Two dimensional gel electrophoresis using narrow pH 3-5.6 immobilised pH gradient strips identifies potential novel disease biomarkers in plasma or serum

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Two-dimensional gel electrophoresis (2-DE) is a protein separation technique often used to separate plasma or serum proteins in an attempt to identify novel biomarkers. This protocol describes how to run 2-DE gels using narrow pH 3-5.6 immobilised pH gradient strips to separate 2 mg of serum proteins. pH 3-6 ampholytes are used to enhance the solubility of proteins in this pH range before the serum proteins are separated in the first dimension by isoelectric point (isoelectric focusing) followed by molecular weight (SDS-PAGE). This approach using the pH 3-5.6 range differs from pH ranges more commonly used for serum or plasma biomarker discovery which span three or more pH units (e.g. pH 3-10 and 4-7), and has the advantage that the pH range lies outside the range of three highly abundant proteins and therefore improves separation and representation of low abundance features. The protocol described takes approximately 8 days.

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INTRODUCTION

There is much interest in discovering biomarkers to assess the pathological states of disease, and blood is the most common sample taken from patients to determine disease severity.[1] In hospital laboratories, plasma or serum is obtained from these blood samples and the levels of the biomarkers are determined using automated immunoanalysers or mass spectrometry. However, for many diseases there are no reliable serum/plasma biomarkers available and as a result occasionally invasive approaches such as biopsies are necessary. Not all biomarkers currently used for clinical diagnoses are reliable (e.g. they may show large variation in marker levels between different individuals with the same pathological state). There is an urgent need for reliable and novel biomarkers for many diseases in order to aid both patients and clinicians for diagnosis as well as for monitoring disease and therapeutic regimens.

Two-dimensional gel electrophoresis (2-DE) separates proteins and is often used to search for novel biomarkers in serum or plasma. This technique separates proteins in the first dimension by isoelectric point (isoelectric focusing) followed by molecular weight (SDS-PAGE). Commonly used pH ranges for isoelectric focusing are pH 3-10 and pH 4-7 which have been used by us and others to successfully discover novel disease biomarkers.[2,3] However, plasma and serum contain high abundant proteins in these pH ranges. These high abundant proteins restrict the amount of protein that can be loaded onto 2-DE gels and therefore decreases the chances of identifying low abundance proteins which potentially could serve as biomarkers.

In the protocol described here we use 2-DE over a narrow pH 3-5.6 range since this lies outside the range of the highly abundant proteins albumin, transferrin and immunoglobulins. The lack of highly abundant proteins in this pH range allows four times more serum or plasma to be loaded compared to a 2-DE gel using a wide pH 3-10 range. Here we present for the first time the detailed protocol for using pH 3-5.6 immobilised pH gradient strips to separate serum and plasma by 2-DE gels.[4,5]

Protocol development included analysis of various narrow pH ranges for 2-DE and their comparison to the more commonly used wide pH range. The use of a narrow pH 3-5.6 range led to an improved separation and visualisation of low abundance proteins. In Gangadharan et al.[4] for example, we demonstrate that using the pH 3–5.6 range with a load of 2 mg 262 additional protein features were detected compared to a pH 3–10 gel across the same pH 3–5.6 range. These extra features are potentially new disease biomarkers which are missed when using 2-DE gels with a wide pH range.

This novel approach described here not only helps in identifying new biomarkers in serum/plasma but would also be of great benefit in the separation and analysis of other samples where high abundant proteins like albumin may pose a problem such as cerebrospinal fluid or urine. A work flow diagram to show how the experimental steps fit together and examples of equipment which can be used is shown in Fig. 1. This work flow could also be adapted for use in 2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE).

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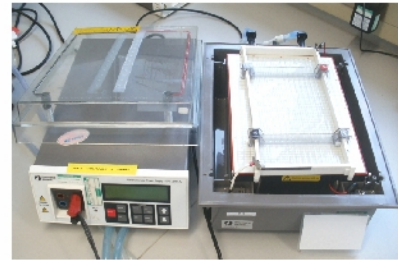

