

A targeted proteomic approach for the identification of tumor-associated membrane antigens using the ProteomeLab™ PF-2D in tandem with mass spectrometry

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Abstract

Mapping differential expression of soluble proteins has become fairly routine using chromatofocusing in combination with the reversed-phase HPLC (ProteomeLab™ PF-2D by Beckman Coulter Inc.); however, identification of membrane antigens has not been reported thus far. In this report, we demonstrate a targeted proteomic approach employing immunoprecipitation, prior to 2D-LC separation, in tandem with MS/MS that can be used to identify tumor-associated membrane antigens. This system is very sensitive and reproducible in that only 1/4th the amount of starting material is required for analysis as compared to gel-based analysis, and permits a focused environment for eliminating non-specific interactions leading to an accurate resolution of the cognate antigen. This system also circumvents the well-known limitations associated with gel-based approaches. This approach has been validated in the identification of ErbB2/HER-2 and was subsequently used to identify CD44E as the cognate antigen for VB1-008, one of our fully human, tumor-specific, monoclonal antibodies.

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The isolation of tumor-specific antibodies produced by cancer patients has been our approach in the design of antibody-based immunotherapeutics, for the treatment of patients with solid tumors. Identification of cognate antigens for such antibodies would enable a treatment regimen to be further tailored and hence provide greater clinical benefit for the patient. The complexity of cell-surface membrane antigens renders routine isolation and purification strategies difficult [1,2]; and attempts to separate intact proteins have been limited to only a small group of soluble proteins [3,4]. Although 2D-PAGE is capable of resolving 1500–3000 proteins in a single separation step, the separation and visualization of higher molecular weight, hydrophobic membrane proteins is generally not possible [5–7].

The ProteomeLab™ PF-2D system works on a principle similar to two-dimensional gel analysis, but uses a (gel-free) liquid phase approach. Hence, the critical parameters necessary for the separation of proteins, i.e., determining isoelectric points, resolving isomers, and their separation on the basis of hydrophobicity, have made antigen fractionation on a PF-2D system more successful and informative when compared to the other approaches [8]. A combination of PF-2D separation followed by final detection by SDS-PAGE has also been suggested for membrane proteins [2,9], but the well-known deficiencies of gel-based approaches still limit these procedures. To date there are no reports of any effective method for the identification of tumor-associated membrane antigens.

Here, we present the applicability of PF-2D fractionation for the separation of membrane antigens and membrane-associated antigens; when coupled to mass

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spectrometry and an additional pre-fractionation step. The method is not only sensitive and reproducible (uses 1/4th the protein concentration required for a gel-based approach), but also is easier to detect, analyze, and compare reactive versus non-reactive antigens simultaneously. In this study, we have used our approach to identify the HER-2 antigen, using a commercial anti-HER2 antibody, as a proof-of-concept, followed by the isolation and identification of CD44E, the cognate antigen for VB1-008, a human IgG Mab, as an example of one of the many antibodies successfully identified by this method. The results suggest that such a system could be used routinely for the rapid and efficient identification of membrane antigens.

Materials and methods

Tumor cell lines and antibodies. SKBR-3 (breast adenocarcinoma) and Hep-G2 (hepatocellular carcinoma) were purchased from ATCC and cultured in accordance with the guidelines and recommendations supplied. Cells were grown and harvested at 90% confluence. SKBR-3 and Hep-G2 were the HER-2-positive and HER-2-negative cell lines, respectively, and anti-HER-2 was purchased from EMD Biosciences (Cat# OP15SP1). VB1-008, an IgG Mab, was generated from the PBLs of a breast cancer patient using our proprietary platform technologies. Anti-CD44 (parent) was purchased from R&D systems (Cat# BBA10); anti-CD44V3 (Cat # NCL-CD44V3); and anti-CD44-isoform2 (parent) (Cat# NCL-CD44 -2) were purchased from Vision Biosystems, anti-AFP (monoclonal) was purchased from Research Diagnostics (RDI—Cat# TRK4F16–5H7) and recombinant h AFP was purchased from Calbiochem/EMD Biosciences, (Cat# 341498).

Membrane protein extraction. Approximately 500 µg of membrane protein, isolated from SKBR-3 and Hep-G2 cells using a combination of detergent-free lysis and differential centrifugation, was used for immunoaffinity purification. Briefly, cells were lysed in 250 mM sucrose, 1 mM EDTA, and 20 mM Hepes, pH 8.0, with a Wheaton homogenizer in the presence of protease inhibitors (Leupeptin, Sigma Cat# L9783; Pepstatin, Sigma Cat# P5318; Aprotinin, Bayer, Cat# 96-070-3; Bestatin, Sigma Cat# B8385; and PMSF, Sigma Cat# P7626 at 0.6 µg/mL concentration) and homogenate centrifuged at 200g to free the lysate of cell debris. The crude lysate was then centrifuged at 1000g, 4000g, and 200,000g to sediment nucleus, mitochondria, and membranes, respectively. The final membrane proteins were solubilized in a buffer containing 20 mM Hepes (pH 8.0) + 0.5% octyl-β-glucoside (OBG) + protease inhibitors.

