

Functional Validation of RNAi Knockdown at the Protein Level, via Sequential Quantitative Proteomics Techniques

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Abstract

RNA-mediated interference (RNAi) offers a mechanism for the study of gene function as well as target validation, but currently available methodologies to interrogate and measure protein levels following gene knockdown are inadequate and imprecise. Indeed, there is a pressing and demonstrable requirement to functionally validate RNAi knockdown by rapid and accurate quantitative surveillance of affected protein(s).

Differential isotopic labeling of tryptic peptides using ^{18}O water is a well-established technique for measuring relative amounts of protein in two related but discrete samples. Through incorporation of the isotopic label, a mass shift is generated in the test sample, thereby allowing it to be easily discriminated from the control sample—via mass spectrometric analysis. Most importantly, ^{18}O labeling is a global strategy in that essentially all peptides in a sample are labeled—so any changes between control and test samples are revealed. Absolute Quantification (AQUA™) represents a complementary quantitative proteomics technique to ^{18}O labeling. PROTEIN-AQUA™ experiments may be performed by employing isotopically labeled internal peptide standards, corresponding to analogous native tryptic peptides of the protein(s) being interrogated. This method is a targeted strategy that exhibits robust efficacy and is being increasingly utilized for a wide variety of quantitative proteomics studies.

A study has been conceived to demonstrate that the ^{18}O labeling and PROTEIN-AQUA strategies can be sequentially coupled to RNAi technology in order to perform true quantitative functional validation of gene knockdown. Following gene knockdown, ^{18}O labeling is performed to observe both the target protein and all other proteins involved in the knockdown cascade. Discrete PROTEIN-AQUA experiments are also executed in parallel to independently verify target knockdown. Subsequent to this comprehensive survey of the perturbed proteome, directed AQUA experiments may be planned to elucidate the absolute concentrations of any affected proteins.

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 ^{18}O water. In addition, we will use this study to show that these techniques can be used to elucidate offsite effects of targeted gene knockdown. 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 ^{18}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 ^{18}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 ^{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 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 ^{18}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. In addition, we have employed ^{18}O labeling to analyze the offsite effects of the gene knockdown.

Materials

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

- Proteomics Grade Trypsin (Catalog No. T6567)
- Anti-GAPDH, Mouse Monoclonal Antibody (Ambion Catalog No. 4300)
- Escort™ V Transfection Reagent (Catalog No. E9778)
- Dulbecco's Modified Eagle's Medium (Catalog No. D5671)
- Fetal Bovine Serum (Catalog No. F6178)
- L-Glutamine solution (Catalog No. G7513)
- Benzonase Endonuclease (Catalog No. E1014)
- Colorburst™ Electrophoresis Markers (Catalog No. C4105)
- Bicinchoninic Acid Kit for Protein Determination (Catalog No. BCA1)
- siGENOME™ SMARTpool Reagent Human GAPDH (Dharmacon Catalog No. M-004253-01)
- Tetramethylbenzidine Liquid Substrate for Membranes (Catalog No. T0565)
- Chemiluminescent Peroxidase Substrate-1 (Catalog No. CPS1120)
- Anti-Mouse IgG Peroxidase (Catalog No. A9044)
- ProteoPrep® Reduction/Alkylation Kit (Catalog No. PROTRA)
- ProteoPrep® TCA Precipitation Kit (Catalog No. PROTPR)
- Trypsin Profile IGD Kit (Catalog No. PP0100)
- ^{18}O Proteome Profiler Kit (Catalog No. P3623)

All of the isotopically labeled peptides used in this experiment were synthesized and purified by Sigma-Genosys (The Woodlands, TX USA).

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 21 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 (Hank's Balanced Salt Solution supplemented with 50 U/mL benzonase) to each culture dish and thoroughly scraping the culture plate.

The cell samples were frozen at $-20\text{ }^{\circ}\text{C}$. 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.

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

Trypsin Digestion (for PROTEIN-AQUA)

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*QNIIIPASTGAAK 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).

The PROTEIN-AQUA samples were then subsequently analyzed by LC-MS.

SDS PAGE and Western Blotting

As determined by BCA analysis, 10 μg of total protein was loaded onto a 4–20% SDS-PAGE gel.

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 $\mu\text{g}/\text{mL}$ 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.

^{18}O Labeling Process

Prior to labeling, the samples were cleaned up using spin columns provided in the ^{18}O Proteome Profiler Kit.

The samples were digested with trypsin (20:1 substrate:enzyme ratio), in the presence of 9% Acetonitrile. 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 h at $37\text{ }^{\circ}\text{C}$, following which time the reactions were stopped by the addition of 5 μL of 1.0% TFA. The samples were then dried in a vacuum centrifuge. The $^{16}\text{O}/^{18}\text{O}$ samples were then analyzed by MALDI-TOF MS and subsequent LC-MS.

