Demonstration of the feasibility of an international effort to develop and distribute analytically validated Multiple Reaction Monitoring (MRM)-based assays to large suites of human proteins

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ABSTRACT

The successful application of MRM in biological specimens raises the exciting possibility that assays can be configured to measure all human proteins, resulting in an assay resource that would promote advances in biomedical research. We report the results of a pilot study designed to test the feasibility of a large-scale, international effort in MRM assay generation. Across three performance sites, 645 novel MRM assays representing 319 proteins expressed in human breast cancer were configured, validated, and made publicly available as a resource for the community. Assays were multiplexed in groups of >150 peptides and deployed to quantify endogenous analyte in a panel of breast cancer-related cell lines. Median assay precision was 5.4%, with high inter-laboratory correlation (R^2 >0.96). Peptide measurements in breast cancer cell lines were able to discriminate amongst molecular subtypes and identify genome-driven changes in the cancer proteome. These results establish the feasibility of a scaled, international effort.

INTRODUCTION

Rapid advances in technology have enabled extraordinarily deep proteomic coverage[1, 2]. This deep coverage comes at the expense of throughput, due to extensive sample processing requirements. Thus, for interesting discovery proteomic leads to be actionable, investigators must be able to verify the results in larger clinical or biological studies[3], requiring targeted methods of analysis enabling higher throughput. Unfortunately, conventional technologies (e.g. ELISA, IHC, Western blotting) are low in throughput, unable to avoid nonspecific interferences, not routinely multiplexed, not quantitative (aside from ELISA), and do not use internal standards (and thus are not readily standardized across laboratories)[4]. Thus proteomics currently lacks critical tools required for success.

Multiple Reaction Monitoring (MRM) Mass Spectrometry (MS) is positioning itself to dramatically improve quantitative proteomics. MRM-MS is an assay platform used for decades in clinical reference laboratories to quantify small molecules[5] (e.g. metabolites in newborn screening) and is being rapidly taken-up by the biology and clinical research communities for quantifying peptides released via proteolysis of biospecimens[6, 7]. MRM-MS was recently selected as the "method of the year" by *Nature Methods*[8], given its potential to promote rapid advances in protein-based research, potentially replacing Western blotting and providing the critical missing link between discovery proteomics and downstream implementation of proteomic findings[9, 10].

MRM-MS is a targeted technique that is completely different from the mass spectrometry approaches widely used in discovery proteomics. MRM is performed on specialized instruments that enable targeting of specific analyte peptides of interest and provides exquisite specificity and sensitivity[11-14]. Background interferences can be detected and avoided, and the use of spiked-in, stable isotope-labeled standards enables precise relative quantification of endogenous analytes in commonly used biospecimens[15]. The National Cancer Institute has invested heavily in the standardization and analytical validation of MRM-based quantification of peptides through its Clinical Proteomic Tumor Analysis Consortium (CPTAC)[16], which has demonstrated robust analytical performance for MRM analyses across laboratories and instrument platforms[17].

For the MRM-based assay technology to meet its potential to promote rapid advances in protein-based biomedical research, the ability to run 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 run in individual laboratories or readily implemented in proteomic core facilities. Towards this end, global assay development projects have been proposed[18-21], and peptide spectral databases[22, 23] (e.g. http://www.srmatlas.org) as well as open-source, vendor-neutral software tools[24-28] (https://panoramaweb.org) are being rapidly developed to support such efforts.

The purpose of this study was to test the feasibility and usefulness of a large-scale, international collaborative effort in MRM-MS assay generation targeting the human proteome, modeling what a global assay development effort might look like. Our approach was to develop a panel of 645 MRM assays covering 319 proteins (~1.5% of the basic human proteome) differentially expressed amongst human breast cancer subtypes from start to finish (i.e. including reagent generation, assay development, analytical validation, assay deployment on biospecimens, and distribution of data and SOPs as a community resource) using state-of-the-art technology and multiplexing capabilities. The results demonstrate feasibility of an international, scaled 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, 3 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[29-31] and because a panel of highly characterized breast cancer cell lines[32-34] was readily available for the study. Although we focused on breast cancer (and on cell lysates) to provide a framework for this pilot, the assays we developed are limited neither to application in cell lysates nor to breast cancer; they are generalizable.

To generate an empirical dataset for selection of target analytes for MRM assay development, unfractionated protein lysates derived from a panel of human breast cancers and breast cancer-derived cell lines (**Supplementary Table 1**) were analyzed by shotgun LC-MS/MS analysis. Over 64,000 unique peptides (representing 9,996 proteins) were identified at a peptide FDR < 0.005 in the combined cell line and tissue data. To enrich for targets that might vary in expression level amongst breast cancer subtypes and thus be of biological interest, potential MRM targets were rank-ordered by differences in their signal intensities amongst the breast cancer subtypes represented in the cell line panel, and identification was required in both the cell

lysate and corresponding cancer tissue. Finally, a target list for MRM assay development was constructed from the filtered list of peptides that were detectable from neat cellular lysate by MRM on a triple quadrupole mass spectrometer. From this rank-ordered list, a set of 318 proteins (represented by 642 proteotypic peptides) were selected for assay development (**Supplementary Table 2**). These proteins were shown to be enriched for breast cancer-specific targets as 73 of these 318 proteins (23%) were also included in a list of 1000 genes of potential functional importance in breast cancer [35]. Although not observed by shotgun LC-MS/MS analysis, 3 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, as shown in **Supplementary Figure 1**.

