

Absolute Quantification as an Effective Tool for Measuring Protein Expression in Targeted Gene Knockdown Experiments

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Abstract

The quantification of protein(s) in a complex mixture is one of the greatest challenges facing proteomics researchers today. While recent advances in differential protein analysis have provided a means for comparing relative protein expression between two populations, there exists a need for the absolute quantification of a single protein in a complex population. Methods for the absolute quantification of proteins have been previously described,¹ which employ the use of an isotopically labeled internal standard corresponding to a tryptic peptide of the protein being quantified. RNA-mediated interference (RNAi) has provided a means for analysis of gene function, as well as target validation, but the techniques used in gene knockdown validation are inadequate. In the present work, we verify a method employing an isotopically labeled peptide for quantification of a single protein in a complex mixture, and examine the usefulness of this method for quantification of protein expression from a gene targeted for knockdown using RNAi.

Introduction

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.¹ This technique, Protein-AQUA, is based on a common principle: the use of an isotopically labeled internal standard. By applying this principle to the quantification of proteins, Gygi's team has advanced the abilities of protein researchers to study complex biological samples quantitatively.

Targeted gene knockdown using small interfering RNA (siRNA) has proven to be a powerful tool for studying gene function in eukaryotes.² Typically, analysis of gene knockdown is performed by Northern blot or quantitative RT-PCR. Unfortunately, reduction in the amount of a specific mRNA does not typically correlate well with protein levels present in the cell. Additionally, methods for studying samples at the protein level, such as Western blot, while sensitive, often lack the ability to discriminate between samples in which the differences in protein levels are minimal.

In the present work, we examine the fundamental principle of the AQUA method and its applicability to complex biological samples. Additionally, we examine the usefulness of this approach to quantitate protein expression levels from a gene targeted for knockdown.

Methods

Peptide Spiking Experiments

Sample 1 – A five protein mixture (containing equal amounts of aldolase, myoglobin, lysozyme, peroxidase, and carbonic anhydrase) was prepared at a final concentration of 1 mg/mL in 50 mM Tris-HCl, pH 8.0. The mixture was digested overnight with Proteomics Grade Trypsin (20:1 substrate:enzyme ratio) to

generate a non-specific peptide background. Equal concentrations of two HPLC purified peptides (GSITEQLLNAR and LTGDQTAALR) and their isotopically labeled (¹⁵N, ¹³C-Leucine) counterparts (GSITEQLL*NAR and LTGDQTAAL*R) were spiked into the five protein tryptic digest.

Sample 2 – A HeLa lysate (1.0 mg/mL) was digested overnight with Proteomics Grade Trypsin (20:1 substrate:enzyme ratio) to generate a non-specific peptide background. Equal concentrations of two HPLC purified peptides (GSITEQLLNAR and LTGDQTAALR) and their isotopically labeled (¹⁵N, ¹³C-Leucine) counterparts (GSITEQLL*NAR and LTGDQTAAL*R) were spiked into the HeLa tryptic digest.

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) 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 two hours 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 hours 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 the BCA assay.

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.

Trypsin Digestion

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

Added 400 picomoles of two isotopically labeled (^{15}N , ^{13}C -Leucine) peptides (GAL*QNIIPASTGAAK and VIPEL*NGK) 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).

Western Blotting

Based on the results of the BCA assay, 10 micrograms of protein from three controls (mock, non-interfering pool, RISC free) and six test samples were separated on a 4–20% SDS-PAGE gel.

The proteins were transferred from the gel to a nitrocellulose membrane. The membrane was blocked with Tris buffered saline (TBS) in 3% nonfat dry milk for 30 minutes. 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 minutes. Then, the blot was incubated with anti-mouse IgG-HRP at a 1:12,500 dilution in TBS-Milk for 30 minutes, and then washed 5 times for a total of 15 minutes in TBS-Tween 20. Finally, the blot was developed for 5 minutes with Tetramethylbenzidine for membranes, before briefly rinsing with water, and capturing the image. Subsequently, the blot was incubated with 0.1 M glycine HCl, pH 2, for 30 minutes 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 incubated with an HRP chemiluminescent substrate, CPS-1, for 5 minutes. The image was directly captured on a Bio-Rad Fluor-S Multimager, and the band density determined using Quantity One software.

LC-MS Analysis of Data

Samples were analyzed by reversed phase LC-MS using a Supelco Discovery C-18 column on 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.

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](#))
- All isotopically labeled peptides used in this experiment were synthesized and purified by Sigma-Genosys (The Woodlands, TX)

Results

Ratio of Native to Stable Isotope Labeled Peptide

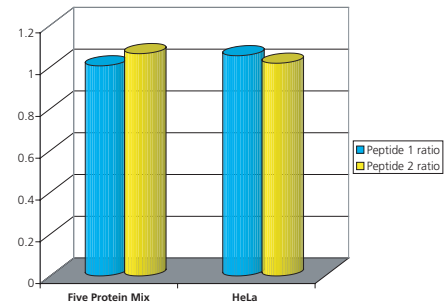


Figure 1: Peptide Spiking Experiments. Peptides were spiked in a 1:1 ratio, and analyzed by the LC-MS procedure described in the Methods section. The area under the RIC peak for the target peptide and its isotopically labeled counterpart was calculated, and a ratio determined. Samples were run in duplicate, and the data above reflects the average of the two samples.

