

TECHNICAL BRIEF

Magnetic bead assisted labeling of antibodies at nanogram scale

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There are currently several initiatives that aim to produce binding reagents for proteome-wide analysis. To enable protein detection, visualization, and target quantification, covalent coupling of reporter molecules to antibodies is essential. However, current labeling protocols recommend considerable amount of antibodies, require antibody purity and are not designed for automation. Given that small amounts of antibodies are often sufficient for downstream analysis, we developed a labeling protocol that combines purification and modification of antibodies at submicrogram quantities. With the support of magnetic microspheres, automated labeling of antibodies in parallel using biotin or fluorescent dyes was achieved.

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Antibodies are considered as essential tools in biological and medical studies and are extensively used to separate, identify, locate, or quantify target molecules in several assays such as immunoprecipitation, Western blot, microarrays, fluorescence microscopy, immunohistochemistry, flow cytometry, and enzyme-linked immunoassay [1, 2]. Antibodies are also known in biochemical and clinical diagnostic tests and as therapeutics [3]. Since each immunoassay mostly relies on the availability of specific and functional antibodies against the desired targets, a vast antibody repertoire targeting numerous different molecules has been produced over decades [4]. In most assays, visualization of antibody-target interaction is achieved via coupling reporter molecules to a detection antibody. Fluorescent dyes, biotin, biolumi-

nescent markers, and enzymes are examples of frequently used reporter molecules [5]. Apparently, efficient reporter molecule coupling would assure the generation of a desired signal in subsequent analysis steps. Therefore, several protocols have been developed for covalent coupling of reporter molecules to antibodies and standard procedures are available for particular applications [6]. Currently, tens of micrograms to milligrams quantity of antibodies are most often recommended per labeling assay [7], even though numerous antibodies can be obtained only at restricted quantities [8]. In addition, antibodies are often provided in buffers containing primary amines (e.g. Tris-based), supplement proteins or stabilizers such as BSA. Such formulations quench many commonly used coupling chemistries such as *N*-hydroxy succinimide esters thus affect labeling efficiency, moreover a certain amount of antibody is needed for efficient purification, which could limit antibody applicability for labeling [9]. Consequently, the application of many antibodies is hampered by

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Abbreviations: BAP, bead-assisted protocol; ISP, in-solution protocol; MFI, melt flow index

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the lack of a labeling protocol that requires only small quantities of antibodies, disregard of the purity level. These facts considerably hinder the use of whole existing antibody repertoire in immunoassays [8], hence the ability to perform labeling on smaller amounts of antibodies can be practically beneficial.

To date, most common antibody labeling protocols are based on performing the reaction in solution, though it is possible to label antibodies on solid phase [10]. A solid-phase approach enables robust labeling by providing control over the reaction conditions and allows for more efficient washing steps resulting in a considerable decrease in initial antibody quantity for a successful labeling reaction [11]. Moreover, the coupling reaction on solid phase is performed in combination with simultaneous purification and concentration of the labeled antibody, which eliminates a separate purification or buffer exchange step and even allows for using impure antibodies and samples at lower concentrations [9]. Nevertheless, one disadvantage with solid-phase labeling is the fact that antibody immobilization can affect the labeling process by steric hindrance of the reactive sites or partial denaturation of antibody due to capture and elution procedures [12]. These challenges were addressed by Staphylococcal protein A coated resin [11] to provide a captured antibody with an oriented immobilization [13] that has allowed fluorescent labeling of as low as ten micrograms of antibodies. Yet, this amount does not cover the available quantity of entire antibodies repertoire, driving the need for methods using less initial amount. Another concern with the existing solid-phase labeling protocols is that large-scale and parallel-labeling reactions would be expensive, laborious and time consuming [14]. Currently, in several solid-phase bioassays, magnetic microspheres have been used as carriers in order to enable automation on robotic workstations. Additionally, using microspheres principally allows for a considerable downscale in the required reagents by providing homogenized mixtures and feasible reactions at lower reagents quantities by higher localized concentrations [15].

