A high throughput pipeline for validation of antibodies

NMETH-BC31689.

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ABSTRACT

Western blotting (WB) is widely used to test antibody specificity, but the assay has low throughput and precision. Here we show that an inverted version of WB allows parallel readout with antibody arrays, shotgun mass spectrometry (MS) and immunoprecipitation followed by MS (IP-MS). The pipeline provides means for large-scale implementation of concepts proposed by an international working group on antibody validation (IWGAV).

Researchers can choose among a plethora of antibodies to the proteins they study, but the quality is unpredictable. There is evidence that 50% of commercially available antibodies fail in the applications they are specified for, and off-target binding is common [1, 2]. The consequence is that large resources are wasted on unproductive experiments, and there are concerns that cross-reactive antibodies contribute to false findings [3-5].

Antibodies are often advertised with images of western blots (WB) to document specificity [6]. However, a WB showing a single band at the expected position is not definitive evidence since many proteins have similar mass. An international working group on antibody validation (IWGAV) has proposed five conceptual pillars for more rigorous quality assessment [7]. Here, we refer to the pillars by numbers, and briefly they involve I) targeted disruption of DNA or RNA to generate negative controls, II) use of mass spectrometry (MS) data as reference, III) use of different antibodies to the same protein as reference for each other, IV) expression of tagged proteins to generate positive controls and V) immunoprecipitation and MS (IP-MS) [7].

We explored the possibility that an inverted version of the WB may allow parallel readout with antibody arrays, shotgun MS and IP-MS (**Fig.1**). This would provide means to implement IWGAV pillars II, III and V in a high throughput format. The first step in the pipeline is stable isotope amino acids labelling in cell culture (SILAC) to allow multiplexing of MS (**Fig. 1a**) [8], **Online Methods**). The sample preparation involves cell lysis, labelling of proteins with amine- and thiol-reactive biotins, denaturing of proteins and size-separation by preparative polyacrylamide gel electrophoresis (PAGE) (**Fig. 1a**) [9]. Three gels with different separation ranges were used to resolve proteins with a mass range of 10-200kDa, and sets with 12 fractions from each of six cell lines were organized in separate microwell plates analogous to "liquid WBs".

An aliquot from each well in the master plate was incubated with bead-based antibody arrays for multiplexed immunoprecipitation with 6307 antibodies and on-bead detection by flow cytometry (**Microsphere Affinity Proteomics, MAP**, **Fig. 1b**) [10]. Analysis by PAGE-MAP resolved the antibody targets as peaks of reactivity across the 72 fractions (**Fig. 1b, Table S1, Table S2**). A second aliquot was analysed by shotgun MS to obtain reference data for 7200 proteins (**Fig. 1c**, **Table S3**). From here on, data series obtained by measuring 12 fractions from each of six cell lines are referred to as PAGE-MAP profiles and PAGE-MS profiles, respectively. The results were imported to an Excel spreadsheet to assess signal to noise ratio in MAP and correlation between PAGE-MAP profiles and PAGE-MS profiles (**Table S2**). Finally, selected antibodies were used for single-plex IP-MS from enriched PAGE fractions for direct assessment of specificity (**Fig. 1d**, PAGE-IP-MS).

IWGAV pillar III involves correlation of results obtained when two antibodies to the same protein are used to measure relative protein abundance in a series of samples [7]. The precision obtained with PAGE-MAP is evident from results showing that antibodies to broadly expressed proteins with similar mass clustered according to their specificity (hereafter referred to as clustering antibodies, **Fig. 2a)**. In total, we identified 1046 clustering antibodies, and the average correlation between PAGE-MAP profiles of nearest neighbours was 0.97 (**Fig. 2b**). When 3544 antibodies were tested in a second

experiment, 74% of those that clustered in experiment 1 also clustered in experiment 2 (**Supplementary Fig. 1).** Those that clustered in a single experiment only had low signal to noise ratios (**Supplementary Fig. 1).** Thus, it appears that sensitivity is linked to reproducibility (**Online Methods**, **antibody scoring**). The average correlation between technical replicates in PAGE-MAP was 0.92 (**Table S4**), while correlations for signal to noise ratios of antibodies were 0.8 and 0.97 for biological- and technical replicates, respectively (**Supplementary Fig. 2, Table S5**). Collectively, these results show that PAGE-MAP yields complex and reproducible signatures for each specificity analogous to fingerprints. The method is therefore ideal for implementation of IWGAV pillar III.

A reference antibody should be validated using an independent method or react with an epitope different from that recognized by the test antibody [7]. Most antibody manufacturers consider the exact immunogen sequence as proprietary information, and among the 6307 antibodies that were tested here, we identified only 210 that clustered with an antibody to a non-overlapping epitope in the same protein (**Fig. 2b**, **Table S2**). These meet the IWGAV criteria for validation, but it seems clear that implementation of pillar III will to a large extent depend on access to independently validated reference antibodies (hereafter referred to as anchor antibodies).

Pillar II involves correlation between antibody reactivity profiles and MS data for the intended target [7]. This approach does not depend on information about epitopes and could potentially be used to identify anchor antibodies for pillar III. The 210 antibodies validated on basis of pillar III served as reference to establish validation criteria. In 75% of cases, the correlation between the PAGE-MAP and PAGE-MS profiles for the intended target was at least 0.7 (**Fig. 2c**). Similar results were obtained with 429 antibodies that clustered with one that was raised against the same protein using a different immunogen formulation (**Fig. 2c**). The average correlation between biological replicates in shotgun MS is also around 0.7 (**Fig. 2d, Table S6**). A cut-off of 0.7 for correlations between PAGE-MAP and PAGE-MS profiles is therefore reasonable and yields a false negative rate of 25% for validation of antibodies.

To estimate the false positive rate for a correlation of 0.7, we assessed frequencies of random correlations between PAGE-MS profiles (**Fig. 2e**). Published MS datasets with one data point per protein and cell type were used as reference [11-13]. The complexity of PAGE-MS profiles with data from six cell types was comparable to that obtained by measuring unfractionated proteins from 11 cell types (**Fig. 2e**). However, a correlation of 0.7 yields a false positive rate of 5%, or 500 candidates if one assumes that cells express at least 10.000 proteins (**Fig. 2e**). The implication is that correlation between PAGE-MAP and PAGE-MS profiles is useful to obtain a rough estimate of specificity, but not as a stand-alone tool to select anchor antibodies for pillar III.