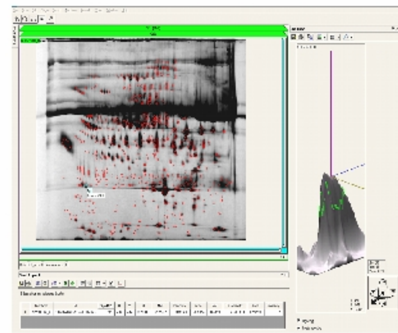

Figure 1 | The work flow used to run 2-DE gels using the pH 3-5.6 range
(a) Serum/plasma samples are initially denatured, solubilised, reduced and prepared with pH 3-6 ampholytes. (b) pH 3-5.6 NL IPG strips are overlaid onto samples in separate lanes of a re-swelling tray. (c) Proteins are separated by charge on a Multiphor using isoelectric focusing. (d) pH 3-5.6 NL strips are transferred onto SDS-PAGE gels and proteins are further separated by molecular weight with a Hoefer DALT running tank. (e) The proteins on the gel are stained and scanned using a Fuji LAS1000Pro camera. (f) Gel images are analysed using the Melanie computer-aided software to identify differences in feature intensity. Features on a pH 3-5.6 serum gel are outlined in red and 3D view of the feature intensity is shown for a selected feature. (g) Features of interest are excised from the gel using a GCM robotic gel excisor. (h) Gel pieces are digested with trypsin using the automated DigestPro workstation. (i) The resulting peptides are analysed by mass spectrometry to identify the proteins of interest.

MATERIALS

REAGENTS

- Blood collection tubes such as P100, plasma or serum tubes (BD).
- Bicinchoninic acid (BCA) assay kit, 3-([3-Cholamidopropyl]dimethylammonio)-1-propanesulphonate (CHAPS), tributyl phosphine, iodoacetamide, pooled human serum (Sigma).
- pH 3-6 SERVALYT® carrier ampholytes (SERVA).
- Urea, dithiothreitol (DTT), -methacryloxy-propyl-trimethoxysilane (Bind-Silane), Repel-Silane ES, dry strip cover mineral oil, Immobiline pH 3-5.6 NL IPG DryStrips (18 cm, 3 mm wide), electrode wicks (GE Healthcare).
- Sodium dodecyl sulphate (SDS), bromophenol blue, ammonium bicarbonate (NH_4HCO_3) (Fluka).
- HPLC grade water, absolute ethanol, hydrochloric acid (HCl), glycine (BDH).
- 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Tris), agarose, sequencing grade bovine trypsin (Roche).
- Thiourea, glycerol (Fisher Scientific).
- Dimethylbenzylammonium propane sulphonate: non-detergent sulphobetaine-256 – NDSB-256 (Calbiochem).
- Acetic acid, acetonitrile (Riedel-de Haën).
- SYPRO Ruby (Invitrogen).
- 96 well reaction plate (Intavis).
- 96 well collection plate – non-skirted 200 µl PCR plate (ABgene).

REAGENT SETUP

- Rehydration sample buffer (5 M urea, 2 M thiourea, 4% (w/v) CHAPS, 65 mM DTT, 2 mM tributyl phosphine, 150 mM NDSB-256, and 0.0012% (w/v) bromophenol blue).
- Equilibration solution (4 M urea, 2 M thiourea, 50 mM Tris HCl (pH 6.8), 30% (v/v) glycerol, 2% (w/v) SDS, 130 mM DTT, 0.002% (w/v) bromophenol blue).
- Laemmli reservoir buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS).

EQUIPMENT

- Reswelling tray, Multiphor II, EPS 3500XL power supply, DALT gradient maker, Hoefer DALT running tank (GE Healthcare)
- LAS1000Pro Intelligent Dark Box II CCD camera (Fuji).
- Melanie image analysis software (Genebio).
- Robotic gel excisor – GCM instrument (Horizon Instruments).
- Dark Reader light box (Clare Chemical Research).
- Savant SpeedVac and vacuum vaporiser (Thermo Electron).
- Automated DigestPro workstation (ABiMED).

PROCEDURE

To avoid keratin contamination of samples it is recommended, although not essential, that all steps below are carried out in clean room conditions using a hair cap bouffant, face mask / beard cover, extended cuff gloves and a non-shedding labcoat.

Sample preparation

1| Collect blood from patients using P100 tubes according to the manufacturer's recommendation or in other serum/plasma tubes as previously described for proteomics.[6,7]

PAUSE POINT Serum / plasma samples can be stored at – 80 °C until required.

2| Determine the total protein concentration of the sample using a protein assay such as BCA protein assay according to the manufacturer's recommendation.

3| Mix 2.4 mg of serum / plasma protein with rehydration sample buffer up to a total volume of 442 µl.

CRITICAL STEP Never heat the samples after adding urea. Elevated temperatures can result in urea hydrolysing to isocyanate. This can cause protein modification by carbamylation leading to artifactual trains of protein features on 2-DE gels.

! CAUTION Tributyl phosphine in the rehydration sample buffer is spontaneously inflammable in air.

