Chapter 5

Model-Based Discovery of Circulating Biomarkers

Maryann S. Vogelsang, Kian Kani, Jonathan E. Katz, and Parag Mallick

Abstract

Proteomic-based biomarker discovery approaches broadly attempt to identify proteins whose basal abundance, or change in abundance in response to a perturbation (e.g., a therapeutic intervention) is able to discriminate between populations of patients. Up until recently, the majority of approaches for discovering circulating biomarkers have focused on directly profiling serum or plasma to identify such proteins. However, the complexity and dynamic range of protein abundance in serum and plasma create a significant challenge for proteomics methods. To overcome these barriers, diverse approaches to simplify or to fractionate serum and plasma have been developed. For some diseases, such as those related to specific organs, there may be useful marker proteins that originate in the organ. Here, we describe an approach for marker discovery that focuses on the profiling of either primary tissue or cell culture models thereof.

Key words: Model-based biomarker discovery, Serum plasma, Quantitative proteomics, Disease biomarkers

1. Introduction

Proteomics has become an important component of biological and clinical research. Numerous proteomics methods have been developed to identify and to quantify the proteins present in biological and clinical samples (1, 2). Differences between cell types or treatment groups have been used to identify cellular functions and pathways affected by disease or perturbations (3, 4), new components and changes in the composition of protein complexes and organelles (5–7), and putative disease biomarkers (8). Serologic markers typically attempt to differentiate classes of patients, such as diagnostic markers that attempt to differentiate patients with a disease from those without a disease, or prognostic markers that indicate which patients are likely to respond to a therapeutic intervention.

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The application of proteomics approaches to the discovery of relevant protein markers from clinical samples has been hampered by sample complexity and variability. To begin to broach this challenge, complex experimental protocols for enrichment, separation, and quantification have been developed for selective or comprehensive proteome analysis (as discussed elsewhere in this book). Though these approaches have been tremendously effective, it is still nontrivial to perform discovery proteomics in serum or plasma. An alternate approach argues that for a subset of biological and clinical questions such as those related to specific organs and their interaction with the circulatory system, a subset of proteins of interest may originate in the specific organ. For example, it is well known that following injury, sets of organ-derived proteins can be found in the circulation. Likewise, in cancer, proteins derived from the tumor are often found in the circulation. In our own studies, we have observed a notable number of tumor-derived proteins in the circulation (unpublished work) (9). In fact, profiling of both tumors and sera revealed that greater than 99% of the tumor-derived proteins identified by serum profiling were also identified by tumor profiling. Furthermore, we observed a notable bias in the serum proteome for cell surface and secreted proteins. Lastly, we have repeatedly observed a strong correlation between quantitative protein abundance changes in the organ, mapping directly to quantitative protein abundance changes in the peripheral circulation. Beyond the significant experimental ease of cell culture models over biofluids, it is also frequently easier to connect discovered markers to biochemical mechanism. Given these observations, here, we present approaches for serologic marker discovery that focuses on the profiling of either primary tissue or cell culture models when available.

Specifically, we begin with a modest adaptation of the liquid-phase-based orthogonal multidimensional intact-protein analysis system (IPAS) for whole cell lysate developed by the Hanash lab (10) for disease biomarker discovery as illustrated in Fig. 1. Additional detailed protocols from the Hanash lab are found in this book (11). Next, we discuss cell surface capture adapted from Faca and Hanash (2009) (12) and last glycocapture, adapted from Zhou et al (2007) (13) for analysis of the secretome. As with many label-based quantification studies, quantification is achieved by comparing the signal intensities of identified peptides from the control and case samples in MS scan events. For cell culture experiments, we typically incorporate SILAC as described below. For tissue analysis, acrylamide-based labeling has been highly successful. When analyzing SILAC-labeled whole cell lysate, we identify more than 5,000 proteins with high confidence and obtained relative quantitative data greater than 65% of identified proteins spanning 6–8 orders of magnitude of protein concentration in the primary substrate and

