Journal of proteome research

Technical Note

pubs.acs.org/jpr

Precision of Multiple Reaction Monitoring Mass Spectrometry Analysis of Formalin-Fixed, Paraffin-Embedded Tissue

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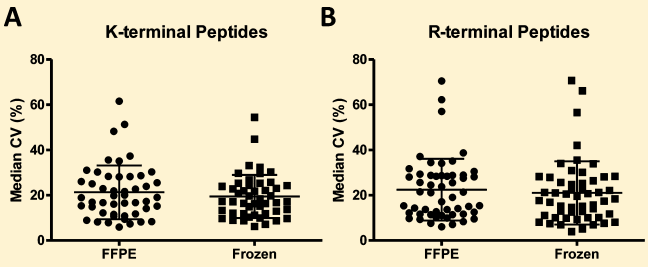
Supporting Information

ABSTRACT: We compared the reproducibility of multiple reaction monitoring (MRM) mass spectrometry-based peptide quantitation in tryptic digests from formalin-fixed, paraffin-embedded (FFPE) and frozen clear cell renal cell carcinoma tissues. The analyses targeted a candidate set of 114 peptides previously identified in shotgun proteomic analyses, of which 104 were detectable in FFPE and frozen tissue. Although signal intensities for MRM of peptides from FFPE tissue were on average 66% of those in frozen tissue, median coefficients of variation (CV) for measurements in FFPE and frozen tissues were nearly identical (18−20%). Measurements of lysine C-terminal peptides and arginine C-terminal peptides from FFPE tissue were similarly reproducible (19.5% and 18.3% median CV, respectively). We further evaluated the precision of MRM-based quantitation by analysis of peptides from the Her2 receptor in FFPE and frozen tissues from a Her2 overexpressing mouse xenograft model of breast cancer and in human FFPE breast cancer specimens. We obtained equivalent MRM measurements of HER2 receptor levels in FFPE and frozen mouse xenografts derived from HER2-overexpressing BT474 cells and HER2-negative Sum159 cells. MRM analyses of 5 HER2-positive and 5 HER-negative human FFPE breast tumors confirmed the results of immunohistochemical analyses, thus demonstrating the feasibility of HER2 protein quantification in FFPE tissue specimens. The data demonstrate that MRM analyses can be performed with equal precision on FFPE and frozen tissues and that lysine-containing peptides can be selected for quantitative comparisons, despite the greater impact of formalin fixation on lysine residues. The data further illustrate the feasibility of applying MRM to quantify clinically important tissue biomarkers in FFPE specimens.



KEYWORDS: *formalin-fixed, paraffin-embedded tissue, multiple reaction monitoring, breast cancer, biomarkers, HER2*

INTRODUCTION

A growing body of literature has provided robust evidence that formalin fixed, paraffin-embedded (FFPE) tissue can be successfully analyzed using mass spectrometry-based proteomic methods, enabling the use of these archival specimens for biomarker discovery through retrospective analysis.[1−5] Although qualitative protein identifications can be obtained from fixed tissue, fixation leads to covalent chemical modification and cross-linking of proteins, DNA and RNA, which may be expected to affect the quantitative reliability of FFPE tissue analyses by targeted proteomic methods, such as multiple reaction monitoring (MRM). Recently, research efforts have begun to demonstrate the technical feasibility of targeted quantitative proteomic analysis in FFPE tissue.[6−8] Huang et al. described the shotgun proteomic analysis of laser capture microdissected FFPE primary and metastatic melanomas and identified 120 proteins as potential markers of metastasis.[7] Label-free quantitation was performed with extracted MS1 signal from an ion trap MS instrument. Güzel et al. analyzed calcyclin peptides by selected reaction monitoring (one transition per peptide) on a triple quadrupole instrument with sequence analogue peptides as reference standards.[6] Similarly, Nishimura et al. quantified two potential prognostic markers for lung adenocarcinoma in FFPE tissue using an endogenous β-actin peptide as an internal standard.[8] Although these reports have demonstrated the application of targeted protein analyses of FFPE tissue, the critical issue of how formalin fixation affects the precision of quantitative analyses in FFPE tissues remains unexplored. Because formaldehyde chemistry significantly affects lysine C-terminal peptides, it is not clear whether quantitative strategies must be adjusted to avoid these.

To assess the impact of fixation on the reproducibility of peptide quantitation, we compared the MRM signals for tryptic peptides derived from paired fixed and frozen clear cell renal cell carcinoma (RCC) tissue from the same tumor. Our analyses describe the precision of measurements of a large set of tryptic peptides across a broad concentration range in both FFPE and frozen specimens. We further evaluated the precision

Received: February 8, 2012
Published: April 24, 2012

of MRM-based quantitation through the analysis of peptides from the HER2 (ERBB2) receptor in FFPE and frozen tissues from a mouse xenograft model of HER2 overexpressing breast cancer. Finally, we used MRM analyses to quantify HER2 protein in FFPE specimens of immunohistochemically confirmed HER2-positive and HER2-negative human breast cancers.

EXPERIMENTAL PROCEDURES

Materials and Reagents

Sub-X xylene substitute was obtained from Surgipath (Richmond, IL). Iodoacetamide (IAM) was from Sigma (St. Louis, MO), *tris*-carboxyethylphosphine (TCEP) was from Pierce (Rockford, IL), sequencing grade trypsin was from Promega (Madison, WI), trifluoroethanol and dithiothreitol (DTT) were from Acros (Geel, Belgium). Trifluoroacetic acid, ammonium bicarbonate, and urea were purchased from ThermoFisher Scientific (Waltham, MA).

