Highly sensitive targeted quantification of ERK phosphorylation dynamics and stoichiometry without affinity enrichment

Tujin Shi[1], Tao Liu[1], Yuqian Gao[1], Matthew J. Gaffrey[1], Thomas L. Fillmore[2], Carrie D. Nicora[1], William B. Chrisler[1], Marina A. Gritsenko[1], Chaochao Wu[1], Jintang He[1], David G. Camp II[1], Karin D. Rodland[1], Richard D. Smith[1], Steven H. Wiley[2], Wei-Jun Qian[1]

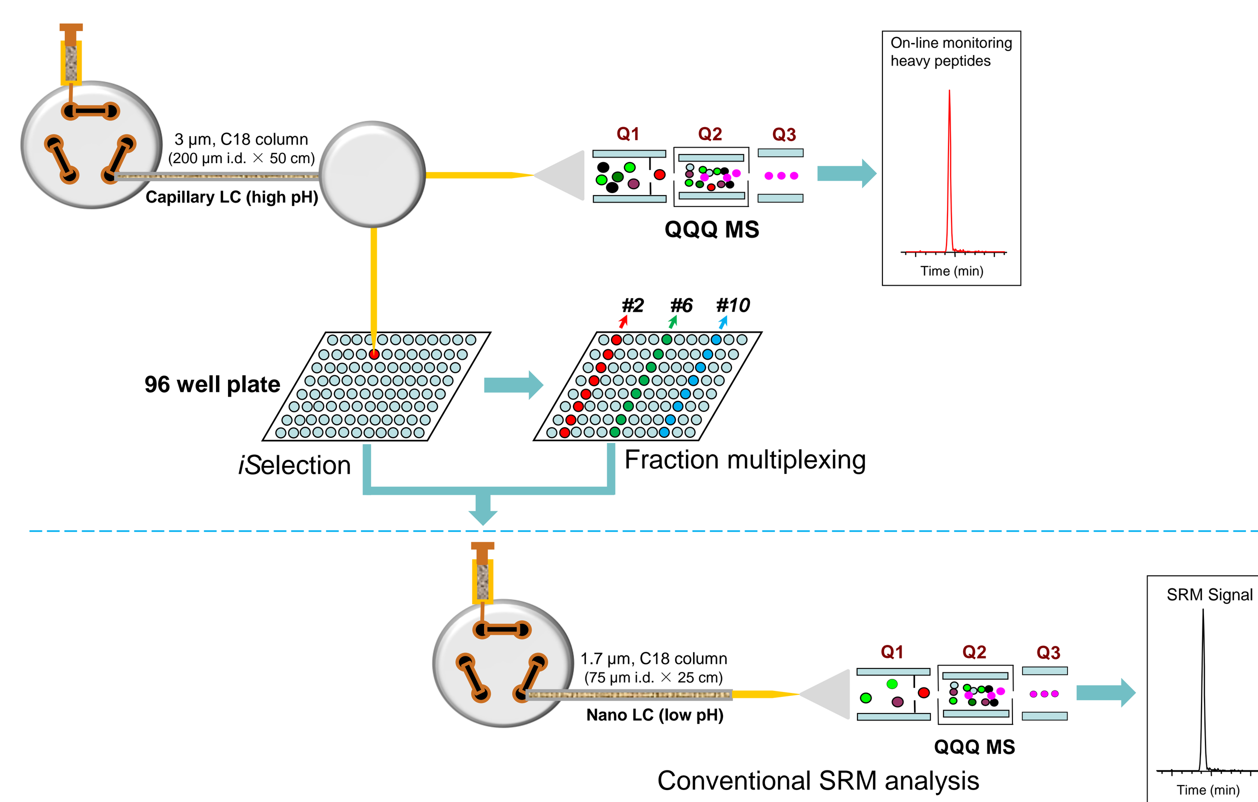
[1]Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA
[2]Environmental Molecular Sciences Laboratory, Pacific Northwest National Laboratory, Richland, WA

Introduction

- Selected reaction monitoring (SRM) coupled with stable isotope dilution is a promising technology for site-specific quantification of protein posttranslational modifications (PTMs). A major constraint is the limited sensitivity for quantifying substoichiometric PTMs in complex biological matrices.
- Sensitive SRM quantification of PTMs primarily relies on affinity enrichment. However, such affinity enrichment approaches often obscure the PTM stoichiometries, and are associated with sample loss, and normally require large amounts of starting material (e.g., ≥1 mg of whole cell lysate).
- To address this limitation, we applied an affinity reagent-independent targeted mass spectrometric capability, termed PRISM (high-Pressure high-Resolution Intelligent Selection and Multiplexing)-SRM, for achieving highly sensitive, accurate quantification of ERK phosphorylation dynamics with minute amounts of starting material (e.g., ~25 µg of cell lysate digests).