Development and characterization of multiplexed assays

For each analyte, synthetic light and heavy stable isotope-labeled peptides were prepared, and optimum MRM transitions and instrument parameters were determined as described in the online methods. The 645 individual peptide assays (**Supplementary Table 2**) were distributed randomly amongst 4 multiplex assay groups (each containing between 156 and 169 peptides). To avoid any bias for performance amongst assay groups, we ensured that each multiplex group contained an equivalent distribution of analyte intensities and retention times (**Supplementary Figure 2**) in addition to LLOQs and CVs (see below and **Supplementary Figure 3**). One of the multiplex assays was randomly chosen to run at all 3 performance sites (the "inter-laboratory" assay), whereas each of the remaining 3 multiplex assays were run at only 1 performance site (the "site-specific" assays).

The analytical performance of the assays was evaluated 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 inter-laboratory assays = 969 total) x 2 matrix dilutions]. All response curves are displayed in Supplementary Appendix A, and assay figures of merit are reported in **Supplementary Table 3**. The majority of assays featured a linear range >3 orders of magnitude. The median assay LLOQs for the inter-laboratory assay group were 0.40, 0.61 and 0.52 fmol/ug (at a cell lysate matrix protein concentration of 1.0 ug/uL), with median CVs of 3.5%, 5.0% and 4.4% for sites 1, 2, and 3, respectively. At this concentration, the site-specific assay groups had median assay LLOQs of 0.37, 0.65 and 0.40 fmol/ug, 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 ≥ 1 transition of the light and ≥ 2 transitions of the heavy peptide and perfect co-elution of heavy and light peptides). Of the 645 assays attempted, 622 (96%) met these criteria and were considered to be successful. Furthermore, 599 (93%) had ≥ 2 transitions and 534 (83%) had all three transitions meeting these criteria.

Deployment of the assays in complete process triplicate to evaluate a panel of 30 human cell lines related to breast cancer

Next, we determined the robustness of the assays when deployed in a common biological setting, characterizing 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 (**Figure 1**). Each lysate was digested in triplicate, so assay variability incorporates the complete processing variability.

A total of 174,420 individual assays were run [(483 site-specific assays + 486 inter-laboratory assays = 969 total) x 30 cell lines x triplicate process replicates x 2 dilutions]. An assay was considered to be informative if the empirically determined concentration of the analyte was above the assay LLOQ (i.e. indicates sufficient sensitivity); 93% (897 of 969) of the assays attempted met these criteria. Endogenous analyte was measured for all 319 proteins. At the individual peptide level, 609 out of 645 peptides (94%) were detected in at least one cell line and 547/645 (85%) were measured in at least half of the cell lines. The empirically determined endogenous levels for each measurement above the analyte LLOQ are plotted in **Supplementary Figure 4**. Transitions measured for each analyte with no interference in any cell line are reported in **Supplementary Table 4**, and all peptide concentration measurements are reported in **Supplementary Table 5.** 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 Figure 5**).

To evaluate precision, the CV across the complete process triplicates was calculated for all endogenous measurements above the LLOQ (**Supplementary Table 6** and **Supplementary Figure 4**). The distributions of CVs for all measurements across the three sites are shown in **Figure 2.** At the three sites, the median assay CVs for the inter-laboratory assay group were 5.0%, 7.3% and 5.1%, with 95% of the results having CVs less than 15%, 25% and 17% for sites 1, 2, and 3, respectively (**Figure 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 (**Figure 2b**). The median CV for all measurements was 5.4%.

The empirically determined endogenous concentration of all analytes constituting the inter-laboratory 152-plex assay, which was run at all 3 laboratories, was used to determine the correlation and agreement across the performance sites. Those measurements that were above the LLOQ at ≥2 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 (**Figure 2c**). There was also excellent agreement in the results amongst the sites, as demonstrated by the slopes from the linear regression of the correlation plots, which ranged from 0.95 to 1.07. To further examine the agreement, a histogram of the percent difference between site measurements is shown in **Figure 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 the known molecular subtypes of breast cancer

The empirically determined MRM-based measurements of 319 proteins were used for hierarchical clustering of the 30 cell lines. As shown in Supplementary **Figure 6**, the cells lines formed 2 major clusters. Of note, the clusters based on the MRM data exactly match the clustering results previously observed for these cell lines using mRNA levels[32-34] (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 provide information not encoded in gene expression profiles

We next asked whether the MRM data revealed any novel information about breast cancer that could not be determined using the genomic profiles of the cell lines. First, to identify proteins that are differentially expressed amongst the molecular subtypes of breast cancer, a Wilcoxon rank test was performed using the MRM dataset. When the false positive rate[36] (FDR) was controlled at 0.01, 4 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 if similar association patterns for this set of proteins can be observed based on their gene expression (mRNA) data (or if the proteomic data provided novel information), we made use of the genomic data of Neve *et al.* (2006)[33], which contains 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 sets of genes showing subtype-association at the mRNA (p-value ≤ 0.01) and the proteomic (using Wilcoxon rank test, FDR cutoff ≤ 0.01) level illustrates that candidate markers could be identified using the MRM/proteomic data that were not detected based on RNA expression profiles. Two, 7 and 11 genes showed RNA expression levels significantly associated with Her2, ER and Basal/Luminal status, respectively, and did not show the same association patterns in their protein abundances, while 0, 44, and 57 genes showed protein abundances significantly associated 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 complementary information to genomic data. **Figure 3 (a-c)** illustrates the associations based on RNA and protein measurements for the significant gene sets. To further demonstrate the complementary information that protein profiling provides, we focused on the 71 genes 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 genes, 28 are believed to be functionally important in breast cancer, based on their inclusion in an independently curated set of 1000 human proteins of relevance to human breast cancer[35]. 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. The number of significant genes associated with the different subtypes, as well as

their association with breast cancer, is illustrated in **Supplementary Figure 7**. Detailed results for all of the 232 genes can be found in **Supplementary Table 7.**

Integrative genomics/proteomics analysis helps to pinpoint 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[37-39]. 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 datasets 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 interpretation of genomic profiles by identifying changes that are ultimately expressed in the proteome, closer to the clinical phenotype.

