Probe the Protein Conformation using Top-down Hydrogen Exchange Mass Spectrometry at Higher Resolution with Electron Transfer Dissociation

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Abstract

Purpose: Study protein conformation by hydrogen/deuterium exchange mass spectrometry (HDX) using electron transfer dissociation (ETD) to get single amino acid resolution.

Methods: A model peptide, referred as P1, was used to confirm the proper ETD experimental condition for low deuterium scrambling and back exchange. Apo- and holo forms of calmodulin were studied by top-down and bottom-up HDX-ETD experiments using a fully automatic HDX workflow station with a Thermo Scientific™ Orbitrap ™ Ascend™ Structural Biology Edition mass spectrometer.

Results: Near 100% sequence coverage was obtained by bottom-up ETD MS2 experiments for apo- and holo- calmodulin, while to-down ETD gave 88% sequence coverage. Overall, the apo calmodulin showed more deuterium incorporation than the holo- calmodulin. Notably, nearly single amino acid deuterium incorporation results were observed from consecutive N and C terminal fragments combining top-down and bottom-up experiment.

Introduction

When HDX is analyzed at the peptide or protein level, only the overall deuterium uptake of peptides or proteins is obtained. Determination of deuterium incorporation at single amino acid resolution requires fragmentation which may induce "hydrogen scrambling". ETD, as a nonergodic, fragmentation technique could yield a low level of hydrogen scrambling and therefore allow single residue localization of incorporated deuterium. Calcium binding protein, calmodulin, was studied with top-down and bottom-up approaches to compare the protein conformation for its apo- and holo- forms.

Materials and methods

Scrambling Optimization

A model peptide P1 was prepared in both H_2O and D_2O at 10 µmol/L and kept overnight allowing complete deuterium exchange. 10 µL of the solution was injected to a C18 trap column with a 2 min short gradient elution for back-exchange and desalting.

Calmodulin ETD HDX workflow

Apo- and holo- calmodulin samples were diluted at a 1:1 ratio with labeling buffer and incubated for various time points. In the bottom-up ETD workflow, the samples were quenched and digested before being loaded onto a Thermo Scientific™ Hypersil GOLD™ C18 column. Conversely, for the top-down ETD workflow, the denatured samples were desalted using the C18 trap and directly eluted to the Orbitrap Ascend MS.

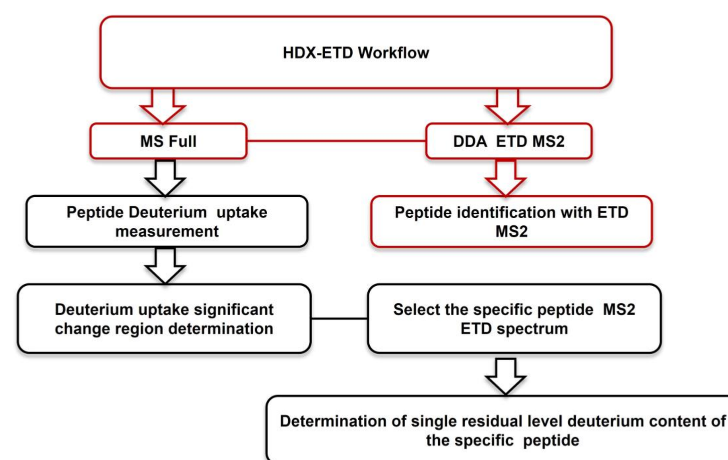

Figure 1. Scheme of bottom-up ETD experiment workflow.

Data Analysis

Peptide identification was performed with Thermo Scientific™ BioPharma Finder™ 5.2 software (BPF). Both top-down and bottom-up HDX experimental data were analyzed with HDExaminer™ software 3.4.1 (Trajan).

Results

ETD Experimental Condition Optimizations

The synthetic peptide P1,HHHHHHIIKIIK, was used to evaluate ETD experimental condition. 10 µL of 10 µMol fully deuterated P1 was injected into a C18 trap column. Multiple MS parameters were evaluated and optimized to reducing scrambling to a negligible level while minimizing the impact on sensitivity on a high-resolution OT

MS full and ETD MS2 spectra were collected during the same injection (**Figure 2**) and MS several transmission voltages, showing in **Figure 3**, were further investigated.