IP-MS (pillar V) can yield strong evidence that an antibody binds the intended target. Currently, the technique is synonymous with antibody pull-down of native proteins from total cell lysates, and the IPs typically contain more than a hundred proteins [14]. Those that bind non-specifically to the antibody solid-phase, or the so called CRAPome, can be identified in databases, but it is difficult to discriminate off-target binding from proteins that interact with intended targets in complexes [7, 14, 15]. The proteins in PAGE fractions are denatured and monomeric, and PAGE-MAP data provided

a map to identify good capture antibodies and fractions enriched for their targets. Thus, singleplex IPs from PAGE fractions were trypsinized for identification of captured proteins by MS. (**Fig. 1d**, **Table S7).** As little as 2ug of fractionated protein was sufficient to identify the antibody target by MS in 46 of the 88 IPs, and the SILAC label served as marker to discriminate sample-derived proteins from contaminants (**Fig. 3a**). When CRAPOME proteins were excluded from the dataset, the SILAC positive signal in 35 of 46 IPs corresponded to a single target (**Fig. 3, Table S7**) [14, 15]. A key feature of PAGE-MAP is therefore that the method paves the way for definitive validation of anchor antibodies by PAGE-IP-MS. Antibodies that cluster with anchor antibodies in PAGE-MAP will have a p-value for specificity around 0.0008, which is near that required for definitive validation (i.e the p-value for a correlation of 0.97, **Fig. 2e**). The pipeline presented here therefore contains all the elements needed to establish array-based validation with high precision, A list of 1110 antibodies identified as good candidates for formal validation by pillar III (i.e. if antibodies to non-overlapping epitopes are found) or by PAGE-IP-MS is provided in **Table S8**. Among these, 43% were polyclonal. Line charts with PAGE-MAP/MS data were uploaded to Benchsci.com.

Future studies should aim at optimizing sample loading to increase the success rate of target identification by PAGE-IP-MS. Protein binding in single-plex IPs as measured by MAP is was predictive of success and is therefore useful as a high-throughput readout (**supplementary Fig. 3**). The same was true for the shotgun MS signal in the cell type used as source for IP. Thus, PAGE-MS serves a dual purpose: one is to obtain a rough estimate of antibody specificity, and the other is to identify cell types and fractions with sufficient levels of the intended target to allow identification by PAGE-IP-MS.

A key question that remains to be addressed in full detail is to what extent capture assays predict the performance of antibodies as detection reagents. An earlier study showed that results from native IP-MS are useful to select antibodies for detection of formalin-fixed native proteins in confocal microscopy [14]. In agreement with this, we found that PAGE-MAP has 88-90% positive predictive value for WB and array-based WB (DigiWest, **Table S9, supplementary protocol**) [16]. Some antibodies that failed in PAGE-MAP were still be useful in WB, and the sample size was too low to assess the negative predictive value (Table S9). It is therefore important to note that antibodies that failed in capture may still be useful as detection reagents. Whether or not WB yields the precision and reproducibility required for implementation of pillar III remains to determine. If not, the validation of antibodies that fail in capture assays will rely on pillar I, which involves targeted disruption of DNA or RNA, and in many cases this is not feasible.

WB is the most commonly used application of antibodies in research, but it seems justified to ask if it is time to retire an assay where antibodies are used one at a time under non-standardized conditions to yield results in the form of an image [17]. We would argue that that WB contributes significantly to the "reproducibility crisis" since published images are not directly comparable and because the assay is inherently difficult to validate. Future studies should therefore determine if PAGE-MAP can replace WB. The positive effects of a successful transition from WB to PAGE-FCM are hard to

overestimate. Researchers would be able to use all their antibodies under identical conditions in every experiment and share numerical data as spreadsheets. Immediate compatibility with IP-MS would facilitate validation in the samples under study and render researchers less dependent on the manufacturers' validation data. Incorrect validation in one study would most likely be discovered as an unexpected inconsistency in the results when others use the anchor antibodies as reference for their own in PAGE-MAP. Manufacturers can be expected to contribute to the transition since they would benefit if researchers use hundreds of antibodies per assay instead of one. They would also have a strong incentive to sell antibodies that recognize non-overlapping epitopes for use in internally referenced MAP assays. Finally, a common platform for antibody- and MS-based detection that yields data in a numerical format would enhance transparency and help to bridge the wide gap that exists between antibody users and proteomics researchers [17].

Author contributions:
-K.S. , A.M., M.I., F.T. : Conducting experiments (PAGE-MAP, PAGE-MAP), data analysis, preparation of figures
-S.K., M.T: DigiWest experiments and data analysis
-T.K, J.S., W.Z. : Data analysis
-T.A.M, M.E.S., G.A.S: Mass spectrometry.
-L.H.: statistical analysis
-F.L-J: Design of experiments, data analysis, preparation of figures, writing of manuscript.

Competing financial interests:
M.T. is founder of NMI Pharmaservices, a company selling DigiWest technology as a service.
W.Z is founder of Seekquence, a company selling information about research reagents including antibodies.

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FIGURE LEGENDS

Fig. 1 A high throughput pipeline for antibody validation
a) Proteins in cell lysates were biotinylated, heated in the presence of SDS and separated by preparative PAGE using the Gelfree 8100 instrument. The instrument yields 12 fractions with size-separated proteins from each sample. **b**) An aliquot from all fractions was incubated with bead-based antibody arrays for multiplexed immunoprecipitation, and captured proteins were labelled with R-Phycoerythrin-conjugated streptavidin for on-bead detection by flow cytometry (Microsphere Affinity Proteomics, MAP) [10, 18]. The analysis resolves antibody targets as reactivity peaks across the fractions. **c**) A second aliquot was immobilized to streptavidin beads and subjected to on-bead trypsin digestion for shotgun mass spectrometry (MS). The analysis yields size distribution profiles for thousands of proteins as peaks across the fractions. **d**) An Excel spreadsheet was used to assess signal to noise ratios and correlations between antibody reactivity patterns and MS data (**Table S2**). Selected antibodies were used in singleplex IP from enriched fractions, and captured proteins were identified by MS. The protocol is described in detail in **Online Methods**.

