Chapter 18

High-Density Serum/Plasma Reverse Phase Protein Arrays

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Abstract

In-depth exploration and characterization of human serum and plasma proteomes is an attractive strategy for the identification of potential prognostic or diagnostic biomarkers. The possibility of analyzing larger numbers of samples in a high-throughput fashion has markedly increased with affinity-based microarrays, thus providing higher statistical power to these biomarker studies. Here, we describe a protocol for high-density serum and plasma reverse phase protein arrays (RPPAs). We demonstrate how a biobank of 12,392 samples was immobilized and analyzed on a single microarray slide, allowing high-quality profiling of abundant target proteins across all samples in one assay.

Key words Reverse phase protein array, RPPA, Serum, Plasma, Affinity proteomics, Noncontact inkjet printer, Protein profiling, Fluorescent detection

1 Introduction

Traditionally there are two main formats of affinity microarrays, forward phase arrays, and reverse phase arrays. In the former, the capture reagent is immobilized on the microarray surface, while in the latter the target analyte is immobilized. In what is now denoted reverse phase protein arrays (RPPAs), the spotted analyte is part of a complex biological sample [1].

RPPAs were first described in the context of printing lysates acquired from laser capture microdissection [2]. Some efforts have been made to apply RPPAs on other sample materials, such as cerebrospinal fluid, serum, and plasma, but the technology is still relatively unexplored in regard to these biofluids [3–8]. The main challenge of analyzing serum or plasma is the complexity of the sample matrix and the dynamic range [9]. Although the sensitivity of RPPAs is limited by the picoliter volumes used for spotting, profiling medium to highly abundant proteins in serum or

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plasma is feasible [10]. Additionally, RPPAs allow high sample throughput by simultaneous analysis of an analyte across thousands of samples [11].

Similarly to all other affinity-based methods, RPPA is dependent on validated high-quality affinity reagents. Today, there is no standardized approach to assessing reagent validity, and it is known that reagent performance is method dependent. However, functional assays such as ELISA, Western blotting, immunohistochemistry, and immunofluorescence are commonly used for determining the specificity, selectivity, and reproducibility of an affinity reagent. Inter- and intra-method reproducibility may also assist in the validation [12].

The most commonly used substratum for RPPAs is nitrocellulose, mainly due to its high binding capacity compared to other substrata [11, 13]. When selecting a reporter molecule, it is important to be aware that nitrocellulose autofluorescence overlap with emission wavelengths of some commonly used fluorescent detection molecules [14].

Different microarray printers are available on the market, and both contact and noncontact printers are suitable for printing RPPAs. Here, we present a protocol in which a noncontact printer is used. The printer uses the piezoelectric effect to eject droplets of 100 pl onto the slides at a distance of 1–5 mm, hence limiting the risk of disturbing the membrane surface [15, 16].

In this protocol, we describe the RPPA technology applied on serum and plasma samples and demonstrate its scalability to thousands of samples within one array. To our knowledge, this is the largest serum RPPA produced to date.

The protocol is divided into the following sections: sample preparation, printing of arrays, assay procedure, and image analysis (*see* Fig. 1). The protocol has been applied to a cohort of 12,392 serum samples (*see* Fig. 2a), which were collected within the TwinGene cohort (2004–2008, Sweden) [17]. The cohort comprises of samples from monozygotic and dizygotic twins, both paired (4851) and individual (2690). An even distribution of females (6764) and males (5628) were included, with an age range of 47–94 and a mean age of 64.9. The array was created with the purpose of studying proteins related to aging and twinness. The primary antibodies applied on the arrays have been produced and validated within the Human Protein Atlas (www.proteinatlas.org) [18] (*see* Fig. 2b, c).

2 Materials

2.1 Sample Preparation

1. Printing buffer: phosphate-buffered saline buffer pH 7.4 (1×PBS), supplemented with 0.1% (v/v) Tween 20 (1×PBS-T), and 50% (v/v) glycerol, stored at +4 °C (*see* **Notes 1** and **2**).
2. Benchtop semiautomatic pipettor: CyBi-SELMA (CyBio).

