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Evaluation of Direct Infusion-Multiple Reaction Monitoring Mass Spectrometry for Quantification of Heat Shock Proteins

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Abstract

Protein quantification with liquid chromatography-multiple reaction monitoring mass spectrometry (LC-MRM) has emerged as a powerful platform for assessing panels of biomarkers. In this study, direct infusion, using automated, chip-based nanoelectrospray ionization, coupled with MRM (DI-MRM) is used for protein quantification. Removal of the LC separation step increases the importance of evaluating the ratios between the transitions. Therefore, the effects of solvent composition, analyte concentration, spray voltage, and quadrupole resolution settings on fragmentation patterns have been studied using peptide and protein standards. After DI-MRM quantification was evaluated for standards, quantitative assays for the expression of heat shock proteins (HSPs) were translated from LC-MRM to DI-MRM for implementation in cell line models of multiple myeloma. Requirements for DI-MRM assay development are described. Then, the two methods are compared; criteria for effective DI-MRM analysis are reported based on the analysis of HSP expression in digests of whole cell lysates. The increased throughput of DI-MRM analysis is useful for rapid analysis of large batches of similar samples, such as time course measurements of cellular responses to therapy.

Keywords

Quantitative Mass Spectrometry; Direct Infusion; Multiple Reaction Monitoring Mass Spectrometry; Heat Shock Proteins; Multiple Myeloma

Introduction

Liquid chromatography-multiple reaction monitoring mass spectrometry (LC-MRM) has emerged as versatile method for quantification of protein biomarkers via tryptic peptide surrogates.[1–5] LC-MRM has been implemented for measurement of biomarker panels,[6–8] modified peptides,[9–12] and detection of mutations.[13] LC-MRM has become the method of choice for evaluation of candidate biomarkers generated by discovery proteomics;[14,15] in addition, standardization has enabled reproducible data to be generated at multiple sites.[16,17] However, most LC-MRM experiments have low to medium throughput (typically 10 to 20 samples per day). To develop additional capability for high throughput protein quantification, direct infusion (DI) can be used for sample introduction prior to MRM analysis. This development may augment the current LC-MRM capabilities in biomarker evaluation and biology.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

Similar to the development trajectory of LC-MRM, direct infusion mass spectrometry has been well established in quantitative analysis of small molecules.[18–22] Quantification of drugs using chip-based nanoelectrospray ionization sample introduction has also been evaluated for regulatory compliance, indicating that these methods can be translated to clinical scenarios.[23] In addition, "top-down" proteomics approaches often use direct infusion nanospray ion sources.[24–26] Applications have also been demonstrated in "bottom-up" or peptide-based proteomics.[27,28] The use of infusion nanospray will complement matrix assisted laser desorption ionization (MALDI) MRM assay development for quantification of small molecules[29–31] and proteins.[32]

In order to evaluate the utility of DI-MRM and define the criteria for assay development, heat shock proteins will be monitored in tryptic digests of whole cell lysates prepared from multiple myeloma cell lines. Heat shock proteins (HSPs) are overexpressed in a wide range of human cancers, particularly multiple myeloma (MM);[33–35] for that reason, they are currently being evaluated both as biomarkers[36] and as therapeutic targets.[37–40] Quantification of these proteins could be used to characterize tumors, evaluate therapeutic responses, and detect acquired drug resistance. High throughput analysis with DI-MRM would enable rapid parallel processing of large numbers of samples from treatment time courses and dose response curves in model systems with potential future translation to clinical samples. For proof of principle, the assessment of HSPs was performed in RPMI-8226 MM cells and the melphalan-resistant cell line, 8226/LR5.[41]

DI-MRM assays were developed from existing LC-MRM data and applied to quantification of standard peptides, standard proteins, and HSPs.[42] The effects of different parameters on DI-MRM data were explored using standard peptides and protein digests to define the steps required for assay development. DI-MRM and LC-MRM quantification of HSPs in a background of digested whole cell lysate were compared to indicate the criteria required to optimize data acquisition against a complex biological background. This feasibility study for DI-MRM indicates its potential and limitations for rapid quantification of panels of protein biomarkers.

