Peptide Immunoaffinity Enrichment and Targeted Mass Spectrometry Enables Multiplex, Quantitative Pharmacodynamic Studies of Phospho-Signaling*

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In most cell signaling experiments, analytes are measured one Western blot lane at a time in a semiquantitative and often poorly specific manner, limiting our understanding of network biology and hindering the translation of novel therapeutics and diagnostics. We show the feasibility of using multiplex immuno-MRM for phospho-pharmacodynamic measurements, establishing the potential for rapid and precise quantification of cell signaling networks. A 69-plex immuno-MRM assay targeting the DNA damage response network was developed and characterized by response curves and determinations of intra- and inter-assay repeatability. The linear range was >3 orders of magnitude, the median limit of quantification was 2.0 fmol/mg, and the median interassay variability was 16% CV. The assay was applied in proof-of-concept studies to immortalized and primary human cells and surgically excised cancer tissues to quantify exposure–response relationships and the effects of a genomic variant (ATM kinase mutation) or pharmacologic (kinase) inhibitor. The study shows the utility of multiplex immuno-MRM for simultaneous quantification of phosphorylated and nonmodified peptides, showing feasibility for development of targeted assay panels to cell signaling networks. Molecular & Cellular Proteomics 14: 10.1074/mcp.O115.050351, 2261–2273, 2015.

Because there is limited correlation between mRNA and protein levels/activity (1), quantification of proteins and post-translational modifications is critical to understanding cellular signaling and determining pharmacodynamic (PD) responses. Phosphorylation is a key post-translational modification used in signaling networks to modulate protein/pathway activity, protein interactions, and protein localization in response to extracellular and intracellular stimuli. Many diseases exhibit dysfunctions in signaling networks, and thus major efforts to identify novel drug targets (e.g. kinase inhibitors) are based on signal transduction pathways (2).

Currently the research community lacks high throughput, quantitative tools for studying phospho-signaling networks, hindering our basic understanding of network biology and hence the translation of novel therapeutics and companion diagnostics. In most experiments, one analyte is measured one Western blot lane at a time in a semiquantitative and often nonspecific manner. These drawbacks limit our ability to extend knowledge beyond individual phosphorylation events to a system-wide study of phosphorylation dynamics, which is critical because signal transduction pathways act as interconnected networks, and the effects of mutations in individual genes (as well as the effects of pharmacologic compounds) spread throughout the network (3). Although Western blotting and related traditional immuno-assay platforms (e.g. ELISA) have been pushed brilliantly to their limits and have formed the basis of many advances in biomedical research, they are inadequate to support the needs of the postgenomic world, in which we need innovative technologies for determining the effects of any experimental condition (e.g. agonist or antagonist exposures, genetic variations) on the major signal transduction networks of the human cell, using precise, standardized, moderate-to-high throughput methods that can be reproduced across laboratories.

Newer technologies, such as planar (4) or bead-based protein arrays (5) and mass cytometry (6) have shown potential for improving our ability to quantify signaling networks. However, like traditional immunoassays, these techniques do not...

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The abbreviations used are: PD, pharmacodynamics; MRM, multiple reaction monitoring; DDR, DNA damage response; MMS, methyl methanesulfonate; IR, ionizing radiation; LCL, lymphoblast cell line; WB, Western blot.
directly detect and quantify the target analyte. Rather, the concentration of the target is inferred from a reporter signal, such as a fluorescent or mass tag on the antibody. As a result, these assay platforms are plagued by interferences present in biological matrices, which undermine the specificity of the assays in all but the most rigorously optimized settings using highly monospecific antibodies (7). Thus, generating such assays and assuring specificity is costly, time-consuming, and very difficult, especially in multiplex.

Technological advancements in MS have enabled an impressive depth of coverage of the phosphoproteome using untargeted (“shotgun”) approaches (8–10). Furthermore, shotgun mass spectrometry has been used to profile signaling pathways by enriching phosphorylated peptides through approaches such as antiphospho-tyrosine antibodies (11), extensive fractionation (12), or panels of antibodies to enrich for signaling nodes (13). Coupling isotopic labeling methods (14, 15) to MS allows relative quantification of detectable peptides between two or a small number of samples, but these methods do not provide the absolute abundances of the peptides detected, nor are they amenable to the analysis of large numbers of biological samples. For example, to achieve substantial depth of coverage, multidimensional biochemical fractionations are required (9), limiting the number of samples that can be analyzed. Relatively large sample consumption is a constraint for analyzing clinical specimens. Under-sampling remains an issue in data-dependent modes, and missing information in the data is substantial. Thus, untargeted mass spectrometry is capable of broad discovery, but does not have adequate throughput or reproducibility for more expansive biological or clinical studies.

