Published in final edited form as:

Proteomics. 2012 April ; 12(8): 1253–1260. doi:10.1002/pmic.201100587.

Multiplexed quantification of estrogen receptor and HER2/Neu in tissue and cell lysates by peptide immunoaffinity enrichment mass spectrometry

Regine M. Schoenherr[†], Jeffrey R. Whiteaker[†], Lei Zhao, Richard G. Ivey, Mary Trute, Jacob Kennedy, Uliana J. Voytovich, Ping Yan, ChenWei Lin, and Amanda G. Paulovich
Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N., Seattle, WA 98109

Abstract

Access to a wider range of quantitative protein assays would significantly impact the number and use of tissue markers in guiding disease treatment. Quantitative mass spectrometry-based peptide and protein assays, such as immuno-SRM assays, have seen tremendous growth in recent years in application to protein quantification in biological fluids such as plasma or urine. Here, we extend the capability of the technique by demonstrating the application of a multiplexed immuno-SRM assay for quantification of estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) levels in cell line lysates and human surgical specimens. The performance of the assay was characterized using peptide response curves, with linear ranges covering approximately 4 orders of magnitude and limits of detection in the low fmol/mg lysate range. Reproducibility was acceptable with median coefficients of variation of approximately 10%. We applied the assay to measurements of ER and HER2 in well-characterized cell line lysates with good discernment based on ER/HER2 status. Finally, the proteins were measured in surgically resected breast cancers, and the results showed good correlation with ER/HER2 status determined by clinical assays. This is the first implementation of the peptide-based immuno-SRM assay technology in cell lysates and human surgical specimens.

Keywords

Estrogen receptor; HER2/Neu; immunoaffinity; peptides; tissue

Diagnostic workup of newly diagnosed cancers frequently includes measurement of the expression of protein biomarkers in tumor cells, where biomarkers are widely used to prognosticate[1, 2], predict response to targeted therapies[3–9], and determine the tissue of origin in carcinomas of unknown primaries[10]. As more targeted therapies come online, the need for companion diagnostics is expected to increase dramatically[11]. For example, the molecular classification of breast cancer according to the expression of hormone receptors and growth factors, such as estrogen receptor (ER) and the human epidermal growth factor receptor 2 (HER2), can guide targeted treatment (e.g. hormone therapy for ER+ and Herceptin for HER2-overproducing tumors). The determination of ER and HER2 status in tissues is typically achieved using immunohistochemistry (IHC), yet, despite tremendous efforts in establishing guidelines and protocols[2, 12, 13], the assays are not quantitative and

*Corresponding author: Dr. Amanda G. Paulovich, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue N., Seattle, WA 98109, USA. apaulovi@fhcrc.org, Office 206-667-1912, Fax 206-667-2277.

[†]These authors contributed equally to this work.

Conflict of interest statement

The authors have declared no conflicts of interest.

they have been difficult to standardize across laboratories, leading to development of alternative nucleic acid-based assays using fluorescence in situ hybridization (FISH) or reverse transcription polymerase chain reaction (RT-PCR)[13, 14].

In this report, we explore the use of a proteomic assay format based on targeted quantitative mass spectrometry using selected/multiple reaction monitoring (SRM/MRM) for classification of human breast cancer cell lines and surgical specimens. SRM/MRM has been used extensively for small molecule quantification and has recently been increasingly applied in the area of quantitative proteomics[15–20] wherein proteins are digested to component peptides using an enzyme such as trypsin, and one or more proteotypic peptides are measured as quantitative stoichiometric surrogates for protein concentration in the sample[21, 22]. Coupled to stable isotope dilution (SID) methods (i.e. using a spiked-in isotopic peptide standard), SRM can be used to measure concentrations of proteotypic peptides as surrogates for quantification of proteins in complex biological matrices[21, 22]. The technique is readily multiplexed, offers good performance, and can be transferred across laboratories[23], although sensitivities are limiting in complex biospecimens. To enhance sensitivity of SRM assays, anti-peptide antibodies can be used to enrich target peptides from complex proteomic samples (Stable Isotope Standards and Capture by Anti-Peptide Antibodies, SISCAPA)[24, 25], resulting in highly sensitive immuno-SRM assays that are readily multiplexed[26], amenable to automation, and have been implemented in a wide array of studies[17, 27–31].