EXPERIMENTAL

Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) at the highest purity, unless otherwise specified. Formic acid was acquired from Fluka (Sigma-Aldrich, St. Louis, MO). HPLC solvents (water and acetonitrile) were from Burdick and Jackson (Honeywell, Muskegon, MI). Bovine serum albumin (BSA) was purchased from (96% purity, A-3912, Sigma, St. Louis, MO).

Synthesis and DI-MRM of Standard Peptides

Standard peptides were synthesized and characterized as described previously.[42] An automated chip-based nanoelectrospray ion source, (NanoMate100, Advion BioSciences, Ithaca, NY), was mounted on a triple quadrupole mass spectrometer (TSQ Vantage, Thermo, San Jose, CA). To evaluate the effects of experimental conditions and instrument parameters on the observed peptide fragmentation pattern, two synthetic peptides, ALLFVPR (containing stable isotope-labeled proline) and IEADSESQEEIIR were diluted to 1 μM in 50% aqueous ACN with 0.1% formic acid. For synthetic peptide infusion, the spray voltage was tuned between 1.5 and 1.9 kV for stable ion signal. The gas pressure was set to 0.3 psi. Data were acquired at 20 ms per transition for 2 minutes using m/z 0.002 scan widths. The quadrupole resolution values were modulated from 0.2 to 0.7.

Standard Protein DI-MRM

As described in the supporting information, a dilution series of BSA digest was created in 50% aqueous acetonitrile with 0.1% formic acid spiked with five synthetic peptide standards (20 nM). In DI-MRM, the spray voltage was set to 1.6 kV, and the gas pressure was set to 0.3 psi. Peptide selection was performed using Q1 = 0.4; fragment ion selection was performed using Q3 = 0.7. Transitions (n = 36) were monitored for 20 ms each with scan width set to 0.002; data were acquired for 5 minutes. Relative and absolute quantification values were calculated using the ratio of the endogenous and standard peptide ion signals observed for all monitored transitions.

Cell Culture, Lysis, and HSP Sample Preparation for Mass Spectrometry

RPMI-8226 and 8226/LR5 cells were maintained as previously described;[41,42] details are provided in the supporting information. The heat shock protein 90 inhibitor, 17-desmethoxy-17-*N*,*N*-dimethylaminoethylamino-geldanamycin (17-DMAG), was dissolved in DMSO (100 μM stock). Based on prior IC50 measurements, the RPMI-8226 cells were treated with 100 nM 17-DMAG for 24 hours, and 8226/LR5 cells were treated with 50 nM 17-DMAG. Cells (n = 10^6) were lysed using in aqueous 100 mM ammonium bicarbonate containing 8 M urea for protein denaturation. Protein concentrations were measured by Bradford assays; consistent aliquots of total protein (380 ng) were injected for LC-MRM, and the same solutions were used for DI-MRM. Digestion, addition of stable isotope-labeled standard (SIS) peptides, and mass spectrometry sample preparation were performed as described for standard proteins (see supporting information).

LC-MRM Quantification

For HSP proteins in cell lysate, 184 transitions from 32 peptides (see Table 1) were monitored as previously described (see supporting information).[42] Both relative and absolute quantification are calculated from the sum of the peak areas for all detected transitions using Skyline[43] or the combination of MRMer[44] and Post-MRMer.[45]