There has been tremendous growth in the application of targeted quantitative MS to quantify proteotypic peptides (16–19). In contrast to untargeted “shotgun” modes of MS, targeted MS focuses the full analytic capacity of the instrument on selected analytes of interest, moving from a stochastic sampling of the complex mixture where the instrument “decides” (nonreproducibly and with low precision) what is analyzed- to targeted, high-precision measurements of suites of proteins of interest that can be context-dependent based on the biological question being asked. The most widely used form of targeted MS is multiple reaction monitoring (MRM). MRM has been the clinical gold standard for decades in pharmaceutical research and in clinical reference laboratories for quantification of small molecules such as drug metabolites or metabolites that accumulate as a result of inborn errors of metabolism (20, 21). Although proteolysis of the biospecimen is a source of preanalytical variation for peptide quantification, careful selection of peptide analytes readily produces MRM-based assays of high precision (%CV ≤ 20%) (17, 22) that enable accurate quantification of reproducibly released tryptic peptides. Also, because MRM assays use internal standards (i.e. synthetic, stable isotope-labeled peptides that are spiked into the biospecimen), assays yield highly reproducible results when shared among laboratories and implemented on different instrument platforms (23), even at high multiplex levels on an international stage (22). The molecular specificity of MRM is very high, as it is conferred by three orthogonal physio-chemical properties of each peptide: its mass, its retention properties on HPLC, and the production of a set of fragment ions of specific mass (of which three to five are usually monitored) detected at characteristic ratios. High specificity, coupled to the large linear range of MRM (>10^4) and the ability to monitor analytes at scheduled times during the MRM run, render MRM assays highly multiplexable. A recent study shows scalability of MRM via analytical validation of four multiplex MRM assays (ranging from 156–169 plex) quantifying 645 human peptides (319 proteins) with median %CV<6 and successful reproduction of assay results internationally across three laboratories (22). Thus, MRM shows many advantages over alternative protein measurement technologies, including the capability to multiplex with ease, the use of internal standards (aiding reproducible quantification and cross-laboratory standardization), high specificity through direct measurement of the analyte, and relatively less time and cost associated with assay development.

Although immuno-MRM has previously been applied to quantify unmodified protein abundances in body fluids (24, 25), cell lysates, and tissues (26), the feasibility and success rate for using immuno-MRM to quantify phosphopeptides and phospho-signaling has not been tested. In this study, we tested the possibility that MRM could be coupled to peptide immuno-affinity enrichment (27, 28) to enable multiplex quantification of phospho-signaling networks. As proof-of-concept, we developed a 69-plex immuno-MRM assay for quantifying phospho-signaling in the DNA damage response (DDR) network. The DDR is critical for maintaining genomic integrity, and mutations in the DDR are among the most frequently identified in tumors (29). In this study we show: (1) simultaneous analysis of modified and nonmodified isoforms of peptides, (2) multiplexed quantitative analysis of signaling events, and (3) applicability to a variety of sample types and conditions. The multiplexed assay presented in this study replaces 69 Western blots with a 40 min MRM-MS run, providing quantitative, precise, specific data, and establishing feasibility for developing targeted assay panels to many cell signaling networks. The ability to quantitatively measure a large array of phosphorylation events would have broad benefits for the biomedical research community, spanning fundamental biological studies through to PD characterizations of novel drug compounds and the development of companion diagnostics.

**EXPERIMENTAL PROCEDURES**

**Materials**—Urea (#U0631), Trizma base (#T2694), and iodoacetamide (#A3221) were obtained from Sigma (St. Louis). Acetonitrile (#A955) and water (#W6, LCMS Optima® grade), tris(2-carboxy-ethyl)phosphine (TCEP, #77720), phosphate buffered saline (PBS, #BP-399–20), and (3-[3-cholamidopropyl] dimethylammonio)-1- propanesulfonate (CHAPS, #28300) detergent were obtained from...
Thermo Scientific (Waltham, MA). Formic acid (#1.11670.1000) was obtained from EMD Millipore (Billerica, MA). Sequencing grade trypsin (#V5111) used for digestion of samples was obtained from Promega (Madison, WI).