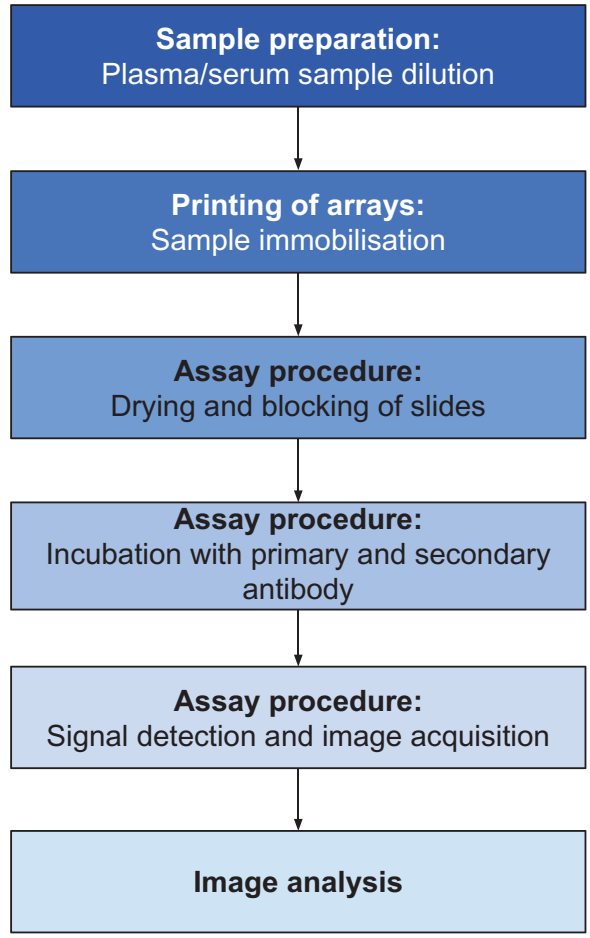

Fig. 1 A flowchart describing the major steps in the described protocol

2.2 Printing of Arrays

1. Microarray printer: Arrayjet Marathon Inkjet Microarrayer with a 12-sample JetSpyder (Arrayjet Ltd.).
2. Plate lids: JetGuard Probe Protector (Arrayjet Ltd.).
3. Slides: nitrocellulose-coated slides, 1 mm × 75.6 mm × 25.0 mm (UniSart 3D nitro, Sartorius Stedim).
4. System buffer: 47% (v/v) glycerol, 0.06% (v/v) Triton X-100 (*see* **Notes 1–3**).
5. Oven: hybridization oven/shaker.

2.3 Assay Procedure

1. Blocking buffer: 1×PBS-T supplemented with 3% (w/v) bovine serum albumin (BSA).
2. Wash buffer 1, WB1: 1×PBS-T.
3. Wash buffer 2, WB2: 1×PBS.
4. Antibody dilution buffer: 1×PBS-T.