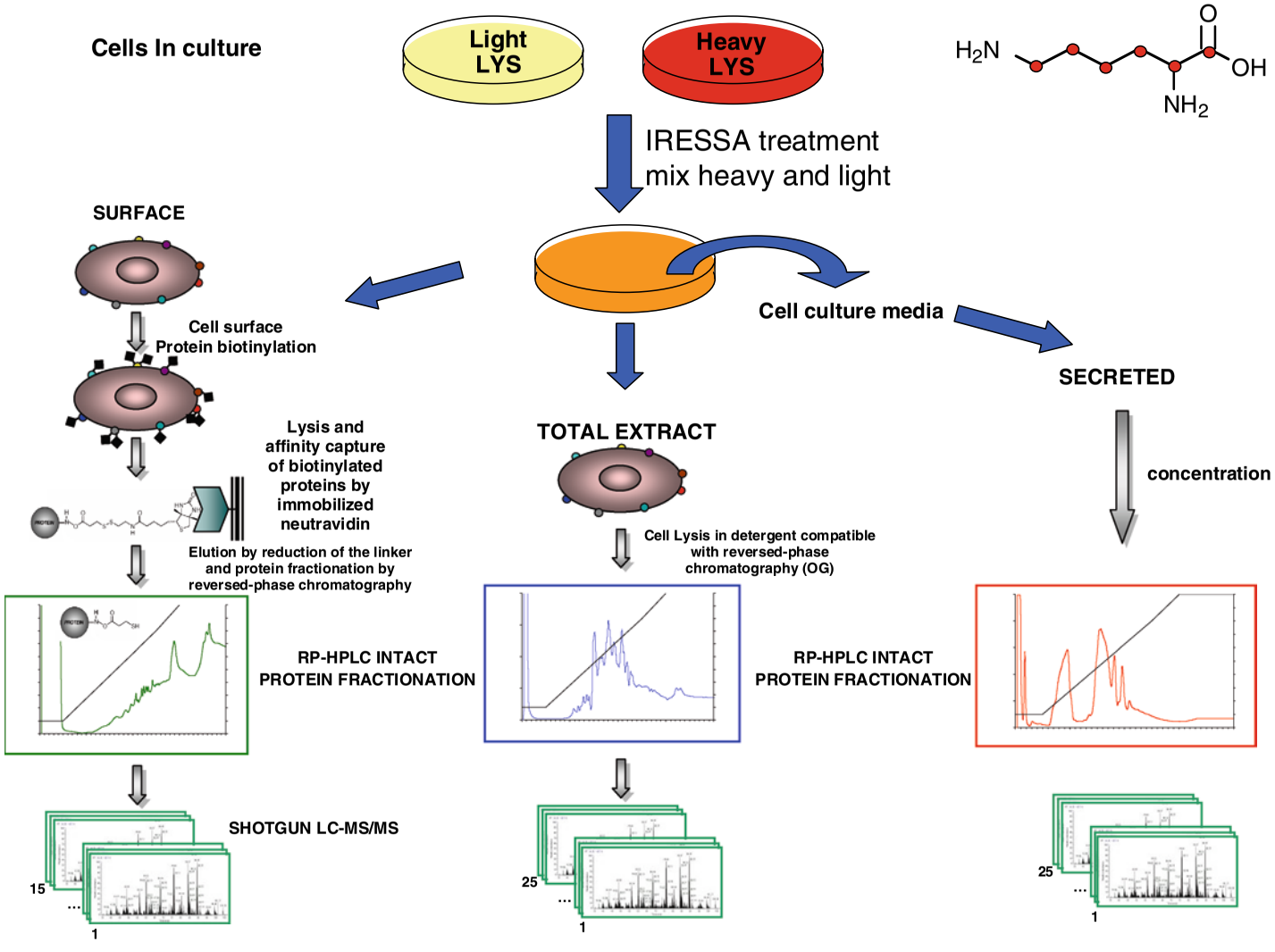

Fig. 1. Experimental design.

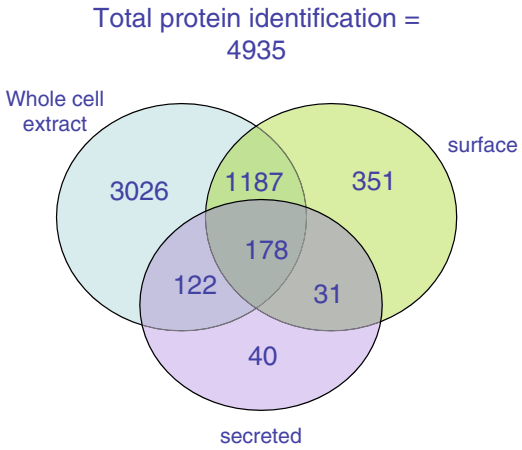

Fig. 2. Summary of proteins identified in A431 in vitro discovery experiment. Number of proteins identified (Peptide and ProteinProphet >0.9) per compartment. Roughly two-thirds of all proteins had SILAC ratio.

more so in the circulation (Fig. 2). By way of example, we use the A431 cell line from ATCC. However, the methods are applicable to other cell lines.

2. Section I Whole Cell Lysate Analysis

2.1. Materials

1. Complete Mini Protease Inhibitor, EDTA-free tablets, (Roche, #11836170). Use one tablet per 7 mL of PBS.
2. *N*-Octylglucoside (Roche, #10634425001).
3. Dithiothreitol, Mass spectrometry grade (DTT) (Sigma, #D5545).
4. Iodoacetamide, Mass spectrometry grade (IAA) (Sigma, #I1149).
5. Guanidine HCl, Mass spectrometry grade (GuHCl; Sigma, #G3272).
6. Ammonium bicarbonate (AMBIC; Sigma, #A-6141).
7. Needles, 1 mL 27½-gauge needle (B-D, #309623).
8. PBS pH 7.4, supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (Gibco, #10010-023).
9. Poros R1 column (4.6 mm × 100 mm, Applied Biosystems, #1-1012-26).
10. BETASIL C18 column (100 mm × 1 mm, 3 µm particle size, Thermo Scientific, #70103-101030).

11. Solvent A: 0.1% Formic acid in water (Honeywell Burdick & Jackson, #BJLC452-1).
12. Solvent B: 0.1% Formic acid in acetonitrile (Honeywell, Burdick & Jackson, #BJLC441-1).
13. Sequencing-grade modified trypsin (Promega, #V5111).
14. Digestion buffer: 0.25 M urea, 50 mM ammonium bicarbonate, and 4% acetonitrile (v/v).
15. BCA Protein Assay Kit (Pierce, #23227).
16. Bovine Serum Albumin Standard Ampules, 2 mg/mL (Pierce, #23209).
17. Quality-control material (see Note 1): Angiotensin II (Sigma A – 9525), Bombessin (Sigma B – 4272), Substance P (Sigma S – 6883), Neurotensin (Sigma N – 6383), and Alpha 1–6 (Sigma T – 2903). The peptides are reconstituted in water to a concentration of 1 mM, for stock solutions. For working standards, the peptides are combined and diluted to a final concentration of 1 μM of each peptide, in 3 M GuHCl, 0.1% formic acid in water (see Note 2).

