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Use of Reverse Phase Protein Microarrays to Study Protein Expression in Leukemia: Technical and Methodological Lessons Learned

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Abstract

Leukemias are well suited to proteomic profiling by RPPA due to the ready accessibility of blasts from the blood or marrow. In this review, we review methodological and procedural issues that affect the quality of RPPA data. We recommend contact printers that minimize sample quantities and evaporation and maximize sample per slide. The impact of sample selection and handling is reviewed as well. Protein is best prepared fresh on the date of acquisition as cryopreservation changes protein expression levels in some diseases. Rapid processing is also required to avoid changes in phosphorylation over time. Sample source, blood vs. marrow does not seem to affect results as long as leukemic blast enrichment procedures are utilized. The choice of the correct “normal” control is important for comparing diseased to “normal” expression. Various means of normalizing the data are discussed.

Keywords

Proteomics; Leukemia; RPPA; Reverse phase protein array

1. Introduction

The leukemias, because they are a “liquid” tumor with readily available malignant cells, are probably the easiest malignancies to study by RPPA. We have generated four different RPPAs from primary patient samples from patients with leukemia. Our first generation AML array had 550 patients’ samples on it and was probed with 51 different antibodies (1). Our second generation array had 719 AML samples from 511 patients and 360 ALL samples (AML719/ALL360) (2). The third array contains matched bulk, CD34+ and CD34+ CD38– stem cell enriched samples from AML and CML cases (manuscript in progress). A fourth array has 260 CD34+ and CD34+ CD38– samples from cases with myelodysplasia and 285 freshly prepared ALL samples. We also utilize a cell line array with 186 different cell lines for validation purposes. Another group has also used RPPA to study B-cell ALL in an abstract presented at the 2008 ASCO meeting (3). The differences in the construction of these arrays represent an evolution in our understanding of how to study protein expression in leukemia. This review discusses technical and methodological issues related to the successful development of leukemia RPPAs. Additional methodological details can be found in the publications from the Kornblau laboratory (1, 4, 5).

2. Technical Aspects of Reverse Phase Protein Microarray Production

2.1. What Printer to Use?

Printers either use contact-based printing methods or inkjet like technologies to spray protein onto the slide. We extensively evaluated both with respect to amount of sample required, printing time, consistency of dot size, reproducibility, etc. While a full comparison of the various available machines is beyond the scope of this chapter a few features guided our decision. We knew that we were going to print 150–300 slides from our samples. Most printers keep all the trays open (lid off) during this process which can lead to problems with evaporation. This makes speed an important consideration. With some machines we noted that samples got sticky toward the end of a long run and did not print as well. Many machines include humidification methods, but with some this led to condensation problems within the chamber and on the slide, and in others this required frequent filling of water chambers. The inkjet technology was markedly slower than direct pin contact methods so we choose to use direct pin-based printing. We evaluated various pin diameter sizes before settling on 175- μ m pins. We observed issues with dot size and reproducibility with pin sizes below 135 μ m. Test data with larger pin sizes did not differ from that with the 175- μ m pins, but obviously consumed more samples and permitted fewer samples per slide. We selected the Aushon 2470 arrayer which had an advantage in that it only uncovers a single tray at a time and it was clearly the fastest in our comparisons.

2.2. Slide Material

We have observed marked variation in technical features of the various commercially available nitrocellulose slides. We have observed uneven membrane thickness so that some portion is higher than others, enough so to interfere with the printer pins. We have seen issues where the machine used to roll the nitrocellulose out onto the slide in the production process was uneven resulting in a rippled effect across the slide (like the middle sheet in corrugated cardboard). There is marked batch to batch variation from the same manufacturer. Some of the membranes have greater autofluorescence than others which is a concern if fluorescent dye based analysis is planned. Others give different degrees of background staining with the DAB precipitate. We currently use ONCYTE[®] nitrocellulose coated film. Slides from Grace Bio-Labs (Bend Oregon, catalog # 305180). The bottom line is that each batch must be carefully checked for quality using a test print, before using them to print arrays with scarce clinical material.