Tissue Digest

The deidentified human tissue samples and experimental protocol were subject to Institutional Review Board exempt approval (Institutional Review Board Protocols 080856 and 110453). Fixed and frozen RCC tissues were obtained from the Cooperative Human Tissue Network-Western Division (Vanderbilt University, Nashville, TN). FFPE human breast cancer tissue blocks were obtained through the Vanderbilt-Ingram Cancer Center Breast Cancer SPORE Tissue Core. Fixed and frozen mouse xenografts of BT474 (HER2+) and Sum159 (HER2 negative) cell lines were kindly provided by the laboratory of Carlos L. Arteaga.

Slices of tissue (30 μm) were placed in separate Eppendorf tubes. Paraffin was removed with three washes of 1 mL of Sub-X and tissue was rehydrated with 2 × 1 mL washes each of 100%, 85%, and 70% ethanol. Deparaffinized, rehydrated tissue slices were resuspended in 100 μL of ammonium bicarbonate (100 mM, pH 8.0) and were heated at 80 °C for 2 h. Tryptic digestion was done by an adaptation of the method of Wang et al.[9] Trifluoroethanol (TFE) (100 μL) was added and the samples were sonicated for 20 s followed by 30 s incubation on ice. The sonication was repeated twice. The resulting homogenate was heated for 1 h at 60 °C followed by a second series of sonication steps, as described above. The homogenate was reduced with *tris*-(carboxyethyl)phosphine (10 mM) and dithiothreitol (25 mM) at 60 °C for 30 min, followed by alkylation with iodoacetamide (50 mM) in the dark at ambient temperature for 20 min. The reduced and alkylated protein mixture was diluted to 1 mL with ammonium bicarbonate (50 mM, pH 8.0) followed by addition of trypsin at 1:50 (w/w). The digest was incubated overnight at 37 °C, followed by freezing at −80 °C and lyophilization. Samples were resuspended in 1 mL of water, desalted over 1 cm^3 (100 mg) Sep-Pak vac C-18 cartridges (Waters Corp., Milford, MA), and evaporated to dryness in vacuo with a Speed-Vac sample concentrator (ThermoFisher, Waltham, MA).

Isoelectric Focusing of Peptides

Isoelectric focusing (IEF) of tryptic peptides was performed by a modification of the method described previously.[5] Four replicate IEF strips were run for digests of FFPE and frozen tissue. RCC tryptic peptides (200 μg) were resuspended in 500 μL of 6 M urea and loaded in an IPGphor rehydration tray. Immobiline immobilized pH gradient strips (24 cm, pH 3.5−4.5) were placed over the samples and allowed to rehydrate overnight at ambient temperature. The loaded strips were focused at 21 °C on an Ettan IPGPhor-3 IEF system (GE Healthcare, Piscataway, NJ) using the following program: step at 300 V for 900 Vh; gradient to 1000 V for 3900 Vh; gradient to 8000 V for 13500 Vh; step to 8000 V for 93 700 Vh. The strips were then cut into 20 (1.2 cm) pieces and placed in separate wells of a 96-well ELISA plate. Peptides were eluted from the strips as follows: 200 μL of 0.1% formic acid (FA) for 15 min; 200 μL of 50% acetonitrile (ACN)/0.1% FA for 15 min; 200 μL of 100% ACN/0.1% FA for 15 min. Solutions of extracted peptides were evaporated in vacuo, resuspended in 1 mL 0.1% trifluoroacetic acid, and desalted over a 96-well C18 Oasis HLB plate 30 μm (10 mg) (Waters Corp., Milford, MA). Peptide solutions were evaporated in vacuo, resuspended in 100 μL of 0.1% FA, and placed in sample vials for liquid chromatography−tandem mass spectrometry (LC−MS/MS) analysis.

Reverse Phase LC−MS/MS Analyses

LC−MS/MS analyses were performed on an LTQ-XL mass spectrometer (Thermo Electron, San Jose, CA) equipped with an Eksigent 1D nanoLC and microautosampler (Dublin, CA). Peptides were loaded on a 100 μm × 5 cm fused silica capillary guard column (Polymicro Technologies, LLC., Phoenix, AZ) packed with 5 μm, 300 Å Jupiter C18 (Phenomenex, Torrance, CA) and resolved on a 100 μm × 11 cm fused silica capillary column (Polymicro Technologies, LLC., Phoenix, AZ) packed with 5 μm, 300 Å Jupiter C18 (Phenomenex, Torrance, CA). Liquid chromatography was carried out at ambient temperature at a flow rate of 0.6 μL min^{-1} using a gradient mixture of 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in ACN (solvent B). Peptides eluting from the capillary tip were introduced into the LTQ source in microelectrospray mode with a capillary voltage of approximately 2 kV. A full scan was obtained for eluting peptides in the range of 400−2000 amu followed by six data-dependent MS/MS scans. MS/MS spectra were recorded using dynamic exclusion of previously analyzed precursors for 60 s with a repeat of 1 and a repeat duration of 1. MS/MS spectra were generated by collision induced dissociation of the peptide ions at a normalized collision energy of 35% to generate a series of b- and y-ions as major fragments.