3. Method

3.1. Reagents for WCL Analysis

1. Prepare 1% OG (w/v) in PBS. For single plate lysis, prepare 1–2 mL. When working with larger-scale experiments, 50 mL or more of buffer is prepared.
2. 6 M GuHCl, plus 1% OG (w/v). (Final concentration will be 3 M).
3. 100 mM Ammonium bicarbonate (AMBIC), pH 8.0.
4. 1 M Iodoacetamide (IAA) in PBS.
5. 1 M Dithiothreitol (DTT) in PBS.
6. 3 M GuHCl in 100 mM AMBIC, pH 8.

3.2. Culture and Isotopic Labeling of A431 Cells

1. Grow A431 in DMEM media (Cellgro) containing 1% dialyzed fetal bovine serum (FBS) (Cellgro) and an antibiotic of choice.
2. Not all of the following are required; choose those necessary for the desired SILAC experimental design: L-arginine-$^{13}C_6$ monohydrochloride (Cambridge Isotope Laboratories, #CLM-2265), L-arginine-$^{13}C_6$,$^{15}N_4$ hydrochloride (Sigma-Isotec, #608033), L-lysine-$^{13}C_6$,$^{15}N_2$ hydrochloride (Sigma-Isotec, #60804), L-methionine-^{13}C,$^{2}H_3$ (methyl-^{13}C,^{2}H) (Sigma-Isotec, #299154).
3. Change medium (using either light or heavy SILAC medium) every 2–3 days if cells are not ready for subculture.

4. After the first passage, begin expansion of light and heavy SILAC cell populations by culturing cells in larger dishes or a larger number of plates, as needed.
5. Grow cells in SILAC media for at least seven passages.
6. Grow plates to 80% confluence.

3.3. Cell Lysis Protocol

1. Start with 15-cm plates at 80% confluence.
2. Wash cells thrice with PBS.
3. Lyse the cells with PBS supplemented with 1% OG, and protease inhibitor cocktail.
4. We typically use between 800 µL and 1.5 mL per plate depending on cell type.
5. Place lysates on ice and disrupt cell membrane by needle treatment or sonication.
6. Centrifuge lysates at 4°C at 15,000 × *g* for 10 min.
7. Decant soluble fraction and centrifuge with a 0.2-µm filter device.
8. Determine protein concentration of lysate solutions by BCA or any equivalent assay (see Note 3).
9. We typically mix 1 mg of heavy lysate solution with 1 mg of light lysate solution.
10. Typical volumes should be around 1 mL.
11. We add DTT to a final concentration of 25 mM in 6 M GuHCl [1% OG (w/v)] to bring the final GuHCl concentration to 3 M.
12. Rotate at 65°C for 1 h.
13. Allow to cool to room temperature for 15 min.
14. Make a solution of 1 M IAA in PBS and add to a final concentration of 55 mM (see Note 4).
15. Typically, we end up at a final volume between 1.5 and 2 mL.
16. Incubate for 1 h, at room temperature in the dark.

3.4. Reversed-Phase Whole Protein Fractionation

1. Protein sample should be approximately 1 mg in about 1–2 mL.
2. Filter lysed cells through a 0.22-µm syringe filter, before loading onto column.
3. Load sample onto a POROS R1/10 column, typically 1 mL volume loaded per run.
4. After column equilibration:

   Gradient: Flow rate: 1 mL/min

   0 min: 5% solvent B

23 min: 50% solvent B

53 min: 80% solvent B

55 min: 95% solvent B

65 min: 100% solvent B

69 min: 5% solvent B

89 min: end run

5. Equilibrate the column for at least 10 min at 1 mL per min with solvent A before injections.
6. Inject the sample and keep the system at the initial solvent concentration of 5% solvent B until absorbance returns to baseline before starting the gradient.
7. Collect fractions of 90-min run.
8. Determine protein concentration of each fraction by BCA or any equivalent assay (see Note 3).
9. Pool the fractions into roughly 100 μg protein per fraction. In our case, typically 20 plus fractions are collected, digested, and then analyzed.
10. The fractions are then placed in a –80°C freezer for approximately 1 h.
11. The fractions are then dried down in a SpeedVac. They are centrifuged under vacuum until dryness (see Note 5).
12. After dryness is reached, the samples may be stored at –80°C.

3.5. Protein Digestion

1. Resuspend individual fractions in 100 μL of 3 M GuHCl in 100 mM ammonium bicarbonate, pH 8.0.
2. Shake for 10 min at room temperature.
3. Add 180–190 μL of ammonium bicarbonate, pH 8.0.
4. Add 1–4 μg of trypsin (from a 0.1 μg/μL trypsin suspension in 100 mM ammonium bicarbonate). The final cell protein extract to trypsin mass ratio is approximately 50:1.
5. Incubate at 37°C for at least 18 h.
6. Interrupt the digestion by adding 9.9 μL of glacial acetic acid.
7. Freeze samples at –80°C for at least 2 h.
8. Centrifuge sample under vacuum to dryness (see Note 5).
9. Digested sample may be stored at –80°C for no longer than 6 months.

3.6. Mass Spectrometry Analysis

1. Resuspend dried fractions into 40 μL of 95:5:0.1 (H_2O:ACN:FA).
2. Load 10 μL/fraction/run (see Note 6).
3. Inject 10 μL of sample onto BETASIL C18 column.