Immunoprecipitation. A pre-clearing step using Protein-G Sepharose alone was the first step in the immunoprecipitation prior to the addition of the antibody. A total of 15–20 µg of antibody was used as the precipitating agent in the mixture. The antigen–antibody mixtures were nutated overnight at 4 °C using a buffer containing 10 mM Hepes, pH 8.0, 0.5% CHAPS, 1.2 mM EDTA, 150 mM NaCl, and 0.01% SDS in the presence of 0.6 µg/mL protease inhibitors. Immune complexes were centrifuged, washed with RIP-A lysis buffer, and eluted with 0.2 M glycine, pH 2.5. The eluate was neutralized with 1 M Tris base and prepared for the chromatofocusing run. All the chemicals used in the study were purchased from Sigma–Aldrich Company, 3050 Spruce Street, St. Louis, Missouri, 63103; and all electrophoresis supplies were from Invitrogen Canada Inc., 2270 Industrial St., Burlington, Ontario, L7P 1A1.

PF-2D fractionation of SKBR-3 and Hep-G2. Using the material generated from the above-mentioned pre-fractionation step, first dimension fractionation on a chromatofocusing HPCF column and the second dimension on a reversed-phase, HPRP column were performed, according to manufacturer's instructions and as reported previously [9]. The cognate binding antigens were eluted with glycine, neutralized, equilibrated with CF start buffer, and separated on the HPCF column using a pH gradient (8.5–4.8). All buffers and columns were purchased from Beckman Coulter Inc., Fullerton, CA. Fractions representing $pI = 5.1 \pm 0.2$ from HER-2-

positive and -negative cell lines and $pI = 5.3 \pm 0.2$ from VB1-008-positive and -negative cell lines, respectively, were selected and fractionated on the HPRP column on the basis of their hydrophobicities.

Fractionation Analysis using ProteoVue™/DeltaVue™ software. Chromatographic profiles obtained for the HPRP column fractions were imported into ProteoVue™ for the final analysis using DeltaVue™. The analyses were combined for the antigen fractionation from both reactive and non-reactive cell lines to generate a comparative antigen expression map for each of the cell lines. Comparative profiling from both antibody-reactive and non-reactive cell lines were performed simultaneously, that clearly visualized differential expression more readily for better analysis.

Dot blot assay. Fractions from the HPRP columns from HER-2-positive and -negative cell lines were concentrated using microcon membranes and spotted on nitrocellulose membrane using a standard dot blot manifold (Schleicher and Schull) and probed with anti-HER-2 and signal detected by chemiluminescence. One blot for each cell line was processed along with SKBR-3 total membranes (5 µg) probed with anti-HER-2 (positive control) and mouse IgG (isotype-matched control). SDS–PAGE and Western blotting subsequently analyzed positives.

Western blot analysis. Fractions (HER2) from approximately eight identical runs of PF-2D were pooled, concentrated, and analyzed by SDS–PAGE and Western blotting. Pre-cast 10% Tris–glycine gels from Novex (Invitrogen) were used to separate proteins that were subsequently transferred onto nitrocellulose membranes for 40 min, electrophoretically. Membranes were blocked overnight in phosphate-buffered saline (PBS) containing 0.5% Tween (PBST) at 4 °C, washed extensively to remove blocking buffer, and incubated with anti-HER-2 or any other antibody at RT for 2 h. Blots were subsequently washed sequentially with PBST and PBS, followed by incubation with secondary antibody coupled to HRP for 30 min at RT. After thorough washing with PBST and PBS, blots were developed with ECL reagent (Amersham Biosciences) and visualized by Chemiluminescence. Images were captured on X-ray films (Kodak MR Film) and developed using a Kodak X-OMAT machine.

2D-PAGE and Western blot analysis. VB1-008 immunoprecipitates were concentrated up to 80 µL and buffered with equal volumes of rehydration buffer (Bio-Rad Cat# 163–2016). The protein solution was loaded onto IPG strips (4–7, Bio-Rad Cat# 163–2001) and subjected to isoelectric focusing on a Protean IEF (Bio-Rad Cat# 165–4000), according to manufacturer's instructions. Following the resolution of proteins on the basis of isoelectric points (pI), the IPG strip was reduced, alkylated (as per manufacturer's instructions), and separated on a second dimension SDS–PAGE gel. After 3 h of electrophoresis at 200 V, proteins from the gel were electro-blotted onto PVDF membranes overnight. The Western blots were then probed with anti-AFP and anti-CD44 (20 µg/mL), and reactivity detected by chemiluminescence.

Antigen identification. Fractions containing differentially expressed protein peaks in the positive cell line, SKBR-3, along with corresponding fractions from Hep-G2 that eluted from the HPRP (second dimension) column at 27, 28, and 29 min; and MDA-MB-435S, along with Panc-1 that eluted from HPRP at 16 and 17.8 min, respectively, were subjected to peptide extraction by in-solution tryptic digestion.