MRM Analyses

MRM analyses were performed on a TSQ Vantage triple quadurpole mass spectrometer (Thermo Electron, San Jose, CA) equipped with an Eksigent Ultra nanoLC and microautosampler (Dublin, CA). Chromatography column and conditions were as described for LC−MS/MS analyses, except that the flow rate was reduced to 0.4 μL min^{-1}. Analyses were performed using the labeled referenced peptide (LRP) method,[10] where peak areas for target peptides were normalized against the isotope labeled β-actin peptide standard U−^{13}C,^{15}N-Arg-GYSFTTTAER, which was added at a concentration of 25 nM. Four transitions were monitored for each peptide and a maximum of 40 peptides were monitored per method. Extracted ion chromatogram peak areas were measured as the sum of the peak areas for the four monitored transitions. Transitions were selected from the most intense y-ions observed in a spectral library derived from shotgun data sets. Spectral libraries were compiled using the Skyline software.[11] All Skyline methods are available as Supporting Information.

dx.doi.org/10.1021/pr300130t | J. Proteome Res. 2012, 11, 3498−3505

Data Analysis

The ScanSifter algorithm read tandem mass spectra stored as centroided peak lists from Thermo RAW files and transcoded them to mzML files.[12] If 90% of the intensity of a tandem mass spectrum appeared at a lower m/z than that of the precursor ion, a single precursor charge was assumed; otherwise, the spectrum was processed under both double and triple precursor charge assumptions. Tandem mass spectra were assigned to peptides from the IPI Human database version 3.37 (May 5, 2008; 69 238 sequences) by the MyriMatch algorithm, version 1.6.75.[13] The sequence database was doubled to contain each sequence in both normal and reversed orientations, enabling false discovery rate estimation. MyriMatch was configured to expect all cysteines to bear carboxamidomethyl modifications and to allow for the possibility of oxidation on methionines. Candidate peptides were required to feature trypsin cleavages or protein termini at one end (semitryptic search); any number of missed cleavages was permitted. A precursor error of 1.25 m/z was allowed, but fragment ions were required to match within 0.5 m/z. The IDPicker algorithm v2.6.165.0[13] filtered the identifications for each LC−MS/MS run to include the largest set for which a 5% identification false discovery rate could be maintained, as described by Qian et al.,[14] and applied parsimonious protein assembly, reporting the smallest list of proteins which could account for the identified peptides. These identifications were pooled for the IEF sample set. Proteins were required to have at least two different peptide sequences observed within an IEF sample set. Database protein entries that could not be distinguished based on the observed peptides were combined into "protein groups" representing the most parsimonious assignment of the spectral count data. False discovery rates (FDR) rates were computed by the formula:[15]

$$\mathrm{FDR} = (2 \times \mathrm{reverse})/(\mathrm{forward} + \mathrm{reverse})$$

The algorithm reported the number of spectra and number of distinct sequences observed for each protein and protein group in each sample set.

RESULTS

Shotgun Proteome Analysis of RCC Tissue

Shotgun proteome analysis of fixed and frozen RCC tissue indicated a qualitative concordance in protein groups identified in both FFPE and frozen tissue (Figure 1A), as we reported previously for comparison of frozen and FFPE tissues.[5] (Spectral count data for the identified proteins is presented in Supporting Information Table S1; a full summary of the data set is provided in the accompanying IDPicker report. Both are provided as Supporting Information.) Of 2165 total protein groups identified, 91% were found in both sample types. However, the resulting peptide identifications obtained from tryptic digests of fixed tissue were biased against lysine C-terminal peptides (Figure 1B). Whereas in frozen tissue the ratio of lysine C-terminal peptides to arginine C-terminal peptides was 1.11, the ratio in FFPE samples was reduced to 0.93. These observations are in agreement with our previous analyses of frozen and FFPE colon adenocarcinoma tissue[5] and are consistent with the known reactivity of aldehydes toward primary amines, which preferentially consumes lysine residues and thus affects the yield of lysine C-terminal peptides. We thus asked whether the modification of lysine residues would affect the quantitation of lysine C-terminal peptides in a tryptic digest of FFPE tissue.

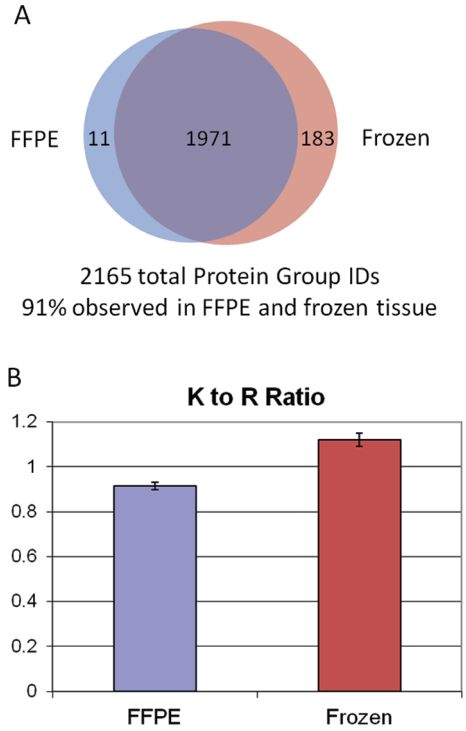


Figure 1. Characteristics of the shotgun proteomic data set. High qualitative concordance was observed for proteins identified in both FFPE and frozen tissue digests. Peptides observed in FFPE tissue were biased against lysine C-terminal peptides, indicated by a lower lysine to arginine peptide ratio. Error bars represent standard deviation from 4 IEF replicates.