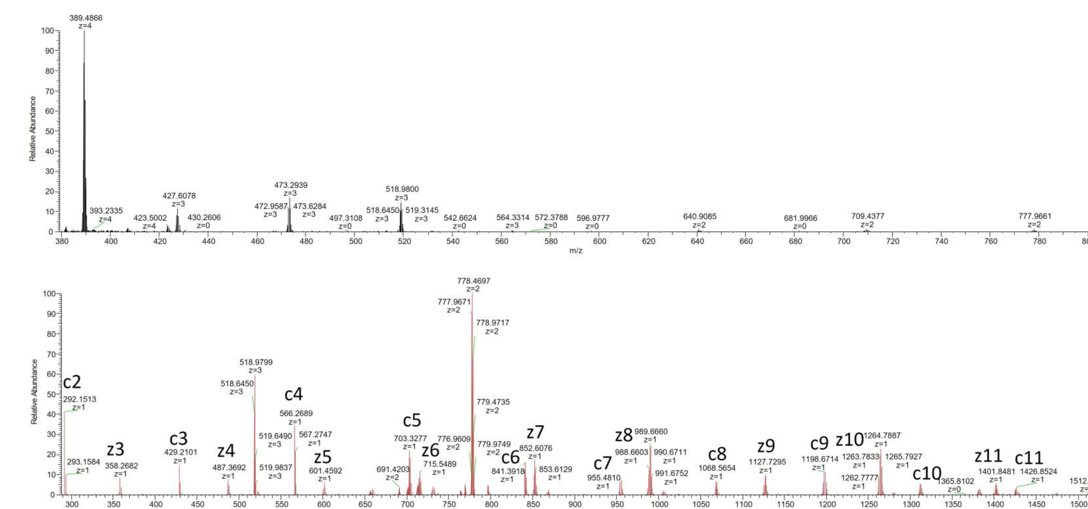

Figure 2. Model peptide P1 MS full scan and ETD spectrum of +3 precursor, *m/z* 518.9800.

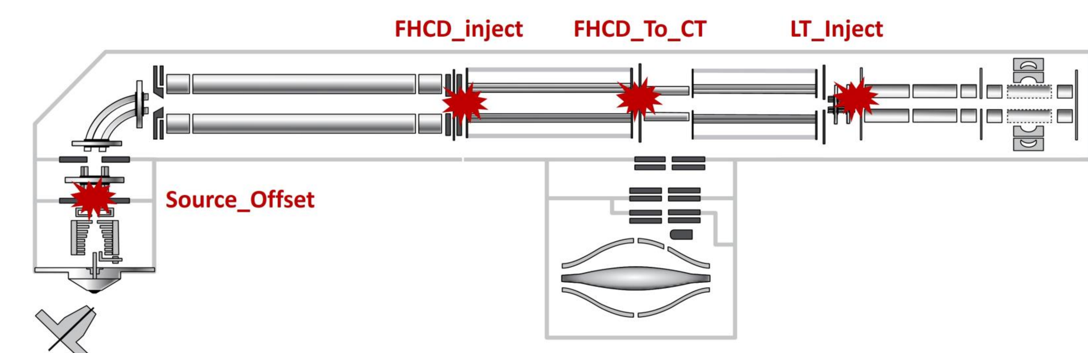

Figure 3. Architecture of Thermo Scientific™ Orbitrap ™ Ascend™ Structural Biology Edition mass spectrometer.

The low deuterium incorporation between c2 and c6 is indicate a low level of deuterium scrambling. The deuterium content for the c fragments of peptide P1 is comparable to what is reported in the literature, as showing in **Figure 4a** for different OT tribrids MS. Additionally, the scrambling level was also evaluated using the ammonia loss as a reporter ion of scrambling, as showing in **Figure 4b**.

