Simplified Method Development for Targeted Hydrogen-Deuterium Exchange Studies of Protein Kinases using MALDI-MS

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Introduction

B-Raf is a serine-threonine protein kinase, which serves as a tumor driver in various forms of cancer due to specific mutations (e.g. V600E). Studies of the functionality of such proteins through structural dynamics[1] can improve the site-specific knowledge of the protein. B-Raf is a protein of low stability prone to aggregation, so it is imperative to design a method for simple and fast analyzes, but other proteins (e.g. ABL1) may be used as test cases for method development.

Hydrogen deuterium exchange (HDX) is a technique to understand higher order structure of proteins by observing the protons exchanged in amino acid side chains and along the amide backbone with heavier deuterium from the solvent. The kinetics of the exchange in peptides/ proteins can be interpreted from MS spectra, which are in turn linked to the structure of the peptides/proteins. Here, HDX reactions are analyzed using matrix assisted laser desorption ionization (MALDI) MS, which delivers fast analysis for biological molecules.

Methods- Protocols

The kinase domains were provided in Tris buffer (**see below**). Initially, Protocol 1 (**Figure 1**) was tested on B-Raf kinase domain to assess sequence coverage. To improve the methods for both sequence coverage and deuteration uptake kinetics, a more stable protein kinase domain of similar mass from ABL1 was used as a test case. Later, the same methods were applied to B-Raf. Using ABL1, optimum environmental conditions for the reaction were tested employing a glove bag with an inert nitrogen atmosphere.

Further an improved protocol (**Figure 2**) was designed to include reversed phase protein clean up, either with C18 or C4, and elution parameters (aqueous and organic buffers) to improve sequence coverage and decrease/prevent back exchange.

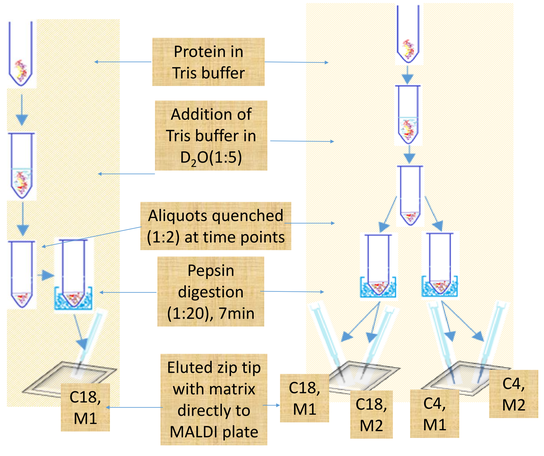

Figure 1: Protocol 1

The reactions are performed inside a glove bag under low humidity conditions with a constant purge of nitrogen gas. **Figure 3** shows the glove bag set up containing pipettes, reaction vials, a dry ice bath for quenching reactions, Zip tips, and MALDI plate.

All reactions were analyzed using a Sciex 4700 MALDI-TOF/TOF MS; mass spectra are manually evaluated.

Figure 2: Improved Protocol 2

Figure 3: Experimental set up

Tris buffer: 20mM Tris, 100mM NaCl, 1mM TCEP
Quench buffer: 0.8% HCOOH
M1: CHCA 10 mg/ml in 50%ACN, 0.1%TFA
M2: CHCA 10 mg/ml in 100%ACN, 0.1%TFA

Results – HDX-MS Method development

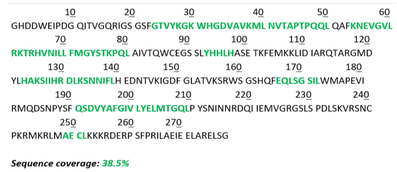

Figure 4: B-Raf Sequence Coverage using Protocol 1

Protocol 1 was performed on B-Raf to briefly analyze the extent of resulting sequence coverage. The obtained sequence coverage was poor.

Following which, ABL1 was provided in similar buffer conditions for development of suitable protocol.

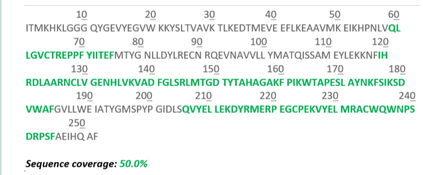

Figure 5: ABL1 Sequence Coverage using Protocol 1

ABL1 was analyzed with Protocol 1 to determine sequence coverage (**Figure 5**) as well as to evaluate the controlled low humidity environment for minimizing back exchange (**Figure 6**).

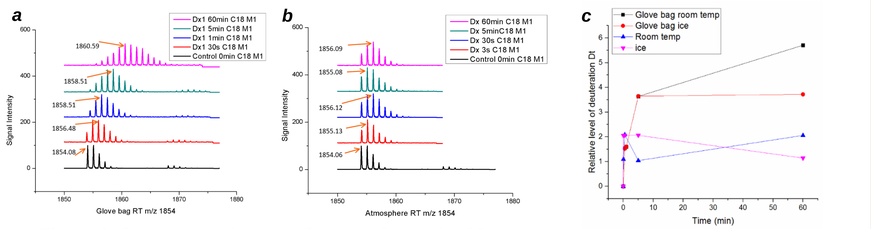

Figure 6: Sample experimental ABL1 data from m/z 1854 VGENHLVKVADFGLSRL. Mass spectra from a) inside the glove bag b) outside the glove bag. c) Deuterium uptake kinetics

$D_t = M_t - M_{UND}$

M_t = Centroid mass of deuterated peak at certain timepoint
M_{UND} = Centroid mass of undeuterated peak
D_t = Relative level of deuteration

To improve sequence coverage, both C18 and C4 stationary phases are used on different aliquots of the samples, which increased observed peptides (**Figure 7**).