**Generation of Immuno-MRM Assay Reagents**—Rabbit polyclonal antibodies were produced by Epitomics-an AbCam Company (Burlingame, CA). Polyclonal antibodies were affinity-purified from 30 ml of antiserum. The serum was first passed over a column with immobilized phosphorylated peptide. The bound antibodies were eluted and passed over a second column with immobilized nonmodified peptide. The flow through was enriched for phospho-specific antibodies and the bound portion contained “total” antibodies. Total antibodies were used for all immuno-MRM experiments in the study. The concentrations of affinity-purified antibodies were determined by Bradford assay. Purified (>95% by HPLC) light (unlabeled) synthetic versions of the modified and nonmodified peptides were obtained from Epitomics-an AbCam company and purified heavy stable isotope labeled peptides were obtained from New England Peptide (Gardner, MA). For stable isotope labeled peptides, the C-terminal arginine or lysine was labeled with [13C and15N] labeled atoms. Peptide stock concentrations of the heavy peptides were determined by amino acid analysis (AAA) at New England Peptide. Aliquots were stored in 3% acetonitrile/0.1% formic acid at −80 °C until use.

**Cells, Tissues, and Treatments**—Cell lines used for proof-of-concept studies were the human mammary epithelial cell line MCF10A, obtained from the ATCC (Manassas, VA) and lymphoblast cell lines (LCL) (GM07057 and GM01526), obtained from the Coriell Institute (Camden, New Jersey). PBMC were obtained from healthy, nonpregnant adults that had not previously received chemotherapy or radiation treatments and were recruited from the Fred Hutchinson Cancer Research Center (FHCRC) or the Seattle Cancer Care Alliance (FHCRC IR #2151). Breast tumor tissue biopsies were obtained under FHCR IR #7077. Cells were grown to 80% confluence prior to treatment with methyl methanesulfonate (MMS, Sigma, #129925) or IR. MMS was diluted in growth medium just prior to addition to cell cultures for a final concentration of 0.5 mm. Irradiation was performed in a JL Shepherd Mark I irradiator using a 152Cs source delivering a dose rate of 4.7 Gy/minute; mock-irradiated cells or tissues were handled in precisely the same manner as the irradiated cells, but the irradiator was not turned on. After incubation for the indicated times, growth medium was removed, cells rinsed, and lifted off the plates by incubation in a fresh aliquot of 0.25% trypsin/EDTA solution at 37 °C, 5% CO2. When cells had lifted from the plate, the trypsin was quenched by the addition of three volumes of DMEM/F12 with 5% horse serum. The cells had lifted from the plate, the trypsin was quenched by the addition of three volumes of DMEM/F12 with 5% horse serum. The tissue was then placed in separate 6 cm plates (Waters #WAT058951) and a positive pressure manifold (Waters #186005521). The eluates were aliquoted by volume and lyophilized, followed by storage at −80 °C until use.

**Western Blots**—Commercial antibodies were purchased and used at the manufacturer’s recommended dilutions: BRCA1 (CST #9025); BRCA1 pS1524 (CST #9009); NBS1 (Epitomics #1507–1); NBS1 pS343 (Epitomics #2194–1); and alpha-Tubulin (Epitomics #1878–1). Protein lysates (10–30 µg/lane) were subjected to SDS-PAGE on Tris-Acetate or Bis-Tris Novex gels (Invitrogen, Carlsbad, CA).

**Peptide Immunoaffinity Enrichment and Liquid Chromatography-Mass Spectrometry**—Enrichment was performed as previously described (30) with the following modifications. The final assay consisted of a mixture of 44 antibodies. Antibodies were crosslinked on protein G beads (1 µm custom-made, Dynabeads® MyOne Protein G, Life Technologies, Grand Island, NY) and peptide enrichment was performed using 1 µg antibody-protein G magnetic beads for each target added to 500 µg of lysate digest resuspended in 200 µl PBS + 0.03% CHAPS (pH was adjusted to 8.0 with 5 µl of 1 N Tris). Beads were mixed in the incubation plate, washed twice in PBS buffer + 0.03% CHAPS, washed once in PBS diluted 1:10, and peptides were eluted in 26 µl of 5% acetic acid/3% acetonitrile. The elution plate was covered with adhesive foil and frozen at −80 °C until analysis. LC-MS was performed as previously described (31) with the following modifications. An Eksigent Ultra nanoLC system (Eksigent Technologies, Dublin, CA) with a nano autosampler was used for liquid chromatography. The LC gradient was delivered at 300 nL/minute and consisted of a linear gradient of mobile phase B developed from 3–40% B in 15 min on a 10 cm × 75 µm column (Reprosil AQ C18, 3 µm; Dr. Maisch, Ammerbuch, Entringen, Germany). The nano-LC system was connected to a hybrid triple quadrupole/ion trap mass spectrometer (6500 QTRAP, ABSciex, Foster City, CA) equipped with a nano electrospray ion interface operated in the positive ion MRM mode. Parameters for declustering potential (DP) and collision energy (CE) were taken from a linear regression of previously optimized values in Skyline (32). Scheduled MRM transitions used a retention time window of 100 s and a desired cycle time of 0.5 s, enabling sufficient points across a peak for accurate quantitation. A minimum of three transitions (six total per peptide pair) were recorded for each light and heavy peptide. MRM data acquired on the 6500 QTRAP were analyzed by Skyline. Peak integrations were reviewed manually and transitions from analyte peptides were confirmed by the same retention times of the light synthetic peptides and heavy stable isotope-labeled peptides, light and heavy peptides coeluted. Data were exported from Skyline for analysis and plotting. Peak areas were calculated using Peak Area + Background. Skyline files containing data used in analysis are available in two compressed documents in supplemental Data 1 (curves data) and supplemental Data 2 (sample data).

