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Ultrasensitive Immunoassays for Combined Detection of CEA+ Extracellular Vesicles and Soluble CEA

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1 Abstract

Colorectal cancer (CRC) is the second leading cause of cancer death in the United States. Carcinoembryonic antigen (CEA or CEACAM-5) is a biomarker commonly used in the management of CRC. It is present at low levels in healthy adults and is elevated in CRC. CEA is a critical part of post-treatment surveillance, and improved CEA assays may enable earlier detection of recurrence. While CEA is known to be secreted from CRC cells in a soluble form, we have observed an alternative form of CRC-derived CEA in plasma samples, in association with extracellular vesicles (EVs). To facilitate measuring EVs that present CEA (CEA*EVs) and/or other CRC-related surface proteins, we developed a high-throughput EV purification method using a multimodal chromatography resin that consistently removes most soluble proteins, which can interfere with downstream EV assays. We employed highly-sensitive, multiplex electrochemiluminescence (ECL) immunoassays for measuring intact EVs presenting CEA or CD73 in purified human plasma and found that plasma levels of CEA*EVs and CD73*EVs were elevated in some late-stage CRC samples in three cohorts. We also assayed CEA/CD73 double-positive EVs (CEA*CD73*EVs) using a three-marker ultrasensitive assay format and found these were elevated in the same CRC samples as CEA*EVs albeit with lower background in control samples, leading to improved specificity. Additionally, approximately 20% of stage IV CRC samples had elevated CEA*EVs and CEA*CD73*EVs while exhibiting low soluble CEA. Receiver operating characteristic analysis (ROC) showed that the combination of CEA*CD73* EVs and soluble CEA assays provided the best discrimination between controls and stage IV CRC samples. These results suggest that assaying both soluble and EV-associated forms of CEA in plasma may improve the value of CEA measurement in CRC. It also shows that assaying EVs that simultaneously present two tumor-related surface markers can increase specificity for the detection of tumor-derived EVs relative to those presenting a single marker.

2 Methods

I. High-throughput (HTP) plasma EV purification using Captocore 400 core-shell resin (Cytiva)

Size Exclusion Principles

- **Pores in the resin shell** exclude extracellular vesicles and other macromolecular structures with molecular weight (MW) > 400kD
- **Ligand-activated core** (octylamine) retains molecules below the MW cutoff that enter the pores
- **High throughput**: Operator can process 8 plates per day (768 samples). This would take several weeks using gravity SEC columns.

Filter Plate Purification Procedure

- 100 µL plasma per sample is diluted and added to the washed resin in deep well plates
- Resin / Samples incubated with mixing for 1h
- Centrifugal processing in 96-well fritted microplates to retain resin with bound proteins and elute purified EVs

II. MSD's ECL-based assays measure the concentration of soluble proteins and intact EVs in complex biological samples

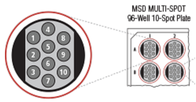
Electrochemiluminescence (ECL) Technology

The MESO SCALE DISCOVERY® (MSD) ECL detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® microplates.

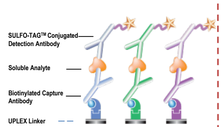
- **High sensitivity:** Multiple excitation cycles can amplify signals to enhance light levels.
- **Broad dynamic range:** High- and low-abundance analytes can be measured without multiple sample dilutions.
- **Low background:** The stimulation method (electricity) is decoupled from the output signal (light) resulting in extremely low background.

U-PLEX® Multiplexed Immunoassays

Biotinylated capture antibodies are each coupled to one of ten unique U-PLEX Linkers, which self-assemble onto spots on the U-PLEX plate. Multiplexing conserves valuable samples by allowing up to ten determinations per well.

