

Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells

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Abstract

Proteomics has the potential to provide answers in cancer pathogenesis and to direct targeted therapy through the comprehensive analysis of protein expression levels and activation status. The realization of this potential requires the development of new, rapid, high-throughput technologies for performing protein arrays on patient samples, as well as novel analytic techniques to interpret them. Herein, we describe the validation and robustness of using reverse phase protein arrays (RPPA) for the analysis of primary acute myelogenous leukemia samples as well as leukemic and normal stem cells. In this report, we show that array printing, detection, amplification, and staining precision are very high, reproducible, and that they correlate with traditional Western blotting. Using replicates of the same sample on the same and/or separate arrays, or using separate protein samples prepared from the same starting sample, the intra- and interarray reproducibility was extremely high. No statistically significant difference in protein signal intensities could be detected within the array setups. The activation status (phosphorylation) was maintained in experiments testing delayed processing and preparation from multiple freeze-thawed samples. Differences in protein expression could reliably be detected in as few as three cell protein

equivalents. RPPA prepared from rare populations of normal and leukemic stem cells were successfully done and showed differences from bulk populations of cells. Examples show how RPPAs are ideally suited for the large-scale analysis of target identification, validation, and drug discovery. In summary, RPPA is a highly reliable, reproducible, high-throughput system that allows for the rapid large-scale proteomic analysis of protein expression and phosphorylation state in primary acute myelogenous leukemia cells, cell lines, and in human stem cells. [Mol Cancer Ther 2006;5(10):2512–21]

Introduction

Understanding cancer physiology and pathogenesis has traditionally focused on alterations at the DNA level that result in the expression of genes that are aberrant in location, altered in expression level, or that harbor mutations. The regulation of protein levels and function, which may also significantly define the phenotype of a cancer cell, occurs at many levels including transcription, mRNA stability, translational regulation, and perhaps, most importantly, by posttranslational modifications (e.g., phosphorylation, prenylation, ubiquitination, etc.). High-throughput technologies such as comparative genomic hybridization and transcriptional profiling provide important data on DNA and RNA levels; however, the functional consequences of these changes cannot be assessed, and confirmatory experiments need to be carried out. Expression arrays, measuring mRNA levels, are routine and informative for some of these alterations but are unable to ascertain the actual level of protein expression (1–3), and are unable to detect the posttranslational modifications of proteins. The development of reliable proteomic characterization is crucial for the more global understanding of cancer cell physiology and pathogenesis at the protein level.

Proteomics can be defined as the large-scale study of proteins, including their structure, function, and activation. The particular challenges are that the proteome differs from cell to cell, changes dynamically over time, and that polymorphisms, splice variants, and posttranslational modifications greatly expand the ascertainable variables for each protein. Attempts at the proteomic characterization of leukemic cells have mainly used matrix-assisted laser desorption/ionization-time of flight analysis after two-dimensional gel electrophoresis. The available evidence is sparse but supports the importance of proteomic analysis of leukemias, e.g., for class distinction (4), target identification (5), apoptosis initiation (6), and stem cell analysis (7). However, the proteins isolated by these

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technologies need to be identified and characterized by other means, and a more comprehensive profiling could not be achieved due to the excessive requirements for material to study and for the time required to perform each analysis. Current techniques are inadequate for high-throughput analysis of primary patient samples.

Understanding the effect and functional significance of the new targeted anticancer agents, directed at functional sites on proteins (often kinases), also requires novel technologies that allow for a sensitive, accurate, and moderate to high throughput assessment of the target of interest. Assessing off-target effects on proteins in the same or neighboring pathways will become part of a comprehensive activity profile of a drug. Application of the promise of functional proteomic analysis to the study of individual cases of leukemia therefore requires a novel, reliable, sensitive, time-, cost-, and sample-sparing, as well as high-throughput functional proteomic technology.

Reverse phase protein (micro)array (RPPA; refs. 8–10) is a new, sensitive, high-throughput, functional proteomic technology that offers many of the advantages needed. It extends the power of immunoblotting to provide a quantitative analysis of the differential expression of active (usually phosphorylated or cleaved) and parental proteins. Total proteins and their corresponding phosphoprotein configuration can be assessed reflecting the activation state/functionality of a given protein or of an entire pathway (e.g., signal transduction pathway). With RPPA, all samples are spotted at the same time, making this method ideally suited for the retrospective analysis of a large number of specimens similar to the idea of gene microarrays. Compared with a conventional Western blot, which uses protein from 5×10^5 cells, RPPA requires nanoliters of protein lysate (picograms to femtograms of protein). The protein equivalent of 200 cells is printed per array slide. Each slide is probed with a single antibody. Thus, samples prepared from only 5,000 to 20,000 cells are sufficient to analyze 100 different protein targets, and from the material previously required for a single Western blot, 2,500 slides (theoretically = 2,500 antibodies) can be printed. This enables the analysis of a much larger number of proteins from each sample and makes this technique suitable for the analysis of cell populations present in low numbers, such as stem cells or leukemic cells that survive chemotherapy ("survivor cells"). Although newer Western blot signal detection reagents, such as SuperSignal West Femto (Pierce, Rockford, IL), have signal sensitivities comparable to RPPA, they cannot compete with the high-throughput nature or miniscule cell numbers and volume requirements achievable with RPPA.

This broad assessment of protein modification and activation of an entire network has the potential to recognize meaningful protein and pathway interactions of known proteins, and can lead to new discoveries and interactions. One limitation of RPPA is that it only identifies known proteins/targets. The potential clinical application and potential benefit of RPPA in solid tumors has been previously shown (10–13).

Herein, we report a systematic validation approach to increase the reliability and reproducibility of this novel high-throughput functional proteomic technology, using primary leukemic specimens. Furthermore, we present the first proof of the utility of RPPA for analysis of human hematopoietic stem cells.

Materials and Methods

Study Group

Peripheral blood, leukopheresis, or bone marrow specimens were collected prospectively from patients with newly diagnosed acute myelogenous leukemia (AML) evaluated at The University of Texas M.D. Anderson Cancer Center between September 1, 1999 and January 1, 2004. Samples were acquired during routine diagnostic assessments in accordance with the regulations and protocols sanctioned by the Investigational Review Board of M.D. Anderson.