**Method Validation**—Response curves were generated in a background matrix consisting of an equal mixture of four cell lines/treatments: MCF10A + 10 Gy IR (2 h), MCF10A + 0.5 mm MMS (3 h), HeLa + 10 Gy IR (2 h), and HeLa + 0.5 mm MMS (6 h). The pooled lysate was digested by trypsin and the heavy stable isotope-labeled peptides were added to aliquots by serial dilution (∼5) covering the
following concentrations: 2000, 400, 80, and 16 fmol/mg protein lysate, then continuing with 2× serial dilution for concentrations 8, 4, 2, and 1 fmol/mg. Light peptide was also spiked into the cell lysate pool at 150 fmol/mg. Blanks were prepared using background matrix with light peptide (no heavy spike). Three process replicates were prepared and analyzed at all concentration points (including blank samples). Linear regression was used to fit the serial dilution data points for each curve. Regression was performed using a 1/y weighting on all points having a correlation coefficient of >0.98. Limits of detection (LOD) for the sum of transition areas were obtained by using the average of the three blank measurements plus three times the standard deviation of the noise. LOQ was defined as the lowest point measured with CV <25%. The upper limit of quantification (ULOQ) was determined by the highest concentration point of the response curve that was maintained in the linear range of the response. Repeatability was determined using the same pooled lysate used in curves. Complete process triplicates were prepared and analyzed on three independent days. Intra-assay variation was calculated as the average CV obtained within each day. Interassay variation was the CV calculated from the average values of the 3 days.

RESULTS

Selection of Analytes and Development of Assay Reagents—There are several considerations for the quantitative analysis of protein phosphorylation. Phosphorylation and dephosphorylation can occur rapidly, requiring rapid cell harvesting and lysis, the presence of protease and phosphatase inhibitors during sample preparation, and appropriate design of controls when studying phospho-signaling (33). In addition, because of their low abundance and ionization properties (34), few phosphopeptides can be quantified by direct MRM analysis of neat cellular lysate, and enrichment is required. Although this can be achieved using the multidimensional biochemical fractionation workflows (10, 33, 35), these workflows require specialized expertise/instrumentation, are low in throughput, and are cost-prohibitive as a workhorse assay platform for the community. We have greatly simplified sample preparation, reduced assay costs, and improved throughput by coupling immunoaffinity enrichment of peptides with quantification by MRM (28). The “immuno-MRM” technique uses antipeptide antibodies to capture the endogenous analyte peptide and a spiked stable isotope-labeled peptide standard from complex biological samples. The method has been shown to be reproducible, sensitive, scalable, and multiplex-able (36–38). Although immuno-MRM has previously been applied to quantify unmodified protein abundances in body fluids (24, 25), cell lysates, and tissues (26), the feasibility and success rate for using immuno-MRM to quantify phosphopeptides and phospho-signaling has not been tested. In this study, we asked the following questions, (1) what is the success rate for developing immuno-MRM assays to phosphopeptides, (2) are the assays of sufficient sensitivity to quantify endogenous levels of phosphopeptides from cells, and (3) can the technology be used to profile pharamacodynamic responses in a variety of sample types and systems?

