Extracellular Vesicle Research Tools

EVix

InCepix – innovative excellence

EVix ELISA tools allow sorting and phenotyping of EVs present in normal samples, revealing EpCAM (CD326) and Neprilysin (CD10) positive urinary EVs.

P18122

M. Wolf, J.W. Orf, P. Everson; InCepix, St. Paul, MN.

ABSTRACT

Determining the physiological role of extra-cellular vesicles (EVs) requires identification and characterization of proteins associated with each type of vesicle. Using EVix tools to capture antigen specific EVs (Capture) followed by detecting these structures in a subsequent antigen specific assay (Detect) we report on the variety of specific EVs isolated from human urine. EVix tools allow for simple and sensitive immune-phenotyping of human EV populations and reveal unique surface antigen population profiles. Normal human urine EVs present CD10 and other cell surface enzymes and specific markers (CD13, CD24, CD 133, CD147 and CD26) in unique combinations. EVix tools also reveal unique distribution patterns of human EV specific antigens when samples are characterized on size exclusion chromatography columns Capture with cholera B toxin also reveals unique detection antigen profiles. Examination of specifically captured EVs reveals the presence of unique populations of EVs within the sample. We show that EpCAM (CD326) is present on normal urine EVs and EVs containing CD326 can be specifically captured and characterized for the presence of other antigens. We show that capture of specific antigen is critical in uncovering certain rare or less prevalent subpopulations within the overall EV population. Capture strategies relying on tetraspan specific antigens (CD9, CD63, CD81) were not successful in revealing EpCAM (CD326) positive EVs. This is most likely because tetraspan positive but EpCAM negative EVs obscure the low level signal from the less prevalent EpCAM positive EVs. Our data also suggests that EpCAM (CD326) is present as a single or very low copy number on the EVs captured, unlike neprilysin (CD10) and other surface markers.

INTRODUCTION

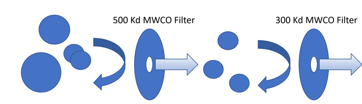
EVix ELISA kits capture extra cellular vesicles (EVs) via monoclonal antibodies immobilized onto microwell plates. Detection of EVs captured onto the microwell is done after washing steps to remove non bound EVs. The capture step selects antigen specific EVs. Anti-tetraspanin antibodies can be used to both capture and detect EVs. Non-tetraspanin cell surface molecules can be targeted to capture EVs. The captured EV population can then be probed for the presence of other cell surface molecules.

We report here the presence of EVs in normal human urine which contain Neprilysin (CD10) and EpCAM (CD326). Additional antigens detected on EVs in urine include Alanine Aminopeptidase (CD13), a putative cancer stem cell marker; Sialoglycoprotein (CD24), Prominin-1 (CD133), Dipeptidyl Peptidase-4 (CD26) as well as Class I and Class II MHC.

Profiles obtained from various capture and detect strategies suggest distinct populations of EVs are present in normal human urine . The detection of Neprilysin (CD10), and other surface enzymes on urinary EVs suggest a role of these enzymes in normal renal physiology, possibly inactivation of signaling peptides. The presence of EpCAM containing EVs in Urine is not surprising given the amount of epithelial cell surface area associated with both the kidney and bladder. The presence of EpCAM EVs may however complicate the use of EpCAM as a readout for possible urinary tract related tumors.

MATERIALS AND METHODS

Human Urine samples were concentrated 100X across 300 kD or 500 kD ultrafiltration membranes (Biomax Ultrafiltration Discs EMD Millipore Corporation, Billerica MA) in stirred cell concentrators under gravity flow. Samples were than dia-filtered three times, by adding 10X volume of tris-glycine buffer to the concentrated samples, and re-concentrating to starting volume. Accumulation of large non filterable species, most likely aggregates of uromodulin present during concentration were removed by centrifugation. No tetraspanin binding activity was seen in 300 MWCO flow through, the majority of tetraspanin containing EVs were retained by the 300 MWCO membrane. In contrast, while 500 MWCO retentate had tetraspanin binding, the 500 MWCO flow through also had tetraspanin activity, suggesting that EVs containing tetraspanins were able to pass through the 500 MWCO membrane.



The 500 MWCO flow through samples were subsequently concentrated 200X against 300 MWCO membranes and subjected to size exclusion chromatography.

Concentrated EV samples were run on a Sephacryl S500HR (Sigma Life Sciences) 40-20,000 kDA (dextrans) 2.5 cm diameter column (40 ml bed volume). 1ml of 200x (500 MWCO flow through) sample volumes were applied to the column and 1 ml fractions were collected. Assaying these column fraction volumes with the EVix ELISA kits revealed unique distribution of various EV populations as they eluted off of the column.