Reproducibility of MRM in FFPE Tissue

To assess measurement precision in FFPE tissue, 114 peptides were selected as MRM targets from a list of peptides identified from shotgun proteomic analysis of IEF-fractionated tryptic digests of FFPE and frozen RCC tissue (Supporting Information Table S2). The peptides selected spanned the entire range of peptide and protein abundance, as determined by spectral count information in the shotgun data set (Supporting Information Table S1). The 114 candidates each were between 7 and 25 amino acids in length; contained no methionine, cysteine, histidine, N-terminal glutamine residues or missed tryptic cleavages; were unique to a single protein in the search database; and were observed predominantly as doubly charged precursors. Transitions were chosen from the 4 most intense y-ions based on reference spectra from the shotgun data set.

The precision with which a peptide can be measured and the intensity of the signal obtained are a function of the peptide concentration, ionization and fragmentation characteristics, variability in yields of sample processing steps (e.g., digestion), and the characteristics of the instrument used to measure the analyte. The measurement precision of the instrument can be empirically assessed through the analysis of an internal standard of known concentration and can provide a reference for the measurement of effects on precision caused by the fixation process. Thus, in addition to the 114 candidate peptides, an endogenous peptide of sequence GYSFTTTAER, corresponding to β-actin, was monitored, as well as an isotope labeled version of the GYSFTTTAER peptide spiked-in at a constant concentration of 25 nM to provide for signal normalization for the targeted peptides and to monitor measurement precision.[16]

dx.doi.org/10.1021/pr300130t | J. Proteome Res. 2012, 11, 3498−3505

Five serial sections of FFPE and frozen RCC tissue were processed in parallel and analyzed using 3 MRM acquisition methods, which monitored 40 peptides and 160 transitions each. The methods analyzed groups of peptide targets corresponding to proteins of different abundances, based on spectral counts from the shotgun analyses. Median spectral counts for the proteins covered in methods 1, 2, and 3 were 190, 129, and 38, respectively. The proteins and peptides comprising the methods are listed in Supporting Information Table S3. Lists of the transitions monitored for each peptide are provided as Skyline (.SKY) files in the Supporting Information. The coefficient of variance (CV) for the LRP-normalized peak area over triplicate MRM runs was determined for each peptide.

Given the possible effects of formaldehyde fixation chemistry on lysine C-terminal tryptic peptides, the precision of MRM measurement was analyzed separately for lysine and arginine C-terminal peptides. The median CVs for the 52 lysine C-terminal peptides were not significantly different between analyses of fixed (median CV = 0.195) or frozen tissue (median CV = 0.190) using the Mann–Whitney U test (Figure 2). Median

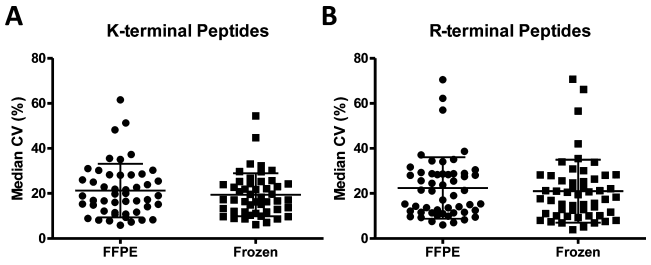


Figure 2. CVs for MRM measurements of 52 lysine C-terminal peptides and 58 arginine C-terminal peptides in 5 serial sections of frozen and FFPE RCC samples. MRM quantitation was done by the LRP method. Comparison of median CVs for peptide measurements from FFPE and frozen tissues indicated no significant effect of fixation on the precision of peptide measurement (Mann–Whitney U test).

CVs for analyses of the 58 arginine C-terminal peptides also indicated no significant difference between fixed (median CV = 0.183) or frozen tissue (median CV = 0.180, Figure 2). This result suggests that there is no discernible effect of formaldehyde fixation chemistry that affects the reproducibility of peptide MRM measurement. In addition, there was no significant difference in median CV between lysine C-terminal peptides (median CV = 0.195) and arginine C-terminal peptides (median CV = 0.183) from FFPE tissue, suggesting that MRM measurements of FFPE tryptic peptides display similar technical variation, regardless of the C-terminal amino acid.

The influence of fixation status on individual peptides was further assessed by considering the median CV obtained for analyses of each peptide across the 5 serial slices (Supporting Information Table S4). A commonly used method to establish the limit of quantitation (LOQ) for an analyte is to determine the average signal at the retention time of interest in a blank sample plus 8 standard deviations of the average blank signal.[17] For MRM analyses, signals from a blank run at a given peptide m/z value and retention time can result in very small values, which can yield theoretical LOQs far below the practical limitations of the analytical system. A more conservative LOQ estimate can be obtained empirically by determining the concentration at which the CV of replicate measurements exceeds 25%.[17] We thus took this latter approach in our studies.

Of the 114 peptides analyzed, 10 were deemed to be undetectable due to lack of a consistent signal among replicates and samples. Of the remaining 104 peptides, 37 yielded median CVs greater than 25% in the FFPE derived samples. These peptides were thus designated as being below the LOQ in these samples. In analyses of frozen tissue digests, 27 peptides exceeded the 25% median CV threshold (Supporting Information Table S3). A greater number of peptides below the LOQ in FFPE tissues may reflect reduced signal intensity, most likely due to reduced tryptic peptide yield in digests of FFPE samples.

