Immobilized Metal Affinity Chromatography Coupled to Multiple Reaction Monitoring Enables Reproducible Quantification of Phospho-signaling*§

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A major goal in cell signaling research is the quantification of phosphorylation pharmacodynamics following perturbations. Traditional methods of studying cellular phospho-signaling measure one analyte at a time with poor standardization, rendering them inadequate for interrogating network biology and contributing to the irreproducibility of preclinical research. In this study, we test the feasibility of circumventing these issues by coupling immobilized metal affinity chromatography (IMAC)-based enrichment of phosphopeptides with targeted, multiple reaction monitoring (MRM) mass spectrometry to achieve precise, specific, standardized, multiplex quantification of phospho-signaling responses. A multiplex immobilized metal affinity chromatography-multiple reaction monitoring assay targeting phospho-analytes responsive to DNA damage was configured, analytically characterized, and deployed to generate phospho-pharmacodynamic curves from primary and immortalized human cells experiencing genotoxic stress. The multiplexed assays demonstrated linear ranges of ≥3 orders of magnitude, median lower limit of quantification of 0.64 fmol on column, median intra-assay variability of 9.3%, median inter-assay variability of 12.7%, and median total CV of 16.0%. The multiplex immobilized metal affinity chromatography-multiple reaction monitoring assay enabled robust quantification of 107 DNA damage-responsive phosphosites from human cells following DNA damage. The assays have been made publicly available as a resource to the community. The approach is generally applicable, enabling wide interrogation of signaling networks. Molecular & Cellular Proteomics 15: 10.1074/mcp.O115.054940, 726–739, 2016.

Cell signaling research is faced with the challenging task of interrogating increasingly large numbers of analytes in “systems biology” approaches, while maintaining the high standards of integrity and reproducibility traditionally associated with the scientific approach. For example, studies interrogating complex systems, such as protein signaling networks, require quantification technologies capable of sensitive, specific, multiplexable, and reproducible application. However, recent reports have highlighted alarmingly high rates of irreproducibility in fundamental biological and pre-clinical studies (1, 2), as well as poor performance of affinity reagents used in traditional proteomic assay and detection platforms (3, 4). There is an imminent need for high quality assays, including highly characterized standards and detailed documentation of processes and procedures (5). To improve the translation of cell signaling discoveries into clinical application, we need reproducible and transferable technologies that enable higher throughput quantification of protein phosphorylation.

Signaling dynamics through post-translational modifications (e.g. phosphorylation) are predominantly measured by Western blotting. Although this technique has led to many discoveries and is the de facto “gold standard,” it suffers from many drawbacks. Western blotting is a low throughput approach applied to individual analytes (i.e. no multiplexing) and is susceptible to erroneous interpretation when applied quantitatively (6). Alternative immunoassay platforms have emerged (e.g. immunohistochemistry, ELISA, mass cytometry, and bead-based or planar arrays), but suffer from similar limitations, namely specificity issues (because of cross-reactivity of antibodies), poor standardization, and difficulties in multiplexing.
One alternative for quantifying phosphorylation is targeted, multiple reaction monitoring (MRM)\(^1\) MS, a widely deployed technique in clinical laboratories for quantification of small molecules (7, 8). MRM is now also well established for precise and specific quantification of endogenous, proteotypic peptides relative to spiked-in stable isotope-labeled internal standards (9–11), and MRM can be applied to phosphopeptides (12–18). MRM assays can be run at high multiplex levels (19–21) and can be standardized to be highly reproducible across laboratories (22–24), even on an international stage (25). Because phosphorylation typically occurs at sub-stoichiometric levels and because phosphopeptides must compete for ionization with more abundant peptides, mass spectrometry-based analysis of phosphorylation requires an analyte enrichment step. Immuno-affinity enrichment approaches using anti-phospho-tyrosine antibodies (26) or panels of antibodies targeting signaling tyrosine enrichment approaches using anti-phospho-tyrosine antibodies (27) have been implemented with shotgun mass spectrometry, enabling robust IMAC-MRM assays without the need for an antibody.

Phosphopeptide enrichment based on metal affinity chromatography has recently matured into a reproducible approach (29). Immobilized metal affinity chromatography (IMAC) is widely used in discovery phosphoproteomic studies to enrich phosphopeptides upstream of shotgun-based mass spectrometry (30, 31). We hypothesized that a subset of the cellular phosphoproteome with favorable binding characteristics to the IMAC resin might be reproducibly recovered for quantification when coupled with quantitative MRM mass spectrometry, enabling robust IMAC-MRM assays without the need for an antibody.

In this report, we: (1) demonstrate the feasibility of generating analytically robust, multiplex IMAC-MRM assays for quantifying cellular phospho-signaling, (2) present a semi-automated, 96-well format magnetic bead-based protocol for IMAC enrichment, (3) provide a catalogue of phosphopeptides that are highly amenable to IMAC-MRM quantification, and (4) make publicly available standard operating protocols (SOP) and fit-for-purpose analytical validation data for IMAC-MRM assays targeting 107 phospho-analysts, providing a community resource for study of the DNA damage response. The data suggest that the IMAC-MRM approach is generally applicable to signaling pathways, enabling wider interrogation of signaling networks.

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\(\text{[13C and 15N] labeled atoms.}\)

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\(^1\) The abbreviations used are: MRM, multiple reaction monitoring; IMAC, immobilized metal affinity chromatography; SOP, standard operating protocols; DDR, DNA damage response; PBMC, peripheral blood mononuclear cell; IR, ionizing radiation; MMS, methyl methane-sulfonate; LOD, limit of detection; LLOQ, lower limit of quantification; WB, Western blot; CPTAC, Clinical Proteomic Tumor Analysis Consortium.

**EXPERIMENTAL PROCEDURES**

**Public Access to All Mass Spectrometry Data—**All LC-MS/MS proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomcentral.proteomexchange.org) via the PRIDE partner repository (32) with the data set identifier PXD002363. The MS/MS spectra and MRM transitions to these 190 phosphopeptide targets can be found in supplemental data File S1.zip. All MRM data (response curves and cell line measurements) are available in the supplemental File S2–S5.zip. Characterization data, SOPs, and assay information are available on the CPTAC Assay Portal (www.assays.cancer.gov).

**Materials—**Urea (#U0631), Trizma base (#T2694), iodoacetamide (IAM, #A3221), iron (III) chloride (FeCl₃, #157740), ammonia (28–30% ammonium hydroxide, #320145), and ammonium bicarbonate (NH₄HCO₃, #A6141) were obtained from Sigma (St. Louis, MO). Acetonitrile (MeCN, #A955), water (H₂O, #W6, LCMS Optimis@ grade), trifluoroacetic acid (TFA, #28901), and phosphate buffered saline (PBS, #BP-399–20) were obtained from Thermo Fisher Scientific (Waltham, MA). Formic acid (FA, #1.11670.1000) was obtained from EMD Millipore (Billerica, MA). Sequencing grade trypsin (#V5113) was obtained from Promega (Madison, WI).

