Demonstrating the feasibility of large-scale development of standardized assays to quantify human proteins

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Multiple reaction monitoring (MRM) mass spectrometry has been successfully applied to monitor targeted proteins in biological specimens, raising the possibility that assays could be configured to measure all human proteins. We report the results of a pilot study designed to test the feasibility of a large-scale, international effort for MRM assay generation. We have configured, validated across three laboratories and made publicly available as a resource to the community 645 novel MRM assays representing 319 proteins expressed in human breast cancer. Assays were multiplexed in groups of >150 peptides and deployed to quantify endogenous analytes in a panel of breast cancer–related cell lines. The median assay precision was 5.4%, with high interlaboratory correlation ($R^2 > 0.96$). Peptide measurements in breast cancer cell lines were able to discriminate among molecular subtypes and identify genome-driven changes in the cancer proteome. These results establish the feasibility of a large-scale effort to develop an MRM assay resource.

For the MRM-based assay technology to meet its potential to promote rapid advances in protein-based biomedical research, MRM-based assays to quantify any human protein (with sufficient sensitivity and throughput) must be made readily available to the target user community (i.e., basic and translational scientists) in the form of validated assays that can be used in individual laboratories or readily implemented in proteomic core facilities. Toward this end, global assay development projects have been proposed8–11, and peptide spectral databases12,13 as well as open-source, vendor-neutral software tools14–18 are being developed. In this study, we tested the feasibility and usefulness of a large-scale, international collaborative effort to generate MRM-MS assays targeting the human proteome, modeling what a global assay development effort might look like. We developed a panel of 645 MRM assays covering 319 proteins (~1.5% of the human proteome) differentially expressed among human breast cancer subtypes. We report our pipeline for reagent generation, assay development, analytical validation, assay deployment on biospecimens, and distribution of data and standard operating procedures (SOPs) as a community resource. The results demonstrate the feasibility of an international, large-scale project to develop MRM assays to all human proteins. We also demonstrate that MRM-based targeted proteomic measurements can recapitulate known biological subtypes of breast cancer, identify genome-driven changes in the cancer proteome, and provide complementary information to that encoded in mRNA or copy-number profiles.

RESULTS

Empirical selection of targets

To model what an international global assay development effort might look like, researchers at three performance sites (Seattle, Boston and Seoul) cooperated to develop 645 MRM assays representing 319 target proteins expressed in human breast cancers.
Breast cancer was chosen as a model system because extensive genomic characterizations have been used to describe well-defined molecular subtypes\(^{19-21}\) and because a panel of highly characterized breast cancer cell lines\(^{22-24}\) was readily available for the study. Although we focused on breast cancer (and on cell lysates) to provide a framework for this pilot project, the assays we developed are limited neither to application in cell lysates nor to breast cancer; they are generalizable.

To generate an empirical data set for selecting target analytes for MRM assay development, we analyzed unfraccionated protein lysates derived from a panel of human breast cancers and breast cancer–derived cell lines (Supplementary Table 1) by shotgun liquid chromatography–tandem mass spectrometry (LC-MS/MS). Over 64,000 unique peptides (representing 9,996 proteins) were identified at a peptide false discovery rate (FDR) < 0.005 in the combined cell-line and tissue data. To enrich for protein targets that might vary in expression level among breast cancer subtypes and thus be of biological interest, we ranked–ordered potential MRM targets by differences in their signal intensities among the breast cancer subtypes represented in the cell-line panel (we required that the target be identified in both the cell lysate and corresponding cancer tissue). From this rank–ordered list, we selected a set of 318 proteins for assay development (Supplementary Table 2). 73 of these 318 proteins (23%) were previously suggested to be of potential functional importance in breast cancer\(^{25}\). Finally, we selected 642 proteotypic peptides to represent these proteins, chosen from a filtered list of peptides that were detectable from 'neat' cellular lysate (that is, without enrichment or fractionation) by MRM on a triple quadrupole mass spectrometer. Although they were not observed by our shotgun LC-MS/MS analysis, three peptides to ESR1 were also included, for a total of 319 proteins represented by 645 proteotypic peptides. The selected proteins map to a variety of cellular compartments and span a range of biological processes (Supplementary Fig. 1).

**Development and characterization of multiplexed assays**

For each proteotypic peptide, we prepared synthetic light and heavy stable isotope–labeled peptides and determined optimum MRM transitions (precursor ion and fragment ion pairs) and instrument parameters as described in the Online Methods. The 645 individual peptide assays (Supplementary Table 2) were distributed randomly among four multiplex assay groups (each containing between 156 and 169 peptides). To avoid any bias for performance among assay groups, we ensured that each multiplex group contained an equivalent distribution of analyte intensities and retention times (Supplementary Fig. 2) in addition to lower limits of quantification (LLOQs) and coefficients of variation (CVs; see below and Supplementary Fig. 3). One of the multiplex assays was randomly chosen to be implemented at all three performance sites (the ‘interlaboratory’ assay), whereas each of the remaining three multiplex assays were acquired at only one performance site (the ‘site–specific’ assays).

We evaluated the analytical performance of the assays at each site by generating response curves in a cell lysate matrix. For the 645 peptides in the study, 1,938 individual reverse response curves were generated ((483 site–specific assays + 486 interlaboratory assays = 969 total) × 2 matrix dilutions). All response curves were plotted (Supplementary Results), and assay figures of merit were calculated (Supplementary Table 3). The majority of assays featured a linear range across more than three orders of magnitude. The median assay LLOQs for the interlaboratory assay group were 0.40, 0.61 and 0.52 fmol/µg (at a cell lysate matrix protein concentration of 1.0 µg/µl), with median CVs of 3.5%, 5.0% and 4.4% for sites 1, 2 and 3, respectively. At the same concentration, the site–specific assay groups had median assay LLOQs of 0.37, 0.65 and 0.40 fmol/µg, with median CVs of 3.5%, 5.4% and 4.4% for sites 1, 2 and 3, respectively.

An assay was deemed successful if it was precise (CV ≤ 20% at the lowest concentration point in the linear range of the assay) and specific (detection of at least one transition of the light and at least two transitions of the heavy peptide and perfect coelution of heavy and light peptides). Of the 645 assays we attempted to generate, 622 (96%) met these criteria and were considered to be successful. Furthermore, 599 (93%) had at least two transitions—and 534 (83%) had all three transitions—meeting these criteria.

**Deploying MRM assays in breast cancer–related cell lines**

Next we determined the robustness of the assays when deployed to characterize human cell lines. Protein lysates from 30 human cell lines representing breast cancer (or normal breast epithelial...
cells; Supplementary Table 1) were prepared at a single site and distributed to all performance sites for MRM analysis (Fig. 1). Each lysate was digested in triplicate, so assay variability incorporates the complete processing variability.

