Automated PTMScan® immunoaffinity enrichment for the capture of KGG modified peptides from complex mixtures

[1]Lilian Phu, [2]Shadie Nimri, [1]Anne Baldwin, [1]Nadia Martinez Martin, [2]Chris Suh, [3]Matthew P. Stokes and [1]Donald S. Kirkpatrick

[1]Department of Microchemistry, Proteomics, and Lipidomics, Genentech, Inc., South San Francisco CA 94080, [2]Phynexus, Inc., San Jose CA 95136, [3]Cell Signaling Technology, Inc., Danvers MA 01923

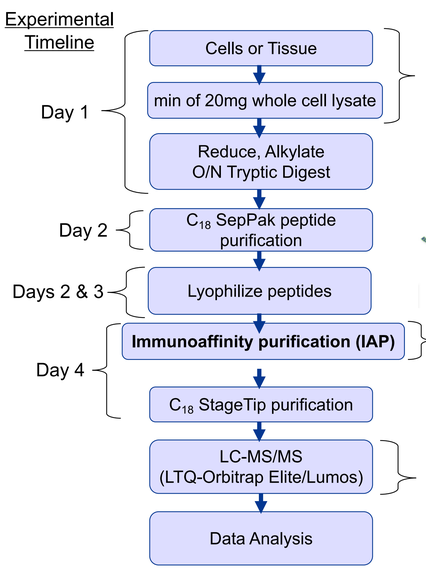
ABSTRACT

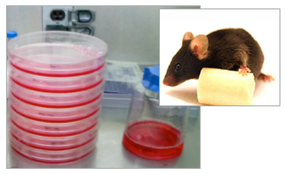
Recent advances in mass spectrometry instrumentation and sample handling have enabled researchers to routinely perform global profiling of many protein post-translational modifications, expanding our knowledge of biological pathways. One key to the success of these experiments is the effective selective enrichment of the modified peptides from complex mixtures before introduction to the mass spectrometer, often via immunoaffinity purification using antibodies that are directed against the PTM of interest. Here, using the ubiquitin remnant motif (KGG) antibody as a model, we expand on the PTMScan® immunoaffinity enrichment protocol by coupling it to the Phynexus MEA robot, developing a robust automated platform that enables the concurrent processing of up to twelve samples with limited manual sample handling. We demonstrate the utility of the automated system in the identification of thousands of KGG peptides from complex biological samples.

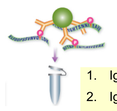
MATERIALS AND METHODS

- ***PTMScan® protocol***

Experimental Timeline



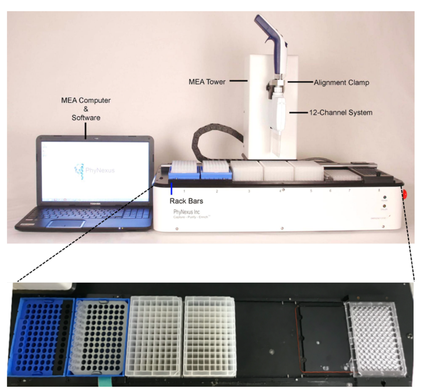




1. IgG beads were equilibrated in IAP buffer.
2. IgG beads incubated with digested peptides on rotator for 1-2hr at 4ºC.
3. IgG beads quickly washed with IAP buffer 2x.
4. IgG beads quickly washed with H_2O 4x.
5. Peptides were eluted with 0.15% TFA.

Kim et.al. Mol .Cell 2011
Gu et.al. Neuromethods 2016
Stokes et al. Meth. in Pharm. Tox. 2016

- ***Phynexus MEA2 Automated Robotic System***

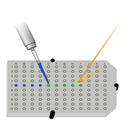


- Entry level automation system
- 12 channel benchtop system (200 or 1000 uL pipette head, variety of tips)
- Streamlined and configurable (accommodates variety of plate options)
- Easy to operate
- Cold room compatibility
- Methods transferable to other automation platforms

- ***Optimization Experiments Performed in Two Stages***

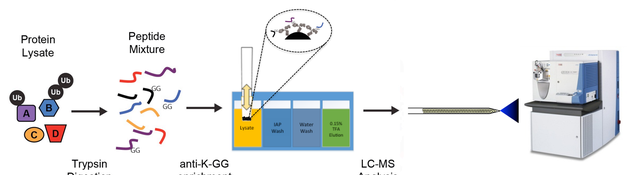
1) Simple 3 KGG peptide mix coupled with MALDI as MS assessment/readout

Peptide Name	Sequence	MH+
UBIQUITIN_HUMAN K27GG	TITLEVEPSDTIENVK(GG)AK	2101.1023
UBIQUITIN_HUMAN K48GG	LIFAGK(GG)QLEDGR	1460.7856
UBIQUITIN_HUMAN K63GG	TLSDYNIQK(GG)ESTLHLVLR	2244.1983