**Synthetic Peptides—**Purified and crude heavy stable isotope-labeled peptides were obtained from New England Peptide (Gardner, MA) and ThermoFisher Scientific. Purified synthetic peptides were >95% pure as measured by HPLC. Peptide stock concentrations were determined by amino acid analysis (AAA) of New England Peptide. Peptide quality control included HPLC chromatograms, MALDI mass spectrum confirming the molecular weight, and AAA analysis to confirm correct amino acid stoichiometries (provided by vendor). Aliquots were stored in 3% MeCN/0.1% FA at −80 °C until use. Crude peptide aliquots were synthesized on a 1.0 μmol scale, purified by solid phase extraction to remove all nonpeptide contaminants (average purity ~75%), and the correct molecular mass verified by MS. Aliquots were shipped lyophilized and resuspended in 1 ml 3% MeCN/0.1% FA or were shipped in 0.4 ml 50% MeCN/0.1% TFA. Aliquots were stored at −80 °C until use. All peptide sequences were synthesized with free N-terminal and C-terminal amino acids. S-carbamidomethylated versions of cysteine residues (CAM-C) were used. The C-terminal arginine or lysine was uniformly labeled with \(^{13}\text{C}\) and \(^{15}\text{N}\) labeled atoms.

**Cell Cultures—**The human mammary epithelial cell line MCF10A was obtained from the ATCC (Manassas, VA) and grown at 37 °C and 5% CO\(_2\) in DMEM/F12 1:1 (Invitrogen #11320) supplemented with 5% horse serum (Invitrogen), 10 mg/ml of insulin (Sigma #I8634), 20 ng/ml of EGF (PeproTech #AF–100–13), 0.5 mg/ml of hydrocortisone (Sigma #H-0888), 100 ng/ml of cholera toxin (Sigma #C-8052), 100 units/ml of penicillin, and 100 mg/ml streptomycin. Cells that were heavy-labeled for the discovery experiment were grown in the presence of 1.0 mg/ml L-Arginine (\(^{13}\text{C}_6\), \(^{15}\text{N}_4\)) and 0.1 mg/ml L-Lysine (\(^{13}\text{C}_6\), \(^{15}\text{N}_2\)) (Cambridge Isotope Laboratories, Tewksbury, MA, # CNLM-539-H-0.1 and CNLM-291-H-0.1) in the medium. Cells were grown to 80% confluence in 100 mm plates before treatment with methyl methane-sulfonate (MMS, Sigma #129925) or ionizing radiation (see below). After incubation for the indicated times, growth medium was removed, cells rinsed in 0.25% trypsin/EDTA solution (Gibco #25200–056), and lifted off the plates by incubation in a fresh aliquot of 0.25% trypsin/EDTA solution at 37 °C, 5% CO\(_2\). When cells had lifted from the plate, the trypsin was quenched by the addition of three volumes of DMEM/F12 with 5% horse serum.

Following informed consent, peripheral blood mononuclear cells (PBMC) were obtained from healthy adults (Fred Hutchinson Cancer Research Center IRB #9026, 8233, and 6421). Whole blood samples were collected by venipuncture into K₂EDTA tubes and maintained at
ambient temperature during transportation to the laboratory. PBMC were isolated from whole blood using Histopaque-1077 (Sigma #10771) density gradient. Whole blood was diluted with an equal volume of PBS, layered over a one-third volume of Histopaque (density = 1.077 g/ml) at room temperature, and centrifuged at 400 x g for 30 min with no brake. PBMCs were harvested from the Histopaque-plasma interface, washed twice in PBS, and treated once with Red Blood Cell Lysis Buffer (5-Prime #2301310). The T-cell population was activated and expanded using a 1:1 cell to bead ratio of CD3/CD28 Dynabeads (Invitrogen # 111-311). Culturing was done in Advanced RPMI 1640 (Gibco #12633012) supplemented with 10% heat-inactivated FBS (Hyclone #SH30071.03HH), 100 units/ml of penicillin, 100 units/ml streptomycin (Gibco #15070-063), 2 mM L-Glutamine (Gibco #25030-081), 100 units/ml IL2 (Life Technologies #PHC0027). Cells were cultured for 9 days at 37 °C and 5% CO2 with fresh growth medium added every two to 3 days. On day 7, cells were resuspended in fresh medium at 1.25 x 10^6 cells/ml in T75 flasks and allowed to equilibrate at 37 °C, 5% CO2 for 36 to 40 h before treatment with DNA-damaging agents.

Cell Treatments—Cells treated with 10 Gy ionizing radiation (IR) were irradiated in a JL Shepherd Mark I irradiator using a 137Cs source delivering a dose rate of 4.7 Gy/minute. All flasks were returned to the incubator for the indicated length of time at 37 °C, 5% CO2, and then cells were harvested and lysates generated. Control cells were mock-irradiated; the mock-irradiated cells were handled in precisely the same manner as the irradiated cells but the irradiator was not turned on. MMS was diluted in growth medium just prior to addition to cell cultures for a final concentration of 0.5 mM. Cells were incubated for the indicated length of time at 37 °C, 5% CO2 before being harvested.

Protein Lysates—Cells were harvested in prechilled tubes, aliquots were removed for counting, and cells were spun down and washed 2 times in an equal volume of ice cold PBS. Cell count was determined with a Beckman Coulter Z1 Particle Counter. Cells were lysed at 5 x 10^6 cells/ml in freshly prepared ice cold Urea Lysis Buffer (6 M Urea, 25 mM Tris (pH8.0), 1 mM EDTA, 1 mM EGTA containing protease and phosphatase inhibitors (Sigma, #P0044, #P5726, and #P8340)). Lysates were sonicated 2 x 12 s and then cleared by centrifugation at 20,000 x g, 10 min at 4 °C. Supernatants were transferred to cryovials, stored in liquid nitrogen and thawed on ice. Protein lysate concentration was measured in triplicate using Micro BCA Protein Assay Kit (Thermo # 23235). Prior to downstream analysis, all samples were blind and the analysis order randomized within and across batches.