Methods

PRISM-SRM[1-4]



Surrogate peptides for ERK1/2 and three best transitions selected for each target peptide. Masses listed are the unlabeled forms.

Isoform	Standard sequence	SRM Transitions Q1	SRM Transitions Q3
ERK1	LADPEHDHTGFLTEYVATR	724.7	839.4 (y7$^+$), 738.4 (y6$^+$), 609.3 (y5$^+$)
	LADPEHDHTGFL*p*TEYVATR	751.3	718.7 ([precursor – 98]$^{3+}$), 821.4 ([y7 – 98]$^+$), 927.9 ([y16 – 98]$^{2+}$)
	LADPEHDHTGFLTE*p*YVATR	751.3	919.4 (y7$^+$), 689.3 (y5$^+$), 976.9 (y16^{2+})
	LADPEHDHTGFL*p*TE*p*YVATR	778.0	745.3 ([precursor – 98]$^{3+}$), 901.4 ([y7 – 98]$^+$), 967.9 ([y16 – 98]$^{2+}$)
ERK2	VADPDHDHTGFLTEYVATR	715.3	952.5 (y8$^+$), 839.4 (y7$^+$), 738.4 (y6$^+$)
	VADPDHDHTGFL*p*TEYVATR	742.0	709.3 ([precursor – 98]$^{3+}$), 934.5 ([y8 – 98]$^+$), 738.4 (y6$^+$)
	VADPDHDHTGFLTE*p*YVATR	742.0	919.4 (y7$^+$), 818.3 (y6$^+$), 689.3 (y5$^+$)
	VADPDHDHTGFL*p*TE*p*YVATR	768.7	736.0 ([precursor – 98]$^{3+}$), 901.4 ([y7 – 98]$^+$), 446.3 (y4$^+$)

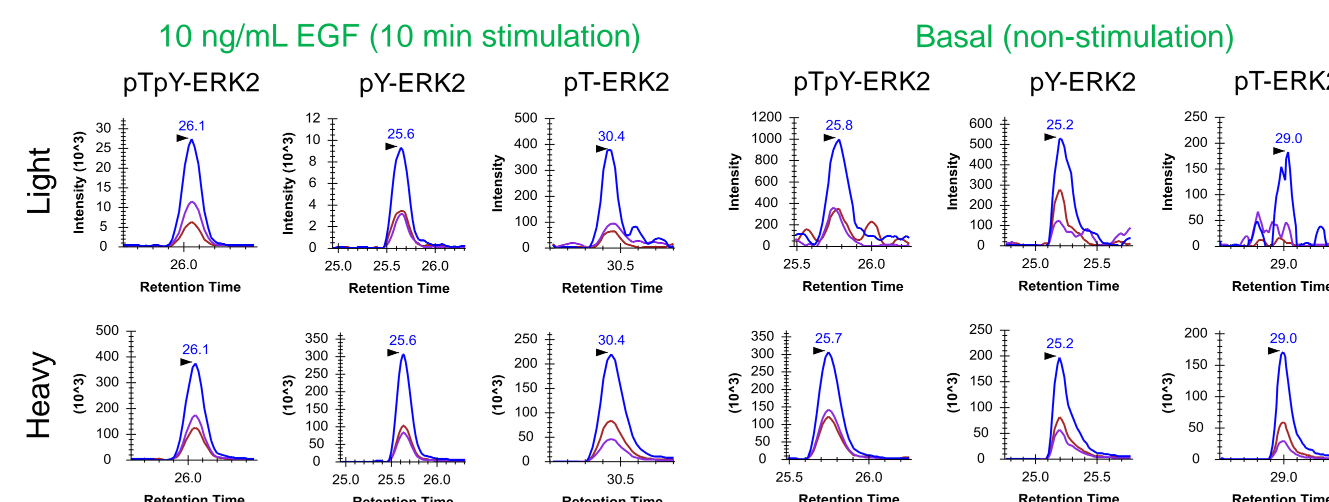
HMEC and stimulations

- human mammary epithelial cells (HMEC 184A1) were cultured until near confluence and depleted of EGF overnight prior to treatment.
- For sensitivity evaluation, HMEC under two conditions were used: the basal level and the stimulated level treated with 10 ng/mL of EGF for 10 min.
- For dose-response experiments, EGF was added directly to culture plates to achieve the required dose (0, 0.1, 0.3, 1.0, 3.0, 10 ng/mL) for either 10 min or 2 h stimulations.

