Chapter 17

A Well-Based Reverse-Phase Protein Array of Formalin-Fixed Paraffin-Embedded Tissue

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Abstract

Biomarkers from tissue-based proteomic studies directly contribute to defining disease states as well as promise to improve early detection or provide for further targeted therapeutics. In the clinical setting, tissue samples are preserved as formalin-fixed paraffin-embedded (FFPE) tissue blocks for histological examination. However, proteomic analysis of FFPE tissue is complicated due to the high level of covalently cross-linked proteins arising from formalin fixation. To address these challenges, we developed well-based reverse-phase protein array (RPPA). This approach is a robust protein isolation methodology (29.44 ± 7.8 μg per 1 mm^3 of FFPE tissue) paired with a novel on electrochemiluminescence detection system. Protein samples derived from FFPE tissue by means of laser capture dissection, with as few as 500 shots, demonstrate measurable signal differences for different proteins. The lysates coated to the array plate, dried up and vacuum-sealed, remain stable up to 2 months at room temperature. This methodology is directly applicable to FFPE tissue and presents the direct opportunity of addressing hypothesis within clinical trials and well-annotated clinical tissue repositories.

Key words Formalin-fixed, Paraffin-embedded, Tissue lysate, Protein extraction, Proteomics, Reverse-phase protein array, Electrochemiluminescence

1 Introduction

The capacity to perform proteomic profiling of formalin fixed, paraffin embedded (FFPE) tissue offers substantial opportunities, leveraging bio-repositories, detailed pathologic diagnosis and clinical annotations. However, the recovery of protein from archival FFPE remains a challenging issue because formalin fixation leads to extensive protein cross-linking through Schiff base formation [1] and results in limited protein extraction, impaired immunoreactivity, and ambiguous identification of protein identification by mass spectrometry. Recent advances in techniques for extracting proteins from FFPE tissue sections have facilitated tissue protein profiling in the clinical proteomics, with varying degree of success [2–8].

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Protein-based array technologies have been used for target identification and characterization [9–11]. Currently there are three types of protein array platforms such as purified recombinant proteins, antibody microarrays, and reverse-phase protein microarrays [9]. Among these platforms, reverse-phase protein array (RPPA) has emerged as a strong candidate proteomic technology for FFPE tissue. Recent studies [7, 8] of FFPE proteome employed RPPA as a validation tool for protein from FFPE tissue specimens. However, limitations are still unsolved and hinder RPPA technology from reaching its full potential. These limitations include the cost of an array printer and complicated study designs where all the specimens to be assayed must be assembled at one time, preventing easy "assay on demand" environments [12]. In addition, the arrays are difficult to store and require extensive antibody and assay validation. In an effort to overcome some of the obstacles of current RPPA, we developed a well-based RPPA platform utilizing an electrochemiluminescence detection system [12]. This platform does not require a printer or arrayer and is applicable to "assay on demand" conditions; however specialized reagents and reader are required. The arrays are stable for over 2 months at room temperature, offering great abilities for antibody affinity validation and alleviating complex study design [12].

As examples of the utility of well-based RPPA, we have demonstrated that a new HER-2 assessment by well-based RPPA significantly correlated with current HER-2 status guideline, suggesting that it could be developed into alternative method for accurately determining HER-2 status in human FFPE breast cancer specimens (*unpublished data*). In addition, we have determined vascular endothelial growth factor expressional levels in archival human FFPE colon cancer tissue specimens by means of this technology [13]. In this study, we showed the new approach could be used for protein profiling analysis in FFPE tissue, with quantification and normalization tools. Especially in secreted proteins, this approach is a sensitive and specific method capable of efficiently unraveling molecular profiles associated with disease status or clinical outcome. Notably, this technology also can be used for an assessment of protein quality in FFPE tissue [14]. Finally, a new proteomic profiling method has the potential to provide better insight into diseases and contribute to the development of clinically applicable biomarkers.

2 Materials

2.1 Protein Extraction from Formalin-Fixed Paraffin-Embedded Tissue

1. Archival formalin-fixed paraffin-embedded (FFPE) prostate tissue specimens.
2. Microtome (Leica, Buffalo Grove, IL, USA).
3. Xylene or Dewaxing reagent (AutoDewaxer, Open Biosystem, Pittsburgh, PA, USA or PROTOCOL™ SafeClear, Fisher Scientific, Pittsburgh, PA, USA) (*see* **Note 1**).