Protein Digestion—Lysates were reduced in 6% TCEP for 30 min at 37 °C with shaking, followed by alkylation with 50 mM IAM in the dark at room temperature. Lysates were then diluted 1:10 with 200 mM TRIS, pH 8, before trypsin was added at a 1:50 trypsin/protein ratio by mass. After 2 h, a second trypsin aliquot was added at a 1:100 trypsin/protein ratio. Digestion was carried out overnight at 37 °C with shaking. After 16 h, the reaction was quenched with FA (final concentration 1% by volume). For MRM studies, a mix of SIS peptides was spiked at 50 fmol (250 fmol/mg protein), levels high enough above the LLOQ so as not to contribute unnecessarily to the assay CV and close to expected endogenous levels so that the peak area ratio was not outside of the range of 100:1 and 1:100. The mixture was desalted using Oasis HLB 96-well plates (Waters #WAT058951) and a positive pressure manifold (Waters Kingfisher #97002540) loaded with 100 μl magnetic beads from 5% bead suspension. The plate containing samples and beads was mixed on the titer plate shaker (Lab Line Instruments) at speed 4 for 30 min at room temperature. A KingFisher magnetic particle processor (Thermo Fisher) with a PCR head was used for all bead handling. Beads were mixed in the incubation plate for 5 min, and then transferred for three washes (1 min each in 0.1% TFA in 80% ACN, 200 μl). Enriched peptides were eluted in 200 μl of 1:1 acetonitrile/1:20 ammonia:water for 5 min. The elution plate was dried using a GenVac vacuum centrifugation system and samples were covered with adhesive foil and frozen at -80 °C until analysis.

Fe3+–NTA magnetic agarose beads were recycled by washing used beads 3 x with 1 ml water and then following the procedure for preparing Ni-NTA-magnetic beads described above.

Nano-liquid Chromatography-Tandem Mass Spectrometry—Targeted LC-MRM-MS analysis was performed by a trap-eluot configuration on a nanoLC-2D and ESI-FLIC-Nanoflex system (Eksigent Technologies, Dublin, CA) coupled to a 5500 QTRAP mass spectrometer (ABSciex, Foster City, CA) by an Advance CaptiveSpray source (Michrom Bioresources, Auburn, CA). Mobile phases consisted of 0.1% FA in water (A) and 90% MeCN with 0.1% FA (B). 4 μl of sample was loaded onto a 200 μm x 0.5 mm ChromXP C18-CL 3 μm 120 Å column (Eksigent) using the following method: hold at 1% B and 10 μl/min for 2 min, gradient from 1 to 50% B and 2 μl/min for 0.1 min, hold 50% B and 2 μl/min for 62.9 min, gradient from 50 to 1% B and 10 μl/min for 0.1 min, re-equilibrate at 1% B and 10 μl/min for 14.9 min. The column temperature was 40 °C. The sample was injected onto the analytical column at 2 min and separated by a 75 μm x 15 cm ChromXP C18-CL 3 μm 120 Å column (Eksigent) using the following gradient method: hold at 1% B for 3 min, gradient from 1 to 10% B for 7 min, gradient from 10 to 25% B for 30 min, gradient from 25 to 40% B for 15 min, gradient from 40 to 60% B for 10 min, gradient from 60 to 90% B for 1 min, hold 90% B for 3 min, gradient from 90 to 1% B for 1 min, re-equilibrate at 1% B for 10 min. The flow rate was 300 nL/min and the column temperature was 40 °C. The MS was used in positive ion mode with parameters consisting of a 1200 V ion spray voltage, curtain gas setting of 10, nebulizer gas setting of 0, and an interface heater temperature of 110 °C. CE was described above, DP was set to 90, EP was set to 10, OXP was set to 10, and Q1 and Q3 were 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 10 points across the peaks.

MRM Mass Spectrometry Data Analysis—MRM peak integration was performed by Skyline (34), and the integrations 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 ≥1 transition from the endogenous peptide exactly co-eluting with ≥2 transitions from the stable isotope-labeled peptide, with the relative intensity of the light transition(s) deviating no more than 20% compared with the relative intensity of the corresponding heavy transitions. Integration results were exported to the program R for linear regression and statistical analysis. Peptide concentrations are calculated as the peak area ratio of the quantifying transition times the concentration of SIS peptide.

Quantitative MRM Assay Development and Characterization—Spectral libraries created in Skyline from discovery proteomics data and synthetic peptide QC data were used to select transitions for

IMAC-MRM Enables Antibody-free Phosphopeptide Quantification (33) with the following modifications. Ni-NTA-magnetic beads (Qiagen, Valencia, CA) were stripped with 100 mM EDTA and then incubated in a 10 μl FeCl3 solution to prepare magnetic Fe3+–NTA-agarose beads. Peptide enrichment was performed out of 200 μg of lysate digest reconstituted in 200 μl of 0.1% TFA in 80% ACN in 96-well plates (Thermo Kingfisher #97002540) loaded with 100 μl magnetic beads from 5% bead suspension. The plate containing samples and beads was mixed on the titer plate shaker (Lab Line Instruments) at speed 4 for 30 min at room temperature. A KingFisher magnetic particle processor (Thermo Fisher) with a PCR head was used for all bead handling. Beads were mixed in the incubation plate for 5 min, and then transferred for three washes (1 min each in 0.1% TFA in 80% ACN, 200 μl). Enriched peptides were eluted in 200 μl of 1:1 acetonitrile/1:20 ammonia:water for 5 min. The elution plate was dried using a GenVac vacuum centrifugation system and samples were covered with adhesive foil and frozen at -80 °C until analysis. Fe3+–NTA magnetic agarose beads were recycled by washing used beads 3 x with 1 ml water and then following the procedure for preparing Ni-NTA-magnetic beads described above.
optimization. The MS/MS spectra to the phosphopeptide targets can be found in supplemental data File S1. Optimal collision energy for a hybrid triple quadrupole/linear ion trap mass spectrometer (5500 QTRAP) for a subset of the peptides was determined by injecting 50 fmol standard peptide solutions into a flow of 30% MeCN, 0.1% FA at a flow rate of 1 μl/min. Optimal values were determined by ramping the potentials. From these results, a regression line was calculated in Skyline and used for the collision energy for all phosphorylated peptides in the assay panel. The top 3 (or more) transitions were selected for method development based on the presence of abundant y-ions at m/z greater than the precursor. In the absence of high m/z y-ions, the most abundant fragment ions were selected. Transitions were also selected that would differentiate between potential phosphorylation sites on the peptide. A mixture of the standard stable isotope-labeled synthetic peptides contained 190 phosphopeptides (77 purified and 113 crude) was prepared to target 165 phosphosites. The list of phosphopeptides and MRM transitions to these 190 phosphopeptide targets can be found in supplemental data File S1. Confirmation of site localization was performed on all peptides by confirmation of equivalent elution times and transition ion profiles of endogenous phosphopeptides and their synthetic standards. All phosphosites with a probability score > 0.8 (35) in the discovery data were confirmed by this approach. Five of the peptides contained multiple potential phosphosites with probability score > 0.8. We confirmed endogenous detection of 2 (out of 5) peptides with phospho-isoforms present. Retention times on the LC platform were empirically determined using the mixture of the standard stable isotope-labeled synthetic peptides in a nonscheduled fashion. Once determined, scheduled retention times were used with 200 s intervals and a target cycle time of 1.5 s.

