

Evaluation of Differential Protein Expression Through the Combination of RNAi and Isotopic Labeling Using ^{18}O Water

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Abstract

The study of differential protein expression is a challenge faced by many researchers. In this study, we show how isotopic labeling by ^{18}O water can be coupled with RNAi knockdown to evaluate differential expression by determining relative protein levels. This work was carried out on pooled protein lysates derived from control cells and cells knocked down by siRNA constructs for the protein GAPDH. After running the samples on an SDS-PAGE gel, the band of interest was excised and the proteins subjected to in-gel tryptic digestion. A second trypsin incubation was used to enzymatically incorporate either ^{18}O or ^{16}O into the samples. Through the incorporation of the isotopic label, a mass shift was generated in the test sample peptides allowing them to be easily differentiated from the control sample analogues via analysis with Matrix-Assisted Laser Desorption Ionization/Time of Flight Mass Spectrometry (MALDI-TOF MS). By analyzing the samples in this manner, relative abundances between experimental and control populations were determined. In combining the technologies of isotopic labeling and RNAi knockdown, we have demonstrated a convenient and versatile approach to examining the effects of differential protein expression.

Introduction

The basis of this study is to show how two biological samples exhibiting differential protein expression can be evaluated through isotopic labeling by ^{18}O water. The labeling process starts with two samples of peptides that have been tryptically digested and dried down. The isotopic label is incorporated when the samples are reconstituted with either ^{16}O water or ^{18}O water in the presence of trypsin. The carboxyl terminus of the peptide receives the label by way of incorporating two ^{18}O atoms. The incorporation of the two ^{18}O atoms creates a +4 Da mass shift (relative to an unlabeled analogous or "control" sample). By mixing the two samples just prior to mass spectrometric analysis, the labeled and unlabeled samples can be analyzed simultaneously and their relative abundances quantified. Because this type of labeling is a global labeling procedure, all protein levels that may have been affected by the stressor (e.g., RNAi knockdown, chemical treatment, etc.) can be evaluated. The workflow is illustrated in [Figure 1](#).

Gene knockdown using small interfering RNA (siRNA) constructs is a powerful tool for studying the biological effects of decreased levels of a specific message (mRNA) and subsequent protein levels. Analysis of RNAi knockdown is typically performed by Northern blot, quantitative PCR, or Western blot. While the Western blot is specific for the protein of interest, it is not amenable for easily discerning minimal differences between protein levels. While relative abundances can be determined from a Western blot, doing so requires expensive equipment and laborious method development in order to ensure reproducible results. In addition, the data obtained is specific to one protein and does not provide information on additional proteins that may have been affected by the gene knockdown.

Isotopic labeling using ^{18}O water is a much more sensitive and versatile method. Using this methodology, relative abundances between a control and test population can easily be quantified. In addition to increased sensitivity, because ^{18}O is a global labeling procedure, effects of the gene knockdown on proteins other than the target protein can also be studied. In short, by coupling ^{18}O labeling with gene knockdown by siRNA, relative abundances of affected proteins can be identified.

Illustration of ^{18}O Labeling to Discern Varying Protein Levels

In order to demonstrate proof of principle for this method, protein mixtures of aldolase and lysozyme were analyzed. The samples were reduced, alkylated, and tryptically digested. Following digestion, the samples were aliquoted such that the two samples contained a protein ratio of 1:1 for aldolase and 1:3 for lysozyme. The samples were then labeled with either ^{16}O or ^{18}O in the presence of trypsin and allowed to incubate at 37 °C overnight. The two samples were mixed just prior to analysis by MALDI-TOF MS. The data generated from this experiment is illustrated in [Figure 2](#).

Experimental Results of Model Protein Experiment:

(ratios given as ^{16}O : ^{18}O).