While the immuno-SRM technology has been prototyped for measuring biomarkers in plasma and other fluids [24, 29, 30], it has not yet been refined and adapted for quantification of proteins in tissues. To our knowledge, this is the first evaluation of peptide-based immuno-SRM in cell lines and tissues, building upon our work in plasma and other reports showing peptide immunoaffinity enrichment from cell line and tissue samples. In one early report, Gobom *et al.* identified and quantified endogenous neurotensin from human hypothalamus lysates using immunoaffinity purification with antibodies coupled to Sepharose G beads. The enriched samples were analyzed by MALDI-TOF-MS and nano-ESI-MS/MS, and the measurements were compared to radioimmunoassay[32]. In another report, Ukena *et al.* characterized an RFamide-related peptide in rat hypothalamus by immunoaffinity enrichment and tandem mass spectrometry on a Q-TOF-MS[33]. Neither report required proteolytic digestion, since the peptides were bioactive peptides and were not part of a larger protein, and neither report combined the enrichment with SRM quantification. Other reports have included proteolytic digestion followed by immunoaffinity enrichment of peptides, yet again not using SRM for subsequent analyses. For example, Rush *et al.* profiled tyrosine-phosphorylated peptides in cancer cell lines by enriching the peptides using a phosphotyrosine-specific antibody and LC-MS/MS[34]. Jiang *et al.* measured a peptide specific to epidermal growth factor receptor (EGFR) in human breast cancer cell lines and tissues, with quantification achieved using an isotopically heavy-labeled peptide and MALDI-MS[35]. In addition, Hoeppel *et al.* used antibodies raised against epitopes based on only three to four amino acids to reduce the complexity of human HEK293 cell lysate digests and characterized the simplified samples by MALDI-TOF-MS/MS[36]. Last, immunoaffinity capture of *proteins* (that is, classic immunoprecipitation) followed by proteolytic digestion and SRM has been reported[37]; our work here focuses on immunoaffinity capture of peptides, not proteins.

To evaluate the utility of the immuno-SRM assay platform for quantification of biomarkers in human cells, we developed assays for the well-established HER2 and ER biomarkers, and tested their performance in a set of highly characterized breast cancer cell lines[38] and surgical specimens. Experimental details are given in the Supplemental Materials. We targeted one peptide from each protein (LLFAPNLLDR for the ER protein and

AVTSANIQEFAGCK for HER2) and developed polyclonal and monoclonal antibodies to each, respectively, as described previously[39–41]. The open-source Skyline software[42, 43] was used to generate a list of transitions and their associated collision energy and declustering potential values for the 2+ and 3+ charge states of the peptides, the peptides were analyzed in pure form by SRM-MS, and subsequently the most sensitive parent peptide charge states and transitions were selected for all further analyses (Table 1). Nine transitions for LLFAPNLLDR and seven for AVTSANIQEFAGCK were included in the SRM method, without incurring appreciable sensitivity losses. The y7 transition ion for LLFAPNLLDR and the y9 transition ion for AVTSANIQEFAGCK were used for quantification, while the additional transitions were used to verify the identity of the peptide ion being monitored.

Performance metrics for the assays were determined by generating response curves using synthetic standard peptides. These curves were not used as calibration curves; rather, the response curves were used to determine the limits of detection, limits of quantification, and linear ranges of the assays. Curves were generated in a trypsin-digested cellular lysate matrix derived from the MCF10A breast epithelial cell line.

For the peptide AVTSANIQEFAGCK (HER2), there were detectable amounts present in the available cell lines, precluding the use of a lysate free of endogenous signal for the curve matrix. Therefore, we prepared a reverse response curve to estimate the limit of detection (LOD), limit of quantification (LLOQ), and linear range[44]. (Of note, these curve parameters were not used to back-calculate concentrations.) The heavy:light peptide ratio was measured and plotted versus the concentration ratio of heavy and light peptide in the sample (Figure 1A and 1B). The assay showed a linear response for all peptide amounts above 0.3 fmol. This linear range covers over 4 orders of magnitude, spanning protein concentrations of 3 to 50,000 fmol/mg lysate. The LOD was 6 fmol/mg lysate determined by calculating three times the signal-to-noise in the blank. The lowest point above the limit of detection with a CV <20% was 12 fmol/mg lysate; this value was used as the LLOQ. The median CV for all points on the response curve was 2.5%, and the CV at the LLOQ was 10.8% (the range of CVs was 1.5% to 10.8%).

The peptide LLFAPNLLDR from the ER protein was not detected in the MCF10A lysate, allowing for generation of a response curve without endogenous interference. However, there was a signal due to a peptide contamination associated with the antibody. Residual peptide was observed even though the peptide was covalently linked to the resin used for affinity purification. Such residual peptides have been reported previously[27, 28, 39]. Although the exact source of the contaminating peptides has not been determined, Hoofnagle *et al.*[27] propose that the peptides hydrolyze slowly from the resin. Because this signal affects the detection of analyte peptide at low concentrations, it must be characterized as part of the assay and thus effectively raises the LOD of the assay. To determine the performance characteristics of the assay, light peptide for LLFAPNLLDR was serially diluted into digested lysate from the MCF10A cell line, keeping the heavy labeled peptide constant. The peptide response curves are plotted in Figure 1C and 1D. The practical LOD (7 fmol/mg lysate) was calculated as the signal producing three times the standard deviation of the background, including the contaminant peptide signal. The coefficient of variation at the limit of detection was 15.9%, so the limit of quantification (defined as lowest point measured with CV<20%) was also 7 fmol/mg. Omitting the lowest points on the response curve, below the practical LOD, resulted in a linear range of nearly 4 orders of magnitude, spanning protein concentrations of 7 fmol/mg to 50,000 fmol/mg. The median CV for all points on the response curve was 4.2%, and the CV at the LLOQ was 15.9% (the range of CVs was 0.8% to 15.9%).