In this study, we present a bead-assisted protocol (BAP) for labeling of antibodies at nanogram scale using protein A coated magnetic microspheres (Fig. 1). As a proof of concept biotinylation and fluorescent dye labeling of antibodies (Supplementary Information Table 1) were performed and functionality of labeled antibodies was investigated. Primarily, the efficiency of magnetic beads as carriers was evaluated for the possible loss of antibodies after capture, washing, and elution procedures. Two micrograms of polyclonal rabbit IgG antibody were immobilized on magnetic microspheres (0.3 mg) according to manufacturer's instructions. Similar washing and incubation steps were performed as in BAP and three times sequential elution was performed. Antibody quantity in three eluates and supernatants after all incubation and washing steps were analyzed through spectrophotometric measurements (Supplementary Information Methods 1). The results showed that >70% of captured antibody was recovered from the beads, demonstrating satisfactory efficiency

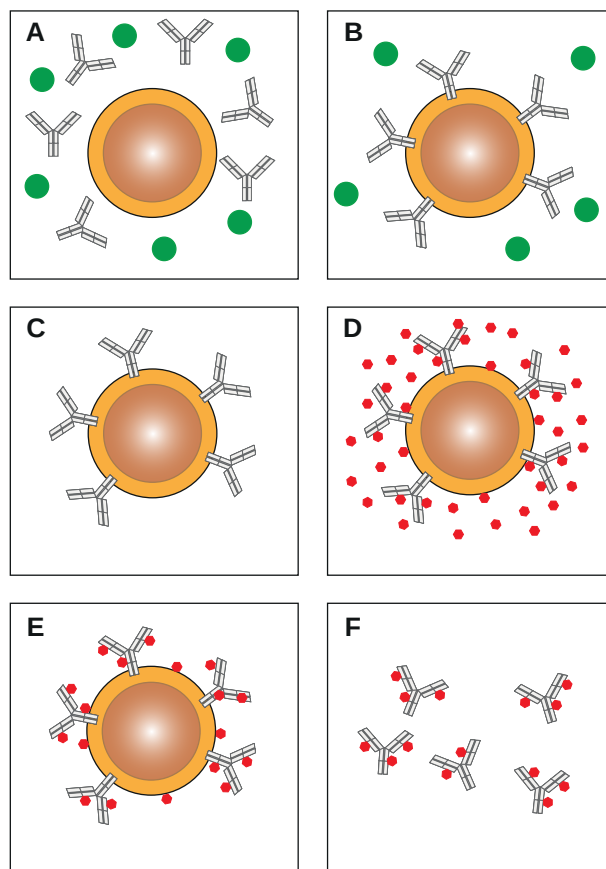


Figure 1. Schematic view of BAP for antibody labeling. (A) Antibodies are incubated with protein A coated magnetic beads and (B and C) unbound impurities (green) are removed by washing the microspheres using a magnetic separator. (D) Labeling reagent (red) is added and reaction occurs via amine-reactive *N*-hydroxy succinimide esters groups. (E) Antibodies are purified from the excess labeling reagent and (F) labeled antibodies are eluted from the beads by lowering the pH, followed by immediate pH adjustment. All steps are set automated on a magnet-equipped robotic workstation.

compared to immobilization in columns [11]. In addition, no antibody was detected (<10 ng) in the last two eluates, i.e. the total recovered antibody can be eluted in a volume of only 10 μ L. Using more beads and longer incubation times may be required to achieve efficient immobilization of antibodies at lower initial concentrations or with weaker-binding efficiency [16]. Therefore, time and volume of capturing step was set as alterable by user in the automated system, for the possibility of specific adjustment to problematic, impure, or low-concentrated antibodies.

