

Verification of RNA Interference at the Protein Level via Quantitative Proteomics Analysis

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Abstract

The MISSION™ TRC shRNA Library is a comprehensive collection of 150,000 pre-cloned lentiviral-based shRNA vector constructs targeting 15,000 annotated human (MISSION TRC-Hs 1.0) and mouse genes for RNA interference-mediated silencing. This lentiviral-based system allows for transduction and integration into non-dividing and primary cells. 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.¹ Indeed, there is a pressing and demonstrable need to functionally validate mRNA knockdown by rapid and accurate quantitative surveillance of affected protein(s).

Absolute Quantification² (AQUA) is a targeted quantitative proteomics technique that exhibits robust efficacy and is being increasingly utilized for a wide variety of quantitative proteomics studies. Utilizing the MISSION TRC shRNA lentiviral-based system, a variety of human proteins have been targeted for gene knockdown. In the present work, we demonstrate that Protein-AQUA™ strategies can be sequentially coupled to RNAi technology in order to perform true quantitative verification of gene knockdown at the protein level.

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. 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 using mass spectrometry.

Targeted gene knockdown using RNA-mediated interference has proven to be a powerful tool for studying gene function in eukaryotes. Transfection of siRNA is the most widely used method for RNAi experiments; however, there are several drawbacks to be considered when employing this approach. To begin with, when employing siRNAs, the cell line being studied must be readily transfected. Although there are a variety of transfectable cell lines which are amenable to siRNA technology, delivering siRNAs to a large number of cell types, including primary cells, has proven to be problematic. Additionally, when using siRNAs, due to the transient nature of siRNA transfection, long-term silencing of a target gene cannot easily be achieved. This is readily attributed to a dilution effect, which occurs as transfected cells continue to divide, and target mRNA levels reaccumulate. The Mission TRC lentiviral library overcomes these issues by stably integrating shRNA into host chromosomes.

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 always correlate well with protein levels present in the cell. Additionally, methods for studying samples at the protein level, such as Western Blotting, while sensitive, often lack the ability to discriminate between samples in which the differences in protein levels are minimal.

In the present work, we have selected two human proteins (vimentin and hsp60) and targeted them for knockdown using a lentiviral-based shRNA vector system. By implementing the Protein-AQUA approach, we have demonstrated the usefulness of this technology to functionally verify gene knockdown at the protein level.

Methods

Lentiviral Particles

Lentiviral particles were obtained from Sigma-Aldrich (St. Louis, MO) for the human protein targets vimentin and heat shock protein 60 (chaperonin). Transduction particles had viral titers (p24-based) of 10⁵–10⁶ transducing units per milliliter.

Transduction

Human lung carcinoma A549 cells were plated at 10⁵ cells/well in a 24-well plate with Ham's Nutrient Mixture supplemented with 10% Fetal Bovine Serum, Heat Inactivated 24 hours prior to transduction. Six microliters of virus was added to each appropriate well along with Hexadimethrine bromide at a final concentration of 8 µg/mL. Each transduction was performed in triplicate. Transductions were conducted for approximately 30 hours, at which time media containing the lentiviral particles and Hexadimethrine bromide was removed and replaced with fresh media containing the selective antibiotic puromycin at a concentration of 2 µg/mL. Media and selective antibiotic were replenished once more at 96 hours. Final cell harvest was 120 hours post-transduction.

Cell Harvest and Sample Preparation

Samples were harvested by adding 300 µL of resuspension reagent (Hank's Balanced Salt Solution supplemented with 50 U/mL benzonase) to each sample well (from the 24 well plate) and thoroughly scraping the culture plate. Urea was added to each sample to obtain a final concentration of 8 M. The protein concentration of each sample was determined by Quanti-Pro 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 100 mM Tris-HCl, pH 8.5. Each sample was digested overnight with Proteomics Grade Trypsin (20:1 substrate:enzyme ratio). Following digestion, each sample was completely dried in a SpeedVac™. Just prior to analysis, each sample was resuspended in 10 µL of 0.1% TFA, containing each AQUA peptide at a concentration of 10 fmol/µL.