In total, 46 peptides yielded median CVs in excess of 25% in either FFPE or frozen tissue; 18 of these had median CVs greater than 25% in both tissue types. Overall, 51% of the peptides targeted (58/114) yielded signals estimated to be above the LOQ. This success rate for analyses in FFPE tissues is comparable to other empirical assessments of proteotypic peptide suitability in semiquantitative MRM assay development for biomarker verification.[17] As is evident in Supporting Information Table S3, methods 2 and 3 produced increasing numbers of peptides exceeding the 25% median CV threshold and this effect was somewhat greater for FFPE tissue. Peptides exceeding the threshold were as follows: Method 2, 16 peptides in FFPE, 9 in frozen; Method 3, 21 in FFPE, 18 in frozen. Peptides targeted by these two methods were from proteins represented with fewer spectral counts in the shotgun analysis (Supporting Information Table S2), indicating lower abundance in the RCC tissues.

The most likely explanation for the effect on median CV in the FFPE samples is the presence of protein cross-links, which result in a lower yield of proteotypic tryptic peptides detectable by MRM analysis, thus decreasing concentrations of the peptides below the limit of quantitation. The influence of fixation appears not to be specific to a particular class of protein, or subcellular compartment, but rather is distributed uniformly among proteins we analyzed. In MRM analyses, effects on peptide yield will have the greatest impact on quantification of peptides that are already near the limit of quantitation in unfixed, frozen samples. This interpretation is consistent with our observation here (Figure 1 and Supporting Information Table 1) and previously[5] that the proteins represented in shotgun data sets with fewer spectral counts yield fewer peptide and protein group identifications in FFPE tissue than in frozen specimens in shotgun proteomic analysis of IEF-fractionated samples.

We also considered the effect of fixation on the magnitude of the peak areas observed during MRM analysis, a more direct measurement of relative peptide abundance. Of the 114 peptides monitored, 70% showed a significant difference in average normalized peak area between fixed and frozen samples (two tailed t test, $p < 0.05$, Supporting Information Figure 1). Figure 3 plots the log2 ratio of LRP-normalized peak areas for peptides from FFPE to frozen tissues. These data demonstrate that peptides from FFPE tissue generally yield lower normalized average peak areas than peptides from frozen tissue, given the predominance of log2 (FFPE:frozen) peptide intensity values below zero in Figure 3. This result further illustrates the significant impact of fixation on the quantitation of proteins in tissue.

MRM Quantification of the HER2 Receptor Protein

A potentially useful application of MRM quantitative analyses in FFPE tissue is the measurement of specific biomarker

dx.doi.org/10.1021/pr300130t | J. Proteome Res. 2012, 11, 3498–3505

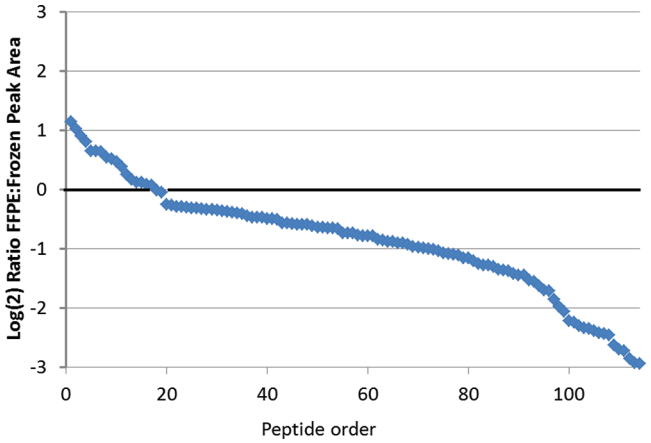


Figure 3. Log2 ratios for average normalized peak areas from FFPE versus frozen tissues. MRM quantitation was done by the LRP method. Peptides are ordered on the *x*-axis in order of decreasing log2 ratios (*y*-axis values). Equivalent intensities yield a value of zero. Log2 ratios less than zero indicate higher peak areas from peptides derived from frozen tissue.

proteins of clinical interest. This is normally achieved by immunohistochemistry (IHC), which can be limited by assay variability, antibody sensitivity, and specificity. In addition, IHC methods are not quantitative and assessment of IHC is subjective. MRM analyses provide quantitative measurements with the potential for improved specificity, thus overcoming key performance limitations of IHC. To explore the possible clinical application of MRM in FFPE tissue, we developed an assay to quantify the HER2 receptor in breast cancer. HER2 overexpression is a clinically important diagnostic factor in the management of breast tumors. Amplification of HER2 is observed in 20% of breast cancer cases and patients harboring HER2-overexpressing tumors are candidates for HER2-targeted therapies.

To monitor multiple peptides unique to HER2, the peptide exclusion criteria were relaxed allowing for the inclusion of peptides containing cysteine, methionine, and histidines. Peptides were still required to be fully tryptic, between 7 and 25 amino acids in length, unique to HER2 and to be represented in a reference spectral library. Twenty-five peptides met these criteria and were targeted by MRM in a tryptic digest of the HER2 overexpressing BT474 human breast cancer cell line. Five transitions were monitored per peptide, as selected from the most intense y-ions observed in a reference spectral library. On the basis of this initial screen, 4 peptides were selected that gave the greatest signal intensity and a CV of less than 25% when analyzing 1 μg of tryptic digest.