We analyzed a total of 174,420 individual assays ((483 site-specific assays + 486 interlaboratory assays = 969 total) × 30 cell lines × triplicate process replicates × 2 dilutions). One quantifying transition was selected to calculate endogenous levels for each measurement above the peptide analyte LLOQ (Supplementary Table 4 and Supplementary Fig. 4) from among the transitions with no interfering signal (Supplementary Table 5). An assay was considered to be informative if the empirically determined concentration of the target was above the assay LLOQ (i.e., indicates sufficient sensitivity); 93% (897 of 969) of the assays attempted met these criteria. We detected all 319 proteins endogenously in at least one cell line. At the individual peptide level, we detected 609 out of 645 peptides (94%) in at least one cell line and measured 547 of 645 (85%) in at least half of the cell lines. The empirical concentrations of the endogenous peptides derived from the same protein showed very high correlation (median of 0.93) in the individual cell lines (Supplementary Fig. 5).

To evaluate precision, we calculated the CV across the complete process triplicates for all endogenous measurements above the LLOQ (Supplementary Table 6 and Supplementary Fig. 4). At the three sites, the median assay CVs for the interlaboratory assay group were 5.0%, 7.3% and 5.1%, with 95% of the results having CVs less than 15%, 25% and 17%. The site-specific assay groups had median assay CVs of 4.7%, 6.3% and 4.7%, with 95% of the results having CVs less than 14%, 20% and 17%, for sites 1, 2 and 3, respectively (Fig. 2a). The site-specific assay groups had median assay CVs of 4.7%, 6.3% and 4.7%, with 95% of the results having CVs less than 14%, 20% and 17%, for sites 1, 2 and 3, respectively (Fig. 2b). The median CV for all measurements was 5.4%.

The empirically determined endogenous concentration of all peptide analytes constituting the interlaboratory 162-plex assay, which was analyzed at all three laboratories, was used to determine the correlation and agreement across the performance sites. Those measurements that were above the LLOQ for at least two sites (90% of measurements) were compared to determine the reproducibility of the measurements across sites. The correlation was excellent, with correlation coefficients ranging from 0.96 to 0.99 (Fig. 2c). There was also excellent agreement in the results among the sites, as demonstrated by the slopes from the linear regression of the correlation plots, which ranged from 0.95 to 1.07. This is also demonstrated by a histogram of the percent difference between site measurements (Fig. 2d). The mean percent difference was 0.9%, with 95% percent of the data within 22% difference and 75% percent of the data within 6.6% difference.

MRM results recapitulate known breast cancer subtypes
The empirically determined MRM-based measurements of 319 proteins were used for hierarchical clustering of the 30 cell lines. The cells lines formed two major clusters (Supplementary Fig. 6), which exactly match the clustering results previously observed for these cell lines using mRNA levels22–24 (into luminal and basal subtypes, largely correlated with estrogen receptor (ER) expression), demonstrating that MRM-based analyses can recapitulate the known molecular subtypes of breast cancer.

MRM results are clearly complementary to genomic data
We next asked whether the MRM data revealed any novel information about breast cancer that could not be determined from the genomic profiles of the cell lines. First, to identify proteins that are differentially expressed among the molecular subtypes of breast cancer, we performed a Wilcoxon rank test using the MRM data set. When the false positive rate26 was controlled at 0.01, four proteins were found to be differentially expressed...
between HER2⁺ vs. HER2⁻ cell lines, 83 proteins were differentially expressed between ER⁺ vs. ER⁻ cell lines, and 118 proteins were differentially expressed between basal vs. luminal cell lines (Supplementary Table 7).

To determine whether similar association patterns for this set of proteins can be observed using their gene expression (mRNA) data, we analyzed the genomic data of Neve et al. [23], which contain gene expression arrays for 28 of the 30 cell lines examined in our project. A total of 232 proteins quantified by MRM in this study also had corresponding gene expression measurements. A comparison between the proteins showing subtype association at the mRNA and the proteomic level illustrates that candidate markers could be identified using the MRM data that were not detected on the basis of RNA expression profiles (Supplementary Table 7 and Supplementary Fig. 7). 2, 7 and 11 proteins showed RNA expression levels significantly associated (P ≤ 0.01) with HER2 (ERBB2 gene product), ER and basal-luminal status, respectively, and did not show the same association patterns in their protein abundances, whereas 0, 44 and 56 proteins showed protein abundances significantly associated (using Wilcoxon rank test, FDR ≤ 0.01) with HER2, ER, and basal-luminal status, respectively, and did not show the same association patterns in their RNA expression signatures. These discrepancies demonstrate that protein profiling provides information complementary to genomic data (Fig. 3). We further focused on the 71 proteins whose protein abundances were significantly associated with HER2, ER, or basal-luminal status but whose RNA expression levels were not (i.e., the protein and mRNA data were discordant). Of these 71 proteins, 28 are likely to be functionally important in breast cancer as determined by their inclusion in an independently curated set of 1,000 human proteins of relevance to human breast cancer [25]. This example demonstrates that information encoded at the proteomic level is different from that at the mRNA level, where no subtype-specific regulation of expression was observed.

**Integrative analysis can identify potential disease genes**

In prior studies of breast cancer, hundreds of genes were found to be associated with patient prognosis at the RNA expression level [27-29]. Although these data suggest candidates, they are not sufficient to identify the primary drivers of clinical behavior of tumors, and many of these mRNA expression differences are not translated into differences at the protein level. Given the complementary information obtained from the mRNA and MRM proteomic results, we hypothesized that proteomic analyses may help identify clinically significant changes. The rationale for this hypothesis is twofold: (i) changes observed in multiple independent data sets using orthogonal technologies (i.e., genomics and proteomics) are less likely to be false positives, and (ii) having protein-level data should greatly augment the resolution of the information available.

**Figure 3** | Protein and RNA expression data show different genes significantly associated with HER2, ER and basal-luminal status. In the heat maps, each row represents a sample and each column represents a gene. The color bars on the left side of each heat map illustrate the subtypes of cell lines. The color bar on the top of each heat map illustrates whether only the protein expression, only the RNA expression or both expression types of the gene were associated with the subtype. (a) Of the four genes shown, all four have significantly different (P value ≤ 0.01) RNA expression levels between HER2⁺ and HER2⁻ cell lines, whereas only two of the four have significantly different (FDR ≤ 0.01) protein expression levels. (b) Of the 69 genes shown, 25 have significantly different RNA expression levels, and 62 have significantly different protein expression levels, between ER⁺ and ER⁻ cell lines, with an overlap of 18 genes. (c) Of the 98 genes shown, 42 have significantly different RNA expression levels, and 87 have significantly different protein expression levels, between basal and luminal cell lines, with an overlap of 31.