To address these questions, we attempted to develop a multiplex assay to quantify phospho-signaling in response to DNA damage. The overall study design is shown in Fig. 1. We mined existing shotgun MS datasets (9, 39) to identify 67 proteotypic phosphopeptides mapping to well characterized proteins involved in the DDR signaling network (supplemental Table S1). The phosphopeptides were prioritized by frequency of observations in the datasets (indicating good response in the mass spectrometer), quality of the MS/MS spectra, and length of peptide (=24 amino acids). No effort was made to identify peptides that may be more immuno-genic. Affinity-purified rabbit polyclonal antibodies were generated using the 67 phosphopeptides as antigens (see supplemental Fig. S1A for antibody yields). Antibodies were capable of enriching both forms of the peptide, the modified and nonmodified (see supplemental Fig. S1B–S1C for an example). Evaluation of the antibody performances and the utility of the assays for measuring endogenous analytes were determined using a two-stage process, described below.
The first stage evaluated the ability of antibodies to capture the target peptide spiked into a background matrix of cell lysate. Stable isotope-labeled versions of each peptide (±phosphorylation) were synthesized, and a mix of all synthetic phosphopeptides and their nonmodified synthetic peptide sequences was prepared and added to a background matrix of 100 µg MCF10A whole cell lysate at four concentrations (1, 10, 100, and 1000 fmol/mg). Light peptides were added at 100 fmol/mg, and the multiplexed antibodies were used to enrich the target peptides. Antibody-peptide pairs were isolated on Protein G coated magnetic beads, and the eluate was analyzed by liquid chromatography (LC)-MRM-MS. Detection of an analyte peptide in any of the spiked samples was considered a success. The success rate in capturing the modified peptides was 49 out of 67 immunogens (73%). The overall success rate in capturing nonmodified peptides was 46 out of 60 possible peptides (77%). The success in a single antibody capturing both the modified and nonmodified versions of the peptides was 38 out of 67 immunogens (57%). Supplemental Table S1 contains a complete listing of detected peptides.

The second stage evaluated the success of the assays for quantifying endogenous levels of analytes from cells exposed to DNA damaging agents. To allow for detection of analytes expressed in different cell types and/or under different conditions, lysates were prepared from two cell lines, each exposed to two DNA damaging agents: (1) MCF10A cells harvested 2 h post 10 Gy ionizing radiation (IR), (2) MCF10A cells harvested following a 1 h exposure to 0.5 mM methyl methanesulfonate (MMS), (3) HeLa cells harvested 2 h post 10 Gy IR, and (4) HeLa cells harvested following a 1 h exposure to 0.5 mM MMS. Five-hundred micrograms and 1 mg aliquots of each lysate (corresponding to an average of 1.25–2.5 × 10^6 cells, respectively) were digested with trypsin, and the labeled synthetic peptide mixture was added at 150 fmol/mg. The multiplexed antibodies were used to enrich peptides from the digested lysate, and the eluates were analyzed by LC-MRM-MS. The success rate in capturing any form of the peptides was 51 out of 67 peptides (76%). Success in capturing endogenous modified peptides was 29 out of 67 targets (43%). The success rate in capturing nonmodified peptides was 44 out of 60 possible peptides (73%). The success in antibodies capturing both the modified and nonmodified versions of the peptides was 24 out of 67 (36%). Of note, these are not purely analytical success rates, as some of the analytes may not be expressed in these specific cell lines.

**Assay Characterization**—Assays detecting the endogenous analyte (supplemental Table S1) were configured into a multiplexed panel for analytical characterization. A total of 44 antibodies (42 antibodies detecting endogenous peptide from this study and two additional antibodies targeting nonmodified peptides to the PCNA gene product) targeting 77 peptides (phospho- and unmodified forms) were multiplexed in the panel. Response curves were generated to characterize the linear range, lower limit of detection (LOD), and limit of quantification (LOQ). Heavy stable isotope-labeled peptides were added to a pool of cell lysates (MCF10A + HeLa cells exposed to 10 Gy IR and 0.5 mM MMS) over several orders of magnitude of concentration. The light peptide was kept constant. Varying the heavy peptide amount enables estimation of the linear range and detection limits directly in the background matrix of interest without interference from the endogenous peptide (40). Specificity was established by comparing the relative peak intensities for transitions in light and heavy peptides and assessing agreement between slopes of response curves for individual transitions. An example response curve is shown in Fig. 2, and figures of merit are reported in Table I for the 48 assays enabling endogenous detection (response curves for all 69 characterized assays are shown in supplemental Fig. S2, and figures of merit for all characterized assays are reported in supplemental Table S2). The linear range for working assays was ≥3 orders of magnitude. The median LOD was 1.4 fmol/mg with a range of 1 to 46 fmol/mg, and the median LOQ was 2.0 fmol/mg.