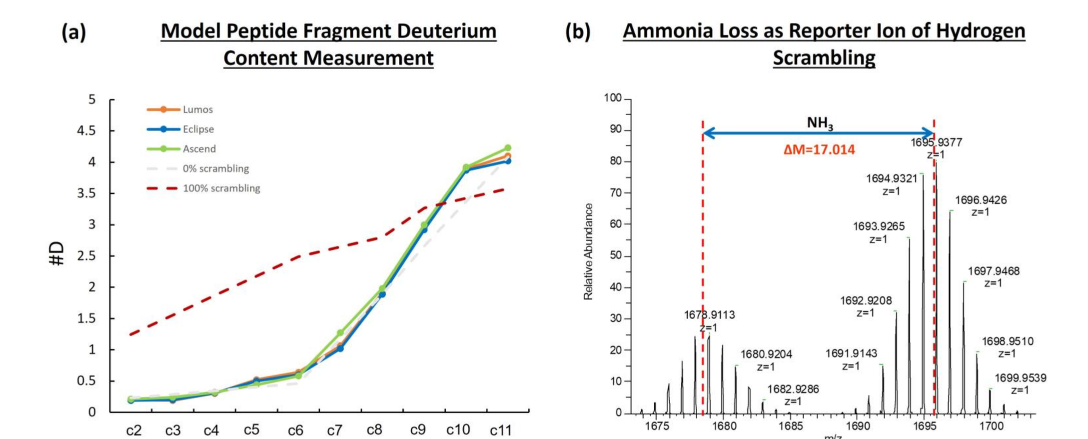

Figure 4. Measurements of P1 ETD Fragment deuterium content on OT Tribrid Mass Spectrometers.

Minimal fragment deuterium scrambling measurement was obtained with optimized instruments and source operation conditions. Recommended settings for tuning OT Ascend are summarized in **Table 1**.

Global Parameters	Ion Transfer Tube Temp.	Vaporizer Temp.*	RF Lens (%)
Recommended Settings	220 ºC	50 ºC	25

Transmission Voltages	Source Offset	FHCD_Inject	FHCD_To_CT	LT_Inject
Recommended Settings	10-11	-3 to -4	4 to 5	-12

* Vaporizer Temp. is recommended based on flow rate at 40 µl/min. Need to be adjusted accordingly for different flow rate

Table 1. Summarized recommending parameter settings.

Calmodulin and Ca^{2+} Binding System

Calmodulin is a calcium binding protein. It is known that once calcium binds to the protein, the conformation has changed. C terminal folding orientation changes more than N terminal after binding.

There are four Ca binding sites at region 21-32, 57-68, 94-105, and 130-141, highlighted in yellow in **Figure 5**.

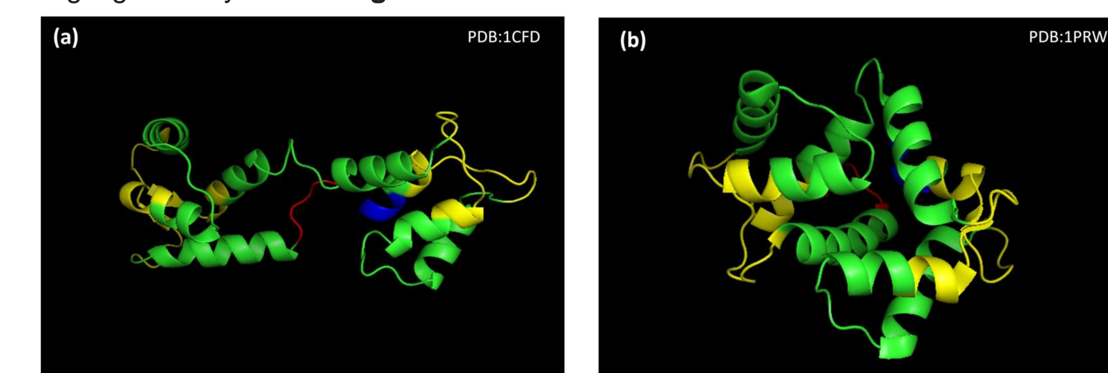

Figure 5. Crystal structure of Apo-, holo- calmodulin forms. Ca^{2+} binding sites are colored in yellow.

Bottom-up ETD MS2 Experiment

Bottom-up ETD at four labeling time points experiments were conducted for the apo-, holo- calmodulin forms. 140 peptides were identified and used to do deuterium uptake measurements for both apo-, holo- calmodulin forms, giving 97% sequence coverage (shown in **Figure 6**). The apo calmodulin showed more deuterium incorporation than the holo- form, and there were more deuterium uptake on the C-terminal peptides than N-terminal peptides.