**Figure 4** | Distribution of protein expression levels, RNA expression levels and DNA copy numbers of the 12 subtype-enriched genes among genomic and proteomic data sets. (a-c) The protein expression levels measured by MRM (a), RNA expression levels (b) and DNA copy-number variation (c) are shown for HER2⁺ and HER2⁻ cell lines and for basal and luminal cell lines. Two proteins, ERBB2 and GRB7, are gene products of the HER2 amplicon on chromosome 17 and show good separation of HER2⁺ and HER2⁻ groups. The other ten proteins show a difference between the basal and luminal subtypes; the corresponding P values from Wilcoxon rank tests are all at or below 1 × 10⁻⁴ with 10,000 iterations. Box plots show the median value plotted as a line with each box displaying the distribution of the inner quartiles and vertical lines showing 95% of the data. RNA, robust multiarray analysis; CGH, comparative genomic hybridization.
the interpretation of genomic profiles by identifying changes that are ultimately expressed in the proteome, which are more directly connected to the clinical phenotype.

We performed an integrative analysis (Online Methods) and identified 31 proteins that show significant correlation (Bonferroni-adjusted $P \leq 0.0001$) between the genomic$^{33}$ (i.e., DNA copy number and mRNA expression) and proteomic (MRM) data (Supplementary Table 8). Furthermore, among the four proteins associated with HER2 status, two have DNA copy-number and gene expression information available, and both proteins (HER2 and GRB7) show significant concordance between genomic and proteomic signatures. Among the 118 proteins associated with basal-luminal status, 30 have corresponding genomic data, and only 10 (ABAT, ANXA1, PLOD3, CDKN2A, HER2, GALK1, CLTC, PRDX3, ALDOA and DPYSL2) show significant concordance scores. Among the 83 proteins associated with ER status, 20 have corresponding genomic data, and only 5 (CLTC, PRDX3, ANXA1, ABAT and PLOD3) show significant concordance scores (Fig. 4). Proteins whose expression is primarily regulated by gene expression showed agreement of measured protein levels with mRNA levels. 14 proteins were identified with protein levels significantly associated with HER2, ER and basal-luminal status; and 2, 4 and 2, respectively, showed the same association patterns in their RNA expression signatures. We observed no proteins showing RNA expression levels significantly associated with HER2, ER and basal-luminal status that did not also have significantly associated protein abundances (Supplementary Fig. 7). On the basis of this analysis, we conclude that the concordance of protein and mRNA levels for the subset of proteins whose expression is primarily regulated by gene expression is high but not perfect.

Although the importance of amplification of the ERBB2 locus (which also contains GRB7) in breast cancer is well established$^{30}$, the clinical relevance of the other nine genes identified above is not known. As it has been shown that the genomic profiles of the cell lines in this study closely recapitulate those of primary breast cancers$^{33}$, we next tested whether these nine genes’ expression levels were associated with outcome in two independent breast cancer data sets (referred to as the van ’t Veer$^{31}$ data set and Loi et al.$^{32}$ data sets) that provide both survival outcome and genomic profiles for large sets of primary human breast cancers. When patients were stratified by either high or low expression levels for each of the nine candidate genes, significant differences between Kaplan-Meier (KM) survival curves of the two patient groups were observed in both data sets for CLTC, DPYSL2 and ABAT (Fig. 5). We next fit a multivariate Cox proportional hazard model to further assess the association between gene expression and survival outcome, accounting for molecular subtype (via prediction analysis of microarray with a 50-gene classifier, or PAM50)$^{33}$, age, tumor size, lymph node status and other clinical covariates (Supplementary Table 9). Again, CLTC and DPYSL2 were found to be significantly associated (CLTC $P$ values = 0.029 and 0.068, and DPYSL2 $P$ = 0.067 and 0.0048 in the two clinical data sets, respectively, with $P$ value significance cutoff of 0.1) with survival outcome. ABAT showed evidence of association with survival outcome in the Loi et al.$^{32}$ data set (ABAT $P$ = 0.012) but not in the van ’t Veer et al.$^{31}$ data set. In summary, as a proof of principle, the above results illustrate the potential advantage of integrating quantitative proteomic data with genomic data to improve our understanding of which of the multitude of genomic alterations are most likely to be translated to the protein level and thus most likely to contribute to clinical phenotypes.

DISCUSSION

All MRM assays developed in this study, including SOPs for sample preparation and analyte-specific instrument parameters for data acquisition, have been made freely available as a resource for the community (see Online Methods). Each assay underwent rigorous analytical characterization and determination of analytical figures of merit, ensuring high quality standards for assay performance, as well as fit-for-purpose validation for the interrogation of human cell lines. The majority of academic centers now have proteomic core facilities with instrumentation to implement MRM-based assays, and all assays developed in

Figure 5 | Kaplan-Meier (KM) survival curves of breast cancer patients are stratified by their expression levels of DPYSL2, CLTC or ABAT. Two independent breast cancer data sets$^{31,32}$ providing both outcome information and genomic profiles were used to determine whether the expression of candidate gene products identified in this study show association with outcome. The data are shown for DPYSL2, CLTC and ABAT. For each gene, the breast cancers were classified into high- or low-expressing groups according to whether the expression of the candidate gene was greater than the median expression of the candidate gene. The $P$ values from log-rank tests comparing the two KM curves are shown above each figure.
this study are readily implementable in such facilities using the SOPs and Skyline files provided (see Online Methods and the Supplementary Protocol).

We used a simple sample preparation protocol for generating cellular protein lysate, without biochemical fractionation or enrichment of the target analytes before MRM analysis. Assuming the success rate found in this study extends to the full range of human proteins whose endogenous levels are detectable by MRM from neat cellular lysates, it is reasonable to estimate that several thousand human proteins might be quantified from cell-line lysates by MRM alone (i.e., without enrichment). This number is highly context dependent, however. For example, although thousands of proteins may be quantifiable in cell lysates without enrichment, in a more challenging matrix (for example, blood plasma), that number is likely to be much lower. In any biospecimen type, MRM assays have greater success for quantifying endogenous levels of more abundant proteins than for less abundant proteins. Thus, to configure MRM assays capable of detecting endogenous levels for the entire human proteome, enrichment strategies will be required for many proteins. For example, major classes of post-translational modifications (for example, phosphorylation) are largely inaccessible by MRM without enrichment. Developing MRM assays for modified proteins may face additional challenges due to enrichment technologies (for example, occasional difficulty enriching a specific modification or in generating an antibody to a specific modification) or peptide characteristics (modifications of interest must reside within proteotypic peptides with suitable size, chromatographic qualities, ionization properties, etc.) for analysis by mass spectrometry.

