High Sensitivity Targeted Quantification of ERK Phosphorylation Dynamics and Stoichiometry without Affinity Enrichment

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Outline

I. Background
II. Proof-of-concept study
   (direct quantification of individual site-specific ERK phosphorylation by PRISM-SRM)
III. Comparison of PRISM-SRM with IMAC-SRM
IV. Quantification of ERK phosphorylation dynamics
V. Conclusions
PRISM for much improved SRM sensitivity

(A) PRISM (high Pressure high Resolution Separation with Intelligent Selection and Multiplexing)

(B) LC-SRM

Shi et al., *Proc Natl Acad Sci USA* 2012, 109, 15395-15400.
Significantly improved LOQs with PRISM

<table>
<thead>
<tr>
<th>Targeted MS platform</th>
<th>LOQs (starting material)</th>
<th>Human plasma/serum</th>
<th>Mammalian cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional LC-SRM</td>
<td>~1 µg/mL (1 µL)</td>
<td>~5,000 copies/cell (?)*</td>
<td></td>
</tr>
<tr>
<td>PRISM-SRM</td>
<td>~1 ng/mL (2 µL)</td>
<td>~100 copies/cell (~25 µg)</td>
<td></td>
</tr>
<tr>
<td>IgY14-PRISM-SRM</td>
<td>~50 pg/mL (10 µL)</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>


**PRISM publications:**
- PRISM-SRM without immunoaffinity depletion: Shi et al., *J Proteome Res*, 2013, 12, 3353-3361.
PRISM-SRM is more sensitive than ELISA for EGFR measurement in MCF7 cells

### ELISA

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>EGFR (pg/µg total protein)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF7 (breast)</td>
<td>0 (ND)</td>
<td>---</td>
</tr>
<tr>
<td>HT29 (colon)</td>
<td>17</td>
<td>14.2</td>
</tr>
<tr>
<td>MDA-MB-231 (breast)</td>
<td>75</td>
<td>12.7</td>
</tr>
<tr>
<td>A431 (skin)</td>
<td>407</td>
<td>15.1</td>
</tr>
</tbody>
</table>

### Conventional LC-SRM

- MDA-MB-231
  - Copies/cell: ~172,000
  - amol/µg: 1696
- MCF7
  - Copies/cell: ~2,500*
  - amol/µg: 21


ERK phosphorylation is ideal for evaluation of analytical platform performance

- Moderate complexity:
  (two isoforms, ERK1 and ERK2; separate or concurrent phosphorylation on proximate T and Y residues in a TEY motif)

- Well-studied both experimentally and theoretically

- Challenging for measuring individual ERK phosphorylation isoforms with antibody-based assays

- Targeted proteomics allows site-specific quantification of PTMs

- However relatively large starting materials required for conventional SRM analysis (~1 mg cell lysate: Tong et al., *Mol Cell Proteomics* 2009, 8, 2131-2144)
Proof-of-concept study

### Surrogate peptides for ERK1/2 phosphorylation analysis

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Standard sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERK1</td>
<td>LADPEHDHTGFLTEYVATR (TY-ERK1)</td>
</tr>
<tr>
<td></td>
<td>LADPEHDHTGFL$^{pT}$TEYVATR (pT-ERK1)</td>
</tr>
<tr>
<td></td>
<td>LADPEHDHTGFL$^{pY}$TEYVATR (pY-ERK1)</td>
</tr>
<tr>
<td></td>
<td>LADPEHDHTGFL$^{pT}p^{Y}$TEYVATR (pTpY-ERK1)</td>
</tr>
<tr>
<td>ERK2</td>
<td>VADPDHDHTGFLTEYVATR (TY-ERK2)</td>
</tr>
<tr>
<td></td>
<td>VADPDHDHTGFL$^{pT}$TEYVATR (pT-ERK2)</td>
</tr>
<tr>
<td></td>
<td>VADPDHDHTGFL$^{pY}$TEYVATR (pY-ERK2)</td>
</tr>
<tr>
<td></td>
<td>VADPDHDHTGFL$^{pT}p^{Y}$TEYVATR (pTpY-ERK2)</td>
</tr>
</tbody>
</table>
Proof-of-concept study