Fig. 2 PAGE-MAP yields high precision for comparison of antibody reactivity profiles.
a) The heatmap to the left shows hierarchical clustering of the reactivity profiles of 3706 antibodies across 12 fractions from each of six human cell lines. Antibodies with signal to noise ratios lower than four were excluded from the dataset. Jurkat: T-ALL, U2OS: osteosarcoma, HeLa: Cervical carcinoma, A431: epidermoid carcinoma, RT4: urinary bladder carcinoma, MCF7: breast cancer. The proteins were separated on a 10% acrylamide gel and analyzed by MAP as shown in Fig. 1a-b. Data were normalized and mean-centered across rows and columns, respectively and subjected to hierarchical clustering in Cluster 3.0 and visualized using Java TreeView. The heatmap to the right is an enlargement of the area marked by a yellow box. Antibody annotations: Gene symbol [predicted mass] # antibody identifier. **b**) Distribution of Pearson correlations between reactivity profiles of antibodies clustering as nearest neighbors with another antibody to the same protein or antibodies that failed to cluster. Whiskers indicate lower and upper quartiles. Numbers in parenthesis indicate numbers of antibodies. **c**) Pearson correlations between PAGE-MAP of antibodies included in Fig. 1b and PAGE-MS profiles for the intended target. **Source data for a-c: Table S2**. **d**) Pearson correlations between replicate protein expression profiles obtained for human cell lines and primary cells in three studies. All datasets contained more than 8000 proteins. **Source data: Table S6. e**) Frequency of random Pearson correlations between PAGE-MS profiles obtained in the present study. Frequencies of random Pearson correlations of protein expression profiles within shotgun MS datasets retrieved from indicated publications are included as reference. All datasets contained more than 8000 proteins. The numbers in brackets indicate the number of cell types included in each dataset. **Source data: Table S3**. Whiskers indicate lower and upper quartiles.

Fig. 3 PAGE-MAP facilitates direct assessment of antibody specificity by PAGE-IP-MS.
a) The line charts show signal intensity (y-axis) measured with MAP (solid lines) and shotgun MS (dashed lines) for indicated proteins in fractions obtained by preparative gel electrophoresis (PAGE) (see Fig. 1). Colors indicate cell type and SILAC label. The antibodies were used in single-plex immunoprecipitation from fractions indicated by arrows. Proteins from SILAC medium and heavy IPs were subjected to on-bead trypsin digestion, and the peptides were analyzed in parallel by MS. The bar graphs show MS signal intensity (log) for all proteins detected by MS in single plex IPs with indicated antibodies. Colors indicate cell type and SILAC label. The data are representative of 46 IP-MS experiments all performed using different antibodies. Each IP was performed once. **Source data**: **Table S7**
b) The heatmap shows relative MS signal intensity values for 57 SILAC- positive proteins detected in IPs obtained with 46 antibodies from PAGE fractions, Proteins (rows) and antibody specificities (columns) were organized in alphabetical order. The predicted pattern for specific binding is therefore distribution of the signal along the diagonal from top left to bottom right. Results from IPs with antibodies to cytokeratins are shown in the middle of the heatmap. These were keratin-specific, but all were reactive with more than one type of keratin. Proteins known to bind non-specifically to polymer beads were excluded from the datasets, and the heatmap was formatted to highlight the most abundant protein in each IP [14, 15] (**Online Methods**). The antibodies correspond to those that were shown to capture the intended target (i.e. 46 of 90 tested). **Source data: Table S7**.

SUPPLEMENTARY TABLES**:**
Supplementary Table 1 contains an overview of all supplementary material. Detailed legends are found in separate worksheets or text boxes in each table.

Supplementary protocol PAGE-MAP vs DigiWest and WB.
Pdf file with images of Western Blots, DigiWest data and the corresponding MAP data. All figures have the following format: the line charts show PAGE-MS and PAGE-MAP signals (y-axis) plotted against Gelfree fraction number (see legend to Fig.3a) . Cell types are indicated by color. Source data are in Table S9. The western blots in the row below show standard WB (digital infrared), and DigiWest side-by side for the same antibodies from indicated cell line (for details see ref. 16).

Online methods

Antibodies: The antibodies used in the study is are listed in **Table S2**. The total number of antibodies tested in experiment 1 was 6307. Collectively, these were monoclonal and polyclonal antibodies from 54 vendors targeting 2723 proteins. The only inclusion criterion was that the antibody was directed against a human protein. Antibodies from

Santa Cruz, ExBio, Millipore and BD biosciences were purchased. All other antibodies were provided as gifts from antibody vendors. We chose to de-identify antibodies that failed to pass our validation criteria. The rationale is that large validation efforts depend critically on manufacturer collaboration, and their support should be risk-free. It is also important to note that the negative predictive value of capture assays for WB has yet to be established. Identifiers data for all antibodies can be obtained on request from the author, but for the reasons mentioned above, we ask that the results are not made public. Antibodies were stored in PBS with 50% glycerol at-20°C. Antibodies annotated with "owl" (one world labs) in the column for Sub-array (E) in Table S2 were obtained from One World Laboratories. A large number are polyclonal antibodies that were not affinity purified. It should be noted that affinity purification greatly enhances performance in IP since non-immune IgG will dilute the specific antibody on the bead surface. The antibodies were therefore only tested in experiment 1. For preparation of antibody arrays (see below), 5μg antibody was transferred to 96 well plate pre-filled with PBS containing 0.4% bovine serum albumin, 10% glycerol and 0.1% sodium azide.

Cell lines and culture conditions: The human Urinary Bladder Papilloma cell line RT4 (cat. no. 300326) and the Human Osteosarcoma cell line U2-OS (cat. no. 300364) were purchased from CLS Cell Lines Service (Germany). The acute T-cell leukemia cell line Jurkat (clone E6-1, cat. no. ATCC TIB-152), the epidermoid carcinoma epithelial cell line A-431 (cat. no. ATCC CRL-1555), the mammary gland adenocarcinoma cell line MCF7 (cat. no. ATCC HTB-22) were purchased from ATCC. The cervical adenocarcinoma cell line HeLa was a kind gift from M.S. Rødland (Oslo University Hospital, Oslo, Norway). The cell lines used in the study were authenticated by STR analysis via an external service provider (Identicell, Aarhus, Denmark). HeLa, RT4, A431, U2-OS, MCF7 and Jurkat cells were grown in RPMI 1640 medium supplemented with 10% FBS and penicillin/streptomycin. The cells were cultivated in a humidified atmosphere with 5% CO_2 at 37°C. The cells were maintained in standard T75 flasks and expanded in T175 flasks prior to harvest. (See also Life Sciences Reporting Summary for further details)

