Development of high sensitive Exosome ELISA by using PS affinity

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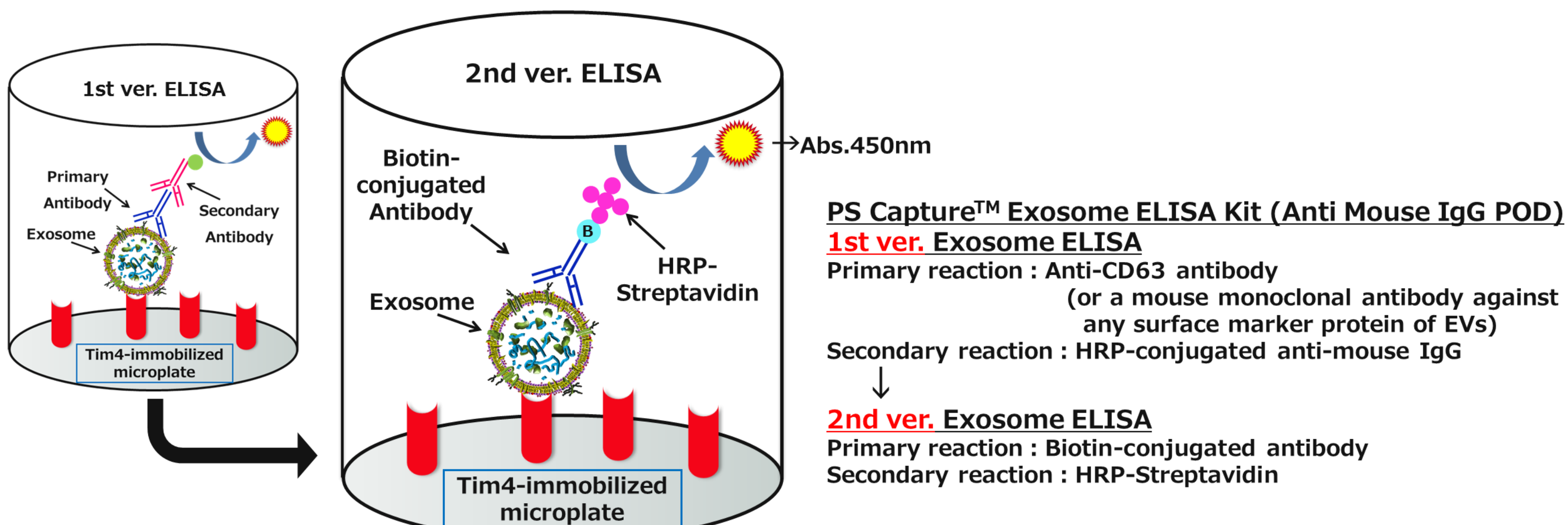
Abstract

Extracellular vesicles (EVs) such as exosome have attracted attention as a messenger of cell-to-cell communication and a biomarker of diseases since they include proteins, mRNAs, microRNAs, and DNAs on their surface or inside and they are stably present in body fluids such as blood, urine, saliva, spinal fluid, and breast milk after being secreted from cells. EVs display phosphatidylserine (PS) on their surface, allowing recognition by PS receptors such as Tim4. Based on the high affinity of Tim4 protein for PS in a Ca^{2+}-dependent manner, we developed a novel method to purify small EVs with Tim4 (PS affinity method). By applying PS affinity method, we developed PS Capture™ Exosome ELISA Kit (Anti Mouse IgG POD) (1st ver. ELISA), which showed higher sensitivity than western blot and conventional ELISA using EV marker antibody-immobilized microplate. However, 1st ver. ELISA cannot detect EVs in serum and plasma directly because of non-specific reaction of secondary antibody. Therefore, we improved non-specific reaction by using biotin-streptavidin detection system and optimizing diluent (2nd ver. ELISA). We confirmed that 2nd ver. ELISA showed good dilution linearity of serum and plasma. In addition, spike and recovery test with serum and plasma showed good recovery rate when EpCAM on the surface of EVs purified from cell culture supernatant of COLO201 human colon cancer cells was detected. These results suggest that this ELISA system will be a powerful tool in investigating new biomarkers on EVs in diagnostic and therapy.

1.Measurement principle

EVs are captured by a plate on which Tim4 protein that specifically binds with PS on the surface of EVs are immobilized. Then, surface marker protein of EVs are detected by using biotin-conjugated antibody against any surface marker protein of EVs as a primary antibody and Poly-HRP Streptavidin as substitute for a secondary antibody.