We performed an integrative analysis and identified 31 genes that show significant correlation (Bonferroni adjusted p-value ≤ 0.0001) between the genomic[33] (i.e. DNA copy number and mRNA expression) and proteomic (MRM) data. Furthermore, amongst the 4 proteins associated with Her2 status (**Supplementary Table 7)**, 2 have DNA copy number and gene expression information available, and both proteins (ERBB2 and GRB7) show significant concordance between genomic and proteomic signatures. Amongst the 118 proteins associated with basal/luminal status, 30 have corresponding genomic data, and only 10 (ABAT, ANXA1, PLOD3, CDKN2A, ERBB2, GALK1, CLTC, PRDX3, ALDOA and DPYSL2) show significant concordance scores. Amongst 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. The genomic and proteomics signatures of these 11 unique genes/proteins (ERBB2, GRB7, CLTC, PRDX3, ANXA1, ABAT, PLOD3, CDKN2A, GALK1, ALDOA and DPYSL2) are illustrated in **Figure 4**, and detailed results of the concordance analysis are provided in **Supplementary Table 8**. Proteins whose expression is primarily regulated by gene expression showed agreement of measured protein levels to mRNA levels. 14 genes were identified with protein levels significantly correlated with its own gene expression (correlation > 0.7 and the Bonferroni adjusted p-value < 0.01). In other words, we can view this as a subset of genes whose protein expressions are primarily regulated by RNA expression. Of these, 2, 7, and 3 genes have protein abundances 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. There were no genes measured showing RNA expression levels significantly associated with Her2, ER and Basal/Luminal status which did not also have significantly associated protein abundances. Based on the above result, 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 Her2/ERBB2 locus (which also contains GRB7) in breast cancer is well established[40], the clinical relevance of the other 9 genes 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 2 independent breast cancer datasets (referred to as van 't Veer *et al.*[41] and Loi *et al.*[42] datasets, respectively) 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 9 candidate genes, significant differences between Kaplan–Meier (KM) survival curves of the 2 patient groups were observed in both datasets for CLTC, DPYSL2 and ABAT (**Figure 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 (PAM50)[43], age, tumor size, lymph node status, and other clinical covariates (**Supplementary Table 9**). Again, CLTC and DPYSL2 were found to be significantly associated with survival outcome (p-value$_{CLTC}$=0.029, 0.068 and p-value$_{DPYSL2}$=0.067, 0.0048 in the 2 clinical datasets, respectively). ABAT showed evidence of association with survival outcome in the Loi *et al.* dataset (pvalue$_{ABAT}$=0.012), but not in the van 't Veer *et al.* dataset. 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

Targeted proteomic assays covering the entire human proteome would alter the state of clinical and biomedical research, promoting rapid advances in protein-based biomedical research by allowing for better translation of basic findings into actionable results. To be useful, such assays must be easily implemented anywhere, with minimal adjustments, while maintaining a high level of performance.

All MRM assays developed in this study, including standard operating protocols (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 this study are readily implementable in such facilities using the SOPs and Skyline files provided in the supplemental materials. Furthermore, the cell lysate sample preparation is straightforward, does not require specialized equipment or expertise, and thus can be easily implemented in any modern biology laboratory. Although we have focused on breast cancer (and on cell lysates) to provide a framework for this study, the assays we develop are limited neither to application in cell lysates nor to breast cancer; they are generalizable.

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-MS [17, 44, 45]. In the present study, we substantially extend the 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 the assays to be highly reproducible, demonstrating international transferability of MRM assays, and thus the potential usefulness of a global MRM assay resource to the international community.

Of great interest and use to clinically-driven research, 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. This demonstrates that panels of MRM assays can effectively contribute to biological characterization of molecular subtypes of cancer. 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 micrograms, making it 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 (e.g. tumor cellularity), as has proven to be feasible in the application of gene expression profiles for breast cancer prognosis[46].

Together, the results of this study demonstrate the feasibility and usefulness of an international effort to develop, analytically validate, and distribute MRM-based assays to large suites of human proteins and demonstrates what could be done if various countries were willing to co-fund a scaled human protein quantification project[18, 19]. One approach to realizing this potential is to develop analytically robust assays to 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 of biology.

This study targeted proteins accessible for MRM-based quantification using a very simple sample preparation protocol for generating cellular protein lysate, without biochemical fractionation or enrichment of the target analytes prior to 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 (i.e. without enrichment or fractionation), it is reasonable to estimate that several thousand human proteins might be quantified from cell line lysates by MRM alone (i.e. without enrichment). Note that this number is highly context-dependent. For example, although thousands of proteins may be quantifiable in cell lysates without enrichment, in a more challenging matrix (e.g. blood plasma) that number is in the hundreds. In all biospecimen types, the MRM assay success rate for quantifying endogenous levels of analyte is higher for

more abundant proteins than for less abundant proteins. Thus, to achieve the vision of configuring 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 (e.g. phosphorylation, etc.) are largely not accessible by MRM without enrichment. In the case of modifications, quantification using MRM may face limitations due to enrichment technologies (e.g. 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 – 10^4 enrichment), offering advantages of improved sensitivity, increased selectivity, and potential for increased throughput (via shorter LC-MRM-MS run times). Enrichment can be achieved either biochemically[47-49] (e.g. using chromatography) or through the use of analyte-specific antibodies for immuno-affinity enrichment[50-54] (producing an immuno-MRM assay). Biochemical enrichments are generally costly and/or labor-intensive procedures that critically limit throughput and require specialized expertise (i.e. are not readily distributable to the general biology community). 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 used[53]).