EVix ELISA kits (InCepix, St. Paul, MN) were used to capture and detect urine EVs. Capture and detect antibodies are indicated in the figure legends.

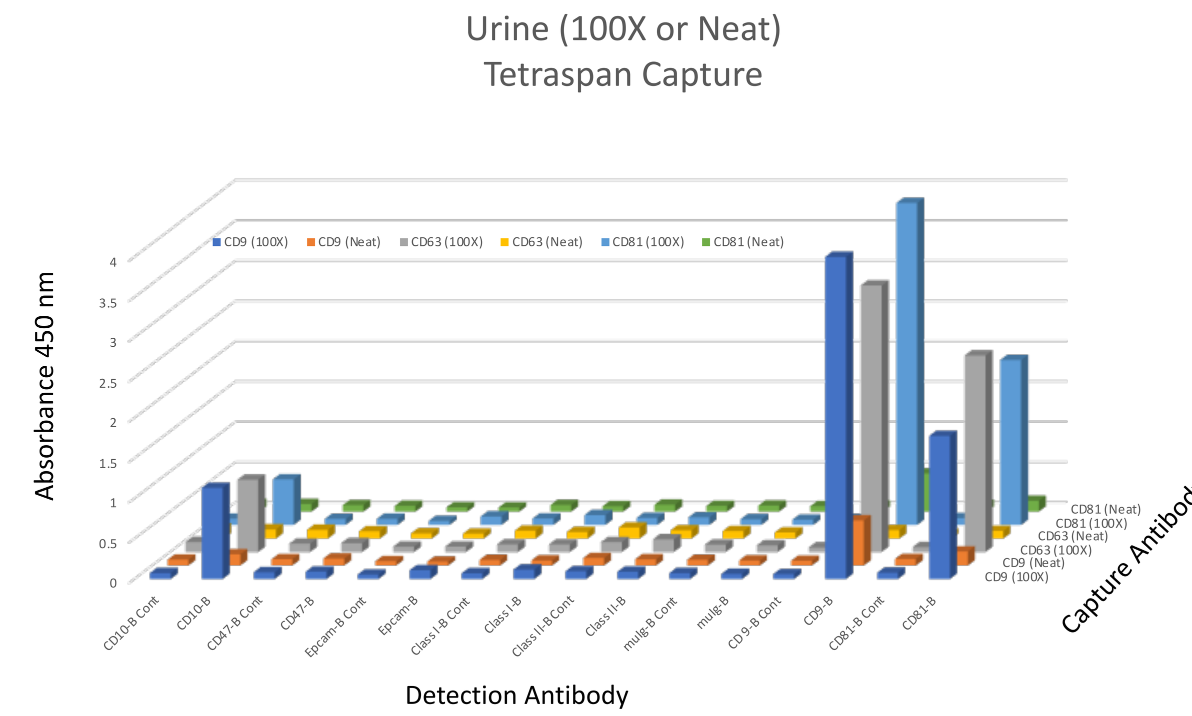


Figure One: Detection of specific antigens on tetraspanin captured EVs. EVs on tetraspanin capture plates yielded positive signals when probed with tetraspanin specific detection antibodies. CD9 signals were always greater than CD81 signals. Only CD10 signals were present above detection levels for the non tetraspanin detection antibodies. Neat urine gave little to no signal except for the tetraspanin capture/detect combinations of CD9 and CD81.