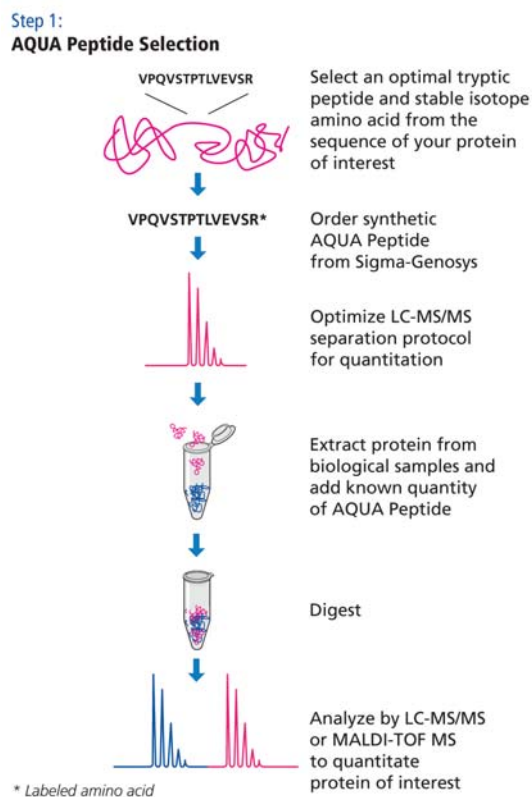


Figure 1: The Protein-AQUA™ Method. This method was developed by Dr. Steve Gygi and colleagues at Harvard Medical School [Stemann O, Zou H, Gerber SA, Gygi SP, Kirschner MW; Dual inhibition of sister chromatid separation at metaphase, *Cell* 2001, Dec 14, 107: 715-726] and is the subject of both U.S. and PCT issued patents. Limited use of this method is permitted under a licensing arrangement with Harvard Medical School.

LC-MS/MS Analysis of Data

An LC-SRM method was developed for peptides corresponding to the protein targets vimentin (FADLSEAANR) and hsp60 (VGLQVVAVK), as outlined in the Results section. The absolute quantity of each peptide (and corresponding protein) was determined, as shown in the Results section. Based on these results, the percent knockdown of each clone was determined.

Materials

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

- MISSION™ shRNA Lentiviral Transduction Particles, targeting human vimentin and heat shock protein 60 (Cat. No. [SHVRS](#))
- Proteomics Grade Trypsin (Cat. No. [T6567](#))
- Benzonase Endonuclease (Cat. No. [E1014](#))
- QuantiPro™ BCA Assay Kit (Cat. No. [QP-BCA](#))
- ProteoPrep® Reduction/Alkylation Kit (Cat. No. [PROT-RA](#))
- ProteoPrep® TCA Precipitation Kit (Cat. No. [PROT-PR](#))
- Human Lung Carcinoma A549 cells (ATCC CCL-185)
- Ham's Nutrient Mixture (Cat. No. [N4888](#))
- 10% Fetal Bovine Serum, Heat Inactivated (Cat. No. [F7678](#))
- Hexadimethrine Bromide (Cat. No. [H9268](#))
- Puromycin (Cat. No. [P9620](#))