Next, we evaluated the performance of the immuno-SRM method using 17 cell lines derived from human breast cancers; the ER/HER2 status of this extensively studied cell line panel has been well-characterized[38]. Samples were analyzed in process triplicate (each sample's triplicate processed on the same day) using the optimized immuno-SRM assays, and the data are reported in Table 2 and plotted in Figures 2A and 2B. Results for the HER2 assay showed good separation between cell lines that do vs. those that do not overproduce HER2 (p-value of 0.0012); the *lowest* HER2-overproducing cell line (MDAMB361) had 3.62E+03 fmol/mg, whereas the *highest* HER2- cell line (MDAMB453) had 1.65E+03 fmol/mg. Results for the ER assay also showed good separation between cell lines that do vs. those that do not express ER (p-value of 0.0019), although there was one outlier ER- sample (HCC1419) that had a relatively high concentration of 258 fmol/mg.

Following demonstration of the assays in breast cancer cell lines, we analyzed eight surgically resected human breast cancers whose ER and HER2 statuses had been determined as part of routine diagnostic workup of the patients using standard clinical assays (e.g. IHC for ER and IHC±FISH for HER2). Samples were analyzed in process duplicate or triplicate (depending on sample availability) using the optimized immuno-SRM assays, and the data are reported in Table 2 and plotted in Figures 2C and 2D (each sample's replicates were processed on the same day). For the HER2 assay, the CVs ranged from 1% to 28% across all cell line and tissue samples (including percent differences for samples measured in duplicate), and the median CV was 7%. The ER assay CVs ranged from 2% to 61% (also including percent differences), with a median CV of 10%. Median CVs for analyses of the cell lines and surgical specimens were slightly higher for the process duplicate or triplicate samples compared to the response curve samples; this is because trypsin digestion was part of the cell line and surgical sample analyses (but not the response curve generation, which used a single common digest as a matrix), indicating a portion of the overall CV is due to trypsin digestion, as described previously[23]. Remarkably, although breast cancer tissues are highly heterogeneous with respect to their composition and cellularity, results for the HER2 assay showed good separation between tumors that do vs. those that do not overproduce HER2 (Figure 2C). The ER assay also showed good separation (Figure 2D), although as with the cell lines (Figure 2B) there was one ER- tumor (080127A1) that showed a relatively high concentration of ER (140 fmol/mg). Significantly, a comparison of the tissue immuno-SRM results with Allred scores obtained for ER showed good agreement, see Table 2 (Allred scores refer to discrete scores (scaled from 0 to 8) based on IHC measurements that correspond to a metric encompassing staining intensities and percentages of positive cells; a higher score corresponds to higher ER levels[45]). The IHC classification for the HER2 tissue samples was also in good agreement with the immuno-SRM results, Table 2. In addition, FISH assays indicating HER2 status were performed on two of the tissue samples, and again those results corresponded well with the immuno-SRM data (Table 2).

These results demonstrate the feasibility of developing immuno-SRM assays of sufficient sensitivity and analytical performance to quantify protein biomarkers in human cell lines and surgical specimens. Additionally, the amount of material used in this demonstration (100 ug) is readily obtainable from core biopsies. However, for immuno-SRM to become a viable platform for tissue-based clinical diagnostics, technical hurdles will need to be overcome. For example, the composition of human tumors is highly heterogeneous, consisting of tumor cells, blood and lymphatic vessels, infiltrating immune cells, stromal elements, etc. Additionally, the proportion of the tumor that corresponds to each of these elements is also highly variable, not only amongst different patients but also amongst different anatomical locations within the same tumor. Because tumor biomarkers generally are produced by only one component of the tumor (commonly the cancer cell component),

simply normalizing the total amount of input material (i.e. tumor protein lysate) in the assay will not account for heterogeneous composition and thus may yield unreliable results.

One possible approach to dealing with tumor composition heterogeneity is to use laser capture microscopy (LCM) to isolate the specific tumor component producing the biomarker. LCM has been used to harvest cells for LC-MS/MS analyses, using anywhere from 30,000–200,000 cells to obtain microgram quantities of protein[46, 47]. The advantage of this technique is that specific populations of cells (e.g. those expressing the biomarker of interest) can be targeted for harvest and immuno-SRM analyses. Disadvantages are that it is labor-intensive, requires specialized equipment, and samples a small amount of tissue that may not be representative of the entire tumor[48]. A second possible approach is to develop a panel of protein markers that are specific to each of the tumor components, and to use the relative abundances of these “component markers” to normalize the biomarker measurements across samples, thus accounting for heterogeneity in tumor composition.