Repeatability was characterized by performing the measurement of endogenous analyte peptide in the background cell lysate mixture over three separate days, carrying out complete process replicates (including digestion) of aliquots of the pooled cell lysates used for response curves. Intra-assay (within day) and inter-assay (between day) variability are reported in Table I. The median intra-assay variability was 10% CV, and the median inter-assay variability was 16% CV. Complete distributions for CVs are shown in Fig. 2C. The assay shows good sensitivity and repeatability over a wide dynamic range. Of note, the recovery of peptides following trypsin digestion is not always quantitative; thus, the assays accurately quantify the reproducibly released trypic phosphopeptide analyte, which may or may not accurately represent full length protein concentrations. Protein standards may help the accuracy of protein-level measurements; however, obtaining labeled protein standards with equivalent physicochemical characteristics to endogenous proteins is very difficult, especially when targeting post-translational modifications. As shown below, accurate quantification of the reproducibly released trypic phosphopeptide analyte enables pharmacodynamic measurements of cell signaling.

**Quantifying Cell Signaling in Primary and Immortalized Human Cells and Human Cancer Tissues**—We conducted proof-of-principle testing to determine whether the multiplex immuno-MRM assay enables PD determinations of the DDR phospho-signaling network in several sample types/conditions. We first profiled the DDR in immortalized (MCF10A) and primary human cells (peripheral blood mononuclear cells, PBMCs) exposed to either IR or MMS to induce genotoxic stress. A heatmap showing the response is plotted in Fig. 3. Analytes are seen to change in both directions (up and down-regulated). As can be seen for representative analytes in Fig.
Peptide Immuno-MRM Enables Phosphopeptide Quantification

3, each responsive analyte shows a characteristic PD curve (data for all analytes are provided in supplemental Fig. S3). Levels of the nonphosphorylated isoforms of the peptides generally trend in the opposite direction of the phosphopeptide, suggesting that there is not an increase in overall abundance of the protein, but an increase in the fraction of phosphorylation at these sites. Phospho-NBS1\textsuperscript{S343} and P53\textsuperscript{S315} are increased in both irradiated (2–6 h) and MMS-treated (6 h time point) samples. However, for TP53, there is an increase in nonmodified peptide in conjunction with the increase in phosphopeptide, where phospho-NBS1\textsuperscript{S343} shows an apparent fractional increase in phosphorylation. The responses of phospho-NBS1\textsuperscript{S343} and TP53\textsuperscript{S315} in MMS-treated cells occurs at a later time point (6 h) compared with the irradiated cells, where they peak at 2 h.

We next tested whether the multiplex assay could also be used to quantify the radiation response of surgically excised breast cancer tissue specimens exposed to IR ex vivo. The modified and nonmodified peptides were successfully detected in the treated tissue lysates as shown in the heatmap in Fig. 4 (see supplemental Fig. S3 for individual profiles of all analytes). Examples of changes in the levels of phospho-ATM\textsuperscript{S367} and phospho-NBS1\textsuperscript{S343} are shown in Fig. 4, showing the capability of the method to measure signaling events directly from excised tissue. The ability to profile pathway activity in tumor biopsies raises the interesting possibility of using direct measurement of pathway activity to tailor therapies to individual tumors.

Quantifying the Effects of a Genomic Variant and a Pharmacologic Agent on Phospho-signaling—We next tested the ability of multiplex immuno-MRM to quantify the effects of genomic mutations or pharmacologic inhibitors that affect kinase activity. To test the effects of a genomic variant on cell signaling, we used lymphoblast cell lines (LCL) derived from ataxia telangiectasia (AT) patients (ATM\textsuperscript{−/−}) and from healthy controls (ATM\textsuperscript{+/+}). AT patients carry bi-allelic mutations in the ATM gene, which encodes a serine/threonine protein kinase that activates checkpoint signaling in response to DNA double strand breaks (41). ATM activity is dependent on dissociation of the ATM homodimer to active monomers via autophosphorylation at several sites, including Ser1981 (42) and Ser367 (43). Cells from AT patients are sensitive to ionizing radiation and defective in phospho-signaling in response to DNA damage (44). Using the 69-plex immuno-MRM assay, we profiled the time-course profile of DDR in cells treated by ionizing radiation (Fig. 5A). We detected endogenous expression of the ATM protein in ATM\textsuperscript{+/+} but not ATM\textsuperscript{−/−} cells (Fig. 5B), consistent with prior reports (45). In ATM\textsuperscript{+/+} cells, autophosphorylation of ATM\textsuperscript{S367} was rapidly induced post-IR, as was phosphorylation of NBS1\textsuperscript{S343}, a known target of the ATM kinase (46, 47). Interestingly, although the ATM\textsuperscript{−/−} cells show an initial defect in the induction of NBS1\textsuperscript{S343} post-IR (Fig. 5B), the phospho-NBS1\textsuperscript{S343} response steadily increases to match and ultimately exceed the ATM\textsuperscript{+/+} response by 5 h, suggesting either residual ATM kinase activity or a compensatory kinase activity.