Stable isotope labeling with amino acids in culture (SILAC): Isotopically labelled amino acids were purchased from Cambridge Isotope Laboratories, Inc. (USA): L-Lysine (13C6, 15N2) – cat. no. CNLM-291-H-PK; L-Lysine (13C6) – cat. no. CLM-2247-H-PK; L-Arginine (D7, 15N4) – cat. no. DNLM-7543-PK; L-Arginine (13C6) – cat. no. CLM-2265-H-PK. Jurkat and A431 cells were labelled with heavy amino acids (Lysine 13C6, 15N2; Arg 15N4, D7). RT4 and HeLa cells were labelled with medium amino acids (Lysine 13C6; Arg 13C6). U2-OS and MCF7 were labelled with light amino acids. First, the cells were adapted to dialyzed FBS. All cell lines were grown in RPMI 1640 (without lysine, arginine and glycine) supplemented with 10% dialyzed FBS (Sigma, cat. no. F0392-100ML), penicillin/streptomycin, 1.1494253 mM light L-arginine, 0.2739726 mM light L-Lysine hydrochloride and 2.0547945 mM light L-glutamine. The cells were passaged at least 5 times to assess the effect of dialyzed FBS on growth and morphology. During this stage the cells were maintained in standard T25 flasks. After adaptation, the cell lines were grown in RPMI 1640 medium (no lysine, arginine, glycine) supplemented with 10%

dialyzed FBS, penicillin/streptomycin and either heavy, medium or light amino acids. The cells were grown for at least 5 population doublings to ensure maximal incorporation of the labels.

Cell lysis: Adherent cells (A431, HeLa, MCF7, U2-OS, RT4) were harvested by trypsinization, followed by two washes in PBS (Sigma, cat. no. D8537). Suspension cells (Jurkat) were washed twice in PBS before lysis. The pellets were then re-suspended in SDS lysis buffer (15mM NaCl, 30 mM HEPES pH 7.4, 1mM EDTA, 2mM MgCl2, 0.3% SDS) supplemented with protease inhibitor cocktail (Sigma, cat. no. P8340-5ML), 1mM TCEP, 1mM PMSF, 1mM NaF, 1mM Na3VO4 and incubated for 10 min at 95°C. Buffer volume used was equal to 15 cell pellet volumes. The lysates were cooled on ice to room temperature and 250 units of benzonase (Semba Biosciences, cat. no. R1006E) was added. The samples were incubated for 30 min at 37°C, centrifuged at 14000g for 5 min, aliquoted and stored at -70°C. Protein concentration was measured using DirectDetect assay free cards using the Direct Detect instrument (MerckMillipore)

Biotinylation of sample proteins: Protein (300 µg) from each cell type was supplemented with sulfo-NHS-LC-Biotin and Biotin-PEG2-maleimide (both at 0.5mg/ml, www.proteochem.com). The samples were incubated 30 min on ice. Free biotin and salts were removed by buffer exchange using 10 kDa Amicon filters (MerckMillipore, cat.no. UFC501096). The sample was added to the filter and centrifuged at 14000 x g for 10 min, and the flow through was discarded. Deionized water (450µl) was added on top of the filter and centrifugation was repeated. The procedure was repeated four times. After the last step, 50µl of water was added to the filter, which was then inverted and placed in a clean collection tube. The filters were centrifuged at 2000 x g for 2 min. Protein concentration was determined using the DirectDetect instrument (MerckMillipore). We have previously shown that biotinylation has little impact on antibody binding [18]. This is also evident from the fact that biotinylation of antibodies rarely affects their performance. The reason is probably that incorporation of the label is stochastic. Thus, any solution will contain a mixture of proteins with a wide range of modifications, and only some of these will interfere with binding.

Preparative gel electrophoresis by Gelfree 8100: A Gelfree 8100 instrument (Expedeon, UK) was used to obtain liquid fractions with size-separated proteins using installed programs for gels with three different separation ranges: Tris-Acetate 5% (80-300kDa), TA 8% (35-90kDa), 10% (15-70kDa). For each separation, a total of 150µg protein was supplemented with SDS-sample buffer for Gelfree separation (Expedeon UK). Fractions (150µl) were harvested at 12 time points as recommended by the manufacturer and transferred to a 96 well plate. The fractions were stored at -70ºC until use.

Solid-Phase-Aided Sample Preparation (Solid-PhASP) of peptides for mass spectrometry (MS): 50µl of each fraction from the Gelfree separation was transferred to a 96 well PCR plate pre-filled with 100µl PBS (Axygen cat no 732-0662). Five microliters of a 50% streptavidin sepharose slurry was added (http://www.gelifesciences.com/). Prior to use,

the streptavidin beads were treated with the 50μγ/ml of Bissulfosuccinimidyl suberate (BS3) for 15 min at 22°C crosslink the streptavidin and thereby minimize release of streptavidin-derived peptides during on-bead trypsin digestion. Microwell plates with sample proteins and streptavidin beads were sealed with caps and rotated for 30 min at 22°C to immobilize biotinylated proteins. The sepharose beads were next washed twice in PBS with 1% dodecyl-maltoside to remove detergents, twice with deionized water and resuspended in 100μl ammonium carbonate buffer. At this point beads with separated proteins from three SILAC-labelled cell types were mixed to allow multiplexed MS. Trypsin (1μg) was added to each well, and the plate was incubated with constant shaking overnight at 37°C. The streptavidin beads were pelleted by centrifugation and the supernatant containing peptides was transferred to a Sep-Pak tC18 μElution filter plate (Waters, cat. no. 186002318). The resin was pre-activated using 100μl acetonitrile (Sigma), followed by equilibration with 200μl of 0.1% formic acid in water. Peptides were passed through the filter plate using a vacuum manifold. The resin was then washed twice with 200μl of 0.1% formic acid in water. The peptides were eluted in two subsequent rounds, each time using 80μl 80% acetonitrile with 0.1% formic acid in water. The samples were dried using a Concentrator Plus vacuum concentrator (Eppendorf) and the volume was adjusted to 12μl using 0.1% formic acid in water. The samples were stored at -20°C until use.