Generation of a Leukemia-Enriched Fraction

Samples were placed on ice immediately after collection and were processed fresh within 2 hours of collection. A leukemia cell-enriched fraction was generated by isolating the mononuclear cell fraction by Ficoll-Hypaque separation (Mediatech, Herndon, VA) followed by the depletion of CD3+/CD19+ B and T cells by magnetic antibody-conjugated sorting (Miltenyi Biotec, Inc., Auburn, CA), as previously described (14). The cells were used fresh to make whole-cell lysates for Western blotting or RPPA arrays. To assess the stability of phosphoepitopes, cryopreserved cells were thawed, kept at room temperature for 2 hours, and an aliquot was lysed for RPPA. The remainder of the cells were re-frozen. The same cycle was repeated once. Cells were then prepared in the usual fashion for RPPA.

Generation of CD34+/CD38–Stem Cell Fractions

To isolate a stem cell-enriched fraction, CD34+ cells were purified from the leukemia-enriched fraction described above by MACS (Miltenyi Biotec) and then separated into CD34+/CD38– and CD34+/CD38+ fractions by flow sorting after incubation with anti-CD34, anti-CD38 antibodies, and IgG controls (Becton Dickinson, San Jose, CA; ref. 15). Cells displaying greater fluorescence intensity than their controls were considered positive. An aliquot of sorted cells was reanalyzed for purity. Sorted and separated cells were lysed in RPPA lysis buffer as described below.

Cell Lines

Leukemia cell lines (U937, HL60, OCI-AML3, KG-1, Mfso7e, and TF1), obtained from the American Type Culture Collection (Manassas, VA), were grown in RPMI 1640 supplemented with 0.5% or 10% fetal bovine serum, 100 mg/nL of penicillin/streptomycin, 4 nmol/L of glutamate (all from Life Technologies, Rockville, MD). Cells were kept at subconfluent levels until harvest, then washed twice in ice-cold PBS and lysed in either standard Laemmli or RPPA lysis buffers for 20 to 30 minutes, centrifuged at 14,000 rpm, and the pellet was discarded. Cell lysates were diluted as described below (see RPPA).

Western Blot

Western immunoblotting analyses were done using material from 4 to 5×10^5 cells and the Bio-Rad Criterion system (Bio-Rad, Hercules, CA), with bone marrow and peripheral blood samples loaded on the same blot with control cell lines (K562 and Jurkat cells) and molecular weight markers as previously described (14). The $2\times$ lysis buffer was composed of 0.5 mol/L of Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 4% β -mercaptoethanol. Numerous Western blot studies (16, 17) have shown that these samples are free of protein degradation (assessed by the absence of actin laddering) and that the phosphoprotein status remains stable for at least a decade (for retinoblastoma protein).

Validation of Antibodies

One of the limiting factors in protein biochemistry is the availability and quality of antibodies. Each candidate antibody was subjected to a stringent validation procedure before being certified for use by RPPA. The antibodies had to have a predominant single band in Western blot against cell lines and patient samples, and not have any nonspecific binding. It is acceptable if the antibodies recognize known cell characteristics, including size variants due to cleavage, mutation, or deletions. Antibodies against phosphorylated epitopes had to show specificity against samples stimulated (e.g., growth factors) or inhibited (specific inhibitors) to yield phosphorylated or nonphosphorylated forms of a protein. Alternatively, genomically altered cells (e.g., transfected or short interfering RNA-inhibited) and cell lines could be used to validate antibodies. Finally, for antibodies passing the above criteria, results using RPPA had to parallel those seen using Western blot.

RPPA

Preparation of Cell Lysate (Adaptable for 96-Well Plate Format). The cells were washed twice in ice-cold PBS, then lysed in 30 μ L of RPPA lysis buffer [1% Triton X-100, 50 nmol/L Hepes (pH 7.4), 150 nmol/L NaCl, 1.5 nmol/L $MgCl_2$, 1 mmol/L EGTA, 100 nmol/L NaF, 10 nmol/L NaPPi, 10% glycerol, 1 nmol/L phenylmethylsulfonyl fluoride, 1 nmol/L Na_3VO_4 , and aprotinin 10 μ g/mL; alternatively, whole proteinase inhibitor tablets could be used (Boehringer/Roche, Mannheim, Germany)] for 20 to 30 minutes with frequent vortexing on ice, centrifuged for 15 minutes at 14,000 rpm, and the supernatant collected. Protein concentration was determined by routine (e.g., Bradford) assays. Lysates were transferred at volumes of 25 to 30 μ L into a PCR 96-well plate. Ten microliters of $4\times$ SDS/2-ME sample buffer (35% glycerol, 8% SDS, 0.25 mol/L Tris-HCl, pH 6.8; with 10% β -mercaptoethanol added before use) was added to each sample well. The plates were covered and incubated for 5 minutes at $95^\circ C$ and then centrifuged for 1 minute at 2,000 rpm.

For quantification purposes, protein cell lysates were serially diluted by transferring 5 μ L (or 10 μ L for 1:2 dilution steps) from each sample to the next row of a 96-well dilution plate (conical-bottomed) followed by the addition of 10 μ L of $4\times$ SDS/2-ME sample buffer and repeated for as many rows as dilutions are planned

(usually six or eight serial dilutions: full strength, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and 1/128) with the addition of lysis buffer immediately prior to array preparation in 96-well plates. The dilution plates were storable at $-80^\circ C$ until processing.

Dilutions were done with multichannel pipettes by hand. Diluted samples were transferred into 384-well plates and heated at $95^\circ C$ for 10 minutes. From these plates, the lysate material was printed onto nitrocellulose-coated glass slides (FAST Slides, Schleicher & Schuell BioScience, Inc., Keene, NH) with an automated robotic GeneTac arrayer (Genomic Solutions, Inc., Ann Arbor, MI). Up to 24 identical slides could be printed at one time. The RPPA transfer method employed is a noncontact method in which ~ 1 nL of protein lysate (corresponding to 10 cell equivalents from full-strength protein lysate) is transferred to the nitrocellulose glass slide per array pin touch. The protein concentration spotted onto the glass slides can be adjusted by varying the number of pin touches from 5 to 10 per dot-spot. This corresponds to 100 cell equivalents at 10 touches in the undiluted sample, down to 0.8 cell equivalents after eight serial dilutions, depending on the original protein concentration in a sample set. Up to 1,152 single dots can be printed onto one slide. Each spot on the array slide represents a certain dilution of the lysate of a particular sample. If six serial dilution steps are used, as many as 192 samples can be spotted on a single slide. Once printed, the slides are stable at $-80^\circ C$ and stainable for at least 6 to 12 months. Diluted protein printing plates (384-well plate) are storable at $-80^\circ C$ for at least 12 to 18 months, and can be used for multiple repeated printing processes of new array slides from the same original samples (18).