Peptide extraction using in-solution digestion. Fractions with identical retention times from both cell lines were processed simultaneously. HPRP fraction volumes were concentrated to a final volume of 100 µL, followed by standard reduction and alkylation using DTT (Sigma Cat# D-8225) and Iodoacetamide (Sigma Cat# I-1149). The samples were then subjected to tryptic digestion at 37 °C for 4–6 h. Peptide samples were then desalted, cleaned, and concentrated using µC18-zip tips (from Millipore Corporation, MA) and analyzed on API-QSTAR pulsar-i mass spectrometer (Hybrid-Quadrupole-TOF LC–MS/MS mass spectrometer, model—026026K, ABI/MDS Sciex, Concord, ON, Canada), equipped with a nanosource. The flow rate was 500 nL/min and spectra were obtained in LC-mode (information-dependent acquisition). The IS voltage was set at 1400 V, CID at 48 V, and pulsar frequency = 6.991 kHz. Spectra were calibrated with Glufibrinopeptide B (Sigma cat # F-3261) at ~5 pmol/µL.

Automated LC–MS/MS analyses were completed using a CapLC (Agilent HP1100) interfaced with a QqTOF-MS (ABI/MDS Sciex, Concord, ON, Canada), with a 150 mM by 300 µM i.d. column packed with

C18 (ZORBAX 300SB-C18- from Agilent). The autosampler loaded 4 μ L aliquots of PF-2D fractions onto a pre-column packed with C18, 0.5 \times 500 mm (Agilent) that was connected in-line to the Cap LC column. Mobile phase A was water:TFA (99.9:0.1) and mobile phase B was acetonitrile:TFA (99.9:0.1). A gradient from 2% to 60% B over 60 min was used at an approximate column flow of 500 nL/min.

Mass spectral analyses. The mass range was 350–1550 amu for 100 cycles. In MS/MS mode, the “enhance all” feature was selected to ensure better detection of the fragmented ions. Collision energy (CE) was set to 48 V at low resolution. MS/MS ion searches were used to sequence peptides and identify the protein using a minimum of three or more significant peptide identifications. MS/MS spectra from the TIC region of each LC–MS experiment were directly uploaded onto the Mascot-based MS/MS ion search engine for analysis; the peptide tolerance was set to 0.2 Da; MS/MS tolerance to 50 ppm, and missed cleavages = 1.

All database searches were based on a PepSea server version 2.2.17; Peptide map score: normal, Copyright (C) 1998-Protana A/S licensed to Applied Biosystems, Foster City.

Results and discussion

It is assumed that cellular progression from a normal to malignant phenotype is reflected in a differential display of specific tumor-associated surface antigens. Therefore, for any given antigen, specific positive and negative cell lines are essential as starting material for the liquid-phase analysis followed by MS analyses. The described antigens were validated extensively by conventional methods and by measuring cell-surface reactivity by flow cytometry.

(A) Known antigen—(HER-2) identification

Proteomelab™ PF-2D separation

The chromatofocusing run was 180 min in duration with the peak fractions representing the pI characteristic of the HER-2 antigen eluting from 123 to 138 min. A single significant peak was observed at pI = 5.1, that was used for fractionation on the second dimension column. The neighboring shoulder-peaks showed no differentially expressed peaks after second dimension analysis on a HPRP column (data not shown).

Fig. 1A represents the comparative profiles of HER-2 in both positive and negative cell lines. The peak table generated by DeltaVue summarizes the characteristics of peaks observed during the run. The negative profile is shown as the red trace (left) and the positive profile is shown as the green trace (right). With respect to HER-2 reactivity, a distinct set of three peaks were differentially up-regulated in SKBR-3 membranes but totally absent in the Hep-G2 membranes (Fig. 1A). The peaks eluted from the RP column with retention times of 27, 28, and 29 min. This is probably due to different hydrophobicities associated with different isoforms of HER-2.

Detection of HER-2 by Dot and Western blot analyses

The dead volume of the reversed-phase column (HPRP) was assumed 6 mL, therefore fractions eluting at 1 mL/min were collected from the sixth minute onwards for each cell line, concentrated using microcon filters, and spotted on

nitrocellulose membranes. One blot for each cell line (34 fractions) was processed in addition to a positive control and a negative control, and was probed with anti-HER-2 antibody. Analysis of the SKBR-3 fractions clearly showed a positive interaction in the fractions with retention times of 27, 28, and 29 minutes (Fig. 1B), corresponding to the peaks seen at 27, 28, and 29-min-elutions (Fig. 1A). The positive and negative controls were SKBR-3 total membranes probed with anti-HER-2 and mouse IgG (isotype-matched control), respectively. Approximately, 5 μ g of SKBR-3 total membrane preparation was used as the starting material for fractionation, spotted into two slots of the dot-blot manifold, and probed with anti-HER-2 and mouse IgG myeloma (isotype-matched control, Calbiochem # 401122), respectively. Positive signals were observed in the positive controls with no signals being observed in the negative control slots thus ensuring that the system and reagents functioned satisfactorily in both sets of experiments.

Eight similar runs of PF-2D fractionation were carried out reproducibly with consistency. Appropriate fractions from eight runs were pooled together, such that all 27th-minute fractions were pooled together for subsequent analysis. All other fractions were pooled appropriately and subsequently resolved by SDS–PAGE and probed with anti-HER2. Fig. 2A and B represent the HER-2 interacting protein profiles obtained on probing the Western blots with the anti-HER-2 antibody. There were 30 fractions with the first fraction collected at the sixth-minute interval; however, no reactivity was observed until the 21st fraction. As can be seen in Fig. 2A and B, no reactivity to HER-2 was observed except for the 27th-min-fraction. This fraction corresponded to the largest of the three peaks eluting at 27, 28, and 29 min from the HPRP column. The molecular weight of the protein (~185 kDa) identified was consistent with that of the HER-2 antigen.