Aldolase ratio = 1.03; (c.f. Theoretical ratio = 1.0)

Lysozyme ratio = 0.34; (c.f. Theoretical ratio = 0.33)

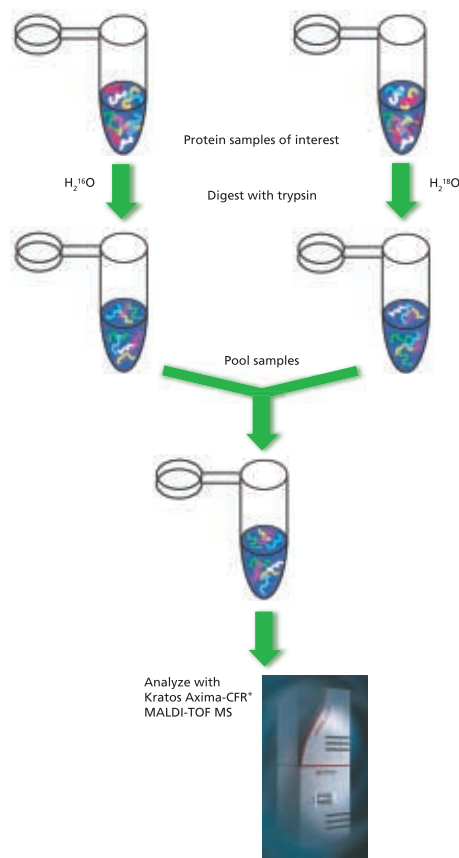


Figure 1.

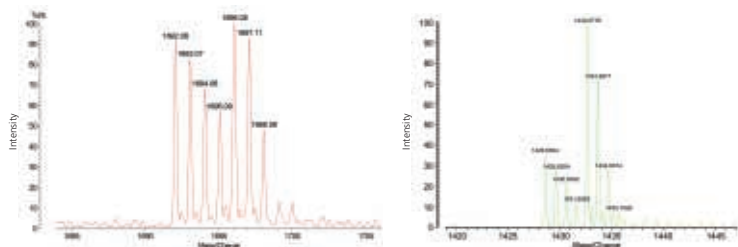


Figure 2. Two samples of known concentration were analyzed using the ^{18}O water labeling method. The samples were derived from a protein mixture of aldolase and lysozyme (hen egg white).

- A.** MALDI-TOF mass spectrum of the peptide YSHEEIAMATVTALR derived from aldolase. This represents expected results obtained when two protein solutions of equal concentration are analyzed using this method. The base peak at m/z 1692.08 (mass +0 Da) has approximately the same intensity or integrated peak area as the peak at m/z 1696.08 (mass +4 Da). The peaks at 1693.07, 1694.06, and 1695.09 (as well as those at 1697.11 and 1698.06) represent the normal isotopic distribution obtained by the mass spectrometric analysis of a peptide.
- B.** MALDI-TOF mass spectrum of the peptide FESNFNTQATNR derived from lysozyme. This represents expected results obtained when two protein solutions, with concentration ratios of 1:3 based on protein amounts, are analyzed using this method. The base peak at m/z 1428.64 has approximately one third of the intensity as the peak at m/z 1432.68.

Methods

All reagents were obtained from Sigma-Aldrich unless otherwise noted.

Culturing of HeLa Cells

HeLa cells were plated at 20,000 cells/mL in 100-cm dishes and allowed to grow for 24 hours in Dulbecco's Modified Eagle's Medium (high glucose) (Product Code [D5671](#)) supplemented with 5% Fetal Bovine Serum (Product Code [F6178](#)) and 4 mM L-glutamine (Product Code [G7513](#)).

Transfection of HeLa Cell Cultures

The spent media was removed and 21 mL of fresh media (DMEM, 5% FBS, 4 mM [L-glutamine]) was added to each culture plate 2 hours before the transfections. Dharmacon siGENOME SMARTpool siRNA (Dharmacon Catalog #M-004253-01) stocks for Human GAPDH, RISC-free (non-functional, non-interfering), two non-interfering sequences, and a non-interfering pool were prepared according to the manufacturer's instructions. The SMARTpool control reagents (non-interfering, non-interfering pool, and RISC-free) were transfected at a final concentration of 19 nM to the HeLa cell cultures using Escort V Transfection Reagent (Product Code [E9778](#)). Each transfection was performed in duplicate. For the test samples, SMARTpool Human GAPDH siRNAs were transfected (at final concentrations of 9.6 nM, 19 nM, and 38 nM) to the HeLa cell cultures using Escort V Transfection Reagent. These transfections were also performed in duplicate. In addition, four mock transfections were performed by following the transfection procedure detailed above without the presence of an RNA duplex. Following the transfection procedures, the HeLa cell culture plates were incubated for an additional 48 hours to allow for sufficient knockdown of the target protein.