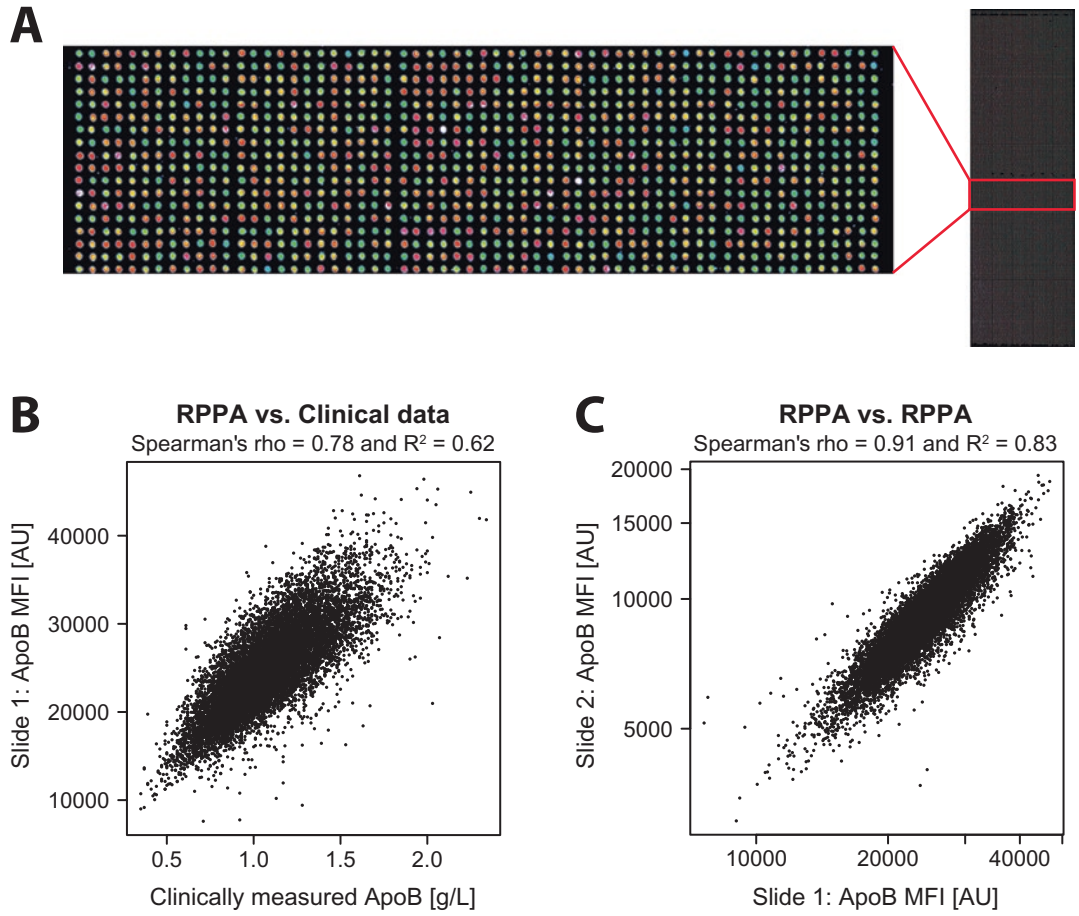

Fig. 2 Example data measuring apolipoprotein B (ApoB) in 12,648 features, consisting of 12,392 unique samples and 256 technical controls, using the serum RPPA arrays. Correlations are calculated using Spearman's rho and the square of Pearson's correlation coefficient. (**a**) Zoom in and full image of array, spots colored by signal intensity (*low-high: blue-green-yellow-red-white*), (**b**) RPPA signals correlated to clinically measured ApoB, and (**c**) correlation between two slides incubated with the same anti-ApoB antibody

5. Primary antibody: anti-ApoB (0.1167 mg/ml, HPA049793, Human Protein Atlas).
6. Secondary antibody: goat anti-rabbit IgG Alexa Fluor 647 (Invitrogen).
7. Slide tray: quadriPERM 4 × 12 (Sarstedt).
8. Orbital shaker: Sky Line DOS-10L (ELMI).
9. Microarray scanner: LuxScan HT24 (CapitalBio Corp.).

2.4 Image Analysis

1. Image analysis software: GenePix Pro 5.1 or later versions (Molecular Devices).
2. Statistical software: Microsoft Excel or R.

3 Methods

3.1 Sample Preparation

In the first part of this protocol, we describe the preparation of crude samples, applicable to both plasma and sera. We will not discuss array design or sample positioning in detail; however, it is important to plan for this prior to sample dilution and transfer to print plates (*see* **Note 4**). We recommend a printing design where samples are randomized within the array and supplemented with controls such as positive controls, negative controls, dilution series, and replicates. To account for local variations in the substratum, the controls should be arrayed in multiple locations and replicates should not be printed next to each other.

1. Place frozen samples at +4 °C to thaw overnight (*see* **Note 5**).
2. Ensure that the thawed samples are free from air bubbles by vortexing and centrifuging them using a benchtop centrifuge (2000 × *g*, 2 min).
3. Distribute printing buffer into print plates using an automated multichannel pipette, 20 μl printing buffer per sample well. Using a benchtop semiautomatic pipettor, dilute samples 1/5 by adding 5 μl sample to the print plates.
4. Prepare controls to be included on the array (*see* **Note 6**). Negative controls: printing buffer. Positive controls: species-specific antibodies (rabbit IgG, mouse IgG, goat IgG, donkey IgG, chicken IgY, and human IgG). Quality control: replicates of individual samples, pools, and dilution series of both individual samples and pools. Transfer each control to its designated position in the print plate.
5. Keep print plates at +4 °C if they are to be used immediately; otherwise, store at −20 °C.