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

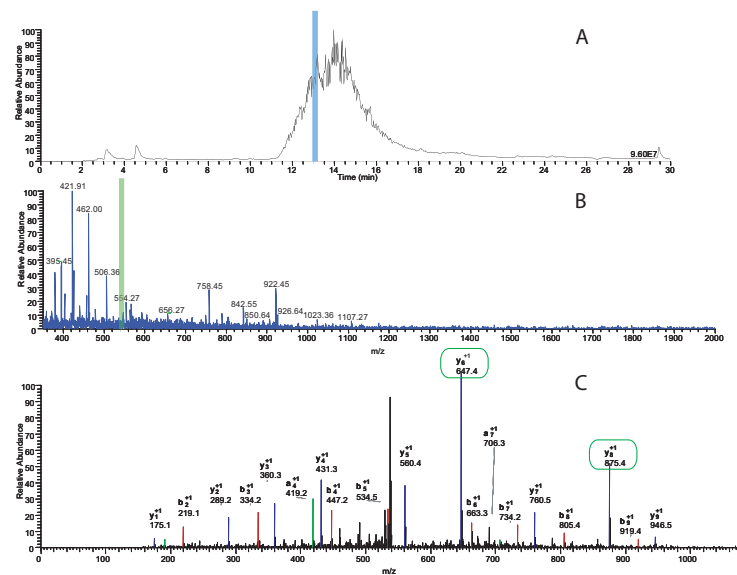


Figure 2: Development of Protein-AQUA LC-SRM for Absolute Protein Quantitation. An injection of a tryptically digested A549 cell lysate (prepared as described in Methods) was made using an Agilent capillary 1100 HPLC. The reverse phase separation was performed on a 15 cm × 2.1 mm Discovery C18 column, using a 30-minute gradient. The mobile phase consisted of formic acidified water and acetonitrile. The separation system was coupled to a Thermo Finnigan LTQ linear ion trap mass spectrometer set to perform tandem MS on the ten most abundant ions in each full scan spectrum. After obtaining tandem MS results on an ion of interest, it was added to an exclusion list to facilitate interrogation of lower abundance ions. The total ion current (**Figure 2A**) shows a complex mixture of species that are not chromatographically resolved. By harnessing the power of tandem MS, more than 100 proteins were confidently identified from the A549 cell lysate. Two of these proteins (vimentin and heat shock protein 60) were chosen for further study. The full scan MS shown in **Figure 2B** demonstrates the relative low abundance of the 547 Da doubly charged ion from one of the vimentin peptides identified. This sequence is then confirmed in **Figure 2C** with an abundance of y and b ions. This experimental approach lays the groundwork for choosing an appropriate AQUA peptide in addition to providing the necessary information for designing the SRM experiment. As shown in **Figures 2B** and **C**, the doubly charged parent ion of this specific vimentin peptide was selected for fragmentation, and the two most abundant y ions present in the tandem MS were selected for detection.

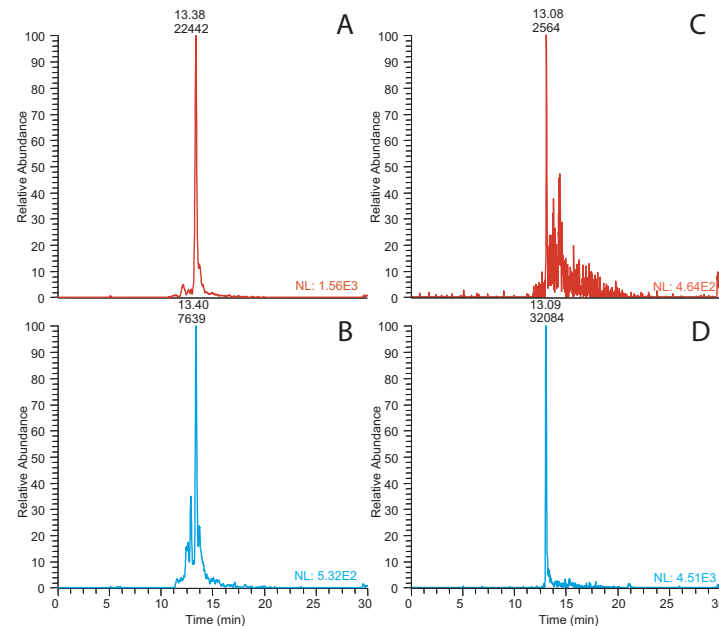


Figure 3: Protein-AQUA Analysis of Vimentin and Hsp60. Selected reaction monitoring (SRM) specifically targets MS detection of the selected parent and daughter ions. **Figures 3A** and **3C** depict the endogenous hsp60 and vimentin peptides, VGLQVVAVK and FADLSEAANR, respectively, as the doubly charged ions fragment to produce two abundant daughter ions. These same daughter ions are monitored in the AQUA peptide standard shown in **Figures 3B** and **3D**, however the absolute mass to charge of both parent and daughter ions in the AQUA spike are of higher mass due to the incorporation of a stable isotope amino acid (K or R, respectively). Quantitation is performed by integrating the co-eluting peak areas shown here at 13.4 and 13.1 minutes. All remaining non-specific signal is

discounted because the reversed phase retention characteristics do not match those of the peptide of interest. By calculating a ratio of the endogenous peptide to that of the known amount of AQUA peptide, an absolute amount of protein in the sample can be determined.