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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 and designed the experiments. J.J.K., S.E.A., K.K., J.S.K., R.G.I., L.Z., Y.L. and H.M. performed the experiments. J.J.K., S.E.A., K.K., P.Y., C.L., Y.Z., and S.W. analyzed the data. M.H.Y., E.G.Y., C.L., H.R., Y.K., S.A.C. and A.G.P. contributed reagents/materials/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.

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FIGURE LEGENDS

Figure 1. Overview of cell line sample preparation, distribution, and MRM analysis. Thirty cell lines related to breast cancer were prepared in complete process triplicate for analysis by quantitative LC-MRM-MS. For each cell line, 3 aliquots of each of 2 cell lysate protein concentrations (1.0 ug/uL and 0.1 ug/uL) were digested by trypsin. A mixture of stable isotope-labeled standards was added prior to desalting the digested peptides. Aliquots were distributed to each performance site where two multiplexed assay groups (one inter-laboratory assay and one site-specific assay) were analyzed on a standardized analytical platform, as described in the Experimental Procedures. The inter-laboratory assay group successfully quantified the endogenous levels of 150 peptides (representing 79 proteins), whereas the site-specific assay groups successfully quantified the endogenous levels of between 147-160 peptides (representing 78-83 proteins; 240 overall) (Supplementary Table 2).

Figure 2. Analysis of cell lysates shows excellent precision of MRM-based measurements in a biological setting, and inter-laboratory assays show high correlation and agreement between sites. CV values for the multiplexed assays measured in complete process triplicates, consisting of **(a)** inter-laboratory targets (150 peptides, 79 proteins) and **(b)** site-specific target groups. At the three sites, the median assay CVs for the inter-laboratory assay group were 5.0%, 7.4% and 5.1%, with 95% of the results having CVs within 15%, 25% and 17%. The site-specific assay groups had median assay CVs of 4.7%, 6.5% and 4.7%, with 95% of the results having CVs within 14%, 22% and 17%. **(c)** Results for individual peptide measurements were correlated by plotting the peptide amounts measured at the Fred Hutchinson Cancer Research Center, Broad Institute, and Seoul National University/ Korea Institute of Science and Technology. For each plot, the x-axis shows the log10 amount of peptide measured at site 1 and the y-axis shows the log10 amount of peptide measured at site 2. **(d)** A distribution of the percent difference for a pairwise comparison of results. Box plots show the median value plotted as a line with each box displaying the distribution of the inner quartiles and vertical lines show 95% of the data.

Figure 3. Heat maps for the protein expressions (left column) and RNA expressions (right column) of genes significantly associated with HER2 (a), ER (b) and basal/luminal (c)[33]. In each heat map, one row represents a sample and one column represents a gene. The color bar on the left side of each heat map illustrates the subtypes of cell lines. The color bar on the top of each heat map illustrates whether only the protein expression, or only the RNA expression, or both expressions of the gene were associated with the subtype. For the 4 genes shown in (a), all have significantly different RNA expression levels between HER2+ and HER2- cell lines; while only 2 out of the 4 have significantly different protein expression levels. For the 69 genes shown in (b), 25 or 62 have significantly different RNA or protein expression levels between ER+ and

ER- cell lines respectively, with an overlap of 18 genes. For the 98 genes shown in (c), 42 or 87 have significantly different RNA or protein expression levels between ER+ and ER- cell lines, with an overlap of 31.

Figure 4. Distribution of protein expression levels (top panel), RNA expression levels (middle panel), and DNA copy numbers (bottom panel) of the twelve subtype-enriched genes showing high concordance amongst genomic and proteomic datasets. Two genes, ERBB2 and GRB7 at chr17, are Her2 amplicon genes that show good separation of Her2+/Her2- groups. The other ten genes show a difference between the basal/luminal subtypes; the corresponding p-values from Wilcoxon rank test are all ≤ 1e-4 with 10k iterations. Box plots show the median value plotted as a line with each box displaying the distribution of the inner quartiles and whiskers show 95% of the data.

Figure 5. **Kaplan-Meier (KM) survival curves of breast cancer patients stratified by their expression levels of DPYSL2, CLTC or ABAT.** Two independent breast cancer datasets[41, 42] providing both outcome information as well as genomic profiles were used to determine whether the expression of candidate genes 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, based on whether or not the expression of the candidate gene was greater than the median expression of the candidate gene. The p-values from Logrank tests comparing the two KM curves are shown above each figure.