2.3. Clinical Material

2.3.1. Sample Quality—Leukemic blasts can be obtained in sufficient numbers from the marrow of nearly all cases and from the blood (or leukapheresis if performed) from the majority of cases of the most common leukemias AML, ALL, CML, CLL, MDS, and MPDs. Samples from granulocytic sarcomas, lymph nodes, or isolated CNS disease are theoretically analyzable, but separation from surrounding stroma or obtaining sufficient numbers of cells presents special difficulties. Blood is collected in heparinized (green top) tubes and marrow into 3 ml of heparinized solution in 15-ml tubes (RPMI + 5% BSM + Pen Strep). The material is stored on ice or in a refrigerator until transported to the laboratory. The effect of delay in processing or temperature on protein concentration is variable. There

is a growing literature (collated by the Office of Biorepositories and Biospecimen Research (OBBR) <https://brd.nci.nih.gov/BRN/search.seam>) documenting that variability in handling results in changes in the characteristics of the samples and that this leads to varied results in profiling arrays and biomarker studies. While mRNA and DNA have been analyzed many times there are few reports assessing changes in protein expression or phosphorylation. Most protein-based studies have analyzed paraffin-fixed material studied by immunohistochemistry; but this methodology has been shown to affect phosphorylation levels (6, 7). Maintaining tissue at room temperature compared to 4°C or 1°C prior to tissue preparation resulted in loss of phosphoprotein signal in cadaveric brain (7). Thus, sample handling prior to processing and methodology both can confound protein-based data. Since phosphorylation status is key to recognizing pathway activation, the ability to accurately ascertain this is crucial to generating consistent results. The OBBR bibliography lists no studies of protein derived from normal or malignant blood or bone marrow cells. We have conducted experiments on this processing material upon arrival, or at 24 or 48 h and we have observed that RB phosphorylation is stable, but that ERK2 phosphorylation is not. As discussed below we know that delay in processing has marked effects on protein expression in ALL. To minimize this possibility we transport material to the laboratory five times per day and rapidly process it upon arrival. There are commercially available materials that purport to stabilize cells but we have not evaluated them.

2.3.2. Enrichment of Leukemic Cells—Protein expression from tumor cells, the surrounding stroma, adjacent normal tissue, and any infiltrated cells (T and B lymphocyte infiltrates) are different. For protein expression studies the quality of the results is obviously dependent on the purity of the material studied: so purification of the malignant material from all other cells is therefore crucial to obtain a representative analysis of protein expression in the tumor. This is a major issue for studies of solid tumors but not for the leukemias. Surface markers are well defined enabling purification or enrichment of leukemic blasts from contaminating non-leukemic cells. Contaminating RBC and neutrophils are easily removed by ficoll separation. For myeloid leukemias contaminating B and T cells are removed by CD3 and CD19 depletion using magnetic antibody cell sorting. Our laboratory has successfully used the Vario, MACS, AutoMacs, and RoboSep systems. We utilize a customized mix of antibodies and find that we can use less antibody (25 µL/2.5 × 10⁷ cell) than the manufacturer specifies. For the CD3/CD19 depletion we use the positive selection column and the leukemia enriched population is in the flow through. For AML and MDS this material may contain non-malignant monocytes, but we usually are processing material within 2 h after collection and the phenotype is usually unknown at that time so that we cannot deplete monocytic markers as this would remove leukemic blasts in FABM4 or M5 cases. If cryopreserved cells are used and phenotype is known, monocytes could be depleted from non-monocytic leukemias. The CD3/CD19 collection can also be processed and can serve as a control. We have used this “bulk” leukemia enriched fraction for the AML550 and AML719 arrays. For ALL purification is generally less of an issue as the percentage of blasts in the marrow and blood is usually much higher at diagnosis than in AML (The average percentage of marrow and blood blasts at MDACC since 2000 were ALL BM 78%, PB 47% and AML BM 51%, PB 27%). If phenotype is known then B or T cells can be removed. Monocytes can be removed from ALL samples as it is rare for ALL

cells to express CD14. For myeloma we utilize a positive selection using anti-CD138 beads. Yields are low, but sufficient for use with RPPA.

