Quantitative screening of serum protein biomarkers by reverse phase protein serum arrays

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Abstract

Protein biomarkers can be key indicators for cancer diagnosis, prediction, and identification of successful cancer therapies. Two of the common methods for protein biomarker detection are antibody arrays, which profile multiple protein expression levels from a patient's serum, or reversed phase protein arrays which measure single or limited sets of proteins from many patients' sera. The reversed phase protein arrays have been widely used for detection of signaling molecules in cell lysates, however, this approach has been difficult to adapt to serum samples. Several years ago, we developed a sensitive method called the enhanced protein array to quantitatively measure serum protein levels from large numbers of patient samples. Here, we further refine the technology on several fronts: 1. simplifying the experimental procedure; 2. optimizing multiple parameters to make the assay more robust; 3. establishing a method for more accurate quantification. Using this technology, we quantitatively measured the protein expression levels of Nidogen-1 and CEACAM-1 from 164 serum samples from hepatocellular carcinoma patients (HCC) and an equal number of controls and found that both Nidogen-1 and CEACAM-1 expression levels are significantly increased in HCC patients. Our work reveals promise for using reverse phase protein serum arrays for biomarker discovery and validation.

Testing of RPPA detection system with different antibody labeling

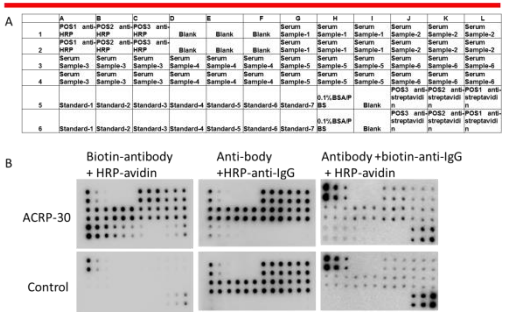


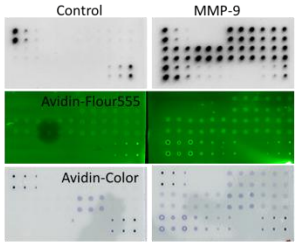
Fig.1 Testing of RPPA detection system with different antibody labeling. Serum samples to be detected and serially diluted standard antigen are printed directly on NC membrane, and determined by corresponding antibodies with different labeling combinations and ECL imagination. A. The map of RPPA array. B ACRP30 in serum samples and serially diluted pure standard with concentration from 2000ng/ml to 31.25ng/ml have been detected by biotin labeled anti-acrp30 antibody and HRP labeled avidin (Left upper), anti-acrp30 antibody and HRP labeled Anti-IgG antibody (Middle upper), anti-acrp30 antibody plus biotin labeled Anti-IgG and HRP labeled avidin (Right upper). The controls use same array and same combination respectively without anti-acrp30 antibody (Lower panel).

Table 1. Sensitivity of different detection methods and concentration of detected targets in serum samples

	Biotin-antibody+ HRP-avidin			Antibody+HRP-anti-IgG			Antibody + biotin-anti-IgG + HRP-avidin		
	ACRP30	MMP-9	VEGF	ACRP30	MMP-9	VEGF	ACRP30	MMP-9	VEGF
Detected minimum concentration (ug/ml)	0.03125	0.03125	0.0625	0.0625	0.125	0.25	0.25	0.25	0.25
FRU	688.0435	3533.214	256	5721.026	1571.579	3302.172	824.1898	266.2696	1914
SD	310.1039	151.2993	48.08352	1937.839	114.9565	628.8396	429.2707	179.6719	83.4386
Detected serum concentration (ng/ml, n=6)	3965.07 ±898.70	77.27 ±29.25	0.49680 ±0.2104	-	-	-	-	-	-

- undetectable because of high background

Testing of RPPA detection system with different imaging agents



	Avidin-HRP	Avidin-Flour555	Avidin-Color
Target	MMP-9	MMP-9	MMP-9
Detected minimum concentration (ng/ml)	31.25	62.5	125
FRU	3461	5184.283	1429.4848
SD	74.24621	1045.8583	103.4423
Detected serum concentration (ng/ml, n=6)	59.390 ±17.393	2.89 ±12.925	0.995353 ±0.5907

Figure 2. Serum samples to be detected and serially diluted MMP-9 standard antigen are printed directly on NC membrane, and determined by biotin labeled anti-MMP-9 antibody with HRP-avidin for ECL development and imaging (upper), Flour555-avidin for laser scanning at 532 nm wavelength (Middle), and HRP-avidin for DAB imaging (lower). The same array and same combination without anti-MMP-9 antibody are used as controls respectively (Left panel).