Mass spectrometry: Peptides were analyzed on QExactive plus Orbitrap mass spectrometer coupled to Easy-nLC1000 liquid chromatographer (both ThermoFisher Scientific). LC was equipped with a 50cm PepMap RSLCC18 column with a diameter of 75μm (ThermoFisher Scientific, cat. no. ES803). Water with 0.1% formic acid was used as solvent A and acetonitrile with 0.1% formic acid was used as solvent B. The gradient was as follows: 2%B to 7%B in 5 min; 7%B to 30%B in 55 min; 30%B to 90%B in 2 min; 90%B for 20 min. Solvent flow was set to 300 nl/min and column temperature was kept at 60°C. The mass spectrometer was operated in the data-dependent mode to automatically switch between MS and MS/MS acquisition. Survey full scan MS spectra (from m/z 400 to 1,200) were acquired in the Orbitrap with resolution R = 70,000 at m/z 200 (after accumulation to a target of 3,000,000 ions in the quadruple). The method used allowed sequential isolation of the most intense multiply-charged ions, up to ten, depending on signal intensity, for fragmentation on the HCD cell using high-energy collision dissociation at a target value of 100,000 charges or maximum acquisition time of 100 ms. MS/MS scans were collected at 17,500 resolution at the Orbitrap cell. Target ions already selected for MS/MS were dynamically excluded for 30 seconds. General mass spectrometry conditions were: electrospray voltage 2.1 kV; no sheath and auxiliary gas flow, heated capillary temperature of 250 °C, normalized HCD collision energy 25%. Ion selection threshold was set to 5e4 counts. Isolation width of 3.0 Da was used.

Analysis of MS data: MS raw files were submitted to MaxQuant software version 1.5.2.8 for protein identification. Parameters were set as follows: no fixed modification; protein N-acetylation and methionine oxidation as variable modifications. When applicable, the following SILAC labels were selected: Lys8; Arg11; Lys6; Arg6. First search

error window of 20 ppm and mains search error of 6 ppm. Trypsin without proline restriction enzyme option was used, with two allowed miscleavages. Minimal unique peptides were set to 1, and FDR allowed was 0.01 (1%) for peptide and protein identification. The reviewed Uniprot human database was used (retrieved June 2015). Generation of reversed sequences was selected to assign FDR rates. All MS data are provided in **Table S3**. Raw MS data were submitted to PRIDE with accession numbers: PXD005945

Microsphere-based antibody arrays. It is worth noting that microspheres with up to 500 fluorescent bar codes are commercially available from Luminex corporation[16]. The procedure for production of the in-house arrays used here has been described in detail previously [10, 18]. Briefly, amine functionalized polymethyl-metha- acrylate (PMMA) microspheres (Bangs Laboratories, IN, USA) first reacted with the hetero-bifunctional crosslinker succinimidyl 3-(2-pyridyldithio)propionate (SPDP, 50μg/ml, Sigma) and reduced with 5mM TCEP (Sigma) to obtain thiol-functionalized beads. The thiol groups were first used as binding sites for maleimide-derivatized Protein A/G (ProSpec-Tany TechnoGene Ltd, IL). Remaining thiols were used to bind serially diluted solutions of malemide-derivatives of fluorescent dyes: Alexa-750 (three levels), Alexa-488 (six levels), Alexa-647 (six levels), Pacific Orange (four levels) and Pacific Blue (four levels). Antibodies from rabbit and goat were coupled directly to protein-A/G beads. For binding of mouse antibodies, the beads were first coupled with goat antibodies to mouse IgG subclasses (Jackson Immunoresearch). Bar-coded microspheres were kept separate in 384 well plates until completion of the antibody coupling step. The beads were next mixed suspended in PBS Casein Block buffer (Thermo Fisher) and stored at -70°C until use.

Analysis with bead-based antibody arrays flow cytometry (PAGE-MAP, Flow ProteoMetry). Aliquots (15μl) of the fractions obtained by GelFree separation (see above) were added to a microwell plate pre-filled with 150 μl PBT. The samples were next supplemented with 10 μl of a solution containing bead-based antibody arrays suspended in PBS casein block buffer (Thermo) supplemented with immunoglobulins (20μg/ml) from human, mouse and goat IgG (Jackson ImmunoResearch). The plate was sealed with plastic film and rotated overnight at 4-8 °C. The plate was next centrifuged at 1000 x g to pellet the beads. The supernatant containing unbound protein was harvested and stored frozen. The beads were next washed twice in PBT and labelled with R-Phycoerythrin-conjugated streptavidin (10 μg/ml in PBS with 0.1% bovine serum albumin, Jackson Immunoresearch). Following two washes with PBT, the beads were resuspended in PBS with 0.1% bovine serum albumin and analyzed by flow cytometry. All data are provided in **Table S2**.

Single-plex immunoprecipitation and mass spectrometry. The antibodies used for IP-MS are listed in **Table S7**. Pipetting was performed in a laminar flow hood, and all solutions were made fresh to minimize keratin contamination. Ten microliters of Protein A/G beads (1% solids) were coupled with a saturating amount of a single antibody

(monitored by staining with fluorochrome-conjugated anti-Ig antibodies and FCM). For mouse monoclonals, the beads were first coupled with Ig subclass-specific goat-anti-mouse IgG. Ten microliters of antibody-coupled beads suspended in PBS casein blocker solution (Thermo) and 25ul of PAGE-fraction was added to a PCR polypropylene microwell plate (Axygen cat no 732-0661) pre-filled with 200ul PBT. We selected proteins from cells that were labelled with medium and heavy amino acids (i.e. Jurkat, HeLa, A431 and RT4 cells). Considering a total load of 150ug per well in the Gelfree 8100 cassettes, 12 fractions and the use of 1/6 fraction, the amount of sample per IP corresponds to approximately 2ug of cellular protein. The plates were capped and rotated overnight at 4°C. The beads were pelleted by centrifugation. The bead pellet was washed three times in PBT. An aliquot corresponding to 1% of the beads was labelled with Phycoerythrin-conjugated streptavidin and analyzed by flow cytometry to assess binding of biotinylated proteins. The remainder were washed twice with PBS with 0.1% dodecylmaltoside and resuspended in 10μl ammonium carbonate buffer with ProteaseMax. Trypsin (100ng) was added, and the beads were incubated at 22°C for 15 min under constant agitation. The conditions for digestion were optimized by measuring reduction in Phycoerythrin-streptavidin fluorescence on the beads at various time points during digestion by flow cytometry. Peptides were purified as described under sample preparation for MS. In each MS analysis, one IP from SILAC heavy and light-labelled lysates were run simultaneously. The LC-gradient was 20 min per IP.