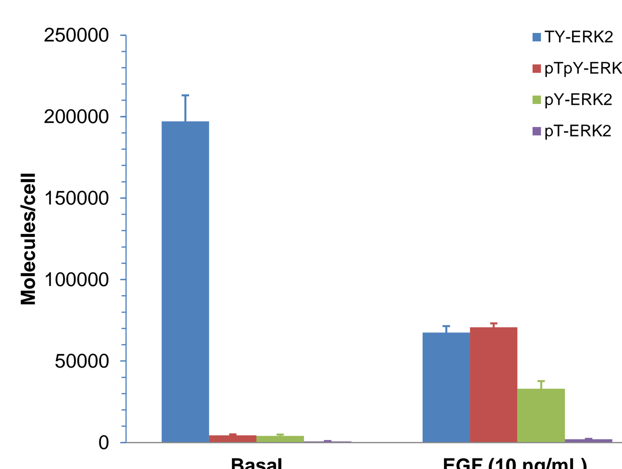
Results

Proof-of-concept

PRISM-SRM quantification of ERK2 phosphorylation isoforms in the stimulated and non-stimulated HMEC cells

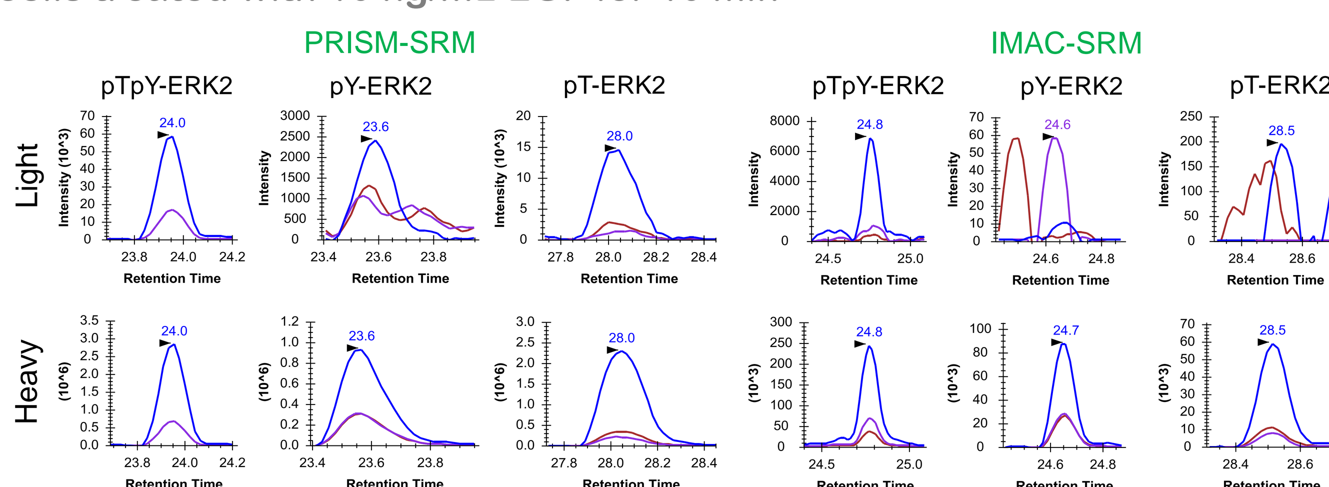


Molar abundance of the non-, mono-, and double-phosphorylated ERK2 isoforms with standard derivation (n = 3) at the basal and 10 ng/mL EGF stimulation conditions



PRISM-SRM *vs* IMAC-SRM

Targeted quantification of ERK2 phosphorylation isoforms in the HMEC cells treated with 10 ng/mL EGF for 10 min