Response curves were performed in a background matrix consisting of a pool of cell lysates (an equal mixture of MCF10A + PBMC cells exposed to 10Gy IR and 0.5 mM MMS + IR-treated PBMC cells). Digestion of the pooled lysate was performed as described above. A reverse curve was prepared in which the SiS peptide concentration was varied over eight concentration points, with the first three points using 10-fold serial dilutions and the rest 2.5-fold, over the range 2000–0.205 fmol per sample (10,000–1.02 fmol/mg protein or 1000–0.102 fmol on column). Response curves using unpurified SiS peptides were prepared using the same dilutions. For unpurified heavy peptide standards, the reported the lower limit of detection (LOD) and lower limit of quantification (LLOQ) concentrations were approximated by assuming a 10% yield of the peptide synthesis. Crude peptide purity was assessed by MALDI-MS. Blanks (no addition of SiS peptide) were prepared and analyzed in addition to the concentration points of the curve. Three process replicates (including digestion) were prepared and analyzed at the eight concentration points (along with blank samples). Linear regression was performed using a 1/y weighting on all points having a correlation coefficient of >0.98. The LOD was obtained by using the average area of the three blank measurements plus three times the standard deviation of the noise. The LOD was defined as the lowest point measured above the LOD with CV <20%. 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. The transition with the largest linear working range, from among those transitions with an average endogenous signal in the response curve data greater than the LOD and greater than ½ of the LLOQ, was selected as the quantifying transition.

Repeatability was determined using the same pooled lysate with addition of SiS peptide concentration of 50 fmol corresponding to 250 fmol/mg. Five complete process replicates per day were prepared and analyzed on each of five independent days. Intra-assay variation was calculated as the average CV obtained within each day, inter-assay variation was the CV calculated from the individual replicates across the 5 days, and the total CV was calculated as the square root of the sum of squares of these two values.

RESULTS

Selection of Phospho-analytes to Target for IMAC-MRM—We sought to identify the hypothetical subspace of the cellular phosphoproteome that is amenable to IMAC-MRM assay development. Our strategy was to select phosphopeptides empirically identified in mass spectrometry data sets and evaluate the success rate in building targeted, IMAC-MRM assays. As proof-of-concept, we focused on the DNA damage response (DDR) network, an important phospho-sensing network associated with numerous diseases (36, 37), including cancer. For target analyte selection, we identified DNA damage-responsive phosphopeptides from three sources: mass spectrometry (MS)-based profiling of IMAC-enriched lysates (this study), deep MS-based phospho-profiling of cell lysates (this study), and well annotated (based on the literature) DDR phospho-proteins that were also detected in published mass spectrometry-based datasets (38, 39). Fig. 1 provides a detailed summary of target selection.

First, a single-step IMAC enrichment was applied to lysates generated from primary human PBMCs exposed to ionizing radiation (IR), as well as an immortalized human breast epithelial cell line (MCF10A) exposed to two DNA damaging agents (IR and the alkylating agent methyl methanesulfonate, MMS) (see supplemental Fig. S1A–S1B for an overview, as well as the Supplemental Methods). A total of 13,950 phosphopeptides were identified and quantified in the experiments (see Table I and supplemental Fig. S1C). The median number of unique phosphopeptides identified in each experiment was 4307 with an enrichment of 87%. There were a total of 142 phosphorylated peptides reproducibly responsive to DNA damage in MCF10A (21 in IR-treated MCF10A cells, 109 in MMS treated MCF10A, and 12 in both IR and MMS-treated MCF10A cells) and 424 phosphopeptides reproducibly responsive in the PBMC sample (see supplemental Methods for filter criteria). From these lists, 85 reproducibly quantified phosphopeptides were prioritized for MRM assay development based on the magnitude of their responses to genotoxic stress and their known association with the DNA damage response (supplemental Table S1 contains an annotated list of selected phosphopeptides).

Next, given the higher sensitivity of MRM compared with shotgun MS/MS, we selected phosphopeptides identified by shotgun MS/MS in more deeply fractionated samples. “Deep” phospho-profiling experiments were conducted in PBMCs from two individual donors exposed to 10 Gy IR (controls were mock-irradiated). Lysates were fractionated by basic reverse-phase chromatography prior to IMAC enrichment and LC-MS/MS analysis to provide deeper coverage of the phosphoproteome (see supplemental Fig. S2A, as well as the Supplemental Methods). In total, 42,186 unique phosphopep-
tides were identified in the deep profiling experiments (Table II). The median number of phosphopeptides identified in a single sample was 19,522 with an enrichment of 92% (Table II and supplemental Fig. S2B). From the phosphopeptides identified and responsive in the deep profiling experiment (supplemental Fig. S2C), we selected an additional 19 phosphopeptides for assay development based on the magnitudes of responses and their known association with the DNA damage response (see supplemental Table S1 for the annotated selection list).

Finally, we selected phosphopeptides identified in published mass spectrometry data. A list of genes associated with the DNA damage response was curated from the literature, and 61 phosphopeptides mapping to the protein products of these DDR gene products and observed in our data sets or by LC-MS/MS in published studies (38, 39) were included in the list for targeted assay development (see supplemental Table S1 for an annotated list).

Fig. 1 summarizes the target selection process. As described above, spanning our one step IMAC experiment, “deep” phospho-profiling experiment, and literature mining, we prioritized 165 unique DNA damage-responsive phosphosites for development of targeted MRM assays; these targets represent 155 unique peptide sequences (some are multiply phosphorylated) mapping to 131 unique genes.

**IMAC-MRM Assay Method Development**—The IMAC enrichment step was refined and partially automated to increase throughput, maintain high reproducibility, and decrease the amount of input material required. The method for the empirical target discovery experiments (described above) used agarose IMAC beads to enrich phosphopeptides from 500 mg of digested protein. For development of IMAC-MRM assays, we refined the IMAC method to use magnetic beads on an automated magnetic particle processor using 200 mg of input protein lysate. The MRM assay was used to compare the performances of the refined versus the original IMAC method with respect to phosphopeptide recovery and assay reproducibility. Fig. 2A shows the comparison of peak area ratios and absolute peak areas using the agarose and magnetic bead methods. Peak areas are generally greater in the magnetic bead method, indicating greater phosphopeptide recovery/enrichment. In addition, the magnetic beads allowed for detection of far greater number of the phosphopeptides targeted by the MRM assay (n = 107 using magnetic beads versus n = 59 with agarose beads). Quantification results based on peak area ratio for phosphopeptides detected in both samples are highly correlated (R² = 0.9784), demonstrating consistency.

