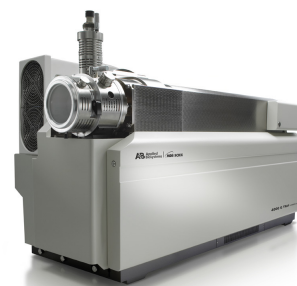


Validating and Quantitating Effects of siRNA-based RNA Interference on GAPDH at the Protein Level

Time Course Experiments Using the mTRAQ™ Reagents and the 4000 QTRAP® System

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RNA interference (RNAi) is a process in which exogenous, double-stranded RNAs that are complimentary to known mRNA's are introduced into a cell. These siRNA molecules cause the specific degradation of target mRNAs, thereby blocking and diminishing the translation and production of the encoded protein. The power and utility of RNAi for specifically silencing the expression of any gene has driven its rapid adoption as a tool for reverse genetics in eukaryotic systems. The decrease in mRNA levels is easily monitored by PCR, and the protein expression changes have been traditionally monitored by Western blot when antibodies are available for specific protein activity assays.

Presented is a targeted mass spectrometric approach using multiple reaction monitoring (MRM) and non-isobaric chemical tags (mTRAQ™ reagents) which enables rapid development of quantitative protein assays to monitor the effects of siRNA on protein expression. This eliminates the requirement for generating protein specific antibodies and stable isotope labeled synthetic peptides. Here, the assay developed for GAPDH will be discussed to show the validity of this mass spectrometric approach. GAPDH is one of the most common “housekeeping genes” used to compare gene expression data¹. Quantitative gene expression data are often normalized to the expression levels of these housekeeping genes. GAPDH has been implicated in several non-metabolic processes, including transcription activation, initiation of apoptosis, and ER to Golgi vesicle shuttling².

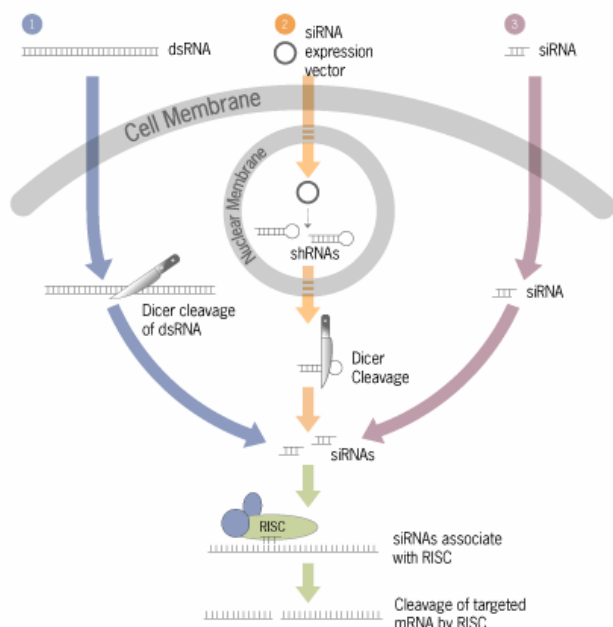


Figure 1. Mechanism of RNAi Interference. There are multiple mechanisms to trigger the RNAi Pathway. In non-mammalian systems, dsRNA (>30 bp) is introduced directly to the cells (1). For mammalian systems, either a siRNA expression vector (2) or a short synthetic interfering RNA (siRNA) molecule (3) can be used. In all cases, this leads to the destruction of the targeted mRNA by RISC (RNA-induced silencing complex) and loss of specific protein expression.

Key Advantages of the mTRAQ™ Reagents for Monitoring siRNA Interference Experiments

- Ease of assay creation
 - No antibodies required for specific analysis of target protein
 - No stable isotope labeled synthetic peptides required for quantitation
 - MRMPilot™ software can be used to directly design MRM assays from the available biological samples
- Highly specific and accurate relative quantitation for time course experiments of protein knock-down
 - mTRAQ™ reagents provides a cost effective strategy for obtaining accurate relative quantitation on multiple peptides for multiple proteins⁵

Experimental Methods

siRNA Transfection: 1×10^6 HeLa cells were transfected with 30nM GAPDH siRNA or negative control siRNA (siRNA with sequences that do not target any gene product). Cells were harvested at various time points (0, 24, 48, 72, 96, 120 hr) in 6 well plates. The samples were divided into 3 tubes: i) for protein activity assays, ii) for RNA extractions and iii) for protein analysis by MRM (Figure 2).