Probing

After slide printing, the same stringent conditions for slide blocking, blotting, and antibody incubation, which were used for immunoblotting, were applied. First, the microarray slides were blocked for endogenous peroxidase, avidin, and biotin protein activity prior to the addition of the primary antibody. The DAKO signal amplification system (Copenhagen, Denmark) was used to detect and amplify antibody-binding intensity. This commercially available catalyzed system kit uses 3,3'-diaminobenzidine tetrachloride and catalyzed reporter deposition of substrate to amplify the signal detected by the primary antibody (19). A biotinylated secondary antibody (anti-mouse or anti-rabbit) was used as a starting point for signal amplification. A streptavidin-biotin complex attached to the secondary antibody and biotinyl-tyramide deposition on this complex is used to amplify the reaction. Tyramide-bound horseradish peroxidase cleaves 3,3'-diaminobenzidine tetrachloride, giving a stable brown precipitate with excellent signal-to-noise ratio. This technique is sensitive and reproducible in the femtomolar sensitivity range (8).

Signal intensity was measured by scanning the slides with ImageQuant (Molecular Dynamics, Sunnyvale, CA) and quantified using the MicroVigene automated RPPA module (VigeneTech, Inc., North Billerica, MA). Using MicroVigene software, the intensity of each spot was

calculated and an intensity concentration curve was calculated with a slope and intercept.⁴ This allows the relative quantification of each sample if the expression intensities are compared with a reference standard curve generated from control lysates, and absolute quantification can be determined by comparison to known quantities of purified peptides. The ratio of signal intensity from phosphorylated and nonphosphorylated antibodies allows for relative quantification of the activation state of a given protein across samples. Differences in loading were assessed and corrected for by normalizing the expression intensities as described below (see Results). For differentially regulated proteins, immunoblotting (WB) was done to confirm the results.

Results

To establish the use of human leukemic cell lines and primary specimens for RPPA analysis, we systematically addressed and validated each experimental step of RPPA. The Leukemia Sample Bank at the University of Texas M.D. Anderson Cancer Center has systematically stored hundreds of AML patient protein samples over the past 15 years. Complete outcome data is available. Therefore, we asked if existing protein samples from the Leukemia Sample Bank would work in RPPA.

Utility and Sensitivity of RPPA

Cell lysates in the Leukemia Sample Bank were prepared using a Western blot lysis buffer containing bromophenol blue (called "blue" lysates) at a cell concentration of 10 cell equivalents per nanoliter. Samples were aliquoted into single-use vials containing 50 μ L before freezing. To assess if the prepared, stored, and ready-to-use Western blot blue lysates could be analyzed by RPPA, we compared these with newly prepared cell lysates ("clear lysates") using cryopreserved specimens from the same patient at a concentration of either 7 or 10 cells/nL equivalents (Fig. 1A). Several observations and results were made. Both blue and clear lysates gave strong signals in the linear part of the dilution curve that could be analyzed for data evaluation (Fig. 1A and D), and intrapatient variability between clear and blue lysates was minimal. Slides in Fig. 1 show an example of phosphoprotein staining from patient samples stained for Stat3 and p-Stat3 (Tyr⁷⁰⁵). Peripheral blood and bone marrow samples obtained on the same day from five different patients are printed in alternating columns with the first row being from the newly prepared clear lysates and the second row from the existing blue lysate.

To detect the limit of resolution and sensitivity, in terms of the smallest detectable difference in cell numbers, we prepared cell lysates at 7 and 10 cell equivalents per nanoliter, respectively. The minimal difference of three cells per nanoliter (equal to 30 cells per dot at 10 touches) between

the two samples could readily be detected, confirming the quantitative nature of the assay, which is illustrated in Fig. 2. The stronger signals in the second row, relative to the top row, are due to higher cell numbers per nanoliter (10 versus 7 cells/nL). If 10 cell equivalents per nanoliter were used for clear lysate preparation and compared with the "original" blue lysates at 10 cells/nL, similar expression intensities were detected (data not shown). The curves from peripheral blood- or bone marrow-derived material are superimposed (Fig. 2), suggesting the equivalency of source material as previously reported (14).

In experiments in which the most concentrated sample had either 1,000 or 500 cell equivalents per dot, and in which the eighth dilution contained protein from eight and four cells, respectively, we were able to detect a difference in signal intensity. The smallest number of cells from which protein was reliably detectable was three cells.

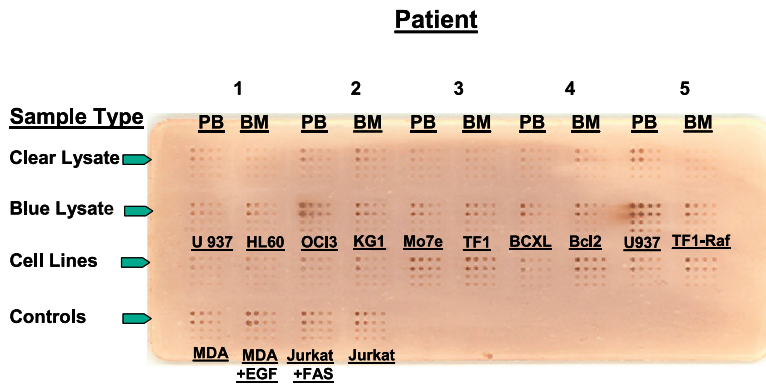
In conclusion, bromophenol blue from the Western blot lysis buffer did not interfere with signal detection and analysis, as illustrated in Fig. 1, indicating that the existing protein lysates in the Leukemia Sample Bank can be used for RPPA. Furthermore, RPPAs are extremely sensitive, and miniscule differences in protein expression, reflecting the smallest differences in cell numbers, were reliably detected in our experiments.

