Optimizing the Conditions of Phospho-peptide Enrichment for Quantification of Phospho-proteins by Liquid Tissue-SRM in FFPE Tissues

Wei-Li Liao, Sheeno Thyparambil, Kathleen Bengali, Joe Abdo, Marlene Darfler, David Krizman, Todd Hembrough, and Jon Burrows

Expression Pathology Inc., Rockville, MD

Overview

The objective of this study is to optimize phospho-peptide enrichment conditions and determine the minimum amount of formalin-fixed paraffin-embedded (FFPE) tumor tissue that could be assayed reproducibly.

HCC827 cells were chosen for our optimization study since they express abnormally high levels of EGFR and have an active EGFR signaling pathway due to an acquired mutation in the EGFR tyrosine kinase. A series of conditions, and various amounts of formalin fixed, Liquid Tissue® solubilized cells were incubated with titanium dioxide (TiO_2) beads. The measured concentrations demonstrated that the enrichment procedure was highly efficient.

The optimal conditions were then applied to the measurement of phospho-peptides from various amounts of FFPE A431 xenograft tissues. Our optimization of phospho-peptide enrichment by TiO_2 beads combined with Liquid Tissue®-SRM platform enables the quantification of phospho-proteins from 25 μg of FFPE A431 xenograft tissues.

Introduction

Protein phosphorylation is critical for cellular activities.

However, detecting the phosphorylation states of multiple signaling pathway components in clinical tumor tissue remains a challenge due to the transient nature of these signals, and the often limited amount of tumor tissue available for analysis.

We have developed a Liquid Tissue®-SRM technology platform which enables relative and absolute quantification of proteins and their phosphorylation status directly in FFPE tissue.

To be applicable to the quantification of phospho-targets in limited amount of tissue, the phospho-peptide enrichment procedure must be optimized.

Methods

- HCC827 cells were washed, fixed with formalin, and subjected to Liquid Tissue (LT) processing.
- A431 xenograft tissue blocks were cut on DIRECTOR slides and processed using standard histological procedures.
- Tissues were collected using laser micro-dissection.
- The collected tissue samples were then solubilized to tryptic peptides using LT technology.

↓ Phospho-peptide enrichment (TiO_2 beads)
(various temperature and incubation time)

- Supplementing with stable isotope-labeled heavy peptides
- Liquid Tissue-SRM Mass Spectrometry Analysis

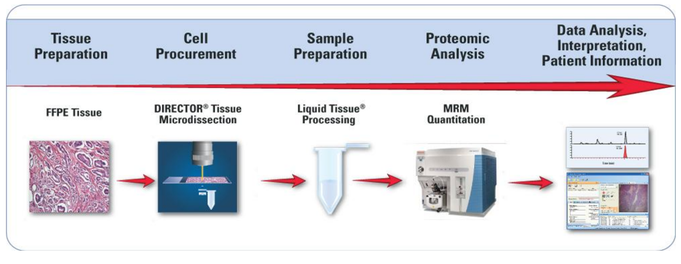

Figure 1. Liquid Tissue®-SRM workflow for analysis of proteins from FFPE tissue.

Results

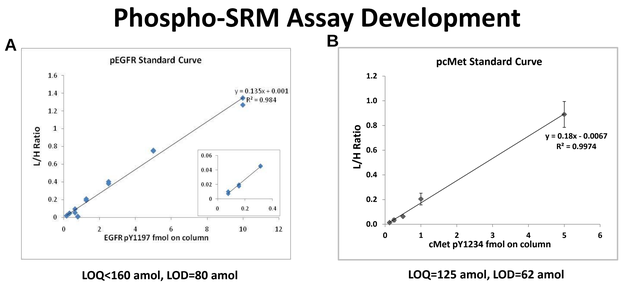

Figure 2. Standard curves for quantification of EGFR pY1197 (A) and cMet pY1234 (B). The area ratio of a corresponding light peptide to heavy peptide was plotted *versus* fmol of light peptide on column.

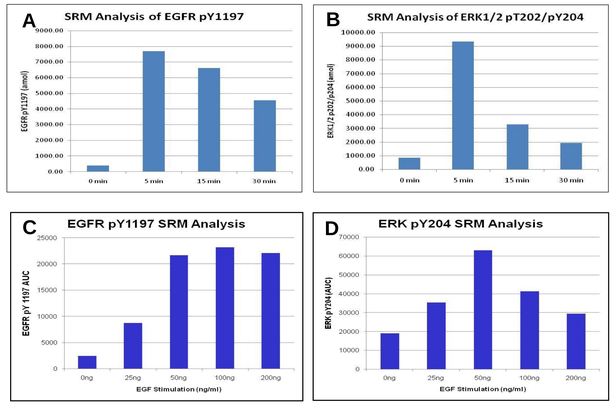

Figure 3. Time course analysis (A, B) and dose response (C, D) of EGF signaling in formalin fixed A431 cells. Cells were incubated for increasing times with 50 ng/ml EGF or incubated for 5 minutes with increasing concentrations of EGF. After washed, cells were fixed with formalin and subjected to Liquid Tissue processing. 100 μg of cellular protein was phospho-enriched with TiO_2. SRM analysis of 1/3 of bound material was performed to quantitate phospho-targets.

