Application Note: Quantitation of Protein Biomarkers in Formalin-Fixed Paraffin-Embedded Tissue

Case Study: Determining Her2 expression levels in invasive breast cancer using FFPE tissue

Measurement of protein biomarkers directly in tissue is of great clinical importance, in particular for personalized medicine. Formalin fixation of tissue samples which are subsequently embedded in paraffin (FFPE) is the gold standard for biopsy and surgical tissue preservation. However, proteins within the tissue are heavily cross-linked as a result of the formalin fixation and thus options for analyzing them are limited.

Her2 is the prototypic diagnostic, prognostic and therapeutic target for the personalized medicine approach to disease treatment. Measurement of Her2 has become an important part of the pathological evaluation of invasive breast cancer due to the success of Her2 antibody-based therapy. Precise quantitation of such protein biomarkers in FFPE tissue will have a major impact on the advancement of personalized medicine.

Introduction

Expression Pathology (EPI) has developed a method which completely solubilizes the entire protein content from cell populations taken from standard FFPE tissue. Using Liquid Tissue® reagents and protocols for sample preparation, the solubilized proteins can be assayed using a variety of methods, including mass spectrometry. Selective Reaction Monitoring (SRM) is an established method for quantitation of proteins using mass spectrometry. Liquid Tissue® preparations of FFPE tissue can be used together with SRM for absolute quantitation of specific protein biomarkers in FFPE tissue. To demonstrate this, EPI, in a collaboration with NextGen Sciences, developed a reproducible, sensitive method to measure expression of Her2 in such tissue. The method is rapid and straight forward, and the results correlate directly with results obtained using traditional methods.

The Assay

Almost one third of invasive breast carcinomas over-express the Her2 protein. Anti-Her2 therapies that block the protein have become important in the management of and in prolonging the survival of patients with aggressive breast cancer. The effectiveness of this therapy depends on accurately evaluating Her2 status in these tumors. This can be done by analyzing the expression of Her2 by immunohistochemistry (IHC) or by evaluating Her2 gene amplification by fluorescent in situ hybridization (FISH). In many cases, both methods are used to evaluate samples. Variability in this approach can occur due to the general drawbacks with IHC, such as low sensitivity, variable reproducibility and subjective interpretation.

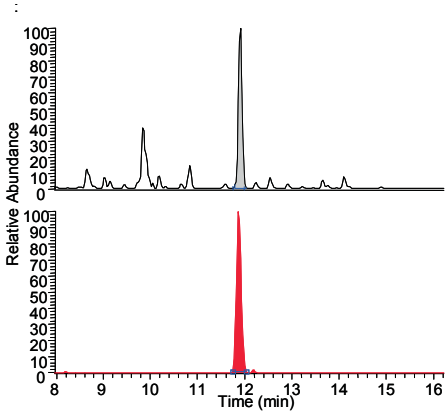

Figure 1: Absolute quantitation of Her2 specific peptide using selective reaction monitoring (SRM). The grey trace is the MS data for a native peptide from a breast cancer tissue sample with an IHC score of +1. The red trace shows the MS data for the synthetic labeled peptide added as an internal standard..

The Liquid Tissue® SRM assay measures a specific peptide derived from the full-length Her2 protein. A synthetic labeled version of the peptide is added to the sample as an internal standard (IS). The amount of Her2 peptide in the sample is determined by comparing the area under the curve for the native peptide (top chromatogram in gray) to the area under the curve for the synthetic IS peptide (bottom chromatogram in red) (Figure 1) and this analyte area to IS area ratio is used to determine the number of fmol of peptide in the sample based on a calibration curve. The concentration of Her2 peptide is then normalized to the amount of Her2 expressed per microgram of total protein.

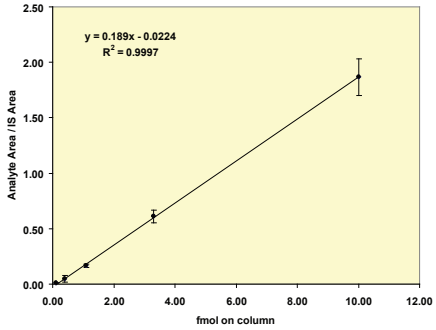

Figure 2: A calibration curve shows the ability to precisely and reproducibly quantitate the specified Her2 peptide by addition of the synthetic internal standard peptide.

The ability to quantitate varying amounts of the native Her2 peptide by addition of a known amount of an internal standard is shown in the calibration curve in Figure 2. A synthetic, non-labeled Her2 peptide was precisely quantitated by comparison to a labeled synthetic internal standard peptide. Results indicate a Lower Limit of Detection (LOD) at 40 attomoles and a Lower Limit of Quantitation (LOQ) at 100 attomoles.