Sample Preparation

The samples were harvested by adding 1 mL of resuspension reagent to each culture dish and thoroughly scraping the culture plate. The resuspension reagent used was Hank's Balanced Salt Solution supplemented with 50 U/mL benzonase (Product Code [E1014](#)). The cell samples were frozen at $-20\text{ }^{\circ}\text{C}$ and then thawed to digest a majority of the chromosomal DNA. The samples were then denatured by the addition of solid urea to each sample at a final concentration of 8 M. The protein content was measured by BCA assay (Product Code [BCA-1](#)).

Gel Electrophoresis and Western Blot Analysis

As determined by BCA analysis, 10 μg of total protein was loaded onto a 4–20% SDS-PAGE gel. Duplicate copies of the gel were run such that samples could be used for in-gel digestion and Western blot analysis. The gels used for ^{18}O labeling were stained with EZBlue™ Gel Staining Reagent (Product Code [G1041](#)) and destained with water.

For the Western blot analysis, the proteins were transferred from the 4–20% SDS-PAGE gel to a nitrocellulose membrane (Product Code [N7892](#)). The membrane was blocked with Tris buffered saline (TBS) with 3% nonfat dry milk (Product Code [T8793](#)) for 30 minutes. The blot was incubated with 1 $\mu\text{g}/\text{mL}$ of the Anti-GAPDH antibody (Ambion Product #4300) in TBS-milk at room temperature with shaking for 30 minutes. The blot was incubated with anti-mouse IgG-HRP (Product Code [A9044](#)) at a 1:12,500 dilution in TBS-milk for 30 minutes. The blot was then washed five times for a total of 15 minutes in TBS-Tween 20 (Product Code [T9039](#)). Finally, the blot was incubated with an HRP chemiluminescent substrate CPS-1 (Product Code [CPS-1-120](#)) for 5 minutes and the image directly captured on a Bio-Rad Fluro-ST™ imaging system. The band density was then determined using the Quantity One software provided.

From the SDS-PAGE gel, the bands of interest containing the GAPDH protein were excised. Each gel slice was then reduced and alkylated using the ProteoPrep™ Reduction and Alkylation Kit (Product Code [PROT-RA](#)) and subjected to an in-gel tryptic digest using the Trypsin Profile IGD Kit (Product Code [PPO100](#)). Following digestion, the samples were vacuum dried in preparation for the labeling procedure.

The Western blot and SDS-PAGE gels are illustrated in [Figure 3](#).

^{18}O Labeling Process

The samples were labeled using the ^{18}O Proteome Profiler Kit (Product Code [P3623](#)). Following digestion, the dried samples were reconstituted with 4 μL of acetonitrile. The isotopic label (either ^{18}O or ^{16}O) was then incorporated into the samples using the Trypsin Singles Proteomics Grade Enzyme (a component of the ^{18}O Proteome Profiler Kit). The trypsin was reconstituted using the appropriate type of water and 16 μL of reconstituted trypsin was added to each sample. This resulted in a concentration of approximately 0.4 μg of trypsin per sample. The samples were allowed to incubate for 18 hours at $37\text{ }^{\circ}\text{C}$, following which time the reactions were stopped by the addition of 1.3 μL of concentrated formic acid. The samples were then dried in a vacuum centrifuge.

The samples were reconstituted with 0.1% TFA immediately prior to analysis and mixed in a 1:1 (control:test) volumetric ratio. These samples were then combined 1:1 with α -cyano-4-hydroxycinnamic acid solution and spotted onto a MALDI target. MALDI-TOF MS data was acquired using a Kratos Axima-CFR™ Plus mass spectrometer in positive ion reflectron mode.