To enable quantitation by stable isotope dilution, labeled peptides corresponding to these 4 sequences were obtained and the limit of quantitation was determined in the absence of biological matrix. Limit of quantitation was defined as the concentration of peptide where the CV for triplicate measurements exceeded 25%. The peptides DPPFCVAR and ELVSEFSR provided the greatest sensitivity, yielding limits of

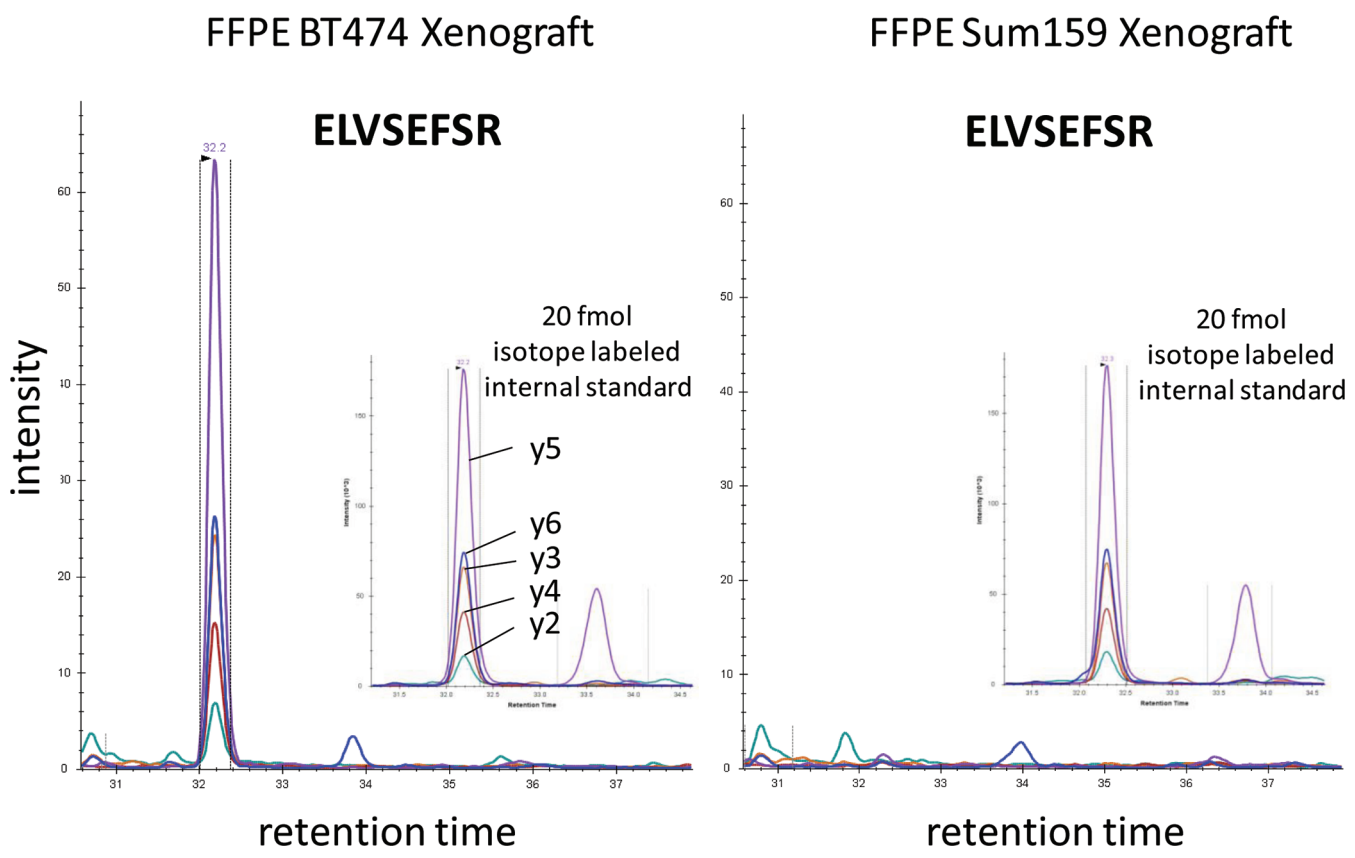


Figure 4. MRM chromatograms for the HER2 intracellular domain peptide ELVSEFSR from analyses of FFPE xenograft specimens derived from the HER2-overexpressing cell line BT474 (left) and the non-HER2-expressing cell line Sum159 (right). MRM quantitation was by stable isotope dilution. Monitored MRM transitions are depicted for the unlabeled, endogenous peptide, and the isotope-labeled internal standards (insets). The intensity scales (*y*-axis) are identical in the two sets of plots.

dx.doi.org/10.1021/pr300130t | *J. Proteome Res.* 2012, 11, 3498–3505

quantitation within a biologically relevant range of 0.33 and 0.1 fmol on-column, respectively (Supporting Information Figure 2). These limits correspond to a lower limit of detection of 12 000 HER2 receptors per cell, assuming 200 pg protein per cell and 1 μg tryptic digest on-column (i.e., 5000 cell equivalents injected for MRM analysis). Detection limits for peptides GQECVEECR and NPQLCYQDTILWK exceeded 1 fmol on-column and were not considered for quantitation.

