



## Abstract

Protein quantification in complex mixtures represents one of the greatest challenges facing proteomics researchers today. Absolute quantification of proteins may be undertaken by employing the use of an isotopically labeled internal standard corresponding to an analogous tryptic peptide of the protein being interrogated. This PROTEIN-AQUA™ method has been previously described in detail by Gygi and co-workers,<sup>1</sup> and is showing increasing efficacy for a wide variety of quantitative proteomics studies. A complementary method to measure relative amounts of protein in two related but discrete samples is differential labeling of tryptic peptides using <sup>18</sup>O water. Through the incorporation of the isotopic label, a mass shift is created in the test sample allowing it to be easily differentiated from the control sample via mass spectrometric analysis. Moreover, the <sup>18</sup>O labeling method can globally label all peptides in a sample so that any changes between control and test samples may be determined. RNA-mediated interference (RNAi) has provided a means for analysis of gene function as well as target validation, but the techniques currently used to probe and measure protein levels of the sample are inadequate. In the present work, we examine the utility of PROTEIN-AQUA and <sup>18</sup>O labeling for quantification of protein expression from several genes targeted for RNAi knockdown.

## Introduction

The basis of this study is to compare various methodologies for examining protein expression of two complex biological samples using Western blot, PROTEIN-AQUA, and isotopic labeling by <sup>18</sup>O water. 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.

The PROTEIN-AQUA and <sup>18</sup>O labeling methodologies utilize similar strategies for protein expression analysis in that they both employ the use of isotopically labeled peptides to be compared to native peptides. In June 2003, Dr. Steve Gygi and his team presented a strategy for absolute protein quantification by employing isotopically labeled peptides for downstream analysis by LC-MS. This technique, PROTEIN-AQUA, is based on a common principle: the use of an isotopically labeled internal standard. The standard is spiked into a peptide sample in known amounts, which allows the researcher to compare the quantity of this internal standard to a peptide of interest and obtain an absolute measure of protein quantification. Similarly, the <sup>18</sup>O labeling method also incorporates the use of isotopically labeled peptides; however, with this technique, the labeling process starts with two discrete samples of peptides, one control and one test. The isotopic label is then incorporated onto the test sample in the presence of trypsin. The incorporation of two <sup>18</sup>O atoms creates a +4 Da mass shift (relative to an unlabeled analog 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 procedure, all protein levels that may have been affected by the stressor (e.g., RNAi knockdown, chemical treatment, etc.) can be evaluated.

In the present work, we compare the use of <sup>18</sup>O water and PROTEIN-AQUA methodologies to Western blot analysis for their utility in analyzing protein expression in a complex biological sample. The sample for all three methods was a total protein extract of HeLa cells. While the same samples were used throughout this experiment, the methods of preparation and quantitative analysis were entirely different. The Western blot and <sup>18</sup>O labeling methodologies both utilized SDS-PAGE for the sample preparation. However, the Western blot utilized antibody detection for the mode of analysis, whereas in the <sup>18</sup>O labeling experiment, the protein sample was in-gel tryptically digested, isotopically labeled, and then analyzed by MALDI-TOF MS. In the PROTEIN-AQUA experiment, the sample was typically digested in solution, separated via C-18 HPLC, and analyzed by electrospray mass spectrometry.

## Materials

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

- Proteomics Grade Trypsin (Product Code T6567)
- Anti-GAPDH, Mouse Monoclonal Antibody (Ambion Catalog #4300)
- Escort V Transfection Reagent (Product Code E9778)
- Dulbecco's Modified Eagle's Medium (Product Code D5671)
- Fetal Bovine Serum (Product Code F6178)
- L-Glutamine solution (Product Code G7513)
- Benzonase Endonuclease (Product Code E1014)
- Colorburst Electrophoresis Markers (Product Code C4105)
- Bicinchoninic Acid Kit for Protein Determination (Product Code BCA-1)
- siGENOME SMARTpool Reagent Human GAPDH (Dharmacon Catalog #M-004253-01)
- Tetramethylbenzidine Liquid Substrate for Membranes (Product Code T0565)
- Chemiluminescent Peroxidase Substrate-1 (Product Code CPS-1-120)
- Anti-Mouse IgG Peroxidase (Product Code A9044)
- ProteoPrep Reduction/Alkylation Kit (Product Code PROT-RA)
- ProteoPrep TCA Precipitation Kit (Product Code PROT-PR)
- Trypsin Profile IGD Kit (Product Code PP0100)
- <sup>18</sup>O Proteome Profiler Kit (Product Code P3623)
- All of the isotopically labeled peptides used in this experiment were synthesized and purified by Sigma-Genosys (The Woodlands, TX)