Analyte enrichment upstream of MRM can reduce sample complexity ($10^3$–$10^6$ enrichment), offering improved sensitivity, increased selectivity and potential for increased throughput (via shorter LC-MRM-MS acquisition times). Enrichment can be achieved either biochemically34–36 (for example, using chromatography) or through the use of analyte-specific antibodies for immuno-affinity enrichment37–41 (producing an immuno-MRM assay). Biochemical enrichments are generally costly and/or labor-intensive procedures that critically limit throughput and require specialized expertise. Immuno-affinity enrichment involves a single-step capture (immunoprecipitation) that is easily implemented in any modern research laboratory using existing expertise and infrastructure (and thus is highly distributable); the major limitations of this approach are a current lack of validated affinity reagents and the up-front cost and time required to generate renewable affinity reagents. Aside from costs, the production of high-affinity anti-peptide antibodies is associated with a 55% per peptide success and a >95% success rate on a protein level (when a multiplex immunization strategy is used49).

Of great interest and utility to clinically driven research, we showed that MRM-based peptide measurements in individual breast cancer cell lines were able to discriminate between molecular subtypes, identify genome-driven changes in the cancer proteome and provide information about cancer cell lines that was not encoded in genomic profiles. Implementation of the assays to clinical samples (i.e., tumor tissue) will require overcoming at least two challenges: the limited yield of protein from a biopsy or surgical specimen and the microheterogeneity of cell types encountered in tumor tissue samples. The protein yields from core biopsies range from 80 to 400 μg, which was feasible for quantification of the analytes in this study; however, these yields may be a challenge when lower-abundance analytes are targeted and enrichment is required. Tissue microheterogeneity can be addressed by strict quality control of the input material (for example, tumor cellularity), as has proven to be feasible in the application of gene expression profiles for breast cancer prognosis42.

The portability of MRM assays across laboratories and instrument platforms has been previously demonstrated in smaller studies aimed at a limited number of peptide analytes quantified by MRM-MS7,43,44. In the present study, we substantially extend this work by demonstrating key requirements for a scaled effort, including a substantial increase in the number of assays configured, an unprecedented level of multiplexing analytically validated assays with internal standards (essential for a scaled effort), and successful international transfer of assays. Strict adherence and attention to SOPs enabled high reproducibility, demonstrating the transferability of MRM assays, and thus the potential usefulness of a global MRM assay resource. Our results demonstrate what could be done if various countries were willing to co-fund a scaled human protein quantification project8,9. One approach to realizing this would be to develop analytically robust assays to study groups of proteins based on biological pathways, cellular localization or other logical groupings in an internationally coordinated fashion. Assay panels targeting whole pathways might be constructed for quantitative interrogation.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. ProteomeXchange: LC-MS/MS proteomics data are available under accession number PXD000246.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.J.K., S.E.A., K.K., J.R.W., P.W., Y.K., S.A.C. and A.G.P. conceived of and designed the experiments. J.J.K., S.E.A., K.K., P.W., C. Liu, Y.Z. and X.W. analyzed the data. M.-H.Y., E.G.Y., C. Lee, H.R., Y.K., S.A.C. and A.G.P. contributed reagents, materials and/or analysis tools. J.J.K., J.R.W. and A.G.P. wrote the paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.
1. Lange, V., Picotti, P., Domon, B. & Aebersold, R. Selected reaction monitoring for quantitative proteomics: a tutorial. Mol. Syst. Biol. 4, 222 (2008).
2. Picotti, P. & Aebersold, R. Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions. Nat. Methods 9, 555–566 (2012).
3. Pan, S. et al. Mass spectrometry based targeted protein quantification: methods and applications. J. Proteome Res. 8, 787–797 (2009).
4. Liebler, D.C. & Zimmerman, L.J. Targeted quantitation of proteins by mass spectrometry. Biochemistry 52, 3797–3806 (2013).
5. Hüttelhain, R. et al. Reproducible quantification of cancer-associated proteins in body fluids using targeted proteomics. Sci. Transl. Med. 4, 142ra94 (2012).
6. Rodriguez, H. et al. Reconstructing the pipeline by introducing multiplexed multiple reaction monitoring mass spectrometry for cancer biomarker verification: an NCI-CPTC initiative perspective. Proteomics Clin. Appl. 4, 904–914 (2010).
7. Addona, T.A. et al. A human proteome detection and quantitation project. Mol. Cell. Proteomics 8, 883–886 (2009).
8. Anderson, N.L. et al. A human proteome project: current state and future direction. Mol. Cell. Proteomics 10, M111.009993 (2011).
9. Legrain, P. et al. The human proteome project: current state and future direction. Mol. Cell. Proteomics 10, M111.009993 (2011).
10. Loo, S. et al. Quantitative, multiplexed assays for low abundance proteins in plasma by targeted mass spectrometry and stable isotope dilution. Mol. Cell. Proteomics 6, 2212–2229 (2007).
11. Stahl-Zeng, J. et al. High sensitivity detection of plasma proteins by multiple reaction monitoring of N-glycosites. Mol. Cell. Proteomics 6, 1809–1817 (2007).
12. Halvey, P.J., Ferrone, C.R. & Liebler, D.C. GelC-MRM quantitation of mutant KRAS oncoprotein in complex biological samples. J. Proteome Res. 11, 3908–3913 (2012).
13. Madian, A.G., Rochelle, N.S. & Regnier, F.E. Mass-linked immuno-selective assays in targeted proteomics. Anal. Chem. 85, 737–748 (2013).
14. Whiteaker, J.R. & Paulovich, A.G. Peptide immunoaffinity enrichment coupled with mass spectrometry for peptide and protein quantification. Clin. Lab. Med. 31, 385–396 (2011).
15. Ackermann, B.L. Hybrid immunoaffinity–mass spectrometric methods for efficient protein biomarker verification in pharmaceutical development. Bioanalysis 1, 265–268 (2009).
16. Keating, J. et al. Evaluation of large scale quantitative proteomic assay development using peptide affinity-based mass spectrometry. Mol. Cell. Proteomics 10, M110.005646 (2011).
17. Anderson, N.L. et al. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring mass spectrometry peptide assays. J. Proteome Res. 13, 346–353 (2014).
18. Witt, A.E. et al. Method development for establishing interlaboratory reproducibility of selected reaction monitoring-based mass spectrometry peptide assays. J. Proteome Res. 9, 6678–6688 (2010).
19. Prakash, A. et al. Platform for establishing interlaboratory reproducibility of selected reaction monitoring-based mass spectrometry peptide assays. J. Proteome Res. 9, 3986–3995 (2012).
ONLINE METHODS

Reagents. Urea (ultra grade), iodoacetamide (IAM, ultra grade), dithiothreitol (DTT), Tris(2-carboxyethyl)phosphine (TCEP), EGTA, EDTA, and phosphatase inhibitor cocktails #1 and #2 were obtained from Sigma-Aldrich. Trypsin Gold was purchased from Promega. MS-grade acetonitrile (MeCN) and water (Optima LCMS, #A955 and #W6, respectively) and ethanol (EtOH) were purchased from Thermo Fisher Scientific. Synthetic heavy and light peptides were purchased from Thermo Fisher Scientific, New England Peptide, and 21st Century Biochemicals.

