Determination of Binding Specificities in Highly Multiplexed Bead-based Assays for Antibody Proteomics* [S]

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One of the major challenges of antibody-based proteomics is the quality assurance of the generated antibodies to ensure specificity to the target protein. Here we describe a single tube multiplex approach to simultaneously analyze the binding of antibodies to a large number of different antigens. This bead-based assay utilizes the full multiplexing capacity theoretically offered by the Luminex suspension array technology. A protocol for an increased coupling throughput for the immobilization of antigens was developed and used to set up complex and stable 100-plex bead mixtures. The possibility of using a two-dimensional multiplexing, in terms of high numbers of both analytes and samples or as in this case antigens and antibodies, enables the specificity of 96 antibodies versus 100 different antigens to be determined in 2 h. This high throughput analysis will potentially have great impact on the possibility for the utilization of different antibody proteomics approaches where the quality assessment of antibodies is of the utmost importance. Molecular & Cellular Proteomics 6:125–132, 2007.

Challenges of the postgenomics era focus on the exploration of the human proteome and aim toward a better understanding of disease-related processes (1). These challenges demand the development of elaborate methods that meet the requirements of improved sensitivity and throughput. The use of specific binding molecules to study target proteins is one possibility when examining expression patterns, subcellular localization, biochemical function, splice variants, and post-translational modifications of proteins.

One antibody-based initiative to study the complexity of human proteins is the human protein atlas for normal and cancer tissues (2). Here a bioinformatics approach is applied to select regions between 100 and 150 amino acids of low homology toward other proteins from genes excluding transmembrane regions and signal peptides (3). These protein epitope signature tags (PrESTs) are expressed together with an affinity handle, purified, and used for the immunization of rabbits. A semiautomated chromatography system is utilized to deplete and affinity purify the polyclonal antisera to obtain so called monospecific antibodies (msAbs) (4). However, before antibodies can be applied to tissue microarrays for immunohistochemical analysis (5) antibody quality has to be quality-assured by differing means. One such method is performed on a planar protein microarray platform used for the specificity analysis of all generated antibodies to 96 antigens (4).

Other approaches for antibody analysis have been described using immobilized proteins (6) or peptides (7) for planar protein microarrays. These systems use an arraying device to create a two-dimension arrangement of immobilized molecules on microscopic slides. The results of performed assays are displayed by the use of reporter dyes, a biochip reader system, and a subsequent image analysis. An alternative platform for a parallelized and miniaturized analysis of proteins is offered by bead-based technologies (8). One available system is built on the principle to use spectrally distinguishable beads (9). A red and an infrared dye are incorporated at different ratios into these microspheres. This creates a set of 100 beads of different color code signatures. Mixtures of these beads are used to create arrays in suspension. A flow cytometer analyzes the co-occurrence of the color code and bead bound reporter dye to display bead assigned interactions.

In the present study, we aimed to develop a highly multiplexed bead-based method for a fast analysis of binding specificities of monospecific antibodies. To facilitate such an analysis, different antigens were coupled to the whole set of available color-coded beads. A coupling procedure was modified to allow an increased amount of loaded beads per preparation. Optimized bead mixtures were subsequently applied to high throughput analysis of antibodies in a robust assay setup.

EXPERIMENTAL PROCEDURES

Antibodies and Antigens—The protocols for antigen selection, cloning, protein expression, immunization of rabbits, and affinity pu-

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* The abbreviations used are: PrEST, protein epitope signature tag; MFI, median fluorescence intensity; msAb, monospecific antibody; HisABP, His6-tagged albumin-binding protein; ABP, albumin-binding protein; AU, arbitrary units; ID, identification; r, correlation coefficient.
distribution of counted beads. The graph shows the distribution of counted beads from two bead mixtures that are derived from the same set of coupled beads. Sets of 100 different beads (ID001–ID100) were mixed and distributed in 96 wells to be used for antibody analysis. The instrument was set to count 100 events per bead ID. The displayed counts represent the mean and S.D. from 96 measurements. The counted events that are derived from applied volumes given by the postcoupling procedure counting (gray diamonds) are distributed unevenly at an average count of 300 ± 100 events per bead ID. This was improved upon a simple trimming procedure by the application a correction factor. The trimmed distribution (black dots) shows an aligned distribution with a bead count of 120 ± 14 events per bead ID.