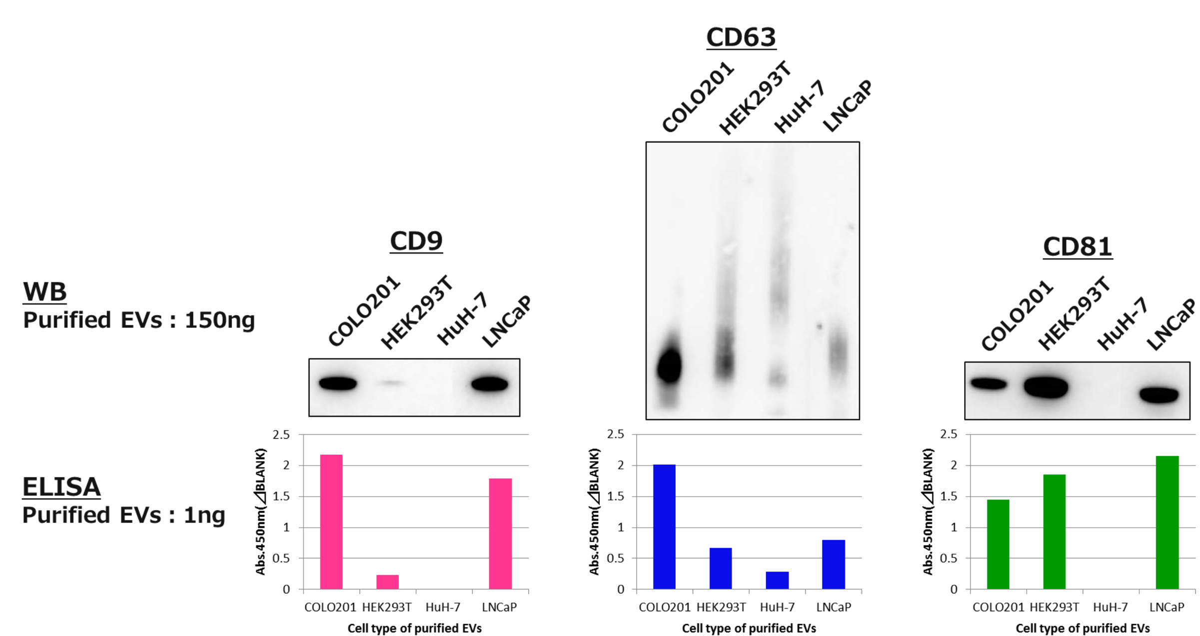
2nd ver. ELISA came to be able to detect EVs in serum and plasma samples directly.

※All samples of serum and plasma used in this report were collected from healthy human and centrifuged at 10,000×g.