Processing of breast cancer tissues. Surgically resected human breast specimens were collected from patients undergoing treatment for breast cancer or reduction mammoplasty by the Fred Hutchinson Cancer Research Center’s and the University of Washington's Breast Specimen Repository & Registry (BSR), after permission and informed consent was obtained under IRB 5306. Deidentified specimens representing each breast cancer subtype (ER+/HER2−, ER−/HER2+, ER+/HER2+ and ER−/HER2−) were selected from the BSR for this study (breast cancer subtype was not blinded to allow pooling of samples by subtype). Marker determinations were made as part of the routine diagnostic workup of these tumors (for example, IHC for ER and IHC−/ISH for HER2). Tissues were weighed while frozen and immediately transferred to ice-cold PBS to thaw. Once thawed, tissues were blotted to remove excess PBS, quickly chopped, and immediately transferred to ice-cold lysis buffer (25 mM Tris, 6 M urea, 1 mM EDTA, 1 mM EGTA, 1 mM TCEP, 1% Sigma phosphatase inhibitor cocktail 1, 1% Sigma phosphatase inhibitor cocktail 2) at a mass ratio of 1:4 tissue:buffer. Tissues were homogenized in 1.5- or 0.5-mL microcentrifuge vials with disposable pestles, as was appropriate for tissue size. After disruption, samples were incubated in lysis buffer on a roller for 15 min at 4 °C. Samples were then spun at 20,800 g for 10 min at 4 °C, and the liquid phase was removed. To remove any residual debris, we spun lysates again at 20,800 g at 4 °C, and the liquid phase was again removed. Lysates were then divided into aliquots and stored at −80 °C.

Lysates were pooled by mass according to a BCA (Pierce) measurement of lysate protein concentrations. Lysate pools for each tumor subtype were processed in parallel. Briefly, lysates were reduced in 20 mM Tris/20 mM DTT for 30 min at 37 °C with shaking, which was followed by alkylation with 50 mM IAM in the dark at room temperature. Lysates were then diluted 1:10 with 100 mM Tris, pH 8, before trypsin was added at a 1:50 trypsin:protein ratio by mass. After 2 h, a second aliquot was added at 1:100 enzyme:substrate. Digestion was carried out overnight at 37 °C with shaking. After 16 h, the reaction was quenched with formic acid to a final concentration of 1% by volume. Digests were desalted using C-18 cartridges (Waters cat. #WAT094225) with vacuum. The C-18 cartridges were washed with three volumes of 80% MeCN/0.1% formic acid (FA) and then equilibrated with four washes of 0.1% FA. The digest was applied to the C-18 cartridge and then washed with four volumes 0.1% FA before being eluted drop by drop with three washes of 80% MeCN/0.1% FA. The eluate was then divided into aliquots by volume, and digests were lyophilized and then stored at −80 °C until use.

Growth of cell lines. All cell lines were obtained from American Type Culture Collection (ATCC). The cell lines were characterized and authenticated by ATCC using short tandem repeat (STR) DNA profiles. Individual cell lines were cultured as follows. Cells were thawed in a 37 °C water bath. The vials were wiped down with 70% EtOH, the cells were spun at 180g for 8 min at 4 °C, the supernatant was discarded and the cells were resuspended in 10 mL of medium (Supplementary Table 1). The cells were transferred to a 100 mm × 20 mm plate (adherent cells) or T25 flask (suspension cells) and incubated at 37 °C, 5% CO2. The adherent cells were split at 80–90% confluence (cell-line dependent) by removing growth medium by aspiration, adding 2 mL of 0.25% trypsin-EDTA per 100 mm × 20 mm plate or 6 mL of 0.25% trypsin-EDTA per T175 flask, and incubating cells at room temperature with occasional mixing until the cells lifted from the surface as seen under the microscope. Two to four volumes of medium containing serum were added to quench trypsin, and, if needed, a cell lifter was used to remove all cells from culture surface. The cells were pooled, and additional medium was added as needed to obtain the optimal split ratio (cell-line dependent, ranging from 1:2 to 1:6 as conditions warranted). The cells were dispensed into culture containers (100 mm × 20 mm plate for a 10-mL final volume, T75 flask for 12–15 mL and T175 flask for 25–35 mL). The cells were grown, lifted from the surface as above, pooled in a 50-mL Falcon tube, and spun at 180g for 8 min at 4 °C. The supernatant was removed, and the cells were resuspended in freezing medium (90% growth medium + 10% DMSO) and distributed as aliquots into 1.8-mL Cryovials. The vials were placed in a Nalgene freezing container (cat. #5100-001), frozen overnight at −80 °C, and transferred for storage in the vapor phase of a liquid nitrogen tank.

Preparation of protein lysates from cell lines. Individual and pooled cell lysates were prepared as needed. Cells were transferred to precooled 50-mL tubes and were spun at 180g for 8 min at 4 °C, and the supernatant was discarded. Cells from the same cell line were resuspended and pooled in 10 mL of ice-cold DPBS, with 50 μL removed for cell counting by hemocytometer. Cells were washed twice by adding ice-cold DPBS to 50 mL, spinning cells at 180g for 8 min at 4 °C and discarding the supernatant. Lysis buffer was added to a final concentration of 0.5 × 108 cells per mL on ice, and the cell suspension was sonicated twice for 10 s (550 Sonic Dismembrator, Fisher Scientific; knob set to 5). The lysates were transferred by pipette tip to microcentrifuge tubes and vortexed twice for 15 s, with a 10-min rest on ice in between. The lysates were centrifuged at 20,000g for 10 min at 4 °C and the supernatants transferred to 1.0-mL CryoTubes (Nunc cat. #377267) and stored in liquid N2. Lysate protein concentration was determined by BCA. For discovery profiling, lysates were pooled by protein mass according to their molecular subtype (ER+/HER2−, ER−/HER2+, ER+/HER2+ and ER−/HER2−). The lysates were reduced in 100 mM Tris/20 mM TCEP for 30 min at 37 °C with shaking, which was followed by alkylation with 50 mM iodoacetamide in the dark at room temperature. Lysates were then diluted 1:10 with 100 mM Tris, pH 8, before trypsin was added at a 1:50 trypsin:protein ratio by mass. After 2 h, a second aliquot was added at 1:100 enzyme:substrate. Digestion was carried out overnight at 37 °C with shaking. After 16 h, the reaction was quenched with formic acid to a final concentration of 1% by volume. Digests were desalted using C-18 cartridges (Waters cat. #WAT094225) with vacuum. The C-18 cartridges were washed with three volumes of 80% MeCN/0.1% formic acid (FA) and then equilibrated with four washes of 0.1% FA. The digest was applied to the C-18 cartridge and then washed with four volumes 0.1% FA before being eluted drop by drop with three washes of 80% MeCN/0.1% FA. The eluate was then divided into aliquots by volume, and digests were lyophilized and then stored at −80 °C until use.