The high affinity of biotin-avidin interaction has driven the development of numerous assays [17] and a wide range of reactive biotin reagents is available for covalent labeling [18]. In order to perform the biotinylation of antibodies using BAP (Supplementary Information Methods 2) and ensure the desired biotin incorporation (2–3 biotins per antibody molecule), two micrograms of polyclonal rabbit IgG antibody

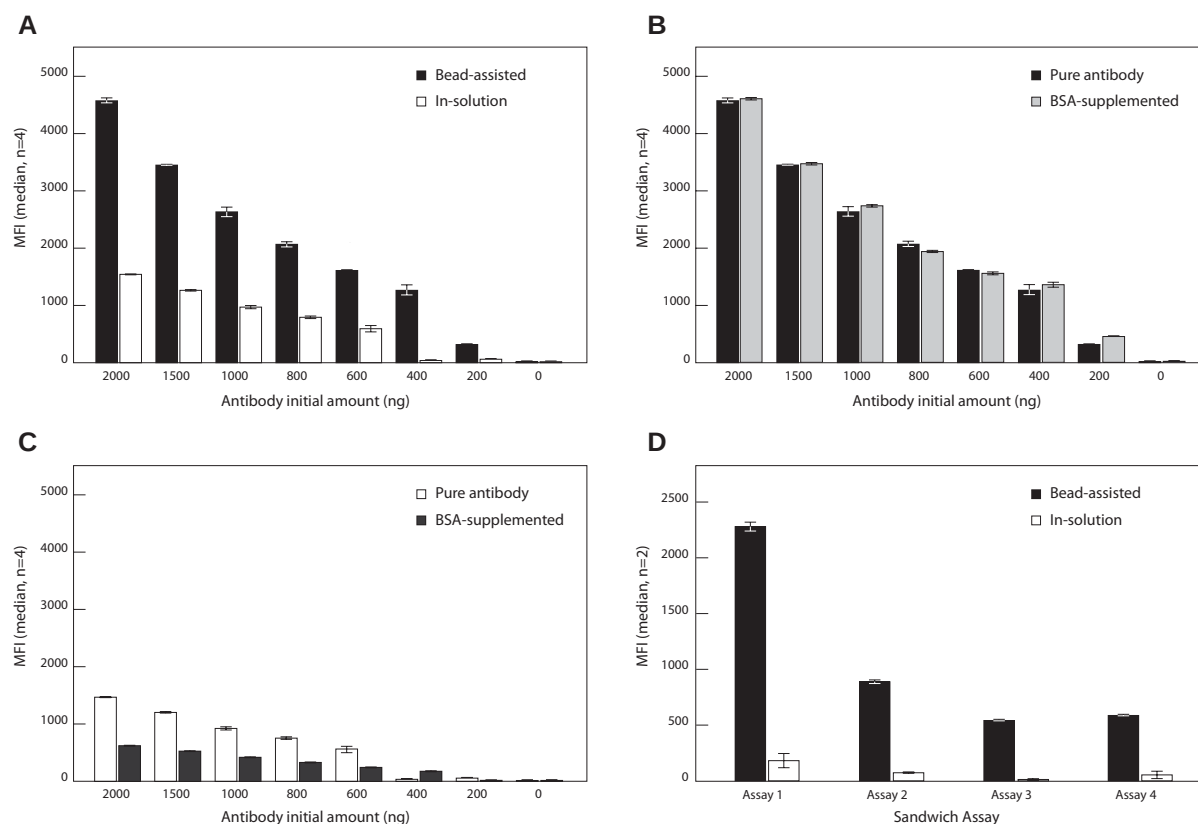


Figure 2. Comparison of BAP and ISP for antibody labeling. (A) Biotinylation of varying amounts of antibody using BAP (black) versus ISP (white) showed higher signals from BAP. Results demonstrated the possibility of labeling as low as 200 ng antibody using BAP. (B) Similar results were achieved from labeling pure (black) and BSA-supplemented antibodies (light gray) using BAP. (C) Signals from biotinylated BSA-supplemented antibodies (dark gray) were significantly lower ($p < 0.05$) than labeling pure antibodies (white) when ISP was employed. (D) Application of labeled antibodies using BAP (black) and ISP (white) as detection antibodies in sandwich assays, confirmed higher signals from BAP.