Of note, there are precedents for measuring tissue biomarkers from grossly dissected tumors, by ensuring that the input material is representative of the tumor *en masse*. For example, the American Society of Clinical Oncology recommends measurement of urokinase plasminogen activator and plasminogen activator inhibitor 1 (uPA/PAI-1) by ELISAs on a minimum of 300 mg of fresh or frozen breast cancer tissue for the determination of prognosis in patients with newly diagnosed, node negative breast cancer[2]. In a second example, the RNA-based Oncotype DX diagnostic test (a clinically validated, high-complexity, multi-analyte reverse transcription–PCR genomic test that predicts the likelihood of breast cancer recurrence in early-stage, node-negative, estrogen receptor–positive breast cancer) selects tissue blocks containing the greatest amount of invasive breast carcinoma that is morphologically consistent with the submitting diagnosis and has the least amount of noninvasive mammary epithelium; samples with metabolically active nontumor elements constituting >50% of the tissue have those elements dissected out before sample extraction[49].

There are limitations of immuno-SRM assays compared with IHC. First, IHC can be used to determine the subcellular localization of proteins (e.g. nuclear versus cytoplasmic). While it may be possible to develop extraction methods to differentially recover nuclear versus cytoplasmic protein from tissues for immuno-SRM analysis, this has not yet been demonstrated. Hence, for rare biomarkers whose diagnostic utility is based not on total protein abundance but rather on mis-localization, IHC may be required. A second potential disadvantage of immuno-SRM assays for tissue diagnosis is that these assays will not be able to distinguish between tumors containing a small subpopulation of biomarker-positive cells versus tumors showing low level biomarker expression in 100% of the cells. Although there may be some instances where this distinction is important, in many cases pathologists have generated composite scores combining the percent of positive cells with a subjective measure of expression level (e.g. the Allred score for ER status in breast cancer[45]). In these cases, immuno-SRM assays may actually be advantageous because the scoring of expression level would no longer be subjective. Third, immuno-SRM measurements depend upon reproducible trypsin digestion. While many proteins are readily digested with trypsin, some are notoriously difficult. Hence, for each proteotypic peptide, the reproducibility of digestion will need to be assessed.

We note that quantification of membrane proteins by immuno-SRM in adherent cell lines presents a unique potential preanalytical variable. This is because adherent cells typically are recovered from the growth plate by trypsinization, which could cleave extracellular protein domains and thus affect the recovery and reproducibility of quantification of membrane

proteins. In such cases, where possible, selecting proteotypic peptides from the intracellular domain may help to mitigate this effect.

Finally, ER and HER2 proteins have been measured in multiplex from breast cancer biopsies using bead-based sandwich ELISA assays[50, 51]. An advantage of these assays is that they can be run in higher throughput compared to currently configured immuno-SRM assays. In addition, up to nine different proteins were assayed as part of the multiplex samples. The plex number was limited, however, by non-specific interference between the individual assays. Conversely, we have previously shown that peptide-based immuno-SRM assays can be readily multiplexed using as many as 31 antibodies[17]. Immuno-SRM relies on mass spectrometry for detection, which provides absolute specificity, a decided advantage over ELISA assays. The need for only one antibody also makes immuno-SRM assays relatively easier to construct, less costly, and more rapid to configure than ELISA assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to the FHCRC/UW Breast Specimen Repository and Registry (BSRR) for specimens used in this study. The BSRR is generously supported by the Breast Cancer Relief Foundation, The Foster Foundation, and Hutchinson Center funds. All BSRR specimens and data have been obtained in accordance with all applicable human subjects laws and regulations, including any requiring informed consent. We also thank Jason Held of the Buck Institute for consultation in choosing proteotypic peptides for estrogen receptor. This work was supported in part by a grant from the Entertainment Industry Foundation (EIF) and the EIF Women's Cancer Research Fund to the Breast Cancer Biomarker Discovery Consortium, by Susan G. Komen for the Cure®, the Avon Foundation, the Paul G. Allen Family Foundation, and the National Cancer Institute (RC2CA148286, U24CA126476, and U24CA160034). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.

Abbreviations

ER	estrogen receptor
FISH	fluorescence in situ hybridization
HER2	receptor tyrosine-protein kinase erbB-2
IHC	immunohistochemistry
SID	stable isotope dilution
SRM	selected reaction monitoring

References

1. Gown AM. Current issues in ER and HER2 testing by IHC in breast cancer. *Mod Pathol*. 2008; 21(Suppl 2):S8–S15. [PubMed: 18437174]
2. Harris L, Fritsche H, Mennel R, Norton L, et al. American Society of Clinical Oncology 2007 update of recommendations for the use of tumor markers in breast cancer. *J. Clin. Oncol*. 2007; 25:5287–5312. [PubMed: 17954709]
3. Goldman JM, Melo JV. Chronic myeloid leukemia--advances in biology and new approaches to treatment. *N Engl J Med*. 2003; 349:1451–1464. [PubMed: 14534339]
4. Dorfman DM, Bui MM, Tubbs RR, Hsi ED, et al. The CD117 immunohistochemistry tissue microarray survey for quality assurance and interlaboratory comparison: a College of American