The magnetic beads can also be recycled for re-use, generating cost savings. Replacing the chelating iron with fresh reagent and using the recycled beads to enrich phosphopeptides from a separate aliquot of the cell lysate demonstrated a
phosphopeptides using magnetic beads. Enrichment was performed from 500 μg lysate for magnetic beads. Enrichment was performed from 500 μg lysate for agarose IMAC beads and magnetic agarose IMAC beads. Enrichment was refined by using magnetic beads to produce a robust, reproducible procedure for phosphopeptide enrichment. A high correlation (R² = 0.9963; peak area ratio slope = 1.01; peak area slope = 0.75–0.86) between the bead batches (Fig. 2C). Based on these data, the magnetic bead IMAC protocol was used for IMAC-MRM assay development, as described below.

**IMAC-MRM Assay Characterization**—Performance figures of merit for the multiplexed MRM assay and enrichment method were determined using response curves and validation samples, according to best practices using a fit-for-purpose approach (40, 41). Response curves were used to characterize the linear range, LOD, and LLOQ. Synthetic, heavy stable isotope-labeled versions of each phosphopeptide analyte targeted in the MRM assay were added to a pool of cellular lysates (an equal mixture of MCF10A + 10 Gy IR, MCF10A + 0.5 mm MMS, and PBMCs + 10 Gy IR) over several orders of magnitude of concentration with no addition of light peptide. The response was measured by evaluating the heavy peptide signals relative to the light endogenous peptides. Varying the heavy peptide amounts enables estimation of the linear range and detection limits directly in the background matrix of interest without interference from the endogenous peptides (40). The transition with the largest linear range was used as a quantifying transition, and the remaining transitions were used to confirm the specificity of the assay for the targeted analyte. Specificity was established by equivalent elution times and equivalent relative peak intensities for transitions for light and heavy peptides. Site localization was confirmed for all targeted phosphopeptides using the synthetic standards as reference (see Experimental Procedures). LOD was calculated as the average signal in blanks plus three times the standard deviation of the noise. LLOQ was reported as the lowest point in the response curve measured with CV<sup>±</sup>20%.

An example response curve is shown in Fig. 3A (response curves are provided for all analytes in supplemental Fig. S3), and performance figures of merit are reported in supplemental Table S2. The majority of working assays had a linear range of ≥3 orders of magnitude. The median LLOQ was 6.4 fmol/mg (0.64 fmol on column) (Fig. 3B). Repeatability was characterized by performing the measurement of endogenous analyte peptide in the background cell lysate mixture over five separate days, including daily complete process replicates (n = 5). Heavy peptides were spiked into the digested lysate at 250 fmol/mg protein lysate (50 fmol per sample based on the pure heavy peptide concentrations). Intra-assay (within day), inter-assay (between day), and total variability are reported in supplemental Table S2. The median intra-assay variability was 9.3%, the median inter-assay variability was 12.7%, and the median total CV was 16.0% (Fig. 3C).

**Fig. 2.** Method optimization for robust IMAC enrichment of phosphopeptides using magnetic beads. IMAC sample handling was refined by using magnetic beads to produce a robust, reproducible procedure for phosphopeptide enrichment. A, Comparison of peak area ratio and absolute peak area for phosphopeptides enriched using agarose IMAC beads and magnetic agarose IMAC beads. Enrichment was performed from 500 μg lysate for agarose and 200 μg lysate for magnetic beads. Spike levels of heavy stable isotope-labeled peptides were adjusted according to input material, so 2.5 times more heavy peptides were added to the agarose bead samples. Peak areas for heavy peptides are plotted in orange, light peptides are plotted in blue. B, Magnetic beads were regenerated by collecting used beads, and recoupling the Fe metal. Beads were re-used to enrich phosphopeptides from two identical aliquots of 200 μg cell lysate. C, Magnetic beads from different manufacturer lots were compared by enriching phosphopeptides from the aliquots of the same lysate. Error bars are the standard deviation of process triplicates. Regression for peak areas was performed using a y-weighted linear fit. The correlation coefficient (R²), slope (m), and number of phosphopeptides measured (n) are reported on each plot.
Successful validation was defined as detection of endogenous signal greater than the limit of detection in more than half of all sample runs with a total variability $\leq 30\%$ CV. Under these stringent criteria, the overall success rate of validating assays was 65% (107 of 165). A list of validated assays is presented in Table III. Thirty phosphopeptides were not detected at endogenous levels in the cell lysate background used for validation, and 28 assays had total variability $>30\%$ CV. Assays that failed validation because of high day-to-day variability were generally associated with endogenous signals at or near the limit of detection or had relatively high hydrophobicity, likely resulting in instability of the peptide standards because of adsorption losses or solubility issues. Characterization data and standard operating protocols for all validated IMAC-MRM assays have been made available as a resource for the community via the CPTAC assay portal (assays.cancer.gov) (42), where in addition to viewing the data, documentation describing the characterization experiments can be browsed and downloaded.

Quantifying Cell Signaling in Primary and Immortalized Human Cells—For proof-of-principle and to demonstrate the utility of the approach, the 107 validated multiplex phosphopeptide MRM assays were applied to measure time-dependent changes in phosphorylation following DNA damage. Human PBMCs were treated with IR, and MCF10A cells were treated with IR or MMS; controls were mock-treated. Cells were harvested at timepoints ranging from 0.25 to 24 h following treatment (7 time points from 0.25 to 24 h for PBMCs, 4 time points from 2 to 24 h for IR-treated MCF10A cells, and 4 time points from 1 to 24 h for MMS-treated MCF10A cells). Three biological replicates for each sample were independently processed in a blinded and randomized fashion using the multiplexed assay protocol on separate days. Endogenous levels of each phosphopeptide are reported in supplemental Table S3. The precision of the assay in the proof-of-principle study was high; endogenous measurements had a median process CV of 10.7%, and a median biological CV across all time points of 19.1% (supplemental Table S4).

Each analyte in the multiplex panel represents a characteristic pharmacodynamic profile in response to DNA damage. Several examples are shown in Fig. 4 (profiles for each peptide are shown in supplemental Fig. S4). Checkpoint signaling is activated by the ATM kinase in response to DNA double strand breaks (43) via autophosphorylation at several sites, including Ser$^{1981}$ (44), Ser$^{367}$ (45), and Ser$^{2996}$ (46, 47). Fig. 4 shows increased phosphorylation of ATM$^{P52996}$ and ATM$^{P5367}$ reaching a maximum within one hour of irradiation. Direct targets of the ATM kinase can also be seen to be induced following irradiation. Phosphorylation of Ser$^{1083}$ on SMC3, a target of ATM (48), follows a similar profile. Phosphorylation of Ser$^{1524}$ on BRCA1, shown to be ATM-dependent following IR-induced DNA damage (49, 50), is seen to be increased for up to four hours following irradiation. Furthermore, phosphorylation of one of the components of the