4| Add 8 µl of pH 3-6 carrier ampholytes (final ampholyte concentration = 1.8%) and vortex mix for 2 minutes. Leave the samples at room temperature for 30 minutes to ensure complete denaturation and solubilisation. Spin samples at 16,000 g for 15 min.

5| Pipette 375 µl of supernatant (containing 2 mg serum/plasma) into separate lanes of a reswelling tray. Place Immobiline pH 3-5.6 NL IPG DryStrips face down onto the protein-containing samples in each lane of the reswelling tray and overlay with 2 ml of dry strip cover mineral oil. Leave to rehydrate for at least 16 h at room temperature.

CRITICAL STEP To ensure successful rehydration, the gel should be swollen to 1 mm thick and the bromophenol blue dye should be stained across the full length of the strip.

Isoelectric focusing (IEF)

6| Drain off excess mineral oil and transfer to the sample tray of Multiphor II apparatus with the gel facing upwards.

7| For each IPG DryStrip, cut two electrode wicks 2 cm in length. Soak electrode wicks with 100 µl HPLC grade water and blot with a cleanwipe to ensure that the wicks are damp but not excessively wet. Place damp wicks on either end of the IPG strips and fix electrode bars onto the wicks at either end of the IPG strips. Pour mineral oil into the Multiphor sample tray until the strips are immersed. Prod wicks gently using tweezers to remove air bubbles and ensure good contact with the IPG gel.

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8| Carry out IEF at 300 V for 2 h, a gradient increase to 3500 V over 3 h and then maintain at 3500 V up to 70 kVh using an EPS 3500XL power supply. For all stages, set the current limit to 10 mA for 12 gels, and the power limit to 5 W. Maintain the temperature of the Multiphor at 17 °C using a recycling thermostatic water bath.

PAUSE POINT Strips can be snap frozen on dry ice and then stored at – 80 °C until required.

Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

9| Gels can be poured using a gradient gel casting machine such as the DALT Gradient maker connected to a peristaltic pump. Pour gels according to the manufacturer's recommendation. Precast gels can also be used such as the ExcelGel system (GE Healthcare). Gels can be either of fixed percentage such as 12% or of a gradient such as 9-16% the latter of which gives better separation for serum / plasma. Gels must be able to accommodate 18 cm IPG strips.

10| Optional step: If pouring gels, the plates can be treated so that the gel covalently binds to one of the glass plates in the gel cassette. This makes it easier to manipulate the gels for fixing, staining, scanning, storage and cutting. One plate needs to be treated with Bind-Silane and the other with Repel-Silane ES according to the manufacturer's recommendations.

11| Immediately post IEF, incubate the IPG strips in 2 ml of reducing equilibration solution for 15 min at room temperature. Drain strips of equilibration solution. Overlay the strips onto the second dimension gels and seal in place with 90 °C, 0.5% (w/v) agarose in Laemmli reservoir buffer. Use the flat end of a spatula to aid placement of the IPG strip.

12| Perform second dimension electrophoresis with Laemmli reservoir buffer using a Hoefer DALT running tank or any other equivalent electrophoresis running tank capable of running large 18 cm by 18 cm gels. Set the current to 20 mA per gel for 1 h, followed by 40 mA per gel for approximately 4 h. Set the power limit to 150 W for a tank containing 6 gels and the voltage limit to 600 V throughout the run. Maintain the temperature at 10 °C using a recycling thermostatic water bath. Terminate electrophoresis once the bromophenol blue tracking dye has reached the bottom of the gel.

13| Remove gels from the running tank and open the glass plates. Discard the IPG strip and the overlay agarose. Wash gels briefly in ultrapure water to remove running buffer and then place into a staining tank such as the Dodeca stainer (BioRad) or equivalent. Fix the proteins on the gels in 40% (v/v) ethanol, 10% (v/v) acetic acid overnight.

14| Gels can then be stained. For highest sensitivity and greatest compatibility by mass spectrometry, fluorescent stains such as SYPRO Ruby are recommended.[8] Perform staining according to the manufacturer's recommendations.

15| Image gels using a scanner/camera appropriate for the stain used. Scanners/cameras are available capable of imaging multiple stains such as the Fuji LAS1000Pro CCD camera which can image either fluorescent, silver or Coomassie stained gels. In the case of SYPRO Ruby stained gels, set the parameters to fluorescence and cool the CCD camera to -25 °C prior to capturing images. Place the gel onto the imaging tray and acquire images over different exposure times (typically between 0.5 to 2 min) until the optimum image has been produced.