# Protein Expression Analysis in Targeted Gene RNAi Knockdown Experiments Utilizing Isotopic Labeling with <sup>18</sup>O Water and PROTEIN-AQUA™ Peptides

## Methods

### Culturing of HeLa Cells

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

### Transfection of HeLa Cultures

The media was removed and 2 mL of fresh media (DMEM, 5% FBS, 4 mM L-glutamine) was added to each culture plate 2 h before the transfections. The plates were 60–70% confluent prior to transfection. Dharmacon siGENOME SMARTpool siRNA stocks for Human GAPDH, RISC-free (non-functional, non-interfering), two non-interfering sequences, and a non-interfering pool were prepared according to the kit instructions.

SMARTpool control reagents (non-interfering, non-interfering pool, and RISC-free) were transfected at a final concentration of 19 nM to the HeLa cultures using Escort V Transfection Reagent. Each transfection was performed in duplicate.

SMARTpool Human GAPDH siRNAs were transfected at final concentrations of 9.6 nM, 19 nM, and 38 nM to the HeLa cultures using Escort V Transfection Reagent. Each transfection was performed in duplicate.

Mock transfections were performed by following the transfection procedure used above without the presence of an RNA duplex. Four mock transfections were performed.

The HeLa plates were incubated for an additional 48 h following the transfection procedures to allow for sufficient knockdown of the target gene. Each plate was 100% confluent at harvest.

### Sample Preparation

Samples were harvested by adding 1 mL of resuspension reagent to each culture dish (Hank's Balanced Salt Solution supplemented with 50 U/mL benzonase) and thoroughly scraping the culture plate.

The cell samples were frozen at –20 °C. Samples were thawed and mixed well to allow for digestion of the chromosomal DNA.

Urea was added to each sample to obtain a final concentration of 8 M. The protein concentration of each sample was determined by BCA assay.

### Trypsin Digestion (for PROTEIN-AQUA)

Each protein sample was reduced and alkylated using the ProteoPrep Reduction/Alkylation Kit.

TCA precipitations were performed on each sample using the ProteoPrep TCA Precipitation Kit.

The precipitated proteins were resuspended in 50 mM Tris-HCl, pH 8.0.

400 picomoles of two isotopically labeled peptides (GAL\*QNIIPASTGAAG and VIPEL\*NGK) were added to each protein sample. These peptides correspond to predicted tryptic fragments of human GAPDH.

Each sample was digested overnight with Proteomics Grade Trypsin (20:1 substrate:enzyme ratio).

### SDS PAGE and Western Blotting (for <sup>18</sup>O Labeling)

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 <sup>18</sup>O labeling were stained with EZBlue™ Gel Staining Reagent and destained with water.

The proteins were transferred from the SDS-PAGE gel to a nitrocellulose membrane. The membrane was blocked with Tris buffered saline (TBS) with 3% nonfat dry milk for 30 min. The blot was then incubated with 1 µg/mL of the Anti-GAPDH antibody in TBS-milk at room temperature with shaking for 30 min. Next, the blot was incubated with anti-mouse IgG-HRP at a 1:12,500 dilution in TBS-Milk for 30 min and then washed 5 times for a total of 15 min in TBS-Tween 20. Finally, the blot was developed for 5 min with Tetramethylbenzidine for membranes, before briefly rinsing the blot with water, and capturing the image. Subsequently, the blot was incubated with 0.1 M glycine, pH 2 for 30 min to strip the membrane.

The blot was processed again using the wash and antibody incubation steps described above for the colorimetric detection. For detection, the blot was then incubated with an HRP chemiluminescent substrate CPS-1 for 5 min. The image was directly captured on a Bio-Rad Fluor-S Multimager, and the band density determined using Quantity One software.

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 and subjected to an in-gel tryptic digest using the Trypsin Profile IGD Kit. Following digestion, the samples were vacuum dried in preparation for the labeling procedure.

The Western blot and SDS-PAGE gels are illustrated in **Figure 1**.