4. Thermomixer.
5. 100, 95, and 70 % ethanol (EtOH).
6. Feather disposable scalpel.
7. SafeSeal microcentrifuge tube.
8. Microcentrifuge tube sealing clips.
9. Disposable Pellet Mixer and Cordless Motor.
10. High pH antigen retrieval buffer (10×, pH 9.9; Dako, Carpentaria, CA, USA). Store at 4 °C.
11. Stock solution of NaN_3 (20×): Prepare 20 % NaN_3 solution in distilled water (DW). Store at room temperature (RT).
12. Stock solution of SDS (20×): 20 % SDS. Store at RT.
13. Stock solution of glycerol (5×): Prepare 50 % glycerol solution in DW. Store at RT.
14. Stock solution of protease inhibitor solution (50×): Prepare 50× protease inhibitor solution (1 tablet/1 mL DW). Store at 4 °C.
15. Protein extraction buffer: 1× high pH antigen retrieval buffer, 1 % NaN3, 1 % SDS, 10 % glycerol, and 1× protease inhibitor solution. Store at RT (*see* **Note 2**).
16. Pascal pressure cooker.
17. Heating block for 1.5 mL microcentrifuge tube.
18. Refrigerated microcentrifuge.
19. Extra-long gel tip.
20. Reynolds Wrap® Aluminum Foil.

2.2 Assessment of Protein Concentration

1. BCA protein assay kit.
2. 96-Well flat bottom plate.
3. Sunrise™ microplate reader.

2.3 Well-Based Reverse-Phase Protein Array

1. Anti-prostate-specific antigen (PSA) and anti-smooth muscle actin antibodies, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies. Goat anti-mouse or rabbit SULFO-TAG™ antibodies (Meso Scale Discovery, Gaithersburg, MD, USA) (*see* **Note 3**).
2. MesoScale Discovery (MSD) Multi-Spot™ plates (MA2400 96 HB Plate, Meso Scale Discovery) (*see* **Note 4**).
3. 37 °C Incubator.
4. Thermowell™ Sealing Tape.
5. Sealing Film Roller Press (LabSource, Romeoville, IL, USA).
6. Multichannel pipets.
7. Stock solution of phosphate-buffered saline (PBS): 10× PBS.
8. Blocking buffer: 1× PBS, 0.1 % Tween-20 with 5 % w/v nonfat dry milk.

9. Wash buffer PBST: 1× PBS, 0.1 % Tween-20.
10. Microplate shaker.
11. Microplate Strip Washer.

2.4 Signal Detection

1. SECTOR Imager 2400 (Meso Scale Discovery).
2. Molecular grade DW (Quality Biological Inc.).
3. MSD read buffer-T (4×) (Meso Scale Discovery).

3 Methods

The basis of this approach is the determination of the protein concentration of lysates, creation of antigen and antibody titration curve, and direct application of the diluted lysates to a carbon surface of the Meso Scale Discovery plate. Lysates are allowed to dry, and proteins are detected by application of antibody choice (Fig. 1). Initial recovery of protein from the FFPE tissue section involves modified heat-induced antigen retrieval procedures using a high-pressure cooker. This methodology has the merits of a speedy procedure (up to 1 h), high-quality protein extraction from FFPE tissue, and permitting the use of phospho-specific antibodies (Fig. 2). In addition, this method results in excellent protein extraction yield from relatively small amounts of materials, with relatively low concentration of detergent and regardless of the deparaffinization step (*see* **Note 5**). Furthermore, this method was applicable to samples obtained with laser capture microdissection (*see* **Note 6**), as demonstrated with three different antibodies [12]. Subsequently, the proteins extracted from FFPE tissues applied to well-based RPPA which is primarily based on electrochemiluminescence detection system [12]. Once an assay is developed, it is only necessary to apply sufficient lysates for detection within the linear range of the assay. The arrays are stable for over 2 months at room temperature (Fig. 3).

3.1 Protein Extraction from Formalin-Fixed Paraffin-Embedded Tissue

1. Cut two 10 μm sections from FFPE tissue blocks using a microtome.
2. Deparaffinize the FFPE tissue section in xylene (3 × 5 min) in glass or plastic coplin jars. Transfer to 100 % EtOH (2 × 5 min), 95 % EtOH (2 × 5 min), 70 % EtOH (1 × 5 min), and then to 1× PBS (2 × 5 min) (*see* **Note 5**).
3. Carefully collect tissue in the SafeSeal microcentrifuge tube using disposable safety scalpel (*see* **Note 7**).
4. Add 1 mL of 1× PBS to the sample and centrifuge the tube for 1 min at 4 °C with maximum speed in a refrigerated microcentrifuge. Remove carefully the PBS solution without disturbing the tissue pellet.