16| Place scanned gels in a plastic bag with approximately 10 ml of 40% (v/v) ethanol, 10% (v/v) acetic acid and seal the bag with a bag sealer. Store at 4 °C until required for excising protein spots.

Differential image analysis and spot excision

17| Perform differential image analysis of the gels. This can be carried out using various commercially available software such as the Medical ELectrophoresis ANalysis Interactive Expert (Melanie) software. Perform differential image analysis according to the manufacturer's recommendations. An optional step is to calibrate all gels internally for pI and molecular weight using the E. coli proteome as a standard[9], typically using 10-15 calibrated landmarks on each gel. Landmarks allow the software to warp the gels so that they can be superimposed onto each other to aid with image analysis. All features displayed as differentially expressed by the software must be validated further by visualising the features across all gels in a montage format.

18| Differentially expressed features can be excised from the gel manually using a clean scalpel or using any software-driven robot. To aid manual excision gels can be visualised in a dark room using either a white light box (for silver or Coomassie stained gels) or a Dark Reader light box (for fluorescent stained gels). For excision of features using a robotic gel excisor (such as a GCM instrument), the co-ordinates of each differentially expressed protein feature is sent to the robot as a list of commands which will programme x and y movements of the robot arms and direct the cutting head to cut and remove features. To avoid contamination between spots, use a new cutter tip on the cutting head for each gel feature. The robot excises gel features by shearing and aspirating actions. Eject the cutter tips with the isolated gel pieces into

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separate wells of a 96 well reaction plate with laser made holes on the bottom.

In-gel trypsin digestion

19| In-gel trypsin digestion can then be performed either manually (see Supporting Information) or using an automated workstation such as the DigestPro as described below (all steps at room temperature unless stated otherwise). The DigestPro workstation adds solutions to the 96 well plate containing the gel pieces using needles and removes liquid through laser made holes on the bottom of the plate by applying nitrogen pressure as previously described.[10] Prepare 18 ng/µl bovine trypsin and place inside the automated robot in its inactive form by storing in acidic conditions (10% (v/v) acetonitrile, 1 mM HCl).

20| Wash gels with 50 µl acetonitrile and 50 µl 50 mM NH_4HCO_3 for 15 min. Remove supernatant. Dehydrate gels with 100 µl acetonitrile for 10 min. Remove acetonitrile.

21| Reduce proteins in the gel with 30 µl 10 mM DTT in 25 mM NH_4HCO_3 for 10 min at 60 °C. Remove supernatant once the samples have cooled (20 min).

22| Alkylate gel proteins with 30 µl 50 mM iodoacetamide in 25 mM NH_4HCO_3 for 15 min. Remove iodoacetamide solution.

23| Wash gel pieces with 50 µl 50 mM NH_4HCO_3 for 15 min. Remove 50 mM NH_4HCO_3. Dehydrate gels twice with 50 µl acetonitrile for 15 min. Remove acetonitrile. Pause workstation for 10 min to allow drying.

24| Activate trypsin by diluting it 2-fold with 25 mM NH_4HCO_3. Add 15 µl of this 9 ng/ml trypsin solution to each gel piece and leave for 10 min to allow gel swelling. Incubate gel pieces at 37 °C for 2 h. Add 10 µl water to compensate for any water loss. Incubate gel pieces at 37 °C for a further 2 h.

25| Add 10 µl 25 mM NH_4HCO_3 to each gel piece and incubate for 10 min. Add 20 µl acetonitrile and leave for 10 min to dehydrate the gel. Transfer the supernatant to a 96 well collection plate.

26| Add 20 µl 10% (v/v) formic acid to the gels and leave for 10 min to extract the peptides. Add supernatant to the collection plate. Dehydrate gels with 30 µl acetonitrile for 15 min. Add supernatant to the collection plate.

27| Dry pooled extracts completely in a SpeedVac and reconstitute peptides by dissolving in 0.1% (v/v) formic acid. Perform mass spectrometric analysis as previously described.[11]

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● TIMING

Collection of samples from patients (Step 1) can take several weeks to months after which 2-DE can be performed with the following timings:

Day 1: Step 2, 2 h; Step 3, 15 min; Step 4, 1 h; Step 5, 30 min (+16 h overnight incubation).

Day 2: Step 6, 10 min; Step 7, 5 min; Step 8, > 23 h overnight run; Step 9, no waiting time (precast gels) or 1-2 days in advance (poured gels), Step 10: 2 h.

Day 3: Step 11, 30-45 min; Step 12, 5 h; Step 13, 30 min (+ 16 h overnight incubation).