- Pathologists Cell Markers Committee Study. Arch Pathol Lab Med. 2006; 130:779–782. [PubMed: 16740027]
5. Paik S, Bryant J, Tan-Chiu E, Romond E, et al. Real-world performance of HER2 testing--National Surgical Adjuvant Breast and Bowel Project experience. J Natl Cancer Inst. 2002; 94:852–854. [PubMed: 12048273]
 6. Saltz LB, Meropol NJ, J LP Sr, Needle MN, et al. Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. J Clin Oncol. 2004; 22:1201–1208. [PubMed: 14993230]
 7. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med. 2004; 350:2129–2139. [PubMed: 15118073]
 8. Paez JG, Janne PA, Lee JC, Tracy S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science. 2004; 304:1497–1500. [PubMed: 15118125]
 9. Pao W, Miller V, Zakowski M, Doherty J, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A. 2004; 101:13306–13311. [PubMed: 15329413]
 10. Oien KA, Evans TR. Raising the profile of cancer of unknown primary. J Clin Oncol. 2008; 26:4373–4375. [PubMed: 18802148]
 11. Papadopoulos N, Kinzler KW, Vogelstein B. The role of companion diagnostics in the development and use of mutation-targeted cancer therapies. Nat Biotechnol. 2006; 24:985–995. [PubMed: 16900147]
 12. Yaziji H, Taylor CR, Goldstein NS, Dabbs DJ, et al. Consensus recommendations on estrogen receptor testing in breast cancer by immunohistochemistry. Appl Immunohistochem Mol Morphol. 2008; 16:513–520. [PubMed: 18931614]
 13. Sauter G, Lee J, Bartlett JM, Slamon DJ, Press MF. Guidelines for human epidermal growth factor receptor 2 testing: biologic and methodologic considerations. J. Clin. Oncol. 2009; 27:1323–1333. [PubMed: 19204209]
 14. Badve SS, Baehner FL, Gray RP, Childs BH, et al. Estrogen- and progesterone-receptor status in ECOG 2197: comparison of immunohistochemistry by local and central laboratories and quantitative reverse transcription polymerase chain reaction by central laboratory. J. Clin. Oncol. 2008; 26:2473–2481. [PubMed: 18487567]
 15. Anderson L, Hunter CL. Quantitative mass spectrometric multiple reaction monitoring assays for major plasma proteins. Mol. Cell. Proteomics. 2006; 5:573–588. [PubMed: 16332733]
 16. Fu Q, Schoenhoff FS, Savage WJ, Zhang P, Van Eyk JE. Multiplex assays for biomarker research and clinical application: translational science coming of age. Proteomics Clin. Appl. 2010; 4:271–284. [PubMed: 21137048]
 17. Whiteaker JR, Lin C, Kennedy J, Hou L, et al. A targeted proteomics-based pipeline for verification of biomarkers in plasma. Nat. Biotechnol. 2011; 29:625–634. [PubMed: 21685906]
 18. Addona TA, Shi X, Keshishian H, Mani DR, et al. A pipeline that integrates the discovery and verification of plasma protein biomarkers reveals candidate markers for cardiovascular disease. Nat. Biotechnol. 2011; 29:635–643. [PubMed: 21685905]
 19. Kuhn E, Wu J, Karl J, Liao H, et al. Quantification of C-reactive protein in the serum of patients with rheumatoid arthritis using multiple reaction monitoring mass spectrometry and ¹³C-labeled peptide standards. Proteomics. 2004; 4:1175–1186. [PubMed: 15048997]
 20. Kuzyk MA, Smith D, Yang J, Cross TJ, et al. Multiple reaction monitoring-based, multiplexed, absolute quantitation of 45 proteins in human plasma. Mol. Cell. Proteomics. 2009; 8:1860–1877. [PubMed: 19411661]
 21. Barr JR, Maggio VL, Patterson DG Jr, Cooper GR, et al. Isotope dilution--mass spectrometric quantification of specific proteins: model application with apolipoprotein A-I. Clin. Chem. 1996; 42:1676–1682. [PubMed: 8855153]
 22. Gerber SA, Rush J, Stemman O, Kirschner MW, Gygi SP. Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proc. Natl. Acad. Sci. U.S.A. 2003; 100:6940–6945. [PubMed: 12771378]