One representative peptide at the binding region was used to show how to use ETD data to obtain near single amino acid resolution, showing in **Figure 7**.

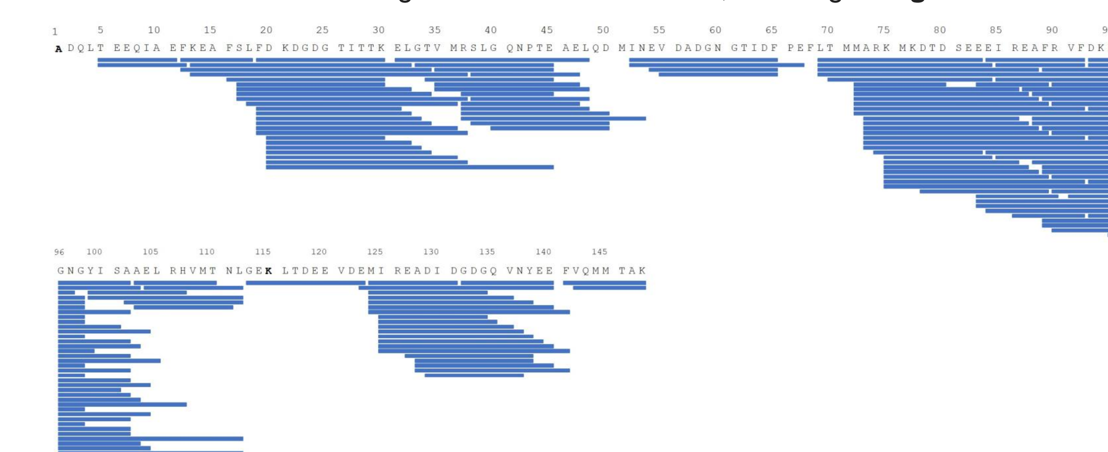

Figure 6. Sequence coverage for bottom-up ETD experiment

One representative peptide, residue 20-32, covering one of the binding regions was used to show how to use ETD data to obtain near single amino acid resolution (Fig.7 b). As showing in **Figure 7 (a)**, c2 to c12 and z3 to z12 except z5, were identified and uptake curves were showed for each one of the c ions in **Figure 7 (c)**.