Flow cytometry. Microsphere-based antibody arrays were analyzed using an Attune flow cytometer (Thermo) equipped with a 96 plate sample loader and four lasers: 405nm (Pacific Blue, Pacific Orange), 488nm (Alexa-488), 567nm (R-Phycoerythrin) and 633nm (Alexa-647, Cy7). The emission filters were standard for the instrument, except for the use of a 520nm band bass filter for detection of Pacific Orange.

Analysis of flow cytometric data. Flow cytometry data were processed using a freely available R-application dedicated for analysis of MAP data [19]. The application identifies microsphere subsets on basis of their color codes and exports values for median R-Phycoerythrin fluorescence for each subset.

DigiWest and Western blotting: The procedures for DigiWest and WB have been described in detail earlier [16]. Proteins in total cell lysates and 83 antibodies were sent from Oslo to Tübingen (Templin lab) for DigiWest and WB. The antibodies were selected from two vendors: NeoBiotechnologies and GeneTex. The vendors were chosen because of the large number of antibodies that performed well, but we also included antibodies that failed in MAP. The results are summarized in **Table S9**.

Analysis of IP-MS data:
Analysis of MS data from IPs was performed as described for PAGE-MS data above except that SILAC negative proteins and proteins known to bind non-specifically to polymer beads were removed from the dataset as described earlier [14, 15]. This is explained in detail in text boxes in **Table S8**. The heatmap in **Fig. 3a** was generated from

data that were normalized along the rows (i.e. between IPs) and mean-centered along columns (i.e. within each IP). MS Dataset ProteomeExchange: PXD010510

Antibody scoring: The numbers in column AI in **Table S2** represent a confidence score to identify the antibodies with the strongest evidence for specificity. Antibodies are rated according to the reproducibility of antibody clustering and correlations between antibody reactivity profiles and MS data in the two experiments. The threshold for sensitivity was set to a signal to noise ratio of four (max/median signal in 12x 6 fractions). We determined the number of experiments where the antibody clustered with another antibody to the same protein (column AG) and the number of experiments where the correlation between the PAGE-MAP and PAGE-MS profiles was at least 0.7 (column AH). The confidence score in column AI is the sum of the values in columns AG:AH. Thus, a score of four is obtained when the antibody clusters in both experiment and the reactivity pattern shows a correlation with the PAGE-MS profile of at least 0.7 in both experiments. We also assessed p-values for correlations between PAGE-MAP profiles of antibodies that clustered as nearest neighbours (columns AC:AD) and for correlations between PAGE-MAP and PAGE-MS profiles (columns AA:AB). Columns AJ and AK list the lowest p-value and the highest signal to noise ratio for the antibody, and the data series with the highest scores are indicated by the value 1 in column AN. It is worth noting that antibodies obtained from One World Labs were only tested in one experiment. The highest score obtainable for these is therefore 2. Moreover, we did not test the predictive value of signal to noise ratios in PAGE-MAP for signal strength in Western blotting or DigiWest. Signal to noise ratios therefore show performance in capture, which is indicative of the probability that the antibody can be validated by PAGE- IP-MS.

Statistics: Text files with data from PAGE-MAP and PAGE-MS were assembled **Table S2**). The data series consist of 72 values obtained by measuring 12 fractions from each of six cell lines with MAP and MS respectively. The PAGE-MAP datasets for each separation (i.e. type of gel cassette) were subjected to hierarchical clustering in Cluster 3.0. The ordering of the clustered datasets was transferred to **Table S2** (see legend in table). Antibodies that clustered according to specificity were identified by sorting all data in Table S2 according to the ordering in the heatmap. If the gene name was the same in the column above or below, the antibody was indicated as clustering (see legend in table).

Standard Excel functions were used to assess signal to noise ratio for MAP and Pearson correlations between antibody reactivity profiles and between antibody reactivity profiles and MS data. The significance of correlation is defined here the frequency of random correlations within single datasets. The procedure for assessing random correlations is explained in detail in text boxes in **Table S3**. This table is source data for **Fig. 2e**.

To determine reproducibility of shotgun MS in this study, the 12 signals obtained by measuring fractions from each cell type were compressed to a single value for

relative protein abundance (**Table S6**, worksheet Sikorski). This was necessary to compensate for variations in protein migration in PAGE.

  To determine reproducibility of PAGE-MAP, we performed per-protein Pearson correlations for signals obtained with 3000 antibodies in two technical replicates (i.e. same fractions analyzed twice) (**Table S4**).

Methods-only references:

19. Stuchly, J. et al. An automated analysis of highly complex flow cytometry-based proteomic data. *Cytometry. Part A : the journal of the International Society for Analytical Cytology* **81**, 120-129 (2012).