were biotinylated using varying molar excess of traceable biotinylation reagent over antibody (50–200 times). Coupling of 2.98 ± 0.13 biotin moles per mole antibody was shown when 150 times biotin molar excess (1.8 nmol) was applied (Supplementary Information Fig. 1). In common practice, when using recommended amounts of antibody and reagents for an in-solution protocol (ISP), 50 times molar excess of biotin is sufficient to obtain 2–3 biotin moles per mole antibody. Here, the presence of protein A during labeling (minimum two times of antibody amount), explains the requirement for using more (three times) biotin to compensate for the presence of another protein (giving 50 times molar excess over total protein quantity). However, since smaller amounts of antibody were used and the protocol is miniaturized, the amount of labeling reagent in BAP is still lower in quantity than for common ISPs.

In order to demonstrate labeling at lower antibody amounts using BAP, biotinylation was performed on varying amounts of initial antibody (200 ng to 2 μ g of polyclonal rabbit IgG). In parallel, a common succinimide-ester protocol was done in solution (Supplementary Information Methods 3). Equal amount of biotinylated antibodies (25 ng

at the concentration of 1 μ g/mL) were applied to suspension bead arrays to be captured by anti-rabbit IgG and detected via fluorescently labeled streptavidin (Supplementary Information Methods 4). The results showed that biotin incorporation by the proposed BAP was superior over ISP, when using lower amounts of antibody than generally recommended for ISP. This could be explained by an inefficient reaction in downscaled ISP resulting in decreased yield or lower degree of labeling (Fig. 2A). In addition, antibodies supplemented with 1% BSA as stabilizer were biotinylated as described above. Similar signals were detected in case of BAP showing the possibility of labeling impure antibodies, whereas a significant (Student's t -test, $p < 0.05$) reduction was observed in the signal intensities of BSA-supplemented antibodies from ISP. This can be explained by the presence of additional protein (BSA) in solution for ISP, which causes extra consumption of the labeling reagent. Though, in case of BAP the impurity would not affect the outcome since the antibody is purified from BSA supplement prior labeling (Fig. 2B and C).

Modification reactions on antibodies may result in altered or loss of function, and thus disrupting the binding

efficiency of labeled antibodies to target antigens [19]. In order to confirm the functionality of labeled antibodies, 1 μ g of three rabbit IgG antibodies A1–A3 (Supplementary Information Table 1) were biotinylated (using 0.9 nmol biotin) by BAP and ISP. Antibodies A2, 3, and 6 were coupled to magnetic beads as capture antibodies. Corresponding antigens were applied and biotinylated antibodies from both BAP and ISP were used as detection antibodies in four sandwich assay combinations (Supplementary Information Methods 4). Results showed higher signal intensities of labeled antibodies from BAP versus ISP (Fig. 2D), where ISP signals were under background level. This indicates a better LOD using labeled antibodies from BAP.

To further investigate the proposed protocol, 1 μ g of ten different antibodies A4–A13 (Supplementary Information Table 1) were biotin labeled in parallel using automated BAP. Distinct Luminex microspheres were coated with corresponding antigens, respectively, and a suspension bead array was prepared by pooling the beads of different color-code at equal concentrations of beads. A dilution series of each biotinylated antibody was used as detection reagent on the array and signal intensities were analyzed (Supplementary Information Methods 4). Results confirmed functionality of labeled antibodies from BAP. Moreover, the melt flow index (MFI) values reached a plateau using more than 25 ng of detection antibody per well. This might indicate the scarce requirement of labeled antibody amount in the final detection assays (Fig. 3A). This amount is in concordance with recommended concentration of labeled antibody (0.5–2 μ g/mL) in commonly used protocols.

