

# <sup>18</sup>O Labeling and Protein AQUA: Combined Isotopic Labeling Strategies for the Evaluation of Protein Response to Hydrogen Peroxide Induced Oxidative Stress

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## Abstract

Many proteomics researchers are faced with the question of how a cell will respond to a certain type of stress. Often, the cellular response involves either up or down regulation of a variety of proteins in an attempt to adapt to this stress. Evaluating the extent of this protein regulation can be quite challenging. In this study, we will show that through a combined analysis using <sup>18</sup>O water and Protein AQUA™, qualitative and quantitative differences in protein populations can be determined. This work was carried out on mammalian cell cultures, which were subjected to oxidative stress through incubation with hydrogen peroxide. Hydrogen peroxide serves as a chemical stimulus used to induce reactive oxygen species (ROS) effects. ROS affect the cell in numerous ways, and are thought to play a role in aging and apoptosis.<sup>2</sup> Proteins exhibiting up or down regulation due to the ROS effects were identified through isotopic labeling with <sup>18</sup>O water. Following identification, specific AQUA peptides were spiked into the samples to obtain absolute quantification data on selected proteins. Through the combination of <sup>18</sup>O labeling and Protein AQUA, we have demonstrated a versatile method for examining changes in protein expression levels. This approach can be utilized for examining cellular responses to not only oxidative stress, but for other stressors such as environmental and chemical insults.

## Introduction

The basis of this study is to show how two biological samples exhibiting differential protein expression can be evaluated through the combined analysis of isotopic labeling by <sup>18</sup>O water and Protein AQUA. By combining these two technologies, the researcher is able to obtain a global analysis of the protein mixtures through <sup>18</sup>O labeling, followed by a targeted interrogation and quantitation of a protein of interest using Protein AQUA.

The <sup>18</sup>O labeling process starts with two samples of tryptically digested peptides. The dried samples are then reconstituted with either <sup>18</sup>O water or <sup>16</sup>O water in the presence of trypsin. The enzymatic activity of the trypsin enables oxygen exchange at the carboxyl terminus of the peptide to incorporate two <sup>18</sup>O atoms, generating a labeled peptide. The incorporation of the two <sup>18</sup>O atoms creates a +4 Da mass shift (relative to an unlabeled "control" sample). By mixing the two samples just prior to mass spectrometric analysis, the labeled and unlabeled samples can be analyzed simultaneously, allowing for the relative abundances of all proteins within the sample to be quantified (Figure 1A).

The Protein AQUA™ technology was developed by Dr. Steve Gygi and his team in 2003. This technology employs the use of an isotopically labeled peptide standard, which corresponds to a known tryptic peptide from the protein of interest. A known amount of peptide standard is then spiked into a protein sample and subsequently analyzed by LC-MS. This allows for absolute quantification of the target protein (Figure 1B).

Oxidative stress is one of many causes for cells to exhibit differential protein expression. For these experiments, CHO cells were subjected to oxidative stress with hydrogen peroxide. In this study, we have used the <sup>18</sup>O labeling technology to identify proteins in subsequent lysates that exhibit differential expression due to oxidative stress. This was followed by Protein AQUA analysis to obtain absolute quantification data for a specific protein.