Calculation of Correction Factors to Account for Interference in DI-MRM

Additional peaks observed in LC-MRM would constitute interference in DI-MRM for transitions from either endogenous peptides or SIS peptides. To evaluate the ability to eliminate these contributions, correction factors were calculated based on LC-MRM data using the following set of equations (Equations 1 and 2):

$$\text{Correction Factor (CF)} = (\text{LC}-\text{MRM Peptide PA})/(\text{TIC PA}) \quad \text{[Eqn. 1]}$$

$$\text{Correction DI}-\text{MRM Ratio} = \frac{(\text{DI}-\text{MRM PA}_{\text{endogenous}}) \times (\text{CF}_{\text{endogenous}})}{(\text{DI}-\text{MRM PA}_{\text{standard}}) \times (\text{CF}_{\text{standard}})} \quad \text{[Eqn. 2]}$$

where PA is peak area and TIC is total ion chromatogram (*i.e.* the total signal observed for that transition throughout the entire LC separation). This correction factor strategy may not be applicable to samples that could have different biological backgrounds (*e.g.* different cell types or tissues) and therefore different interference contributions.

RESULTS AND DISCUSSION

Stability of Peptide Transition Ratios under Different Conditions

In DI-MRM, verification of the identity of the target peptide can only be achieved using the ratios of the fragment ion intensities. For that reason, the effects of different parameters on

the transition ratios were investigated. The effects of quadrupole resolution settings on fragment ion transmission and transition ratios were investigated using two synthetic peptides: ALLFVPR (containing labeled proline) and IEADSESQEEIIR (see Figure S-1). Decreasing the values set for Q1 and Q3 resolution from 0.7 to 0.4 and 0.2 lead to progressively lower amounts of signal. Q1 resolution did not have an effect on fragment ion ratios, but modulation of Q3 did. The changes in transition ratios due to Q3 resolution are most notable when comparing fragment ions with low m/z values (< 300) to those with higher m/z values (> 500). Therefore, Q1 and Q3 settings should not be varied during an experiment. If narrower values are selected for Q3 resolution, the transition ratios must be re-evaluated. Variations in the solvent system (from 10% to 90% ACN) did not have any effect on fragmentation patterns. To enable comparison with LC-MRM, 50% aqueous acetonitrile with 0.1% formic acid was selected as the solvent system for DI-MRM. Data were acquired for the BSA digest (50 nM) at 7 different spray voltages (from 1.5 to 2.0 kV). Loss of low intensity ion signals was noted at the two lowest spray voltages, but transitions with sufficient intensity (above 20 a.u.) did not show significant differences in fragmentation patterns (transition ratio CV values < 2. In order to eliminate ion signal loss, higher spray voltages were selected for the rest of the experiments (1.6 to 1.9 kV for standard peptides and protein digests and 1.9 kV for digested whole cell lysate).

Next, nine different concentrations (from 0.4 to 80 nM) of the BSA digest were prepared to evaluate DI-MRM reproducibility, sensitivity and linearity of response. Prior to sample analysis, the solvent blank was analyzed as a control. Among the 36 transitions monitored for endogenous and spiked standard BSA peptides, most intensity values were less than 1 a.u with the maximum observed value ~3 a.u. For peptides with the lowest amounts of ion signal, DDSPSLPK and QTALVELLK, the CV values for transition ratios were below 10% for all samples with concentrations greater than or equal to 2 nM. For the peptides with the highest ion signals (peak intensity > 300 a.u.), AEFVEVTK and YLYEIAR, the CV values for the transition ratios measured across the entire dilution series were less than 7%; for concentrations ≥ 2 nM, the CV values of the transition ratios were less than 2%, indicating the high degree of reproducibility for the transition ratios in DI-MRM. DI-MRM sensitivity was established using consistency of the transition ratios. The fragmentation pattern of the AEFVEVTK peptide in DI-MRM data was consistent with LC-MRM for all concentrations except 0.4 nM, indicating the threshold for transition ratio verification, and thus for quantification of the peptide (Figure S-2A). At high concentrations of endogenous peptides, saturation effects were notable (Figure S-2B), so matched SIS peptides are required for DI-MRM quantification. However, this DI-MRM assay has high linearity ($R^2 = 0.9946$) over this range of concentrations (Figure S-2C).