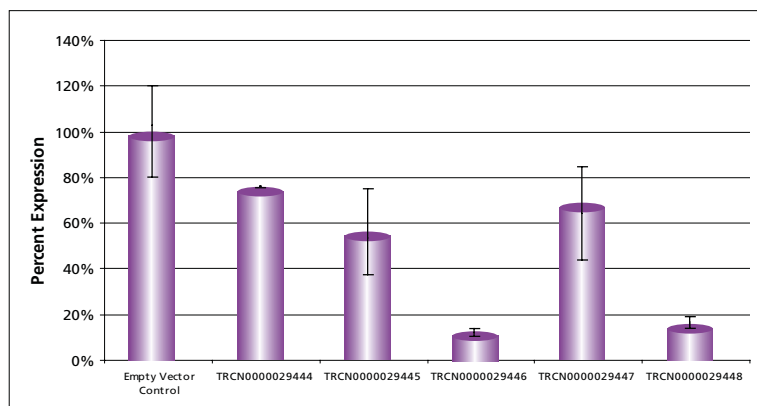


Figure 4: Silencing of Heat Shock Protein 60 Using MISSION shRNA Constructs.

Expression of heat shock protein 60 (chaperonin) following infection with various MISSION shRNA viral constructs. Percent expression was determined from the absolute protein levels calculated using the Protein-AQUA method. The data derived from this experiment indicates that at least two of the five clones achieved knockdown at the protein level greater than 80%.

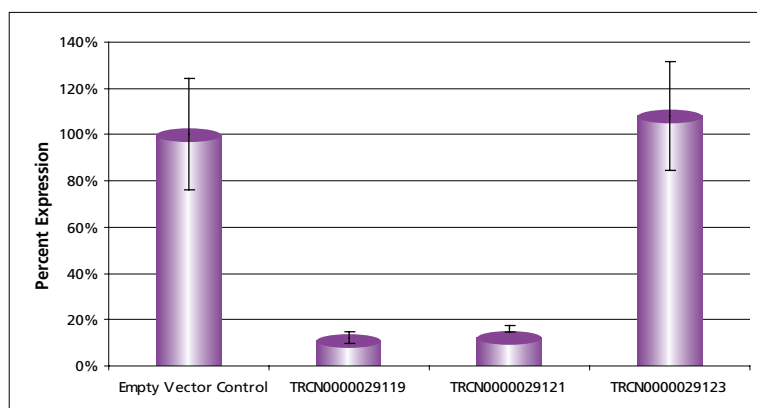


Figure 5: Silencing of Vimentin Using MISSION shRNA Constructs. Expression of vimentin following infection with various MISSION shRNA viral constructs. Percent expression was determined from the absolute protein levels calculated using the Protein-AQUA method. The data derived from this experiment indicates that at least two of the three clones achieved knockdown at the protein level greater than 85%.

Conclusions

RNA interference technology has rapidly become a powerful and vital tool for molecular biology research. In addition to providing a means for the study of gene function, as well as performing target validation, RNAi may potentially provide a new option for therapeutics. The MISSION TRC shRNA libraries overcome the limitations of synthetic siRNAs by providing a system for long-term silencing, by utilizing lentiviral particles for transduction and integration into primary cells, dividing cells, and non-dividing cells. To our knowledge, this is the first example employing an AQUA-based approach for quantification of a protein targeted for knockdown using lentiviral-based shRNA vectors. We believe this initial dataset strongly suggests that Protein-AQUA strategies can be sequentially coupled with RNAi technology to perform true verification of gene knockdown at the protein level.

References

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