Peptide extraction and LC–MS/MS analysis of HER-2

From another set of PF-2D separations, HER-2-positive fractions—27, 28, and 29 min were centrifuged in separate microcon filters (MWCO—10K) to remove acetonitrile traces and the supernatants were used for in-solution tryptic digestion using sequencing grade trypsin, as mentioned in the methods section. MS/MS analysis and database searching of the sequenced peptides resulted in the identification of HER-2 antigen that matched with 9/12 unique peptide IDs (scores = 45, 49, 55, 41, 31, 55, 47, 54, and 36) by MS/MS ion searches (Fig. 2C) and a total score of 410. The statistically significant threshold score (with $p < .05$) was calculated as 76 for proteins and 26 for peptides.

(B) Unknown antigen ID for VB1-008

Comparative analysis of VB1-008 cell surface reactivity has shown preferential binding to the breast cancer tissues through high-density tissue micro-arrays (TMA) and

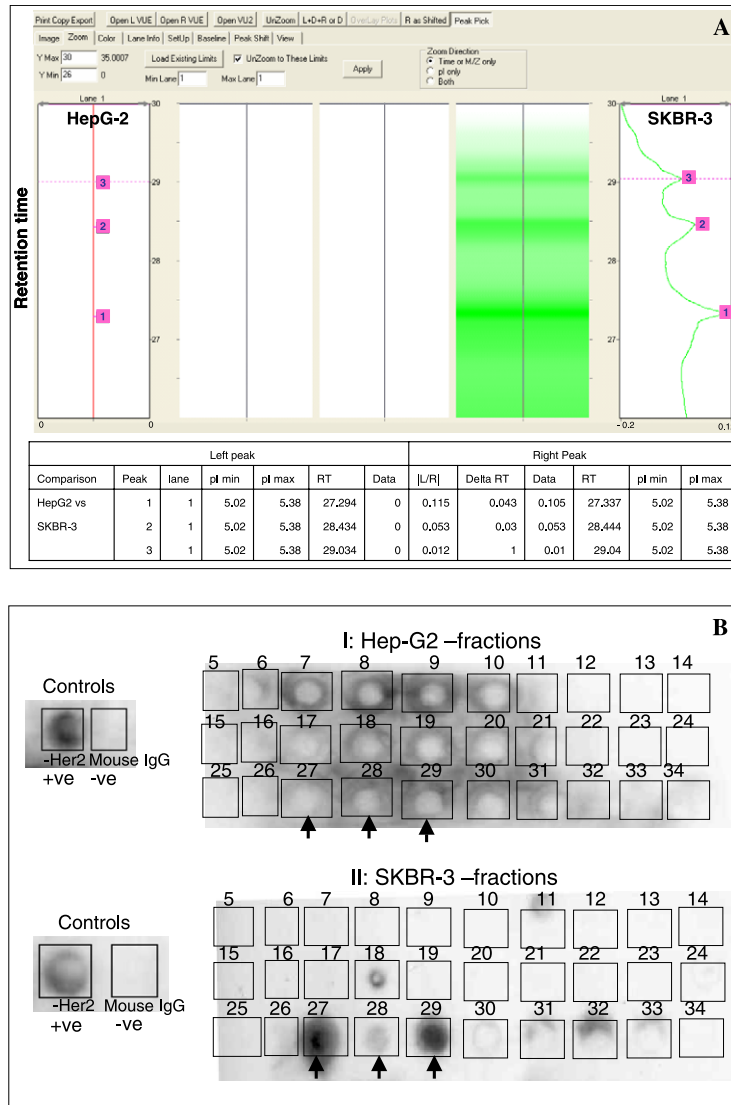


Fig. 1. PF-2D separation profiles have HER-2-reactive and non-reactive cell lines (A). Fractions corresponding to pI 5.3 ± 0.5 , for HER2, and $pI = 5.15$ in the chromatofocusing separation were equilibrated and separated on the reversed-phase column. The gradient was set between 0% and 100% acetonitrile over 36 min at the rate of 1 min/fraction. Separated antigens are visible after analysis of the profiles using DeltaVue software (Beckman Coulter Inc.). The attached peak table generated by DeltaVue shows the characteristics of the peaks illustrated in the figure. In each case, the negative samples are designated as the left peak (red trace) and positive samples are designated as the right peak (green trace). SKBR-3 and Hep-G2 fractions were concentrated and spotted on 34 slots, each representing 1 min of retention on the column. Blots were thereafter probed with anti-HER-2 for reactivity (B).

tumor cell line profiling by flow, but shows low normal tissue reactivity.

