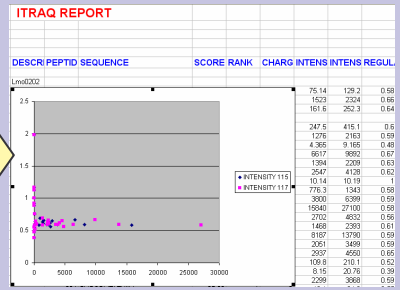


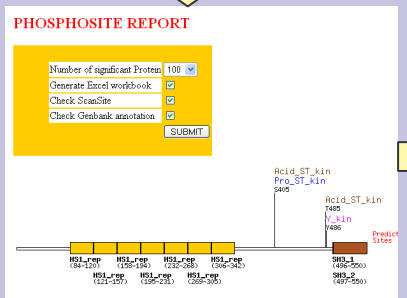


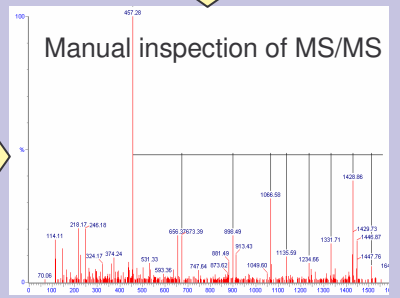
Differential labeling of phosphopeptides with **iTRAQ™**

Nano-HPLC-Q-TOF MS/MS

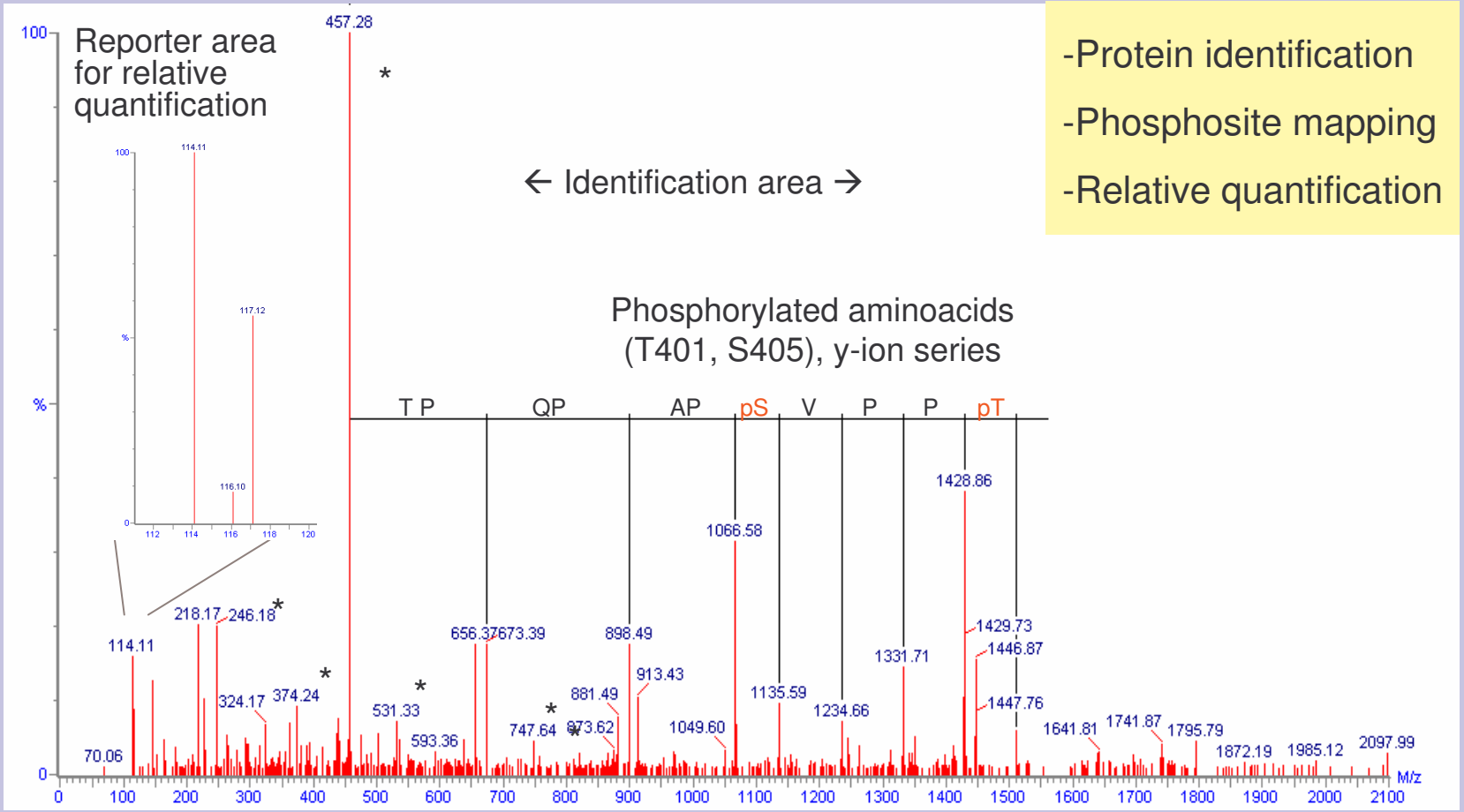








iTRAQ link peptide sequencing (MS/MS) with the process of relative phosphopeptide quantification