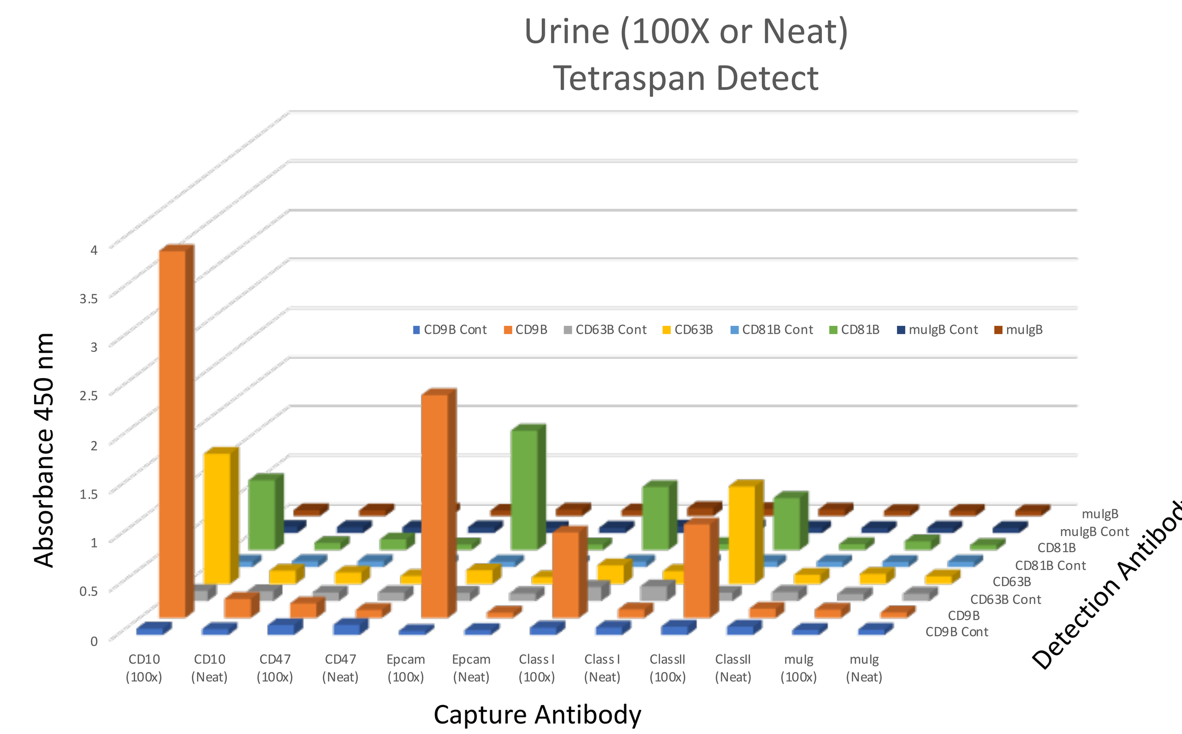


Figure Two: Detection of tetraspanin antigens on captured EVs. Non-tetraspanin capture plates yielded positive signals when probed with tetraspanin specific detection antibodies. CD10 (Neprilysin), CD326 (EpCAM), Class I MHC and Class II MHC captured EVs were detected with anti-tetraspanin detection antibodies. Neat urine gave much reduced signals compared to concentrated urine.