Proteomelab™ PF-2D separation

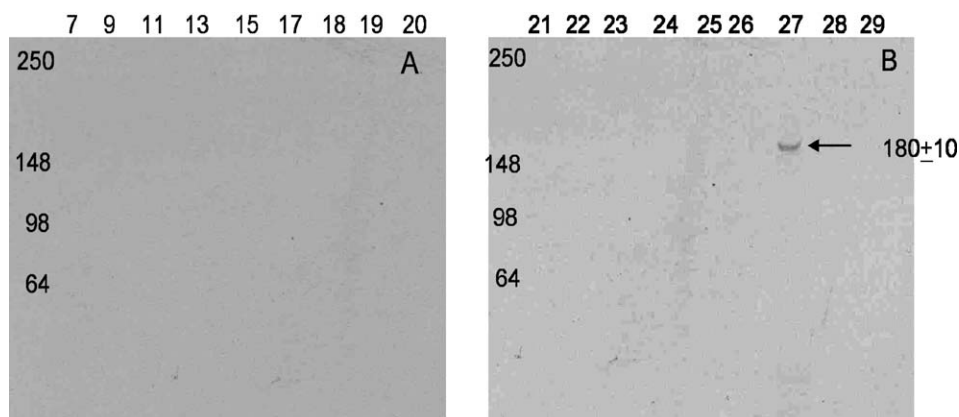
A chromatofocusing run of 180 min eluted fractions for VB1-008-Ag between 118 and 133 min. Two significant peaks at $pI = 5.3$ and 5.2 were found to elute sharply in two distinct fractions, respectively. Reacting fractions were pooled for further analysis on HPRP—reversed-phase chromatography.

Fig. 3A represents the comparative profiles of VB1-008 Ag in both positive and negative cell lines. Two strong peaks eluting with retention times of 16 and 17.8 min were observed to be up-regulated in MDA-MB-435S (MB-435S/435S) that seemed to be negligible or absent in Panc-1

membranes. The peak table generated by DeltaVue summarizes the characteristics of the peaks in the PF-2D run. The negative profile is shown as the left peak (red trace) and the positive profile is shown as the right peak (green trace).

Peptide extraction and LC–MS/MS analysis of VB1-008 Ag

VB1-008-positive fractions eluting at 16 and 17.8 min were centrifuged in separate microcon filters (MWCO/10K) to remove acetonitrile traces and the supernatants were used for in-solution tryptic digestion using sequencing grade trypsin. MS/MS analysis and database searching of the sequenced peptides resulted in the identification of CD44 and α -fetoprotein (AFP), each of which matched the protein with at least three unique peptides (Fig. 3B,



C: Peptide summary for HER-2 antigen (erbB-2 isoform b [Homo sapiens] [gi|54792098](#); Score: 410; Queries matched: 12) identified by MS analyses

Observed	Mr(calc)	Score	Error	Start	End	Peptide
478.78	955.5226	26	0.0274	900	907	R.EIPDLLEK.G
567.81	1133.5903	45	0.0197	858	866	K.WMALESILR.R
839.91	1226.6295	49	-0.0295	707	717	K.GIWIPDGENVK.I
839.91	1677.8185	55	-0.0185	128	140	R.NPQLCYQDTILWK.D
896.93	1791.8647	41	-0.0247	787	801	R.LGSQDLLNWCMQIAK.G
617.03	1848.0508	21	0.0292	707	723	K.GIWIPDGENVKIPVAIK.V
631.99	1892.916	18	0.0336	570	585	R.CPSGVKPDLSYMPIWK.F
684.02	2049.0349	31	0.0051	911	927	R.LPQPPICTIDVYMIMVK.C
1133.62	2264.0266	55	0.0134	1043	1066	R.SPLAPSEGAGSDVFDGLGMGAAG.G
1185.64	2367.1746	47	-0.0446	956	976	R.FVVIQNEDLGPASPLDSTFYR.S
1184.57	2367.13	54	0.0254	956	976	R.FVVIQNEDLGPASPLDSTFYR.S
895.78	2684.3149	36	-0.0049	660	683	R.LLQETELVEPLTPSGAMPNQAQMR.I

Fig. 2. Detection of HER-2 by Western blot analysis. Concentrated PF-2D fractions from SKBR-3 were used for SDS-PAGE and subsequent Western transfers. Blots were probed with anti-HER-2 antibody and the reaction detected with chemiluminescence. (A,B) List of peptides recovered for HER2 identified by LC-MS/MS analysis. HER-2 antigen (gi| 119533) was identified with a score = 410; peptides matched = 12 with nine significant peptide IDs (C).

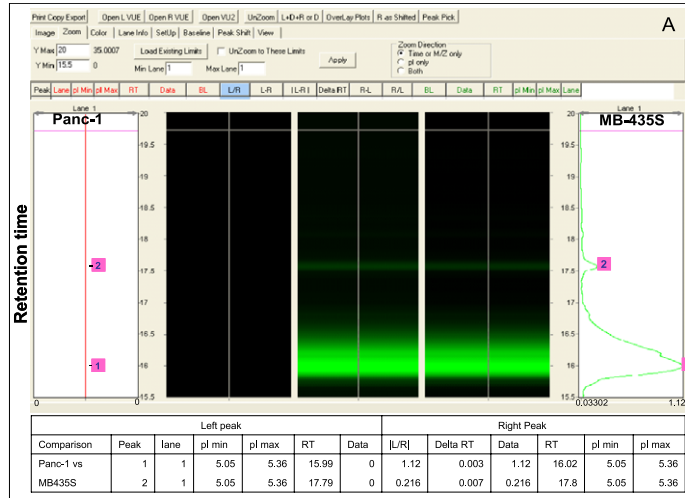
C, and D). More specifically, CD44 isoform-3 was further identified with a score of 377, with seven unique peptide IDs (scores = 42, 45, 44, 53, 50, 80, and 63); CD44E with a score of 311 with five unique peptide IDs (scores = 45, 58, 63, 65, and 80) and AFP with a score of 338 with seven unique peptide IDs (scores = 42, 27, 39, 33, 29, 26, 29, and 30). The statistically significant threshold score (with $p < .05$) was calculated as 76 for proteins and 26 for peptides. The highlighted row represents the peptide sequence from the unique exon5-v8 junction, identified with highly significant peptide scores of 58 and 53, present exclusively in two CD44 isoforms, CD44E and CD44-isoform-3, respectively (Fig. 3B and C).