2.Correlation of EV marker detection with WB and ELISA

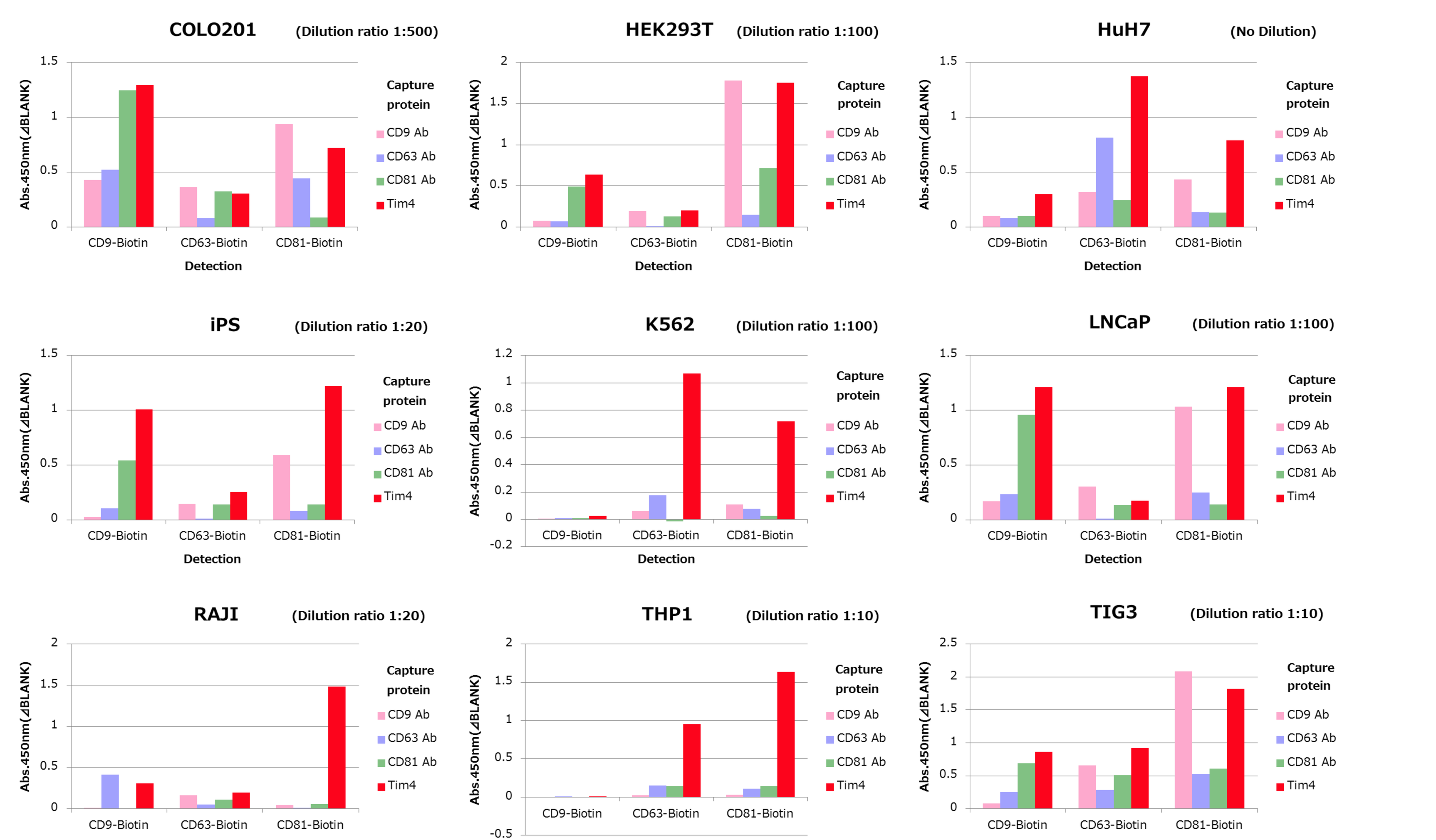
EVs purified from various cell culture supernatant were detected by western blot and 1st ver. ELISA using anti-CD9, anti-CD63 and anti-CD81 antibodies.

These results showed that the amount of each marker protein correlated between WB and ELISA.



3.Comparison of 2nd ver. ELISA with conventional ELISA

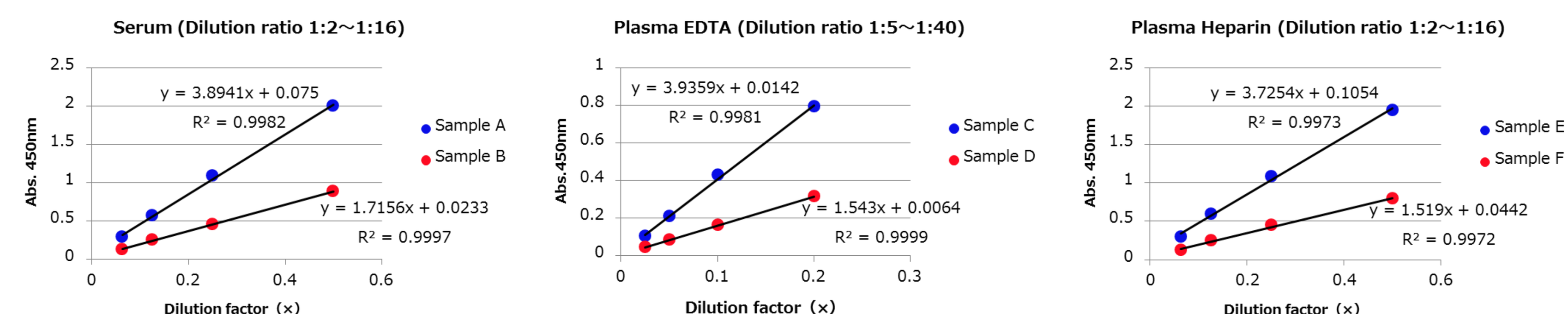
EVs in various cell culture supernatant were diluted and incubated in each well of microplate immobilized anti-CD9, anti-CD63, anti-CD81 antibodies or Tim4. Bound EVs are detected with biotin-conjugated antibodies against EV surface marker CD9, CD63 or CD81.



These results indicated that Tim4-immobilized ELISA could detect EV surface marker protein more efficiently and widely than antibodies-immobilized ELISA in various cell lines.