4. After injection, the sample is desalted directly on the column, for 20 min, prior to elution.
   Gradient: Flow rate: 65 µL/min

Time (min)	Pump	Start (%)
0.01	B	5
3	B	8
7	B	15
60	B	35
65	B	95
75	B	95
78	B	5
80	B	5
80.01	Controller	Stop

5. Acquire spectra in a data-dependent mode in *m/z* range of 400–1,800. Select the six most abundant +2 ion or greater for each MS spectrum for MS/MS analysis. Mass spectrometer parameters are as follows: electrospray ionization voltage of 3.75 kV, capillary temperature of 200°C, resolution of 60,000, and FT target value of 1,000,000.

   Scan event details:

1. ITMS + p norm o(400.0-1800.0)
   CV = 0.0V
2. FTMS + p norm res = 60000 o(400.0-1800.0)
   CV = 0.0V
3. ITMS + c norm Dep MS/MS Most intense ion from (2)
   Activation type: CID
   Min. signal required: 500.0
   Isolation width: 2.00
   Normalized coll. energy: 35.0
   Default charge state: 2
   Activation Q: 0.250
   Activation time: 30.000
   CV = 0.0V
4. ITMS + c norm Dep MS/MS second most intense ion from (2)
   Activation type: CID
   Min. signal required: 500.0
   Isolation width: 2.00
   Normalized coll. energy: 35.0
   Default charge state: 2

Activation Q: 0.250
Activation time: 30.000
CV = 0.0V

5. ITMS + c norm Dep MS/MS third most intense ion from (2)
   Activation type: CID
   Min. signal required: 500.0
   Isolation width: 2.00
   Normalized coll. energy: 35.0
   Default charge state: 2
   Activation Q: 0.250
   Activation time: 30.000
   CV = 0.0V
6. ITMS + c norm Dep MS/MS fourth most intense ion from (2)
   Activation type: CID
   Min. signal required: 500.0
   Isolation width: 2.00
   Normalized coll. energy: 35.0
   Default charge state: 2
   Activation Q: 0.250
   Activation time: 30.000
   CV = 0.0V
7. ITMS + c norm Dep MS/MS fifth most intense ion from (2)
   Activation type: CID
   Min. signal required: 500.0
   Isolation width: 2.00
   Normalized coll. energy: 35.0
   Default charge state: 2
   Activation Q: 0.250
   Activation time: 30.000
   CV = 0.0V
8. ITMS + c norm Dep MS/MS sixth most intense ion from (2)
   Activation type: CID
   Min. signal required: 500.0
   Isolation width: 2.00
   Normalized coll. energy: 35.0
   Default charge state: 2
   Activation Q: 0.250
   Activation time: 30.000
   CV = 0.0V

3.7. Data Analysis

The acquired data is automatically processed by the Computational Proteomics Analysis System – CPAS (14). Tandem mass spectra are searched against the human IPI database (60,428 protein entries) with sequences for human and bovine trypsin added. Searches are typically performed with X!Tandem (2005.12.01) using a mass tolerance for precursor ions set to 20 ppm. The mass

tolerance for fragment ions is set to 0.5 Da. A fixed modification of 6.020129 mass units was added to lysine residues for database searching to account for incorporation of the heavy lysine isotope. All identifications with a PeptideProphet (15) probability greater than 0.75 are submitted to ProteinProphet (16), and the subsequent protein identifications are filtered at a 1% error rate with tryptic fragments (one missed cleavage), with an allowance for fixed modification on $C=57.021$ and variable amino acid modifications on $C=-17.027$, $E=-18.011$, $K=6.020$, $M=15.995$, and $Q=-17.027$.

The quantitative approach consisted of differential labeling of proteins in culture with lysine isotopes (heavy or light) (17). Quantitative information is extracted using a script designated "Q3" that was developed originally to obtain the relative quantification for each pair of peptides identified by MS/MS that contains acrylamide-labeled peptides (18). Only peptides with a minimum of 0.75 PeptideProphet score and mass deviation less than 20 ppm were considered in the analysis. All normalized peptide ratios for a specific protein were averaged to compute an overall protein ratio. A single sample *t*-test is used to assess confidence in the protein quantitation ratio (19). In addition, protein quantification values are manually verified to ensure that quantified peptides have clear elution profiles and isotope envelopes at the appropriate mass spacing.

4. Section II Cell Surface Analysis

The cell surface proteome is of substantial interest, especially in being rich with therapeutic targets. Gaining a better understanding of the surface membrane proteins and their role in cellular growth can help lead to the development of new drug targets. Here, we introduce a cell surface protein capture technique that employs biotinylation of proteins of interest.

4.1. Materials

1. Epidermoid carcinoma cell line A-431 (ATCC, #CRL-1555).
2. DMEM F-12 medium (Invitrogen, #11320-033).
3. SILAC DMEM media (Invitrogen, #MS10030).
4. Dialysed FBS (Invitrogen, #26400-044).
5. Trypsin (with 0.05% EDTA) (Cellgro, #25-052-Cl).
6. Dulbecco's PBS (D-PBS, Invitrogen, #14190-144).
7. EZ-Link sulfo-NHS-LC-biotin (Pierce, #21217).
8. Glycine (Sigma-Aldrich, #241261).
9. NP40 (Sigma-Aldrich, #100511-472).

10. HALT cocktail protease inhibitor (Pierce, #PI78430).
11. D-Biotin (US Biological, #B1750).
12. Pierce Monomeric Avidin Kit (Pierce, #20227).
13. Sodium Orthovanadate (Vanadate, New England Biolabs, #P0758L).
14. Centricon YM-3 columns (Millipore, #42404).