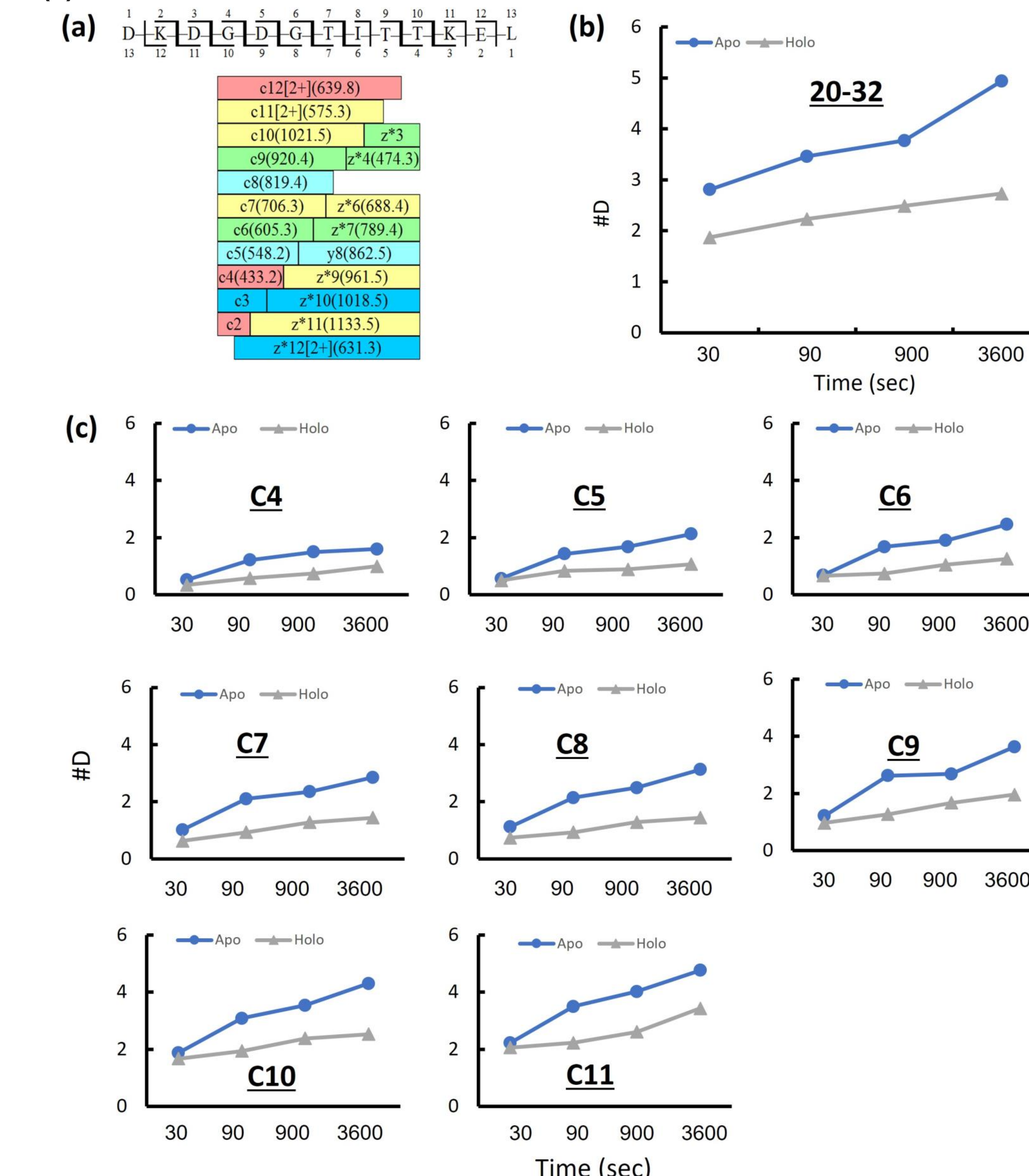

Figure 7. Representative peptide 20-32. (a) Fragment coverage map; (b) Peptide level uptake curve; (c) Uptake curve of c4 to c11 ions.

With the obtained fragment ion deuterium incorporation information, the fragment verses deuterium content could be plotted out as in **Figure 8**. For this particular peptide, deuterium incorporation was obtained at near single amino acid resolution by ETD fragment ions.

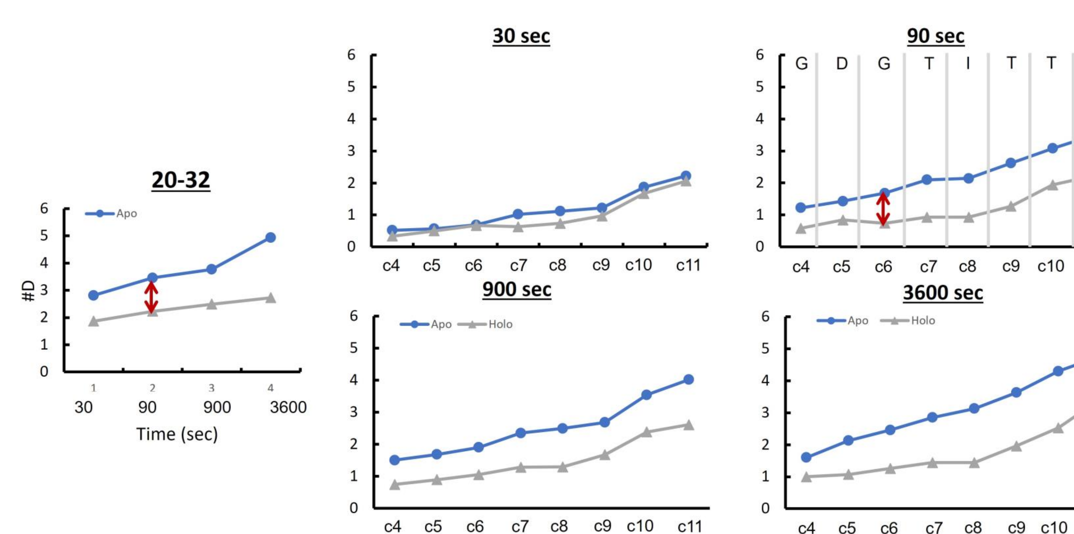

Figure 8. Fragments deuterium difference between holo- and apo- states.