Protein and Phosphoepitope Stability

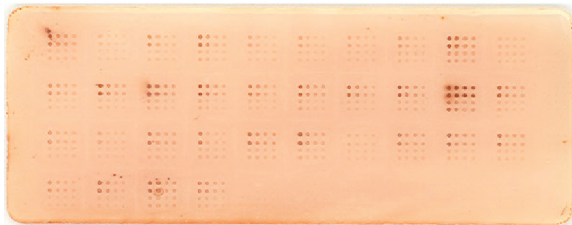
One of the main variables in measuring protein and phosphoprotein expression is the stability of a given protein *ex vivo*. Both in terms of time from specimen collection until processing, and the "storage" (freezing) of a sample. We wanted to assess if processing of properly stored, cryopreserved cells by RPPA gave similar values compared with specimens prepared at the time of collection. Therefore, we tested whether the handling of cells used to generate protein affected protein and phosphoepitope stability and detection. Vials of cryopreserved blood and marrow-derived cells, obtained on the same day from a patient, were thawed and a portion was removed to make a whole cell lysate, and the remaining cells were re-frozen. This was repeated for two cycles, after which, a second lysate was prepared. These freeze-thaw specimens, along with the freshly prepared lysate, were printed onto slides and probed with eight total phosphoproteins: ERK1/2, p-MAPK 42/44, Stat3, p-Stat3 (Thr⁷⁰⁵), Akt, p-Akt (Ser⁴⁷³), p38, and p-p38 (Thr¹⁸⁰/Tyr¹⁸²). There were minor, but no statistically significant, differences in phosphoepitope expression intensity between the freshly prepared blue lysate and the samples prepared from cryopreserved samples (either peripheral blood or bone marrow) from the first or third thawing. Similar levels of expression of (phospho)proteins were observed in peripheral blood and bone marrow samples consistent with our findings using Western blot or flow cytometry (14), indicating that leukemia-enriched peripheral blood and bone marrow samples can be used in the same analysis when only one is available. The above observation was further confirmed in a third sample set of 23 AML samples with simultaneously collected peripheral blood and bone

⁴ J. Hu, K. Baggerly, K. Lu, C. Joy, S. Neeley, B. Hennessy, G. Mills, and K. Coombes. Supercurve quantification of reverse-phase protein lysate arrays, submitted manuscript, 2006.

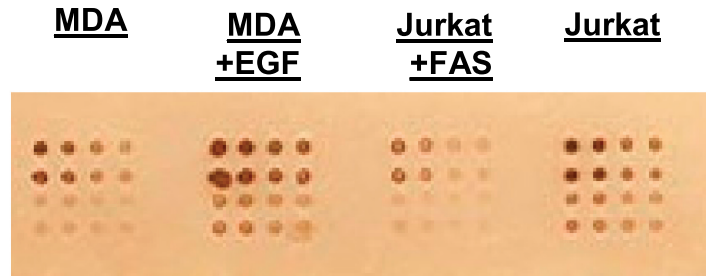
A Total Stat 3



B p-Stat 3 (Tyr 705)



C p-Akt473



D

“Clear Lysate”

“Blue Lysate”

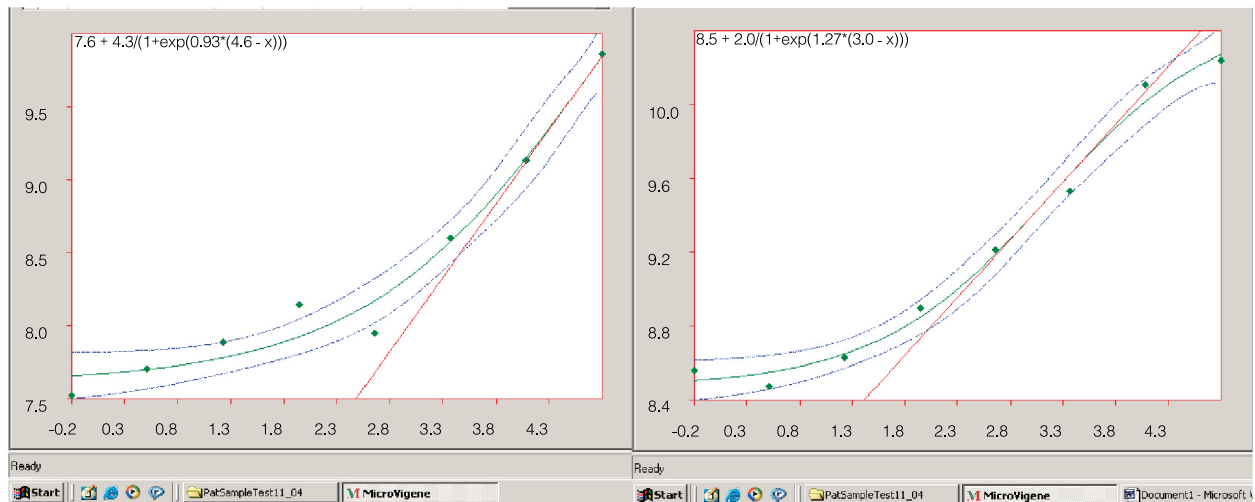


Figure 1. **A**, total Stat3 (top) and p-Stat3 (Tyr⁷⁰⁵) protein expression from five different patients (bottom left). Top row, newly prepared clear cell lysates from peripheral blood (PB) and bone marrow (BM). Second row, blue lysates of the same specimen. Third row, leukemia cell lines. Fourth row, MDA-468 ± EGF, Jurkat ± FAS ligand stimulation. **B**, of note is the small to absent change of Stat3 in the control cell lysates. Sample arrangement in the p-Stat3 (Tyr⁷⁰⁵) slide is identical to **A**. **C**, bottom right, the same control cell samples (same experiment) printed onto a different slide and probed for p-Akt473 clearly showing an increase in p-Akt473 levels with EGF stimulation of MDA-468 cells (MDA) and decrease in Fas ligand – treated Jurkat cells. **D**, dilution curves and log-linear representation by MicroVigene. Analysis of representative curves from MicroVigene for blue and clear peripheral blood lysate samples from the same patient. Each spot represents a dilution of the sample. An optimized curve (green line) with standard deviations (blue line above and below) is automatically plotted through the data points. The software program “fits” a linear curve (red straight line) onto the dilution curve and calculates a function. The EC 30 or 50 of that curve gives a log number which is used for processing of the data.