CD44 is remarkable for its ability to generate alternatively spliced forms, many of which differ in their activities. The human CD44 gene is composed of a 6-kb DNA segment containing at least 20 exons [10]. Exons 1–5 encode a constant region of the extracellular domain, exons 6–14, very commonly known as v1–v10, encode for the variable region of the exons, also in the extracellular region and are subjected to alternative splicing of the molecule [11]. One of the CD44 isoforms containing the last three exon-products of the variable region (CD44V8–10), known as epithelial CD44 or CD44E, is preferentially expressed on

epithelial cells [12]. CD44-isoform-3 and CD44E are almost identical to each other except for two amino acid substitutions at positions 221 and 230; however, conserve the unique junction of exon5–v8 present in these two isoforms exclusively, thus distinguishing them from the rest of the spliced variants. A peptide sequence, TNMDSSH, derived from the unique region of CD44E and CD44 isoform-3 (exon5–v8 junction) showed strong binding by ELISA and competed most of VB1-008 binding to MDA-MB-435S cells, by flow cytometry (manuscript in preparation).

Validation of VB1-008 antigen

(1) *WB analyses of VB1-008 Ag with anti-AFP and anti-CD44.* Separation of the VB1-008 immunoprecipitates by 2D-PAGE, and subsequent Western blot analysis detected two distinct spots, “a” and “b”, in the pI range of 5.2–5.4, and molecular weight 51 ± 3 kDa, respectively, when probed with VB1-008. LC-MS/MS analysis of these two spots revealed the identities of AFP and CD44 (manuscript in preparation). Identical 2D-blot, when probed with anti-AFP and anti-CD44, confirmed the identities of AFP (spot ‘a’) and CD44 (spot ‘b’), visualized with the respective antibodies at the appropriate positions of mass and pI (Fig. 4A



B: Peptide summary for CD44-3 antigen (CD44 antigen isoform 3 [Homo sapiens] [gi|48255939](#); Mass: 53410; Score: 377; Queries matched: 7) identified by MS analyses – for VB1-008

Observed	Mr(calc)	Score	Error	Start	End	Peptide
313.70	624.69	42	0.01	42	46	R.YSISR.T
517.59	1033.18	45	0.01	30	38	R.FAGVFHVEK.N
1010.186	3027.56	44	0.44	4	29	K.FWWHAAWGLCLVPLSLAQIDLNITCR.F
941.003	2824.978	53	0.04	219	248	R.IPATNMDSHSHITLQPTANPNTGLVE
454.20	906.41	50	0.037	47	54	TEAADLCK
672.32	1342.68	80	-0.06	433	445	LVINSNGAVEDR
930.40	1858.78	63	0.03	466	472	ESSETPDQFMTAETR

C: Peptide summary for CD44-E antigen (CD44E protein, epithelial [Homo sapiens] [gi|105583](#); Mass: 53595; Score: 311; Queries matched: 6) identified by MS analyses – for VB1-008

Observed	Mr(calc)	Score	Error	Start	End	Peptide
517.50	1033.18	45	0.01	30	38	R.FAGVFHVEK.N
972.66	2915.06	58	0.03	222	248	R.TNMDSSHSTTLQPTANPNTGLVEDLDR.T
1010.25	3027.56	63	0.44	4	29	K.FWWHAAWGLCLVPLSLAQIDLNITCR.F
1194.32	3579.74	65	0.05	187	218	R.SSTSGGYIFYTFSTVHPIDEDSPWITDSTR.I
1442.73	4325.46	80	0.05	249	288	R.TGPLSMTTQQSNQSFSTSHGLEEDKDHPPTSTLTSSNR.N

D: Peptide summary for Alpha-fetoprotein (unnamed protein product (AFP) [Homo sapiens] [gi|31351](#); Mass: 68677; Score: 338; Queries matched: 24; with 7 significant peptide IDs) identified by MS analyses – for VB1-008

