Use of high-capacity membranes for simple, rapid extracellular vesicle isolations with high yield and purity

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Abstract

Introduction

Despite their small size, extracellular vesicles (EV) play important roles in normal physiological processes (e.g., immune response, neuronal function, and stem cell maintenance) and diseases (e.g., cancer and liver disease). A key bottleneck in EV research is the isolation of the vesicles, which has historically been accomplished via ultracentrifugation. However, ultracentrifugation is time-consuming, is not scalable, requires specialized equipment, may damage vesicles during the high-speed spins, and suffers from low yield. More recently, precipitation solutions have been utilized to simplify EV isolation protocols, but these techniques are often inconsistent, with low yield and reduced purity. Thus, there is a significant need for a method to rapidly isolate EVs without compromising purity or yield.

Methods

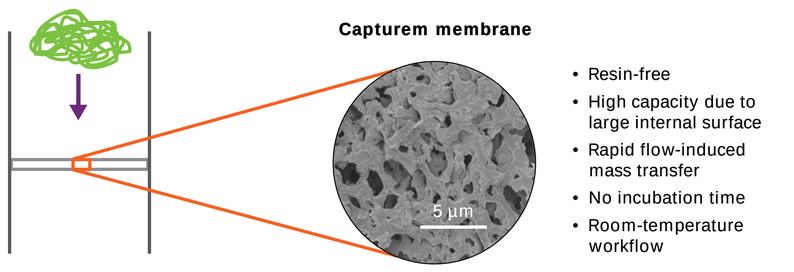
Here we describe the use of novel membranes conjugated to a proprietary, nonantibody-based EV-binding compound to selectively isolate EVs. The membranes, which we have named Capturem™ membranes, have been chemically modified to have increased surface area, which allows higher binding capacity while providing highly pure and concentrated samples. Additionally, the membranes have been assembled into benchtop centrifuge-compatible spin columns which can be used to isolate EVs in under 30 minutes.

Results and conclusions

Isolations performed with the Capturem EV isolation spin columns produced EVs of sizes comparable to experimental values reported in the literature; contained the key EV protein markers CD63, CD9, and Alix; and showed little or no expression of the EV-negative markers calnexin and albumin. Furthermore, we were able to collect ~3 ng total RNA from exosomes isolated from a single spin column (using an input of as little as 50 µl of plasma), enough total RNA to generate multiple cDNA libraries for downstream NGS analysis.

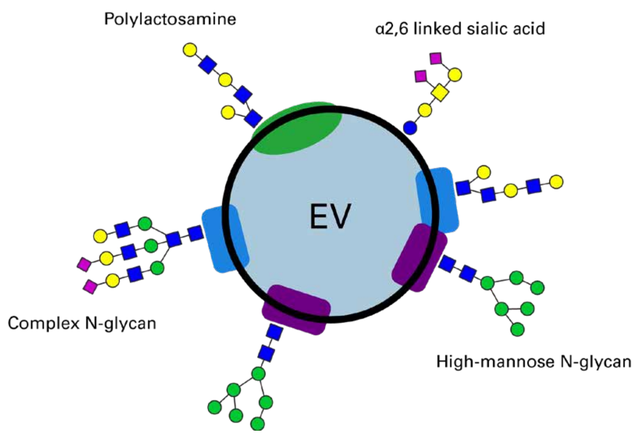
These Capturem columns enable researchers studying EVs to accelerate the pace of their research by obtaining high yields of noncontaminated exosomes in a simple and rapid manner.