3.2 Printing of Arrays

A range of different printers are available that may be suitable for printing serum and plasma samples on nitrocellulose slides. This protocol describes the printing procedure using the noncontact printer Marathon Inkjet Microarrayer from Arrayjet.

1. Start the printer and perform the instrument-specific start-up routine according to the printer's manual. Ensure that the humidity and temperature stabilize at 50% and +20 °C, respectively, before starting the printing process. Empty waste and refill system buffer bottles (*see* **Note 3**).
2. Thaw printing plates and let them reach room temperature. Vortex and centrifuge the plates until no air bubbles are present (2000 × *g*, 5 min). Attach plate lids to limit evaporation and contamination of wells during printing. Mount ready plates in the printer and fill up the plate tray with up to six print plates at a time.
3. Load the printer with unused slides, up to 100 slides per run (*see* **Note 7**).

4. Array the samples according to the desired printing design (*see* **Note 4**). Spot three drops per sample, which gives a final spot volume of 300 pl and a diameter of 200 μm.
5. During print run: Keep a log over the humidity, temperature, and instrument pressure. Empty waste and refill the system buffer bottles as needed. For consecutive loadings, thaw and prepare print plates as in **step 2**.
6. When the printing is finished, remove all slides from the printer and dry them in an oven at +37 °C for 16 h or until dry. If slides are not dried properly, tailing or bleed off may occur in the consecutive steps (*see* **Note 8**).
7. Store printed slides in air and light tight boxes at +4 °C.

3.3 Assay Procedure

Slides with printed samples are incubated with an antibody targeting the analyte of interest, and detection is enabled using a far-red-fluorescent dye antibody (*see* **Note 9**). The following instructions describe the usage of 1-pad slides for the screening of one analyte across thousands of samples. The protocol can be adjusted to also suit slides with multiple pads (e.g., 8- or 16-pad slides) (*see* **Note 10**).

1. Take out slides from +4 °C and let them reach room temperature.
2. Submerge each slide in 15 ml blocking buffer and incubate on an orbital shaker (100 rpm, 1 h).
3. For each slide, prepare a slide tray filled with WB1 and using tweezers quickly dip the slides into the buffer. Proceed by submerging each slide in a new slide tray filled with ca 15 ml WB1 and let wash on an orbital shaker (100 rpm, 5 min). Continue by repeating the dip and washing step in consecutive order, three times (*see* **Note 11**).
4. Dilute primary antibody 1/2350: 1.7 μl anti-ApoB in 4 ml antibody dilution buffer (*see* **Note 12**).
5. Incubate slides with 4 ml diluted primary antibody on an orbital shaker (65 rpm, 1 h). Cover the slides to avoid dust contamination.
6. Wash slides as described in **step 3**.
7. Dilute secondary antibody 1/60,000: 0.25 μl goat anti-rabbit IgG Alexa Fluor 647 in 15 ml antibody dilution buffer (*see* **Note 12**).
8. Incubate slides with 15 ml diluted secondary antibody on an orbital shaker (90 rpm, 1 h). Cover slides from light to protect fluorophores.
9. Wash slides as described in **step 3**. Keep slides covered from light.
10. In order to wash away any residual Tween 20, wash slides with WB2 on an orbital shaker (100 rpm, 5 min). Keep slides covered from light.

11. Remove potential residual salts by carefully rinsing the slides in deionized water.
12. Spin-dry each slide before loading them into a microarray scanner (*see* **Note 13**).

3.4 Image Analysis

In order to interpret the assay, numerical data has to be extracted from the scanned images. Different software products are available and different approaches can be used. Here, we describe the first steps in one approach.

1. Import the acquired gray-scale image into the image analysis software GenePix Pro (5.1 or later versions) and assign excitation wavelengths to the image.
2. Create an array list, such as a GenePix Array List file (GAL file), that contains information of which sample is located in which spot. This may be done in the printer software, the GenePix software, or manually in, for example, Microsoft Excel (*see* **Note 14**).
3. Load the array list, manually align the grid roughly, and then let the software automatically align the grid features to the spots. Use the setting "Find irregular features" to better fit spot morphology. Assess the alignment, adjust grid features if necessary, and flag spots that will require attention during data analysis. Analyze the array and save the result file.
4. The result file contains a numerical matrix with spot features per row and measurement parameters per column. The data can be analyzed using software products such as Microsoft Excel or R. The latter may be preferred for big data sets.
5. Before interpreting the data, it is important to assess its quality. Check if the printed controls give expected signals. Adjust for varying background signals and their potential influence on the spots by subtracting the local background of each spot. Exclude data points from spots that are flagged or give a signal below three standard deviations of the buffer signals plus buffer mean. If possible, assess the quality of the data by correlating it with measurements from a different method and between replicate slides.