Data availability statement: Supplementary Tables 2-9 contain all data, and an overview is found in Table S1. Raw MS data were uploaded to PRIDE accessions: PXD005945, PXD010510. Line charts with PAGE-MAP data for antibodies that passed validation and MS data for the intended targets were uploaded to Benchsci.com.
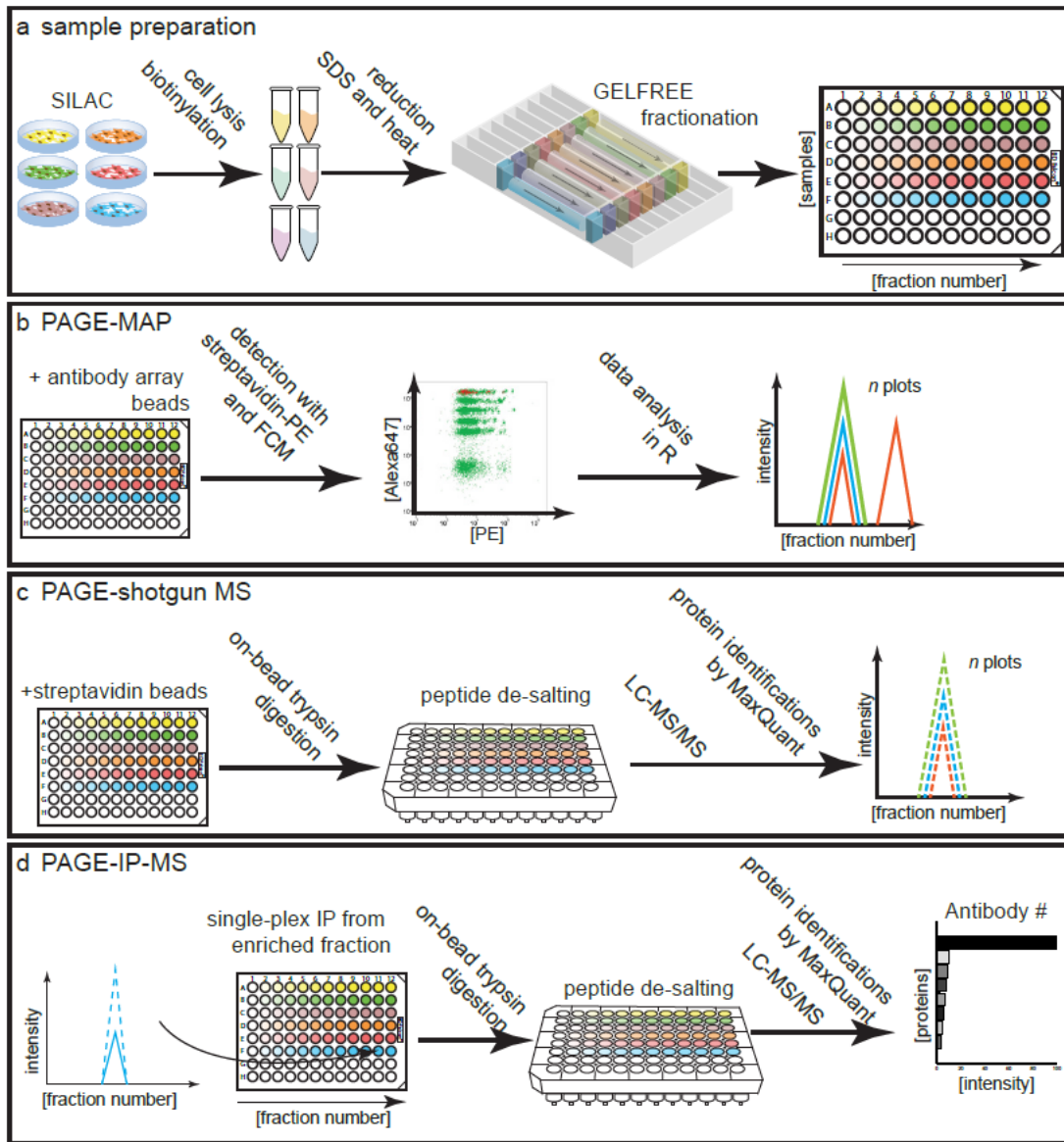
Figure 1

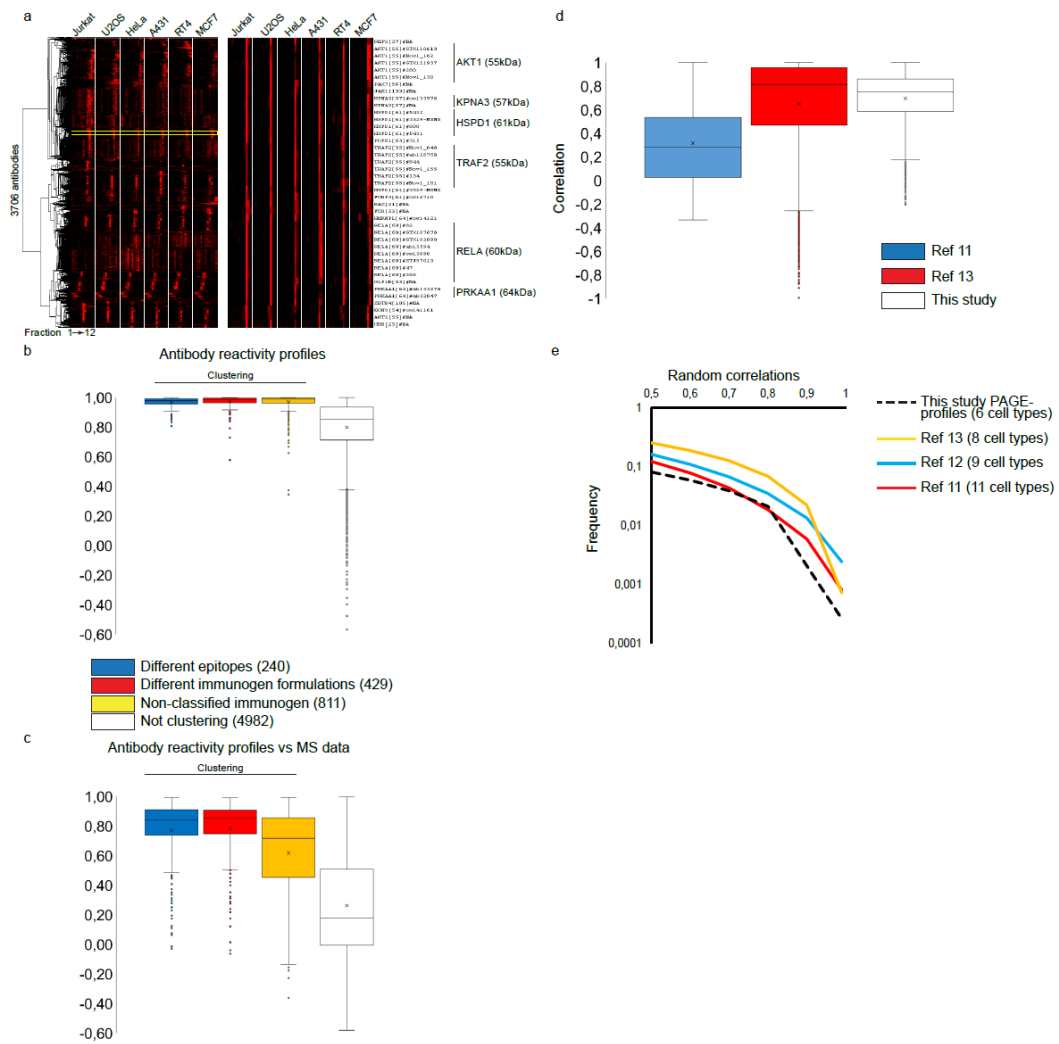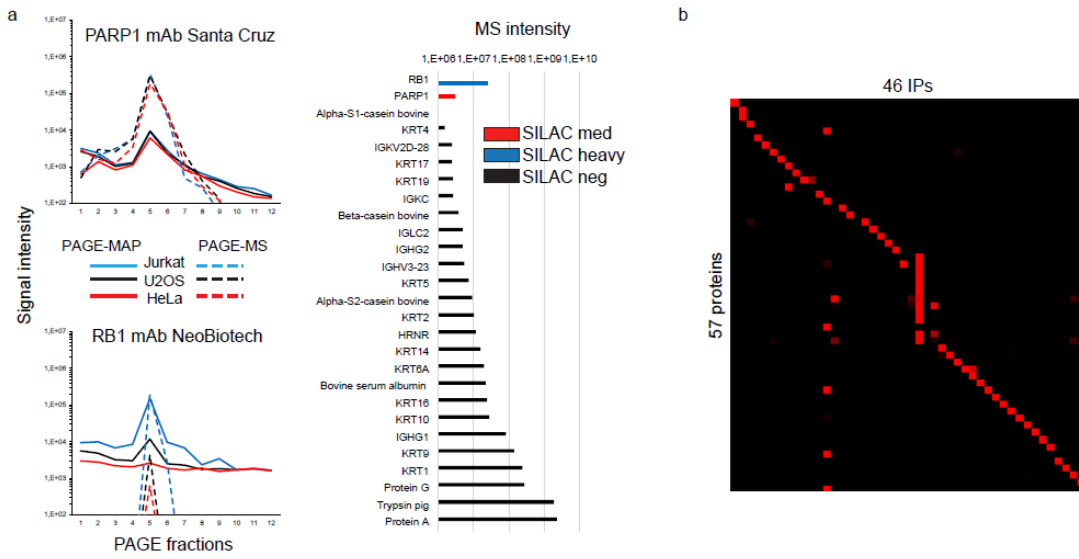**a** sample preparation