We then analyzed FFPE and frozen xenograft tissue samples from human BT474 and Sum159 (HER2-negative) breast cancer cell lines. Whereas signals from HER2 peptides were readily discernible in BT474 xenografts, HER2 peptide signals were absent in Sum159 xenografts, consistent with their low level of HER2 expression (Figure 4). Estimates of HER2 receptor numbers from quantification of the DPPFCVAR peptide were 60% higher than from quantification of the ELVSEFSR peptide. The DPPFCVAR peptide is derived from the ectodomain of the HER2 receptor, whereas ELVSEFSR is in the intracellular activation domain of the receptor. This result could be the result of proteolytic shedding of the receptor ectodomain. Indeed, BT474 xenografts have been reported to shed the HER2 extracellular domain into the circulation.[18]

To explore the application of MRM for analyses of HER2 in FFPE human breast tumor tissues, we analyzed 5 HER2-overexpressing breast cancer tissues and 5 HER2-negative breast cancers, which had been classified based on previous IHC analysis in the Vanderbilt clinical immunohistochemistry laboratory. MRM analyses of these specimens (Figure 5B) demonstrate a clear difference in signal intensity for HER2 peptides between the HER2+ and HER2 negative tumors and indicate a wide range of biological variability in receptor expression levels. Quantification of receptor levels was performed by stable isotope dilution. Assuming a yield of 200 pg protein from an average cell, we estimated HER2 receptor levels ranging from 110 000 to 468 000 receptors per cell in the HER2 positive cancers and fewer than 14 000 receptors per cell in the HER2 negative cancers. Because of the higher limit of quantification for the DPPFCVAR peptide, it was not possible to use this peptide to quantify receptor levels in all specimens. In tissues most highly overexpressing HER2, quantification of receptor levels based on DPPFCVAR yielded the same result as ELVSEFSR quantification, suggesting no evidence of receptor ectodomain shedding in the tissues analyzed.

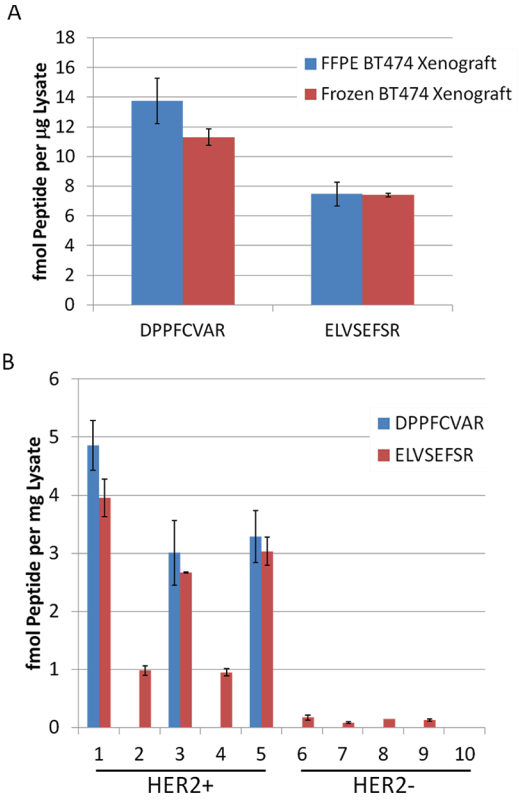


Figure 5. (A) Quantification of HER2 receptor protein in frozen (red bars) and FFPE (blue bars) BT474 xenograft tissues. HER2 protein was quantified based on MRM of peptides representing the extracellular (DPPFCVAR) and intracellular (ELVSEFSR) domains of the receptor. MRM quantitation was by stable isotope dilution. Plotted values are mean ± SD for 3 process replicates of one frozen and one FFPE tumor. (B) Quantification of HER2 receptor protein in 5 human HER2-positive and 5 HER2 negative human FFPE breast tumor tissues. MRM quantitation was by stable isotope dilution analysis of peptides representing the extracellular (DPPFCVAR) and intracellular (ELVSEFSR) domains of HER2. Plotted values are mean ± SD for 3 process replicates of each specimen.

DISCUSSION

The goal of this work was to assess the performance characteristics of protein quantitation by MRM in FFPE tissue and to apply MRM to analyze a clinically relevant biomarker in human FFPE tissue specimens. Our studies addressed the hypothesis that protein cross-linking in FFPE tissues would add to variability in MRM measurements, particularly for lysine C-terminal peptides. Although peptide yields, particularly for lysine C-terminal tryptic peptides, are lower in FFPE tissues than in frozen tissues, measurement variation is not significantly different between these specimen types. Median CVs for analyses of tryptic peptides in FFPE and frozen tissues were all below 20% and were not significantly different between specimen type or between lysine- and arginine C-terminal peptides. The data not only demonstrate that MRM analyses can be performed with equal precision on FFPE and frozen tissues, but also indicate that lysine C-terminal peptides need not be excluded as MRM candidates when working with FFPE tissue. Our analyses of HER2 protein in FFPE breast tumor xenografts and in human FFPE breast tissue specimens illustrated the feasibility of applying MRM to quantify clinically important tissue biomarkers.