23. Addona TA, Abbatiello SE, Schilling B, Skates SJ, et al. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. *Nat. Biotechnol.* 2009; 27:633–641. [PubMed: 19561596]
24. Anderson NL, Anderson NG, Haines LR, Hardie DB, et al. Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). *J Proteome Res.* 2004; 3:235–244. [PubMed: 15113099]
25. Whiteaker JR, Zhao L, Zhang HY, Feng LC, et al. Antibody-based enrichment of peptides on magnetic beads for mass-spectrometry-based quantification of serum biomarkers. *Anal Biochem.* 2007; 362:44–54. [PubMed: 17241609]
26. Whiteaker JR, Zhao L, Anderson L, Paulovich AG. An automated and multiplexed method for high throughput peptide immunoaffinity enrichment and multiple reaction monitoring mass spectrometry-based quantification of protein biomarkers. *Mol. Cell. Proteomics.* 2010; 9:184–196. [PubMed: 19843560]
27. Hoofnagle AN, Becker JO, Wener MH, Heinecke JW. Quantification of thyroglobulin, a lowabundance serum protein, by immunoaffinity peptide enrichment and tandem mass spectrometry. *Clin Chem.* 2008; 54:1796–1804. [PubMed: 18801935]
28. Kuhn E, Addona T, Keshishian H, Burgess M, et al. Developing Multiplexed Assays for Troponin I and Interleukin-33 in Plasma by Peptide Immunoaffinity Enrichment and Targeted Mass Spectrometry. *Clin Chem.* 2009; 55:1108–1117. [PubMed: 19372185]
29. Neubert H, Gale J, Muirhead D. Online high-flow peptide immunoaffinity enrichment and nanoflow LC/MS/MS: assay development for total salivary pepsin/pepsinogen. *Clin. Chem.* 2010; 56:1413–1423. [PubMed: 20472821]
30. Whiteaker JR, Zhang H, Zhao L, Wang P, et al. Integrated pipeline for mass spectrometry-based discovery and confirmation of biomarkers demonstrated in a mouse model of breast cancer. *J. Proteome Res.* 2007; 6:3962–3975. [PubMed: 17711321]
31. Berna M, Schmalz C, Duffin K, Mitchell P, et al. Online immunoaffinity liquid chromatography/tandem mass spectrometry determination of a type II collagen peptide biomarker in rat urine: Investigation of the impact of collision-induced dissociation fluctuation on peptide quantitation. *Anal Biochem.* 2006; 356:235–243. [PubMed: 16797470]
32. Gobom J, Kraeuter KO, Persson R, Steen H, et al. Detection and quantification of neurotensin in human brain tissue by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal. Chem.* 2000; 72:3320–3326. [PubMed: 10939406]
33. Ukena K, Iwakoshi E, Minakata H, Tsutsui K. A novel rat hypothalamic RFamide-related peptide identified by immunoaffinity chromatography and mass spectrometry. *FEBS Lett.* 2002; 512:255–258. [PubMed: 11852091]
34. Rush J, Moritz A, Lee KA, Guo A, et al. Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. *Nat. Biotechnol.* 2005; 23:94–101. [PubMed: 15592455]
35. Jiang J, Parker CE, Hoadley KA, Perou CM, et al. Development of an immuno tandem mass spectrometry (iMALDI) assay for EGFR diagnosis. *Proteomics Clin. Appl.* 2007; 1:1651–1659. [PubMed: 21136662]
36. Hoeppe S, Schreiber TD, Planatscher H, Zell A, et al. Targeting peptide termini, a novel immunoaffinity approach to reduce complexity in mass spectrometric protein identification. *Mol. Cell. Proteomics.* 2011; 10:M110.002857. [PubMed: 20962300]
37. Domanski D, Murphy LC, Borchers CH. Assay development for the determination of phosphorylation stoichiometry using multiple reaction monitoring methods with and without phosphatase treatment: application to breast cancer signaling pathways. *Anal. Chem.* 2010; 82:5610–5620. [PubMed: 20524616]
38. Neve RM, Chin K, Fridlyand J, Yeh J, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer. Cell.* 2006; 10:515–527. [PubMed: 17157791]
39. Whiteaker JR, Zhao L, Abbatiello SE, Burgess M, et al. Evaluation of large scale quantitative proteomic assay development using peptide affinity-based mass spectrometry. *Mol. Cell. Proteomics.* 2011; 10:M110.005645. [PubMed: 21245105]
40. Schoenherr RM, Zhao L, Whiteaker JR, Feng LC, et al. Automated screening of monoclonal antibodies for SISCAPA assays using a magnetic bead processor and liquid chromatography-