PCR Assay: The RNA was purified using the PARIS™ Kit (Protein And RNA Isolation System, (Ambion). RNA was reverse transcribed using High Capacity Reverse Transcription Kit (Applied Biosystems) and qPCR was performed for both 18S (housekeeping gene) and GAPDH on all samples.

Enzyme Assay: The amount of GAPDH protein activity was determined based on the KDAAlert™ GAPDH Assay Kit (Ambion). The KDAAlert™ Kit is a fluorescence based method for measuring the enzymatic activity of GAPDH from cultured cells. The transfected cells were disrupted in the lysis buffer and then mixed with KDAAlert™ Master Mix. The fluorescence was read on a plate reader at 0 and 4 minutes. The fluorescence difference between 0 and 4 minutes divided by the concentration of protein was used to determine the units of enzyme per mg of protein.

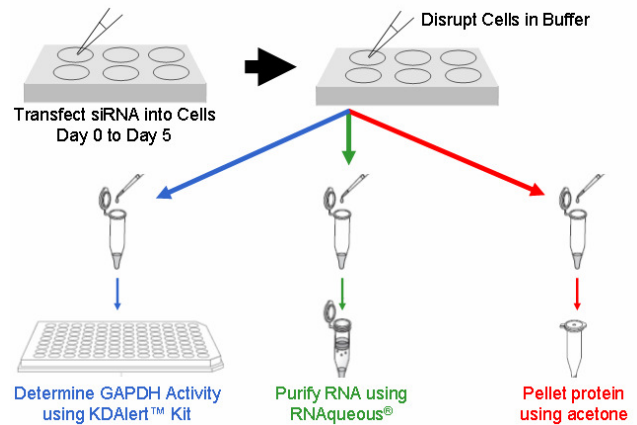


Figure 2. The Sample Preparation Flowchart. Each time point from the siRNA experiment is split into 3 parts for RNA, enzymatic activity and protein analysis.

Protein Preparation: The protein concentration was determined using BCA Protein Assay Kit (Pierce). Proteins extracted from each of the samples were reduced/alkylated, tryptically digested and labeled using mTRAQ reagents. Peptides from time 0 (both GAPDH transfected and negative control) were labeled with mTRAQ reagent light. All other time points were labeled with mTRAQ reagent heavy, and then mixed in 1:1 with the light labeled Day 0 sample (Figure 3). The peptide mix was fractionated by SCX into two fractions (120mM and 500mM ammonium acetate).

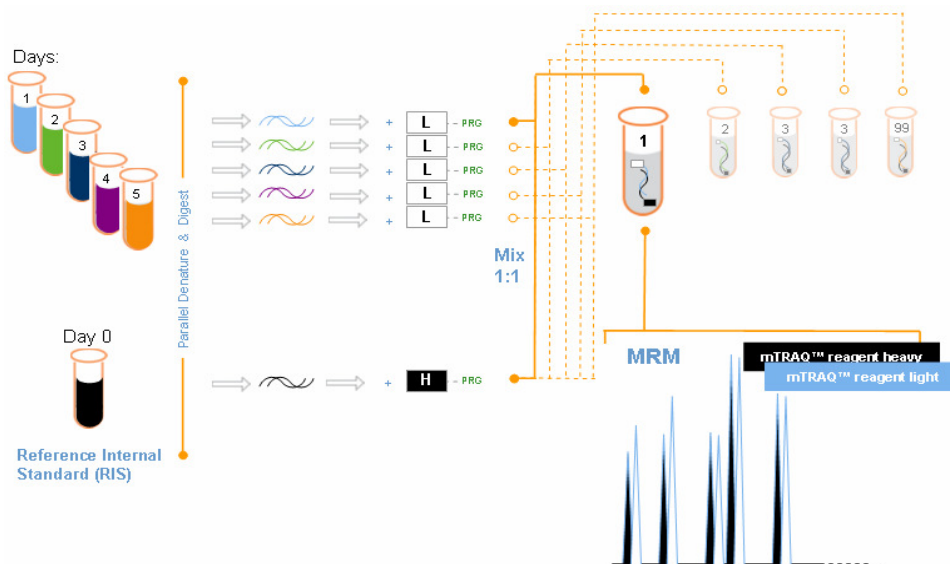


Figure 3. Non-isobaric peptide labeling workflow. The zero time point (Day 0) was used as the reference standard in both the GAPDH transfected and negative control experiments, and was labeled with the light mTRAQ™ reagent. Addition of this labeled standard into each individual labeled sample (mTRAQ reagent heavy) of the time course enables normalization of the MRMs across multiple samples. Each labeled time point is mixed at a ratio of 1:1 with Day 0. MRM analysis using MRM transitions to both the light and heavy labeled peptides was performed and the ratio vs. Day 0 computed for all time points.