- Enabled quick experiments to
  - ✓ Select appropriate tip format
  - ✓ Evaluate temperature effects on peptide capture
  - ✓ Initial testing of covalent crosslinking of KGG IgG to Protein A resin

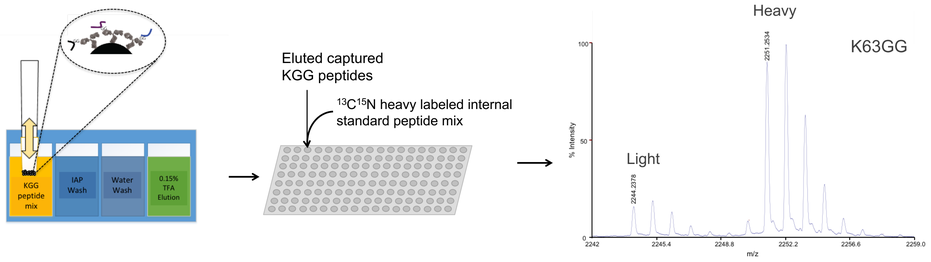
2) Complex peptide mixtures with LC-MS/MS analysis

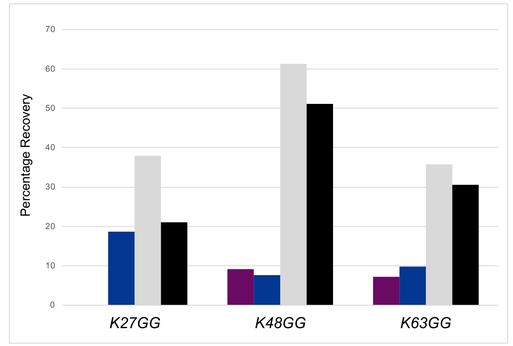


- Refinement of protocol

RESULTS

- ***Investigating the effect of temperature on peptide capture***

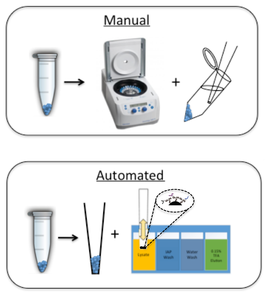




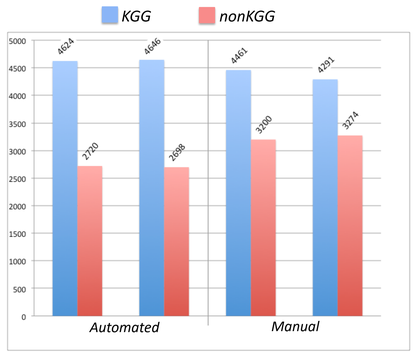
- Increased peptide capture efficiency at 4ºC

- ***Automated and Manual IAP protocol are comparable***

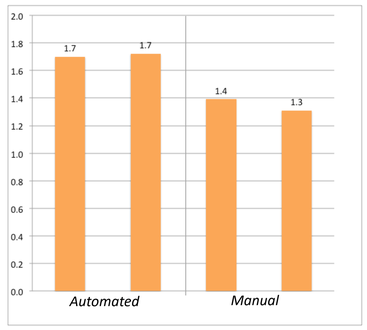
Anti-KGG enrichment



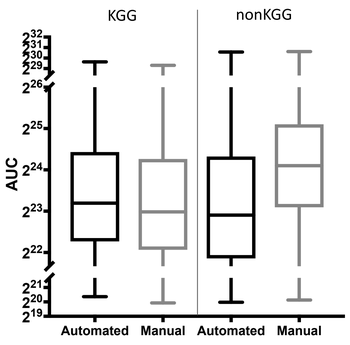
Unique Peptide Identifications



KGG:nonKGG peptide ID ratio



Peptide Abundances



- Automated processing yielded more efficient washing → more complete removal of liquid from resin at each step

- ***Evaluating performance of different Protein A resins in automated protocol***

Phytip ProA

- Standard Protein A
- *incubated with KGG IgG**

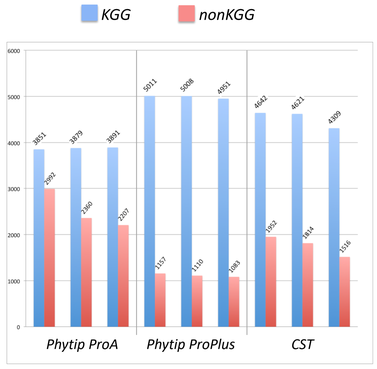
Phytip ProPlus

- recombinant alkali tolerant Protein A
- Protein A coupled to resin matrix in orientation for maximal capacity
- improved capacity for IgG (by ~10%) as compared to ProA
- *incubated with KGG IgG**