A leukemia stem cell phenotype, minimally defined as lineage⁻, CD34⁺, CD38⁻, has been defined for AML (8). Protein expression may differ between the “bulk” leukemic cell and an AML stem cell raising the question of whether it would be superior to study leukemic stem cells instead of bulk cells. The critical issue is whether sufficient numbers of stem cells can be produced for use in RPPA. To print a sample set of about 200 slides once (without replicate), with five serial dilutions, requires ~300,000 cells as we utilize 30 µL of protein lysate at a concentration of 10,000 cells/µL. We have generated AML stem cells, defined as CD34⁺ CD38⁻ by sequential CD34 positive selection and CD38 depletion of the ficolled material. The median yields of CD34⁺ cells was ~20% of the starting material and the median yield of CD34⁺/CD38⁻ stem cells was about 1%. This percentage is much higher than the 1/1,000 to 1/10,000 frequency of normal stem cells. For the stem cell array we started with a median of 4×10^7 ficolled cells, we generated a median of 2×10^5 stem cells. The yields of CD34⁺ and stem cells from a larger consecutive series of samples are shown in Table 1. For MDS the markers to select for are the same as for AML, but the percentage of blasts in the BM and PB is by definition much lower than in AML (<20% vs. >20%). We have used a similar schema for MDS, generating CD34⁺ and CD34⁺/CD38⁻ stem cells. As expected yields are lower as we are typically starting with less material and that material has a lower percentage of leukemic blasts (Table 2). We can successfully generate a CD34⁺ fraction from all AML and MDS samples, but we were only successful in generating stem cells in 60/97 fresh AML and 97/237 cryopreserved and 32/68 fresh MDS samples. Consequently, studying protein expression in only stem cells creates a bias in sample selection as not all samples produce a stem cell fraction. One alternative is to use fewer dilutions. Since we use five serial 1:2 dilutions omitting the highest cell concentration reduces the sample need by half. For CML we also have utilized CD34⁺/CD38⁻ selection to generate CML SC. This debate is important as analysis of our stem cell array probed with 112 antibodies revealed highly significant difference in expression between stem cells and bulk cells for 70/112 antibodies (manuscript in preparation). A potential compromise is that most of these changes are also detected when CD34⁺ cells are compared to bulk cells, suggesting that CD34⁺ cells might serve as a partial surrogate for stem cells. Our initial study suggests that it would be superior to study CD34⁺ or stem cells. We are currently collecting a larger collection of matched samples to study this in a larger cohort.

2.3.3. Impact of Patient Pretreatment on Sample Quality—Most of our work has utilized diagnostic samples, collected prior to the patients receiving any chemotherapy, and samples obtained at relapse. Many patients with high WBC may have received hydra prior to referral to us. We prefer to collect a new sample after a couple of days of washout if possible, but if therapy is to be initiated and the WBC count is still high it is unlikely that limited exposure to hydroxyurea will have large effects on protein expression. So such samples could be utilized with caution. We do not have enough paired naïve vs. on hydroxyurea samples to test this. We also try to collect relapse samples. Since many relapses are discovered unexpectedly during routine BM examinations the percentage of blasts is often much lower than at diagnosis and PB involvement is much less frequent. To increase

our capture of relapse specimens we collect all follow-up marrows but for financial and workload efficiency we only process those with >5% blasts. This requires keeping the sample refrigerated for a longer period of time (hours) until the differential is known. As a consequence far more of our relapse samples are BM derived. If samples are obtained shortly after the initiation of therapy it might be possible to collect cells surviving to that point by removing “doomed” annexin V positive cells. The yield is likely to be very low beyond 1–2 days after the start of chemotherapy. At day 14 from the initiation of therapy over 85% of bone marrows on AML patients have too few cells to count making it impractical to try and collect sufficient cells at that time point. Samples could be collected from primary refractory patients from day 21, 28, or 35 BM. We have not collected remission samples on patients to try and compare expression in their normal cells vs. their leukemic cells. The percentage of leukemic blasts among CD34+ CD38– cells would be variable and unless a leukemia-specific phenotype has been identified there would not be a way to separate malignant from normal blast. Furthermore, these cells are likely to be present at very low frequencies of 0.1–0.01% of cells, making recovery of sufficient cells for RPPA (or any assay) very difficult.