4.Dilution linearity of serum and plasma

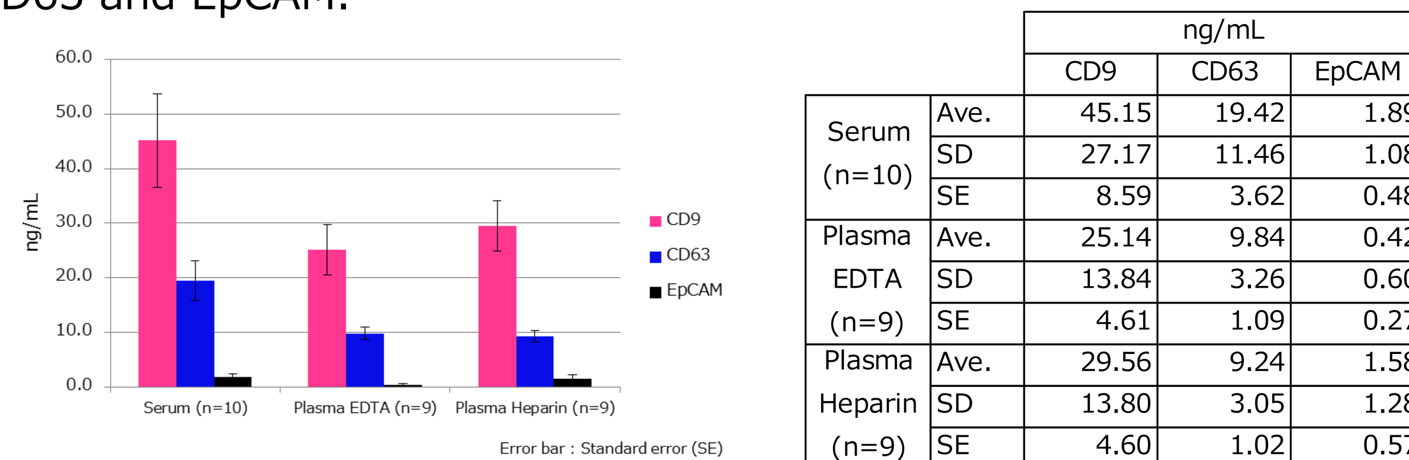
The dilution linearity of 4-step dilution samples of serum (1:2 to 1:16), plasma EDTA (1:5 to 1:40) and plasma heparin (1:2 to 1:16) was evaluated. (Detected by biotin-conjugated anti-CD63 antibody)



This analysis confirmed that 2nd ver. ELISA showed good dilution linearity in the assay using serum and plasma samples.

5.Detection of EV markers in normal serum and plasma

Serum, plasma EDTA and plasma heparin samples were added to each well and surface markers were detected by using biotin-conjugated antibody which recognized EV marker CD9, CD63 and EpCAM.