### LC-MS Analysis of Data

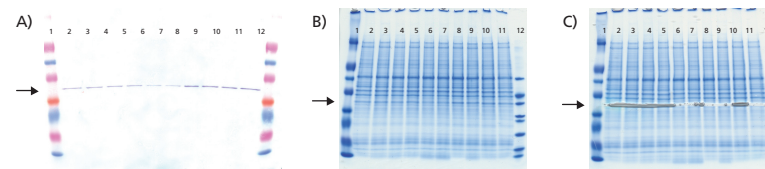
Samples were analyzed by reversed phase LC-MS using an Agilent 1100 Capillary LC followed by a Finnigan LCQ Classic Ion Trap. The mass spectrometer was equipped with an electrospray ionization source. Positive ion mass spectra were collected over a 150–2000 Da range.

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

The samples were labeled using the <sup>18</sup>O Proteome Profiler Kit. Following digestion, the dried samples were reconstituted with 4 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 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 h at 37 °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.

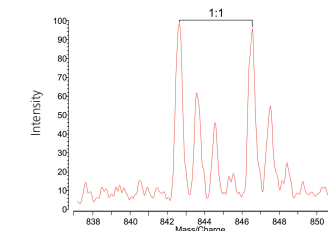
## Results



**Figure 1. All gels were loaded in the following manner:**

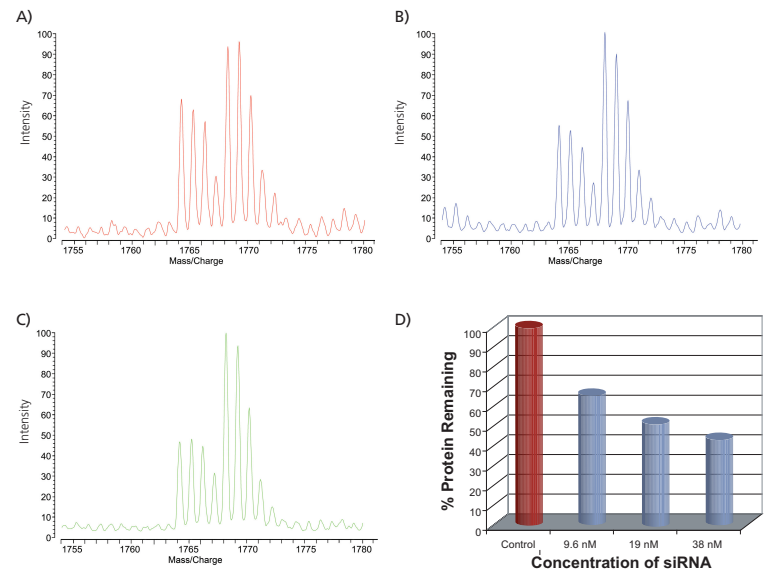
Lane 1: ColorBurst Marker (Product Code C4105); Lanes 2 and 3: 9.6 nM siRNA; Lanes 4 and 5: 19 nM siRNA; Lanes 6 and 7: 38 nM siRNA; Lane 8: mock transfection; Lanes 9 and 10: non-interfering siRNA; Lane 11: pool of non-interfering siRNA; Lane 12: ColorBurst Marker for Western blot; Low Range Marker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

- 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.
- SDS-PAGE of HeLa cell culture extracts. The band of GAPDH is at a molecular weight of about 36 kDa.
- SDS-PAGE of HeLa cell culture extracts with bands of GAPDH removed for in-gel tryptic digestion and subsequent <sup>18</sup>O labeling procedure.



**Figure 2. Typical data for unmodified proteins labeled with <sup>18</sup>O.**

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.

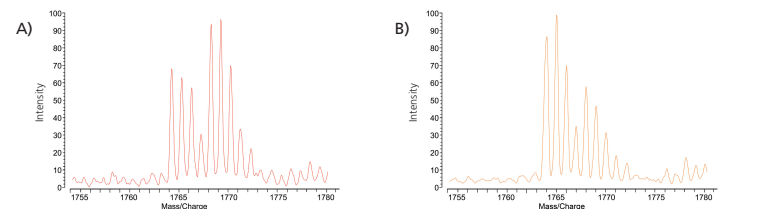


**Figure 3. MALDI-TOF MS analysis of a tryptically digested mixture of two samples of HeLa extracts.**

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 <sup>18</sup>O water (control). The peptide shown is LISWYDNEFGYSNR from GAPDH.