Capturem membrane technology overview



- Resin-free
- High capacity due to large internal surface
- Rapid flow-induced mass transfer
- No incubation time
- Room-temperature workflow

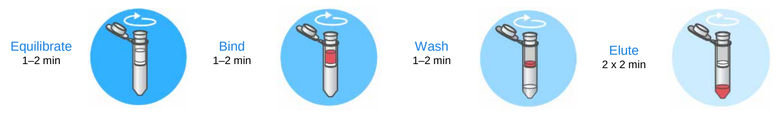
Glycosylation of extracellular vesicles



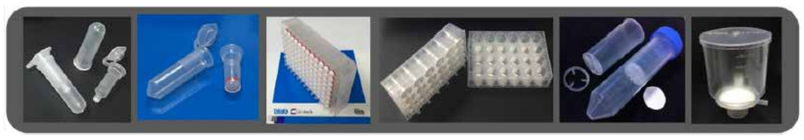
- Surfaces of EVs are enriched in carbohydrates and glycosylated proteins
- Lectin-based assays have proven ability to capture EVs for glycome analyses
- Capturem EV isolation utilizes an immobilized lectin-based compound

Image adapted from Figure 1, Williams, C., *et al.*, Glycosylation of extracellular vesicles: current knowledge, tools and clinical perspectives. *J Extracell Vesicles* **7,** 1442985 (2018).

Capturem EV isolation workflow



Capturem miniprep workflow for EV purification. With the Capturem Extracellular Vesicle Isolation Kit (Mini), EVs are first bound to the equilibrated membrane, then washed and eluted with the appropriate buffers. Each step is followed by spinning the tube for 1–2 min at 500*g*. This entire purification is complete in <30 min.



Different column formats. The Capturem product line includes a wide range of formats and sizes. Yields will vary based on loading concentration and sample details. Pictured from left to right: nanoprep, miniprep, 96-well, 24-well, maxiprep, and large-volume Capturem formats. Current capacity for Capturem EV isolation includes mini spin columns, with maxi spin columns expected to follow.

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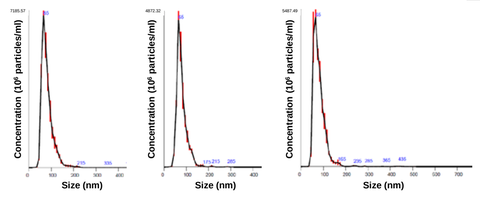
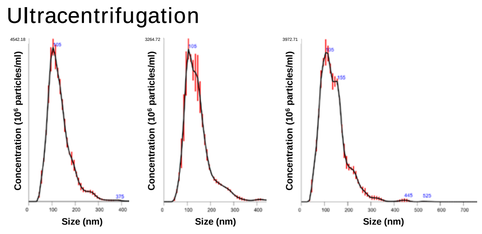
1 Consistent isolation of appropriately sized extracellular vesicles

A Capturem kit



Ultracentrifugation



B

Size	Capturem kit		
	Mean	Mode	D90
R1	82.9	70.7	114.0
R2	81.4	71.2	108.7
R3	78.2	67.8	108.4
Avg	**80.8**	**69.9**	**110.4**

Size	Ultracentrifugation		
	Mean	Mode	D90
R1	129.2	103.8	190.5
R2	134.4	107.2	205.9
R3	140.8	107.7	212.8
Avg	**134.8**	**106.2**	**203.1**

C Operator 1

Conc.	Capturem kit	Ultracentrifugation
R1	2.63	4.02
R2	2.53	4.27
R3	2.66	4.04
Avg	**2.61 x 10^{10} /ml**	**4.11 x 10^{10} /ml**
Total	**2.61 x 10^{9}**	**4.11 x 10^{9}**

Operator 2

Conc.	Capturem kit	Ultracentrifugation
R1	2.33	2.02
R2	2.83	2.03
R3	2.63	1.91
Avg	**2.60 x 10^{10} /ml**	**1.98 x 10^{10} /ml**
Total	**2.60 x 10^{9}**	**1.98 x 10^{9}**

Figure 1. Nanoparticle tracking analysis (NTA) of EVs isolated by the Capturem EV isolation kit or ultracentrifugation. Panel A. The calculated size distribution for each method is depicted as a mean (black line) with standard error (red shaded area). **Panel B.** Particle size is shown for each preparation. **Panel C.** Final concentration is shown for each preparation as performed by two different operators.