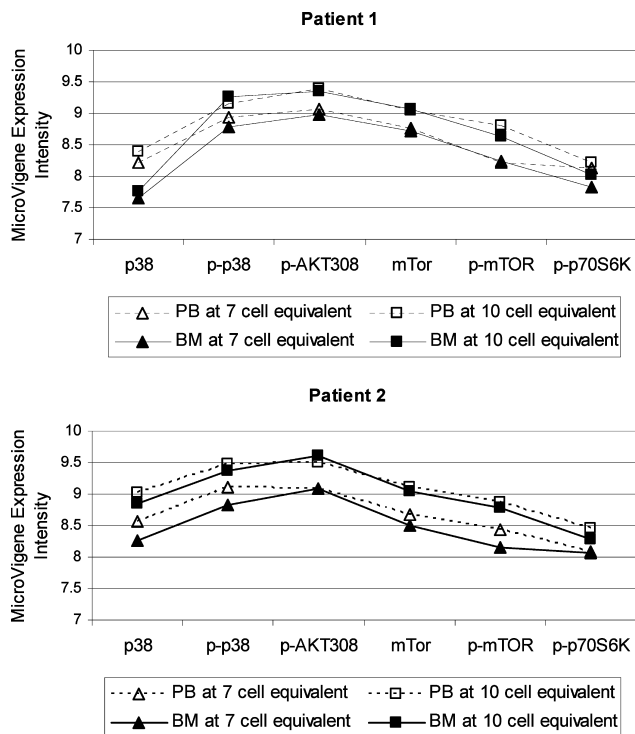


Figure 2. Sensitivity of RPPA. Protein lysates were prepared from the leukemia-enriched fractions from two patients with simultaneously obtained blood and marrow samples at calculated concentrations of 7 and 10 cells/nL equivalents. Lysates were printed (10 touches) onto RPPA arrays and assayed with six antibodies. The relative signal intensity of the curve of each is shown. For both patients, the signal strength for each protein was similar regardless of source (blood or marrow). For both patients, the signal strength of the 7 cell/nL sample was consistently lower than that of the 10 cell/nL sample demonstrating that RPPA could detect quantities at a 3 to 30 cell difference, depending on the number of touches (10 touches).

marrow samples. The expression profiles of these specimens showed no statistically significant difference between peripheral blood or bone marrow from the same patient on unsupervised hierarchical clustering ($P = 0.67$ for 23 samples and 37 antibodies; ref. 20). In ongoing work, we confirmed that membrane proteins (e.g., Bcl-2, Bax, membrane tyrosine-kinase receptor proteins) could be reproducibly measured in patient samples and cell lines (20).

Reproducibility and Precision of Printing/Spotting

To assess the reproducibility and variability of RPPA, the variation between lysate preparation as well as the variation between plate and experiment setups and array runs were tested (inter- and intrasample and intra- and interarray variation).

First, to test the effect of array setup and preparation, the same lysate was prepared twice (two array plates) and printed onto the same array slide (interplate preparation). High correlations between the expression intensities of corresponding samples were observed. Correlation coefficients for 10 paired samples were: 0.99, 0.96, 0.89, 0.95, 0.99, 0.95, 0.93, 0.91, 0.96, and 0.97.

We next tested the variability of preparing new lysates from the same leukemia specimen (interexperiment). When these were printed on the same array slide, high correlations were observed, demonstrating reproducibility within protein lysate production. Pearson's correlation coefficients were 0.99, 0.99, 0.99, 0.96, 0.95, 0.83, and 0.93 (three sample pairs could not be evaluated due to staining artifacts). Figure 3 graphically depicts the precision and reproducibility of RPPA and illustrates some of the findings: blood and marrow from the same patient generally had similar protein expressions (with some exceptions, mostly in patient 5); repeated analysis of the same sample again showed a high concordance with the first array setup, arguing for the robustness of the assay.

Lastly, interarray variation (the same sample spotted onto different slides) is high, with coefficients of variation of <15%. The assayed proteins in all experiments were: p38, p-p38, Stat3, p-Stat3 (Tyr⁷⁰⁵), ERK, p-MAPK 40/42, m-Tor, p-mTOR (Ser²⁴⁴⁸), p-Akt S473, and p-p70S6 kinase (Thr³⁸⁹).

Duplicate spotting (printing the same sample from a plate twice onto the same slide), is used by most groups but reduces the number of different samples that can be printed on a single slide. The correlation between duplicate spots was tested for six different antibodies and extremely high concordance was observed [mean R^2 , 0.9926; ERK1/2, 0.9973; p-MAPK (42/44), 0.9919; Stat3, 0.9825; pStat3 (Thr⁷⁰⁵), 0.9979; Akt, 0.9920; and p-Akt (Ser⁴⁷³), 0.9941; Fig. 4]. This lack of variability suggests that duplication on the same array slide is not necessary.

Protein Quantification

To accurately determine the absolute concentrations of proteins in a sample, we generated standard signal intensity-concentration curves (Fig. 5) for purified proteins or recombinant peptides of known concentration for comparison with the samples in which protein concentrations are unknown. Using these peptide standard reference curves, the unknown protein concentration of each sample/lysate can be calculated. First, the protein concentration for control cell lysates printed onto each slide (e.g., U937, HL60, Jurkat), is determined to serve as a reference point for the signal intensity of a "slide." Each slide can then be normalized to the protein expression intensities of the control cell lysates. From that, the absolute protein concentration can be determined reading off the peptide standard curve. An example is shown in Fig. 5. Purified activated Akt protein and p-Akt 473 peptide were arrayed by RPPA. The signal intensity (Y axis) versus protein concentration [protein (pg) log scale] was plotted. There was a linear relationship between the concentrations of purified activated AKT and phospho-AKT peptides and signal intensity. The RPPA assay detected activated AKT protein to picogram, and phospho-AKT peptide to femto-gram levels, complementing our observation of the ability to detect minimal differences in cell numbers or protein concentration.