Quantification of Heat Shock Proteins in Digests of Whole Cell Lysates

DI-MRM assay development is illustrated using the endogenous and SIS peptides for ALLFVPR from HSP90α (Figure S-3). Total ion signal and potential interferences are evaluated for each peptide and each transition. A cutoff value of 40% interference was selected to enable evaluation of transitions with little interference as well as the utility of correction factors to reduce or eliminate the contribution of noise and interfering peptide peaks. The level of interference in each transition is included in Supplementary Table S-1, and a heat map of the error induced in relative and absolute quantification plotted against interferences (in %) in both endogenous and standard transitions is shown in Supplementary Figure S-4.

The next step in the development of the DI-MRM assay was to evaluate the amount of ion signal observed for each transition and assess whether it was sufficient for quantification. First, a solvent blank was analyzed to observe the level of background noise in each of the

184 monitored transitions; the maximum value for peak intensity was ~3 a.u. Then, DI-MRM peak intensity was plotted against LC-MRM peak area (Figure 1A). Noise contribution can be significant for peptide ion signals at or below 10 a.u. in DI-MRM intensity. A trend line was fitted to the data, which indicated a strong correlation between the two methods ($R^2 = 0.9732$) when DI-MRM intensity values were greater than 10 a.u. For the peaks with low intensity in DI-MRM (< 10 a.u.), the correlation of DI-MRM intensity with LC-MRM peak area was poor ($R^2 = 0.2796$). Because of the noise contribution, quantification will not be reliable for peaks below a certain intensity threshold; theoretical calculations for the effect of baseline noise on the relative and absolute quantification derived from DI-MRM data are shown in Supplementary Figure S-5. For comparison, the peak intensity values for each peptide (given as the sum of all transitions) are shown in Supplementary Table S-2. Each peak must be evaluated, because the limits of detection and quantification as well as the accuracy of the values will depend on the amount of background noise observed in each transition.

Selection of DI-MRM Acquisition Times

After transition evaluation, the total sample analysis time was selected (Equation 3).

$$\text{Total Acquisition Time} = (\#\text{of transitions})(\text{scan time per transition})(\#\text{of observation}) \quad \text{[Eqn. 3]}$$

For BSA (monitoring 36 transitions), one minute acquisitions obtained more than 80 observations for each transition and generated reproducible data with CV values typically less than 10%. For analysis of HSP proteins in digests of whole cell lysates, the effect of acquisition time on CV was evaluated using data acquired over spans of 30 seconds to 10 minutes (see Supplementary Figure S-6). Using 20% CV as a maximum cutoff value, data could be acquired in as little as two minutes for this set of transitions (n = 184, each sampled 32 times). CV values correlate well with DI-MRM peak intensity; higher intensity values produce more precise measurements. One noteworthy advantage of DI-MRM is that data acquisition time can be lengthened to improve sampling and assay performance, whereas in LC-MRM the peptides can only be detected over their elution profiles. DI-MRM experiments described here typically include > 30 observations of each transition. If improved precision was required (*e.g.* CV < 10%), data could be acquired for 5 minutes for HSP monitoring.

Comparison of LC-MRM to DI-MRM Measurements of HSPs in Digests of Whole Cell Lysates

HSP expression levels were monitored in RPMI-8226 and 8226/LR5 cells following 17-DMAG treatment. In order to examine the trends in protein expression and compare LC-MRM and DI-MRM data sets, heat maps were generated for the relative expression of each protein using ratios to the pretreatment control (Figure 1B). No proteins decreased significantly, so darker shading indicates increases in protein expression. Overall, the same trends are observed by both LC-MRM and DI-MRM. Most notably, the upregulation of HSP90α, HSP90β, and HSP71 are diminished and delayed in the melphalan-resistant 8226/LR5 myeloma cells, when compared to RPMI-8226 cells. Here, DI-MRM is consistent with LC-MRM in the pattern of changes in protein expression, but the use of DI-MRM for relative and absolute quantification must be further explored. DI-MRM expression ratio measurements of HSPs from RPMI-8226 cell lysates correlate well ($R^2 = 0.9368$) to the corresponding LC-MRM values (Figure 1C). The slope of the trend line (1.5) is driven mainly by the differences in ratios with higher fold change values; most DI-MRM ratio values were lower than those from LC-MRM data. Comparison of the CV values for the same data set acquired for RPMI-8226 cells is shown in Figure 1D, illustrating that DI-