- 9.6 nM siRNA. The ratio of test to control was 65.7% (i.e., 34.3% knocked down).
- 19 nM siRNA. The ratio of test to control was 50.8% (i.e., 49.2% knocked down).
- 38 nM siRNA. The ratio of test to control was 43.0% (i.e., 57.0% knocked down).

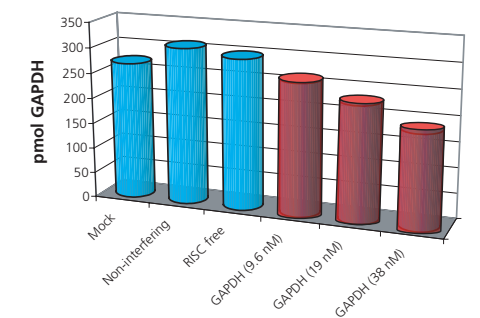
D. Graph comparing the protein level from the three siRNA knockdown experiments to the control sample.



**Figure 4. 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 <sup>18</sup>O and <sup>16</sup>O, respectively, while in **B**, the test and control samples were labeled with <sup>16</sup>O and <sup>18</sup>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.

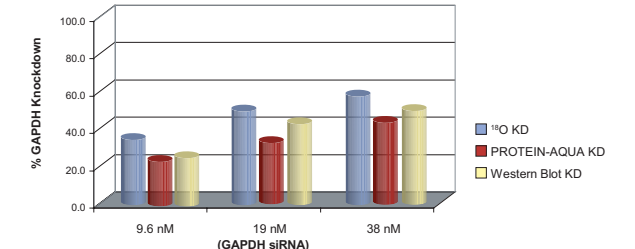
### PROTEIN-AQUA Quantification Following siRNA Knockdown



**Figure 5. Samples were analyzed by the LC-MS procedure described in the methods section.**

The area under the RIC peak corresponding to each target peptide and its isotopically labeled counterpart was determined, and a ratio was calculated. Based on the known amount of isotopically labeled peptide in each sample, the concentration of GAPDH was determined. For every sample, the data from each of the two peptides was averaged. For samples where n>4 (mock and non-interfering), the high and low data points were removed before calculating the average.

### Comparison of Quantification Methods for siRNA Knockdown



**Figure 6. The samples of the HeLa extracts expressing GAPDH were analyzed independently using PROTEIN-AQUA, <sup>18</sup>O, and Western blot methodologies.**

Knockdown percentages obtained from PROTEIN-AQUA and <sup>18</sup>O methodologies were 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.

## Conclusions

To our knowledge, this is the first example employing either a PROTEIN-AQUA or <sup>18</sup>O labeling based approach for quantification of a protein targeted for knockdown using RNAi. We believe that these methods will provide definite advantages in the future study of protein expression analysis. The utility of the <sup>18</sup>O labeling method lies in the fact that it is a global labeling procedure. By employing this method, virtually all of the peptides within the sample were labeled. While the majority of the peptides exhibited a 1:1 ratio of the test versus control samples, this result is extremely useful. These peptides were not affected by the GAPDH knockdown, and served as convenient internal controls. Peptides that did not exhibit a 1:1 ratio were affected by the knockdown of the GAPDH-specific siRNA. While other approaches directed at quantifying protein levels are suitable for calculating relative protein concentrations, the PROTEIN-AQUA methodology has enabled us to absolutely quantify the concentration of our target protein in each sample. The agreement between the data obtained in our analysis with other widely used techniques for relative protein expression analysis lends further credibility to our results.

Analysis of data from all three methods showed that increasing concentrations of transfected siRNA correlated to progressively decreasing amounts of GAPDH protein observed in the test samples. The differences in protein levels could be quantified using the both the PROTEIN-AQUA and the <sup>18</sup>O labeling methods. The percent knockdown recorded for both of these methodologies, as well as that recorded for Western blot densitometry analysis were consistent to within 20% of each other. The high correlation between these three completely different and distinct methods indicates that each method yields valid results. In addition to the high correlation between the knockdown percentages, the data obtained from these techniques is complementary to each other. The Western blot can be used to indicate that the protein is of the correct molecular weight as determined by SDS-PAGE, <sup>18</sup>O labeling can be used to discover additional unknown protein aberrations from the siRNA treatment, and PROTEIN-AQUA can verify the exact amount of target protein both in the sample and between experiments. Finally, we believe that the use of the PROTEIN-AQUA technique or the <sup>18</sup>O labeling method will enable a high-throughput, multiplexed approach to proteomics analysis.

## References

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