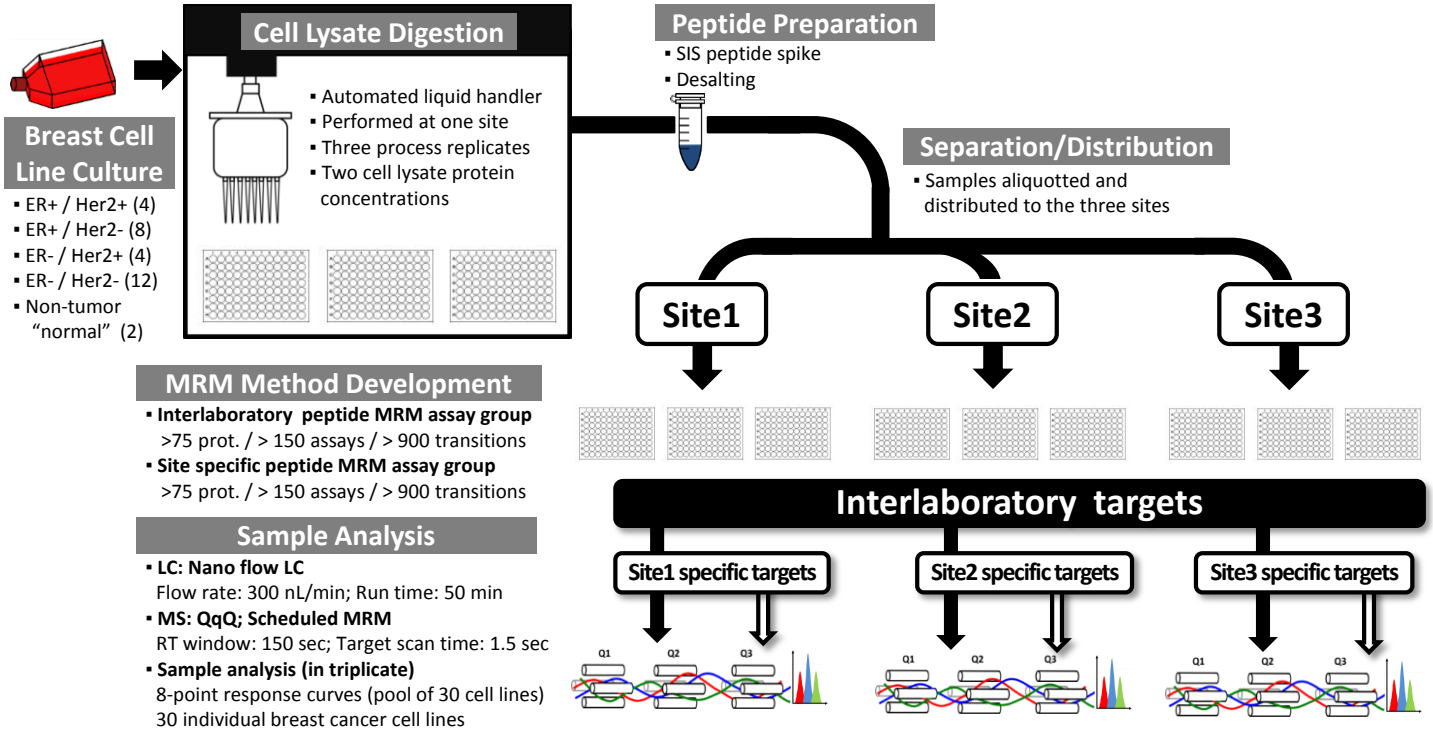
Breast Cell Line Culture
▪ ER+ / Her2+ (4)
▪ ER+ / Her2- (8)
▪ ER- / Her2+ (4)
▪ ER- / Her2- (12)
▪ Non-tumor "normal" (2)
Cell Lysate Digestion
▪ Automated liquid handler
▪ Performed at one site
▪ Three process replicates
▪ Two cell lysate protein concentrations
Peptide Preparation
▪ SIS peptide spike
▪ Desalting
Separation/Distribution
▪ Samples aliquotted and distributed to the three sites
Site1
Site2
Site3
MRM Method Development
▪ Interlaboratory peptide MRM assay group
>75 prot. / > 150 assays / > 900 transitions
▪ Site specific peptide MRM assay group
>75 prot. / > 150 assays / > 900 transitions
Sample Analysis
▪ LC: Nano flow LC
Flow rate: 300 nL/min; Run time: 50 min
▪ MS: QqQ; Scheduled MRM
RT window: 150 sec; Target scan time: 1.5 sec
▪ Sample analysis (in triplicate)
8-point response curves (pool of 30 cell lines)
30 individual breast cancer cell lines
Interlaboratory targets
Site1 specific targets
Site2 specific targets
Site3 specific targets
Q1
Q2
Q3

Figure 1.