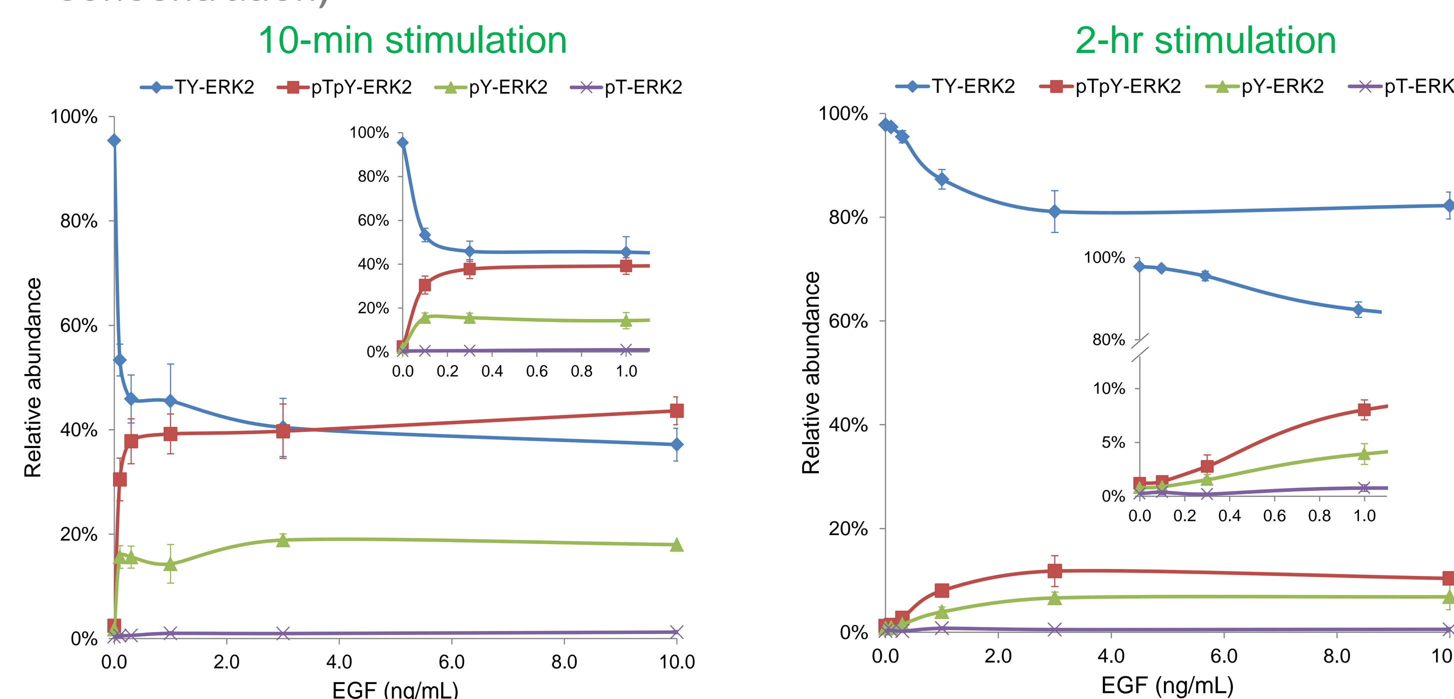


Evaluation of phosphopeptide recovery following IMAC enrichment assuming peptide lossless in the PRISM fractionation

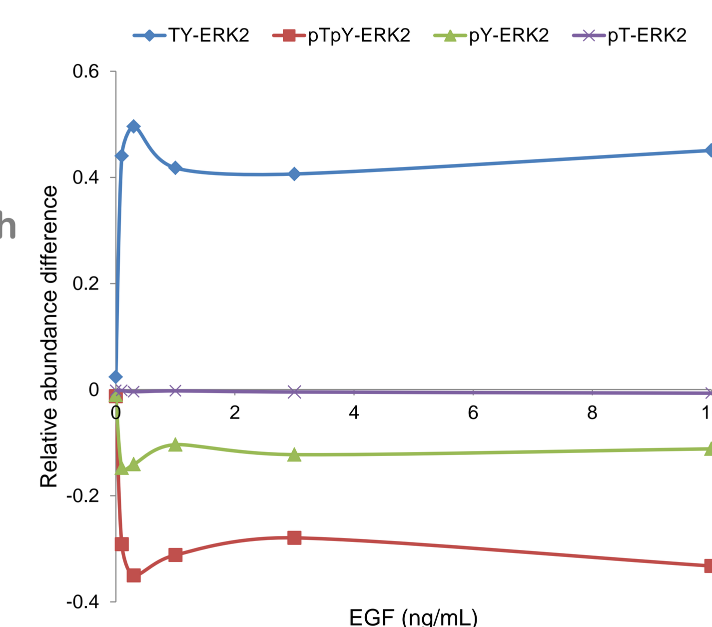
	Isoform	Surrogate peptide	pTpY 1	pTpY 2	pY 1	pY 2	pT 1	pT 2
IMAC	ERK1	Light	2800	1600				
		Heavy	2.50E+05	3.00E+05	6.50E+04	6.00E+04	4.50E+04	4.00E+04
	ERK2	Light	7000	5000		200	200	220
		Heavy	2.50E+05	2.20E+05	9.00E+04	8.50E+04	6.00E+04	6.50E+04
PRISM	ERK1	Light	2.00E+04		1500		3.00E+03	
		Heavy	2.10E+06		9.50E+05		5.50E+05	
	ERK2	Light	6.00E+04		2500		1.50E+04	
		Heavy	2.90E+06		9.50E+05		2.30E+06	
Recovery (%) (IMAC/PRISM)	ERK1	Light	14.0	8.0				
		Heavy	11.9	14.3	6.8	6.3	8.2	7.3
	ERK2	Light	11.7	8.3		8.0	1.3	1.5
		Heavy	8.6	7.6	9.5	8.9	2.6	2.8
Average recovery (%) of each isoform across two replicates			10.6		7.9		4.0	