Nano Liquid Chromatography: Samples were separated by reverse phase LC using the Tempo™ nanoMDLC System (AB SCIEX) and PepMap100 C18, 75 µm x 15cm nanoLC column (Dionex). Chromatographic method employed a 10 min online trapping and desalting step followed by a 1 hour gradient of 5-40% mobile phase B (mobile B = 98% acetonitrile, 0.1% formic acid).

Mass Spectrometry: Optimized MRM transitions for labeled GAPDH peptides were developed using MRMPilot™ software (Figure 4). Mass spectrometric analysis was performed with the 4000 QTRAP® system (AB SCIEX) using the MIDAS™ Workflow. The sequence of GAPDH protein was loaded into MRMPilot™ Software and MRM transitions were computed *in silico*. A MIDAS™ Workflow acquisition method was created and run on the labeled peptide fractions. Once the data is acquired, the MS/MS is used to confirm the signal detected from the desired peptide and then the MRM is evaluated for signal quality. If better MRMs were required, another round of analysis was performed using the experimentally determined MS/MS to choose better fragment ions. When good MRM transitions were determined for

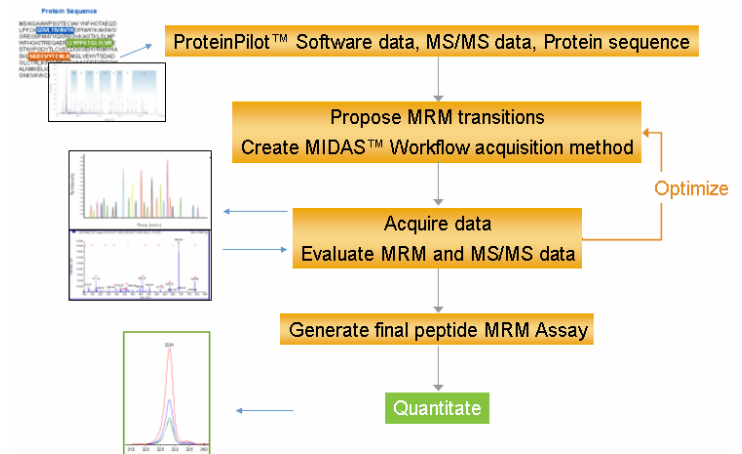


Figure 4. Workflow for MRM Assay Development with MRMPilot™ Software. Previous MS/MS data or protein sequence information can be loaded into MRMPilot™ Software as the starting information for assay development. MRM transitions are created and tested experimentally on QTRAP® systems using the MIDAS™ Workflow. The quality of the MRM signal is assessed for quantitative purposes and the full scan MS/MS is used to confirm the peptide ID. From this data, MRMs can be iteratively improved until the final MRM assay is created.

three peptides from the GAPDH protein, the final MRM assay was created.

Triplicate runs were performed for quantitative accuracy and to test reproducibility. Quantitation of the relative changes in GAPDH protein expression in each sample was determined using MultiQuant™ software.

PCR Analysis to Confirm mRNA Knockdown

RNA was reverse transcribed then quantitative PCR (qPCR) was performed for the housekeeping gene, 18S well as GAPDH in both the Negative control and GAPDH transfected samples. The difference between GAPDH transfected samples and the corresponding negative control was used to calculate the percent remaining GAPDH mRNA. This was normalized to the signal at Day 0 (Figure 5). The siRNA knocked down GAPDH mRNA by >90% until Day 3. A small amount of recovery of mRNA was observed at Day 4 as seen by an increase in mRNA levels.