MRM variability is higher than LC-MRM in these assays applied to digests of whole cell lysate. Even so, most measurements (33/40) still have CV values below 20%, indicating that DI-MRM can still obtain sufficient precision.

Then, a more detailed examination of relative quantification was performed to evaluate the use of raw DI-MRM data and correction factors. Different methods for relative quantification have been compared for evaluating HSP90α expression at 24 hours after treatment (see Supplementary Table S-3). In the LC-MRM data, selection of different sets of transitions ($y_3 - y_6$, $y_3 - y_5$, or just y_3) produced the same results, approximately 2.2-fold increase in RPMI-8226 and 1.2-fold increase in 8226/LR5 cells. For RPMI-8226 cells, none of the DI-MRM measurements was significantly different from LC-MRM ($p > 0.05$). Selection of the single transition (y_3) with the least interference in LC-MRM resulted in the highest CV value (30%) and the most disparate value for the change in relative expression. In 8226/LR5 cells, all values for relative quantification of the change in HSP90α were significantly different from the LC-MRM data ($p < 0.05$). The values for the changes in expression of HSP90α were calculated to be 1.1- fold in DI-MRM versus 1.2-fold in LC-MRM. Again, the use of the single transition produced the most disparate data (0.76-fold change in expression). From both cell lines, selection of the unique transition from ALLFVPR with no background peaks in LC-MRM is not the best strategy for DI-MRM. Correction factors did improve the agreement between DI-MRM and LC-MRM, but some differences were still significant.

Based on these results, the use of correction factors to eliminate the contribution of interference in DI-MRM data for other peptides was evaluated. Relative quantification of the other HSPs was calculated using all LC-MRM transitions with correction factors for elimination of the contribution of interference (see Table 2); in addition, relative quantification was also calculated using selected LC-MRM transitions (below 40% interference contribution) with interference correction (see Supplementary Table S-4). Most of the LC-MRM and DI-MRM data are concordant; those values that show significant differences based on Student's t tests had very high precision in both LC-MRM and DI-MRM (CV values typically less than 2%). Those measurements also still showed the same trends in protein expression, as noted above (see Figure 1). Agreement in the values for relative quantification is noted for the HSP peptides that had the highest ion signals, but deviations are observed in peptides with lower amounts of DI-MRM ion signals. Similar to relative quantification, the interference-corrected values for absolute quantification (Table 3) were reliable for peptides with higher intensity values, but discordant when the peak intensities were low. The expression levels of HSP90α, HSP90β, CDC37, HSP7C, and HSPA5 were consistent by LC-MRM and DI-MRM ($p > 0.05$), but some of the errors in the average values were > 20% when comparing against LC-MRM data. DI-MRM measurements of the expression levels of proteins with low intensity peptides, such as HSP71, HSP74, and HSP7E, were significantly different from the LC-MRM results. Therefore, DI-MRM is most appropriate for high intensity peptides (> 100 a.u.); however, data for proteins like CDC37 indicate that lower intensity DI-MRM measurements can be consistent with LC-MRM when corrected for the contributions from interference.