**Fig. 3.** IMAC-MRM assay validation. A, Representative response curve for the phosphopeptide NYPpSQELIK (pS1524 on BRCA1). Each point measures the peak area ratio of heavy to light peptide (heavy spiked into 200 µg aliquots of cell lysate digest; light measured as endogenous analyte). The inset shows the response curve plotted on logarithmic scale. Error bars are the standard deviation of three process replicates. B, Distribution of the LLOQ for 107 validated assays. Limits of quantification were determined by the lowest point measured with CV less than 20%. C, Assays were validated by measuring endogenous phosphopeptide in five replicates on each of 5 days. Intra-assay variation reports the distribution of average %CV of five within-day process replicates. Inter-assay variation is the distribution of the square root of the sum of squares for intra- and inter-assay variation. Data for 107 validated assays are plotted. Box plots show the median (bar), inner quartiles (box), 5–95th percentiles (line), and outliers (whisker).
### TABLE III

**List of validated IMAC-MRM assays**

| Gene symbol | Modified peptide sequence | PO4 site | Gene description |
|-------------|---------------------------|----------|------------------|
| ABLIM1      | STS(ph)QGSINSVPYSR         | pS452    | actin binding LIM protein 1 |
| AHNAK       | LPS(ph)SSGASAAPTSGAVDIR    | pS210    | AHNAK nucleoprotein |
| ATM         | NLS(ph)DIDQSNFK            | pS2996   | ataxia telangiectasia mutated |
| ATM         | SLEIS(ph)QSYTTTQR          | pS367    | ataxia telangiectasia mutated |
| ATRIP       | LS(ph)DGDMTSALR            | pS518    | ATR interacting protein |
| ATRIP       | LAAPSVHSV(ph)PR            | pS224    | ATR interacting protein |
| BCA1        | NYPS(ph)QEELIK             | pS1524   | breast cancer 1, early onset |
| BCA2        | KPS(ph)YNQLASTPIIFK        | pS70     | breast cancer 2, early onset |
| BRI1        | ATPELGSSSASS(ph)PPR        | pS1032   | BCA1 interacting protein C-terminal helicase 1 |
| C16orf54    | SEDWYG(ph)AVPLLDR          | pS98     | Transmembrane protein C16orf54 |
| C17orf70    | APS(ph)PLGPITR             | pS667    | Fanconi anemia-associated protein of 100 kDa |
| C20orf50    | LPS(ph)TDGGYIDLQFK         | pS24     | Transmembrane protein 230 |
| CD3EAP      | QEDINTEPLEDVTLS(ph)PTK     | pS285    | CD3e molecule, epsilon associated protein |
| CD3EAP      | FSCPNFTAKPPASES(ph)PR      | pS27     | CD3e molecule, epsilon associated protein |
| CDC42EP3    | ANS(ph)TSDVFTETSPVSLK      | pS389    | CDC42 effector protein (RhD GTPase binding) 3 |
| CHEK1       | YSS(ph)QPEPR               | pS317    | CHK1 checkpoint homolog (S. pombe) |
| CLSPN       | SLS(ph)SDSTLLLFK           | pS720    | clasin |
| CTN         | LPPS(ph)PVYDAAASK          | pS418    | cortactin |
| DOCK2       | VEONEPSI(ph)PGSTLPEVK      | pS1685   | dedicator of cytokinesis 2 |
| DYN111      | KPVTVSPPTTTPS(ph)PTEGAEAS  | pS516    | dynein, cytoplasmic 1, light intermediate chain 1 |
| ELAV1       | NVALLSQYHSP(ph)PAR         | pS202    | ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen?) |
| EPS15       | LDS(ph)PDPFK               | pS796    | epidermal growth factor receptor pathway substrate 15 |
| EPS15       | IGT(ph)TPRPCPLPPGK         | pT777    | epidermal growth factor receptor pathway substrate 15 |
| FAM129B     | AAPEASS(ph)PPASPLQHLLPGK   | pS692    | family with sequence similarity 129, member B |
| FANC1       | SADFS(ph)OSTSIIGK          | pS730    | Fanconi anemia, complementation group I |
| GI2FY2      | S(ph)FDEVEGFGR             | pS139    | GRB10 interacting GYF protein 2 |
| HP1B3       | TIPSWATLAS(ph)QLAR         | pS142    | heterochromatin protein 1, binding protein 3 |
| HSPB1       | GPS(ph)WDPPR               | pS15     | heat shock 27kDa protein 1 |
| IL16        | LLS(ph)TQAEEQGVPVLK        | pS846    | interleukin 16 (lymphocyte chemoattractant factor) |
| JUN         | NSDLT5S(ph)PVDGGLLK        | pS63     | jun proto-oncogene |
| KIF4A       | TFS(ph)LTEVR               | pS801    | kinesin family member 4A |
| LASP1       | GFSWADTP(ph)PELOR          | pT104    | LIM and SH2 protein 1 |
| LAT2        | LLOFYPS(ph)LEDAPSSR        | pS86     | linker for activation of T cells family, member 2 |
| LCK         | NLDNGFYF(ph)JISPR          | pY192    | lymphocyte-specific protein tyrosine kinase |
| LIG1        | EGEDGDOPTT(ph)PPKPLK       | pT183    | ligase I, DNA, ATP-dependent |
| LMNA        | S(ph)VGGSGGSGFDNLVTR       | pS628    | lamin A/C |
| LMNA        | SVGGS(ph)VGGSGFDNLVTR      | pS632    | lamin A/C |
| MAPK4       | AASSNLNS(ph)NGETESVK       | pS805    | mitogen-activated protein kinase kinase kinase kinase 4 |
| MARCKS      | GEPPAAEAPEAGAS(ph)PVEK     | pS101    | myristoylated alanine-rich protein kinase C substrate |
| MAVS        | GPVs(ph)PSVSFGPLAR         | pS222    | mitochondrial antiviral signaling protein |
| MDC1        | AQPFGFID(S)DTDAEER         | pS329    | mediator of DNA-damage checkpoint 1 |
| MDC1        | GPGAPGLHLOSEQAS(ph)TDVEEGK | pS376    | mediator of DNA-damage checkpoint 1 |
| MED24       | LLS(ph)SNEIIANLSPTDR       | pS862    | mediator complex subunit 24 |
| MLL2        | ASEPLS(ph)PFPGESR          | pS2274   | myeloid/lymphoid or mixed-lineage leukemia 2 |
| MOCOS       | SLS(ph)POEDALTSR           | pS530    | molybdenum cofactor sulfatase |
| MYBBP1A     | EIPSATG(S)PhIPSK           | pS1163   | MYB binding protein (P160) 1a |
| MYO9B       | VSPPAGS(ph)APETPDEK        | pS1267   | myosin IXB |
| MYO9B       | VQQEKPS(ph)PGSTQIQR         | pS1290   | myosin IXB |
| NBN         | TTPGPSLS(ph)PGVSDKE        | pS343    | nibrin |