In addition to biotinylation, fluorescent techniques have an important role in many biological assays [14]. The proposed protocol was therefore applied for dye labeling of three different antibodies A14–A16 using two fluorescent dyes (Supplementary Information Table 1). Functionality of dye-labeled antibodies was investigated using planar microarrays (Supplementary Information Methods 5) and detected signal to noise ratios confirmed the specificity of dye-labeled antibodies (Fig. 3B).

In summary, we have described an automated protocol for antibody labeling using nanogram quantities of antibodies. This aids current requirements within affinity proteomics toward higher throughput and multiplexing of immunoassays. Dye and biotin labeling together with in-procedure purification of antibodies have been confirmed for the *N*-hydroxy succinimide esters chemistry at as low as 200 ng using protein A coated magnetic microspheres as solid support. Though, the observed MFI value of $\sim$ 200 AU for 200 ng labeling might not suffice for some applications. Hence, this has to be evaluated for each detection assay in order to define the lowest threshold for antibody amount based on requirements of the subsequent detection method. The proposed concept can be coupled to magnet equipped robotic workstation with a higher capacity for parallel labeling of hundreds of antibodies in less than 3 h. The automated set-up is flexible with regard to quantities, timing, temperature, and volumes, there-

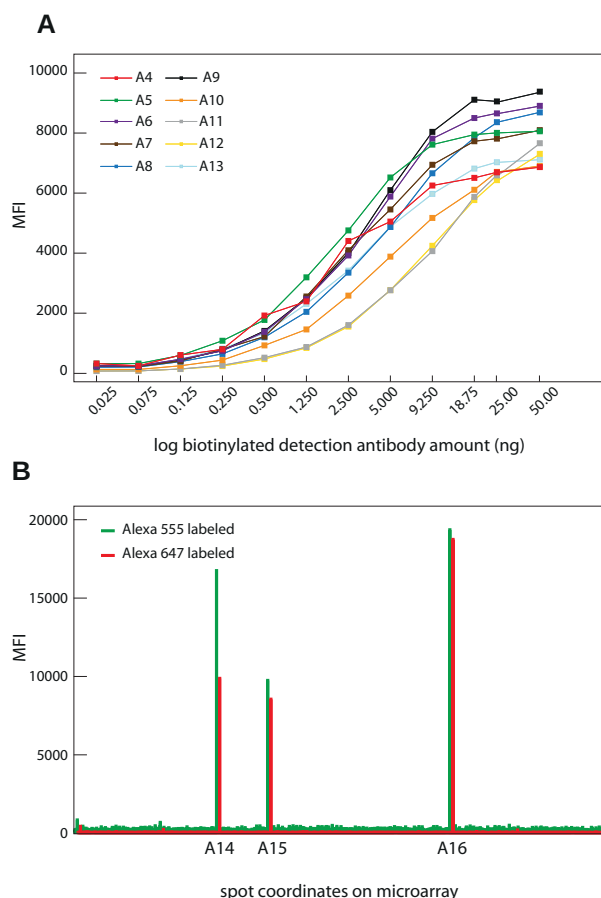


Figure 3. Evaluation of labeled antibodies using BAP. (A) Luminex microspheres coated with antigens incubated with different concentrations of biotinylated antibodies (median, $n = 2$) showed specificity of labeled antibodies to target antigens. The signal does not increase using higher concentrations than 1 μ g/mL of detection antibody. (B) Functionality investigation of Alexa 555 (green) and Alexa 647 (red) labeled antibodies on planar microarrays showed strong fluorescent signal to noise from correct spot coordinates on the array. This confirmed successful labeling procedure and showed retained functionality of dye-labeled antibodies.

fore can be facily adapted to many reactions with analogous labeling chemistries. The range of coatings for magnetic microspheres as well as customized surface functionalization offers coupling to label any desired molecule, antibody isotypes, or other proteins. In conclusion, labeling nanograms of antibodies serves both small and large-scale analysis and opens possibilities for high throughput and multiplexed applications in the antibody-based proteomics field.