4 Notes

1. Prepare buffers in ultrapure water, such as Milli-Q water (Merck Millipore).
2. Adjust the glycerol level in the printing buffer in order to accommodate for different dilution factors. Aim for a final glycerol percentage of 40–50%, and make sure it is the same for all wells in each print plate. If using a different printer than

a Marathon Inkjet Microarrayer, check instrument requirements regarding buffers before preparing them.

3. Handle Triton X-100 and waste with care since these are harmful to the environment and may carry biological hazards.
4. If unsure of printing design, do a test print with different distances between spots to make sure all samples will fit and still be distinguishable. Different membrane pad sizes are available, e.g., 1, 8, or 16 pads per slide. The choice of slide format depends on the number of samples, inclusion of replicates, and the number of analytes to be profiled. The uptake and printing order of samples depend on printer type; therefore, ensure that the printing plates are filled appropriately.
5. If a large number of samples are to be printed, thaw and dilute them in batches. When returning samples to freezer storage, take out the next batch to thaw. Do not thaw or freeze samples unless necessary. Keep samples on ice when working at room temperature and use appropriate protective wear (gloves, laboratory coat, and safety goggles).
6. To allow flexible usage of secondary antibodies from different species, print various species-specific antibodies to ensure the inclusion of a positive control on the array. Replicates should be present in a print plate as well as printed several times and distributed throughout large arrays for printing quality control.
7. Always handle slides with gloves and/or tweezers. Slides are preferably held by the edges or the barcode area, if present. Make sure not to touch the membrane surface or to damage the barcode if present, especially if using tweezers. Note the positions of all slides in the printer so that potential technical variability during printing can be tracked and accounted for.
8. An initial quality control can be done by observing the spots in a regular benchtop microscope. Spots are mainly visible before they have dried. Drying status, alignment, and morphology can be assessed in this way. Before assay usage, dried slides may also be roughly assessed with the naked eye in regard to alignment. Non-dried content may spread outside spot area, especially when submerged into blocking buffer, causing tailing of spots that complicates grid alignment during image analysis or bleed off that might cause increased local background.
9. Use fluorophore at red, far-red, or infrared wavelengths since the nitrocellulose membrane has a natural high autofluorescence at green wavelengths. Use a maximum of one fluorophore per detection channel in the scanner you will use.
10. If using 1-pad slides: Perform washes and incubations in slide trays, one tray per slide. Optimize buffer and antibody volumes

depending on the tray size. If using slides with multiple pads: block slides as described in the protocol (**steps 1–3**). Using a slide holder with a silicone mask, create a separate chamber for each pad during primary antibody incubation. Wash pads by first rinsing with WB1 by pipetting in and out five times and then adding WB1 for 5 min wash incubation; repeat four times. Take out slides from the holder, quickly dip in WB1, and submerge in secondary antibody for incubation and follow detailed protocol (from **step 7**). Optimize buffer and antibody volumes depending on the incubation chamber size.

11. Blocking buffer and antibody residues maintained within the nitrocellulose membrane can contribute to uneven background levels. This can be alleviated by varying the position of the slides on the shake table to ensure an even washing of the membranes.
12. A small-scale antibody dilution optimization and validation test is recommended to be performed beforehand, in order to determine a suitable working concentration of each antibody. Slide area and slide tray volume should be taken into consideration. If a large array is to be analyzed, it is recommended to also print a subset of the samples onto smaller pads to be used for the antibody dilution optimization.
13. Adjust laser power and photomultiplier-tube (PMT) settings in scanner to avoid saturated signals and for the acquisition of a suitable signal dynamic range. Use appropriate channel(s) for the fluorophores you have used. We recommend also including the channel for green wavelengths during scanning if available, since the autofluorescence may serve as support for grid alignment as well as identification of contaminations or membrane and printing irregularities.
14. For array list formatting, see the manual of the printer software, the GenePix software or Molecular Devices Knowledge Base (http:// mdc.custhelp.com).