| NBN         | IPNYOLS(ph)PTK             | pS432    | nibrin |
| NCL         | GGFVFDFNS(ph)EDEAK         | pS619    | nucleolin |
| NFATC1      | GLGACTLLGS(ph)PR           | pS233    | nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 1 |
| NSUN2       | ESTQLS(ph)PADLEKGPTDPSK    | pS456    | NOP2/Sun domain family, member 2 |
| NUFIP2      | NDS(ph)WGSGFSLR            | pS652    | nuclear fragile X mental retardation protein interacting protein 2 |
| Gene symbol | Modified peptide sequence | PO4 site | Gene description |
|-------------|---------------------------|----------|------------------|
| NUMA1       | VSLEPHQGPGT(ph)PESK        | pT2000   | nuclear mitotic apparatus protein 1 |
| NUP107      | SGFGEISS(ph)PVIR           | pS11     | nucleoporin 107kDa |
| PALB2       | TVEEDCDSL(ph)QDQLSPQLK     | pS59     | partner and localizer of BRCA2 |
| PALLD       | IAS(ph)DEEEEQGTK           | pS893    | palladin, cytoskeletal associated protein |
| PGM1        | AIGGILLAS(ph)HNPGGNPGDFGIK | pS117    | phosphoglucomutase 1 |
| PHACTR2     | ASIANSODGPTAGSTQ(ph)PPFK   | pT25     | phosphatase and actin regulator 2 |
| PKP3        | AGGLWDWEAVE(ph)PSPR        | pS238    | plakophilin 3 |
| PLEC1       | S(ph)GGGAGGSGVLDPAER       | pS42     | PLEC_isoform2 |
| PLEKHA2     | SI(ph)LTRPGSSLSGPNFLCR     | pS314    | pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 2 |
| PPP1R10     | VLS(ph)PTAAKPSFEGK         | pS313    | protein phosphatase 1, regulatory (inhibitor) subunit 10 |
| PRKRA       | EDS(ph)GTFSGLK             | pS18     | protein kinase, interferon-inducible double stranded RNA dependent activator |
| PWP1        | NSS(ph)SGFGGSR             | pS485    | PWP1 homolog (S. cerevisiae) |
| RAD18       | NDLOTEIS(ph)PIS           | pS471    | RAD18 homolog (S. cerevisiae) |
| RAD18       | NHLLOFALES(ph)PAK          | pS99     | RAD18 homolog (S. cerevisiae) |
| RANBP2      | FESPAQGIL(ph)PVR          | pS955    | RAN binding protein 2 |
| RAVER1      | LLS(ph)PLSSAR              | pS676    | RAVER1_isoform2 |
| RB1         | TAVIPING(ph)PR             | pS249    | retinoblastoma 1 |
| RB1         | IPGNNIYSI(ph)PLK           | pS807    | retinoblastoma 1 |
| RBM7        | SFS(ph)SPENFQR             | pS136    | RNA binding motif protein 7 |
| RCSD1       | SQS(ph)DCGELGDR           | pS179    | RCSD domain containing 1 |
| RDBP        | SI(ph)ADDQLGESSR          | pS115    | RD RNA binding protein |
| REV3L       | SGTL(ph)PFEIFEK           | pS1724   | REV3-like, catalytic subunit of DNA polymerase zeta (yeast) |
| RFTN1       | GDHAS(ph)LENEKPGTGDCVSAAPGR| pS199    | raftlin, lipid raft linker 1 |
| RPL12       | IGPLGS(ph)JK              | pS38     | ribosomal protein L12 |
| SAMHD1      | NFTKPDQGDIAPLIT(ph)PQK     | pT592    | SAM domain and HD domain 1 |
| SAPS3       | I/OQFDGGGS(ph)DEEDEWEEK   | pS617    | protein phosphatase 6, regulatory subunit 3 |
| SGOL2       | DNFSSL(ph)PJK             | pT845    | shugoshin-like 2 (S. pombe) |
| SH2D4A      | TLS(ph)SSAQEDIR           | pS315    | SH2 domain containing 4A |
| SH3BP1      | TSEVPPPAPAS(ph)PK          | pS544    | SH3-domain binding protein 1 |
| SLC9A1      | CLS(ph)DGPHPEGEPFPPK      | pS796    | solute carrier family 9 (sodium/hydrogen exchanger), member 1 |
| SMC3        | GSGS(ph)QSSVPDQFTVGIR     | pS1083   | structural maintenance of chromosomes 3 |
| SNRPN70     | GGGGGGDNGLEGLGDNDS(ph)P    | pS410    | small nuclear ribonucleoprotein 70kDa (U1) |
| SNRPC       | FCYDCYTDLTHDS(ph)PSVR     | pS17     | small nuclear ribonucleoprotein polypeptide C |
| SRRM2       | ENS(ph)FGSPLEFR           | pS1326   | serine/arginine repetitive matrix 2 |
| STAU2       | VISGTTLYLS(ph)PJK          | pS440    | staufen, RNA binding protein, homolog 2 (Drosophila) |
| STMN1       | ASGQAFEULIS(ph)PR         | pS25     | stathmin 1 |
| STMN1       | AS(ph)QGAFEULISPR         | pS16     | stathmin 1 |
| TACC2       | FSS(ph)PTELDLYR           | pS2512   | transforming, acidic coiled-coil containing protein 2 |
| TMP0        | HAS(ph)PILITEFSIDPR       | pS306    | thymopoietin |
| TNKS1BP1    | YESQEPLAQEQAES(ph)PLLATR  | pS601    | tankyrase 1 binding protein 1, 182kDa |
| TNKS1BP1    | S(ph)OEVADVQDOEWR         | pS836    | tankyrase 1 binding protein 1, 182kDa |
| TOPBP1      | NAVALSA(ph)POLK           | pS888    | topoisomerase (DNA) II binding protein 1 |
| TP53        | ALPNNTSSS(ph)PQPK         | pS315    | tumor protein p53 |
| TP53BP1     | NIS(ph)PEDLGLSLTDGSDK     | pS500    | tumor protein p53 binding protein 1 |
| TP53BP1     | SGTATEPVEQDS(ph)SQPSLPLVR | pS830    | tumor protein p53 binding protein 1 |
| TPX2        | SSDQPLTVPVS(ph)PJK        | pS738    | TPX2, microtubule-associated, homolog (Xenopus laevis) |
| USP1        | ALDFTDS(ph)QENEK          | pS42     | ubiquitin specific peptidase 1 |
| VIM         | ETNLD(ph)LPLVTDHSDK       | pS430    | vimentin |
| VIM         | SLYSAS(ph)PGGVYATR        | pS56     | vimentin |
| YLPM1       | GASPQYITPSTSL(ph)PJR      | pS634    | YLP motif containing 1 |
| ZC3H11A     | TVLVTVPES(ph)PEEEKV       | pS108    | zinc finger CCHC-type containing 11A |
| ZCCHC8      | GT(ph)PPPVPFPLPK          | pT479    | zinc finger, CCHC domain containing 8 |
| ZYX         | S(ph)PGAPGPLTLK           | pS344    | zyxin |
NBS1pS343, is seen to rapidly increase following irradiation, consistent with prior reports (52, 53). Activation of downstream targets is also evident in the kinetic profiles. Phosphorylation of Ser315 on TP53 is seen to increase at later timepoints compared with ATM. TP53pS315 is phosphorylated by cyclin B1/CDK1 (54, 55), which is activated indirectly by ATM (56).