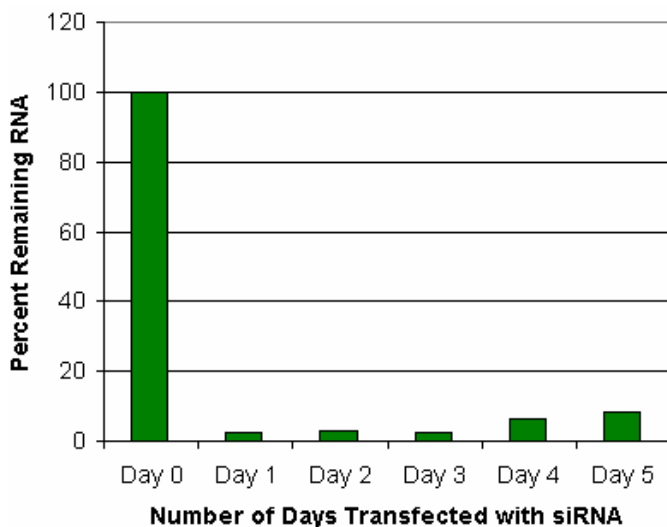


Figure 5. RNA levels after siRNA transfection. qPCR was first performed on the GAPDH and housekeeping gene, 18S. The comparative C_T method ($\Delta\Delta C_T$) is used to determine relative quantitation of gene expression. C_T s were normalized to its control, 18S, which is a housekeeping gene. The difference between GAPDH transfected samples and the corresponding negative control was used to calculate the percent remaining GAPDH RNA, in relation to Day 0. Percent remaining = $(2^{-\Delta\Delta C_T[\text{Day}]}) / (2^{-\Delta\Delta C_T[\text{Day 0}]})$. $\Delta\Delta C_T = (C_T \text{ GAPDH} - C_T \text{ 18S}) \text{ GAPDH Transfected} - (C_T \text{ GAPDH} - C_T \text{ 18S}) \text{ Negative Control Transfected}$.

GAPDH Enzymatic Activity

The GAPDH protein activity was determined using the KDAIert™ Kit. As expected, the percent remaining protein activity decreased with time after siRNA transfection. Similar to the mRNA levels, the enzymatic activity observed increased in the later timepoints, showing some recovery at Day 5 (Figure 6). However unlike the sharp decrease in the mRNA level after 1 day of transfection, the decrease in protein activity was more gradual. This is because the half life at protein level is >35 hrs compared to 8 hrs at the mRNA level^{3,4}.

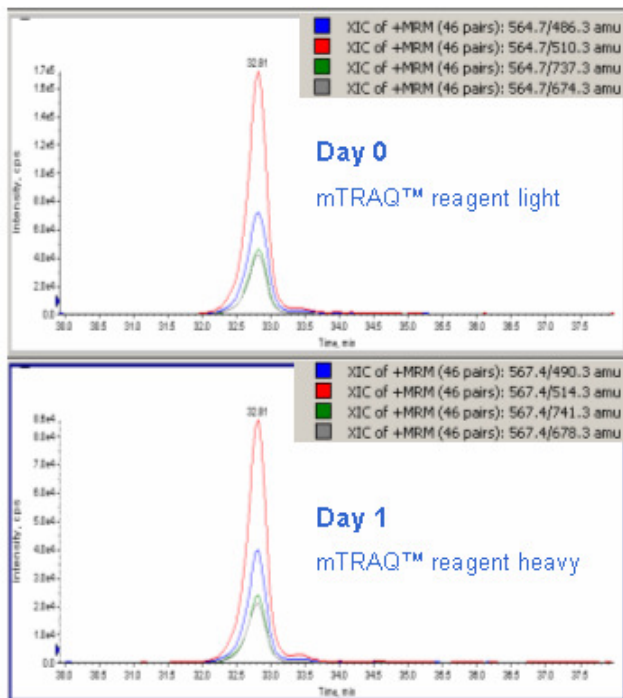


Figure 7. MRMs transitions of light and heavy mTRAQ™ reagent labeled GALQNIIPASTGAAK peptide. Shown are the MRM transitions of the Day 0 time point (top) labeled with light reagent and the Day 1 time point (bottom) labeled with heavy reagent present in a 1:1 mixture of the samples. Four MRM transitions for each form of the labeled peptides are monitored. The intensity ratios between each transition within the light labeled peptide correlates with the ratios for the heavy labeled peptide. This ensures that the signal obtained from MRM transitions of each specific peptide has no contribution from isobaric interferences which would skew the quantitation result, adding quantitative robustness to the assay. The difference in intensity between heavy and light provides information on the difference in protein content between Day and Day 0.

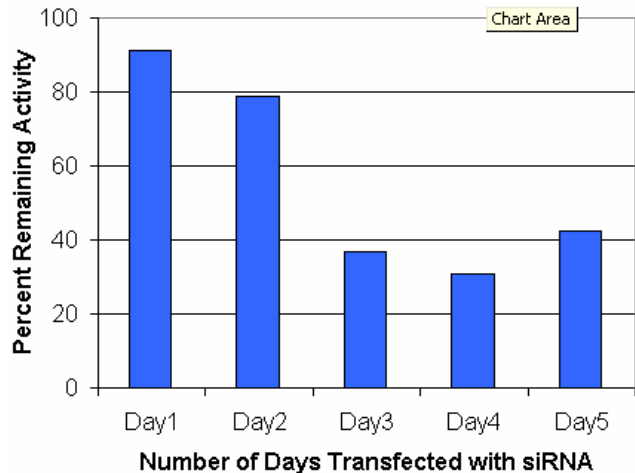


Figure 6. GAPDH Protein Activity after siRNA transfection. The cells were first disrupted in lysis buffer and then mixed with KDAIert Master Mix. The fluorescence was read on a plate reader at 0 and 4 minutes. The difference between 0 and 4 minutes was determined and divided by the concentration of protein to obtain the units of enzyme per mg of protein. The percent remaining activity of the protein is equal to the ratio between the enzyme activity of GAPDH transfected samples and its corresponding negative control. The protein activity decrease by 10% by day1 and kept decreasing until day 4 after which the activity began to rise.