CONCLUSIONS

DI-MRM assay design and evaluation were examined using standard peptides and proteins as well as heat shock protein expression measurements in digests of whole cell lysates. While, DI-MRM can significantly reduce analysis time per sample (2 minutes/sample vs. ~2 hours for LC-MRM), additional steps are required in assay development and evaluation. DI-MRM and LC-MRM assay results can be similar when the peak intensity is sufficient or when corrected to eliminate the contribution of noise and interfering peptide peaks. DI-

MRM is appropriate for high abundance proteins within a sample, and interference(s) in each MRM transition will not be differentiated from the ion signal of interest. Applications involving fractionated peptides or affinity-enriched samples (*e.g.* immunoprecipitates) could eliminate this issue. Furthermore, the use of DI-MRM was explored for samples with highly similar proteomes (*i.e.* time courses of drug treatment in cell lines), which should not have changes in background noise and interference; translation to tissue specimens or other clinical materials requires further study due to potential changes in other protein constituents.

During these experiments, stable ion signal could be routinely obtained from 5 μl samples for 70 minutes. Multiplexed assays could be developed for 6,000 transitions using the sampling rates described above (see Equation 3), enabling high content screening. The expansion of DI-MRM to measuring unique features in the tandem mass spectrometry landscapes generated by MS^e,[46] all ion fragmentation (AIF),[47] or sequential windowed acquisition of all theoretical fragment ions (SWATH-MS or MS/MS^{ALL}) could prove to be useful as proteomics transfers its focus from cataloging to quantification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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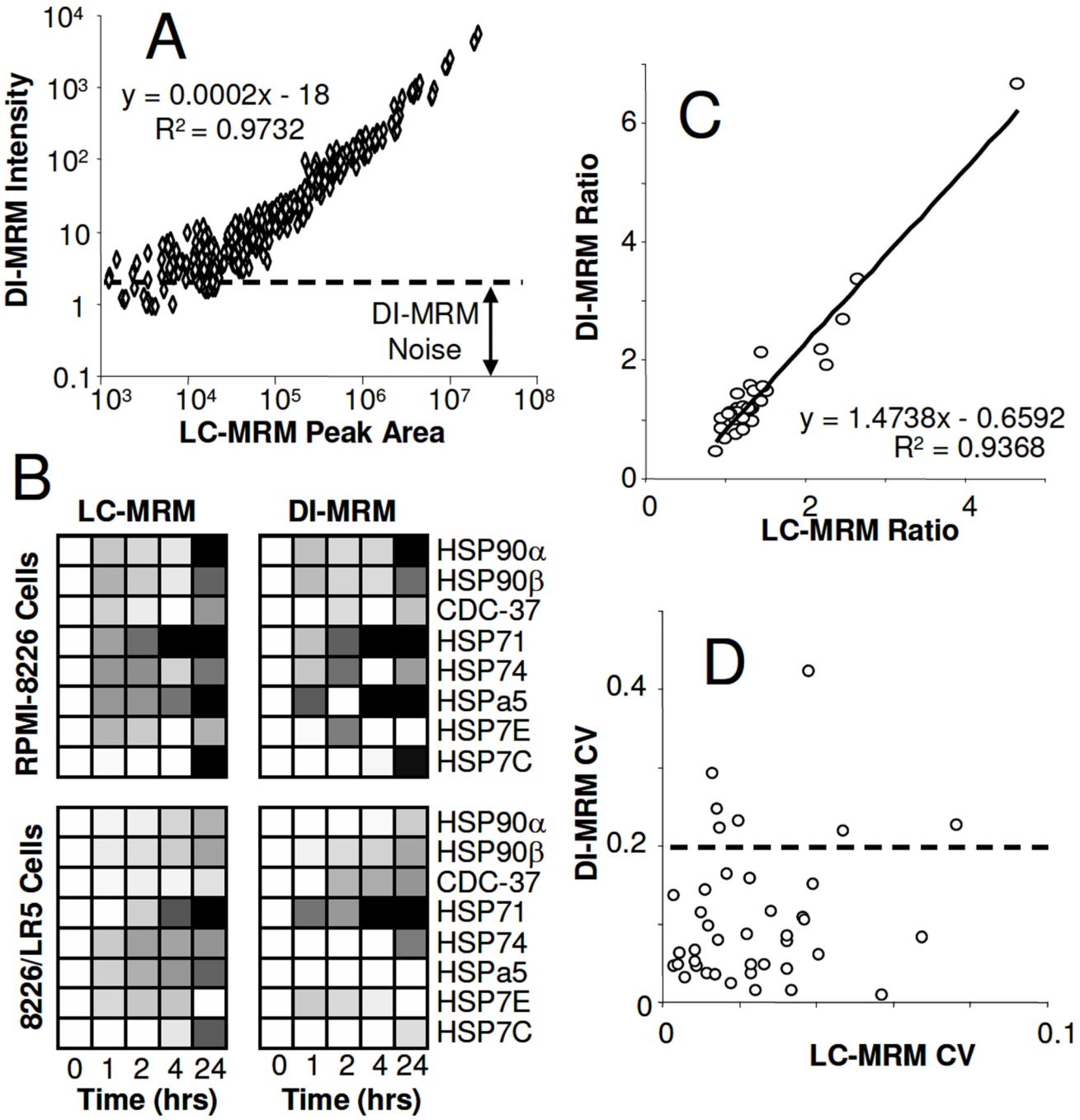