Analysis of Hematopoietic Stem Cells by RPPA

Most analyses of protein expression in leukemia use the bulk population of leukemic cells in the marrow or blood,

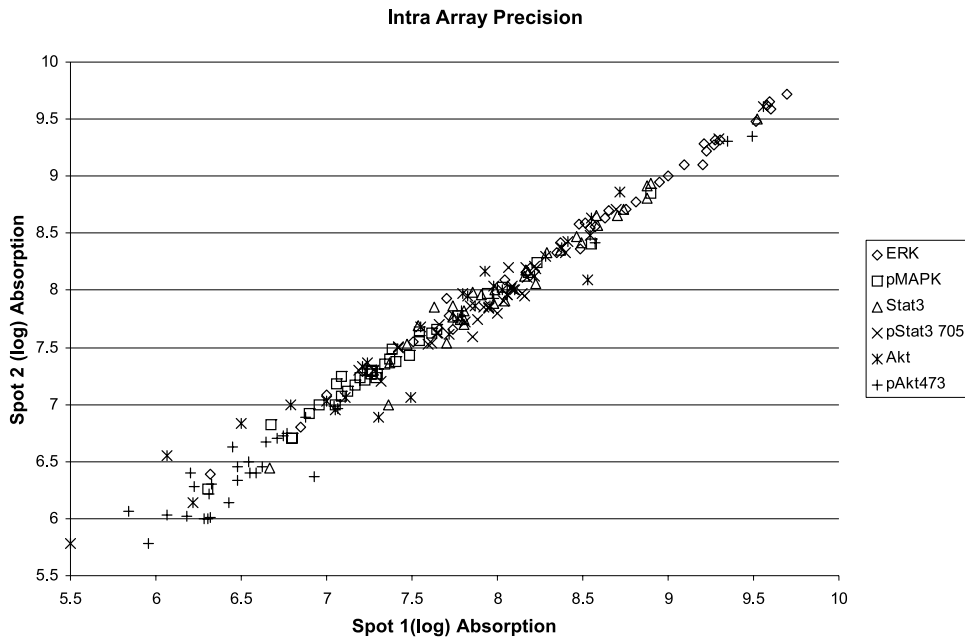


Figure 3. Protein lysates were printed in replicate on the same RPPA and probed with six antibodies. The correlation between the replicates is shown. Mean R^2 , 0.9926; ERK1/2, 0.9973; p-MAPK (42/44), 0.9919; Stat3, 0.9825; pStat3 (Thr⁷⁰⁵), 0.9979; Akt, 0.9920; p-Akt (Ser⁴⁷³), 0.9941.

rather than the rare stem cell population. An unresolved question is whether protein expression in stem cells is similar or distinct from that of the bulk population of leukemia cells. Because only 10 to 20,000 leukemic stem cells or hematopoietic stem cells can realistically be isolated from a sample (0.001% of 1×10^7 cells = 10,000 stem cells)

traditional methods (Western blot, flow) have not been able to analyze protein expression in stem cells. In contrast, a single slide for RPPA analysis requires only ~200 cell protein equivalents for a series of eight dilutions. Consequently, RPPA is ideally suited for the analysis of low-abundance populations of cells and 10 to 20,000 stem cells

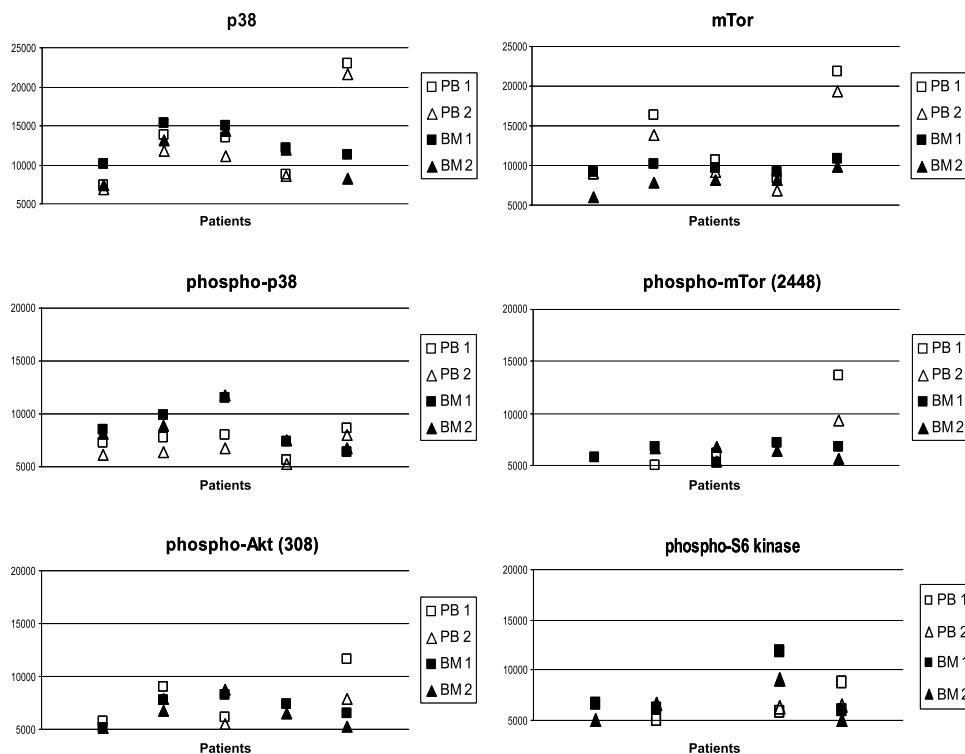
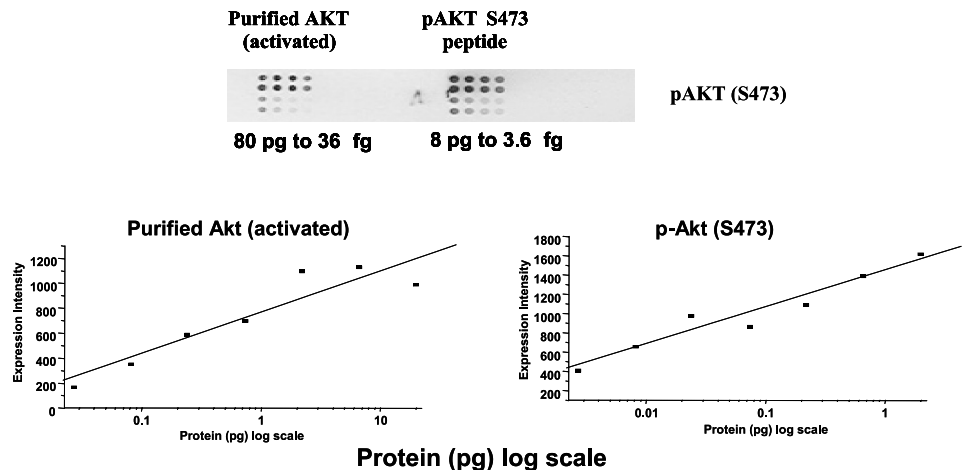