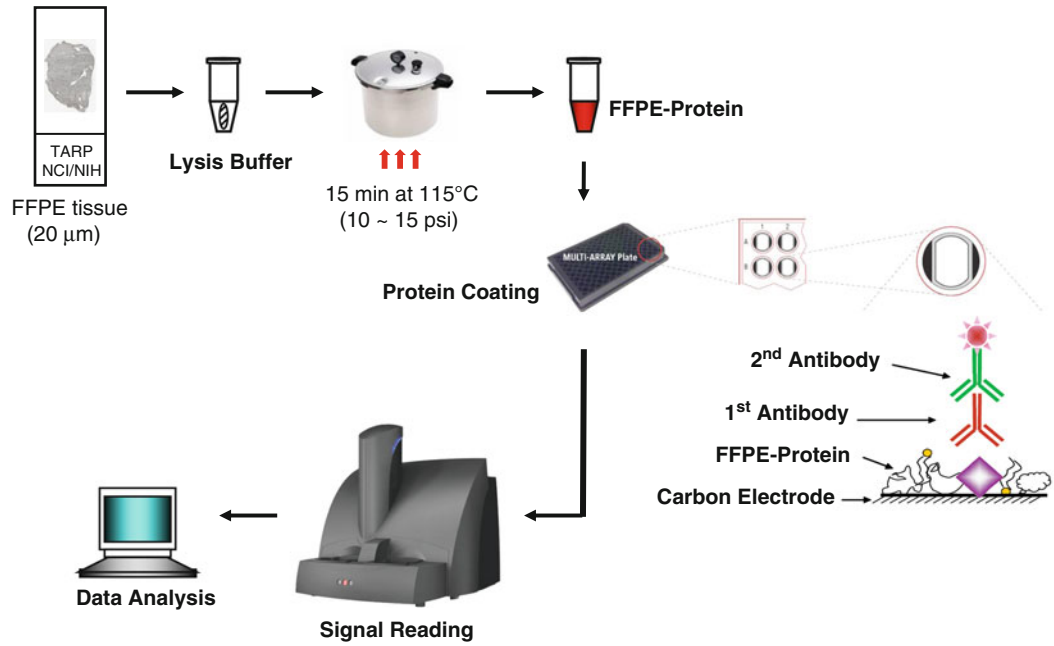

Fig. 1 Schematic diagram of novel protein array from FFPE tissue. Two ten-micrometer-thick formalin-fixed paraffin-embedded (FFPE) tissue sections were trimmed of excess wax and homogenized using a Disposable Pellet Mixer in 200 μL protein extraction solution, followed by incubation for 15 min at 115 °C within a pressure cooker. After incubation, the tissue lysates were centrifuged at 15,000 × *g* for 30 min at 4 °C. The supernatants were collected and used for the protein array. Five microliters of protein extract from FFPE tissue specimen at predetermined protein concentrations was added to Multi-Spot™ plates (MA2400 96 HB Plate, MSD, Gaithersburg, MD, USA), the plate was allowed to dry at room temperature for 90 min, and the plates were subsequently further incubated at 37 °C for 30 min. The antigen-coated plates were preincubated with 5 % BSA in PBST before incubation with specific antibodies at 4 °C for overnight. After washing with PBST, the plates were incubated for 1 h with goat anti-mouse or rabbit SULFO-TAG™ antibodies at a dilution of 1:1,000 (0.5 μg/mL). The plates were then aspirated and washed three times with PBST. Finally, MSD-T read buffer was added to the plates and they were read on the Sector Imager 2400 (MSD, Gaithersburg, MD, USA)

5. Dry the sample for 10 min at 55 °C using Thermomixer (*see* **Note 8**).
6. Add 200 μL of protein extraction buffer to the deparaffinized tissue. Homogenize immediately using the disposable pellet mixers and cordless motor until the sample is uniformly homogeneous.
7. Fix the sealing clips to the microcentrifuge tube and place the sample in a heating block. Subsequently, wrap the heating block with aluminum foil.
8. Place the heat block into a pressure cooker and incubate for 15 min at 115 °C (*see* **Note 9**).
9. Turn off pressure cooker and wait for the pressure to reach 0 psi (*see* **Note 10**).