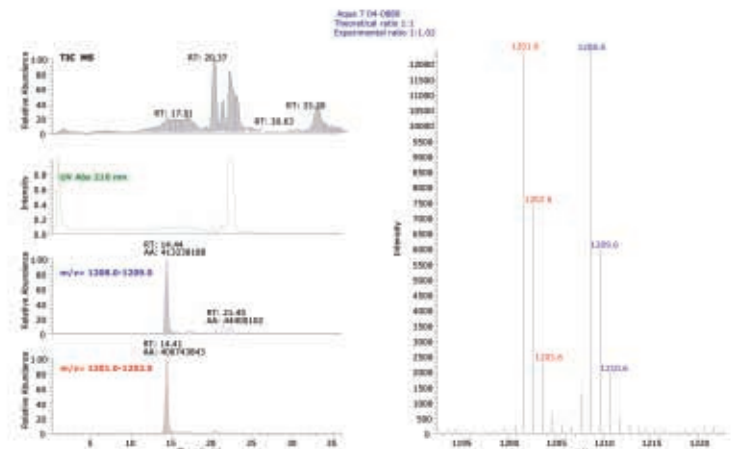


Figure 2: LC-MS Analysis of the Five Protein Digest With Spiked Peptides. Restricted ion current data is presented showing co-elution of the native and isotopically labeled peptides. Using the area under the RIC peak for each peptide and its isotopically labeled counterpart, an experimental ratio was determined (as depicted in Figure 1).

Analysis of Transfected HeLa Cells (GAPDH knockdown)

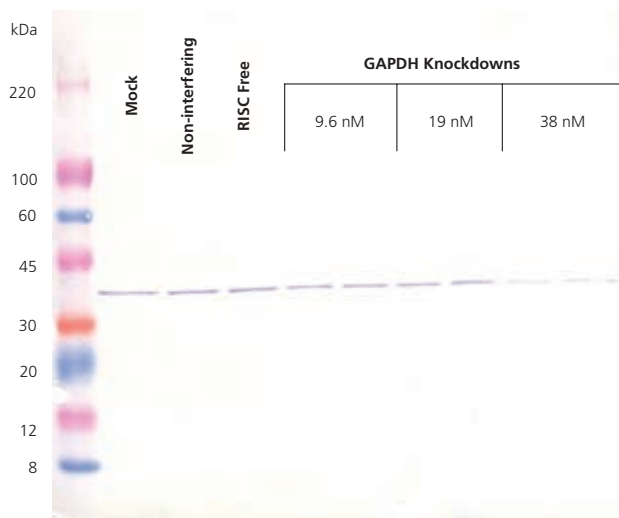


Figure 3: Colorimetric Detection of GAPDH by Western Blot. There is an evident decrease in the presence of GAPDH in the samples targeted for gene knockdown using RNAi. With increasing amounts of transfected siRNA, there is a visible decrease in signal. Quantitative RT-PCR analysis of the HeLa transfections indicate a similar decrease in total GAPDH mRNA (data not shown).

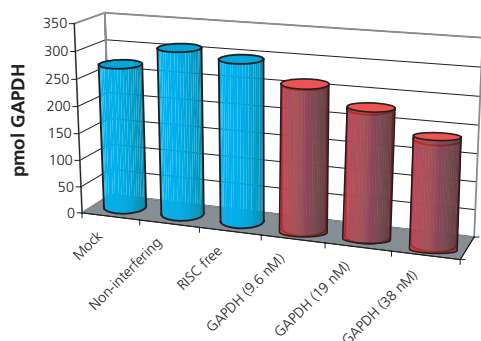


Figure 4: Absolute Quantification of GAPDH from HeLa Transfections. Samples were analyzed by the LC-MS procedure described in the Methods section. The area under the RIC peak corresponding to each target peptide and its isotopically labeled counterpart was determined and a ratio calculated. Based on 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.

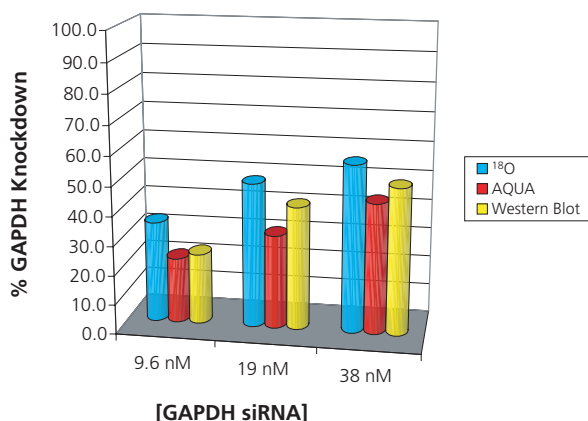


Figure 5: Comparison of Methods for Measuring Protein Expression in Gene Knockdown Experiments. For each method, data from each knockdown was compared to the non-interfering pool to determine percent GAPDH knockdown. Methods and data analysis for the ^{18}O labeling can be found on poster P169-M.

Conclusions

To our knowledge, this is the first example employing an AQUA-based approach for quantification of a protein targeted for knockdown using RNAi. While other approaches directed at quantifying protein levels are suitable for calculating relative protein concentrations, the 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. Finally, we believe the AQUA technique, which incorporates the use of an LC-MS separation, will enable a high-throughput, multiplexed approach to both proteomics analysis and RNAi functional validation experiments.

References

- Gerber, S. A.; Rush, J.; Stemman, O.; Kirschner, M. W.; and Gygi, S. P. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, 100, 6940–6945.
- Hannon, G. *RNAi: A Guide to Gene Silencing*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2003.