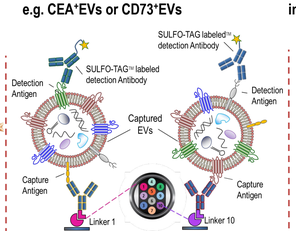


Assay Format "A" for soluble analytes: e.g. CEA, CD73, Albumin



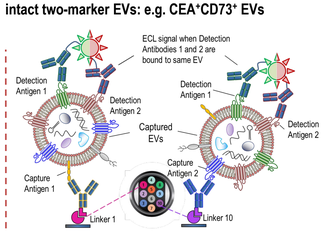
Soluble analytes are captured and detected using a sandwich immunoassay format with SULFO-TAG ECL labels.

Assay Format "B" for intact EVs: e.g. CEA*EVs or CD73*EVs



Intact EVs are captured by antibodies targeting distinct surface antigens, e.g. CD73 or CEA. Bound EVs are detected using SULFO-TAG labeled detection antibodies that recognize common antigen(s) on EVs; usually this is a cocktail of antibodies targeting common EV proteins, e.g. CD81, CD9 and CD63.

Assay format "C," ultrasensitive assays for intact two-marker EVs: e.g. CEA*CD73* EVs



Bound EVs are detected using a pair of detection antibodies targeting two distinct antigens. Ultrasensitive assay format only generates signal when both detection antibodies bind to the same EV. At least one of the two detection antibodies typically targets CD81, CD9, or CD63. MSD's ultrasensitive technology allows even rare EV populations to be detectable by this assay format.

3 Human plasma samples tested

All plasma samples used in this study were collected in EDTA tubes and were immediately centrifuged twice after collection to remove platelets.

1. EV purification study: 16 convenience plasma samples were used to assess the performance of Captocore-mediated EV purification.

2. CRC Sample Testing, Discovery Cohort 1: 23 plasma samples purchased from iSpecimen were assayed for soluble CEA, CEA*EVs and CD73*EVs. This set included 8 healthy and 15 Stage IV CRC samples.

3. CRC Sample Testing, Discovery Cohort 2: 37 Plasma samples from the Vanderbilt University Medical Center (VUMC) Cooperative Human Tissue Network (CHTN) were assayed for EVs and soluble markers. This set included 14 healthy and 23 Stage IV CRC samples.

4: CRC Sample Testing, Validation Cohort: 105 plasma samples from the VUMC CHTN were assayed for EVs and soluble markers. This set included 23 healthy, 17 Stage I CRC, 18 Stage II CRC, 23 Stage III CRC, and 24 Stage IV CRC samples.

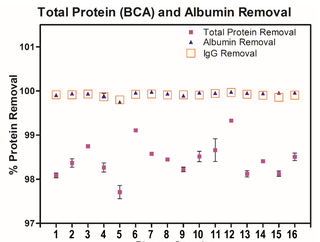
4 HTP purification of high- and low-abundance EV populations

I. Over 99.9% removal of soluble proteins under the MW cutoff (400kD)

EV purification was performed on 16 samples in triplicate. Soluble protein removal is reported as the ratio of protein concentration measured in plasma before and after EV purification. Variability was assessed by comparing protein removal between technical replicates and across samples. Albumin and IgG removal is above 99.9%. IgM is larger than 400kD and thus was not efficiently removed. Total protein removal is above 98%.

Protein	Size (kD)	Assay	16 plasma (triplicates)		Captocore Variability (triplicate): %CV Removal		
			Mean % Removal	StDev %Removal	Mean	Min	Max
Total Protein	NA	Pierce BCA assay [1]	98.47	0.39	0.10%	0.01%	0.3%
Albumin	66	R-PLEX® Human Albumin [2]	99.92	0.05	0.01%	0.001%	0.06%
IgG	150	MSD® Panel 1 Human/NHP [3]	99.92	0.03	0.01%	0.001%	0.1%
IgM	1000		56.75	12	5.9%	0.9%	20.8%

Assay catalog numbers: [1]: Pierce A55864; [2]: MSD K1514VR; [3]: MSD K15203D

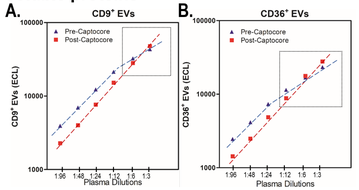


II. EV purification eliminates assay interference due to soluble proteins

When assaying specific EV populations using intact-EV assays like those shown in formats "B" or "C," the presence of abundant soluble forms of the capture antigen(s) can compete with EVs for the capture antibodies.