<table>
<thead>
<tr>
<th></th>
<th>SRM Transitions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Q1</td>
</tr>
<tr>
<td>TY-ERK1</td>
<td>724.7 839.4 (y7⁺), 738.4 (y6⁺), 609.3 (y5⁺)</td>
</tr>
<tr>
<td>pT-ERK1</td>
<td>751.3 718.7 ([precursor – 98]³⁺), 821.4 ([y7 – 98]⁺), 927.9 ([y16 – 98]²⁺)</td>
</tr>
<tr>
<td>pY-ERK1</td>
<td>751.3 919.4 (y7⁺), 689.3 (y5⁺), 976.9 (y16²⁺)</td>
</tr>
<tr>
<td>pTpY-ERK1</td>
<td>778.0 745.3 ([precursor – 98]³⁺), 901.4 ([y7 – 98]⁺), 967.9 ([y16 – 98]²⁺)</td>
</tr>
<tr>
<td></td>
<td>Q3</td>
</tr>
<tr>
<td>TY-ERK2</td>
<td>715.3 952.5 (y8⁺), 839.4 (y7⁺), 738.4 (y6⁺)</td>
</tr>
<tr>
<td>pT-ERK2</td>
<td>742.0 709.3 ([precursor – 98]³⁺), 934.5 ([y8 – 98]⁺), 738.4 (y6⁺)</td>
</tr>
<tr>
<td>pY-ERK2</td>
<td>742.0 919.4 (y7⁺), 818.3 (y6⁺), 689.3 (y5⁺)</td>
</tr>
<tr>
<td>pTpY-ERK2</td>
<td>768.7 736.0 ([precursor – 98]³⁺), 901.4 ([y7 – 98]⁺), 446.3 (y4⁺)</td>
</tr>
</tbody>
</table>

- Sensitivity evaluation: the basal level and the stimulated level treated with **10 ng/mL** EGF for **10 min**
- Dose-response experiments: different EGF doses (**0, 0.1, 0.3, 1.0, 3.0, 10 ng/mL**) for either **10 min** or **2 h** stimulations
PRISM-SRM enables direct quantification of site-specific ERK phosphorylation in HMEC cells

10 ng/mL EGF stimulation (10 min)

Light

pTpY-ERK2  pY-ERK2  pT-ERK2

Heavy

pTpY-ERK2  pY-ERK2  pT-ERK2

Basal (no stimulation)

Molar abundance of individual ERK2 isoforms with standard derivation (n = 3)

Estimated LOQ for pT-ERK2: ~1000 copies per cell
PRISM-SRM vs. IMAC-SRM

~50 µg EGF-treated HMEC cell lysate digest

Addition of internal standard (heavy-isotope labeled peptides)

PRISM (~25 µg)

LC-SRM

IMAC (~25 µg)

LC-SRM
PRISM-SRM provides ≥10-fold higher sensitivity than IMAC-SRM

**PRISM-SRM**

- **pTpY-ERK2**
- **pT-ERK2**

**IMAC-SRM**

- **pTpY-ERK2**
- **pT-ERK2**

**Recovery of IMAC (relative to PRISM):**

- **pTpY (10.6%)**
- **pY (7.9%)**
- **pT (4.0%)**

![Graph showing peak intensity comparison between PRISM and IMAC for pTpY-ERK2, pT-ERK2, and pY-ERK2](chart)
Distributive vs. processive phosphorylation model for ERK

Distributive model


Processive model
ERK phosphorylation dynamics as a result of EGF-induced dose response

Maximal ERK activation: **0.3-1 ng/mL** EGF at peak activation (10-min); **3 ng/mL** EGF at steady state (2-h)

EGF dose at the peak activation close to physiological levels in humans (~0.5 ng/mL)

Increase of pY-ERK2 mirrors that of pTpY-ERK2, but pT-ERK2 does not increase significantly
ERK phosphorylation dynamics as a result of EGF-induced dose response

**Relative abundance changes**
(10-min vs. 2-h)

**Time-dependent ERK2 phosphorylation**
(1 ng/mL EGF stimulation)

- Similar stoichiometry changes for pTpY-ERK2 and pY-ERK2 from peak activation to steady state; however pTpY-ERK2 changes more.
- Time-course analysis: the activation pattern of pY-ERK2 is similar to that of pTpY-ERK2, but often with lower stoichiometry.
ERK is phosphorylated in a processive, rather than distributive manner in mammalian cells.

Conclusions

- PRISM-SRM enables sensitive, site-specific quantification of low level protein phosphorylation without affinity enrichment.
- Compared to IMAC, PRISM provides at least 10-fold improvement in SRM sensitivity.
- Phosphorylation dynamics data (dose responses and timecourse) confirms that ERK is phosphorylated in a processive manner in mammalian cells.
- PRISM-SRM has great potential for simultaneous quantification of multiple PTMs in a single analysis (i.e., without serial or parallel enrichment).
Acknowledgments

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