To test the potential for PD studies of pharmacologic agents, we profiled the response of an ATM\textsuperscript{+/+} LCL follow-
ing exposure to 5 Gy IR ± KU55933 (ATM kinase inhibitor) (Fig. 5A). Nonphosphorylated ATM peptide is detected at similar levels ± KU55933, showing that basal expression of the ATM kinase is not affected by the inhibitor (Fig. 5C). Autophosphorylation at ATM Tyr367 was undetectable in KU55933-treated cells, but showed a robust induction in con-
trol cells. Additionally, phosphorylation of an ATM kinase target, NBS1S343 was severely muted in the presence of the inhibitor, consistent with inhibition of the ATM kinase. Interestingly, phospho-NBS1S343 is induced in the KU55933-treated cells (albeit at reduced levels compared with controls) in the absence of any detectable phosphorylation of ATM, and BRCA1S1524 also shows a delayed response in the KU55933-treated samples compared with the control.

The current workhorse technology for assessing the activation state of cell signaling pathways is Western blotting. For comparison, several targets quantified by immuno-MRM in this study were also examined by Western blotting (Fig. 5D–5E). Qualitatively, the shapes and magnitudes of the responses measured by Western blotting and immuno-MRM are very similar, as are the sensitivities of the two techniques. However, the multiplex MRM platform greatly increases throughput (69 Western blots would need to be performed to equal the data generated by one multiplex immuno-MRM analysis), and produces highly specific and quantitative results that set an analytical bar that is impossible to achieve with Western blotting. The quality of data obtained by the
immuno-MRM is superior to the blots in quantification (verifiable internal standards are available) (48), specificity (multiple metrics of peptide identity are utilized), and performance (analytical figures of merit are established and characterized), as has been outlined in previous publications (49).

**DISCUSSION**

This study shows the feasibility of developing targeted immuno-MRM-based assay panels to cell signaling networks. The overall sensitivity for assays targeting phosphorylated peptides (as measured by LLODs and LOQs) is comparable to assays previously characterized in other systems (25, 26), indicating that even low abundance signaling molecules can be enriched and analyzed from samples. However, there may be some limitations. Although the MRM approach is generally applicable to measuring unmodified proteins through quantifying multiple analyte peptides representing each target (50), constructing assays for specific phosphosites is constrained to the sequence containing the targeted modification. This constraint renders some modifications sites challenging for MRM-based targeting (e.g. if the peptide sequence containing the modification is not proteotypic), although using alternative proteolytic enzymes can be useful for expanding measurement to these difficult sequences (51). Nonetheless, it is likely that some sites will not be amenable to MRM measurement.

The DNA damage response (DDR) is a highly regulated signal transduction network that orchestrates the temporal and spatial organization of protein complexes required to repair (or tolerate) DNA damage (e.g. nucleotide excision repair, base excision repair, homologous recombination, non-homologous end joining, postreplication repair) (52). Interestingly, despite its evolutionary conservation, there is a high degree of inter-individual variability among humans in the functionality of the DDR network (53). Because the DDR network is a major defense system against environmental exposures linked to diseases, as well as a major target of somatic mutational inactivation in human cancers, being able to quantify and understand this variation has major clinical and public health implications (54). Unfortunately, there are few examples of translating knowledge of the DDR into the clinic or public health arenas, due in part to a paucity of quantitative tools for monitoring DDR activity (54, 55). This study shows the feasibility of configuring highly multiplex, MRM-based assays to support basic, clinical, and epidemiological studies.