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Table S1. Information on antibodies and target antigens used in labeling assays.

Affinity-purified mono-specific polyclonal antibodies (rabbit IgGs) and corresponding target antigens, Histidin-tagged protein fragments (ca. 100 amino acids) denoted protein epitope signature tags (PrESTs) were acquired from the Human Protein Atlas (HPA). Antibody A3 was supplied by Aviva Systems Biology Corporation (San Diego, CA).

Antibody	Antibody ID from provider	Target Antigen	Gene Accession Number
A1	HPA023317	P1	EFCAB3
A2	HPA030717	P2	EFCAB3
A3	ARP53251_P050	-	EFCAB3
A4	HPA060274	P4	ZNF432
A5	HPA060253	P5	SYNE4
A6	HPA060247	P6	ZNF554
A7	HPA060363	P7	FAM195A
A8	HPA060368	P8	SULT1C3
A9	HPA060290	P9	SENP3
A10	HPA060291	P10	AC006486.1
A11	HPA060387	P11	AL354898.1
A12	HPA060395	P12	C7orf61
A13	HPA060399	P13	OR10R2
A14	HPA049577	P14	EMILIN2
A15	HPA049579	P15	C5orf50
A16	HPA049588	P16	IFI27L2

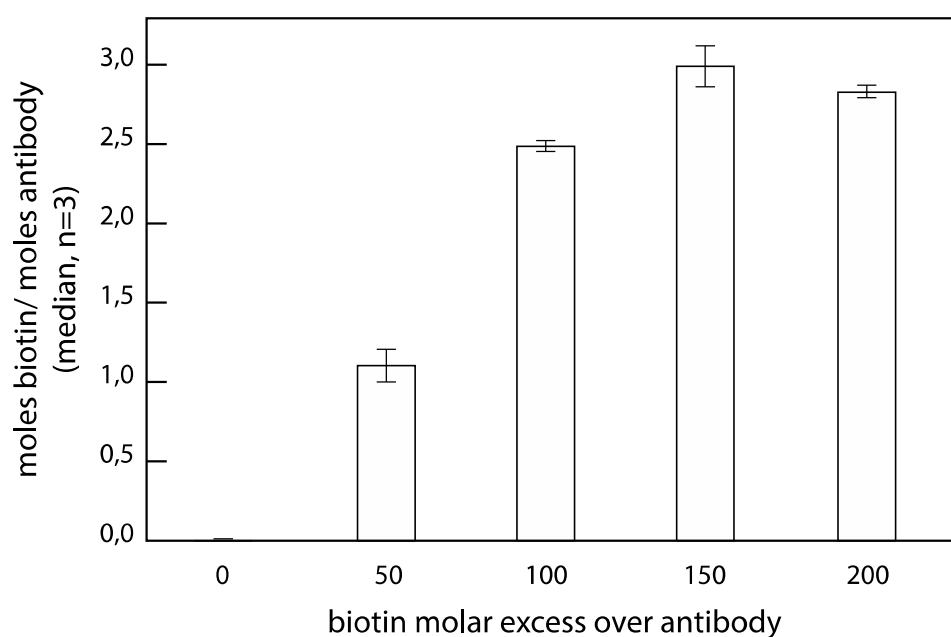


Figure S1. Investigation of degree of labeling (DOL) using BAP with varying molar excess of biotin over antibody.

Results from biotinylation of 2 µg rabbit IgG antibody using traceable biotinylation reagent showed coupling of 2.98 ± 0.13 biotin moles per mole antibody, using 150 times biotin molar excess (1.8 nmol). Using more biotin quantity does not give a higher degree of labeling.