MALDI-TOF MS

MALDI mass spectra were acquired using a Shimadzu-Biotech Axima-CFR+ instrument in positive ion reflectron mode. Samples were mixed 1:1 with α -cyano-4-hydroxy-cinnamic acid (5 mg/mL in 90% acetonitrile, 0.1% TFA). Aliquots (1 μL) were spotted on the MALDI target and dried under low vacuum. Mass spectra were acquired by summation of approximately 100 shots.

LC-MS

Lysate samples were separated using an Agilent 1100 capillary LC system interfaced with a Finnigan LCO Classic ion trap instrument. Mass spectra were acquired in positive ion mode using an electrospray source equipped with a metal needle kit. Data was acquired in MS mode over an m/z range of 150–2000.

GLOBAL PROTEOMIC ANALYSIS

For global proteomic analysis, lysate samples were analyzed using a Thermo LTQ ion trap instrument. Prefractionation was again performed using an Agilent 1100 capillary LC system. Data was acquired in positive ion mode using a triple-play acquisition methodology. Proteins were identified using Sequest through the BioBrowser software interface.

Results

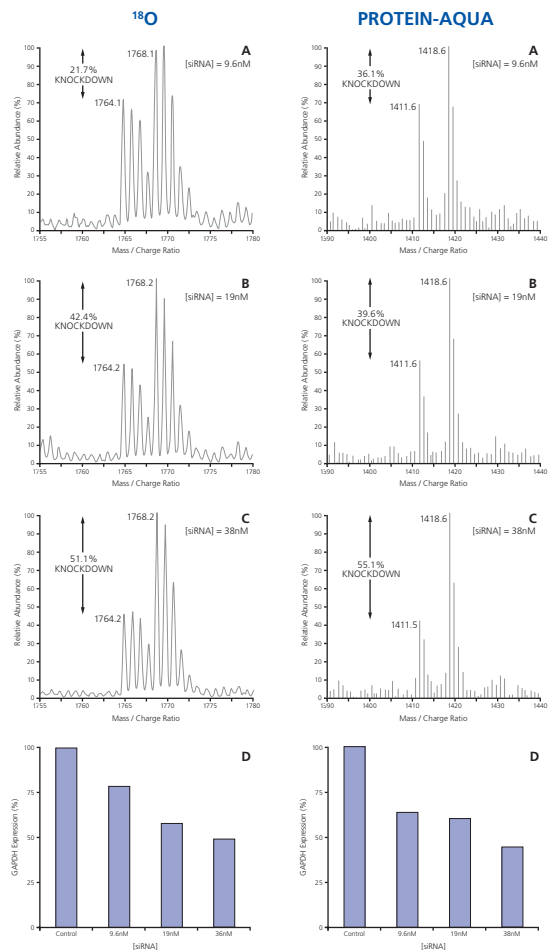
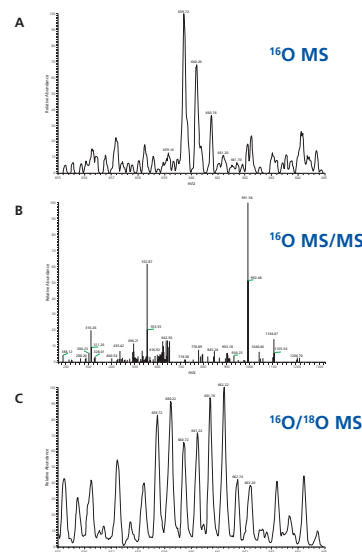


Figure 1: LC-MS Analysis of ¹⁸O and PROTEIN-AQUA data

The above figures show the percent knockdown for each of the transfected samples as determined by ¹⁸O labeling (figures on left side) and by PROTEIN-AQUA (figures on right side).

- A. GAPDH samples transfected with 9.6 nM siRNA
- B. GAPDH samples transfected with 19 nM siRNA
- C. GAPDH samples transfected with 38 nM siRNA
- D. Comparison charts showing the percent of GAPDH expression in the transfected samples vs. a control sample (i.e., no knockdown) of siRNA samples vs. a control



D

Peptide Mass	Peptide	I ₂ /I ₀	I ₄ /I ₀	¹⁸ O/ ¹⁶ O
659.866	ITLPVDFVTADK	0.72	1.16	1.21
659.866	ITLPVDFVTADK	0.85	1.23	1.31
	Average:		1.20	1.26

Figure 4: MS Data for the Phosphoglycerate Kinase Analysis

- A. Mass spectra of a ¹⁶O peptide (ITLPVDFVTADK) from phosphoglycerate kinase.
- B. MS/MS analysis of the peptide, confirming the sequence, which is used for identification of the corresponding protein.
- C. Mass spectra of the same peptide (ITLPVDFVTADK) from a mixture of ¹⁶O (control, non-interfering pool) and ¹⁸O (test, 38 nM siRNA) samples, showing an increase in expression of the corresponding enzyme.
- D. Calculations used to determine the ratio of test to control for the targeted peptide.