Testing of RPPA detection system with different blocking buffers

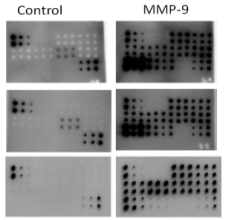
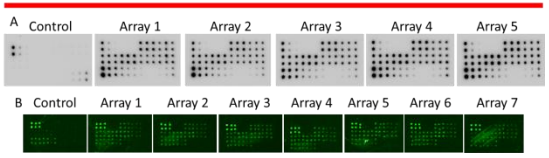


Figure 3. Serum samples to be detected and serially diluted MMP-9 standard antigen are printed directly on NC membrane, and determined by biotin labeled anti-MMP-9 antibody with HRP-avidin for ECL imagination. 1%BSA / 1XPBS (upper), 5%BSA / 1XPBS (Middle), 10%BSA /25%Casein/ 1XPBS (lower) are used as blocking buffer and the same array and same detection system without anti-MMP-9 antibody are used as controls (Left panel) .

The Intra assay and Inter assay CV on membrane and glasses



	Arrays on NC membranes				Arrays on glass slides	
Target	MMP-9				MMP-9	
Type	Intra-assay precision			Inter-assay precision	Intra-assay precision	Inter-assay precision
Repeat times (n)	2	4	6	6	6	7
Mean (ng/ml 6samples)	49.51-83.05	48.82-84.71	48.53-81.75	41.54-83.28	122.68-895.87	118.18-844.12
Standard Derivation (6 samples)	2.24-5.51	1.998-5.99	1.665-6.30	0.9929-5.26	27.13-327.53	56.06-248.60
CV(%,6 samples)	2.69-5.52	3.65-5.07	3.03-7.15	2.39-6.34	21.37-40.19	16.33-131.47

Detection of proteins in serum samples

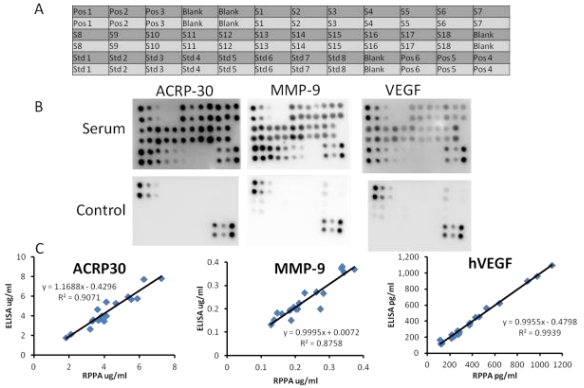


Fig.5 Detection of proteins ACRP30, MMP-9, VEGF in 18 serum samples by established RPPA system and investigation of correlation of RPPA and ELISA. A. The map of RPPA array for serum samples detection. B. Results of ACRP30, MMP-9, and VEGF detection by RPPA method in 18 serum samples. C. Correlation of RPPA and ELISA assay in ACRP30, MMP-9, and VEGF detection in 18 serum samples.

Testing Nidogen-1 and CEACAM-1 in human serum by RPPA assay

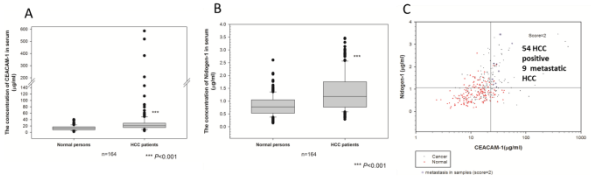


Figure 6 Potential biomarker levels of Nidogen-1 and CEACAM-1 measured in HCC and control patient serum by RPPA assay. 164 pairs of serum samples from HCC patients and normal subjects were measures and concentration of CEACAM (A), Nidogen-1 (B) or combination of Nidogen-1 and CEACAM-1 (C) are indicated.

Conclusions

1. **Comparing data from the inter and intra CV between NC membrane and glass slide supports, RPPA assay using NC membrane as solid support shows the best stability and consistency with CV range from 3 - 7%.**
2. **To detect proteins in serum samples, the RPPA system has been optimized by investigating combination of antibodies, signal reporting and detection methods and with different blocking buffers.**
3. **With our established RPPA system, proteins in serum samples have been detected and were found to correlate well with ELISA results.**
4. **Detection of Nidogen-1 and CEACAM-1 in human serum by our RPPA assay has identified the potential of RPPA assays as a new diagnostic method to detect proteins in serum samples.**