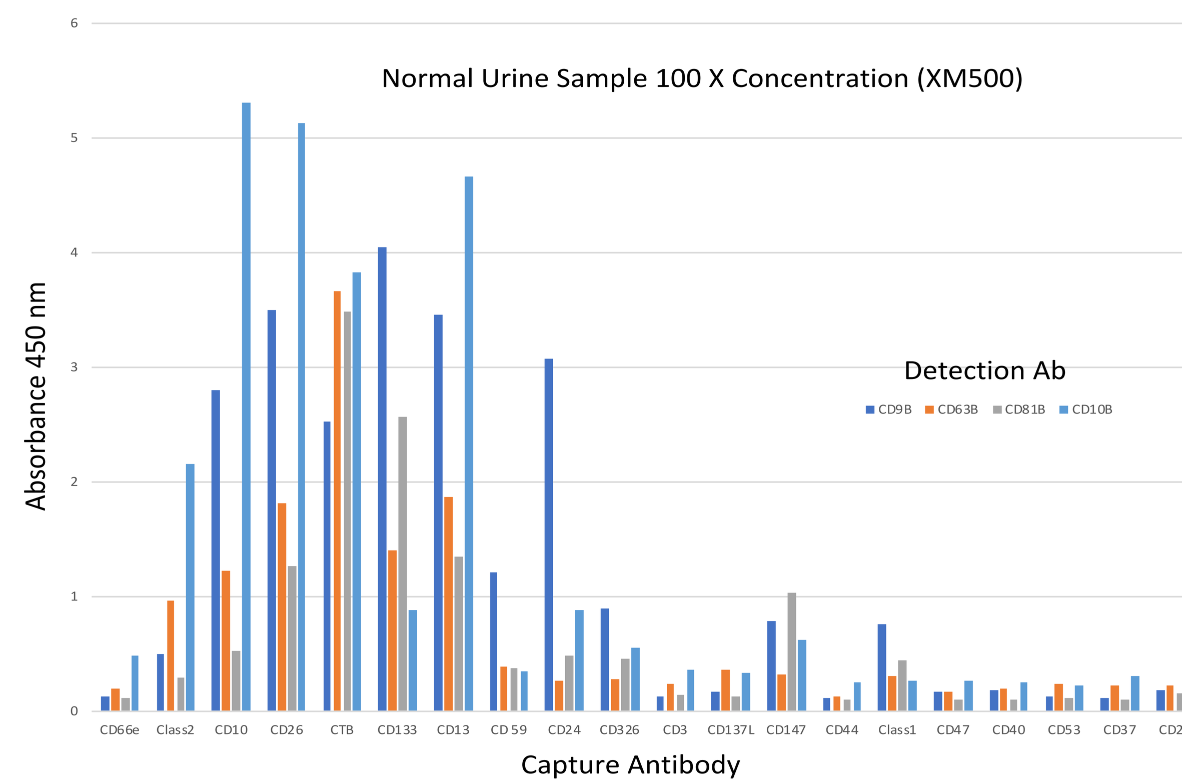


Figure Three: Detection of tetraspanin antigens and CD10 (Neprilysin) on captured EVs. Non-tetraspanin capture plates yielded positive signals when probed with tetraspanin and CD10 (Neprilysin) specific detection antibodies. CD10 detection yielded higher signals compared to CD9 for certain populations compared to others. CD24 captured EVs had higher CD9 signal than CD10 signal. In contrast Class II captured EVS had a much higher CD10 signal compared to CD9.