Day 4: Step 14, 1 h to overnight (depending on staining method used); Step 15, 1 h; Step 16, 15 min.

Day 5: Step 17, 1 day (can take several days – timing depends on software used and experience of user).

Day 6: Step 18, 1 h; Step 19, 15 min; Step 20, 35 min; Step 21, 40 min; Step 22, 20 min; Step 23, 50 min; Step 24, 4-5 h; Step 25, 30 min; Step 26, 35 min

Days 7 and 8: Step 27, 1-2 days.

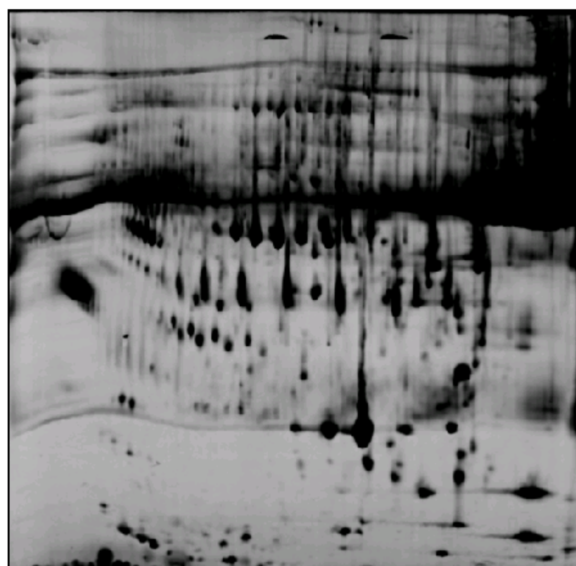

Figure 2: Anticipated results

Two milligrams of normal human serum (Sigma) run on a 2-DE gel using the pH 3-5.6 range.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Solution
No features or reduced feature number	Sample load is insufficient	Re-check sample concentration.
	Not all the sample entered the IPG strip	Sample needs to be solubilised by adjusting the amounts of detergent, reducing agent and ampholyte in the rehydration sample buffer.Check that the IPG strip is placed on the Multiphor the correct way around.
	Stain is not sensitive enough	If using Coomassie no spots are seen, try more sensitive silver or fluorescent stains.
Horizontal streaks on gels	Protein remains at the origin of the 2nd dimension	Change sample preparation approach. Adjust concentations of urea, detergents, ampholytes, and reducing agent.
	Gels overloaded with protein	Use less sample.
	Nucleic acids are bound to the proteins	Treat samples with an endonuclease to reduce viscosity and increase protein uptake into the IPG strip.
	Over or Under-focusing	Reduce or prolong focusing time.
	Ionic impurities in sample	Desalt/dilute the sample so that salts are less than 10 mM.
Vertical streaks on gel	Pinpoint streaks = Particles in buffer	Use purified water for all buffers.
	Broad streaks connected to features = Protein aggregation	Possible incomplete reduction/alkylation. Use more reducing agent in buffer. More SDS for equilibration (0.1% w/v) or increasingequilibration time may also help.
Vertical gaps in gel	Air bubble	Ensure there are no air bubbles between the agarose and polyacrylamide gel when applying the IPG strip to the gel.
	High salt concentration	Desalt/dilute the samples before sample preparation for 2-DE.
	Excessive DTT	Reduce concentration of DTT to 50 mM or less.
Individual features seen as multiple spots	Protein carbamylation	Do not heat the sample after making it up in rehydration sample buffer.
	Spots vertically twinned	IPG strip needs to be placed properly on the 2nd dimension gel with the plastic backing of the IPG strip against the glassplate.
Distortion of features	Top surface of 2nd dimension is not flat	Immediately overlay gel with water-saturated butanol after casting.
	Incomplete/too rapid polymerisation	Degas solutions before casting. Increase or decrease the concentration of TEMED/APS to accelerate or slow down polymerisation.

ANTICIPATED RESULTS

Typically 500 to 600 features are seen when using 2 mg of serum / plasma protein on a 2-DE gel over the pH 3-5.6 range. Fig. 2 shows a typical image when running a gel following the conditions described using normal human serum from Sigma. Anticipated results can also be referred to in our publications.[4,5]

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AUTHOR CONTRIBUTIONS B.G. developed the approach to use pH 3-5.6 IPG strips for biomarker discovery and wrote the initial manuscript. B.G. and N.Z. analysed the results and contributed to writing the paper.

COMPETING FINANCIAL INTERESTS The authors declare that they have no competing financial interests.

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