2.3.4. Comparison of Protein Profiles from Blood and Bone Marrow Samples—

We have examined whether the source, blood vs. marrow affects protein expression patterns. In the AML719/ALL 360 array there are 140 AML and 22 ALL samples where we have paired same day blood and marrow specimens. We have analyzed this array with 176 antibodies permitting a wide ranging comparison of expression within these two compartments. In general, for both AML and ALL the majority of the antibodies tested showed no difference between the two compartments. For AML 25 were significant at a *p*-value of <0.01 (a more stringent criteria was used since we were performing 176 analyses) with 10 being higher in the blood and 15 higher in the marrow. For the majority of these the fold difference was <25% so whether these statistically significant differences have biological differences are questionable. Supporting the idea that some of these are real and others spurious is the observation that four of seven proteins with compartmental differences in the AML550 array produced similar results in the AML719 analysis. So for practical purposes blood and marrow produce similar results and can probably be used interchangeably. We were somewhat surprised to see as little difference as we did. At our institution research samples are collected during diagnostic procedures to avoid performing additional bone marrows on patients (who likely would decline to participate at a much higher rate if additional procedures were required, thereby introducing a selection bias). The research sample is typically the fourth pull, after the diagnostic pulls for hematopathology, cytogenetics, and flow cytometry. Although the needle is repositioned between aspirates the degree of peripheral blood contamination is likely to increase with pull number. Since we cannot estimate the degree of dilution it is possible that there are more significant differences in expression between these compartments that are obscured by the dilution.

2.3.5. Freshly Prepared vs. Cryopreserved Samples—Protein preparations can be prepared from samples on the day they arrive in the laboratory (“fresh”) or from cryopreserved (“frozen”) material. We asked whether protein expression changes in cells as part of the cryopreservation process. When we prepare protein from cryopreserved cells we

rapidly thaw the cells, store in 20% FCS and 10% DMSO, and then dilute them in warmed media with 20% FCS. We allow the cells to stabilize for 2 h to recover from shock and then perform a ficoll separation to remove dead cells (which sink). The viable cells are then washed in TBS, counted, and then lysed at the appropriate cell concentration in the protein lysis buffer. For AML there was no significant difference in global expression across all the antibodies tested (Fig. 1a) as the distribution histograms of Fresh and frozen preps were similar. The results were distinctly different for ALL, where the “frozen” preps had significantly lower expression levels compared to the “fresh” preps (Fig. 1b). In the AML719/ALL360 RPPA, we made a frozen protein prep for 58 AML and 12 ALL cases permitting direct comparison. For AML the majority of proteins did not show a significant change between the paired samples, whereas most of the ALL samples did show a change. For ALL this was not merely a proportional reduction in protein levels, with a sample having similar expression by rank in both fresh and frozen samples. Instead the rank order was scrambled. Based on this we determined that protein expression in ALL blasts changes as part of the cryopreservation/thawing process and determined that we could not use protein prepared from cryopreserved ALL blasts for analysis of protein expression patterns. For AML this does not seem to be an issue. ALL cells are notoriously harder to cryopreserve and this may be another reflection of that fragility.

2.3.6. Delays in Sample Processing Affect Protein Profiles—The AML719/ALL360 array also contained 70 samples from the Italian Giemma cooperative (provided by Dr Agostino Tafuri). These samples were processed at a central laboratory, but came from across the country, so they were subject to varying time delays between collection and processing, and possibly different handling conditions (temperature). When we compared the global expression of the Italian samples to our ALL samples which were processed within 2 h, we again observed marked differences in global expression across all the tested antibodies (Fig. 1c). The yellow “rug” along the bottom shows lower expression for the Italian samples than the pink “MDACC” samples which included both fresh and frozen samples, and more closely resembles the protein prepared from cryopreserved cells. This suggests that delays in processing and possibly handling also affect protein expression in ALL.

We conclude that optimally, protein should be prepared fresh on the day of acquisition, preferably with minimal delay between collection and processing. For AML properly stored cryopreserved cells can be utilized but cryopreserved cells are not adequate for protein studies of ALL. We have not performed similar studies with CML or MDS cells, but since these are myeloid diseases they may behave more like AML than ALL.