Also, a non-aqueous matrix solution[2], M2, was added to elute peptides and reduce back exchange during matrix crystallization. Data are presented in **Figure 8**.

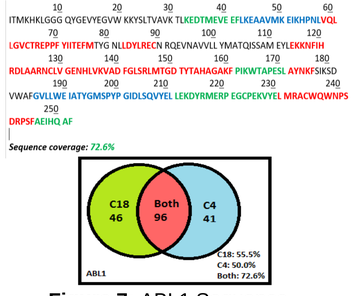

Figure 7: ABL1 Sequence Coverage Using Protocol 2

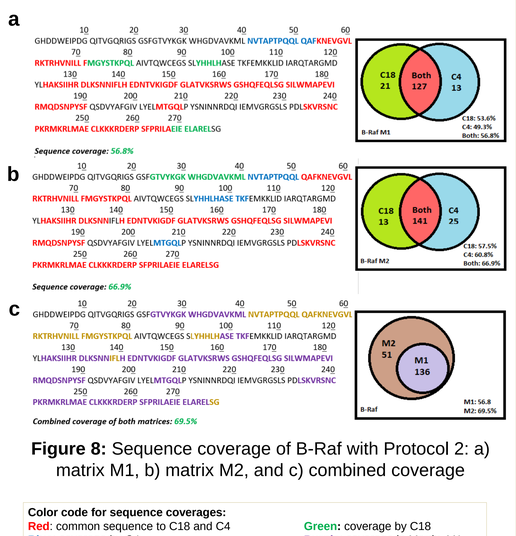

Figure 8: Sequence coverage of B-Raf with Protocol 2: a) matrix M1, b) matrix M2, and c) combined coverage

Color code for sequence coverages:
Red: common sequence to C18 and C4
Blue: coverage by C4
Brown: coverage in Matrix, M2 and both matrices.
Green: coverage by C18
Purple: coverage in Matrix, M1

Results: Example Data

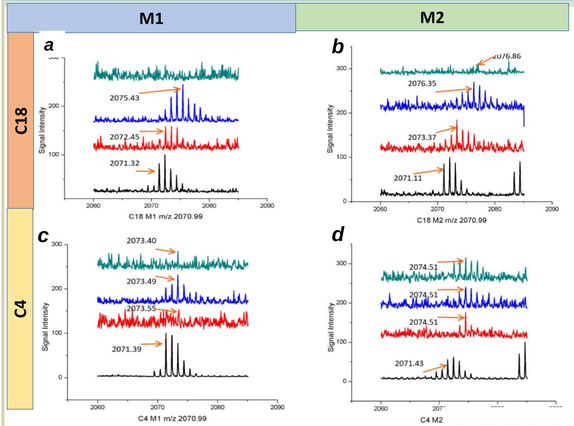

Figure 9: Deuterium uptake in various conditions for peptide of m/z 2070 (WMAPEVIRMQDSNPYSF) a) C18 M1, b) C18 M2, c) C4 M1, d) C4 M2

In **Figure 10**, differences in M1 and M2 elution demonstrate the relative variation in deuterium uptake for the same peptide m/z 2070 from BRaf. Elution with M1 shows considerable back exchange in comparison to M2 which occurs most obviously at the 5 min timepoint.

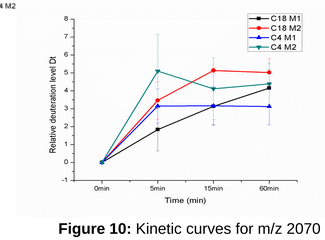

Figure 10: Kinetic curves for m/z 2070 deuterium uptake.

Green: ***60min***
Blue: ***15min***
Red: ***5min***
Black: ***0min control***

Exchange reactions performed on B-Raf kinase domain using protocol 2 gave data represented by **Figure 9.** It shows a sample experimental data of a peptide at m/z 2070 obtained from pepsin digest of B-Raf in various conditions according to protocol 2. The variation in method of clean up, C18 or C4, and matrix solution used show a change in deuteration.

Conclusions

- The modified method (protocol 2) developed on ABL1 improved results in sequence coverage which were also seen when translated to B-Raf.
- Non aqueous matrix solution significantly reduces back exchange during matrix crystallization.

Future Work

- Optimize the conditions for reproducible deuteration rates and kinetics.
- Explore protein modifiers and other analysis techniques like Differential Ion Mobility Filtration

Acknowledgements and References

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1. Hentze, N., Mayer, M.P. Analyzing Protein Dynamics Using Hydrogen Exchange Mass Spectrometry. J. Vis. Exp. (81), e50839, doi:10.3791/50839 (2013).
2. Gregory F. Pirrone, Heather Wang, Nicole Canfield, Alexander S. Chin, Timothy A. Rhodes, and Alexey A. Makarov. Use of MALDI-MS Combined with Differential Hydrogen−Deuterium Exchange for Semiautomated Protein Global Conformational Screening, Anal. Chem. 2017, 89, 8351−8357, DOI:10.1021/acs.analchem.7b01590