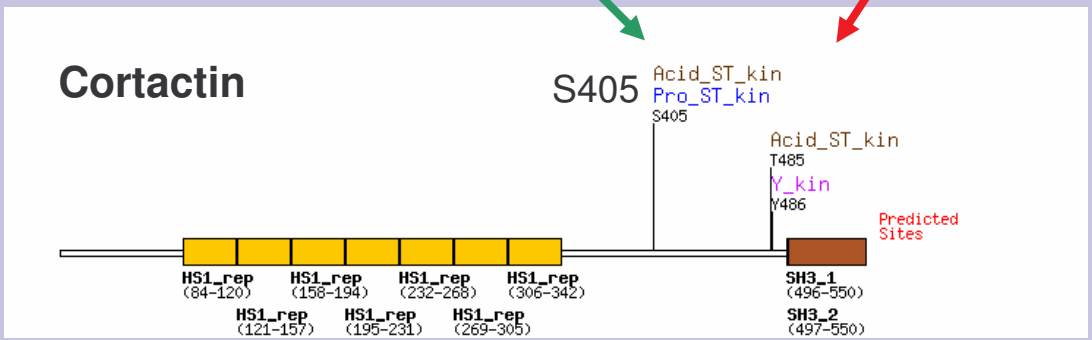
-Protein identification

-Phosphosite mapping

-Relative quantification

Cortactin: Regulation of a Erk1 dependent phosphosite after InlB and HGF induction

Erk1 Src

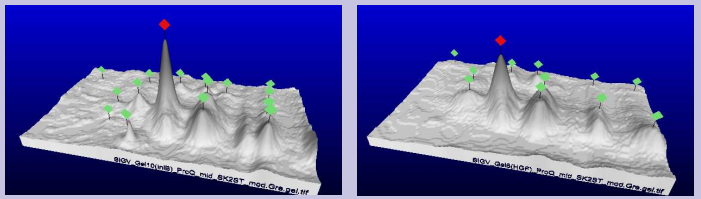


N-WASP

Arp2/3

T401/S405 phosphorylation:

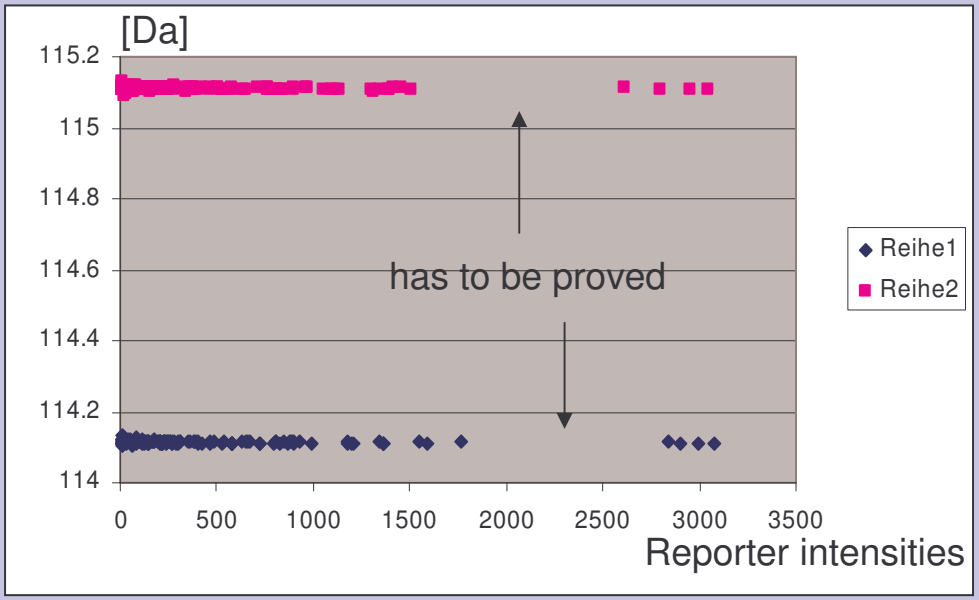
total phosphorylation:



Martinez-Quiles et al., Molecular and Cellular Biology, 2004

Dynamic of quantification signals

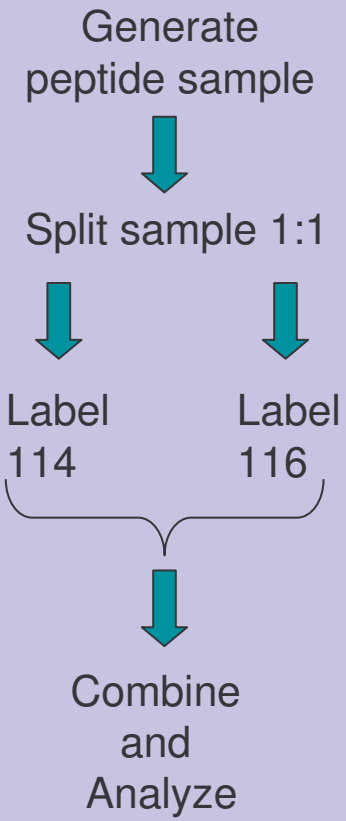
Reporter intensities the iTRAQ channels 114.1 & 115.1 Da