Response of the DDR network, as measured by the multiplex IMAC-MRM assay, is shown more globally by plotting heatmaps of the relative response for each assay in the three sample types/treatments (Fig. 5). All but one of the 107 phosphorylated peptides targeted by MRM were measured in at least one of the time points in the three time-course studies. Overall, there are notable differences in both the responsive analytes and their kinetic patterns that vary by cell type (MCF10A versus PBMC) and DNA damaging agent (IR versus MMS). In comparing the MCF10A 10Gy IR and MMS treatments, the response generally occurs at later timepoints in the MMS-treated samples. In IR-treated samples, most analytes have returned to mock-irradiated levels by 24 h, whereas in MMS-treated samples they are continuing to increase/decrease between 6 and 24 h post-treatment. There are a larger number of analytes with large responses in the PBMCs samples compared with MCF10A cell lines.

DISCUSSION

This study demonstrates the feasibility of quantifying cell signaling through coupling a simple, one-step IMAC enrichment of phosphopeptides with quantitative MRM mass spectrometry. The optimized enrichment approach requires a relatively small amount (200 µg/H9262) of input material, samples can be processed in parallel, and sample handling is largely automated using magnetic beads. The high specificity and use of internal standards for quantification in MRM assays offer substantial advantages compared with the current gold standard, Western blotting (WB). Nonspecific bands in WB and uncertainties in phosphosite quantification (6) are alleviated in the IMAC-MRM assays, because the assay uses internal standards, and the mass spectrometer directly detects the analyte of interest (i.e. not a surrogate signal such as a fluorescent tag). In addition, the IMAC-MRM assays can be transferred across laboratories and instrument platforms through the use of the internal standards, enabling standardization and improving the reproducibility of results. Of note, the IMAC-MRM assays validated in this study could replace 107 Western blots with a single 80 min MRM run, which produces a small flat file of highly specific and quantitative data consisting of ratios of the intensities of the endogenous analytes relative to intensities of their respective spiked-in stable isotope-labeled standards.

Given the success of IMAC-MRM assay validation for the phospho-analyses targeted in this study, it is reasonable to expect that additional phospho-peptides empirically observed in the shotgun MS/MS data could also be targeted by IMAC-MRM. For example, mining the empirical MS/MS data-sets from this study alone, we identified 8190 phosphopeptides that met the following filters: (1) unique to the protein of interest ("proteotypic"), (2) no missed trypsin cleavages, (3) no oxidized Met, no n-term Cys, Pro, or Gln, (4) hydrophobicity...
score between 10 and 40 (57), and (5) length between 8–25 amino acids (supplemental Table S5). This list of MRM-amenable phosphopeptides (2407 from MMS-treated MCF10A, 3132 from IR-treated MCF10A, 3,015 from IR-treated PBMC IMAC shotgun experiments, and 5793 from the “deep” phospho-profiling experiments) are considered to occupy the hypothetical space of the phosphoproteome amenable to quantification using the IMAC-MRM approach (supplemental Table S5). (Of note, additional phospho-analytes amenable to IMAC-MRM quantification may be identified using different cell types and perturbations.) Extrapolating from the success rate of IMAC-MRM assay validation in this study (which applied the same filters for analyte selection), it is reasonable to expect a 65% or better success rate (i.e. /H11022 5,300 phosphopeptides) for configuring validated assays from the larger set. This subspace of the phosphoproteome, amenable to IMAC-MRM assays, represents a large variety of biological pathways. For example, analysis of KEGG pathways enriched

![Heatmap of DDR activity](image)

**Fig. 5.** Activity of the DDR measured by the multiplexed IMAC-MRM assay. Response following DNA damage is plotted as a heat map for endogenous-detected analytes in the multiplex IMAC-MRM panel (using R script heatmap.2. Values are the log2 median phosphopeptide concentration of three biological replicates relative to the concentration measured in the mock-treatment. For visualization in the heat maps, data points from endogenous levels below the LLOQ were imputed using the LLOQ peak area for the endogenous signal.**
(FDR < 1%) in this putative MRM target list identified 57 networks with at least two proteins represented on the list (supplemental Table S6), indicating that broad range of signaling networks might be quantified using single step IMAC as an enrichment method. Furthermore, 5759 of these phosphosites contained motifs mapping to 42 kinases based on the GPS 3.0 prediction system (58) (supplemental Table S7), suggesting that many kinase motifs are well represented in the space of the phosphoproteome amenable to IMAC-MRM assay development.

IMAC is a well-established single-step procedure for highly sensitive and specific phosphopeptide enrichment. Our results show high coverage (>4000 phosphopeptides in a single enrichment, >19,000 when coupled with fractionation) and good specificity (enrichment of >85% in single step and >90% when coupled with fractionation). However, other enrichment strategies could be employed to provide complementary coverage of phosphosites (e.g. TiO2, Ga-IMAC, Zr-IMAC, or methyl esterification) (59–61).

Although the assay has many performance advantages, there are some challenges. Some sites will be difficult for MRM-based targeting (e.g. multiple phosphosites on very large tryptic peptides or nonproteotypic sequences). Using alternative enzymes (62) or expanding the assay to incorporate alternative enrichment strategies could overcome some limitations; however, it is likely that some sites will not be amenable to MRM development. Additionally, occupancy of some phosphosites near trypsin cleavage sites may affect the efficiency of trypsin digestion. In order to quantify phosphosite stoichiometry, all possible phospho-isoforms (and the corresponding nonmodified peptide) must be targeted separately in the MRM assay. Because phosphosites can be clustered on an analyte peptide and not all possible phospho-isoforms may be detected, quantitative conclusions about phosphosite stoichiometry must be made with caution, and should be characterized on an individual analyte basis.

Finally, MRM-based assays are highly distributable among laboratories (22, 25), and the assays validated in this study have been made publicly available as a resource to the community via the Clinical Proteomic Tumor Analysis Consortium (CPTAC) Assay Portal (http://assays.cancer.gov) (42), where SOPs, reagents, and assay characterization data for these assays can be viewed and downloaded. The downloadable assay information enables any research laboratory to prepare samples, which can be transferred to core facilities for MRM analysis, as is currently done for most genomic analyses. Furthermore, open-source tools for data analysis are also available (34).

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