Results

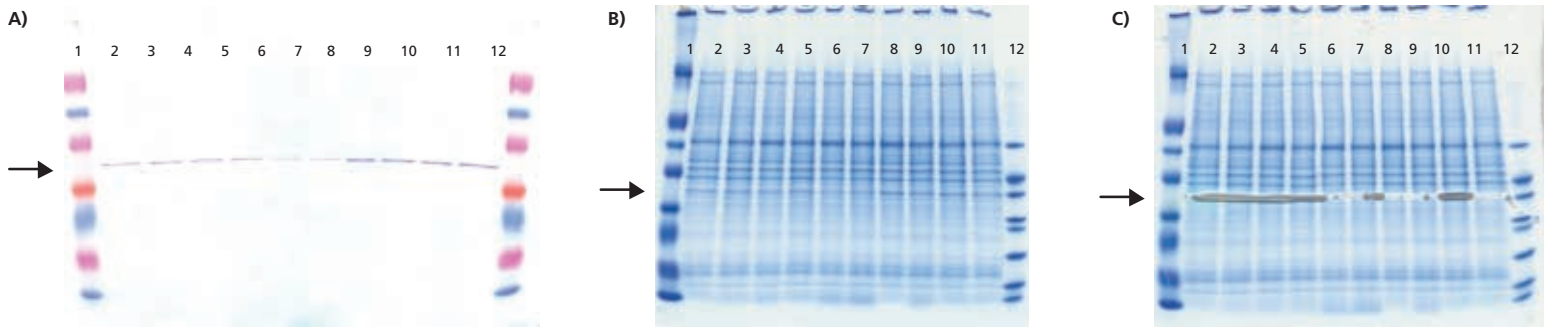


Figure 3. All gels were loaded in the following manner: Lane 1: ColorBurst Marker (Product Code [C4105](#)); Lanes 2 and 3: 9.6 nM siRNA; Lanes 4 and 5: 19 nM siRNA; Lanes 6 and 7: 38 nM siRNA; Lane 8: mock transfection; Lanes 9 and 10: non-interfering siRNA; Lane 11: pool of non-interfering siRNA; Lane 12: ColorBurst Marker for Western blot; Low Range Marker (Product Code [M3913](#)) for SDS-PAGE. The arrows indicate location of GAPDH protein.

A. Western blot showing bands of GAPDH. The bands in which the protein has been knocked down with siRNA are less intense than the control bands. **B.** SDS-PAGE of HeLa cell culture extracts. The band of GAPDH is at a molecular weight of about 36 kDa. **C.** SDS-PAGE of HeLa cell culture extracts with bands of GAPDH removed for in-gel tryptic digestion and subsequent labeling procedure.



Figure 4. MALDI-TOF MS analysis of a tryptically digested mixture of two samples of HeLa extracts. One sample was subjected to various levels of knockdown by siRNA, the other sample served as a control. For these studies, the non-interfering pool was used as the control. Following knockdown, the samples were isotopically labeled using either ^{16}O water (test) or ^{18}O water (control). The peptide shown is LISWYDNEFGYSNR from GAPDH.

- A.** 9.6 nM siRNA. The ratio of test to control was 65.7% (i.e., 34.3 % knocked down).
- B.** 19 nM siRNA. The ratio of test to control was 50.8% (i.e., 49.2 % knocked down).
- C.** 38 nM siRNA. The ratio of test to control was 43.0% (i.e., 57.0% knocked down).
- D.** Graph comparing the protein level from the three siRNA knockdown experiments to the control sample.

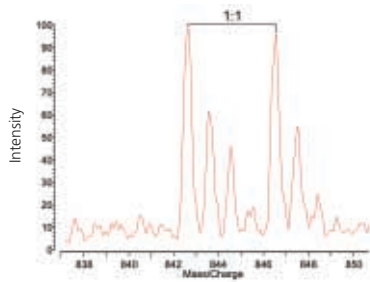


Figure 5. Typical data for unmodified proteins. MALDI-TOF MS analysis of an observed (non-GAPDH) peptide that was obtained from the mixture of HeLa extracts. The GAPDH protein in the samples was subjected to knockdown by siRNA. All proteins present in the sample that were not effected by the siRNA knockdown, should exhibit peptides in a 1:1 ratio, thereby serving as internal controls. This phenomenon was observed in the mass spectra obtained from these experiments.