Protein	¹⁸ O/ ¹⁶ O (KD/Control)	Number of Data Points	Standard Deviation
Aldolase	0.999	6	0.19
Phosphoglycerate Kinase	1.262	2	0.07
Phosphofruktokinase	0.729	1	0.00
GAPDH	0.572	3	0.05
Human Pyruvate Kinase	0.813	6	0.08
Filamin 1	1.378	2	0.01
Eukaryotic Translation Elongation Factor	0.896	12	0.11

Figure 5: Summary Table of Differentially Expressed Proteins

Based on the ¹⁸O labeling analysis, the following proteins showed differential expression (>10% change in expression relative to control sample). The proteins listed in red are associated with the glycolysis/gluconeogenesis pathway. Aldolase, an example of a protein that was not affected by the knockdown event, shows a 1:1 ratio of the ¹⁶O and ¹⁸O labeled peptides.

Conclusion

In the present work, we have employed a variety of methodologies for quantifying the knockdown of a target protein by RNAi and evaluated the concentrations of additional proteins that may have been affected by the knockdown event. By utilizing ¹⁸O labeling, PROTEIN-AQUA, and Western Blot analysis, we were able to quantify the knockdown of GAPDH by RNAi at the protein level. Analysis of data from these three independent methods showed that increasing concentrations of transfected siRNA correlated to progressively decreasing expression of GAPDH. The percent knockdown calculated from the individual testing methods was consistent to within 20% of each other. The high correlation between these three completely distinct methods indicates that each method yields valid results.

Each method of analysis has its own set of advantages and serves a certain utility. While Western blot analysis can be used to indicate that the protein is of the correct molecular weight as determined by SDS-PAGE, PROTEIN-AQUA is the only approach that allows for absolute quantification, and can be used to determine the exact amount of target protein both in the sample and between experiments. While both of these methods allow for a targeted proteomics analysis, ¹⁸O labeling can be used to discover additional unknown protein aberrations from the siRNA treatment. Analysis of these additional knockdown effects was the second focus of this study.

By employing the ¹⁸O labeling methodology, virtually all of the peptides within the sample were labeled. Analysis of the data showed that the majority of peptides within the sample exhibited a 1:1 ratio of the test vs. control samples, indicating that these peptides were not affected by the GAPDH knockdown. However, the focus of this study is on the peptides that do not exhibit a 1:1 ratio, because the proteins from which they are derived may have been affected by the knockdown of the target protein. Through analysis of these peptides, seven proteins were identified which were up- or down-regulated by the targeted knockdown of GAPDH. Several of these proteins, including phosphoglycerate kinase, can be found in the glycolysis/gluconeogenesis pathway, of which GAPDH is a fundamental enzyme. It is assumed that the up or down regulation associated with these proteins is a result of the knockdown of GAPDH.

In summary, through the combination of PROTEIN-AQUA, ¹⁸O Labeling, and Western blot analysis, we were able to absolutely quantify the concentration of our target protein and obtain relative protein concentrations for additional proteins affected by the knockdown cascade. We believe that the combination of PROTEIN-AQUA and ¹⁸O labeling will enable a high-throughput, multiplexed approach to proteomics analysis.

References

1. This method was developed by Dr. Steve Gygi and colleagues at Harvard Medical School [Stemmann O, Zou H, Gerber SA, Gygi SP, Kirschner MW; Dual inhibition of sister chromatid separation at metaphase, Cell, **2001**, Dec 14, 107: 715-726]. Limited use of this method is permitted under a licensing arrangement with Harvard Medical School.
2. Yao, X. et al. Proteolytic ¹⁸O Labeling for Comparative Proteomics: Model Studies with Two Serotypes of Adenovirus., Anal. Chem., **2001**, 73, 2836

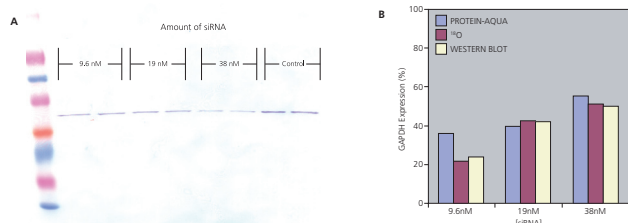
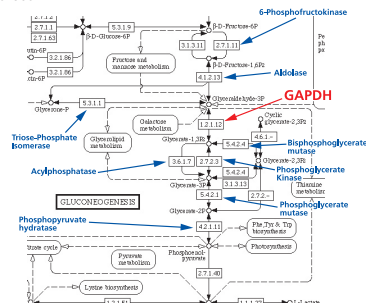


Figure 2:

- A. The gel for the Western blot was loaded in the following manner: Lane 1: ColorBurst Marker (Cat. No. 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; Lane 9: non-interfering siRNA.
- B. The HeLa extracts expressing GAPDH were analyzed independently using the ¹⁸O and PROTEIN-AQUA methodologies. Knockdown percentages were also compared to densitometer 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 vs. the control samples agreed with 20% between the methods.



<http://www.genome.ad.jp/kegg/pathway/map/map0010.html>

Figure 3: Glycolysis/Gluconeogenesis Pathways

Enzymes involved in the pathway may potentially be affected by the targeted knockdown of GAPDH.