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References

1. Aguilar-Mahecha A, Hassan S, Ferrario C, Basik M (2006) Microarrays as validation strategies in clinical samples: tissue and protein microarrays. OMICS 10:311–326. doi:10.1089/omi.2006.10.311
2. Paweletz CP, Charboneau L, Bichsel VE et al (2001) Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. Oncogene 20:1981–1989. doi:10.1038/sj.onc.1204265
3. Caiazzo RJ, Maher AJ, Drummond MP et al (2009) Protein microarrays as an application for disease biomarkers. Proteomics Clin Appl 3:138–147. doi:10.1002/prca.200800149
4. Ahmed F, Gyorgy A, Kamnaksh A et al (2012) Time-dependent changes of protein biomarker levels in the cerebrospinal fluid after blast traumatic brain injury. Electrophoresis 33:3705–3711. doi:10.1002/elps.201200299
5. Janzi M, Odling J, Pan-Hammarström Q et al (2005) Serum microarrays for large scale screening of protein levels. Mol Cell Proteomics 4:1942–1947. doi:10.1074/mcp.M500213-MCP200
6. Janzi M, Sjöberg R, Wan J et al (2009) Screening for C3 deficiency in newborns using microarrays. PLoS One 4:e5321. doi:10.1371/journal.pone.0005321
7. Aguilar-Mahecha A, Cantin C, O'Connor-McCourt M et al (2009) Development of reverse phase protein microarrays for the validation of clusterin, a mid-abundant blood biomarker. Proteome Sci 7:15. doi:10.1186/1477-5956-7-15
8. Kobayashi M, Nagashio R, Jiang SX et al (2015) Calnexin is a novel sero-diagnostic marker for lung cancer. Lung Cancer 90:342–345. doi:10.1016/j.lungcan.2015.08.015
9. Solier C, Langen H (2014) Antibody-based proteomics and biomarker research—current status and limitations. Proteomics 14:774–783. doi:10.1002/pmic.201300334
10. Ayoglu B, Häggmark A, Neiman M et al (2011) Systematic antibody and antigen-based proteomic profiling with microarrays. Expert Rev Mol Diagn 11:219–234. doi:10.1586/erm.10.110
11. Akbani R, Carragher N, Goldstein T et al (2014) Realizing the promise of reverse phase protein arrays for clinical, translational and basic research: a workshop report. Mol Cell Proteomics 13:1625–1643
12. Bordeaux J, Welsh AW, Agarwal S et al (2010) Antibody validation. BioTechniques 48:197–209. doi:10.2144/000113382
13. Sauer U (2011) Impact of substrates for probe immobilization. In: Korf U (ed) Protein microarrays: methods and protocols, Methods in molecular biology, vol 785. Springer Science+Business Media, LLC: Humana Press, Heidelberg, Germany, pp 363–378
14. Tighe P, Negm O, Todd I, Fairclough L (2013) Utility, reliability and reproducibility of immunoassay multiplex kits. Methods 61:23–29. doi:10.1016/j.ymeth.2013.01.003
15. Mcwilliam I, Kwan MC, Hall D (2011) Inkjet printing for the production of protein microarrays. In: Korf U (ed) Protein microarrays: methods and protocols, Methods in molecular biology, vol 785. Springer Science+Business Media, LLC: Humana Press, Heidelberg, Germany, pp 345–361
16. Tisone TC, Tonkinson JL (2005) Non-contact dispensing for protein microarrays. In: Schena M (ed) Protein microarrays. Jones and Bartlett Publishers, Sudbury, MA, pp 169–185
17. Magnusson PKE, Almqvist C, Rahman I et al (2013) The Swedish twin registry: establishment of a biobank and other recent developments. Twin Res Hum Genet 16:317–329. doi:10.1017/thg.2012.104
18. Uhlén M, Björling E, Agaton C et al (2005) A human protein atlas for normal and cancer tissues based on antibody proteomics. Mol Cell Proteomics 4:1920–1932. doi:10.1074/mcp.M500279-MCP200