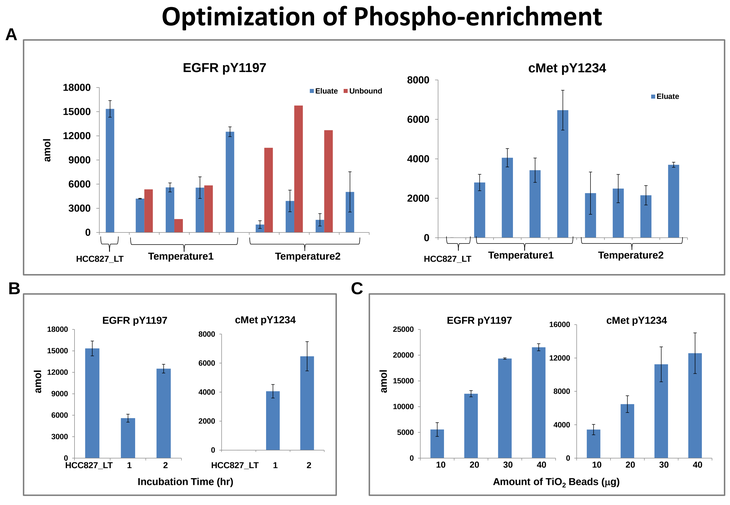

Figure 4. Optimization of phospho-peptide enrichment in HCC827 cells. Different temperature (A), incubation time (B), and amount of TiO_2 (C) were tested. 50 μg of formalin fixed HCC827 cell protein was Liquid Tissue processed, and subjected to optimization of multiple different experimental parameters.

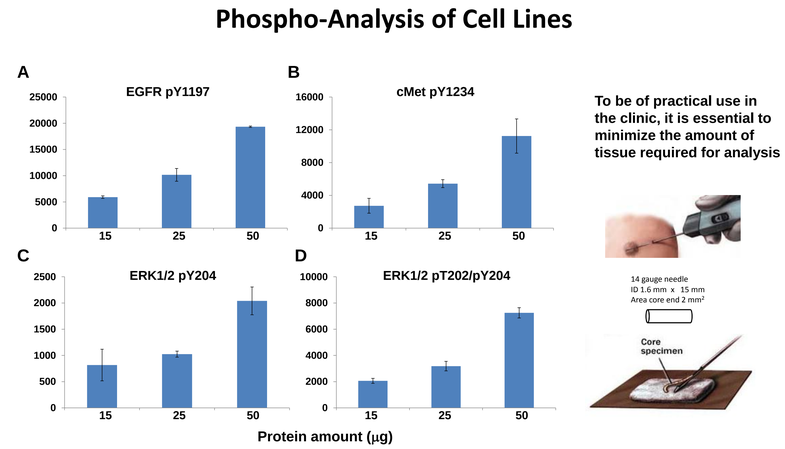

Figure 5. Applying the optimal condition to quantify EGFR pY1197 (A), cMet pY1234 (B), ERK1/2 pY204 (C), and ERK1/2 pT202/pY204 (D) in various amount of HCC827 cells. 15, 25, or 50 μg of formalin fixed HCC827 cell protein was Liquid Tissue processed and subjected to phospho-peptide enrichment using the optimal condition.

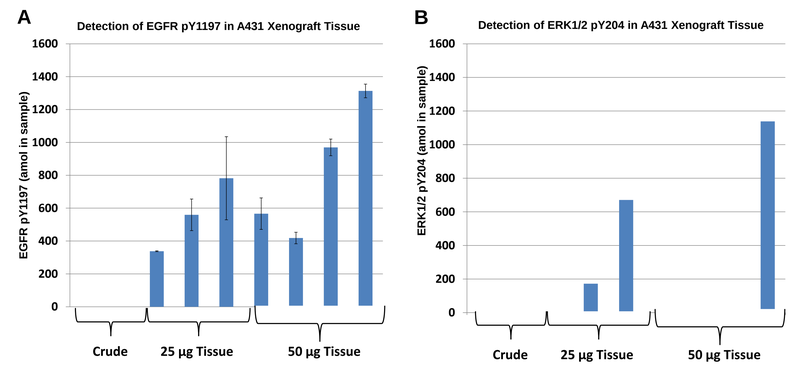

Figure 6. Quantitation of EGFR pY1197 (A) and ERK1/2 pY204 (B) in FFPE A431 xenograft tissue. 25 or 50 μg of FFPE tumor protein was used for phospho-enrichment and 1/3 of the bound material was subjected to mass spectrometry. Using the best conditions, we were able to reproducibly quantitate multiple phospho-peptides from 1/3 of a 25 μg tissue sample.

Conclusions

- Our platform, combining phospho-peptide enrichment and Liquid Tissue-SRM assays, allows the quantification of phospho-targets from 25-50 μg of FFPE tumor protein, far less than current methods use.
- Optimization of phospho-enrichment should enable us to quantitate phospho-pathway activity in very small sources of tumor tissue, such as core biopsies, where immediate fixation of these tissues is possible, and the impact of pre-analytical variables will be minimized.

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