5. Methods

5.1. Reagents for Cell Surface Analysis

1. 50 mM Tris–HCl (pH 7.5)
2. 2 mM Biotin in PBS
3. 0.1 M glycine (pH 2.8)
4. Prepare SILAC media according to the manufacturer's instructions:
   (a) Prepare 1 L of complete medium as follows:
   - Replace 100 mL of basal medium with dialyzed FBS.
   - Add 10 mL of 100× L-glutamine, if basal medium does not contain glutamine.
   - Add 10 mL of 100× penicillin–streptomycin, if needed.
   - Add any additional growth factors required for your cell line.
   - Mix well and filter-sterilize the medium using a 0.22 μm filtration device.
   - Store the complete medium at 4°C protected from light until use.
5. 100 mL of NHS-SS-BIOTIN (at 1 mg/mL) in PBS, pH 8.0 at 4°C.

5.2. Culture and Isotopic Labeling of A431 Cells

1. Grow cells in SILAC media containing 1% dialyzed fetal bovine serum (FBS) (Cellgro) and penicillin–streptomycin (or antibiotic of choice).
2. Not all of the following are required; choose those necessary for the desired SILAC experimental design: L-arginine-$^{13}C_6$ mono-hydrochloride (Cambridge Isotope Laboratories, #CLM-2265), L-arginine-$^{13}C_6$,$^{15}N_4$ hydrochloride (Sigma-Isotec, #608033), L-lysine-$^{13}C_6$,$^{15}N_2$ hydrochloride (Sigma-Isotec, #60804), L-methionine-^{13}C,$^{2}H_3$ (methyl-^{13}C,^{2}H) (Sigma-Isotec, #299154).
3. Change medium (using either light or heavy SILAC medium) every 2–3 days if the cells are not ready for subculture.

4. After the first passage, begin expansion of light and heavy SILAC cell populations by culturing the cells in larger dishes or a larger number of plates, as needed.
5. Grow the cells in SILAC media for at least seven passages.
6. Grow plates to 80% confluence.
7. The cells are typically expanded to 50 cm × 15 cm plates of H1975 (Light) and 50 cm × 15 cm plates of H1975 (Heavy).
8. The cells are maintained in SILAC, 1% FBS until 100 plate expansion is reached (see Note 7).
9. Starting with 50 cm × 15 cm plates of H1975 and 50 cm × 15 cm plates of H1975.
10. Maintain in SILAC, 1% FBS.
11. Split the cells with 1 mL trypsin.
12. Spin the cells 1,000 × *g* for 10 min.
13. Aspirate the media.
14. Add PBS.
15. Spin the cells 1,000 × *g* for 10 min.
16. Count the cells and separately seed equivalent light- and heavy-labeled cells.
17. Aspirate the media.
18. Resuspend in SILAC media.

5.3. Gefitinib (Model Therapeutic) Treatment

1. When the cells reach 65–90% confluency, wash the cells with PBS.
2. Resuspend the cells in either SILAC media with DMSO or SILAC media with 100 nM Iressa.
3. Treat 25 heavy plates with DMSO, 25 heavy plates with Gefitinib, 25 light plates with DMSO, and 25 light plates with Gefitinib.
4. Follow cells through a 16 h time course (see Note 8).

5.4. Cell Surface Protein Biotinylation

1. At 1 h time points per 12 plates, aspirate cell media and wash cells with 15 mL of PBS three times on ice.
2. Add 5 mL of NHS-SS-BIOTIN in PBS pH 8.0 at 4°C to each plate.
3. Incubate for 20 min on ice.
4. Aspirate NHS-SS-BIOTIN solution.
5. To stop biotinylation, wash cells with 5 mL of 100 mM glycine, chilled at 4°C (made in 50 mM Tris–HCl).
6. Incubate cells in glycine–Tris–HCl for 5 min.
7. Aspirate the glycine–Tris–HCl.

8. Wash with ice-cold PBS twice.
9. Harvest the cells by scraping in PBS (supplemented with 1% NP40, HALT protease inhibitor, and Na Vanadate).
10. The cells may be flash-frozen in dry ice and stored at –80°C until further processing.
11. If continuing on, the cells may be further disrupted by a brief sonication cycle to initiate cell lysis.

5.5. Cell Lysis

1. Sonicate the cells (according to manufacturer's recommended settings).
2. Pipette up and down to suspend the cells.
3. Use lower power to prevent foaming and disrupt cells by sonicating on ice using five 1 s bursts.
4. Incubate cells for 30 min on ice, vortexing every 5 min for 5 s.
5. To improve solubilization efficiency, 2–3 additional sonication cycles may be added during incubation.
6. Centrifuge cell lysate at 20,000 × *g* for 20 min at 4°C.
7. Transfer clarified supernatant to a new tube.
8. Keep on ice until ready to load onto an avidin column.

5.6. Purification of Biotinylated Surface Proteins

1. Wash the avidin (2 mL) column with at least 8 mL of PBS.
2. Wash the column with 6 mL of 2 mM biotin in PBS. This blocks any nonreversible biotin binding sites on the column.
3. Remove excess biotin by washing the column with 12 mL of 0.1 M glycine (pH 2.8).
4. Wash the column extensively with at least five column volumes of PBS (10 mL).
5. Load clarified solubilized supernatant to the column.
6. Pass supernatant through the column at least three times.
7. Wash unbound protein by applying 8 mL of PBS containing 1% NP40.
   Elute bound biotinylated proteins from the column with 12 mL of 5 mM biotin in PBS containing 1% NP40. These may be collected as 6 × 2 mL fractions (see Note 9).
8. Load samples into Centricon YM-3 columns and spin at 5,000 × *g* until volume is approximately 500 µL–1 mL (see Note 10).