Figure 1. Correlation between DI-MRM and LC-MRM Quantification of HSPs in Digests of Whole Cell Lysate

Correlation between signal intensity in DI-MRM and LC-MRM peak area for all transitions used to monitor HSPs in RPMI-8226 lysates (**A**). Lower intensity signals have higher DI-MRM intensity values due to noise-broadening. The dashed line indicates the maximum level of LC-MRM noise observed in a solvent blank; however, each transition has a different level of noise (the range is indicated with the arrow). In most cases, the intensity of the noise is ~1 on this QqQ mass spectrometer. Heat maps of protein expression indicate changes in relative expression of HSPs after treatment with 17-DMAG (**B**). Expression ratios determined by LC-MRM and DI-MRM are highly correlated, as shown for protein

expression in RPMI-8226 cells (**C**). CV values for DI-MRM measurements for protein expression in RPMI-8226 cells are typically higher than LC-MRM, but the majority (33/40) are still below 20% (**D**).

Table 1

LC-MRM and DI-MRM Assays Developed for Measuring the Expression of Heat Shock Proteins

For each protein, the Uniprot accession, peptide(s), label, and transitions for LC-MRM analysis are listed. Transitions for DI-MRM were selected using a cutoff value of 40% for interference contribution from other ion signals observed in that transition during LC-MRM. GAPDH was used as a control for evaluation of protein loading.

Protein (Uniprot)	Peptide	Label	LC-MRM Transitions	DI-MRM Transitions
HS90A	ALLFVPR	P_6: $^{13}C_5$, ^{15}N	$y_3 - y_6$	$y_3 - y_5$
	DQVANSAFVER	V_9: $^{13}C_5$, ^{15}N	$y_4 - y_9$	$y_5 - y_9$
HS90B	ALLFIPR	P_6: $^{13}C_5$, ^{15}N	$y_3 - y_6$	$y_3 - y_5$
	EQVANSAFVER	V_9: $^{13}C_5$, ^{15}N	$y_4 - y_9$	y_5, y_6, y_8, y_9
CDC37	LQAEAQQLR	L_8: $^{13}C_6$, ^{15}N	$y_4 - y_7$	y_7
	EGEEAGPGDPLLEAVPK	P_{16}: $^{13}C_5$, ^{15}N	y_4, y_5, y_8, y_9, $y_{11} - y_{13}$	y_8, $y_{11} - y_{13}$
HSP71	NQVALNPQNTVFDAK	V_{11}: $^{13}C_5$, ^{15}N	y_3, y_4, $y_9 - y_{12}$	y_3, y_4, $y_9 - y_{12}$
HSP7C	GTLDPVEK	V_6: $^{13}C_5$, ^{15}N	$y_3 - y_7$	y_4, y_6
HSP74	AFSDPFVEAEK	V_7: $^{13}C_5$, ^{15}N	y_4, $y_6 - y_9$	y_7, y_9
HSPA5	VEIIANDQGNR	V_1: $^{13}C_5$, ^{15}N	$y_4 - y_9$	y_7, y_9
HSP7E	FTVLFPSGTPLPAR	P_{12}: $^{13}C_5$, ^{15}N	y_3, y_5, $y_7 - y_{11}$	$y_9 - y_{11}$
GAPDH	VGVNGFGR	$G_7 \rightarrow A$	$y_3 - y_7$	$y_3 - y_7$