The major question regarding the use of proteomic methods to analyze FFPE tissue is the impact of protein cross-linking that occurs during formalin fixation. The terms "antigen retrieval" and "crosslink reversal" have been applied to describe the various protocols to prepare FFPE tissue for IHC analysis.[19−23] Although these approaches facilitate IHC, there is no molecular or chemical evidence to demonstrate that formaldehyde-derived cross-links have been reversed. Indeed, given the chemical stability of many formaldehyde-derived covalent cross-links in proteins,[24,25] it is unlikely that any method or reagent could quantitatively reverse cross-links while sparing peptide bonds and other functional groups.

Our results suggest the chemical modifications induced by formalin fixation decrease the sensitivity of MRM measurements. Thus, somewhat fewer targets are accessible to MRM when FFPE tissue is analyzed. The fractional loss of peptide signal is negligible for high-abundance proteins, but proteins near the limit of quantitation in frozen tissue are less likely to be accurately quantified by MRM in FFPE tissue. This limitation may be overcome to some extent through increased

dx.doi.org/10.1021/pr300130t | J. Proteome Res. 2012, 11, 3498−3505

sample input and possibly through the use of larger capacity chromatography columns, permitting injection of larger amounts of tryptic digest. However, our data indicate that the overall impact of formalin fixation on peptide yield and signals was significant, yet modest and does not preclude application of MRM to analyze proteins in FFPE tissues. We should also point out that, despite the validity of this general conclusion, certain peptides may be unusually sensitive to formalin fixation and display high measurement variation in FFPE samples. Thus, MRM assay development should consider multiple peptides during assay development and optimization.

We were unable to assess the accuracy of quantitation in FFPE tissues because the concentrations of the proteins analyzed were not known. Spike-in experiments with known amounts of target proteins to assess quantitative accuracy are not possible with FFPE tissues, as the spiked proteins would not control for the formalin fixation steps, which probably have the greatest effect on protein yield. Assessment of accuracy in MRM analyses of any sample type is complicated by uncontrolled variability in protein digestion and recovery.[26] However, the goal of MRM analyses usually is comparison of protein levels, rather than assessment of absolute amounts and the underlying assumption is that deviations from accuracy are evenly distributed across all measurements. Precise and reproducible measurement of relative differences between sample classes can enable biomarker verification and validation studies.

To evaluate MRM analyses of FFPE tissues to quantify a clinically relevant protein biomarker, we analyzed HER2 receptor expression levels in HER2 positive and HER2 negative breast tumors. HER2 is an important diagnostic and predictive factor, as patients with HER2-positive tumors are candidates for anti-HER2 therapies. HER2-positive tumors are defined by intense membrane staining in the majority of tumor cells (3+ by IHC) using antibodies directed against the HER2 C-terminus or by expression of ≥2.2 copies of the *HER2* gene, as determined by fluorescent in situ hybridization (FISH).[27] IHC staining or HER2 copy number determination in FFPE tissue sections is semiquantitative in nature and results are dependent on antibody performance and operator experience. Further, the rate of discordance between IHC and FISH can approach 20%, which is significantly below the maximum rate of 5% recommended by the American Society of Clinical Oncologists.[27]

An MRM-based approach to analysis of tissue biomarker proteins offers potential advantages over IHC. Most significantly, MRM would allow a systematic approach to configuration of targeted assays for tissue proteins, even when antibody reagents validated for IHC are unavailable. This would remove a major barrier to analysis of protein biomarkers in FFPE specimens. MRM analyses also provide additional molecular detail that may not be accessible through IHC. For example, analyses of HER2 protein with MRM quantitation of both extracellular and intracellular sequences can extend the specificity of molecular characterization of HER2-positive breast cancers. A potential mechanism of resistance to the HER2-targeted drug trastuzumab is shedding of the receptor ectodomain, as HER2 is subject to proteolytic processing at the plasma membrane by an ADAM or MMP.[28] HER2 measurement based on MRM analysis of intracellular and extracellular peptide sequences provides a more informative assessment of the status of the HER2 target than does IHC and FISH. MRM could further enable analysis of modified or variant protein forms, which may be difficult to detect selectively with antibody reagents.

Our analyses of HER2 in FFPE tissue specimens illustrate the potential utility of MRM for analysis of a clinically relevant tissue protein biomarker. The data demonstrate that MRM analyses are concordant with HER2 status as measured by IHC. An assessment of the performance of MRM for HER2 or other tissue biomarkers is beyond the scope of this preliminary study. Nevertheless, our results demonstrate that MRM analyses in FFPE and frozen tissues display similar performance characteristics and that measurement precision in the two tissue types is essentially identical. Although target peptide yields are lower in FFPE tissues, this has a modest effect on assay sensitivity. Our studies were limited to measurements of unmodified peptides and may not be applicable to post-translationally modified (e.g., phosphorylated, N-acetylated) sequences, where the stability of labile modifications during tissue harvest and fixation may affect the results. Our findings indicate that effects of formalin fixation on tissue proteins does not present major potential barrier to the development of MRM-based tissue assays for protein expression for research purposes. The data also suggest that further development of MRM instrumentation and approaches may provide powerful new diagnostic tools for the surgical pathology laboratory.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

R.W.S. was supported by a Canary Foundation and American Cancer Society postdoctoral fellowship in the early detection of cancer. This work was supported in part by the National Cancer Institute through the Clinical Proteomic Technology Assessment for Cancer program (U24CA126479 to D.C.L.), the Vanderbilt SPORE in Breast Cancer (P50CA098131 to C.L.A.) and the Tennessee Valley Cooperative Human Tissue Network (U01CA091664 to M.K.W.).

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