Following isolation and mass spectrometric and computational analyses, proceed as described (Section I, Subheadings 3.4–3.6) with the following modification: residue mass alteration must include the mass of the linked portion of the NHS-Sulfhydryl that remains post biotin cleavage.

6. Section III Glycocapture

As noted (Sections I–II), analysis of conditioned media and the secretome can provide significant insight into which proteins are likely to be found in the circulation. Here, we introduce the glycocapture technique for secretome analysis.

6.1. Materials

1. 10DG Desalting Columns (Econo-Pac; Bio-Rad Laboratories, #732-2010).
2. Vial 1 mL glass with polyethylene snap cap (Waters, #WAT025054C).
3. Affi-Prep Hydrazide resin (Bio-Rad, #156-001 6). Supplied as a 50% suspension in isopropanol.
4. Trizma base (Sigma-Adlrich, #T1503-500G).
5. Sodium dodecyl sulfate, SDS (Sigma-Aldrich, #436143-25G).
6. Urea (Fisher Scientific, #BP169-212).
7. Ethylenediaminetetraacetic acid disodium salt dihydrate, EDTA (Sigma-Aldrich, #E5134-50G).
8. Ammonium bicarbonate (AMBIC; Sigma, #A-6141).
9. Sodium acetate trihydrate (Fisher, #BP334-500).
10. Sodium chloride (Sigma-Aldrich, #S3014-5KG).
11. Hydrochloric acid, HCl (Fisher Scientific, #A144-500).
12. Acetic acid, glacial (Sigma-Aldrich, #A6283-500ML).
13. TCEP HCI (Pierce, #20490); used as a 200 mM stock solution, 5 mM final concentration in sample.
14. Sodium periodate (Pierce, #20504).
15. lodoacetamide, Mass spectrometry grade (IAA) (Sigma, #I1149).
16. Sequencing-grade modified Trypsin, (Promega, #V5113) 20 μg/tube, 100 μg total
17. *N*-glycosidaseF(PNGaseF,RocheDiagnostics,#11365185001); dissolve in double-distilled water to get a 1 U/μL solution.
18. Spin column (MoBiTec, #M1002).
19. 10-μm filter (MoBiTec, #M2210).
20. Spin columns with 10-μm filter (MoBiTec, #M1002S).
21. SepPak Ci 8 column (Waters, #WAT023590); C-18 Vydek (The Nest Group, #SNS SS18V).
22. Trifluoroacetic acid, sequencing grade (Pierce, #28904).
23. Methanol (Honeywell Burdick & Jackson, #BJLC230-1).

7. Methods

7.1. Reagents to Be Prepared

Following items must be prepared fresh each time:

1. Urea buffer: 8 M urea, 200 mM Tris pH 8.3, 0.1% SDS, 5 mM EDTA in water; each sample requires approximately 10 mL (see Note 11).
2. 50 mM Ammonium bicarbonate buffer in water, pH 7.8, 0.1% SDS.
3. 250 mM stock Iodoacetamide in water. Prepare this solution immediately prior to use and keep it in dark (light sensitive).

Following items may be made as stock solutions:

4. Oxidation buffer: 20 mM Sodium acetate in water, 150 mM Sodium chloride, pH 5.0. May be stored at room temperature (see Note 12).
5. Coupling buffer: 100 mM Sodium acetate in water, 1 M Sodium chloride, pH 4.5. May be stored at room temperature (see Note 12).
6. 80% (v/v) Acetonitrile in water. May be stored at room temperature.
7. 1.5 M Sodium chloride in water. May be stored at room temperature.
8. 0.4% (v/v) Acetic acid in water. May be stored at room temperature.

7.2. Glycocapture

1. Equilibrate desalting columns with 20 mL of Oxidation Buffer (OB). Add samples to the columns and drain to waste. Add OB to the columns to bring total volume to 3 mL. Add 120% sample volume of OB and collect flow-through.
2. Prepare 50 mM Sodium periodate solution in water. This solution is light sensitive. Protect from light, by wrapping tube in foil.
3. Add the periodate solution at one-fifth sample volume (10 mM final concentration).
4. Oxidize samples in darkness (typically wrapped in foil) at room temperature for 1 h on an end-over-end rotator.
5. Equilibrate the desalting columns with 20 mL of Coupling Buffer (CB). Add samples to the columns and drain to waste. Add CB to the columns to bring total volume to 3 mL. Add 120% sample volume of CB and collect flow-through in clean tubes.
6. Prepare 100 μL of hydrazide resin (200 μL of a 50% slurry in isopropanol) by washing resin with ten CV (column volumes)

water (or three times three CV water), followed by ten CV of CB. Remove washes by centrifuging at 1,000 × *g* for 2 min. After washing, resuspend the hydrazide resin in 50% slurry of CB. Add the oxidized samples to hydrazide resin slurry. Conjugate the glycoproteins to the hydrazide resin at room temperature with mixing for 10–24 h (see Note 13).