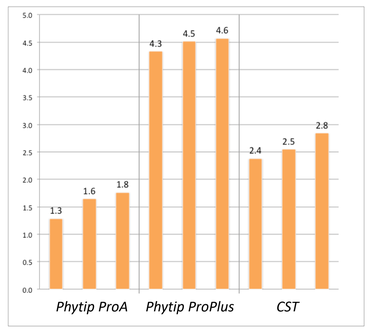
CST (2x resin volume)

- *Precoupled to KGG IgG*

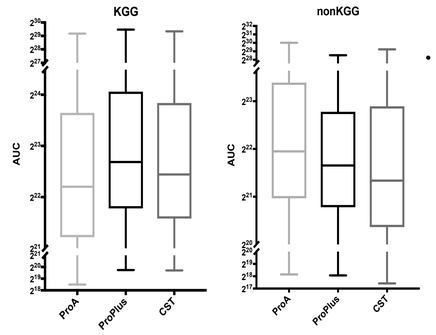
Unique Peptide Identifications



KGG:nonKGG peptide ID ratio



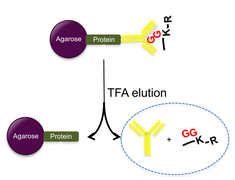
Peptide Abundances



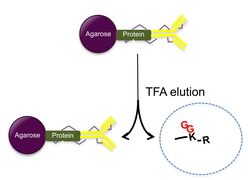
- ProPlus resin yielded highest recovery of KGG peptides and reduced nonspecific binding

- ***Exploring use of covalently crosslinked IgG in automated protocol***

Non-crosslinked Antibody



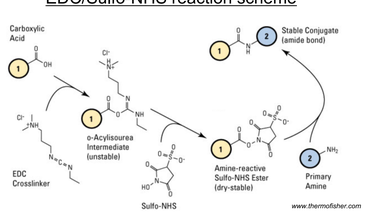
Crosslinked Antibody



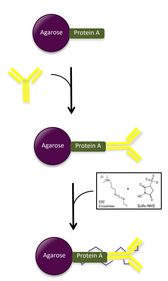
EDC/Sulfo-NHS chemistry

- Zero length carbodiimide crosslinker
- Stable (nonreversible) amide bond formed between carboxylate and primary amine group

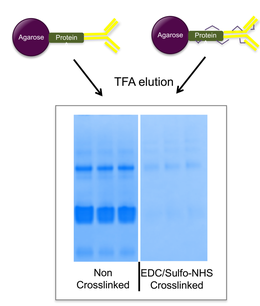
EDC/Sulfo-NHS reaction scheme



Crosslinking Protocol

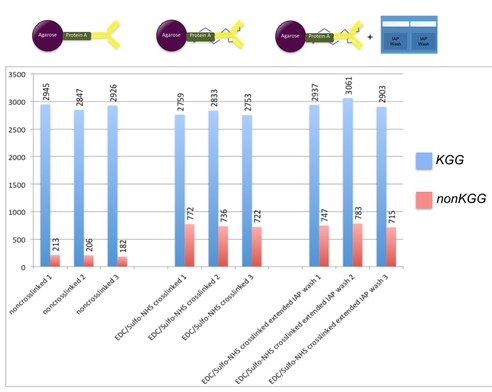


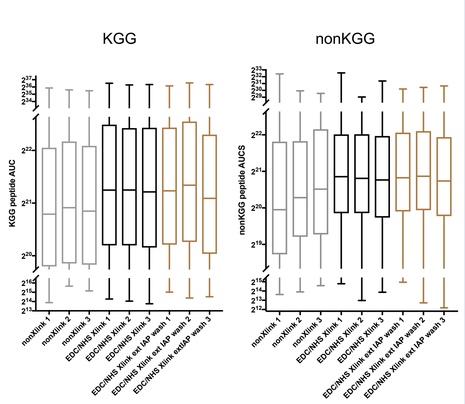
SDS-PAGE to assess crosslinking efficiency



- Reduced IgG eluted along with peptides which interferes with downstream MS analysis (column clogging, chromatography deterioration)

Udeshi et.al. MCP 2012
Udeshi et.al. Nature Prot. 2012





- Three-fold increase in nonspecific peptides observed (not mitigated with additional IAP buffer washes) with EDC/Sulfo-NHS crosslinking which has the potential to negatively impact KGG-peptide identification rates

SUMMARY

- KGG automation matches manual performance with increased throughput and efficiency

	MANUAL	AUTOMATED
MANUAL HANDLING TIME	~1 to1.5 hr	~15 min
REPRODUCIBILITY	Variability (individual tubes handled sequentially)	Uniformity (all columns processed concurrently)
CAPACITY	8-10 per 1.5 hr hands-on time	60 per 1.5 hr hands-on time

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