Table 2

Comparison of Relative Quantification of HSPs Using LC-MRM and Interference-Corrected DI-MRM Data

For each protein measurement, the fold change and CV (%) values are shown for LC-MRM and DI-MRM. Student's t tests were used to calculate p values.

Protein (Uniprot Accession)	RPMI-8226 Cells (24 hrs after treatment vs. control)					8226/LR5 Cells (24 hrs after treatment vs. control)				
	LC-MRM		DI-MRM		p value	LC-MRM		DI-MRM		p value
	Fold Change	CV (%)	Fold Change	CV (%)		Fold Change	CV (%)	Fold Change	CV (%)	
HS90A	2.21	1.0	1.86	10	0.089	1.23	0.9	1.12	0.9	0.00023
HS90B	1.53	1.2	1.46	6.9	0.24	1.28	0.2	1.23	2.7	0.12
CDC37	1.33	1.5	1.08	20	0.18	1.08	3.3	1.24	12	0.22
HSP71	4.67	2.6	6.10	4.2	0.0031	3.54	2.0	5.50	2.3	0.032
HSP7C	2.28	1.4	1.52	3.3	0.00021	1.56	0.3	0.94	3.3	0.00084
HSP74	1.45	1.7	1.22	18	0.21	1.34	20	1.33	38	0.99
HSPA5	2.66	2.2	3.43	5.5	0.022	1.53	1.9	0.86	18	0.018
HSP7E	1.23	0.3	0.87	20	0.07	0.99	1.2	0.93	7.8	0.28

Table 3

LC-MRM and DI-MRM Measurements of Baseline HSP Protein Expression Levels in RPMI-8226 and 8226/LR5 Cells

For each protein measurement, the average amounts and standard deviations (in femtomoles per microgram of total protein) are shown for LC-MRM and DI-MRM. Student's t test was used to calculate p values.

Protein	RPMI-8226 Cells (fmol/μg total protein)				8226/LR5 Cells (fmol/μg total protein)			
	LC-MRM	DI-MRM	Error (%)	p value	LC-MRM	DI-MRM	Error (%)	p value
HS90A	110.3 ± 4.5	113.4 ± 6.9	2.8	0.56	134.5 ± 2.2	151.8 ± 4.8	12.9	0.011
HS90B	78.0 ± 0.47	77.5 ± 2.5	−0.6	0.75	97.9 ± 0.79	97.5 ± 2.2	−0.4	0.76
CDC37	9.0 ± 0.61	9.2 ± 0.76	2.2	0.74	12.3 ± 0.58	10.1 ± 2.5	−17.9	0.44
HSP71	128.3 ± 6.01	72.1 ± 15.8	−43.7	0.01	113.6 ± 5.3	37.8 ± 11.3	−66.7	0.0018
HSP7C	76.2 ± 3.0	101.9 ± 15.4	33.7	0.11	77.2 ± 0.6	138.1 ± 7.4	78.9	0.055
HSP74	7.16 ± 0.27	2.8 ± 1.2	−60.9	0.025	5.2 ± 0.4	1.82 ± 0.48	−65	0.00059
HSPA5	481.6 ± 5.7	661.3 ± 71.2	37.3	0.18	432.0 ± 9.0	954.2 ± 158.4	120.9	0.13
HSP7E	1.92 ± 0.15	0.93 ± 0.21	−51.6	0.0026	2.61 ± 0.07	1.53 ± 0.97	−41.4	0.19