2.3.7. Finding Appropriate Controls for Clinical Profiling—Another question is what is the appropriate control to use? Expression controls are required to verify that the antibody works as well as to provide something to compare expression to. We desire to have a positive control for each antibody as well as a negative control. It would be desirable to have a cell line known not to express each protein, but this would consume a large portion of the sample space on the array and would require foreknowledge of each epitope to be tested. Furthermore, known negatives are not available for all epitopes. As a compromise for the

negative control we use protein lysis buffer (Biorad Lamelli buffer, catalog # 1610737). For the positive control we have made a protein prep from a mixture of 11 different AML cell lines. To date this has shown expression of all 190+ antibodies tested. We made a large 50 ml protein prep of this so we would have something that would be standard from array to array, thereby enabling a comparison of expression within and across different printing. We are willing to provide some of this to others using RPPA technology to facilitate comparison of results from laboratory to laboratory. We use this for both the variable slope and topographical normalization procedures described below.

In an attempt to define what can be used as a normal expression control we have included normal peripheral blood lymphocytes, Granulocyte-colony stimulating factor stimulated peripheral blood CD34+ cells collected by apheresis and bone marrow derived 34+ collected from normal (unstimulated) donors. The first two are more readily available and more economical to obtain. Unfortunately, when we compared expression between PBL, G-primed PB-CD34+, and the BM CD34+ there were significant differences between all three potential “normals” with differences varying in both directions (higher and lower) (Fig. 2). The two CD34+ controls also showed significant differences between each other, reflecting either the consequences of G-CSF priming, or potentially compartmental differences. We have concluded that normal unprimed BM derived CD34+ cells make the best comparator to bulk and CD34+ AML and MDS leukemic cells. It would be preferable to include normal BM CD34+ CD38– stem cells as well but this is economically impractical. With normal SC comprising 1:1,000 to 1:10,000 of normal marrow mononuclear cells collecting 30,000 cells would require a starting cell number of between 6×10^7 and 6×10^8 [3×10^4 cells needed $\times 2$ (assuming a 50% selection efficiency) $\times 1 \times 10^3$ or 1×10^4 population frequency]. This would require a substantial portion of a normal marrow collection from a normal donor for allogeneic stem cell transplant or 6–60 vials of commercial CD34+ cells at \$650 for 1×10^6 cells. For reasons of practicality we have resorted to use normal BM CD34+ cells.

3. Reverse Phase Array Data Normalization

3.1. Normalization Based on Protein Concentration

We have utilized standardization of the processing of our samples as one method of normalization and have also employed statistical measures as well. If sufficient material is available then methods to determine the concentration of protein in a sample could be utilized to determine protein concentration and sufficient buffer added to reach a consistent concentration. However, in cases where material is scarce, such as in the stem cell array it would require too much material to determine concentration. This also assumes that the concentration of protein is uniform from sample to sample which is unlikely. We have been able to deal with differences in concentration by recognizing when a protein has low levels of expression across all the tested antibodies and making adjustments using “Variable Slope” normalization techniques (this and the other algorithms mentioned below are available at <http://bioinformatics.mdanderson.org/Software/OOMPA>) (9).

3.2. Impact of Background Variation

Another problem is how to deal with uneven background staining. While the majority of arrays have fairly even and negligible background sometimes there is marked difference in background staining. We have also developed a technique to account for background staining differences through a technique called topographical normalization (Fig. 3). By printing the dilution series of the control cell line lysate mix described above repeatedly across the slide in every sixth column (there are eight repetitions from slide top to bottom in each column $\times$ 24 vertical blocks = 196 times on our standard 6,912 dot array) we essentially have six topographical maps showing what should be a consistent elevation across the array at six different “altitudes” (full strength, 1/2, 1/4, 1/8, 1/16th, and empty protein lysis buffer). These elevations map variation across the slide and can be used to correct for this. Topographical normalization has negligible effect on the majority of slides with minimal background variation but is very helpful on the 10–15% of slides with more significant background variation.

3.3. Using Dilution Series for Data Normalization

We utilize a dilution series consisting of several points from each sample. This allows us to build a dilution curve to assess the sensitivity of the antibody and the linearity of the detected signal. We utilize “SuperCurve” algorithms built on the curves of all samples on a slide. The SuperCurve software is freely available at <http://bioinformatics.mdanderson.org/Software/OOMPA>. This defines confidence intervals for the signal strength of each dilution and allows us to recognize when a misprint or large dot has occurred and to eliminate that point from the dilution curve. The signal from each sample is then determined from the curve fitted to the dilution series. This has the advantage that a value is not based on a single dot on the slide.