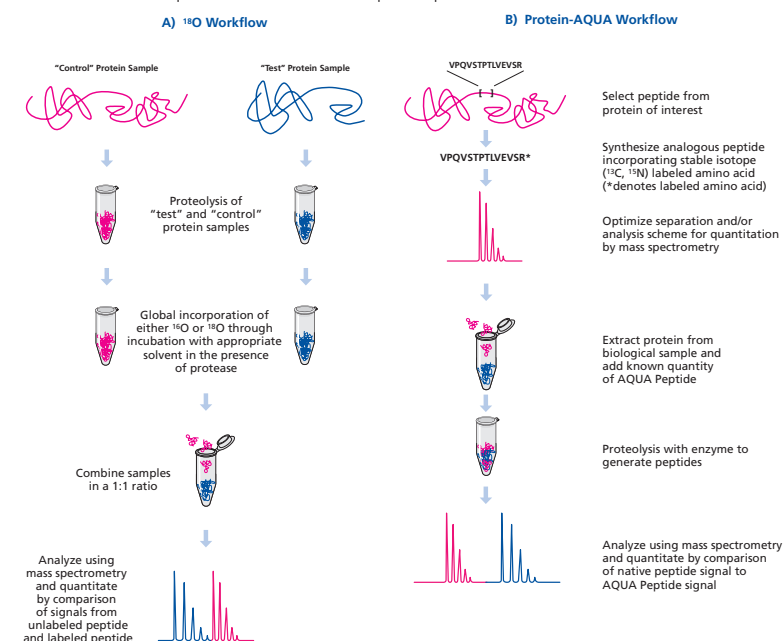


Figure 1: Overview of the <sup>18</sup>O and Protein AQUA procedures.

## Materials

All products were obtained from Sigma-Aldrich (St. Louis, MO), unless otherwise noted.

- Proteomics Grade Trypsin (Cat. No. T6567)
- EX-CELL™ ACF CHO Medium (Cat. No. C5467)
- Protein Extraction Reagent (Cat. No. C0356)
- Spermine (Cat. No. S3256)
- TCEP 0.5 M (Cat. No. 646547)
- Bradford Reagent (Cat. No. B6916)
- ProteoPrep Reduction/Alkylation Kit (Cat. No. PROTRA)
- Trypsin Profile IGD Kit (Cat. No. PP0100)
- ProteoPrep TCA Precipitation Kit (Cat. No. PROTPR)
- <sup>18</sup>O Proteome Profiler Kit (Cat. No. P3623)
- All of the isotopically labeled peptides used in this experiment were synthesized and purified by Sigma-Genosys (The Woodlands, TX)

## Methods

### Culturing of CHO Cells

CHO K1 cell suspensions were grown in six culture flasks for 24 hours at 1 x 10<sup>6</sup> cells/mL. The cells were grown in EX-CELL™ ACF CHO Medium.

### Induction with H<sub>2</sub>O<sub>2</sub>

The H<sub>2</sub>O<sub>2</sub> was prepared for use by diluting 30% H<sub>2</sub>O<sub>2</sub> with high purity water to a final concentration of 9.87 mM. The solution was then filtered with a 0.22 µm PVDF filter. Three flasks of CHO K1 cells were induced with 100 µM H<sub>2</sub>O<sub>2</sub>. Following three hours of incubation, a 5 mL aliquot was removed from each of the cultures. The remainder of the cultures were allowed to incubate for a total of 24 hours, after which time a second 5 mL aliquot was removed. Following removal from the flasks, the aliquots were centrifuged, and the supernatant discarded. The proteins were extracted from each of the cell pellets through the addition of 1 mL of extraction reagent. The samples were stored at -20 °C prior to analysis.

The extraction reagent was prepared by adding 10 M spermine, and 5 mM TCEP to one bottle of Protein Extraction Reagent, and then diluting the solution with 14.75 mL of high purity water. The samples were then incubated for 1 hour with mixing at room temperature, followed by centrifugation for 30 minutes. The resulting supernatant contained the soluble proteins.

The protein concentration of each sample was determined by Bradford assay. The samples were diluted to 1 mg/mL with Protein Extraction Reagent. Following dilution, the samples were reduced and alkylated using the ProteoPrep Reduction and Alkylation Kit.

### Gel Electrophoresis and Analysis

An aliquot of the reduced and alkylated samples was removed for analysis by 2D gel electrophoresis. IPG strips (11 cm, pH 7.4) were rehydrated with 200 µg of sample for 3 hours at room temperature. The strips were focused overnight at 8000 V for 85,000 Vhr. Following focusing, each strip was equilibrated in 2 mL of IPG Equilibration Buffer and then loaded onto 4-20% Criterion Gels. The gels were electrophoresed at 80 V for 10 minutes followed by 170 V for 70 minutes. The gels were then stained with EZBlue Gel Staining Reagent and scanned using a UMAX PowerLook 2100XL scanner.

The gels images were then analyzed using Phoretix software to determine relative differences between the test and control samples.