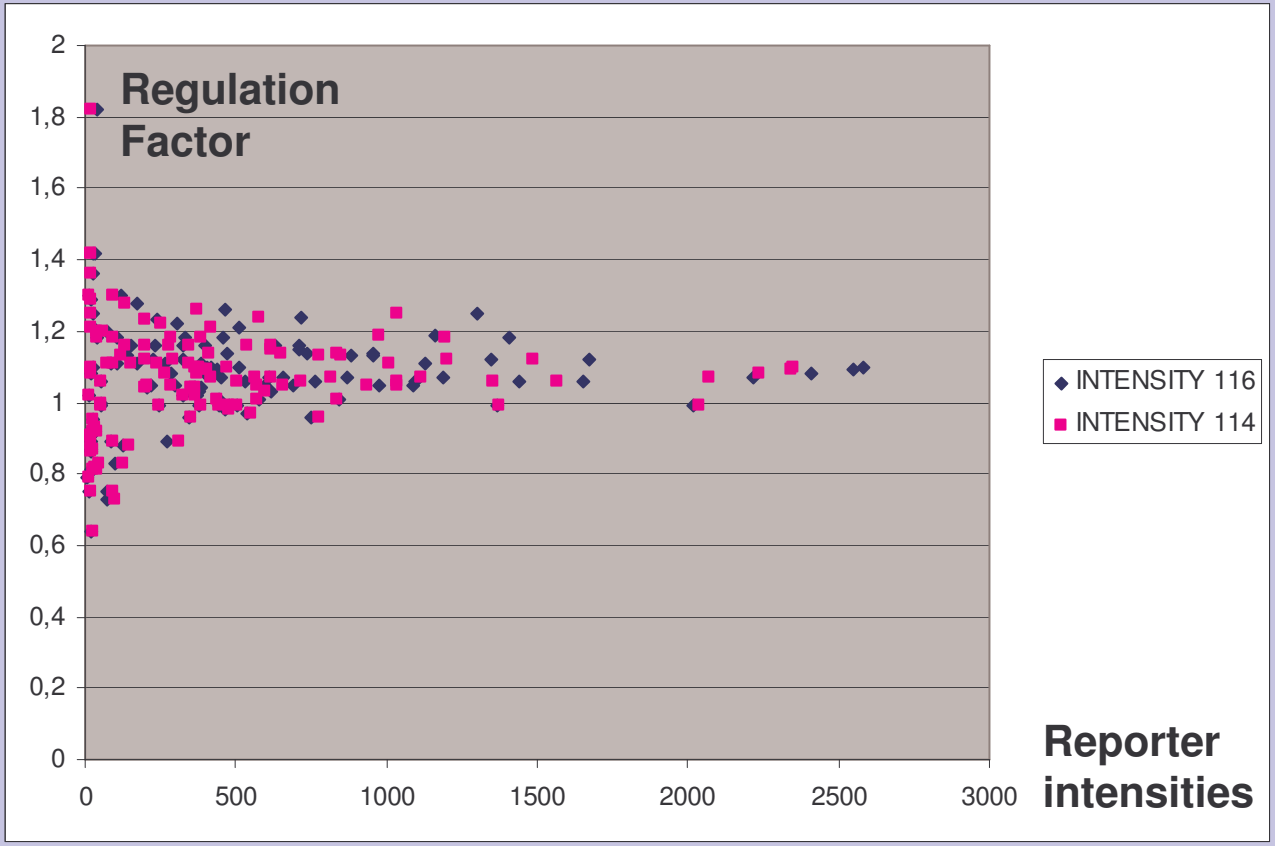


Most intense reporters are:

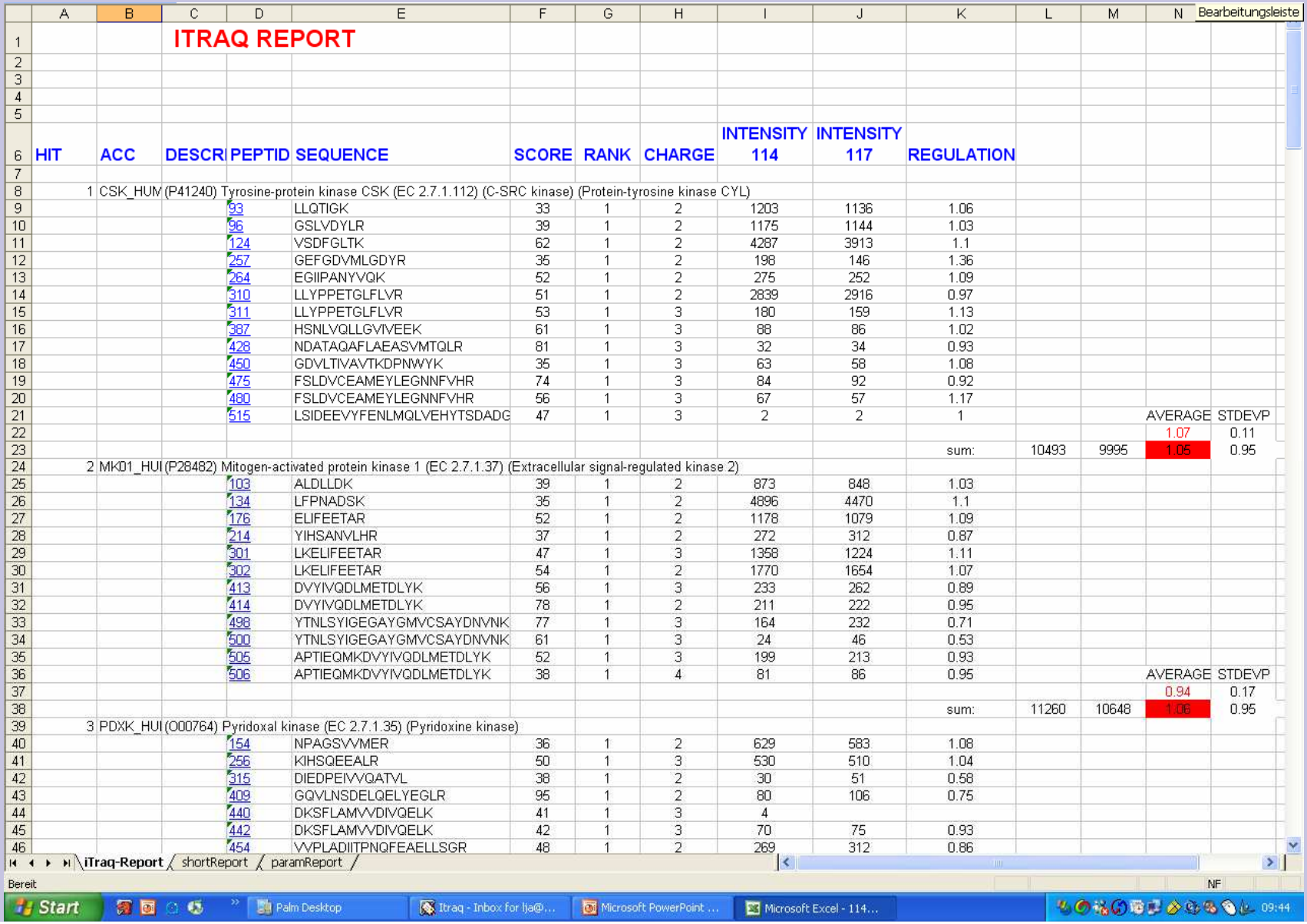
- Tryptic peptides ending on K (harboring two labels)
- Short peptides (due to ion suppression in longer peptides)
- has to be specified...