4. Data Consistency and Data Reproducibility

A major question for RPPA analysis is whether these results are reproducible? The AML550 and the AML719 shared 187 samples and 47 samples but used different vials from the same protein prep and were printed over a year apart. As shown in Table 3 the majority of proteins showed highly significantly correlated expression between the two arrays using either the Pearson and Spearman rank tests. This demonstrated that protein preps stored at -80°C are stable over time. We have looked at the range of expression in samples stored for various durations of time (up to 15 years) and not observed evidence of differences. From this we conclude that the methodology is robust and reproducible within a single laboratory using the same samples. The methodology needs to have a separate laboratory, using separate samples that demonstrate similar patterns of protein expression to be fully validated.

This review did not focus on issues like antibody validation or staining techniques, which are more generic to all RPPA methodology, but these are obviously equally crucial to obtaining valid results.

5. Conclusions

RPPA is a valuable and facile tool for the study of protein expression in AML, but like all array methodologies obtaining accurate data requires care and attention to detail at all steps. Variability in samples selection and processing and the appropriate selection of controls is crucial. With care, repeatable results can be obtained, suggesting that RPPA could become a more generalized tool for the study of protein expression in leukemia. We are using our RPPAs to define protein expression signatures in AML and AML stem cells with the goal of developing the ability to classify the various types of AML based on the protein expression, with the idea that this can be used to match targeted therapies to the setting where the relevant pathway is crucial to the survival of leukemic cells. These are retrospective studies, but if signatures are to be used for classification and treatment selection then it is more likely that “forward phase” arrays will be required. In FPPA, numerous key antibodies, identified by RPPA, are printed on a slide, which can then be probed with protein from an individual case to provide a timely readout of protein expression and activation. Newer array technology may make protein-based classification a reality.

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Fig. 1.

Comparison of overall protein expression. These three figures all show the 75th percentile expression of 176 proteins for all cases. The *histograms* show the frequency distribution and the *colored "rugs"* along the bottom shows the distribution of the two conditions being compared. (a) Compares expression in AML protein preps made on the day of acquisition (fresh, *blue*) vs. those made from cryopreserved cells (frozen, *green*). The rugs show an equal distribution. (b) Shows a comparison between fresh (*blue*) and frozen (*green*) protein preps for ALL for the patients collected at MDACC. It is clear that there are two separate frequency distributions with lower levels observed in the frozen specimens. (c) Compares the distribution between all the samples from MDACC, both fresh and frozen, (*yellow*) and

those from Italian collaborators (*pink*). The Italian samples were shipped to a central laboratory and are known to have various delays between collection and processing, but were all processed from fresh cells. It is evident that the Italian samples do not appear at the upper end of the scale and also have a wide of distribution with many samples overlapping with the frozen MDACC samples.

Fig. 2.

Protein expression in “normal” controls varies by cell type and condition. The range of expression of normal peripheral blood lymphocytes (*red*), normal bone marrow-derived unstimulated CD34+ cells (*green*), and G-CSF mobilized CD34+ cells obtained by pheresis (*blue*) is shown for four different proteins. The numbers in *black* are in log₂ and cover the range of expression of the AML samples for each of the four proteins listed. For Bad, all three show a similar range of distribution. For Catenin- α , G-CSF primed CD34+ cells have higher expression than the unstimulated BM CD34+ cells. For Bad phosphorylated on amino acid 112 levels of G-primed CD34+ cells are similar to the normal PBLs and lower than the unstimulated CD34+ cells and for AMPK α both CD34+ samples have a similar range of expression that is higher than that of normal PBLs. This highlights the need to select an appropriate population of cells for if expression within a diseased condition is to be compared to that of normal cells.

Fig. 3. Topographical normalization examples of a typical slide with low background and a slide with atypically high background and variation are shown. An example of a topographical elevation map showing variation at six altitudes (full, 1/2, 1/4th, 1/8th, 1/16th, and blank) is shown.