### <sup>18</sup>O Labeling Process

Samples from the 24 hour incubation were labeled using the <sup>18</sup>O Proteome Profiler Kit. Following reduction and alkylation, the samples were cleaned up with the provided spin columns, and digested according to the manufacturer's directions. The samples were then dried and reconstituted with 10 µL of acetonitrile. The isotopic label (either <sup>18</sup>O or <sup>16</sup>O) was then incorporated into the samples using the Trypsin Singles Proteomics Grade Enzyme (a component of the <sup>18</sup>O Proteome Profiler Kit). The trypsin was reconstituted using the appropriate type of water, and 40 µL of reconstituted trypsin was added to each sample. This resulted in a concentration of approximately 1 µg of trypsin per 50 µg sample. The samples were allowed to incubate for 18 hours at 37 °C, following which time, the reactions were stopped by the addition of 5 µL of 1% TFA. The samples were then dried in a vacuum centrifuge.

Samples were redissolved with 80% acetonitrile / 0.1% formic acid and the test and control samples were combined in a one-to-one ratio. The samples were run individually through a 2-step 2D Liquid Chromatography (LC) system. The samples were first injected on a CTI SCX column (5 cm x 320 µm) and then eluted with successive salt steps onto a LC packings trap (MGV-80C18 PM, 2 mm X 800 µm). Peptides were eluted from the trap onto a Micro-Tech Scientific C18 column (MC10-C18W-150MS, 10 cm x 150 µm) with a 1 hour 0-80% linear gradient (A: 0.1% formic acid, B: 100% acetonitrile/0.1% formic acid) after each salt step. The eluted peptides were analyzed by MS.

The MS data was evaluated with BioWorks™ 3.2 to determine the identity of peptides present. Raw files were then evaluated with XChange using the BioWorks™ results as a filter.<sup>3</sup> Data were eliminated from consideration if a BLAST search could not identify the peptide in mouse or rat databases, or if the <sup>18</sup>O/<sup>16</sup>O ratio failed either the Q-test or Chauvenet's criteria.

### AQUA Procedure

One protein, α-enolase was chosen for Protein AQUA analysis. Following reduction and alkylation, the protein samples were TCA precipitated using the ProteoPrep Protein Precipitation Kit. The protein pellets were redissolved in 100 µL of 50 mM Tris HCl pH 8.0. Trypsin was added at a 1:80 enzyme-to-substrate ratio, and the samples were allowed to digest overnight at 37 °C. Trypsin was again added at a 1:80 ratio and allowed to digest for an additional three hours. The samples were then dried in a vacuum centrifuge. Samples were analyzed on a Thermo Electron linear ion trap mass spectrometer.

## Results

### 2D Gel Analysis of Differential Expression

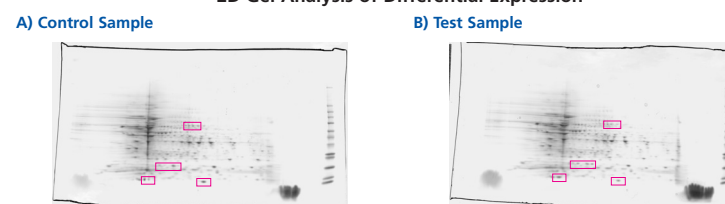


Figure 2: Samples from the three hour induction time point were subjected to 2D gel electrophoresis. The gels were analyzed using Phoretix software. The boxes highlight some proteins that showed differential expression between the control and test samples. Cofilin, Pyruvate Kinase and Ubiquitin were some of the proteins identified as showing differential expression between the two samples (highlighted in red).  
 A. 2D gel of control sample (non-induced).  
 B. 2D gel of test sample (induced with H<sub>2</sub>O<sub>2</sub>).