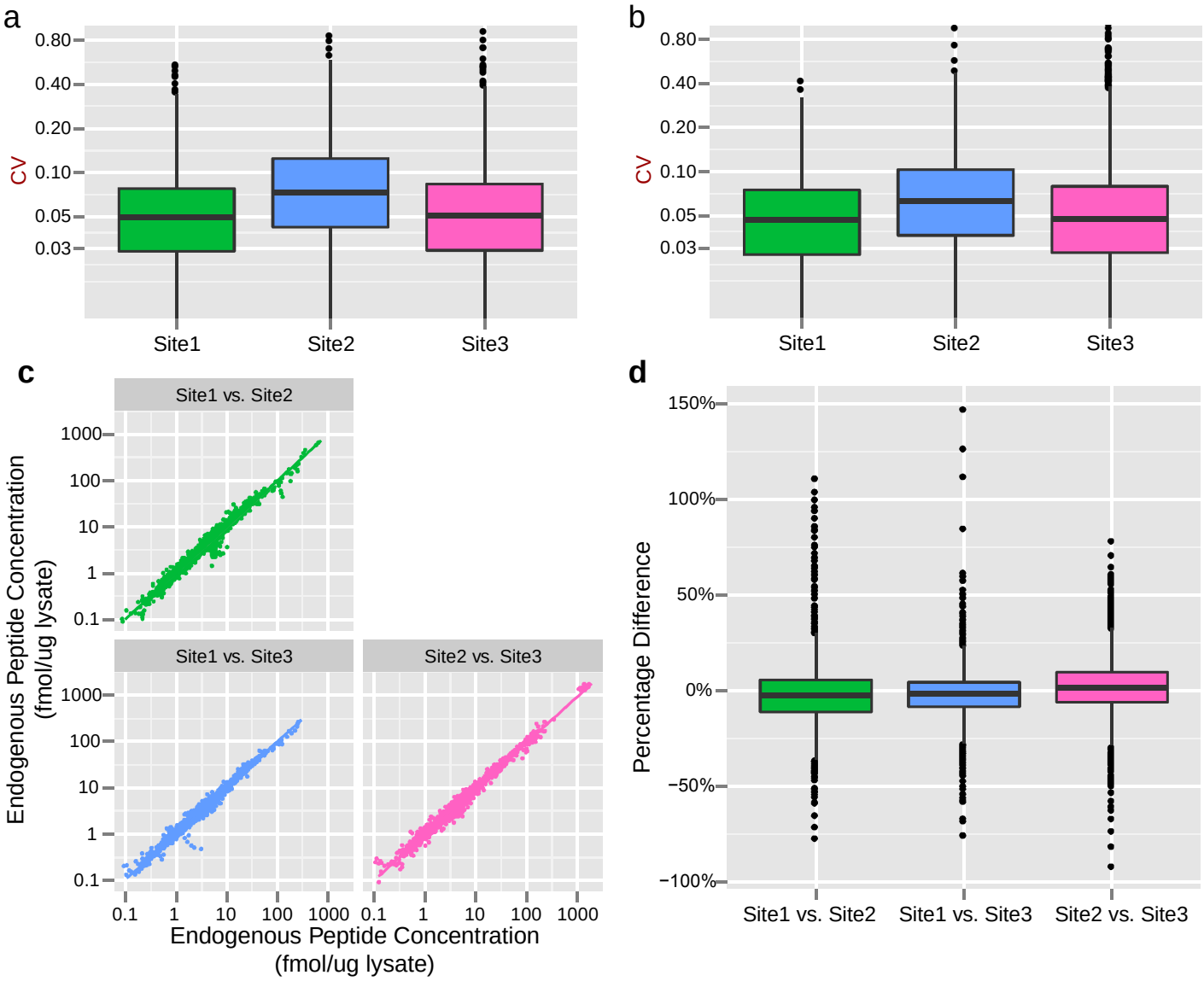
a
CV
0.80
0.40
0.20
0.10
0.05
0.03
Site1
Site2
Site3
b
CV
0.80
0.40
0.20
0.10
0.05
0.03
Site1
Site2
Site3
c
Site1 vs. Site2
Site1 vs. Site3
Site2 vs. Site3
Endogenous Peptide Concentration (fmol/ug lysate)
1000
100
10
1
0.1
0.1
1
10
100
1000
Endogenous Peptide Concentration (fmol/ug lysate)
d
Percentage Difference
150%
100%
50%
0%
−50%
−100%
Site1 vs. Site2
Site1 vs. Site3
Site2 vs. Site3

Figure 2.