Correction factor. The trimmed distribution (black dots) shows an aligned distribution with a bead count of 120 ± 14 events per bead ID.

Multiplexed Analysis of Binding Specificities

Bead Coupling in 96-Well Plates—Based on the manufacturer’s protocol a bead coupling procedure was developed using a filter membrane-bottomed microtiter plate (MultiScreen-HTS, Millipore) and a vacuum device (MultiScreen Vacuum Manifold, Millipore). For each coupling, up to 10⁶ beads per ID were applied to separate wells. All beads were washed and resuspended by sonication. The plate was placed into a plastic tray (Bio-Rad) containing buffer. This was placed into an ultrasonic cleaner and sonicated for 5 min to resuspend beads from the filter membrane. For coupling 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (10 μl, 50 mg/ml) and N-hydroxysuccinimide (10 μl, 50 mg/ml) were prepared and applied to activate the beads. The plates were incubated in a shaker (Thermomixer, Eppendorf) for 20 min. The liquid was removed, and beads were washed and subsequently sonicated for 5 min. PrEST proteins were added to the corresponding well to a final concentration of 40 μg/ml. Coupling took place over 120 min under permanent mixing. Wells were washed with and beads were resuspended with sonication to be transferred to microcentrifuge tubes (Starlab) for storage.

Preparation of Bead Mixtures—Protein-coupled beads were counted using the Luminex LX200 instrument. The beads were vortexed, sonicated, and applied to the bead mixture using 500 beads per ID and analyzed. The bead mixture was resuspended in PBST, sonicated, and stored at 4 °C in the dark. After each series of analysis initially applied volumes of each bead ID were modified according to the counted bead events. The applied volume per ID was trimmed for subsequent mixtures of beads. The average number of events for each ID was determined and used to divide 120. This ratio was then used as a correction factor for the previously used volumes for the respective bead ID.

Determination of Antibody Specificity—All antibody dilutions were prepared in PBST. The applied msAb concentration was on average around 30 ng/ml. Two different anti-HisABP antibodies were available, either rabbit- (diluted 1:26,500) or chicken-generated (diluted 1:60,000). The antibody dilutions (45 μl) were added to the bead mixture (5 μl) and incubated for 60 min under permanent mixing (Thermomixer) in a 96-well plate (Corning). Subsequently R-phycoerythrin-labeled anti-rabbit IgG antibody (0.5 μg/ml, 25 μl, Jackson Immunoresearch) or anti-chicken IgY (0.5 μg/ml, 25 μl, Jackson Immunoresearch) was added for a final incubation of 60 min. Wash assays performed in a filter membrane-bottomed microtiter plate (Millipore) were carried out under the above stated conditions and concentrations. Washing was performed postantibody incubation on a vacuum device (Millipore) with PBST (3 × 75 μl).

Readout and Data Analysis—Measurements were performed on Luminex LX200 instrumentation using Luminex IS 2.3 software counting 100 events per color code ID for each single specificity analysis. To display antibody-antigen interactions the median fluorescence intensity (MFI) was chosen. Data analyses and graphical representations were performed using Microsoft Office Excel 2003 or R, a language and environment for statistical computing and graphics (11).
RESULTS

Bead Mixtures—The setup of a multiplexed bead-based analysis requires a controllable input of bead mixtures because the creation of such suspension arrays is based on the combination of beads with different color code IDs. Here all 100 currently available IDs were chosen for the immobilization of antigens. Once coupled, the beads were counted for the determination of the coupling yield. This is required to indicate how many beads were recovered from the coupling procedure and to define the quantity of beads per volume. In the presented assay system, an amount of 500 beads per ID was sufficient for the detection of at least 100 events. This requirement resulted in the finding that beads from the plate coupling procedure could be used in more than 1500 experiments. Moreover slight tendencies toward tailing or agglutination were prevented by using such an optimized bead mixture as shown by the bead maps (Supplemental Fig. 1). In addition, beads appeared at their respective region with only minor scattering. In the present study, no signs of bleaching were found.