ERK phosphorylation dynamics in EGF-induced dose responses

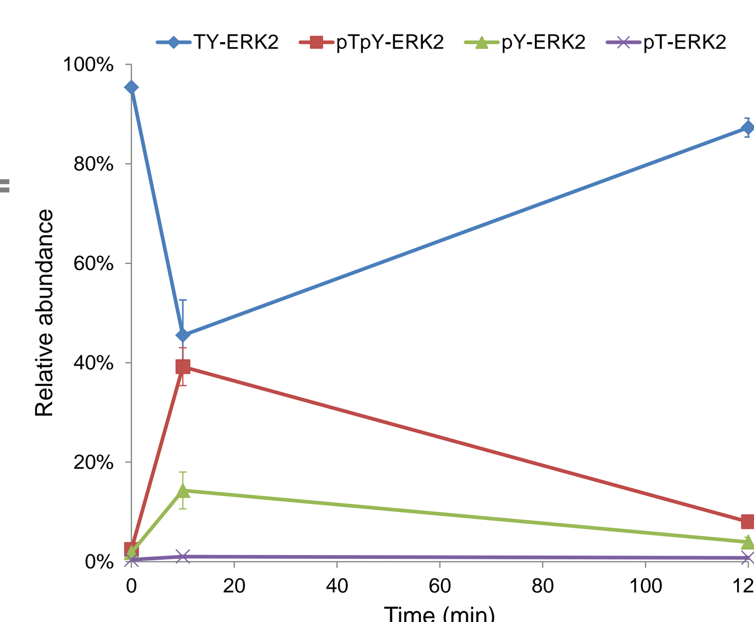
Quantification of ERK phosphorylation dynamics in the HMEC treated with different EGF concentrations (three biological replicates for each concentration)



Relative abundance changes in the ERK2 phosphorylation stoichiometries between 10-min and 2-h stimulation



Time-dependent ERK2 phosphorylation following 1 ng/mL EGF stimulation with SD (n = 3)



Conclusions

- Direct PRISM-SRM (i.e., without affinity enrichment) enables for sensitive quantification of protein phosphorylation dynamics in mammalian cells starting with only ~25 µg of whole cell lysate.
- Compared to IMAC-SRM, direct PRISM-SRM can provide at least 10-fold improvement in SRM sensitivity, which was primarily due to the high peptide recovery in the PRISM workflow.
- Accurate quantification of ERK phosphorylation dynamics in response to different doses of EGF showed the maximal ERK activation in response to 0.3 ng/mL EGF at the peak activation (10-min) but 3 ng/mL EGF at the steady state (2-h). These doses matches well to physiological levels of EGF in humans.
- Comparison of the time-dependent and dose-dependent levels of individual phosphorylated ERK isoforms provided strong support for a processive, rather than distributed, model ERK phosphorylation.[5]
- PRISM-SRM potentially offers a new approach for simultaneous quantification of multiple PTMs in a singe sample.

Acknowledgments

Portions of the research were supported by the NIH Director's New Innovator Award Program DP2OD006668 (to WQ), NCI Early Detection Research Network Interagency Agreement Y01-CN-05013-29 (to KDR and DGC), U24-CA-160901 (to RDS) from the National Cancer Institute Clinical Proteomic Tumor Analysis Consortium (CPTAC), and P41GM103493 (to RDS). Proteomics portions of this work were performed in the Environmental Molecular Sciences Laboratory, a U.S. Department of Energy/BER national scientific user facility at Pacific Northwest National Laboratory.

References

1. Shi T, et al. *Proc Natl Acad Sci USA* 109:15395-15400 (2012).
2. Shi T, et al. *J Proteome Res* 12: 3353-3361 (2013)
3. Shi T, et al. *J Proteome Res* 13: 875-882 (2014).
4. He J, et al. *Mol Oncol* (2014), in press.
5. Aoki K, et al. *Proc Natl Acad Sci USA*, 108: 12675-12680 (2011).

CONTACT
Tujin Shi, Ph.D.
tujin.shi@pnnl.gov

omics.pnl.gov