In such cases, the quantitation of EV concentration is underestimated at low dilutions, as illustrated by CD9+ EVs (**A**) and CD36+ EVs (**B**).

Removal of interfering proteins by Captocore allows samples to be accurately quantified with less dilution where signals are higher – an important consideration when assaying low abundance EVs.



III. Consistent recovery of high- and low-abundance populations of plasma EVs

EV recovery was determined using intact-EV assays to measure the EV concentration in the 16 plasma samples from Section I before and after purification. Purification of each sample was performed in triplicate. Pre- and post- purification samples were diluted sufficiently to ensure dilution linearity and thus accurate relative quantitation.

Recovery of five high-abundance EVs was measured using the intact-EV assay format "B." EV recovery is consistent across phenotypes but varies between samples. Each technical replicate was assayed separately.

Recovery of six low-abundance EVs was measured using the ultrasensitive assay format "C." Here, technical replicates were pooled. Recovery of low-abundance EVs is slightly lower and more variable between samples than high-abundance EVs.

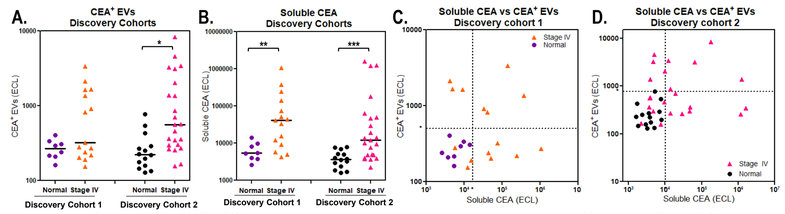
EV populations	% EV Recovery (ratio of ECL signal Post- / Pre- Captocore Purification)										
	High-abundance EVs					Low-abundance EVs					
Capture Antigen	CD36	HLADR	CD63	CD81	CD9	CD105	CD15	CD33	CD38	CD44	CD73
Plasma Samples 1	61.1	64.1	61.1	54.0	62.0	49.2	49.0	48.2	71.7	54.0	55.4
2	57.4	65.5	61.8	54.4	58.7	50.0	34.9	31.4	46.0	52.2	47.5
3	58.6	60.0	61.7	50.3	59.5	42.1	16.9	34.8	46.9	38.3	38.5
4	79.7	72.7	79.7	57.1	82.8	54.0	33.3	41.9	52.4	52.3	59.1
5	77.7	76.1	81.0	67.7	75.5	73.6	71.1	79.7	75.1	88.0	67.9
6	64.6	64.5	65.9	61.7	68.3	37.5	36.6	34.6	43.7	43.9	36.1
7	64.3	67.9	60.7	61.7	68.1	46.3	56.2	NA	56.1	57.6	36.5
8	75.3	72.1	69.6	56.2	76.9	50.7	50.9	48.7	57.2	58.9	49.0
9	67.5	61.9	63.2	54.0	66.0	51.3	34.2	34.4	63.4	57.1	46.2
10	57.3	57.6	54.8	43.0	57.1	48.3	34.4	37.1	37.2	50.4	31.4
11	52.5	50.1	40.7	34.6	47.5	38.1	28.7	32.2	40.8	36.8	41.3
12	39.7	35.5	33.0	28.5	39.9	24.4	26.3	25.0	25.3	24.0	27.1
13	50.0	57.3	59.2	45.5	53.1	40.7	44.7	40.0	47.2	50.9	38.1
14	63.5	58.9	69.7	51.2	72.4	46.2	41.0	56.4	50.5	57.0	43.6
15	39.6	40.9	44.4	39.0	41.5	30.1	38.0	26.3	34.0	35.9	25.6
16	43.3	47.3	50.7	42.2	48.5	33.5	33.4	23.7	48.7	44.9	33.5

%CV of triplicates of Captocore purification: Min = 0.9%, Max=22.2%, Median=5.1%; NA: Not Available