- selected reaction monitoring-mass spectrometry. *J. Immunol. Methods.* 2010; 353:49–61. [PubMed: 19961853]
41. Razavi M, Pope ME, Soste MV, Eyford BA, et al. MALDI immunoscreening (MiSCREEN): a method for selection of anti-peptide monoclonal antibodies for use in immunoproteomics. *J. Immunol. Methods.* 2011; 364:50–64. [PubMed: 21078325]
42. MacLean B, Tomazela DM, Shulman N, Chambers M, et al. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics.* 2010; 26:966–968. [PubMed: 20147306]
43. MacLean B, Tomazela DM, Abbatiello SE, Zhang S, et al. Effect of collision energy optimization on the measurement of peptides by selected reaction monitoring (SRM) mass spectrometry. *Anal. Chem.* 2010; 82:10116–10124. [PubMed: 21090646]
44. Campbell J, Rezai T, Prakash A, Krastins B, et al. Evaluation of absolute peptide quantitation strategies using selected reaction monitoring. *Proteomics.* 2011; 11:1148–1152. [PubMed: 21365756]
45. Harvey JM, Clark GM, Osborne CK, Allred DC. Estrogen receptor status by immunohistochemistry is superior to the ligand-binding assay for predicting response to adjuvant endocrine therapy in breast cancer. *J Clin Oncol.* 1999; 17:1474–1481. [PubMed: 10334533]
46. Zang L, Palmer Toy D, Hancock WS, Sgroi DC, Karger BL. Proteomic analysis of ductal carcinoma of the breast using laser capture microdissection, LC-MS, and 16O/18O isotopic labeling. *J. Proteome Res.* 2004; 3:604–612. [PubMed: 15253443]
47. Baker H, Patel V, Molinolo AA, Shillitoe EJ, et al. Proteome-wide analysis of head and neck squamous cell carcinomas using laser-capture microdissection and tandem mass spectrometry. *Oral Oncol.* 2005; 41:183–199. [PubMed: 15695121]
48. Moeder CB, Giltman JM, Harigopal M, Molinaro A, et al. Quantitative justification of the change from 10% to 30% for human epidermal growth factor receptor 2 scoring in the American Society of Clinical Oncology/College of American Pathologists guidelines: tumor heterogeneity in breast cancer and its implications for tissue microarray based assessment of outcome. *J Clin Oncol.* 2007; 25:5418–5425. [PubMed: 18048824]
49. Cronin M, Sangli C, Liu ML, Pho M, et al. Analytical validation of the Oncotype DX genomic diagnostic test for recurrence prognosis and therapeutic response prediction in node-negative, estrogen receptor-positive breast cancer. *Clin Chem.* 2007; 53:1084–1091. [PubMed: 17463177]
50. Sauer G, Schneiderhan-Marra N, Kazmaier C, Hutzl K, et al. Prediction of nodal involvement in breast cancer based on multiparametric protein analyses from preoperative core needle biopsies of the primary lesion. *Clin. Cancer Res.* 2008; 14:3345–3353. [PubMed: 18519762]
51. Schneiderhan-Marra N, Sauer G, Kazmaier C, Hsu HY, et al. Multiplexed immunoassays for the analysis of breast cancer biopsies. *Anal. Bioanal Chem.* 2010; 397:3329–3338. [PubMed: 20640897]

Figure 1. Response curves for HER2 and ER peptides in cell lysate

Peptides were serially diluted in digested lysate of MCF10A cells. 100 ug lysate was used for each antibody capture. For the HER2 peptide AVTSANIQEFAGCK, the response was determined by varying the heavy stable isotope-labeled peptide relative to the constant light peptide plotted in linear space (A) and as log10 transformed values (B). The ER peptide LLFAPNLLDR response was determined by varying the light synthetic peptide relative to a constant heavy peptide amount and plotting in linear (C) and log10 space (D). Weighted (1/y) linear regression was performed using R. Insets magnify the low concentration portion of the curves. Error bars indicate the range of measurements.

Figure 2. Quantification of HER2 and ER in cell lines and tissues

The concentration determined by immuno-SRM was plotted for the cell lines (A and B) and tissues (C and D) reported in Table 2. HER2 values were plotted on a log10 scale due to the wide range in values. For ER, samples measured below the LLOQ were plotted at the LLOQ.

Protein and peptide information for ER and HER2

Table 1

For LLFAPNLLDDR, the C-terminal arginine (bold and underlined) was ¹³C and ¹⁵N isotopically labeled. For AVTSANIQEFAGCK, the label consisted of only the ¹³C isotope. The cysteine in AVTSANIQEFAGCK was carbamidomethylated. Collision energies (CE) and declustering potentials (DP) were obtained using the regression equations in Skyline for an AB Sciex triple quadrupole.