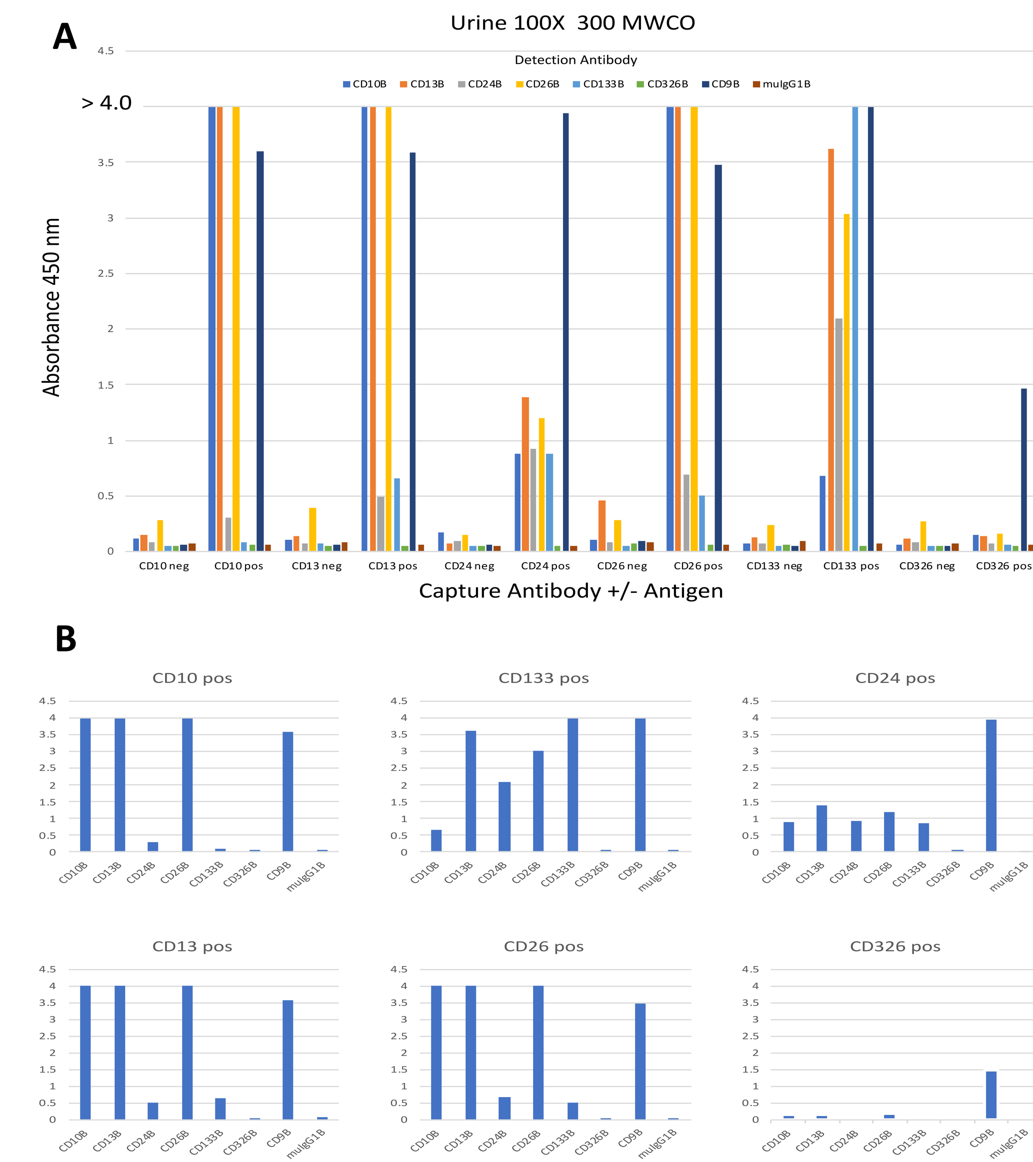


Figure Four: **A.** Detection of specific antigens on captured EVs. CD10, CD13, CD24, CD26, CD133, and CD326 capture plates yielded positive signals when probed with CD10, CD13, CD26, CD133, CD326, and CD9 detection antibodies. Non-specific binding shown as CD neg. **B.** Different population phenotypes were observed for specific capture antibodies. CD10, CD13 and CD 26 displayed similar population phenotype. In contrast CD24, and CD 133 captured EV populations were dissimilar. CD326 (EpCAM) captured EVs displayed little of the markers seen in the other populations. CD326 did not appreciatively detect CD326 captured EVs. Competition for epitope availability may explain this, suggesting that CD326 levels per EV are less than for other antigens. Less CD9 signal is suggestive that CD326 positive EVs may be fewer in number than the other populations.

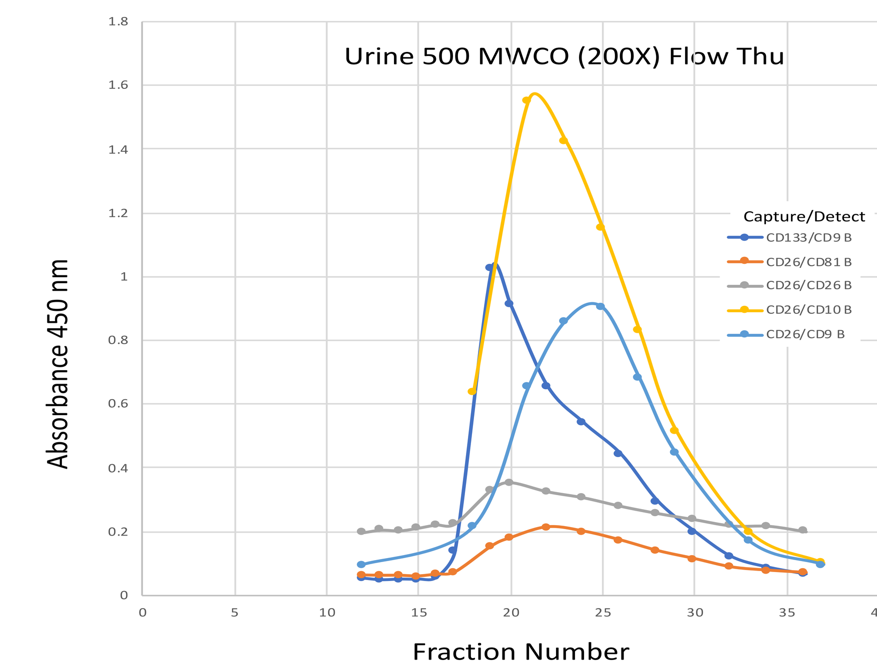


Figure Five: Size Exclusion Chromatography of the 500 kD MWCO membrane flow through concentrated 200X with a 300 kD MWCO membrane. Column bed was Sephacryl S500 HR (40,000 to 20,000,000 MW). A 1 ml volume was applied to column and 1 ml fractions collected. Capture and detect antibody pairs are shown in legend. CD10 (Neprilysin) was detected on CD26 captured EVs. CD133 captured EVs appear to be larger than CD26 captured EVs when both populations were detected with CD9. As these EVs passed through the 500 kD MWCO membrane it is assumed that these EVs are smaller species.

CONCLUSIONS

1. Human Urine contains different antigen positive populations of EVs.
2. Neprilysin and EpCam are present on EVs captured from urine. These molecules have different distribution patterns dependent upon antibody used to capture the EV populations.
3. CD13, CD24, CD26 and CD133 are also present on EVs captured from urine.
4. Size exclusion chromatography reveals different retention times for antigen specific EVs. This will be useful in further profiling urine EVs.
5. EVix Dual antigen capture/detect ELISA kits are useful in phenotyping EV populations in urine.