5 CEA+ EVs and soluble CEA are elevated in distinct sets of CRC samples

I. Observation from two discovery cohorts

CEA+ EV levels were assessed using the intact-EV assay format "B" in purified plasma samples. Soluble CEA was measured using the R-PLEX CEA assay on unpurified plasma. In the two discovery cohorts, CEA+EV (**A**) and soluble CEA (**B**) levels are markedly increased in stage IV CRC individuals compared to normal controls. In both cohorts, a subset of stage IV CRC individuals (3/15) (**C**), and (4/23) (**D**) showed elevated CEA+EVs but low soluble CEA.

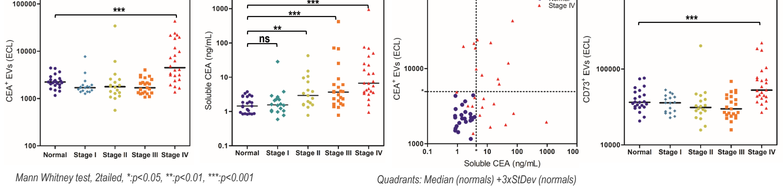


*Mann Whitney test, 2tailed, *:p<0.05, **:p<0.01, ***:p<0.001*

Quadrants: Median (normals) +3xStDev (normals)

II. Confirmation of the observations in a validation cohort, and detection of CD73+ EVs

EVs were assayed using ultrasensitive intact EV assay format "C" using common EV proteins for detection antigens 1 and 2. Elevated CEA+ EV (**A**) and soluble CEA (**B**) levels were confirmed in stage IV CRC samples. **C.** A subset of stage IV CRC (5/24) showed elevated CEA+ EVs but low soluble CEA. **D.** CD73+ EVs were elevated in stage IV CRC with a high correlation to CEA+ EVs (correlation not shown).

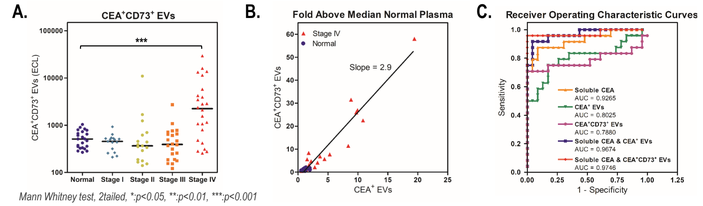


*Mann Whitney test, 2tailed, *:p<0.05, **:p<0.01, ***:p<0.001*

Quadrants: Median (normals) +3xStDev (normals)

6 Detection of intact CRC-derived EVs in addition to soluble CEA improves Stage IV CRC sample identification

A. CEA*CD73* EV levels were assessed using the ultrasensitive intact-EV assay format "C" with CEA capture and CD73 as one of the detection antigens. CEA*CD73* EVs were significantly elevated in stage IV CRC. **B.** CEA*CD73* EV assay has lower background in normals than CEA*EVs and thus shows improved separation of CRC samples from controls. **C.** ROC curves show that the combination of soluble CEA with CEA*EVs or CEA*CD73*EVs improves specificity and sensitivity for classification of stage IV CRC and controls.



*Mann Whitney test, 2tailed, *:p<0.05, **:p<0.01, ***:p<0.001*

7 Conclusion

Our high-throughput EV purification method was effective at removing soluble protein interferants, allowing for strong quantitative comparison of EVs using ECL-based intact-EV immunoassays. We applied these methods to three CRC sample sets and showed that CEA*EVs were detectable in a subset of late-stage samples, including some (~20%) that did not exhibit elevated plasma levels of soluble CEA. We also demonstrated that an ultrasensitive assay for CEA*CD73* EVs may represent an improved method for quantifying colorectal tumor-derived EVs by improving specificity relative to CEA*EVs. The combination of assays for intact tumor-derived EVs with existing assays for soluble CEA can improve discrimination between CRC and control samples and might ultimately improve the current limited accuracy of CEA detection in monitoring of late stage CRC tumors.

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