Table 1
Yield of CD34+ and CD34+ CD39– “stem cells” from 93 consecutive AML samples

	# Cells sorted	# Samples	34+				CD34+ CD38–			
			# with cells	Median yield	# with cells	Median yield	# with cells	Median yield	# with cells	Median yield
BM	<2 ^{∧7}	8	8	9.20E+ ⁵	8	9.20E+ ⁵	5	3.93E+ ⁵		
	<3 ^{∧7}	10	10	1.18E+ ⁶	10	1.18E+ ⁶	6	2.00E+ ⁵		
	<4 ^{∧7}	16	16	8.15E+ ⁵	16	8.15E+ ⁵	9	2.00E+ ⁵		
	<7 ^{∧7}	9	9	1.56E+ ⁶	9	1.56E+ ⁶	6	3.00E+ ⁵		
	>7 ^{∧7}	5	5	6.85E+ ⁶	5	6.85E+ ⁶	5	6.00E+ ⁵		
PB	<2 ^{∧7}	4	4	2.00E+ ⁶	4	2.00E+ ⁶	2	5.50E+ ⁵		
	<3 ^{∧7}	11	11	7.36E+ ⁵	11	7.36E+ ⁵	5	2.70E+ ⁵		
	<4 ^{∧7}	3	3	8.80E+ ⁵	3	8.80E+ ⁵	2	2.00E+ ⁵		
	<7 ^{∧7}	17	17	1.63E+ ⁶	17	1.63E+ ⁶	14	4.00E+ ⁵		
	>7 ^{∧7}	9	9	7.94E+ ⁶	9	7.94E+ ⁶	6	1.00E+ ⁶		

Table 2

Yield of CD34+ and CD34+ CD38- stem cells from fresh and cryopreserved blood and marrow samples from patients with MDS

		CD34+				CD34+ CD38-			
	Starting # of cells	#	# with CD34+	Median yield	Med% yield	#	# with CD34+ CD38-	Median yield	Med% yield
Cryopreserved	Marrow	$<5 \times 10^6$	8	$1.70E+^5$	8.5	1	$3.00E+^5$	18.9	
		$0.5-1 \times 10^7$	50	$1.75E+^5$	3.1	9	$2.00E+^5$	6.0	
		$1-3 \times 10^7$	73	$4.30E+^5$	2.9	32	$2.90E+^5$	2.5	
		$>3 \times 10^7$	22	$1.20E+^6$	3.4	15	$4.00E+^5$	1.0	
	Blood	$<5 \times 10^6$	0						
		$0.5-1 \times 10^7$	30	$1.20E+^5$	2.0	4	$2.00E+^5$	6.7	
		$1-3 \times 10^7$	30	$4.20E+^5$	2.5	20	$2.00E+^5$	1.5	
		$>3 \times 10^7$	24	$7.20E+^5$	2.5	15	$4.20E+^5$	0.8	
Fresh	Marrow	$<5 \times 10^6$	5	$3.80E+^5$	7.6	1	$2.00E+^5$	5.0	
		$0.5-1 \times 10^7$	10	$2.80E+^5$	4.4	2	$2.00E+^5$	11.0	
		$1-3 \times 10^7$	32	$6.40E+^5$	4.1	14	$2.50E+^5$	1.3	
		$>3 \times 10^7$	14	$2.20E+^6$	4.8	10	$4.80E+^5$	0.7	
	Blood	$<1 \times 10^6$	0	$1.08E+^6$	10.8		$3.20E+^5$	0.0	
		$1-5 \times 10^6$	1	$2.40E+^6$	4.8	0	$8.00E+^5$	0.7	
		$0.5-1 \times 10^7$	3	$2.05E+^6$	2.4	2	$4.15E+^5$	0.1	
		$<5 \times 10^7$	3	$1.40E+^5$	2.9	3	$3.40E+^5$	5.0	

Table 3

Comparisons of expression between the AML550 and AML719 arrays for 187 shared patients and 47 shared antibodies

<i>p</i> -Value	Pearson	Spearman	Protein (Spearman)
>0.1	1	2	P70S6K, S6RP
<0.01	1	2	MCL1* STAT3*
<0.001	3	2	Badp136, Stat1p701
<0.0001	1	4	
<0.00001	6	5	
>0.000001	1	2	
<0.000001	36	32	

For two antibodies, MCL1, and STAT3, different antibodies were used and provided poorly correlated proteins