### <sup>18</sup>O Labeling Analysis of Differential Expression

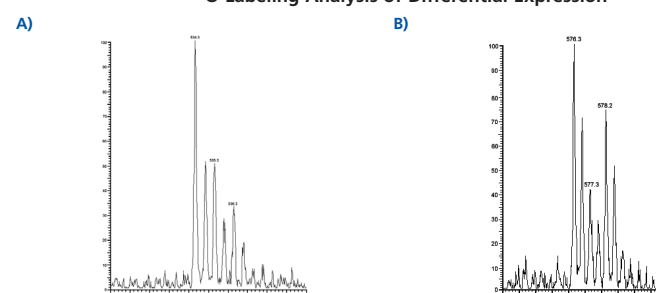


Figure 3: LC-MS Analysis of two tryptic peptides identified from the <sup>18</sup>O labeling samples. During analysis of the <sup>18</sup>O labeled samples, the <sup>18</sup>O/<sup>16</sup>O ratio was determined using an algorithm that takes into account the normal isotopic distribution of the peptides as well as efficiency of <sup>18</sup>O incorporation. The Xchange program that was used to quantitate the data adds an additional feature to this algorithm by compensating for baseline noise.  
 A. Ubiquitin peptide ESTLHLVLR (m/z 534.301). The test sample (<sup>16</sup>O) shows up-regulation over the control sample (<sup>18</sup>O). This result is also corroborated by several literature references that mention the regulation of ubiquitin in response to oxidative stress.<sup>4</sup>  
 B. YIDQEELMK from Heat Shock Protein 90B (m/z 576.269). The ratio of test-to-control is 1.26 as calculated by the Xchange program.

### Relative Quantitation of Six Proteins

Protein	Peptide	Peptide m/z	<sup>18</sup> O/ <sup>16</sup> O	Average	Xchange Ratio	Average
Alpha Enolase	IEEELGSK	452.721	1.49		1.11	
Alpha Enolase	IEEELGSK	452.721	1.36	1.43	1.16	1.14
Cofilin	EILVGDVGVQTVDDPYTFV	1098.543	1.56		1.97	
Cofilin	KSSTPEEVK	502.753	1.54	1.55	1.46	1.72
Heat Shock Protein 71 KDa	EEFEHQK	537.732	2.09		1.45	
Heat Shock Protein 71 KDa	IINEPTAAAIAYGLDK	830.438	1.44	1.77	1.57	1.51
Ubiquitin	ESTLHLVLR	534.301	2.19		1.59	
Ubiquitin	ESTLHLVLR	534.301	1.83	2.01	1.47	1.53
Pyruvate Kinase	GVNLPAAVADLPAVSEKK	818.935	1.55		1.40	
Pyruvate Kinase	TATESFASDPILYR	785.878	1.67	1.61	1.96	1.68
Heat Shock Protein 90B	YIDQEELMK	576.269	1.17		1.26	
Heat Shock Protein 90B	ADHGPEIGR	476.223	2.91	2.04	1.56	1.41

Figure 4: Data from six proteins that have been regulated in response to induction by H<sub>2</sub>O<sub>2</sub>. The table shows two averages for each protein. The <sup>18</sup>O/<sup>16</sup>O ratio was calculated by using the inverse of the following equation:  $^{18}\text{O}/^{16}\text{O} = [(I_1 - (M_1/M_2)_0) - (M_2/M_1)_1] / [I_2 - (M_2/M_1)_0]$ . (Reference 5) The second ratio was calculated using the Xchange program.

### Differential Regulation in Response to H<sub>2</sub>O<sub>2</sub> Induction

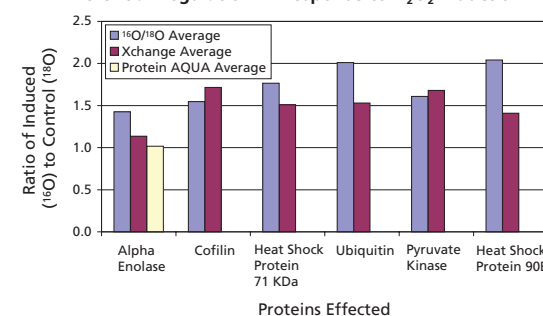


Figure 5: Graph highlighting the proteins mentioned in Figure 4. The graph shows the ratio of test versus control sample as measured by the formula for quantifying <sup>18</sup>O labeling (detailed in the figure legend for Figure 4), as measured by the Xchange program, and where applicable, as measured by Protein AQUA.