Deviation of regulatory data





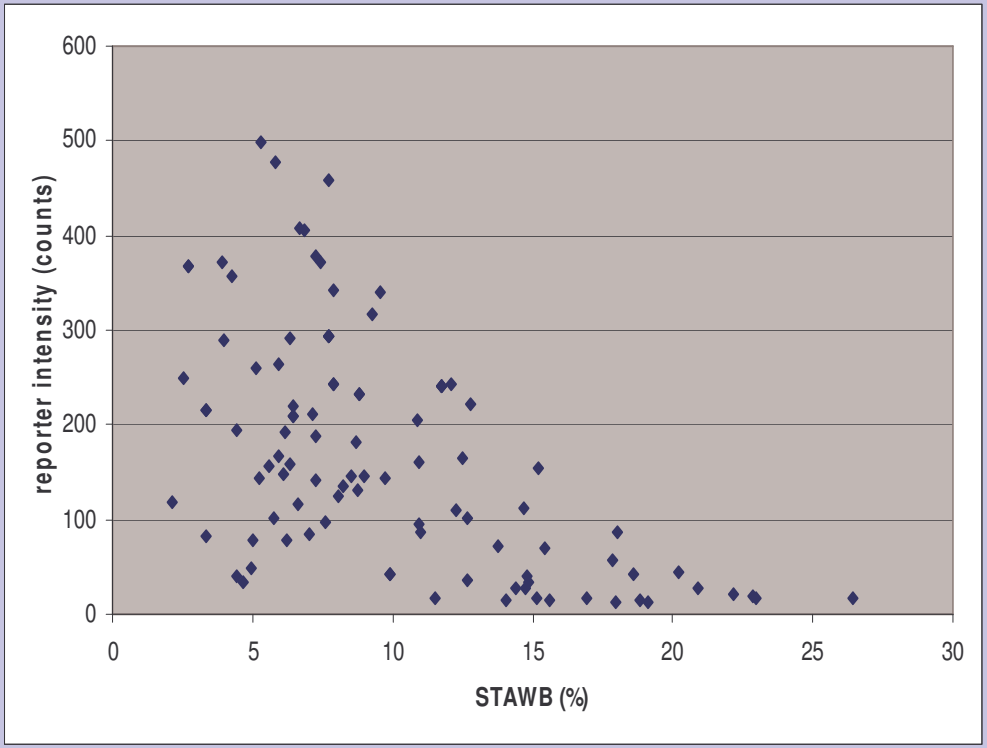
Quantification of kinase fractions by iTRAQ