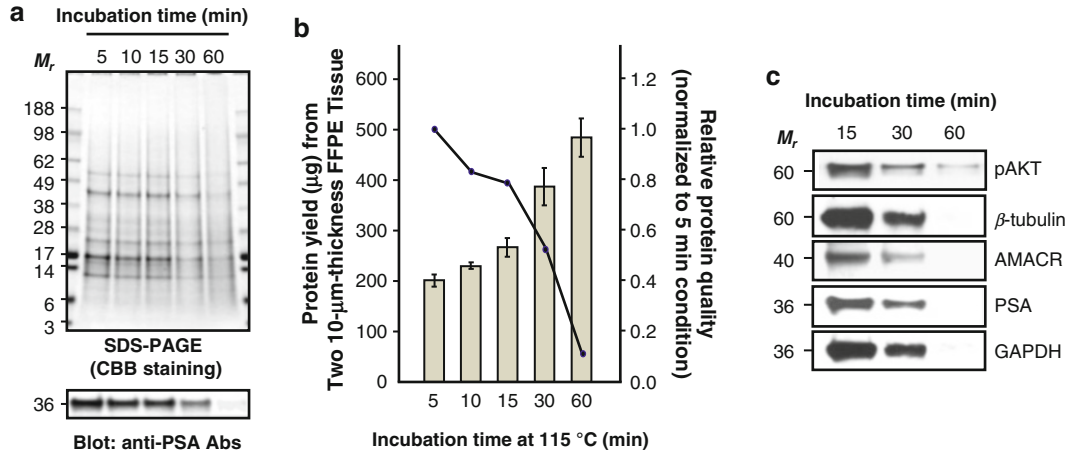

Fig. 2 FFPE-protein quality according to incubation time within a pressure cooker. (**a**) Non-deparaffinized archival human prostate FFPE tissue section was trimmed of excess wax and homogenized using a Disposable Pellet Mixer in 200 μL protein extraction solution, with incubation for 5, 10, 15, 30, and 60 min at 115 °C within a pressure cooker. The FFPE-proteins were separated by 4–12 % reducing SDS-PAGE (CBB staining), electroblotted to nitrocellulose membrane, and probed with anti-PSA antibodies (1:200). (**b**) The amount of protein extracted from each condition was measured using BCA Protein Assay Kit (Pierce). The bar graph shows the relative averages of protein yield; average ± SD. Relative protein quality of each entity is normalized to 5 min incubation condition (1.00). (**c**) Protein integrity of FFPE-derived protein by western blotting. FFPE-proteins were extracted from FFPE tissue specimen with 15, 30, and 60 min incubation within a pressure cooker. 20 μg of FFPE-proteins were subjected to a 4–12 % gradient polyacrylamide gel under reducing condition. After transfer to nitrocellulose membrane, the membrane was probed with anti-pAKT, anti-β-tubulin, anti-AMACR, anti-PSA, and anti-GAPDH antibodies. The signal was detected with a SuperSignal Chemiluminescence kit (Pierce) (*Mr*: protein molecular marker) (reproduced from [12] with permission from Willey)

10. Carefully take out the heating block from pressure cooker and place the tube on ice for 10 min (*see* **Note 11**).
11. Centrifuge the tube for 30 min at 4 °C with maximum speed in a refrigerated microcentrifuge. Carefully transfer supernatant solution to a fresh tube using gel loading tip.

3.2 Assessment of Protein Concentration

1. Centrifuge the tube for 10 min at 4 °C with maximum speed in a refrigerated microcentrifuge (*see* **Note 12**).
2. Take 3 μL of the sample and dilute tenfold with DW.
3. Take 25 μL of the diluted sample and add the sample to flat bottom 96-well plate. Measure protein concentration using BCA protein assay kit.
4. Read absorbance of the sample using a microplate reader and calculate protein concentration based on standard curve.
5. Check the quantity and quality of the protein sample (*see* **Note 13**).
6. Take 10 μg of the protein sample and apply to SDS-PAGE. An example of the results produced is shown in Fig. 2.