2 Higher EV purity with Capturem kit vs. ultracentrifugation

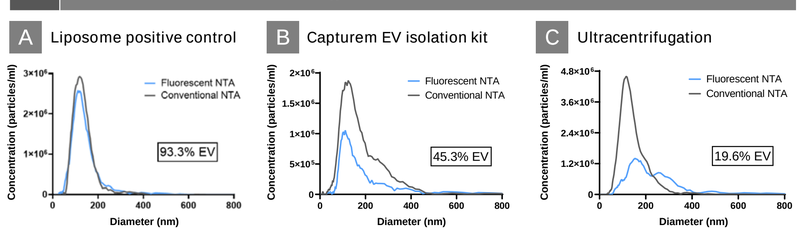


Figure 2. Fluorescent NTA demonstrates higher EV purity delivered by the Capturem kit compared with ultracentrifugation. A proprietary fluorescent dye was allowed to react specifically with the surface of intact vesicles, and quantification followed via fluorescent microscopy. **Panel A.** Liposome positive control. **Panel B.** EVs isolated using the Capturem EV isolation kit. **Panel C.** EVs isolated via ultracentrifugation. Analysis was performed by Alpha NanoTech.

3 Capturem EV isolation kit delivers high-purity exosomes

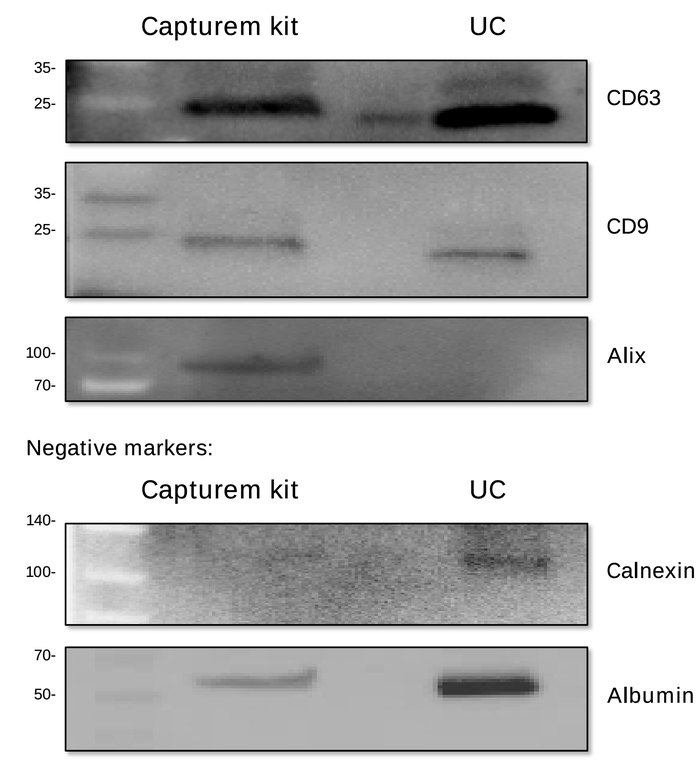


Figure 3. Western blot characterization of exosome preparations by Capturem kits and ultracentrifugation (UC). Western blotting shows that the Capturem kit contains higher levels of exosome-specific markers CD63, CD9, and Alix and lower levels of the non-exosomal protein calnexin and carryover protein albumin. In contrast, the sample prepared using UC contains considerably higher levels of albumin and calnexin. Each lane was loaded with 5 µg of total protein as measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

4 Morphological characterization of EV preparations by TEM and immunoelectron microscopy

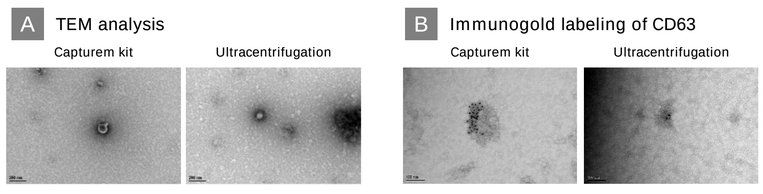
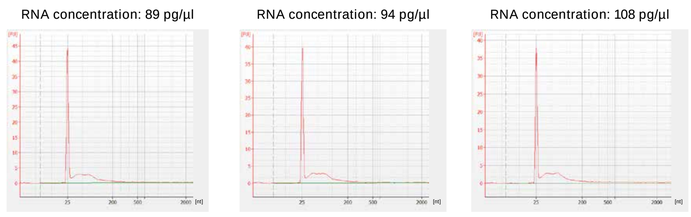