Top-Down ETD Experiment

Top-down workflow can aid in distinguishing variants. As showing in **Figure 9**, calmodulin with a N-terminus His-tag displayed a higher charge distribution compared to the variant without it. The His-tag introduces more charges to the N-terminus, resulting in increased coverage at the N-terminus but reduced coverage at the C-terminus.

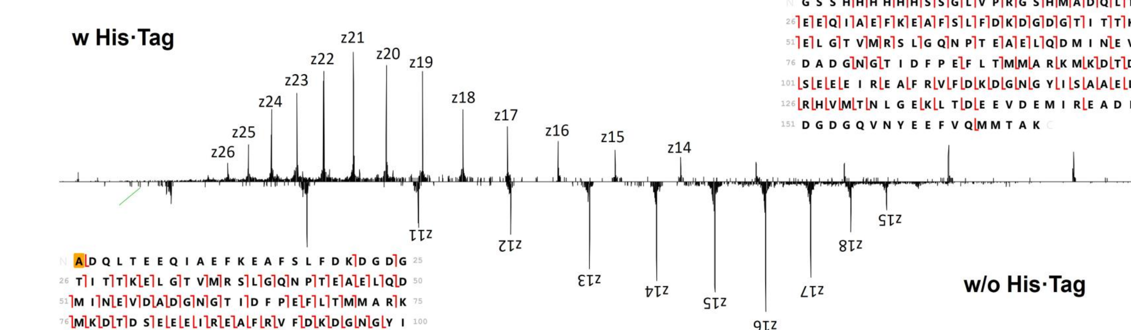

Figure 9. Top-down ETD MS full scan and sequence coverage map for two Calmodulin variants. Top panel: with His-Tag; Bottom-panel: without His-Tag.

Top-down MS full scan with targeted ETD MS2 was performed for apo-, holo- calmodulin (no His-Tag) at different labeling time points. The deuterium incorporation of the intact level was measured. The apo- calmodulin had more deuterium incorporation than the holo- form for all the measured time points. HDExaminer was used to calculate the deuterium incorporation for each fragment.

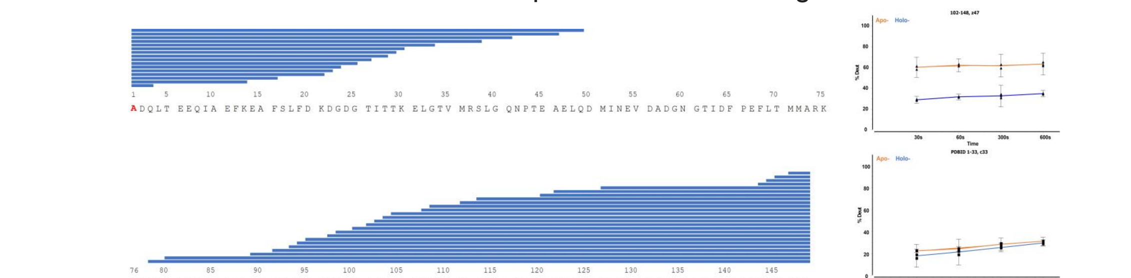

Figure 10. Sequence coverage for top-down ETD experiment and uptake curves of two representative fragments.

Conclusions

- The low deuterium scrambling HDX-ETD calibration procedure using P1 peptide as standard was developed for OT Ascend.
- HDX-ETD top-down and bottom-up experiments were successfully applied to study apo-, holo- calmodulin forms conformation. Consistent results were obtained from the two approaches. Overall, the apo- calmodulin had more deuterium incorporation than the holo- calmodulin form.
- Nearly single amino acid resolution was obtained for N-, and partial of C-terminals from the top-down experiment. Bottom-up ETD MS2 experiment data provided complementary information on nearly amino acid resolution to the top-down experiment.

References

1. Kasper D. Rand and Thomas J. D. Jørgensen, Analytical Chemistry 2007 79 (22), 8686-8693, DOI: 10.1021/ac0710782
2. Kasper D. Rand, Martin Zehl, Ole N. Jensen, and Thomas J. D. Jørgensen, Analytical Chemistry 2010 82 (23), 9755-9762DOI:10.1021/ac101889b

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