Quantitative MRM Analysis of Protein Levels

Three tryptic GAPDH peptides, GALQNIIPASTGAAK, LTGMAFR, and LSWYDNEFGYSNR, were tracked for the various siRNA interference time points. Four MRM transitions for each peptide were monitored to increase robustness to the MRM assay (Figure 7).

To ensure 1:1 mixing ratio between Day 0 and other time points, peptides from proteins that are known to be unaffected by the interference experiment were used to normalize the sample mixing. Two peptides from actin-2, GYSFTTTAER and GILTLK, were chosen and monitored in each experiment. The ratio of these peptide between Day 0 and each time point is expected to be 1:1, if a deviation was observed, it was used to correct the GAPDH ratios for sample mixing.

For all 3 peptides monitored by MRM, the relative level of each decreases to <70% in the first 24 hrs and gradually decreased as time progressed. The protein expression MRM data (Figure 8) correlates well with the protein activity data as expected. After Day 4, the protein expression also shows slight increase in level which corroborates both the protein activity response and the observed recovery of mRNA from the qPCR results. As expected, there were very minor protein level changes (Figure 8) for the various time points with negative control siRNA (siRNA with sequences that do not target any gene product).

Conclusions

A targeted MRM approach using mTRAQ™ reagent was developed for monitoring the effect of GAPDH siRNA on the relative protein expression levels. Maximal knockdown of the mRNA took place in 1 day and started to recover after 3 days. The protein activity also decreased over time and the activity started recovering after 4 days of the transfection. This parallels the pattern obtained for the protein expression level from mass spectrometric analysis. This study provides a proof of concept for the use of MRM analysis and the mTRAQ™ reagents for monitoring protein expression levels from siRNA knockdowns.

This type of analysis is cost effective to undertake because it does not require antibodies to be created or stable isotope labeled peptides to be synthesized. Many proteins and multiple peptides per protein can be easily monitored with this strategy at no extra expense to add increased robustness to the quantitative assay.

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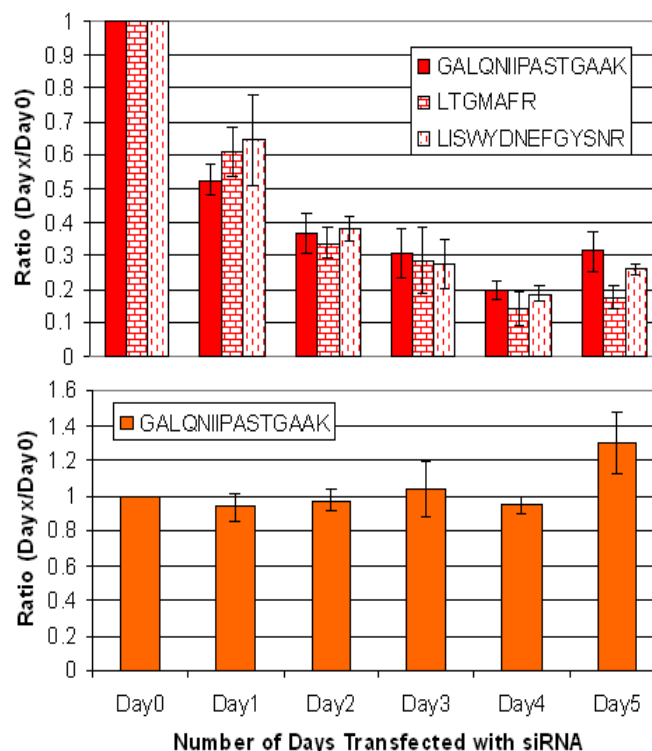


Figure 8. Time course study of GAPDH protein level with siRNA transfection. Shown are the GAPDH levels for 3 different GAPDH peptides at various timepoints of siRNA transfection (Top). As a reference, Day 0 is set to 1 (100%) and the various time points are compared to Day 0. In addition, GAPDH was monitored in the negative control siRNA transfection (Bottom) and very little change was observed as expected.

References

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