Assay Properties—A total number of 84 different msAbs

**Fig. 2. Analysis of bead coupled proteins.** PrEST proteins immobilized on beads were analyzed using anti-HisABP directed antibodies to provide evidence of coupling. A, a rabbit IgG antibody was utilized to detect immobilized HisABP. On all PrEST-coupled beads (black columns) HisABP was detected above background (red line). An anti-rabbit IgG antibody on bead ID001 (gray column) displayed the presence of the applied anti-HisABP antibody. Displayed data are derived from mean values from a triplicate analysis performed in separate cavities; error bars represent S.D. HisABP signals varied less than 40% relative to an average MFI of 824 AU and were more than a factor of 10 above background. B, the correlation of the HisABP determination was studied in independent experiments and displayed a correlation of \( r = 0.99 \). C, the presented rabbit anti-ABP-based system was compared with a different HisABP detection system using an anti-HisABP chicken IgY antibody. Both systems also differed in the applied species-specific, labeled detection antibody but correlated with \( r = 0.95 \).
Fig. 3. **Overview of specificity analysis.** Binding specificities of 84 msAbs were determined in a multiplexed assay against PrEST antigens. The results were summarized in a heat map to display the signal intensities in a color scale. Each antibody (msAb001 to msAb083; msAb097) had been raised against a respective antigen (PrEST-001 to PrEST-083; PrEST-097) and was incubated at a concentration of less than 0.1 μg/ml. The diagonal line of dots indicates binding of the antibodies to their PrEST antigens, whereas signals appearing to the left or right of this line indicate unspecific binding to other PrESTs. In total, 13 of 84 (15%) msAbs showed cross-reactivity to other antigens than the one used for immunization. The vertical colored line on the far right-hand side of the map stems from anti-rabbit IgG signals and displays the presence of the msAbs in each test. All shown results are average values from three separately performed experimental runs.
were tested for specificity in an assay with antigen-coupled beads. 100 different bead IDs were utilized to create a bead mixture that contained 98 bead IDs coupled with different PrEST antigens, one bead ID carrying the common tag protein HisABP (ID080) and one bead ID coated with an anti-rabbit IgG antibody (ID001). The anti-rabbit IgG antibody beads were used to determine the presence of rabbit IgG molecules and to display failures in specific binding or the absence of antibody. To verify coupling of PrEST proteins to the beads the immobilized antigens were detected via their HisABP tag by respective antibodies as shown in Fig. 2. This test was performed in triplicates for each run and used to determine a correlation coefficient \( \rho \) between separately performed experiments. The resulting correlations for rabbit antibodies were \( \rho = 0.99 \). When comparing the results between assays utilizing rabbit or chicken antibody-based ABP detection the correlation was \( \rho = 0.95 \). A blank incubation of the bead mixtures without msAbs present was used to display potential cross-reactivity of the applied detection system and bead-derived cross-talk. The background signals determined in PBST buffer containing anti-HisABP from chicken as shown in Fig. 2A averaged at 43 AU. Upon incubation without chicken IgY antibodies, the average background signal dropped to 8 AU (see also Fig. 4). Further indications that the increase in background derived from the IgY could be drawn from correlating these signals with the HisABP detection. Here \( \rho > 0.9 \) showed that the applied anti-rabbit IgG detection antibody can bind IgY to a minor extent.

The order in which the antibodies were measured by the instrument was varied and did not affect the specificity analysis. No carryover effects from previous wells could be detected.

**Determination of Antibody Specificity—**For the purpose of antibody specificity analysis, all affinity-purified antibodies were diluted to a concentration of less than 85 ng/ml (<30 fmol of total protein per experiment) and analyzed in three separately performed assays. Mean values of these experiments were used to display the binding properties. Results were summarized in a heat map format shown in Fig. 3. 85% of all tested msAbs were specific and only recognized their respective antigen. To assure that msAbs were able to bind their target with an adequate strength, the resulting specific signal intensities should be above a signal-to-noise ration of 10 or 430 AU. The determined average signals were then grouped based on this value and integrated into a density plot (Fig. 4). At the chosen cutoff level a total number of 107 signals from 84 analyzed antibodies were measured above the threshold of 430 AU. For less than 5% (4 of 84) of all tested antibodies the specific signal intensity was below cutoff. The major proportion of 8209 signals had a signal-to-background ratio of less than 10. When the distributions of these signals and those of background signals without any applied msAb were compared only a slight shift to increased signal intensity was observed when monospecific antibodies were present.