Supplementary Information- Methods

Magnetic Bead Assisted Labeling of Antibodies at Nanogram Scale

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1. Evaluation of magnetic microspheres efficiency as solid support

Two micrograms of polyclonal rabbit IgG (Bethyl laboratories, Montgomery, USA) in 30 μ l PBS (pH 7.4, Medicago AB, Uppsala, Sweden) were captured on 10 μ l (0.3 mg) of recombinant protein A coated magnetic microspheres (Dynabeads^R, Life Technologies, New York, USA) according to manufacturer's instructions. After 2 h incubation on ice, two times washes were performed in 100 μ l PBST (PBS, 1% Tween 20, pH 7.4) and once in PBS. The antibody was eluted by lowering the pH for two minutes in 10 μ l acetate solution (0.2 M, pH 3.3). Three sequential elutions were performed and 2.5 μ l (quarter eluate volume) of Tris buffer (1 M, pH 11) was immediately added to each eluate to raise the pH. The concentration of antibody was determined in three eluates and all microsphere supernatants from incubation and washing steps by using an absorbance spectrum at 280 nm ($\epsilon=170000$ M⁻¹ cm⁻¹) on a NanoDrop 8000 Uv-Vis Spectrophotometer (ThermoFisher Scientific, California, USA). Efficiency was calculated as the portion of antibody recovered in eluates in regards to initial antibody amount.

2. Magnetic bead assisted protocol (BAP) for antibody labeling

Protein A coated magnetic microspheres (adjusted amount to antibody quantity) were washed twice in 100 μ l of PBST and buffer exchanged to 100 μ l PBS. The supernatant was removed from the beads using a magnetic separator and the antibody solution (desired quantity in 30 μ l) was incubated with the beads pellet for 30 minutes at room temperature with mixing intervals for antibody capture. The beads were washed twice with 100 μ l of PBST to purify antibodies and once in PBS to exchange the buffer. Labeling reagent (adjusted amount to antibody quantity, in 30 μ l PBS) was then added to the pellet and incubated for 2 h with mixing intervals on ice (unless otherwise stated). A final purification step to remove the excess of labeling reagent was performed by two times washing in 100 μ l of PBST and once in PBS. Elution and pH raise was done as described above and 22.5 μ l of PBS was added to each eluate. All steps were set fully automated on a robotic workstation (MagnatrixTM 1200, NorDiag AB, Hägersten, Sweden) for parallel labeling of 12 antibodies.

Traceable amine reactive ChromaLink Biotin (Solulink, California, USA) was dissolved in anhydrous N,N-dimethylformamide (DMF) (Solulink) to the final concentration of 10 mg/ml. Two micrograms of Polyclonal rabbit IgG antibody were biotinylated via BAP using varying molar excess of ChromaLink biotin over antibody (50- 200 times) and incorporation of biotin was investigated according to manufacturer's instructions.

Sulfo-N-Hydroxysuccinimide-polyethyleneglycol biotin (NHS-PEG4-Biotin) from Thermo Scientific (Illinois, USA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Missouri, USA) to the final concentration of 10 mg/ml. Polyclonal rabbit IgG antibody solutions containing 200 ng to 2 µg antibody quantity in PBS were biotinylated using 50 times molar excess of biotin over total protein quantity. Antibodies A1-13 were biotinylated at 1 µg using 0,9 nmol of biotinylation reagent. Amine reactive Alexa Fluor 647 and Alexa Fluor 555 succinimidyl esters (Life Technologies) were dissolved in anhydrous DMF (Sigma-Aldrich) to the final concentration of 10 mg/ml and antibodies A14-16 were dye labeled at 1 µg using 0,8 nmol [S1] of dye-labeling reagent. Dye-labeling reaction was performed at room temperature in dark. Biotinylated and dye-labeled antibodies were stored at +4°C upon further analysis, dye-labeled antibodies were protected from light.