ITRAQ REPORT

HIT	ACC	DESCR	PEPTID	SEQUENCE	SCORE	RANK	CHARGE	INTENSITY 114	INTENSITY 117	REGULATION				
1	CSK_HUM	(P41240) Tyrosine-protein kinase CSK (EC 2.7.1.112) (C-SRC kinase) (Protein-tyrosine kinase CYL)												
			93	LLQTIGK	33	1	2	1203	1136	1.06				
			96	GSLVDYLR	39	1	2	1175	1144	1.03				
			124	VSDFGLTK	62	1	2	4287	3913	1.1				
			257	GEFGDVMLGDYR	35	1	2	198	146	1.36				
			264	EGIIPANYVQK	52	1	2	275	252	1.09				
			310	LLYPPETGLFLVR	51	1	2	2839	2916	0.97				
			311	LLYPPETGLFLVR	53	1	3	180	159	1.13				
			387	HSNLVQLLGVIVEEK	61	1	3	88	86	1.02				
			428	NDATAQAFLAEASVMTQLR	81	1	3	32	34	0.93				
			450	GDVLTIVAVTKDPNWYK	35	1	3	63	58	1.08				
			475	FSLDVCEAMEYLEGNNFVHR	74	1	3	84	92	0.92				
			480	FSLDVCEAMEYLEGNNFVHR	56	1	3	67	57	1.17				
			515	LSIDEEVYFENLMQLVEHYTSDADG	47	1	3	2	2	1			AVERAGE	STDEVP
													1.07	0.11
										sum:	10493	9995	1.05	0.95
2	MK01_HUI	(P28482) Mitogen-activated protein kinase 1 (EC 2.7.1.37) (Extracellular signal-regulated kinase 2)												
			103	ALDLLDK	39	1	2	873	848	1.03				
			134	LFPNADSK	35	1	2	4896	4470	1.1				
			176	ELIFEETAR	52	1	2	1178	1079	1.09				
			214	YIHSANVLHR	37	1	2	272	312	0.87				
			301	LKELIFEETAR	47	1	3	1358	1224	1.11				
			302	LKELIFEETAR	54	1	2	1770	1654	1.07				
			413	DVYIVQDLMETDLYK	56	1	3	233	262	0.89				
			414	DVYIVQDLMETDLYK	78	1	2	211	222	0.95				
			498	YTNLSYIGEGAYGMVCSAYDNVNK	77	1	3	164	232	0.71				
			500	YTNLSYIGEGAYGMVCSAYDNVNK	61	1	3	24	46	0.53				
			505	APIEQMKDVYIVQDLMETDLYK	52	1	3	199	213	0.93				
			506	APIEQMKDVYIVQDLMETDLYK	38	1	4	81	86	0.95			AVERAGE	STDEVP
													0.94	0.17
										sum:	11260	10648	1.06	0.95
3	PDXK_HUI	(O00764) Pyridoxal kinase (EC 2.7.1.35) (Pyridoxine kinase)												
			154	NPAGSVVMER	36	1	2	629	583	1.08				
			256	KIHSQEEALR	50	1	3	530	510	1.04				
			315	DIEDPEIVVQATVL	38	1	2	30	51	0.58				
			409	GQVLNSDELQELYEGLR	95	1	2	80	106	0.75				
			440	DKSFLAMVVDIVQELK	41	1	3	4						
			442	DKSFLAMVVDIVQELK	42	1	3	70	75	0.93				
			454	VVPLADIITPNQFEAELLSGR	48	1	2	269	312	0.86				

Deviation of regulatory data (*

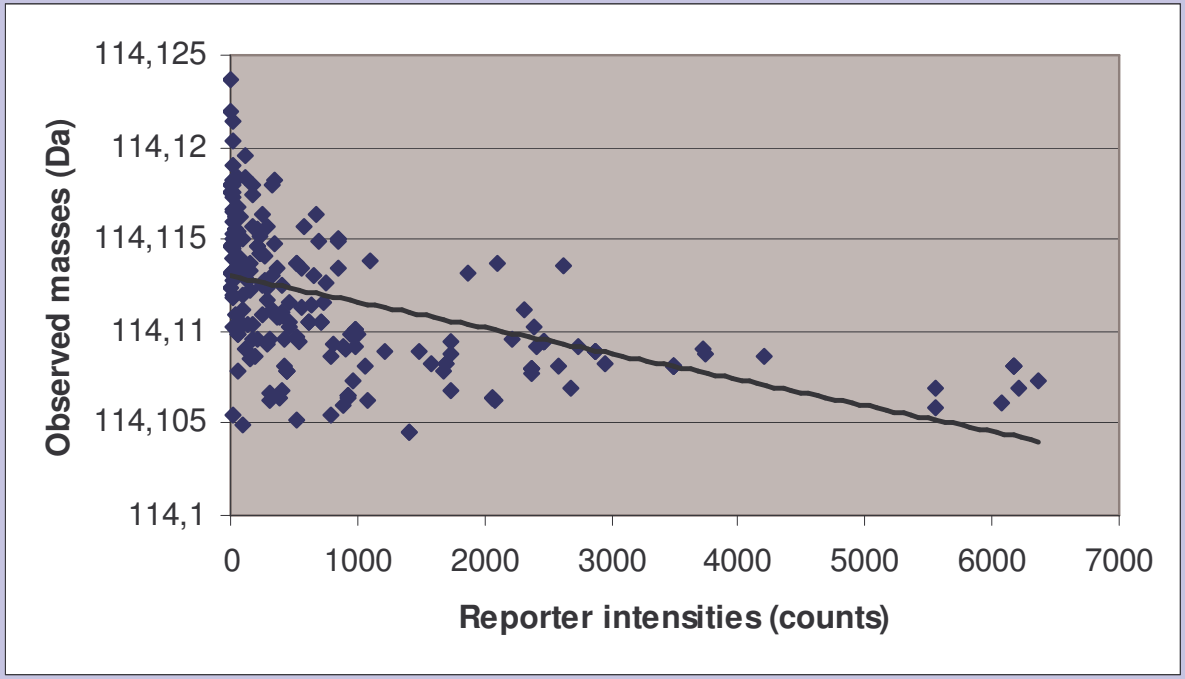


Reporter-intensity:	Deviation:
< 50	up to 30%
< 100	up to 18%
< 200	up to 13%
< 300	up to 10%
< 400	up to 7%
> 500	≈ 5%

* : Data certainly contain false-positive reporter signals and final deviations will be significantly lower in well defined workflows

Characterization of quantification signals

iTRAQ reagent 114:



- Mass delta of the reporter masses also depend on their intensities
- Maximum mass delta of ± 0.01 Da has to be considered