**Confirmation of Binding Pattern for Specificity Validation—**To confirm the results obtained from the presented bead-based approach, antibodies were also tested on planar microarrays. The same antibody solutions were applied to an array of 96 PrEST antigens matching those from the bead-based system. Specific binding was confirmed by both systems. In terms of cross-reactive or unspecific binding, a congruent binding pattern could be displayed with the two approaches as shown in Fig. 5. The presented examples show that binding occurred to their target antigen as well as to further PrEST proteins. Alignment of the target sequences to two additionally detected PrESTs revealed a noticeable sequence homology (data not shown). In a single case, specificity could only be shown on planar microarrays; although the antibody was tested in different bead-based assays, no binding to the correct antigen could be detected. These also included experiments with a reduced number of different bead IDs such as single or duplex assays (data not shown). The related target antigen (PrEST-071) was 55 amino acids long and had a high content of lysine and arginine (see “Discussion”).
Because the present bead-based assay procedure was built up to manage antibody analysis without washing steps, the permanent presence of the binding molecule may also display interactions of lower affinity. To prove specificities of antibodies in an alternative setup, a procedure was developed in which the monospecific and detection antibodies were removed after each incubation step and beads were washed in between. Antibodies showing cross-reactivity in the primary screening were reapplied to reconfirm their binding pattern in wash and no-wash experiments. A correlation between wash and no-wash using the rabbit antibody-based anti-ABP test assays resulted in $r = 0.99$. The effect of the washing steps slightly improved the signal-to-noise ratio compared with the no-wash setting. To ensure that mixtures of beads are stable once created, a bead mixture was stored over 3 months and reused for specificity analysis as shown in Fig. 5. The results from this stored bead mixture cover all previously indicated binding events and therefore prove to serve as a stable source for the reproduction of such specificity analysis. Additional experiments were performed on all three assay platforms using increased msAb concentrations exceeding 100 ng/ml (Supplemental Fig. 2). At higher concentrations the antibodies tended to bind to more antigens, and the cross-reactive binding events became more pronounced. But even at the lowest antibody concentration binding toward the major cross-reactive antigens was detectable.

Expanding the Range of Parameters and Reinsetion of Beads—To study the possibility to scale up the total number of parameters on which a binder can be tested, more than one 100-plexed bead mixture was tested. To study such a scenario a second bead mixture was created from a different set of antigens. Another 96 PrEST proteins were coupled to beads and mixed with beads that were used for the first approach such as anti-rabbit IgG (ID001), HisABP (ID080), PrEST-097 (ID071), and PrEST-098 (ID070). PrEST-097 was further coupled to a second bead (ID072) to confirm binding on a second bead ID. By the reutilization of beads in different mixtures identical material can be used in various experiments. Antibody msAb097 that was raised against PrEST-097 was studied with two differing sets of beads against a total number of 193 antigens (Fig. 6). In both cases the antibody was shown to be very specific to its target antigen irrespective of the bead mixture, the set of antigens, or the bead ID to which the antigen was coupled.

DISCUSSION

The presented bead-based assay allows a fast and direct determination of binding specificities in a highly multiplexed fashion. All 100 currently available bead IDs were used to set up a procedure to analyze the interaction of antibodies with 99 different PrEST antigens. A bead mixture was optimized to allow an even amount of counted beads including one bead ID to report the presence of IgG molecules. A simple assay procedure was developed without the need of any washing steps. Studies carried out in 96-well plates allowed the analysis of up to 96 binders. We studied 84 msAbs at a time and combined our experimental setup with control experiments. In less than 2 h postincubation a complete set of 9600 data points to study antibody-antigen interactions was available for analysis. To our current knowledge no other reports on using
We further describe a method to increase the bead coupling throughput using the microtiter plate format. This reduces the overall time to set up bead mixtures of higher complexity and can be implemented into available liquid handling systems for automation. The overall yield from coupling 10^6 beads in a microtiter plate allowed the antigens to be applied in more than 1500 separate experiments. For each experiment around 500 beads per ID were used to yield 100 counted events.