Figure 4. Concordance of peripheral blood (PB) and bone marrow (BM). The same lysate of peripheral blood and bone marrow from two different array setups showed close concordance in expression intensities in 10 samples from the five initial patients. There were some minor variabilities between experiments 1 and 2, especially for patient 5, which could reflect experimental variability or a reduced signal intensity, although overall total and phosphoprotein expression strength were well maintained.

Figure 5. Absolute protein quantification by RPPA. *Top*, magnification of dilution curves from a protein/peptide reference RPPA slide. Below the RPPA slide section is the amount of protein determined from the log scale. Each spot is plotted as the densitometric absorption number against the protein concentration of a dilution spot of a sample. Because the absolute protein concentration of purified AKT and p-AKT (S473) peptide are known, the unknown protein concentration of any lysates can be calculated according to the Akt standard curves.



would provide sufficient lysate to allow for the printing of 50 to 200 slides (equal to 50–200 different antibodies) from one sample. This number could be doubled using fluorochrome probes, Cy3 and Cy5, for each slide. As a pilot, we generated protein lysates from CD34+/CD38+ or CD34+/CD38– sorted cells from leukemic and normal specimens (21). Figure 6 illustrates an example of how normal and leukemic CD34+/CD38+ progenitor and CD34+/CD38– stem cells can be assayed by RPPA. Notably, different levels of protein expression were observed when normal and leukemic CD34+/CD38+ and CD34+/CD38– cells were compared, or when leukemic and normal stem cells were compared, which were not always apparent when normal and leukemic CD34+/CD38+ cells were compared. In preliminary results of 22 antibodies, leukemic stem cells seem to have a different protein signature compared with normal stem cells (data not shown). Full analysis and confirmatory experiments are ongoing.

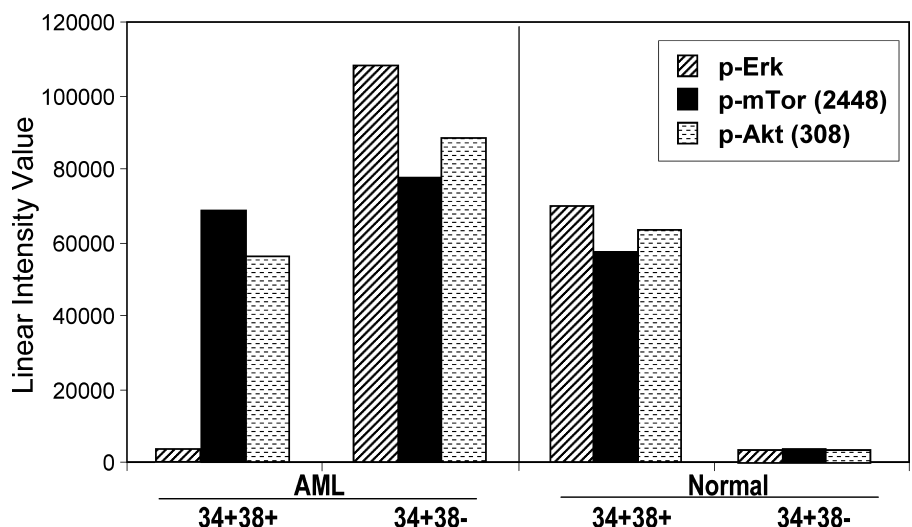
Correlation of RPPA with Western Blotting

The correlation of RPPA with conventional techniques was assessed in human (leukemia) cell lines Jurkat, K562,

HL60, and HeLa. The same lysates were analyzed by RPPA and concurrently by Western blots. The expression intensities of proteins measured by RPPA highly correlated with the expression levels determined by Western blotting. There was an excellent correlation between the results of WB and RPPA in the four cell lines for five antibodies: Pearson's correlation coefficients for these experiments were Akt, 0.93; p-Akt (473), 0.98; Erk, 0.89; p-Erk (42/44), 0.94; and Bcl-2, 0.95.

Approach to Data Normalization and Loading Control Interarray Comparison. One challenge in array analysis is the variability and comparability of staining between arrays (interarray). One approach to facilitate interslide comparison is to run positive controls, consisting of unstimulated, stimulated, and inhibited cell lysates (e.g., MDA-468 ± EGF, U937, HL60, Jurkat ± FAS, or mixtures of cell lysates) on each slide. These serve as a reference point for the signal intensity of a slide. Individual samples can be compared relative to the average slide intensity as well as in relation to the intensity of a particular sample on a slide stained for a different antibody. Thus,

Figure 6. Examples of HSC analysis by RPPA. Several pairs ($n = 4$) of an equal number of CD34+/CD38+ and CD34+/CD38– cells from patients with AML and from normal bone marrow donors were assayed by RPPA and probed with antibodies for phosphorylated forms of ERK, mTor, and AKT(Ser³⁰⁸). Although there was no apparent difference in the expression levels of phosphor-, mTor, and AKT between normal and leukemic CD34+/CD38+ progenitor cells, AML stem cells (CD34+/CD38–) showed increased levels of activation of all three signal transduction proteins relative to leukemic or normal CD34+/CD38+ progenitor cells or normal CD34+/CD38– stem cells. Analysis of HSC by RPPA is feasible and that important differences in protein expression may exist between leukemic stem cells and the bulk population of leukemic cells.



cross-comparison of samples and antibodies from distinct arrays can be compared.