3. Biotinylation of antibodies using in-solution protocol (ISP)

Polyclonal rabbit IgG antibody solutions containing 200 ng to 2 µg antibody in PBS were mixed with the biotinylation reagent (50 times molar excess over protein quantity) in a total volume of 30 µl and incubated on ice. The reaction was stopped after 2 h by addition of 5 µl Tris buffer (1 M, pH 8) and 20 min incubation at room temperature. Labeled antibodies were purified using protein A coated magnetic microspheres as described above. In addition, antibodies A1- A3 were biotinylated at 1 µg using ISP. Labeled antibodies were stored at +4°C upon further analysis.

4. Evaluation of biotinylated antibodies using suspension bead array assays

Biotinylated antibodies were evaluated using Luminex 200™ suspension bead array system (Luminex Corporation, Austin, USA). Polyclonal cross-adsorbed goat anti-rabbit IgG antibody (ThermoFisher Scientific), target antigens P4- P13 and antibodies A1- A3 were coupled to distinct color-coded carboxylated Luminex MagPlex microspheres (Luminex Corporation) using a previously established protocol [S2]. Target antigens P1- P2 at the concentration of 0.8 ng/ml were incubated with antibody coated Luminex microspheres (100-150 particles/well) in total volume of 30 µl in PBS for 30 min at room temperature. Microspheres were washed three times in 100 µl PBST (PBS, 0.05% Tween 20, pH 7.4). Biotinylated antibodies A1- A3 were incubated with the captured antigens on Luminex microspheres in four different sandwich assay combinations of capture/ detection antibodies (Assay1: A2/ A2, Assay 2: A6/ A2, Assay 3: A2/ A6, Assay 4: A3/ A3). Biotinylated polyclonal rabbit IgG antibodies from ISP and BAP approaches were incubated with the anti-

rabbit antibody coated Luminex microspheres (100- 150 particles/well) in total volume of 30 μ l in PBS. In addition, a dilution series ranging from 1 to 2000 ng/ml biotinylated antibodies A4- A13 were incubated with target antigen coated Luminex microspheres (100- 150 particles/well) at a total volume of 30 μ l in PBS. A control sample using non-coated Luminex microspheres was also included in each assay to investigate noise level and define background threshold (average + three times standard deviation of noise level). The binding reactions were performed in a sealed plate for 1 h at room temperature on a microplate shaker. R-Phycoerythrin modified streptavidin (SAPE) (Life Technologies) was diluted in PBST to the concentration of 1.33 μ g/ml and protected from light. After three times washing in 100 μ l PBST, the Luminex microspheres were incubated with 50 μ l SAPE solution for 20 min with shaking. The microspheres were washed three times in 100 μ l PBST, resuspended in 100 μ l PBST and were processed in the Luminex instrument according to the manufacturer's instructions. Median fluorescence intensity (MFI) from detected Luminex microspheres was used for data analysis.

5. Evaluation of dye-labeled antibodies using planar protein array assays

Functionality of dye-labeled antibodies A14-A16 was evaluated using planar protein microarrays. 384 target antigens (including the corresponding targets for the labeled antibodies, P14- P16) were printed in 21 repeated subarrays of 384 spots on functionalized glass slide (OPEpoxySlide, CapitalBio, Beijing, China) using Arrayjet Marathon arrayer (Arrayjet) by a previously established protocol [S3]. A 24-well mask (Arrayit, California, USA) was applied to the slide and labeled antibodies were added to four separate wells (0.5 μ g/ml in 60 μ l PBST). The slides were sealed and incubated on a sample stirrer (110 rpm) for 1 hour and washed twice in PBST (PBS, 1% Tween 20, pH 7.4) (5 min), once in PBS (5 min) and rinsed in deionized water. The slides were spin-dried and scanned using Agilent G2565AA Microarray Scanner System (Agilent, California, USA). GenePix Pro 5.0 software (Axon Instruments Inc, California, USA) was employed for image analysis and signal quantification.

Supplementary References

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