Protein Description	Prot.	Gene Symbol	Accession Number	Peptide sequence	Parent ion m/z	Parent ion charge state	Fragment ion m/z	Ion type	Fragment ion charge state	CE (V)	DP (V)
Estrogen receptor alpha	ER	ESR1	P03372	LLFAPNLLDDR	642.8874	2+	1171.683	y10	1+	32.4	78
							1058.599	y9	1+	32.4	78
							911.5309	y8	1+	32.4	78
							840.4938	y7	1+	32.4	78
							743.441	y6	1+	32.4	78
							629.3981	y5	1+	32.4	78
							516.314	y4	1+	32.4	78
							420.7505	y7	2+	32.4	78
							445.2809	b4	1+	32.4	78
							1181.692	y10	1+	32.4	78
							1068.608	y9	1+	32.4	78
							921.5392	y8	1+	32.4	78
							850.502	y7	1+	32.4	78
Receptor tyrosine-protein kinase erbB-2	HER2	ERBB2	P04626	AVTSANIQEFAGCK	748.3641	2+	753.4493	y6	1+	32.4	78
							639.4064	y5	1+	32.4	78
							526.3223	y4	1+	32.4	78
							425.7547	y7	2+	32.4	78
							445.2809	b4	1+	32.4	78
							1325.615	y12	1+	38.4	85.7
							1066.499	y9	1+	38.4	85.7
							952.4557	y8	1+	38.4	85.7
							839.3716	y7	1+	38.4	85.7
							582.2704	y5	1+	38.4	85.7

Protein Description	Prot.	Gene Symbol	Accession Number	Peptide sequence	Parent ion m/z	Parent ion charge state	Fragment ion m/z	Ion type	Fragment ion charge state	CE (V)	DP (V)
				AVTSANIQEFAGCK	751.3742	2+	435.202	y4	1+	38.4	85.7
							663.3114	y12	2+	38.4	85.7
							1331.636	y12	1+	38.4	85.7
							1072.519	y9	1+	38.4	85.7
							958.4758	y8	1+	38.4	85.7
							845.3917	y7	1+	38.4	85.7
							588.2906	y5	1+	38.4	85.7
							441.2222	y4	1+	38.4	85.7
							666.3214	y12	2+	38.4	85.7

Table 2
Quantification of HER2 and ER in cell lines and tissues

The multiplexed immuno-SRM assay was applied to a set of highly characterized cell lines and tissue samples. For tissues, HER2 and ER status were determined by immunohistochemistry and confirmed by FISH for HER2 when intermediate. Concentration values were based on a single point calibration of the immuno-SRM assay relative to the amount of heavy stable isotope-labeled peptide. 100 ug of lysate was measured for each sample in process triplicate. Tissue samples with an asterisk were analyzed in duplicate, for which CV was reported as the percent difference. For entries marked <LLOQ, peak areas were below the limit of quantification.

Sample	HER2			ER			HER2 status	ER status
	fmol/mg lysate	CV	fmol/mg lysate	CV	fmol/mg lysate	CV		
HCC1419	3.47E+04	16%	2.58E+02	6%			+	-
ZR7530	3.45 E+04	5%	3.04E+02	8%			+	+
HCC1569	2.71E+04	12%	<LLOQ				+	-
BT474	1.98E+04	18%	9.68E+02	17%			+	+
HCC1954	1.70E+04	4%	1.04E+02	7%			+	-
UACC812	1.44E+04	7%	2.94E+02	16%			+	+
HCC202	1.42E+04	4%	9.7E+01	10%			+	-
MDAMB361	3.62E+03	1%	2.27E+02	6%			+	+
MDAMB453	1.65E+03	28%	<LLOQ				-	-
ZR751	1.17E+03	11%	7.86E+02	9%			-	+
T47D	7.72E+02	4%	6.45E+02	2%			-	+
CAMA1	6.66E+02	24%	1.74E+02	12%			-	+
HCC1428	6.10E+02	11%	3.67E+02	18%			-	+
MCF7	2.45E+02	7%	6.66E+02	8%			-	+
HCC70	2.32E+02	6%	7.9E+01	16%			-	-
HS578T	1.30E+02	5%	7.2E+01	10%			-	-
HCC1395	1.02E+02	11%	<LLOQ				-	-
tissue 080127A1*	1.19E+04	16%	1.40E+02	15%			+	- ^a (2 of 8) ^c
tissue 090163B2	4.37E+03	3%	1.25E+02	11%			+	+
tissue 080189A1	1.01E+03	10%	<LLOQ				-	- ^a (4 of 8) ^c
tissue 080077A1	8.72E+02	2%	6.71E+02	5%			-	- ^a (0 of 8) ^c
tissue 080028A1*	3.84E+02	4%	2.71E+02	61%			- ^a (3+ of 3+) ^a	+
							- ^a (not amplified) ^b	+
							- ^a (8 of 8) ^c	

Sample	HER2		ER		CV	HER2 status	ER status
	fmol/mg lysate	CV	fmol/mg lysate	CV			
tissue 090252A1	2.69E+02	16%	<LLOQ			− ^a (not amplified) ^b	− ^a (0 of 8) ^c
tissue 090023E1	2.30E+02	11%	<LLOQ			− ^a	− ^a (0 of 8) ^c
tissue 080031A2	1.67E+02	5%	<LLOQ			− ^a	− ^a

^a determined by IHC

^b intermediate by IHC, confirmed by FISH

^c Allred scores, which refer to discrete scores (scaled from 0 to 8) based on IHC measurements that correspond to a metric encompassing staining intensities and percentage of positive cells; a higher score corresponds to higher ER levels.