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Empirical identification of targets for MRM assay development. Breast cancer samples (tissue and cell-line lysates) representing four molecular subtypes based on ER and HER2 status (Supplementary Table 1) were analyzed by shotgun LC-MS/MS. Four pooled tissue samples (one from each of the four subtypes) and ten pooled cell line samples (two pools each from the four subtypes and two non-tumor ‘normal’) were analyzed by 1D and 2D LC-MS/MS. Results from the 2D LC-MS/MS analysis were used to measure the relative abundances of the proteins in the various subtypes and to distinguish those that were differentially expressed. From this list of candidates, proteins with two proteotypic peptides that were observed by 1D LC-MS/MS (considered to be at a sufficient intensity to be seen reliably in MRM) and observed in the corresponding pooled tissue sample were prioritized for assay development.

Two analytical systems were used for analysis of the tissue and cell-line lysates. For the first system, the LC system consisted of a nanoAcquity HPLC (Waters) with high-pH mobile phases of 20 mM ammonium formate at pH 10 in water (A1) and 100% acetonitrile (B1) and low-pH mobile phases of 0.1% formic acid in water (A2) and 0.1% formic acid in acetonitrile (B2). For the 2D LC-MS/MS analyses, 10 µg of protein digest was injected at high pH onto a 300 µm × 50 mm XBridge C18, 130-Å, 5-µm column (Waters cat. #186003682). A step gradient was used to elute the sample off of the high-pH reverse-phase column in six distinct fractions using 11.1, 14.5, 17.4, 20.8, 45.0 and 65.0% B. Fractions were eluted from the high-pH column into 20 µL/min of low pH (A2) and onto a 180 µm × 20 mm C18, 100-Å, 5-µm column (Waters cat. #186006527) by the following method: hold 3% B for 0.5 min, gradient from 3% to step% B for 0.5 min, hold step% B for 4 min, gradient from step% B for 0.5 min, re-equilibrate at 3% B for 15 min. The flow rate was 2 µL/min. 2D LC-MS/MS samples eluted from the trap column and separated by a 100 µm × 100 mm C18, 130-Å, 1.7-µm column (Waters cat. #186003546) by the following method: gradient from 3% to 40% B for 120 min, gradient from 40% to 90% B for 2 min, hold 90% B for 10 min, re-equilibrate at 3% B for 20 min. The flow rate was 1,000 nL/min. 1D LC-MS/MS analyses were carried out as described without a trap column, using direct injection of 2 µg of protein digest. The HPLC was coupled to an LTQ-Orbitrap Xevo hybrid mass spectrometer using an Advance CaptiveSpray source (Michrom Bioresources) operated in positive ion mode. A spray voltage of 1,700 V was applied to the nanospray tip. MS/MS analysis consisted of one full-scan MS from 300 to 2,000 m/z at resolution 30,000 followed by 15 data-dependent MS/MS scans. Dynamic exclusion parameters included repeat count 1, exclusion list size 500, and exclusion duration 15 s. Five replicate injections were performed.

Data were searched against version 3.69 of the Human International Protein Index (IPI) sequence database with decoy sequences using Spectrum Mill, OMSQA, and the X!Tandem database search engine with a previously described score plugin. All searches were performed with tryptic enzyme constraint set for up to two missed cleavages, oxidized methionine set as a variable modification and carbamidomethylated cysteine set as a static modification. For X!Tandem, peptide MH+ mass tolerances were set at ±2.0 Da with post-search filtering of precursor mass to 50 p.p.m., and fragment MH+ mass tolerances were set at ±0.5 Da. For OMSQA, peptide MH+ mass tolerances were set at ±2.0 Da, and fragment MH+ mass tolerances were set at ±0.5 Da. For Spectrum Mill, peptide MH+ mass tolerances were set at ±2.0 p.p.m., and fragment MH+ mass tolerances were set at ±0.7 Da. Identification from three search engines were made with an FDR (false discovery rate) < 0.005 on the basis of a decoy database search. The LC-MS/MS proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org/) via the PRIDE partner repository with the data set identifier PXD000246. The intensity of each peptide in each subtype (including normal) was measured by two methods (MS1 and MS2). MS1 measures the precursor-ion intensity for each acquisition (reported by SpectrumMill), and MS2 measures the sum of intensities of matched fragment ions for each acquisition. The intensity values were normalized by the median values of each individual acquisition and then log2 transformed. For each peptide, the reported intensity value for a given subtype was the maximum value between the two pools and between the MS1 and MS2 measurements.

Results from the 2D LC-MS/MS analysis (wherein the method is more sensitive to detection of the analytes) were used to measure the relative abundances of the proteins in the various subtypes and to distinguish those that were differentially expressed. Absolute differential expression of a peptide was measured by the ratio of the highest intensity of the samples from a given subtype to the highest intensity of the samples from a comparator subtype (for example, ER+ vs. ER−). Relative differential expression was measured by a differential score (DScore), which took the ratio and normalized it to the sum of the s.d. of all intensities associated with the two comparator subtypes. Proteins were ranked according to the weighted average of the ratio and DScore of the individual peptides from that protein. From this list of candidates, proteins with two proteotypic peptides that were observed by 1D LC-MS/MS (considered to be at a sufficient intensity to be seen reliably in MRM) and observed in the corresponding pooled tissue sample (maximizing the value of the assay as a resource to
the community as well as providing a suite of validated assays to be used to develop approaches for dealing with the difficulties of tissue-based analysis) were prioritized for assay development.

**Qualification of potential targets on a triple quadrupole platform.** The detectability of prioritized targets was verified on a triple quadrupole (QQQ) system. Proteotypic peptides and suitable transitions could be optimized directly from the discovery libraries, but to ensure that we could successfully develop an MRM assay, we confirmed detection using a QQQ mass spectrometer before final peptide selection. Each candidate peptide was qualified by detection of endogenous analyte in a pool of all cell-line lysates used in the profiling experiments using MRM (4000 or 5500 QTRAP (Sciex)). Peptides were filtered to include only those that were proteotypic, had a hydrophobicity calculated by SSRCalc between 8 and 57, did not contain methionine, and did not contain N-terminal cysteine or glutamine. For peptides meeting these criteria, the ten most intense transitions were selected from a consensus spectral library of all identifications and exported into a scheduled MRM method on the basis of retention-time prediction using SSRCalc 3.0 (100A). The exported transition list was separated into multiple instrument methods so that there were no more than 1,000 transitions per MRM analysis.