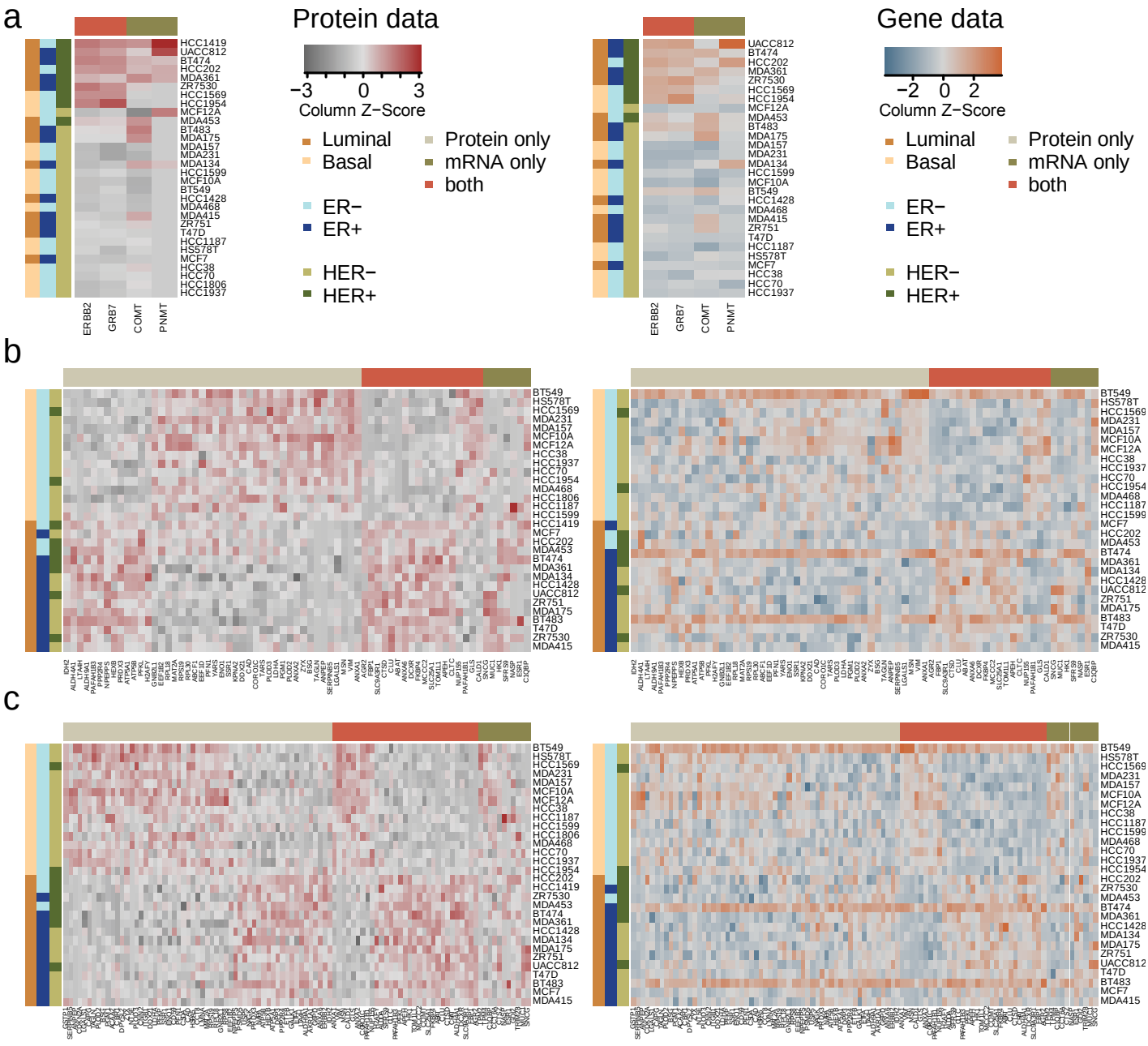
a
Protein data
−3 0 3
Column Z−Score
Luminal
Basal
ER−
ER+
HER−
HER+
Protein only
mRNA only
both
HCC1419
UACC812
BT474
HCC202
MDA361
ZR7530
HCC1569
HCC1954
MCF12A
MDA453
BT483
MDA175
MDA157
MDA231
MDA134
HCC1599
MCF10A
BT549
HCC1428
MDA468
MDA415
ZR751
T47D
HCC1187
HS578T
MCF7
HCC38
HCC70
HCC1806
HCC1937
ERBB2
GRB7
COMT
PNMT
Gene data
−2 0 2
Column Z−Score
UACC812
BT474
HCC202
MDA361
ZR7530
HCC1569
HCC1954
MCF12A
MDA453
BT483
MDA175
MDA157
MDA231
MDA134
HCC1599
MCF10A
BT549
HCC1428
MDA468
MDA415
ZR751
T47D
HCC1187
HS578T
MCF7
HCC38
HCC70
HCC1937
b
c

Figure 3.

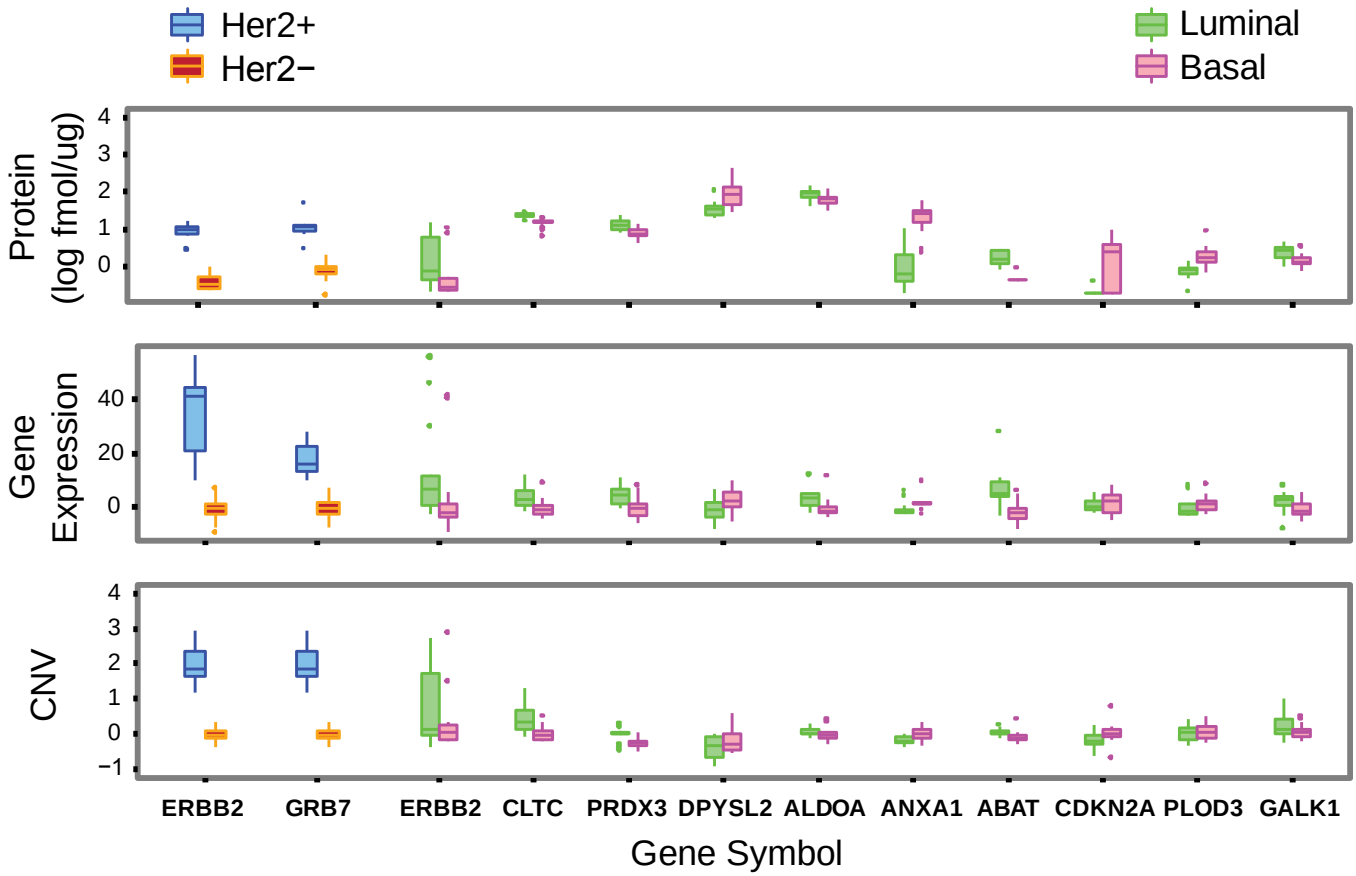
Her2+
Her2−
Luminal
Basal
Protein (log fmol/ug)
Gene Expression
CNV
ERBB2
GRB7
ERBB2
CLTC
PRDX3
DPYSL2
ALDOA
ANXA1
ABAT
CDKN2A
PLOD3
GALK1
Gene Symbol