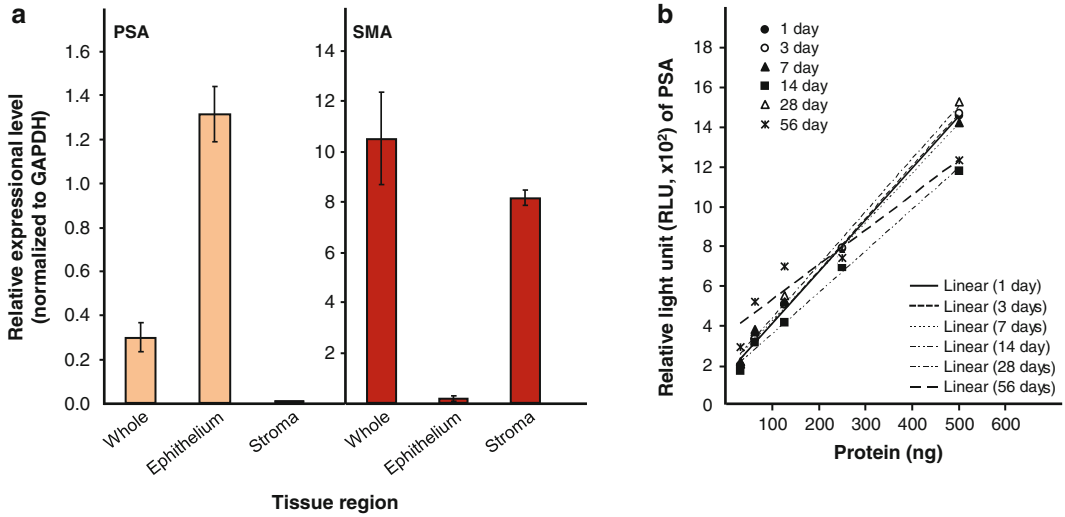

Fig. 3 Sensitivity and stability of the well-based RPPA. (**a**) Five-hundred shots (15-μm diameter laser beam) were microdissected from each of epithelial and stromal region within a 10-μm-thick prostate hematoxylin stained FFPE tissue. Protein extracted from an adjacent whole section of the same prostate FFPE tissue block was also included in this assay. The well-based RPPA using 500 ng extracted protein per well was performed. Primary antibodies were diluted 1:500 (prostate-specific antigen; PSA) or 1:1,000 (smooth muscle actin; SMA & GAPDH) with 3 % BSA. After normalization with GAPDH level, relative expressional signals were represented as ratio. The bar graph shows the average ± SD of three replicated wells. (**b**) Stability of a novel reverse-phase protein array platform. In order to examine the stability of well-based RPPA platform using FFPE-proteins, we stored the vacuum-sealed 96-well plate at RT after protein coating and measured PSA signal over 2 months (1, 3, 7, 14, 28, and 56 days, $R^2 \geq 0.93$). Dynamic ranges in plot are based on the standard curve; results given are the mean of three replicated wells (reproduced from [12] with permission from Willey)

3.3 Well-Based Reverse-Phase Protein Array

1. Pipet 5 μL (1,000–10 ng/well) (*see* **Note 14**) of tissue lysate preparation in wash buffer (PBST) to each well in the Multi-Spot™ plates (MA2400 96 HB Plate, MSD) making sure to touch the bottom of each well with the tip of the pipet and then releasing the solution slowly.
2. After applying the tissue lysate separation to the plate, allow the plate to dry at room temperature for 90 min (*see* **Note 15**).
3. Place the plate in an incubator at 37 °C for 30 min to make sure all fluid has completely evaporated.
4. Remove the plate from the incubator and apply 150 μL of blocking buffer to each well. Then cover the plate with sealing tape using Sealing Film Roller Press.
5. Allow the plate to incubate with the blocking solution for 1 h. Lightly tap the plate to make sure you remove all air bubbles and then place on a plate shaker for 1 h.
6. Discard the blocking buffer and wash the plate 5× with wash buffer using a microplate strip washer.

7. After washing the plate, apply 25 μL of the primary antibodies to each well (*see* **Note 16**). Then cover the plate with sealing tape using Sealing Film Roller Press.
8. Incubate the plate with primary antibody overnight at 4 °C with mild shaking.
9. Discard the primary antibody solution and wash the plate 5× with wash buffer using a microplate strip washer.
10. Apply 25 μL of the sulfo-tagged secondary antibodies to each well and then cover the plate with sealing tape using Sealing Film Roller Press.
11. Incubate the plate for 90 min at RT with mild shaking (*see* **Note 17**).
12. Discard the primary antibody solution and wash the plate 5× with wash buffer using a microplate strip washer.
13. Apply 150 μL of MSD read buffer-T (diluted 1:4 in molecular grade DW) to each well and lightly tap the plate to make sure you remove all air bubbles.
14. Incubate the plate for 2 min and then insert the plate into the SECTOR Imager 2400 instrument for reading (*see* **Note 18**).