Verification of the MRM detectability of the endogenous peptide signals was performed using an Eksigent nanoLC-Ultra 2Dplus (Eksigent Technologies) coupled to a 5500 QTRAP mass spectrometer (AB Sciex). Mobile phases consisted of 0.1% formic acid in water (A) and 90% acetonitrile with 0.1% formic acid (B). The pool of cell-line lysates (1 µg) was loaded onto a 0.2 × 5 mm Chromolith CapRod RP-18e column (EMD Chemicals) for 3 min at 10 µL/min with 5% mobile phase B. The peptides were then be separated by a 0.1 × 150 mm Chromolith CapRod RP-18e column (EMD Chemicals) by the following gradient method: hold 5% B for 9 min, gradient from 5% to 40% B for 100 min, gradient from 40% to 90% B for 1 min, hold 90% B for 5 min, re-equilibrate at 5% B for 14 min. The flow rate was 1,000 nL/min. The trap column was back flushed during the last 5 min of an acquisition using 5% B at 10 µL/min. The source employed was an Advance CaptiveSpray source (Michrom Bioresources). The MS was used in positive-ion mode with parameters consisting of a 1,200 V ion spray voltage, curtain gas setting of 10, nebulizer gas setting of 0, and an interface heater temperature of 110 °C. CE was set by Skyline, DP was set to 100, EP was set to 10, CXP was set to 10 and Q1 and Q3 set to unit/unit resolution (0.7 Da). Throughout the method, the actual cycle time remained at or below 2 s, allowing for measurement of at least ten points across the peaks.

**Quantitative MRM assay development and characterization by response curves.** Heavy stable isotope–labeled standards (SIS) and matched light versions were synthesized and purified to >95% purity by HPLC. Heavy peptides incorporated a fully atom labeled \(^{13}\text{C}\) and \(^{15}\text{N}\) isotope at the C-terminal lysine (K) or arginine (R) position of each (tryptic) peptide, resulting in a mass shift of +8 or +10 Da, respectively. Peptides were quantified by amino acid analysis, and aliquots were stored in 30% acetonitrile/0.1% formic acid at −80 °C until use. Each synthetic peptide was analyzed by MS/MS on an LTQ mass spectrometer using infusion with an Advion TriVersa interface. The spectral library from these analyses was used to select transitions for optimization. Optimal collision energy for a hybrid triple quadrupole/linear ion trap mass spectrometer (5500 QTRAP) for each of the peptides was determined by injecting 50 fmol of standard peptide solutions into a flow of 30% acetonitrile, 0.1% formic acid at a flow rate of 1 µL/min. Optimal values were determined by ramping the potentials and evaluating the results through DiscoveryQuant (Sciex). The top three transitions were selected for method development on the basis of the presence of abundant y ions at \(m/z\) greater than that of the precursor. In the absence of high-\(m/z\) y ions, the most abundant fragment ions were selected.

The analytical performance of the biomarker candidate assays was characterized via generation of a response curve in a background matrix consisting of an equal mix (by protein mass) of all of the cell lines. Because the concentration of a number of analytes was expected to exceed the practical linear range of the assay, response curves were generated in pooled lysate and a 1:10
Deployment of analytically validated MRM assays on a panel of cell lines. Digestion of the individual cell lines at two concentrations (1.0 µg/µL and 0.1 µg/µL) was performed in an automated fashion as described above. After digestion, a mix of SIS peptides was spiked into the individual cell lysates at the concentrations shown in Supplementary Table 4. The ‘heavy’ peptides were spiked at one of three possible concentrations, depending on the LLOQ of the peptide MRM assay as well as the expected endogenous signal. Spike-in levels were high enough above the LLOQ so as not to contribute unnecessarily to the assay CV and were designed to be within two orders of magnitude of expected endogenous levels. Three complete process replicates were prepared and analyzed for the 30 individual cell lines. As with the response curves, retention times on the LC platform were empirically determined using a mixture of the standard stable isotope-labeled synthetic peptides in a nonscheduled fashion. Once the retention times were known, scheduled MRMs were set up using a 150-s MRM detection window and a target cycle time of 1.5 s. The quantitative LC-MRM-MS analysis was performed using the method described above. MRM data were processed using Skyline. All data were manually inspected to ensure correct peak detection, absence of interferences, and accurate integration. Reported peak areas are the sum of the peak area and background area reported by Skyline. Peak specificity between the light (or endogenous) and heavy (or SIS) MRM signal was defined as the detection of at least one transition from the endogenous peptide exactly coeluting with at least two transitions from the stable isotope-labeled peptide, with the relative intensity of the light transition(s) deviating no more than 20% compared to the relative intensity of the corresponding heavy transitions. Peptide concentrations are reported from the results of one quantifying transition, defined as the transition with the lowest LLOQ with no interferences for a given peptide. When a working assay had two or more transitions that met these criteria, the quantifying transition was defined as the transition with the lowest CV at the LLOQ. Working assays were described as ‘informative’ if they were sufficiently sensitive to precisely quantify the endogenous analyte in at least one cell line. Endogenous levels were calculated by integrating peaks from the heavy and light signals and measuring the peak area ratio against the isotope-labeled analog, which was spiked at a known concentration in the lysates. Peak integration was performed by Skyline, and the integrations were manually checked. For the individual data sets, 8% of the integrations were changed after manual investigation. Integration results were exported to the program R for linear regression and statistical analysis.

Cell lysate samples were analyzed in complete process triplicate (including digestion) at two cell lysate protein concentrations (1.0 µg/µL and 0.1 µg/µL). All reported results are from the 1.0 µg/µL data set unless the measurements were above the ULOQ or were missing. In those cases, the results from the 0.1 µg/µL data set were normalized to the lysate protein amount and reported in place of the 1.0 µg/µL results. Endogenous levels that were reported at 0.1 µg/µL, which make up 4.2% of the reported measurements, are marked with an asterisk in Supplementary Table 4.

Results from the peptide quantification were used to report protein concentrations, assuming 100% recovery of the peptides. From the results of the two peptides associated with a given protein, the median values of the triplicate measurements were considered. Concentrations of 0.5 × LLOQ or 2 × ULOQ were imputed for missing values below LLOQ and above ULOQ, respectively. For a given protein, the peptide with the least number of missing values among the 30 cell lines was used to calculate protein concentration. If multiple peptides had the same number of missing values, the most intense peptide was used. Clustering was performed with these protein concentrations using the R function “heatmap” with complete linkage. All MRM data (response curves and cell line measurements) are available at https://proteomics.fhrc.org/CPAS/project/Published%20Experiments/International%20MRR%20Assays/begin.view?