SILAC → cell lysis / biotinylation → SDS and heat / reduction → GELFREE fractionation → [samples] / [fraction number]

b PAGE-MAP

+ antibody array beads → detection with streptavidin-PE and FCM → [Alexa647] / [PE] → data analysis in R → intensity / [fraction number] — *n* plots

c PAGE-shotgun MS

+streptavidin beads → on-bead trypsin digestion → peptide de-salting → LC-MS/MS → protein identifications by MaxQuant → intensity / [fraction number] — *n* plots

d PAGE-IP-MS

intensity / [fraction number] → single-plex IP from enriched fraction → [fraction number] → on-bead trypsin digestion → peptide de-salting → LC-MS/MS → protein identifications by MaxQuant → [proteins] / [intensity] — Antibody #

Figure 2

Figure 3

a

PARP1 mAb Santa Cruz

PAGE-MAP   PAGE-MS
- Jurkat
- U2OS
- HeLa

RB1 mAb NeoBiotech

Signal intensity

PAGE fractions

MS intensity

1,E+06 1,E+07 1,E+08 1,E+09 1,E+10

RB1
PARP1
Alpha-S1-casein bovine
KRT4
IGKV2D-28
KRT17
KRT19
IGKC
Beta-casein bovine
IGLC2
IGHG2
IGHV3-23
KRT5
Alpha-S2-casein bovine
KRT2
HRNR
KRT14
KRT6A
Bovine serum albumin
KRT16
KRT10
IGHG1
KRT9
KRT1
Protein G
Trypsin pig
Protein A

- SILAC med
- SILAC heavy
- SILAC neg

b

46 IPs

57 proteins

Supplementary figure S1

496   1431   535

Experiment 1   Experiment 2

Signal to noise ratio
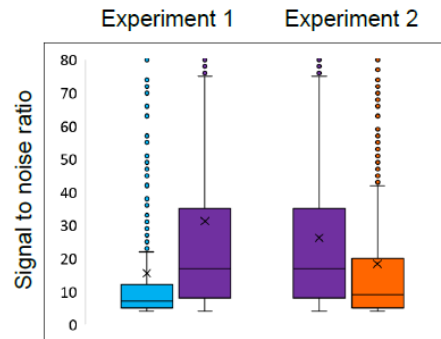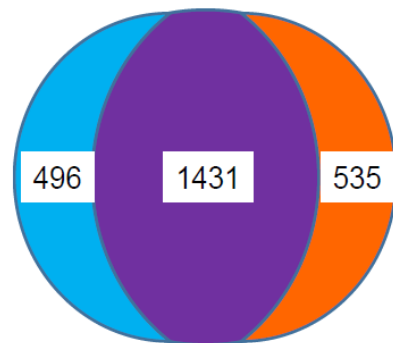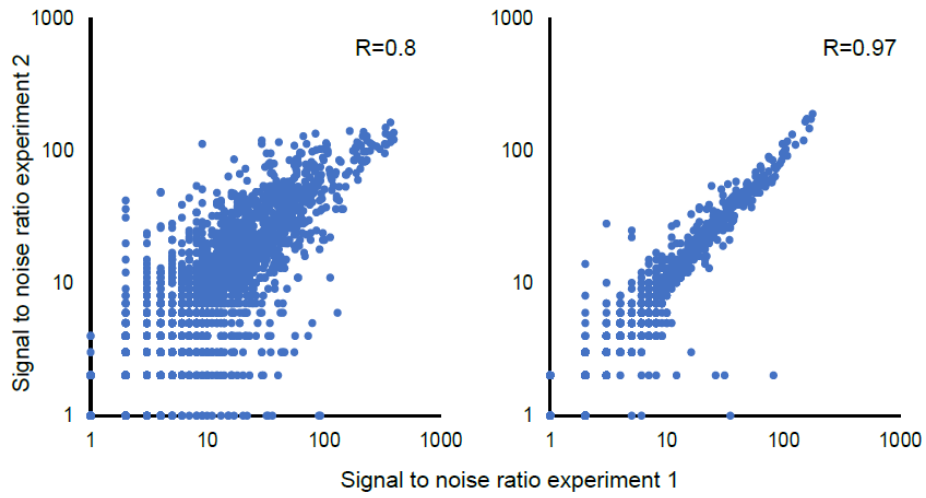
- Clustering in experiment 1 only
- Clustering in experiments 1 and 2
- Clustering in experiment 2 only

Supplementary figure S2

Supplementary figure S3

Target identified by MS (46)
Target not identified by MS (44)