Observed	Mr(calc)	Score	Error	Start	End	Peptide
589.69	589.68	42	0.01	206	211	K.AATVTK.E
593.64	593.63	18	0.01	229	233	K.NFGTR.T
606.68	606.67	11	0.01	333	337	R.FLGD.R
381.63	762.98	(15)	-0.00	188	194	K.IIFSCCK.A
383.52	768.88	12	0.00	156	161	R.ETFMK.F
456.22	910.42	18	0.01	162	168	K.FIYIAR.R
467.15	969.12	25	0.01	122	129	R.HNCFLAHK.K
490.22	978.10	23	0.01	250	257	K.VNFTIQK.L
979.10	979.08	12	0.01	376	383	K.GYOELLEK.C
504.79	1007.17	39	0.01	234	242	R.TFQAITVK.L
579.72	1156.17	13	0.01	338	347	R.DFNQFSSGK.N
619.20	1239.35	13	0.01	195	205	K.AENAVFCQTK.A
646.39	1291.38	11	0.01	272	283	R.GDVLDCLODQGEK.J
483.59	1387.74	39	0.02	361	372	R.RHPQLAVSVILR.V
511.09	1530.77	9	0.01	215	228	R.ESLLNQHACAVMK.N
518.79	1554.70	11	0.01	384	396	K.CFQTEPLECQDK.G
518.92	1557.58	(12)	0.01	108	121	K.YGHSDCCSQSEGR.H
791.84	1581.66	18	0.02	348	360	K.NIFLASFVHYSR.R
815.28	1630.89	3	0.01	258	271	K.LVLDVAHVHEHCRR.G
866.97	1730.96	15	0.01	284	298	K.IMSYICSQDQLSNK.I
638.33	1916.07	8	0.01	582	598	K.CCQGOEVEVCFAEQGK.L
642.32	1923.95	29	0.03	169	184	R.RHPFLYAPTILLWAAR.Y
811.33	2434.64	12	0.03	311	332	R.GCIIHAENDEKPEGLSPNLR.F
968.66	2904.26	18	-0.02	258	283	K.LVLDVAHVHEHCRRGDVLDCLDQGEK.J

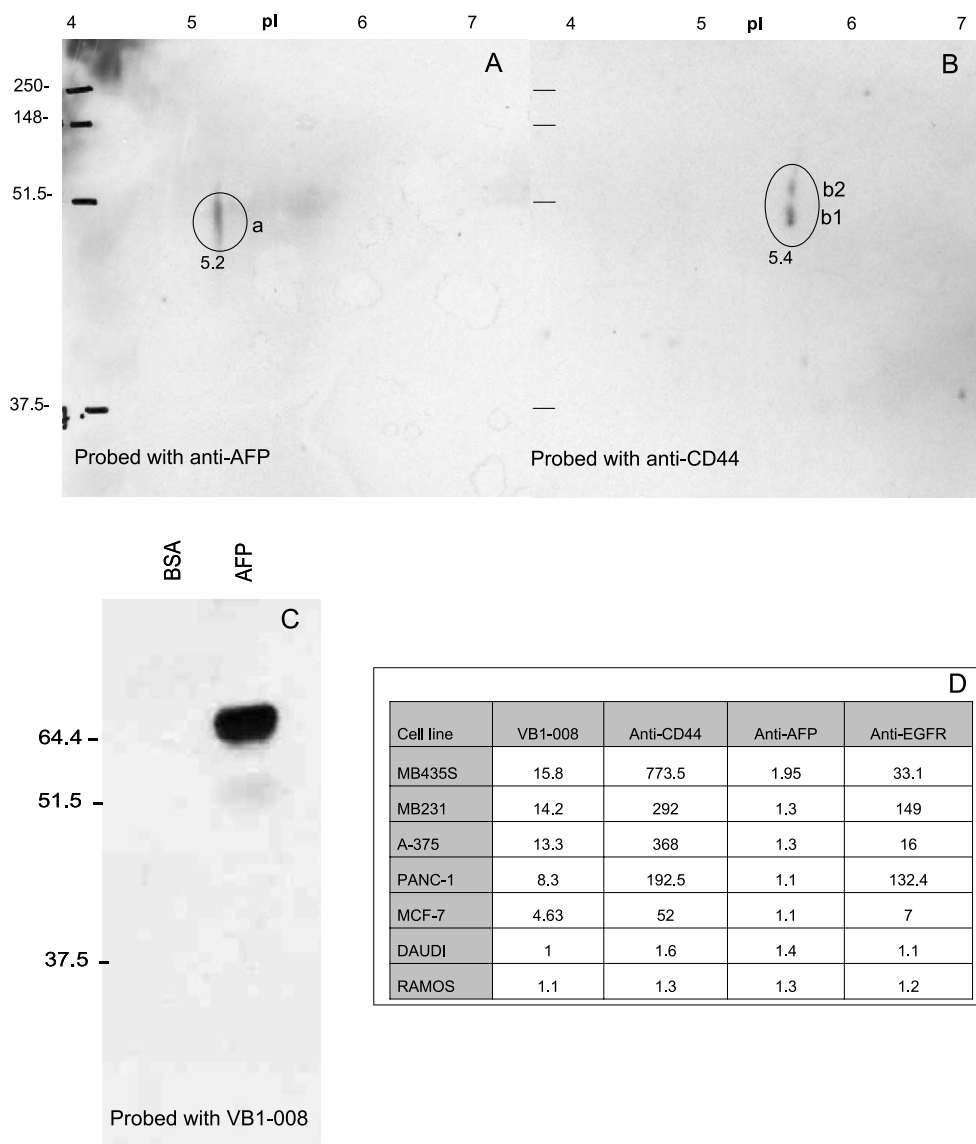


Fig. 4. The 2D-WB profiles obtained upon analysis of the VB1-008 immuno-precipitates were probed with anti-CD44 (A) and anti-AFP (B), both mouse-monoclonal antibodies, respectively. Recombinant AFP molecule, commercially available from RDI systems, was electrophoresed, transferred onto nitrocellulose membrane, and probed with VB1-008 (C). A panel of cell lines expressing different levels of VB1-008 Ag was selected for comparative cell surface reactivity experiments. Approximately, 300, 000 cells from each cell line were used and the fold-increase in median fluorescence of VB1-008/anti-CD44/anti-AFP was measured and compared to the respective isotype-matched controls (D). The isotype-matched control for VB1-008 was 4B5-IgG and the control for anti-CD44, anti-AFP, and anti-EGFR was mouse IgG, since the latter three antibodies were mouse monoclonal antibodies.