4 Notes

1. We found aqueous based *dewaxers* such as AutoDewaxer (Openbiosystems) or PROTOCOL™ SafeClear (Fisher Scientific) could be used as a deparaffinizing agent in place of xylene at high temperature with equal results and greater safety (*see* ref. 15). The temperature of xylene should be controlled under 65 °C.
2. Protein extraction buffer cannot be stored at 4 °C due to precipitation. For this reason, we recommend preparing solution fresh before use.
3. Primary antibody concentration depends on target antibody. Before the assay develops, we recommend a titration test of antigen and antibody, like enzyme-linked immunosorbent assay. The primary antibodies which are suggested for western blotting as well as immunohistochemistry are compatible in this platform.
4. Wells of the plate coated with carbon particles and incorporated in the bottom of each well (for more detailed information *see* www.mesoscale.com).
5. When handling over penny-sized FFPE tissue, the protein extraction yield is consistent regardless of deparaffinization (*see* ref. 12).

6. Protein extraction from laser capture microdissection sample was successful, with average total protein yield 3.38 ± 1.2 μg per 500 shots. We performed the new reverse-phase protein array using 500 ng of the protein extracts against PSA, smooth muscle actin, and GAPDH (Fig. 3a). Expression levels of PSA and smooth muscle actin were in accordance with expected compartments of the tissue (*see* ref. 12).
7. Do *not* dry the deparaffinized tissue slide. Tissue should be collected under wet condition to prevent tissue loss.
8. The pellet should become white.
9. We find that the protein extraction buffer containing 1 % SDS combined with a higher temperature (at 115 °C) and moderate pressure (10–15 psi) resulted in a greater protein yield without change of quality of protein as measured by SDS-PAGE (*see* ref. 12). The protein extraction yield is increased with longer antigen retrieval time; however the protein quality is rapidly dropped after 30 min at 115 °C within a pressure cooker (Fig. 2a, b). In addition, the PSA signal from FFPE-protein extract from prostate tissue after 15 min incubation was approximately 80 % of that probed from 5 min incubation (Fig. 2a, b). To optimize conditions for both protein quality and yield, we utilized a 15 min antigen retrieval time for the protein extraction protocol.
10. When the pressure of pressure cooker reached a low pressure (<5 psi), the user should gently tilt the weight (petcock) located on the lid to one side. This will release residual pressure and enable a safe environment in which to open Pascal press cooker post run.
11. Remove aluminum foil cover from heating block and carefully take out the tube using a pincet. Do *not* touch or handle a heating block with naked hand because heating block is very hot.
12. Precipitation can vary depending on storage times at 4 °C. Recentrifuge the tube and transfer the clear supernatant solution to the fresh tube.
13. Although the extracted protein showed a smearing pattern on SDS-PAGE, the lysate contained relatively broad range of molecular weight proteins ranging from 10 to 180 kDa. The average total protein yield was 29.44 ± 7.8 μg per 1 mm^3 of archival human FFPE tissue. We confirmed that the protein recovery yield from FFPE tissue was approximately 90 % of that recovered from fresh tissue (*see* ref. 12).
14. Optimal amount of coated protein should be determined by research after antigen and antibody titration. The signal in the well-based RPPA correlates with that of western blots ($R^2 = 0.931$) and had greater sensitivity. We have confirmed the

sensitivity that the assay can detect multiple markers using 10 or fewer cells when applied to fresh cell lines (*see* ref. 12).

15. One can tell the plate is dry once the wells no longer appear shiny and all fluid has evaporated. The drying times can be extended depending on tissue lysate dilutions.
16. The typical concentration ranges from 1 to 3 μg/mL. Primary antibody concentration depends on target antibodies. Optimal concentration should be determined by the user for each application.
17. The typical concentration ranges from 0.2 to 2 μg/mL for initial assay development. As a starting point, we recommend diluting secondary antibody to 1 μg/mL. If background signals are higher than desired, reduce the concentration of secondary antibody to 0.5 μg/mL. Optimal concentration should be determined by the researcher for each application.
18. Turn on the SECTOR imager before secondary antibody incubation step. Once the instrument is turned on and software is started, it takes approximately 45–60 min for the CCD chip to stabilize. The SECTOR imager should be operated in a dust-free environment with an ambient temperature between 20 and 26 °C, and humidity levels between 10 and 80 %. Environment or locations with high levels of vibration should be avoided.

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