Specificity of the reporter channels 114.1, 115.1, 116.1 and 117.1 Da

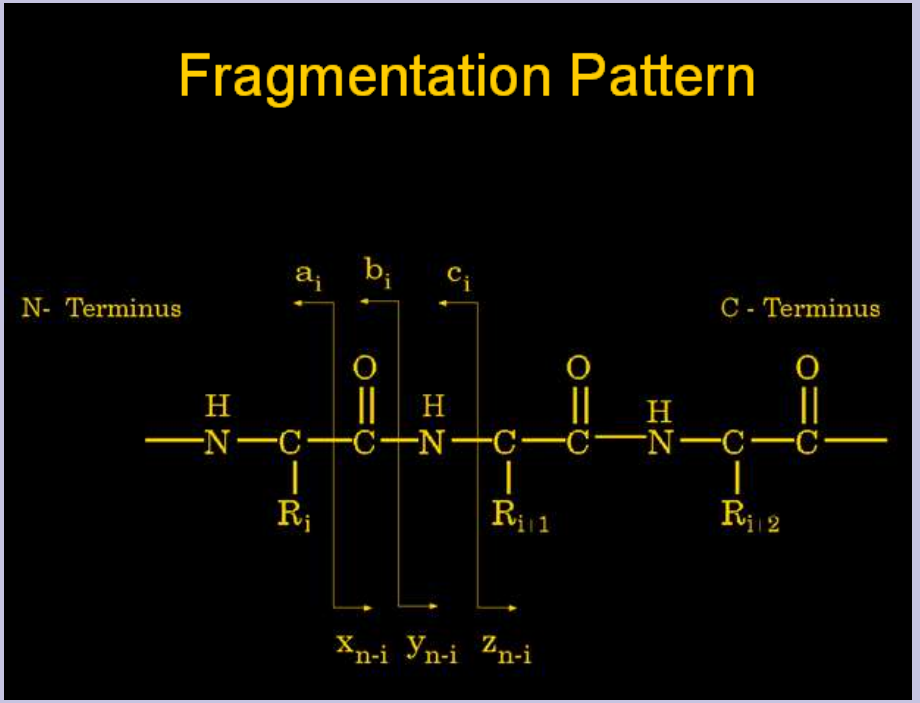
PTM by Ornithyl:
Monoisotopic Mass Change:114.079

AATPQPVTMFR Alanine **a-ion** = 115,09 Da

Proline at the C-terminus of the protein (Lmo1388) **y-ion** =116.063 Da

C-terminus **Asparatic Acid**
z-ion = 117.04 Da

Amide of Valine
y-ion = 117.09 Da



Specificity of the reporter channels (only 117.1 Da reagent used)

SEQUENCE	SCORE	RANK	CHARGE	INTENSITY 114.1	INTENSITY 115.1	INTENSITY 116.1	INTENSITY 117.1	MASS 114.1	MASS 115.1	MASS 116.1	MASS 117.1
TVLGK	18.29	1	2	0	0	13.4	595.6	0.00	0.00	116.09	117.10
LTELK	17.02	1	2	0	0	37.17	1418	0.00	0.00	116.11	117.10
AGYTFK	24.49	1	2	0	0	16.33	443.7	0.00	0.00	116.10	117.10
GWYDAK	20.66	1	2	0	0	0	59.14	0.00	0.00	0.00	117.10
EGYTFK	15.19	1	2	2.141	0	16.21	407.2	114.11	0.00	116.10	117.10
LDISSNK	42.68	1	2	0	0	91.22	1848	0.00	0.00	116.11	117.10
GWYDEK	16.16	1	2	0	0	0	16.15	0.00	0.00	0.00	117.11
WDFATSK	37.72	1	2	0	2.104	10.38	207.7	0.00	115.10	116.11	117.10
WDFATDK	38.2	1	2	0	0	9.193	455.3	0.00	0.00	116.10	117.10
VSDISVLAK	8.84	1	2	0							
VSDISVLAK	52.38	1	2	5.175	11						
LFFYNNK	52.02	1	2	0							
ANVSIPNTVK	24.72	1	3	0	0	0	7.234	0.00	0.00	0.00	117.10
ANVSIPNTVK	24.41	1	2	0	0	0	4.129	0.00	0.00	0.00	117.10
KWDFATDK	5.07	1	2	0	0	0	9.431	0.00	0.00	0.00	117.10
STTQAVDYQGLLK	38.18	1	2	0	0	0	18.48	0.00	0.00	0.00	117.10
STTQAVDYQGLLK	30.54	1	3	0	0	0	9.236	0.00	0.00	0.00	117.09
GTTTFSGTVTQPLK	45.07	1	2	0	0	0	31.26	0.00	0.00	0.00	117.11
GTTTFSGTVTQPLK	18.06	1	3	2.209	0	0	24.27	114.07	0.00	0.00	117.11
TGGDKWDFATSK	11.92	1	2	0	0	0	13.33	0.00	0.00	0.00	117.10
TGGDKWDFATSK	22.25	1	3	0	5.13	26.59	537.7	0.00	115.10	116.10	117.10
LDISSNKVSDISVLAK	49.19	1	3	0	0	0	28.4	0.00	0.00	0.00	117.10
EGHTFVGWFDAQTGGTK	27.47	1	3	0	0	2.104	7.104	0.00	0.00	116.12	117.10
EVEAGNLLTEPAKPVK	34.6	1	3	0	0	0	23.28	0.00	0.00	0.00	117.10
STTQAVDYQGLLKEPK	50.56	1	3	0				0.00	0.00	116.13	117.10
ITQLGLNDQAWTNAPVNYK	51.47	1	2						0.00	0.00	117.10
TNVTDTVSQTDLDQVTTLQADR	99.37	1	3						0.00	0.00	117.11
TNVTDTVSQTDLDQVTTLQADR	95.1	1	2						0.00	116.12	117.11
NLTYLTLYFNNISDISPVSSLTK	57.98	1	3	0	1.104	0	8.095	0.00	115.10	0.00	117.10
SIDGVEYLNNLTQINFSNNQLTDITPLK	82.99	1	3	0	0	8.225	103.5	0.00	0.00	116.11	117.10
SIDGVEYLNNLTQINFSNNQLTDITPLK	29.02	1	4	0	0	0	0	0.00	0.00	0.00	0.00
SIDGVEYLNNLTQINFSNNQLTDITPLK	32.01	1	3	0	1.104	0	57.12	0.00	115.06	0.00	117.11
SIDGVEYLNNLTQINFSNNQLTDITPLK	31.74	1	3	0	0	0	8.113	0.00	0.00	0.00	117.11
IGTLASLTNLTDLDLANNQISNLAPLSGLT	58.15	1	3	0	0	0	0	0.00	0.00	0.00	0.00
IGTLASLTNLTDLDLANNQISNLAPLSGLT	30.28	1	4	0	0	0	7.621	0.00	0.00	0.00	117.11
IGTLASLTNLTDLDLANNQISNLAPLSGLT	91.9	1	3	0	0	4.172	56.67	0.00	0.00	116.11	117.11
IGTLASLTNLTDLDLANNQISNLAPLSGLT	20.11	1	4	0	0	0	2.104	0.00	0.00	0.00	117.10
VSSLANLTNINWLSAGHNQISDLTPLAN	25.69	1	4	0	0	0	3.156	0.00	0.00	0.00	117.10