To demonstrate how well this assay works, 10 breast cancer samples which had already been analyzed for Her2 using IHC and FISH were analyzed by the Liquid Tissue® SRM assay. Cancerous epithelium was collected from each sample utilizing EPI's Director® slides and laser microdissection technology. Liquid Tissue® protein lysates were prepared from the microdissected cell populations for analysis.

Results

Results from the Liquid Tissue® SRM assay indicate a range of Her2 peptide from Not Detected (samples #1, 2 and 3) to 3.8 fmol/µg total protein analyzed for samples with an IHC score of 3+ (sample #10). Her2 expression data obtained using SRM was compared to IHC and FISH data (Table 1).

Table 1: Absolute quantition of Her2 specific peptide using SRM, compared to IHC and FISH scores.

Sample	IHC Score	FISH Score	SRM Score (fmol/µg)
1	0	1.07	ND
2	0	1.10	ND
3	0	1.08	ND
4	1+	1.07	0.06
5	1+	1.33	0.12
6	1+	1.23	0.15
7	2+	1.28	0.29
8	2+	2.10	0.48
9	3+	3.38	0.99
10	3+	12.5	3.80

To better demonstrate this comparison graphically, the SRM and FISH data were normalized by dividing the value for each sample by the average of all samples within each assay. This allows disparate forms of data to be directly compared (Figure 3).

Results from both the FISH and the SRM assays correlate closely with each other, and both follow the IHC score; however, the SRM assay demonstrates a broader dynamic range than either IHC or FISH. The sensitivity of each method is similar.

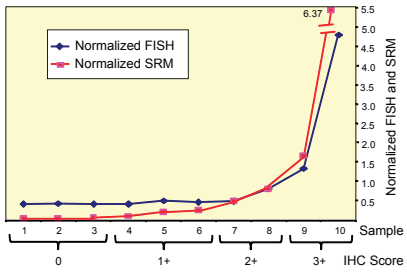

Figure 3: Direct comparison of normalized SRM and FISH data.

The most important aspect of any Her2 assay is found in patients with breast cancers scored as 2+ by IHC. It is in these patients that the decision whether to prescribe anti-Her2 therapies is most challenging. Current guidelines rely on FISH data to determine the course of therapy. Patients whose tumors demonstrate FISH ratios of <2 have no gene amplification and those with a ratio of ≥2 are designated as having gene amplification and are candidates for receiving anti-Her2 therapy. The Liquid Tissue® SRM data precisely correlated to the FISH data in the IHC 2+ patient samples, suggesting the potential use of the SRM assay for improved treatment decisions, in this case to help classify Her2 IHC 2+ patients based on accurate protein analysis.

Demonstration of the intra-sample reproducibility of the assay was done by performing the assay on three separate Liquid Tissue® lysates prepared from three serial sections cut from the same tissue block. The same histological area was microdissected from each of three serial sections using Director® slides, and Liquid Tissue® lysates were prepared for analysis.

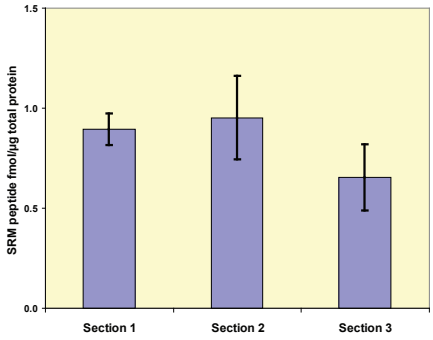

Figure 4: Reproducibility of the SRM assay in a tissue sample with an IHC score of 2+.

Results indicate the ability to generate comparable data between the serial sections. Duplicate analysis of each lysate indicates generally low variation in the assay (error bars represent 2 standard deviations). (Figure 4)

Conclusion

This study shows that FFPE tissue can be used for direct measurement of clinically important protein biomarkers by mass spectrometry-based SRM protocols. SRM assays can be multiplexed (mSRM) indicating the potential to develop assays where multiple proteins are measured simultaneously, for example cancer therapy targets or cell signaling pathway proteins.

Expression Pathology specializes in discovery and quantitation of protein biomarkers in FFPE tissue. In addition to our own discovery and development program we partner with pharmaceutical and diagnostic companies as well as with academic institutions to develop assays based on our technology.

NextGen Sciences provides quality service in biomarkers, proteomics and protein characterization for research and development. The biomarker services, biomarkerexpress™ is a suite of mass spectrometry-based biomarker services that utilize proprietary methods to significantly decrease timelines and increase the success rates traditionally associated with biomarker development. The services include discovery of protein biomarkers, development of protein biomarker assays, testing biological samples utilizing the assays to determine levels of protein biomarkers, and a biomarker knowledgebase.

Acknowledgements

Tissue samples, Her2 and FISH scoring

MDR Global Systems, LLC.

Liquid Tissue technology is protected by U.S. Patent 7,473,532 and patents pending and foreign equivalents thereof.

DIRECTOR technology is protected by U.S. Patents 7,294,367 and 7,381,440 and foreign equivalents thereof.

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