and B). Each of the commercially available antibodies, anti-AFP and anti-CD44, reacted specifically with the appropriate spots identified by MS analysis as AFP and CD44. An additional spot, identified with anti-CD44 in Fig. 4B was seen consistently in all experiments where

anti-CD44 was used to probe VB1-008 immunoprecipitates and resolved on 2D-PAGE. This additional spot ('b2') most likely represents a differentially glycosylated variant of spot 'b1', that is only seen with high-affinity antibodies. Except for the anti-CD44 (parent), no other variant of

Fig. 3. Fractions corresponding to $pI = 5.15$ in the chromatofocusing separation were equilibrated and separated on the reversed-phase column. The gradient was set between 0% and 100% acetonitrile over 36 minutes at the rate of 1 min/fraction. The attached peak table generated by DeltaVue shows the characteristics of the peaks illustrated in (A). Negative sample is designated as the left peak (red trace) and positive sample as the right peak (green trace). List of peptides recovered for CD44E, CD44-isoform-3, and AFP, identified by LC-MS/MS analysis is given in (B), (C), and (D). All figures show error data, start and end of the peptide sequences in alignment with the respective proteins, score of the MS/MS fragmentation, and masses. A minimum of three unique peptide IDs were required to establish a successful protein ID.; (B,C) CD44 antigen isoform-3, viz., gi|48255939; mass, 53410; score, 377; unique peptide IDs = 7; and for CD44E (epithelial isoform), viz., gi|105583; mass, 53076; score, 311; unique peptide IDs = 5; (D) α -fetoprotein (AFP), gi|4051989; mass, 68633; score, 411; unique peptide IDs = 7.

CD44 (commercially available) showed any significant reactivity to the VB1-008 immunoprecipitates (data not shown). In addition, recombinant AFP protein band reacted strongly on a Western blot probed with VB1-008, our fully human antibody (Fig. 4C). Further experiments from our laboratory have also shown that anti-CD44 immunoprecipitates reacted strongly to anti-AFP on Western blots, thus confirming a specific interaction between AFP and CD44. Under these conditions, VB1-008 detected a single band at ~110 kDa, which on MS analysis identified AFP and CD44E (manuscript under preparation).

(2) *Cell surface reactivity of anti-CD44 and anti-AFP by flow cytometry.* Cell-surface orientation of the two components, CD44 and AFP, was determined by flow reactivity on a panel of cell lines, to VB1-008, anti-CD44, anti-AFP, and anti-EGFR. Since anti-CD44 and anti-AFP were mouse monoclonal antibodies, anti-EGFR was chosen as an unrelated antibody control and mouse IgG was used as an isotype-matched control. As seen in Fig. 4D, the same rank order of binding was observed with anti-CD44; however, not surprisingly, the level of reactivity for VB1-008 was far less as only a single CD44 isoform is the target. As predicted by its intracellular localization, anti-AFP did not show any detectable binding over the isotype-matched control.

Successful fractionation and specific detection of a known membrane-associated antigen, (HER-2), has been clearly demonstrated using the current targeted approach. The introduction of a pre-fractionation step, prior to PF-2D, reduces sample complexity, thus making it possible to distinguish minute isoform differences between membrane antigens. Concentration of peptide extracts after a long separation regime can in most cases prove futile. Introduction of the capillary-LC system, allows for fractions in the 96-well plate collected from the PF-2D to be digested and directly loaded onto the LC-MS/MS system in workable micro-quantities. This system, when coupled to our technologies, affords a novel discovery and screening opportunity for identifying new tumor-associated antigens, in step with therapeutic drug development. One such example depicted here is CD44E/CD44-isoform-3, which was shown to be the cognate membrane antigen for the novel tumor-specific VB1-008 antibody. The cognate nature of this antigen has been thoroughly validated using conventional gel-based approaches as well as competitive inhibition up to 96% by flow cytometry (manuscript in preparation). In addition, antigen identification for another of our antibodies, VB1-050 that had proven difficult by gel-based methods (both by 1D- and 2D-PAGE) was successfully established by this methodology. Gel-based analysis of the VB1-050 immunoprecipitates detected a single band in both the reactive or non-reactive cell lines, hence proved inconclusive. However, PF-2D analysis clearly eliminated protein stacking and distinguished the reactive from the non-reactive cell lines. One of the two peaks

detected in the reactive cell line by PF-2D resulted in the ID of the antigen, while the other peak characterized by similar molecular weight and *pI* to the first peak was common to both reactive and non-reactive cell lines (manuscript in preparation). But for this methodology, the antigen for VB1-050 could not be identified. This methodology will not only prove useful for the identification of simpler antigens but will be an important resource for the identification of more complex membrane tumor antigens possessing high levels of hydrophobicity and complex post-translational modifications.

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