### Protein AQUA Quantitation for α-Enolase

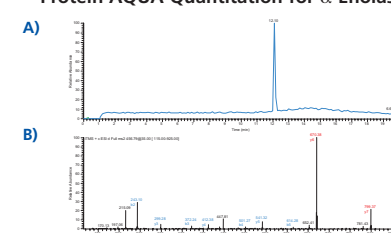


Figure 6: An 8 µL injection of previously described trypsin digested cell lysate was made onto an Agilent capillary 1100 HPLC using a simple 30 minute linear gradient. This reverse phase separation was performed on a 15 cm x 2.1 mm Discovery C18 column. The mobile phase consisted of formic acidified water and acetonitrile. This separation system was coupled to a Thermo Electron linear ion trap mass spectrometer set to perform tandem mass spectrometry. Figure 6A depicts the AQUA peptide. Figure 6B depicts its fragment ions from which y<sub>6</sub> and y<sub>7</sub> were selected for selective reaction monitoring (SRM).

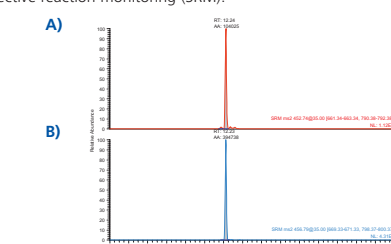


Figure 7: Selective reaction monitoring specifically targets MS detection of the specified parent and daughter ions. Figure 7A depicts the endogenous α-enolase peptide IEEELGSK as the doubly charged 456 m/z ion fragments to produce two abundant daughter ions y<sub>6</sub> and y<sub>7</sub>. These same daughter ions are monitored in the AQUA peptide standard shown in Figure 7B, however, the absolute mass to charge of both parent and daughter ions in the AQUA spike was 10 Da greater due to the incorporation of the stable isotope K (<sup>41</sup>K/<sup>39</sup>K). Quantitation was performed by integrating the co-eluting peak areas shown here at 12 minutes. All remaining non-specific signal is discounted because the reversed phase retention characteristics do not match those of the peptide of interest. The average ratio from analysis of replicate AQUA samples was 1.02:1 (test-to-control). This compared favorably to the <sup>18</sup>O average as determined by Xchange for the same peptide which showed an average ratio of 1.14:1.

## Conclusion

In this series of experiments, three independent methods were used to evaluate the effects of a biological stressor on cellular proteins: 2D gel analysis, <sup>18</sup>O labeling and Protein AQUA.

- The conventional method (analysis by 2D gel electrophoresis), allowed for the identification of several proteins that exhibited differential protein expression. Some proteins identified by included cofilin, pyruvate kinase, and ubiquitin. The proteins that had been identified from the 2D gel analysis were also identified by <sup>18</sup>O labeling.
- Because <sup>18</sup>O labeling is a global labeling technique, all peptides within the sample (except for the C-terminal peptide of each protein) had an isotopic label associated with them.
- Peptides that exhibited a non 1:1 ratio were assumed to have been affected by the treatment with H<sub>2</sub>O<sub>2</sub>.
- The <sup>18</sup>O labeling offers a simplification over 2D gel analysis in that all affected proteins can be evaluated without having to perform individual in-gel digestions and analysis. This allowed for easy identifications of additional proteins that had not been identified during analysis by 2D gel electrophoresis, namely Heat Shock protein 71 KDa, and Heat Shock Protein 90 Beta.
- The <sup>18</sup>O/<sup>16</sup>O ratios obtained by the Xchange program correlated well with the ratios obtained by the currently accepted formula for <sup>18</sup>O quantitation.
- Protein AQUA was used to obtain absolute quantification data on a protein of interest, namely α-enolase. Evaluation of the peptide IEEELGSK by both <sup>18</sup>O labeling and Protein AQUA confirmed that in this sample the protein exhibited a near 1:1 ratio, and was therefore not likely affected by the stressor.
- By combining the complimentary technologies of <sup>18</sup>O labeling and Protein AQUA, we have shown a multiplexed approach to evaluating differential protein expression. By utilizing <sup>18</sup>O labeling we were able to evaluate the global changes of the protein sample in response to a biological stressor. When followed by Protein AQUA, we were able to verify our <sup>18</sup>O data with absolute quantification data on a targeted protein.

## Acknowledgements

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## References

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