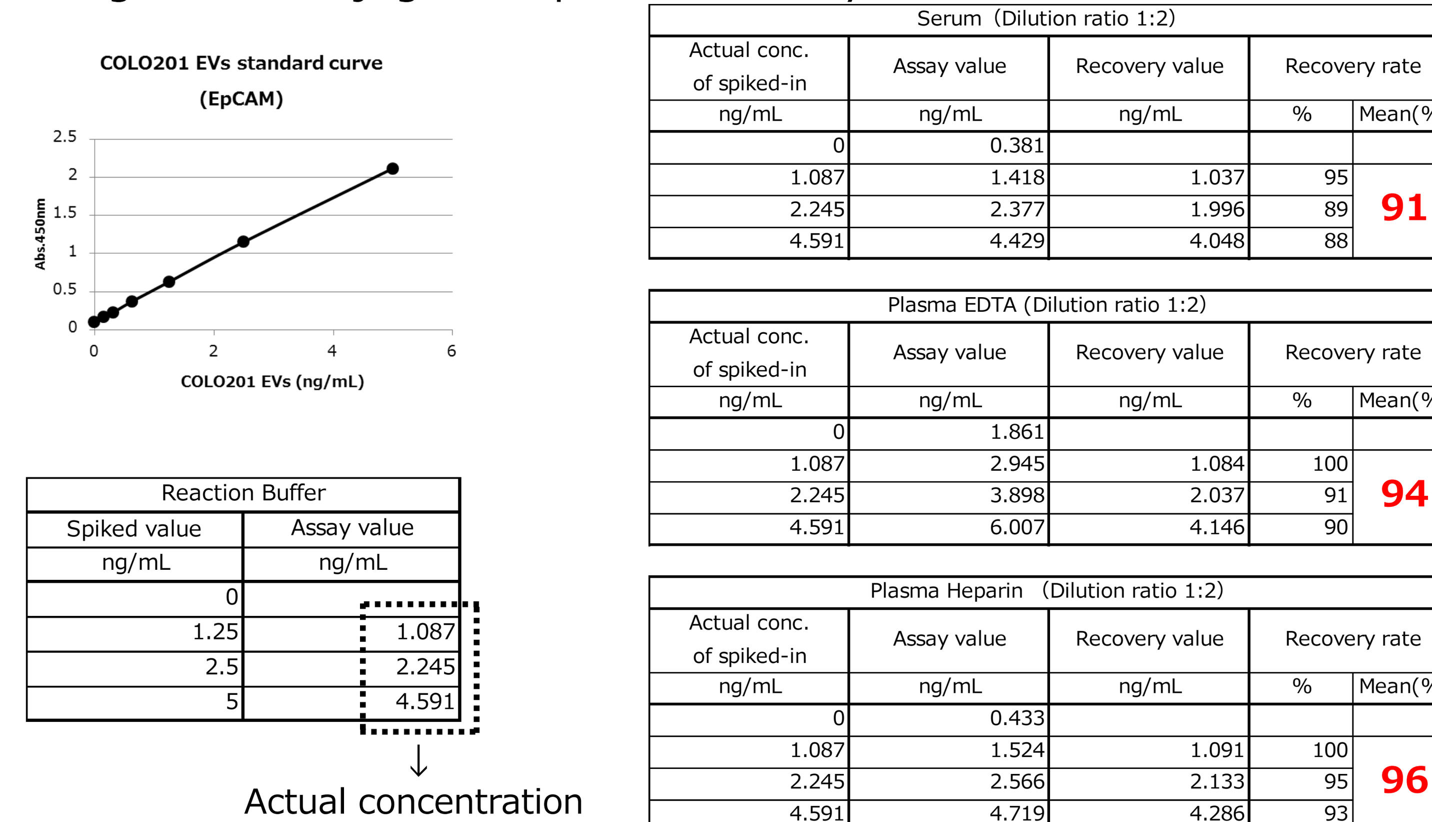


		ng/mL		
		CD9	CD63	EpCAM
Serum (n=10)	Ave.	45.15	19.42	1.89
	SD	27.17	11.46	1.08
	SE	8.59	3.62	0.48
Plasma EDTA (n=9)	Ave.	25.14	9.84	0.42
	SD	13.84	3.26	0.60
	SE	4.61	1.09	0.27
Plasma Heparin (n=9)	Ave.	29.56	9.24	1.58
	SD	13.80	3.05	1.28
	SE	4.60	1.02	0.57

The data showed that EVs in serum and plasma expressed CD9 and CD63, but EpCAM level was very low.

6.Spike and recovery test of cancer EVs

Three concentrations of EVs purified from cell culture supernatant of colorectal cancer cell line, COLO201 cells were added to 1:2 diluted samples of serum, plasma EDTA and plasma heparin. Then, concentrations of samples were measured to calculate the recovery rate by using biotin-conjugated EpCAM antibody.



Reaction Buffer	
Spiked value	Assay value
ng/mL	ng/mL
0	
1.25	1.087
2.5	2.245
5	4.591

↓ Actual concentration

Serum (Dilution ratio 1:2)				
Actual conc. of spiked-in	Assay value	Recovery value	Recovery rate	
ng/mL	ng/mL	ng/mL	%	Mean(%)
0	0.381			
1.087	1.418	1.037	95	91
2.245	2.377	1.996	89	
4.591	4.429	4.048	88	

Plasma EDTA (Dilution ratio 1:2)				
Actual conc. of spiked-in	Assay value	Recovery value	Recovery rate	
ng/mL	ng/mL	ng/mL	%	Mean(%)
0	1.861			
1.087	2.945	1.084	100	94
2.245	3.898	2.037	91	
4.591	6.007	4.146	90	

Plasma Heparin (Dilution ratio 1:2)				
Actual conc. of spiked-in	Assay value	Recovery value	Recovery rate	
ng/mL	ng/mL	ng/mL	%	Mean(%)
0	0.433			
1.087	1.524	1.091	100	96
2.245	2.566	2.133	95	
4.591	4.719	4.286	93	

These results showed good recovery rate of EpCAM on the surface of EVs, and therefore, it suggested that specific EV surface markers could be detected by using this ELISA system quantitatively.

Conclusions

- Tim4-immobilized ELISA system can extensively detect EV surface markers in various cell lines.
- This ELISA system can detect EV surface protein in serum and plasma quantitatively.
- Our ELISA system will be a powerful tool in EV studies including research of biomarkers.