Once the signal intensity of a sample (corrected for different variables) is determined, the absolute concentration can be determined from the peptide standard curve, similar to an ELISA assay. Alternatively, if only relative protein expression levels are compared and the number of absolute protein concentrations are not required, a set of samples/slides/experiments can be compared based on the correction for the difference in signal intensity to the control cell lysates only. We routinely use both approaches depending on the specific question.

Alternatively, if the absolute concentration of a protein does not need to be determined, building the ratio of the phospho- over the corresponding total protein is another way to compare expression results among samples. Ratios factor out protein loading, reflecting the change in the activity of proteins relative to the total amount of that particular protein. Either corrected numbers or ratio numbers can then be used for hierarchical clustering or other means of data analysis and representation.

Identification of Molecular Targets and Markers

Many on and off target effects of conventional and novel drugs are unknown. To illustrate the advantage of RPPA for specimen analysis and target identification, we applied our system to screen the effect of a novel National Cancer Institute-sponsored agent, XK469, on protein expression in six standard leukemia cell lines at three concentration curves and several time points. Surprisingly, while screening cell lines for >25 protein targets (antibodies), we found a decrease of S6 ribosomal protein, suggesting a potential inhibition of the upstream S6 ribosomal kinase (22). Given the high number of samples (160 at one time) and antibodies (20–40) that can be assayed in a time- and cost-saving way, this makes the system ideally suited for *in vitro* and *in vivo* target identification, validation, and drug discovery.

Discussion

The presented studies show that RPPA is a rapid, high-throughput, reliable, and reproducible methodology for the study of protein expression levels and protein activation states in primary AML samples as well as normal and AML stem cells. Given the limitation of genomic arrays and conventional protein processing methods, protein micro-arrays such as RPPA have the potential to complement transcriptional profiling by offering a new means to quantify the level and activation status of cancer-associated proteins (8).

In our studies, we first established that human leukemia specimens can be used for RPPA analysis. We showed that the existing AML lysates from the Leukemia Sample Bank of the M.D. Anderson Cancer Center could be analyzed by RPPA, providing the same results as newly prepared samples with a dedicated/special RPPA buffer. The stability of protein/phosphoepitopes over time was shown by similar findings obtained from freshly prepared protein

samples made from cryopreserved cells when compared with protein samples prepared from the same specimens years before and stored at -80°C since preparation. Furthermore, these results showed that phosphoprotein epitopes in cryopreserved cells were relatively stable to repetitive freeze-thawing and to the variability of specimen processing. Similar observations were made by Xu et al. (23), although in fewer samples.

These results are important for several reasons. First, they increase the confidence in our current sample processing and storage procedures; results are reproducible over time, samples (e.g., from a clinical trial) can be stored and assayed at one time point on the same array, reducing some of the main variables in any type of array. Furthermore, this is important as it increases the confidence in the results of AML profiling, which we are currently attempting, and will enable us to carry out large-scale proteomic analyses of AML.

The sensitivity of RPPA was shown by the ability to detect protein expression in primary samples at levels down to the femtomolar range using comparisons to identify purified protein preparation standards. We have obtained >100 different phospho- and total peptides sufficient for the quantification of thousands of patient samples. The peptides can be arrayed on each slide allowing reference peptide curves from each array. We are currently expanding the peptide standard curves and the cross-array quantification comparisons. The ability to detect differences in expression intensities in as few as three cell equivalents shows the robustness and sensitivity of the RPPA system. Neither WB nor matrix-assisted laser desorption/ionization-time of flight achieve this sensitivity on a large-scale basis. As mentioned earlier, the advantage of RPPA, besides its' sensitivity, lies more in the unique feature to measure protein expression in miniscule amounts of material in a rapid high-throughput fashion. Arraying all samples on one array at the same time reduces experimental variability to a great extent. For example, specimens from 40 to 60 patients, at several different time points from an entire phase II study could be assayed under identical conditions for dozens of antibodies in a time-sparing manner. Immunohistochemistry assesses single cells, but is less suitable for a large-scale comparison of many cells for many proteins. Fixation and protein stability are issues and the entire proteome is not as reliably represented as with RPPA.

The printing precision and reliability of the RPPA technology are extremely high and the experimental variability can be kept very low. This is most likely due to the intrinsic factors of RPPA such as the greater precision of the technology because sample handling and preparation are similar to WBs. Interslide/array comparison was likewise very high. One emerging feature is that the greatest reliability and least variability is achieved when samples are assayed together on one array/slide. The very high correlation between replicate printings of the same sample on the same slide suggests that duplicate printing could be omitted to permit a greater number of individual

samples to be printed on the same slide and to reduce costs. Furthermore, better comparability of a greater number of samples can be achieved as more samples are handled under identical conditions on one array reducing experimental bias.

For the first time, we show that it is possible to analyze human hematopoietic stem cells on a large-scale proteomic basis and compare their expression profile to bulk leukemic cells. In the limited pilot analysis, we observed differences between the bulk and stem cell population. Because resistance and recurrence are likely to emerge from the stem cell population analysis of this low-abundance population using RPPA, this could provide insights into the mechanisms employed by stem cells to resist therapy and could suggest therapeutic targets. This tremendous potential of RPPA, by itself, needs to be fully exploited in an attempt to learn more about normal and leukemic stem cells, and the ways to eradicate leukemic stem cells.

Finally, we propose a new approach to overcome some of the normalization and quantification difficulties in proteomic analysis. As not all samples may have protein concentration or cell numbers available, a protein loading procedure needs to correct for potential uneven loading. We found that correcting each spot/sample for an average of five to eight stably expressed proteins (housekeeping), or all proteins, can factor out to a large part, printing, detection, and staining variability before analyzing the data and still allow detection of differentially regulated total and phosphoproteins. At the same time, this highlights the need for the standardization of protein sample collection and processing.

In summary, we were able to show that RPPA enables the comprehensive study of leukemic specimens through the higher sensitivity and increased capacity of this platform, thereby furthering the understanding of leukemogenesis. Our aim is to discover new molecular mechanisms and interactions in leukemia, validate these as potential therapy targets, and ultimately target them as a step towards the goal of personalized molecular medicine.

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