The data show that immuno-MRM offers substantial analytical advantages over the current gold standard (Western blotting) for monitoring cellular phospho-signaling. Whereas Western blots (WB) are plagued by nonspecific bands (usually cut out of published figures) and uncertainties about which band represents the analyte of interest, MRM uses the mass spectrometer to detect the actual analyte of interest, rendering specificity of the assays near absolute. Thus, cross-reactivity of the antibodies does not affect specificity. Although WB is semiquantitative at best and has not been standardized across laboratories, MRM uses synthetic, internal stable isotope-labeled standard peptides (spiked into the biospecimen at a known concentration) to enable precise quantification of target analyte and standardization across laboratories.

Another advantage of the immuno-MRM platform is the ability to measure phosphorylated and nonmodified peptides with a single antibody. The immuno-MRM assay has relaxed specificity requirements for the antibody, because the mass spectrometer can easily distinguish the phosphorylated and nonmodified isoforms of a given peptide, providing a significant advantage in affinity reagent generation. In our findings, several nonmodified peptides (e.g. BRCA1 and NBS1 in Fig. 3) were seen to decrease in proportion with an increase in the phospho-isoform. Quantitative conclusions about phospho-site stoichiometry must be made with caution, because phospho-sites can be clustered on an analyte peptide, and not all possible phospho-isoforms may be detected by the mass spectrometer. If multiple isoforms of the analyte peptide exist, each corresponding mass must be targeted separately in the MRM assay to quantify site occupancy. Additionally, phosphorylation events in the vicinity of trypsin cleavage sites may...
Fig. 5. **PD studies of genetic effects and kinase inhibition.** LCLs were exposed to 5 Gy IR and harvested over a time-course following exposure (0.25, 1, 3, 5, 8, 24 h); controls were mock-irradiated and harvested at 1 h. Whole cell lysates were analyzed using the multiplexed assay panel. Dashed line indicates the lower limit of quantification. 

A, Heatmap showing response of individual analytes as the mean log₂ fold change relative to mock-treated samples. 

B, Results from normal (ATM+/+, gray line) and mutant (ATM−/−, black line) cell lines for phosphorylated and nonmodified peptides. Error bars are the range of biological duplicates. 

C, Time-course profiles measured in ATM+/+ cells (untreated, gray line; exposed to KU55933 kinase inhibitor, black line). Error bars are the standard deviation of three biological replicates. 

D, Western blots for corresponding targets in ATM+/+ and ATM−/− cell lines following IR or mock-treatment (m). Lysates are the same as those analyzed in Fig. 5A. Blot images are cropped to the molecular weight region of interest. 

E, Western blot for ATM+/+ cells responding to IR in the presence or absence (mock) of the KU55933 inhibitor. Lysates are the same as those analyzed in Fig. 5B.
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affect digestion efficiency. Thus, the MRM-based assays may accurately quantify the reproducibly recovered tryptic peptide analyte targeted by the MRM method, but do not accurately quantify all peptide phospho-isomers that are not released by proteolysis, not efficiently captured by the antibody, or whose masses are not targeted by the MRM method.

Immuo-MRM also offers substantial advantages in terms of throughput and costs. Unlike the WB (and other immunoassay platforms), MRM assays can be highly multiplexed with ease. In this study, we showed a 69-plex MRM assay using a mixture of 44 antibodies for immunoaffinity enrichment. The LC-MRM run time described herein is 40 min per sample, including wash and re-equilibration time. Thus, >25 samples/day could be analyzed on a single instrument, producing over 1500 precise measurements, the equivalent of >150 Western blots. When considering the high multiplex level, the time and cost per analyte are significantly lowered. We estimate that it costs $40–$50 per sample to run the immuno-MRM assay. This corresponds to below one dollar per analyte when multiplexing. To run an equivalent number of Western blots would cost approximately $500 per sample, roughly $7 per analyte. Although sample processing for immuno-MRM requires 2 days (including overnight trypsin digestion and overnight incubation with antibodies), it can be performed offline in parallel, making the MRM-MS run time the limiting factor in increasing throughput. Faster run times could be accomplished by using higher flow rates (with some sacrifice of sensitivity) or targeting fewer analytes.

Finally, standardizing proteomic measurements to harmonize results across the community has been extremely challenging, and a reliance on poor-performing affinity reagents (56) and a small number of Western blots have been explicitly cited as contributors to the inability of industry to reproduce preclinical research results (57, 58). MRM assays use internal standards and can be transported across laboratories and instrument platforms, enabling harmonization of proteomic results among investigators and facilitating the reproducibility of results and the regulatory evaluation of novel drug compounds.

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[This article contains supplemental Figs. S1 and S2, Tables S1 to S3, and Data S1 and S2.]

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