7. After coupling reaction is complete, spin down the resin and remove supernatant. Wash thrice with 1 mL of Urea buffer; remove by centrifuging at 1,000 × *g* for 2 min. Resuspend in 200 µL of Urea buffer after last wash.
8. Add 5 µL of TCEP solution (5 mM final concentration) and incubate at room temperature for 30 min. During the incubation, prepare the 250 mM iodoacetamide solution in water; add 8 µL (10 mM final concentration) of prepared solution to the hydrazide resin slurry. Incubate in darkness at room temperature for another 30 min.
9. Wash the resin thrice with 1 mL of Urea buffer; remove by centrifuging at 1,000 × *g* for 2 min. Resuspend in 150 µL of Urea buffer after last wash.
10. Incubate the resin for 30 min at 55°C to denature proteins. After incubation, wash thrice with 1 mL of Urea buffer; remove by centrifuging at 1,000 × *g* for 2 min.
11. Wash resin thrice with 1 mL of a diluted form of Urea buffer (prepare a 1:4 dilution in water).
12. Remove by centrifuging at 1,000 × *g* for 2 min. Resuspend the hydrazide resin in 150 µL of the 1:4 diluted Urea buffer and add 20 µg of trypsin solution. Check pH; it should be approximately pH 8.3. Incubate overnight at 37°C with gentle agitation.
13. Remove the trypsin-released peptides by washing the resin thrice, each time with 1 mL of 1.5 M NaCl, 80% Acetonitrile, methanol, and 50 mM Ammonium bicarbonate buffer, pH 7.8.
14. Remove supernatant by centrifuging at 1,000 × *g* for 2 min at each step.
15. Resuspend the resin with 150 µL of 50 mM Ammonium bicarbonate buffer, pH 7.8. Transfer the resin to a 0.65-mL tube.
16. *N*-linked glycopeptides are released from the resin by adding 2.5 µL of the 1 U/µL *N*-glycosidase F solution to the resin and by incubating at 37°C with mixing overnight.
17. Centrifuge at 1,000 × *g* for 2 min. Transfer supernatant to a clean glass vial.
18. Wash the resin twice with 200 µL of 80% acetonitrile (Be mindful to keep the washes for the next step). Centrifuge at 1,000 × *g* for 2 min.

19. Combine the washes with the initial supernatant in the glass vial, and dry the samples using a SpeedVac system (see Note 5).
20. Perform C18 cleanup on samples.

Following isolation and mass spectrometric and computational analysis, proceed as described above with the following modification: asparagine residue mass alteration must include the mass of the glycosylation anchor.

8. Notes

1. Quality control: As an internal control for properly running instruments and samples, we typically recommend quality-control runs every 4–5 samples using a known mixture. One simple mixture that can be effective is an equimolar mix of angiotensin II, bombesin, substance P, neurotensin, and alpha 1–6. For microspray experiments, 2 µL injections of 1 µM of each peptide as a final concentration in water are used. For nanospray experiments, 2 µL injections of 0.1 µM of each peptide as a final concentration in water are used.

   The following are the intensities of the above-mentioned peptides. These peptides represent the dominant H+ adducts for the five quality-control peptides (Fig. 3):

   Angiotensin II: 523.7747

   Neurotensin : 558.3107

   Substance P : 674.3715

   Bombesin : 810.4148

   Alpha 1–6 : 882.4628
2. Depending on the application in our lab, the five-peptide mixture is reconstituted in different final buffers. For application as standards between runs, the final concentration of each peptide is 1 µM, in 3 M GuHCl. For standard infusion mix, the concentration is 0.1 µM in 50:50:0.1% (acetonitrile–water–formic acid).
3. Due to our small sample size, we follow the microplate procedure in the Pierce BCA Assay kit instructions manual for kit #23277.
4. Make up 1 M DTT and add about 10 µL of 1 M to 1 mL of cell solution. This renders a solution approximately 10 mM DTT final.
5. The samples are dried down in a vacuum centrifuge, with a −110°C cooling trap. This step helps in two ways:first, we boil-off the solvents from the fractionation run; second, we have smaller volumes to work within the subsequent digestion steps.