PrEST-coupled beads were mixed to create differing bead mixture compositions including some beads that were common. One prerequisite for highly multiplexed sets of protein-coupled beads is the homogeneous suspension of beads. In the presented study, no cross-talk or agglutination between the antigen-coupled beads was discovered. Once created, a bead mixture was stable for at least 3 months without indications of bleaching or bead coagulation. Moreover the binding pattern of tested cross-reactive antibodies resembled that of previous analysis using planar microarrays and different bead-based setups.

Our bead-based assay system was found to be a flexible and reproducible tool. Beads can easily be inserted or omitted in certain bead mixtures when being created. This is beneficial because controls can be reapplied in different experimental setups to enable comparability of results. Furthermore less complex experimental setups such as single or duplex assays are feasible when confirmation of binding events is demanded. The number of available bead IDs is currently 100. This reduces the total number of parameters that antibodies can be tested on at one time. This limitation can yet be overcome in the use of more than one bead mixture to analyze specificities as presented by a feasible reinsertion of beads to a different bead mixture. When screening a large number of samples for a limited amount of parameters the presented bead-based assay could add to planar microarray approaches. In addition, the applied platform neither needs the setup of an arraying and scanning facility nor requires a post-assay image analysis.

Other bead-based systems have been used previously to characterize monoclonal antibody utilizing a competitive approach (12). In our presented assay system, different proteins were coupled to discrete beads and utilized in different binding reactions. This assay system holds a great potential to be used for many different kinds of interaction analysis and may supplement approaches to screen antibody libraries (13) or those of binding molecules with alternative scaffolds.

When the bead-based assay system was compared with a planar microarray approach, very similar results could be achieved concerning specificity of antibodies and their binding pattern. The few differences that were also discovered may stem from differing immobilization procedures, surface
and surface treatment, storage conditions, and applied detection antibodies and their dyes as well as the different readout systems. As a result of this the absolute signal intensity and sensitivity can be affected. For both systems similar dynamic ranges and sensitivity could be observed.

We discovered a case where a shorter PrEST antigen failed to be recognized in our bead-based approach but was bound by its respective antibody using the planar microarray. A possible explanation for this was that the epitope of the antibody was masked during PrEST immobilization using N-hydroxysuccinimid ester chemistry because 11 of the 55 amino acids of the antigen were either lysine or arginine. In contrast to this, antigens were immobilized on glass slides via epoxy chemistry where different functional groups from the protein can act in the covalent attachment to the surface. As a future alternative for the bead coupling, specific affinity interaction such as biotin-avidin or His₆-chelator can be utilized to get a directed immobilization. However, the stability of such non-covalent strategies requires further investigation to assure that no antigenic information is transferred from one bead to another during storage of highly multiplexed bead mixtures.

In conclusion, our bead-based approach provides a promising tool for specificity analysis of binding molecules and allows approaches such as antibody-based proteomics that demand a certain throughput to select and utilize suitable binders. A protocol for an increased coupling throughput for the immobilization of antigens was developed and used to set up complex and stable bead mixtures of 100 different bead IDs. The analysis of monospecific antibodies was performed in highly multiplexed fluorescence-based assays that did not require additional washing steps but was shown to be highly reproducible. When compared with a planar protein microarray approach congruent results in terms of the binding patterns were obtained. To conclude this, the characterization of antibodies by a bead-based assay is a promising method allowing the specificity determination of 96 antibodies versus 100 different antigens in 2 h. The possibility to utilize such a two-dimensional multiplexing in terms of high numbers of both analytes and samples or as in this case antigens and antibodies is shown. This high throughput analysis will potentially have great impact on the possibility for the utilization of different antibody proteomics approaches where the quality assessment of antibodies is of utmost importance.

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The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

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