Cellular compartment and biological process characterization of proteins included in the study. A total of 5,416 Gene Ontology (GO) annotations were obtained for the 319 proteins in the dilution of the pooled lysate (1.0 µg/µL and 0.1 µg/µL background matrix). Digestion was performed in an automated fashion as described above using an Eppendorf epMotion 5075 automated pipetting system. A reverse curve was prepared in which the SIS peptide concentration was varied over eight concentration points using threefold serial dilutions over the range 20–0.091 nM. Light peptide was also spiked into the cell lysate pool at 5 nM to ensure that the heavy-to-light peak areas were within two orders of magnitude. Blanks and double blanks were prepared and analyzed in addition to the concentration points of the curve. Three process replicates were prepared and analyzed at the eight concentration points (along with blank and double-blank samples). Retention times on the LC platform were empirically determined using a mixture of the standard stable isotope-labeled synthetic peptides in a nonscheduled fashion. Once the retention times were known, scheduled MRMs were set up using a 150-s MRM detection window and a target cycle time of 1.5 s. The quantitative LC-MRM-MS analysis was performed using the method described above. MRM data were processed using Skyline. All data were manually inspected to ensure correct peak detection, absence of interferences, and accurate integration. Reported peak areas are the sum of the peak area and background area reported by Skyline. Peak specificity between the light (or endogenous) and heavy (or SIS) MRM signal was defined as the detection of at least one transition from the endogenous peptide exactly coeluting with at least two transitions from the stable isotope-labeled peptide, with the relative intensity of the light transition(s) deviating no more than 20% compared to the relative intensity of the corresponding heavy transitions. Peptide concentrations are reported from the results of one quantifying transition, defined as the transition with the lowest LLOQ with no interferences for a given peptide. When a working assay had two or more transitions that met these criteria, the quantifying transition was defined as the transition with the lowest CV at the LLOQ. Working assays were described as ‘informative’ if they were sufficiently sensitive to precisely quantify the endogenous analyte in at least one cell line. Endogenous levels were calculated by integrating peaks from the heavy and light signals and measuring the peak area ratio against the isotope-labeled analog, which was spiked at a known concentration in the lysates. Peak integration was performed by Skyline, and the integrations were manually checked. For the individual data sets, 8% of the integrations were changed after manual investigation. Integration results were exported to the program R for linear regression and statistical analysis.

Cell lysate samples were analyzed in complete process triplicate (including digestion) at two cell lysate protein concentrations (1.0 µg/µL and 0.1 µg/µL). All reported results are from the 1.0 µg/µL data set unless the measurements were above the ULOQ or were missing. In those cases, the results from the 0.1 µg/µL data set were normalized to the lysate protein amount and reported in place of the 1.0 µg/µL results. Endogenous levels that were reported at 0.1 µg/µL, which make up 4.2% of the reported measurements, are marked with an asterisk in Supplementary Table 4.

Results from the peptide quantification were used to report protein concentrations, assuming 100% recovery of the peptides. From the results of the two peptides associated with a given protein, the median values of the triplicate measurements were considered. Concentrations of 0.5 × LLOQ or 2 × ULOQ were imputed for missing values below LLOQ and above ULOQ, respectively. For a given protein, the peptide with the least number of missing values among the 30 cell lines was used to calculate protein concentration. If multiple peptides had the same number of missing values, the most intense peptide was used. Clustering was performed with these protein concentrations using the R function “heatmap” with complete linkage. All MRM data (response curves and cell line measurements) are available at https://proteomics.fhrc.org/CPAS/project/Published%20Experiments/International%20MRR%20Assays/begin.view?

Cellular compartment and biological process characterization of proteins included in the study. A total of 5,416 Gene Ontology (GO) annotations were obtained for the 319 proteins in
Proteogenomic integration. DNA copy-number data and RNA expression data were obtained from Neve et al. Gene expression arrays were available for 232 of the 319 proteins and 28 of the 30 cell lines targeted in this study. Differential expression among the molecular subtypes of breast cancer was determined by calculating the \( P \) value and FDR. The \( P \) value reported is from the nonparametric Wilcoxon rank-sum test based on the \( Z \) statistic. The FDR was calculated using R package “qvalue” to control the familywise error due to multiple hypothesis testing.

Both DNA copy-number and mRNA expression data were available for 90 of the 319 proteins and 27 of the 30 cell lines targeted in this study. Global normalization was performed for both raw CGH array and expression array data, and copy-number amplifications and deletions were inferred using R package “cghFlasso”. Then, for each gene, the average of the pairwise Spearman’s rank correlations among its DNA copy number, RNA expression, and protein expression across the 27 cell lines was calculated. To assess the significance of these average correlation scores, we performed permutation testing (10,000 permutations), in which the null distributions were estimated through permuting the sample orders in the RNA expression data set and protein expression data set.

Survival analysis. The van ’t Veer et al. data set was downloaded from the Stanford University public repository. The data set contains gene expression arrays, molecular subtype, and patient clinical outcome information for 244 breast cancer tumors. We normalized the raw data for each array to have median 0 and MAD (median absolute deviation) 1. To investigate the prognostic property of the candidate genes, we first partitioned the 244 samples into two groups by using the median gene expression level of each gene, and performed a log-rank test to compare the KM curves of the two patient groups. For the two genes giving significant \( P \) values in the log-rank test, we further fit a multivariate Cox proportional hazard model (R function “coxph”) to assess the association between gene expression and the survival outcome, accounting for the molecular subtype, age, tumor size, lymph node status, and whether the patient has received chemotherapy. Because two independent data sets were used in the Cox analysis, we applied a \( P \) value cutoff of 0.1, declaring a gene to be significantly associated with survival if its \( P \) values from both data sets were below 0.1.

The Loi et al. data set, including gene expression data and clinical information for 414 breast cancer tumors, was obtained from http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE6532. After the global normalization was performed, R package “genefu” (version 1.8.0) was used to determine the molecular subtypes of the breast cancer patients in the Loi et al. data set. As described above for the van ’t Veer et al. data set, a log-rank test and Cox proportional hazard model were performed.

Public access to mass spectrometry data. The LC-MS/MS proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org/) via the PRIDE partner repository with the data set identifier PXD000246. All MRM data (response curves and cell line measurements) are available at https://proteomics.fhcrc.org/CPAS/project/Published%20Experiments/International%20MRM%20Assays/begin.view?

45. MacLean, B., Eng, J.K., Beavis, R.C. & McIntosh, M. General framework for developing and evaluating database scoring algorithms using the TANDEM search engine. Bioinformatics 22, 2830–2832 (2006).
46. Vizcaíno, J.A. et al. The PRoteomics IDENTifications (PRIDE) database and associated tools: status in 2013. Nucleic Acids Res. 41, D1063–D1069 (2013).
47. Griffin, N.M. et al. Label-free, normalized quantification of complex mass spectrometry data for proteomic analysis. Nat. Biotechnol. 28, 83–89 (2010).
48. Krokhin, O.V. Sequence-specific retention calculator. Algorithm for peptide retention prediction in ion-pair RP-HPLC: application to 300- and 100-Å pore size C18 sorbents. Anal. Chem. 78, 7785–7795 (2006).
49. Storey, J.D. A direct approach to false discovery rates. J. R. Stat. Soc. Series B Stat. Methodol. 64, 479–498 (2002).
50. Tibshirani, R. & Wang, P. Spatial smoothing and hot spot detection for CGH data using the fused lasso. Biostatistics 9, 18–29 (2008).