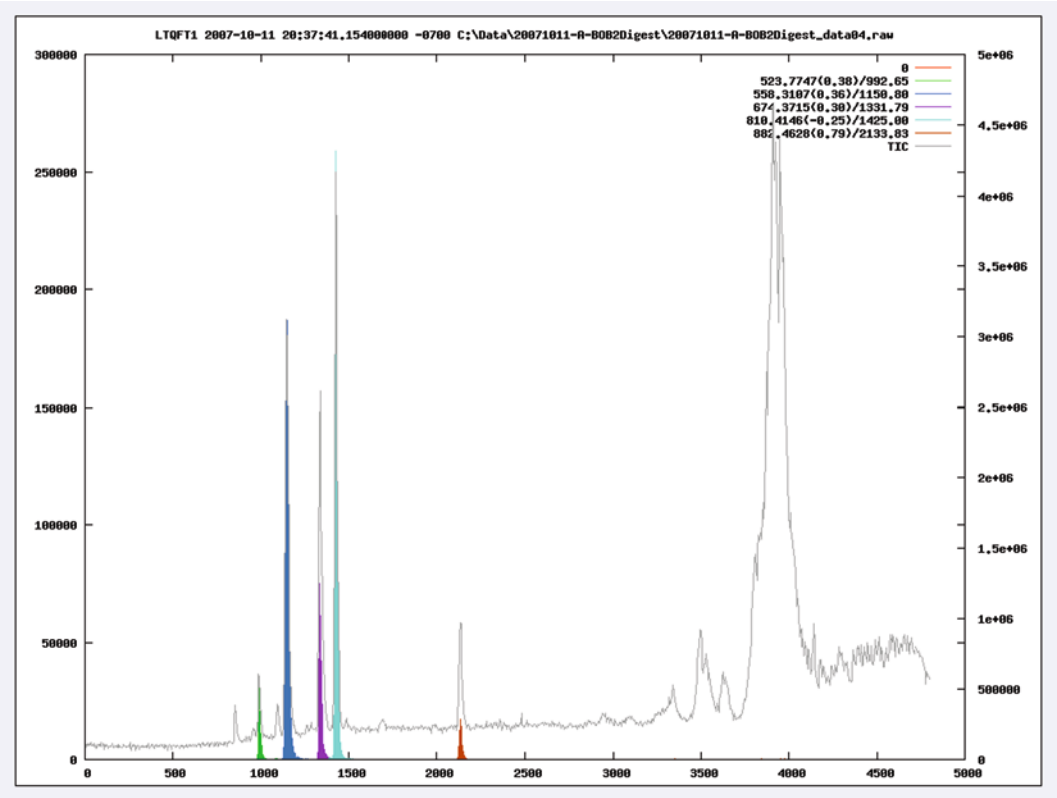

Fig. 3. An example of an automatically-generated picture created at the end of each of our 5-peptide quality control (QC) runs. Shown is the total ion chromatogram (scan # vs. intensity) on top of which is overlayed 5 extracted ion chromatograms for the m/z values (+/- 8 ppm) of the dominant charge states for each of the five peptides that make up our QC mixture. From a quick inspection the investigators can rapidly determine if the chromatography and sensitivity are within acceptable tolerances.

6. Our system utilizes two-column setup on a ten-port valve. This enables a sample to be analyzed on one column while the offline column is washed and having the next sample applied, as shown in Fig. 4.
7. In this example experiment, we scale up to 100 plates to (1) account for lower abundance of protein at the cell surface level. A significant level of protein is lost during the cell surface protein capture experiment, and to account for that loss and to ensure that we have enough protein for mass spectrometry analysis, we increase the number of plates we lyse. (2) 100 plates provide us with a quantity to work within the varying drug treatment conditions. As a side note, for nonenriched protein preparations, a typical plate yields approximately 500 μg to 2 mg of protein.
8. We divided our plates as follows:

   24 plates H (+ Gefitinib) vs. 24 plates L (– no treatment)

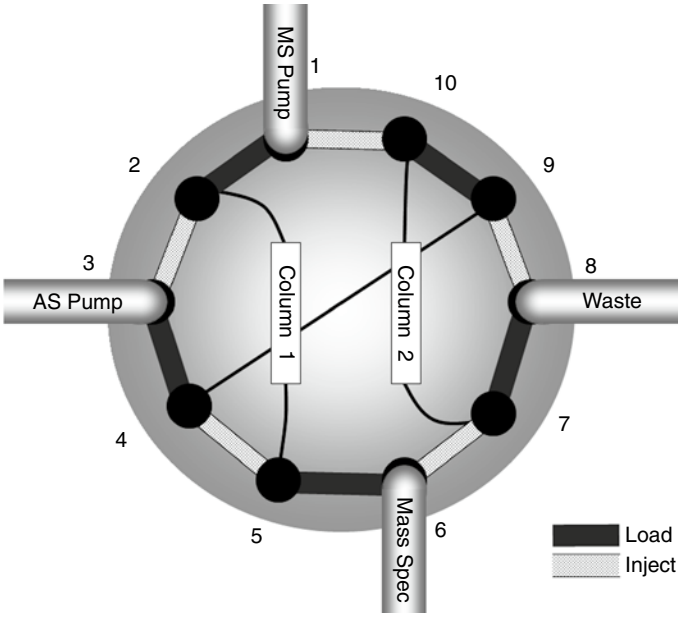

Fig. 4. Electrospray double column setup.

24 plates L (+ Gefitinib) vs. 24 plates H (– no treatment)

2 plates H (+ Gefitinib) vs. 2 plates L (– no treatment)

2 plates L (+ Gefitinib) vs. 2 plates H (– no treatment)

9. Column may be regenerated with 8 mL of 0.1 M glycine (pH 2.8).
10. Samples are concentrated using Centricon centrifugal filter device to concentrate samples to a workable volume without a concomitant increase in salt concentration as would occur in evaporative concentrator. Following concentration, the samples may be digested as described in Section I, Subheading 3.4, with few exceptions. Since this preparation renders the sample in solution, you need to add 3 M GuHCl in 100 mM AMBIC, to a final working concentration of GuHCl is 1 M.
11. First, prepare the 200 mM Tris buffer, pH 8.3. For a 1-L stock solution, add 24.24 g of Trizma base to approximately 400 mL of water and titrate to pH 8.3 with concentrated HCl, with stirring. Then, add remaining water to a final volume of 1 L. Alternatively, it is useful to prepare a 2× stock solution. Follow the above preparation, but use 48.48 g Trizma base in a final volume of 1 L. To complete a 50-mL preparation of Urea buffer, weigh 24.02 g of urea, 93 mg of

EDTA, and 50 mg of SDS and add these items to one appropriately sized container, then add 200 mM Tris buffer to a final volume of 50 mL.

12. To prepare 500 mL of oxidation buffer, add 1.36 g of sodium acetate to a beaker. Add about half the water (200–250 mL) and titrate the solution with glacial acetic acid until pH 5.0. Add 4.38 g of NaCl, stir, and check pH. Then, add remaining water to a final volume of 500 mL. 500 mL of coupling buffer is prepared as above, but the following are used: 6.8 g of sodium acetate (for 100 mM) and 29.22 g of NaCl (for a 1 M concentration). The coupling buffer is titrated to pH 4.5 with glacial acetic acid.
13. The binding capacity is ~1 mg pure beads per 10 mg bound protein.

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