Figure 4.

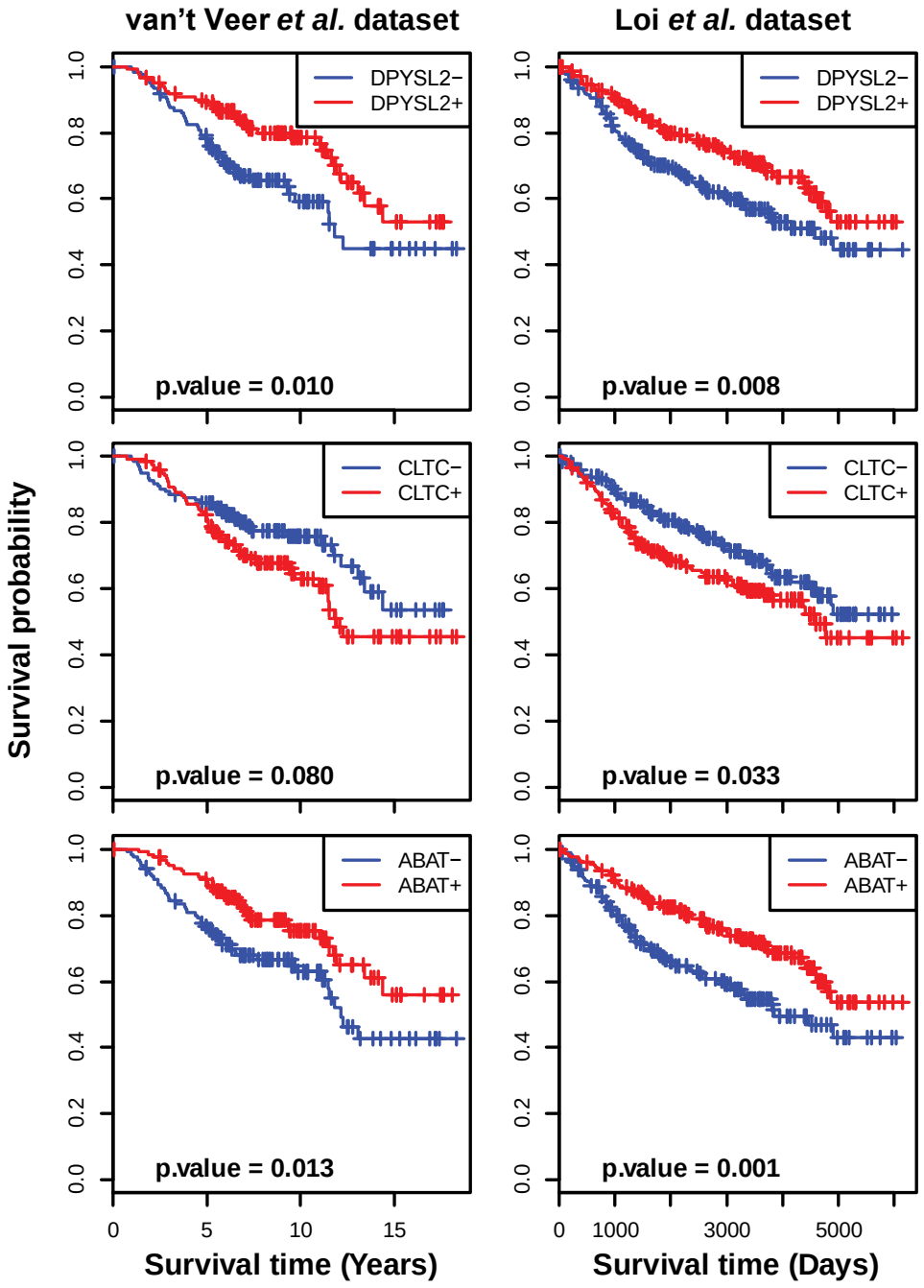
van't Veer et al. dataset
Loi et al. dataset
Survival probability
DPYSL2−
DPYSL2+
CLTC−
CLTC+
ABAT−
ABAT+
p.value = 0.010
p.value = 0.008
p.value = 0.080
p.value = 0.033
p.value = 0.013
p.value = 0.001
0.0
0.2
0.4
0.6
0.8
1.0
0
5
10
15
1000
3000
5000
Survival time (Years)
Survival time (Days)

Figure 5.