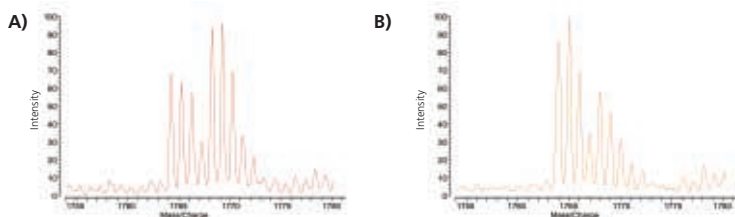


Figure 6. Inverse Labeling. MALDI-TOF MS spectra of the peptide LISWYDNEFGYSNR derived from GAPDH. The samples were inversely labeled such that in **A**), the test and control samples were labeled with ^{16}O and ^{18}O , respectively, while in **B**), the test and control samples were labeled with ^{18}O and ^{16}O , respectively. The inverse labeling was performed to show that the differences between the samples were a specific effect of the knockdown and not an artifact of the labeling process.

Comparison of Quantification Methods for siRNA Knockdown

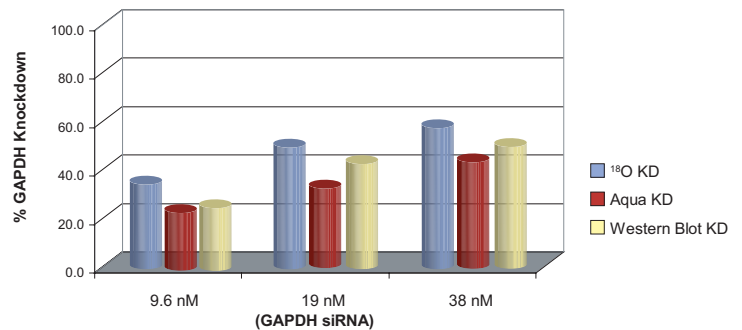


Figure 7. The samples of the HeLa extracts expressing GAPDH were analyzed independently using the ^{18}O and AQUA methodologies. Knockdown percentages were also compared to densitometry data from a Western blot analysis. The comparison data shows the percentage of knockdown found for the three levels of siRNA (i.e., 9.6, 19, and 38 nM) as compared to a non-interfering pool. The amounts of protein determined to be in the test samples versus the control samples were consistent within approximately 20% between the methods. For specific details of the AQUA experiments and methodologies, refer to poster P177-S.

Conclusions

- In a model $^{16}\text{O}/^{18}\text{O}$ differential protein labeling experiment, the observed ratios correlated very closely to expected ratios.
 - aldolase: expected ratio: 1.0; observed ratio: 1.03
 - lysozyme: expected ratio: 0.33; observed ratio: 0.34
- Isotopic labeling using ^{18}O confers high utility because it is a global labeling technique. All peptides in the sample (except for the C-terminal peptide of the protein) should have an isotopic label incorporated.
 - The ^{18}O label was observed in nearly all peptides within the sample.
 - In an RNAi experiment, in which GAPDH was knocked down, the majority of the peptides exhibited a 1:1 ratio of the test versus control samples. These peptides served as convenient internal controls.
 - Peptides that did not exhibit a 1:1 ratio were affected by the knockdown of the GAPDH-specific siRNA, and these peptides corresponded either to GAPDH or another protein affected by the knockdown cascade.
- Analysis of the data showed that increasing concentrations of siRNA correlated to progressively decreasing amounts of GAPDH protein observed in the test samples.
 - Relative differences in protein levels could be quantified using the ^{18}O labeling method.
 - The percent knockdown recorded in each test sample was consistent within 20% of that determined by AQUA technology and Western blot analysis.

References

1. Yao, X.; et al. Proteolytic ^{18}O Labeling for Comparative Proteomics: Model Studies with Two Serotypes of Adenovirus. *Anal. Chem.* **2001**, *73*, 2836–2842.
2. Wang, Y. K.; et al. Inverse ^{18}O Labeling Mass Spectrometry for the Rapid Identification of Marker/Target Proteins. *Anal. Chem.* **2001**, *73*, 3742–3750.