Expected reporter intensities close by 117.10 Da

Unexpected reporter intensities

Dynamic range of iTRAQ

A kinase fraction was splited in 4 equal ratios, labeled individually with iTRAQ reagents (114, 115, 116, 117), mixed in variable ratios and were analyzed by LC-MS/MS

Expected ratios:

114	115	116	117
1	1	1	1
1	2	1	2
0	0	4	1
16	1	1	1

Found ratios (sum of 338 peptides):

114	115	116	117
1	0.96	1.03	0.95
1	1.79	1.11	1.82
0.005	0.25	3.47	1
17.8	2.12	1.42	1

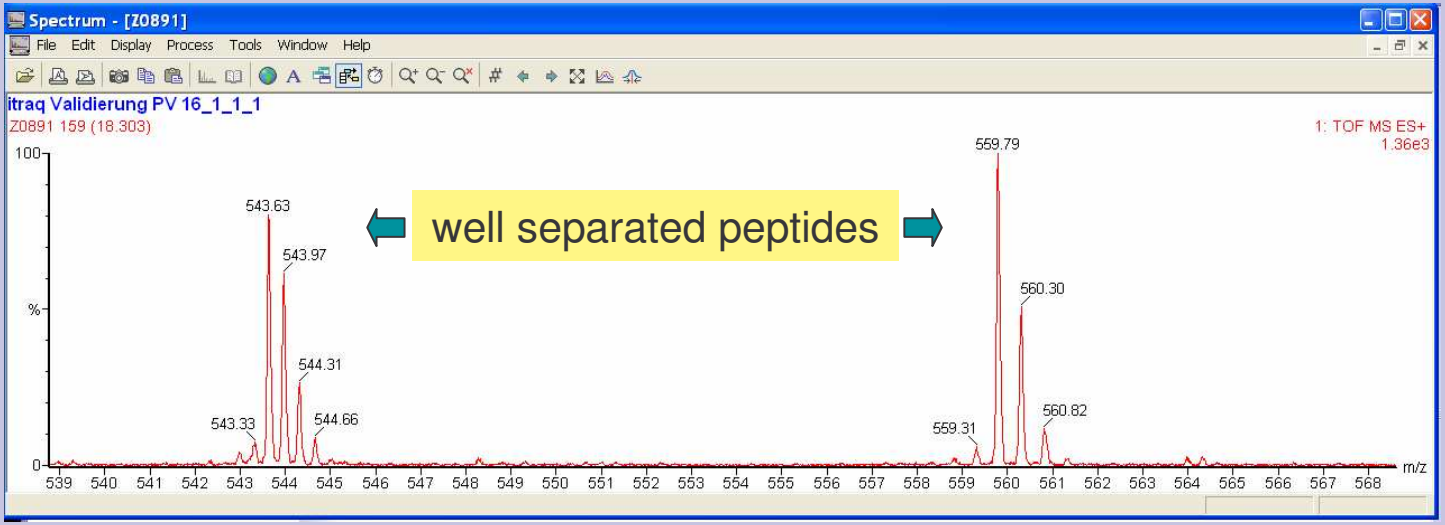
Dynamic range of iTRAQ

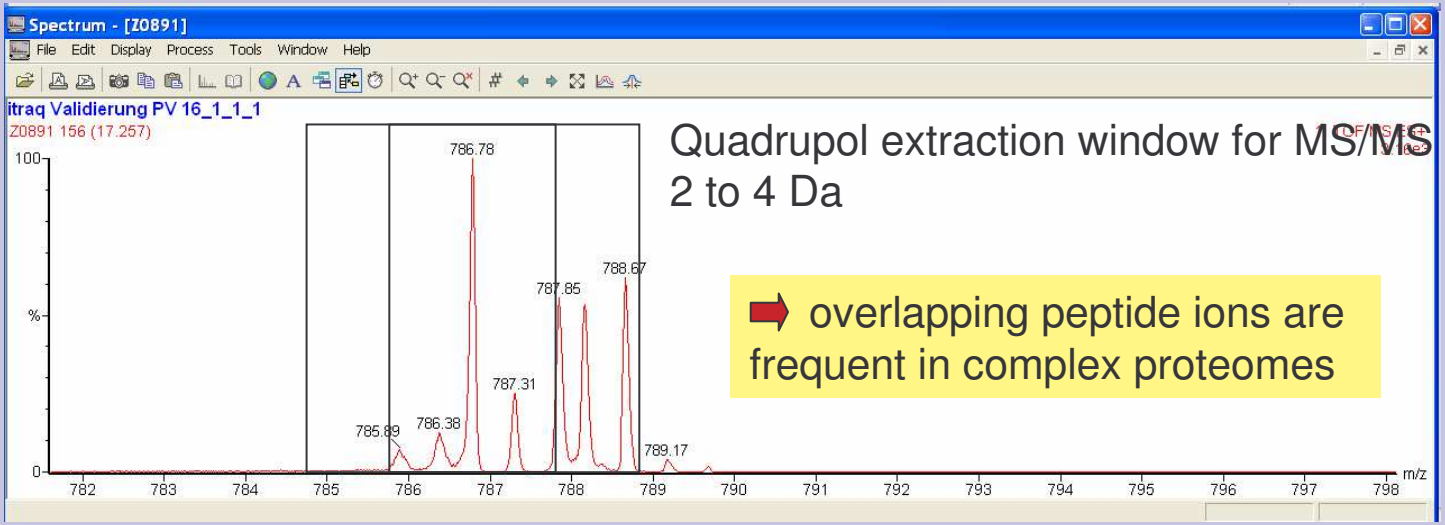
Observed ratios were caused predominantly by By-products of the iTRAQ reagents:

Reagent	% of -2	% of -1	% of +1	% of +2
iTRAQ™114			5.9	0.2
iTRAQ™115		2.0	5.6	0.1
iTRAQ™116	0.0	3.0	4.5	
iTRAQ™117	0.1	4.0		

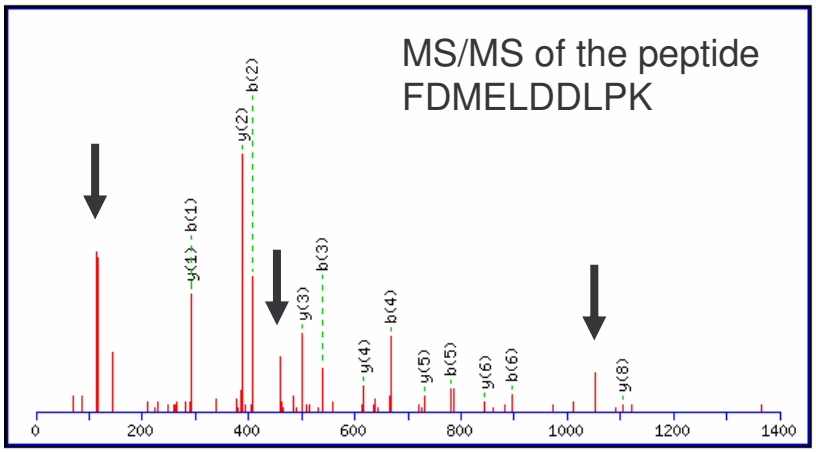
By-product certification taken from a typical product sheet (Applied Biosystems)

Specificity of the MS/MS analyses





Specificity of the MS/MS analyses



Unassigned fragmentation ions indicate to co-extracted peptides

Reporter intensities interfere with peptide sequencing

Monoisotopic mass of neutral peptide (Mr): 1509.76
Fixed modifications: iTRAQ (K),iTRAQ (N-term)
Ions Score: 45 **Matches (Bold Red):** 13/82 fragment ions using 26 most intense peaks

#	b	b^{++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y^{*}	y^{*++}	y^{0}	y^{0++}	#
1	292.18	146.59			F							10
2	407.21	204.11	389.19	195.10	D	1219.60	610.30	1202.57	601.79	1201.59	601.30	9
3	538.25	269.63	520.24	260.62	M	1104.57	552.79	1087.55	544.28	1086.56	543.79	8
4	667.29	334.15	649.28	325.14	E	973.53	487.27	956.51	478.76	955.52	478.27	7
5	780.37	390.69	762.36	381.68	L	844.49	422.75	827.46	414.24	826.48	413.74	6
6	895.40	448.20	877.39	439.20	D	731.41	366.21	714.38	357.69	713.40	357.20	5
7	1010.43	505.72	992.42	496.71	D	616.38	308.69	599.35	300.18	598.37	299.69	4
8	1123.51	562.26	1105.50	553.25	L	501.35	251.18	484.33	242.67			3
9	1220.56	610.79	1202.55	601.78	P	388.27	194.64	371.24	186.12			2
10					K	291.22	146.11	274.19	137.60			1

Fragment ion masses that can be predicted based on the peptide sequence. Experimentally observed masses are assigned in bold red

Systematic quantification by iTRAQ (Outlook)

1. Define the mass accuracy of the four reporter masses
2. Extract and delete the reporter intensities from the MS/MS spectra

Identify peptide sequences by MASCOT DB search

Reject those sequences for quantitative analyses that…

- exhibit a high ratio of un-assigned / assigned ions
- potentially produce contami-nating fragmentation ions

remaining

Recalculate reporter intensities according expected by-products

Annotate regulatory information to individual peptides

“Score” the reliability based on reporter intensities and mass accuracy

Acknowledgments

Dr. Josef Wissing

Kinome Analysis

Dr. Guido Dieterich

Bioinformatics

Tobias Reinl

InlB / HGF signaling

Roman Fischer

Phosphorylation site analysis
InlA / E-cadherin signaling

Prof. Jürgen Wehland,
and the Department of Cell Biology

Department of Structural Biology
Dr. Manfred Nimtz

MS-facility

Prof. Klawonn, FH Wolfenbüttel

Interpretation of Peptide Fragmentation pattern

Dr. Hendrik Daub, MPI Munich

Kinome mapping