Figure 4. TEM analysis of EVs isolated by the Capturem EV isolation kit and ultracentrifugation. Panel A. Negative staining using uranyl acetate. Representative images of EVs isolated from the same donor. **Panel B.** Exosomes stained with 10 nM of gold-conjugated anti-CD63 antibody followed by uranyl acetate counterstaining. Scale bar: 200 nm (Panel A), 100 nm (Panel B). Analysis was performed by Alpha NanoTech.

5 High and consistent RNA content in EV preparations

A 50 µl plasma on one Capturem EV mini column



B 300 µl plasma, ultracentrifugation

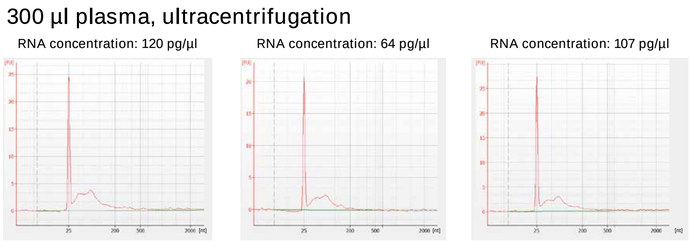
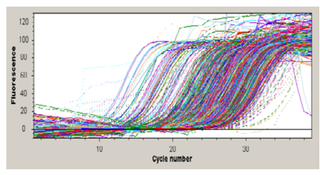


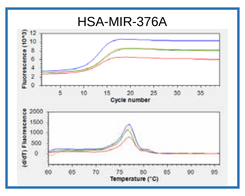
Figure 5. RNA analysis of EVs isolated by the Capturem EV isolation kit or ultracentrifugation. Representative bioanalyzer profiles of RNA isolated (via NucleoSpin miRNA Plasma, Takara Bio, Cat. # 740981.50) from the same donor and analyzed with the RNA 6000 Pico Kit (Agilent, Cat. # 5067-1513). The Capturem EV miniprep (**Panel A**) was performed with just 50 µl of plasma, while ultracentrifugation (**Panel B**) used 300 µl of plasma. The y-axes show fluorescence units (FU) and the x-axes show nucleotide lengths (nt) of the RNA. The peaks at 25 nt are internal standards.

6 Analysis of purified EV RNA on SmartChip™ miRNA panel

A Amplification curves from SmartChip qPCR



- 101 out of ~1,200 (quadruplicates) gave a Ct<28 with clean NTCs
- Verified off-chip



B Verify hits off-chip (QuantStudio)

Assay	NTC Ct	Sample Ct
GAPDH_var3_0301	Undetermined	22.0
hsa_miR376a-3p	Undetermined	28.0
hsa_miR_3679-3p	Undetermined	35.3
hsa_miR_302a-3p	Undetermined	34.3
hsa_miR_496	Undetermined	29.9
hsa_miR_548w	Undetermined	23.5
hsa_miR_744-3p	Undetermined	31.8
hsa_miR_660-3	Undetermined	31.3
hsa_miR_3167	39.7	27.9

Figure 6. SmartChip miRNA analysis of RNA purified from EVs. Panel A. RNA isolated from EVs using the Capturem EV isolation kit was preamplified with Takara PreAmp Master Mix. miRNA was analyzed using the SmartChip Real-Time PCR System. **Panel B.** 101 of 285 assays with a Ct<28 had clean no-template controls (NTCs) on the NTC chip. The top eight assays were tested off-chip along with *GAPDH*. miRs 548w, 496, 744-3p, 660-3, and 3167 had the highest copy numbers. Off-chip verification was done by QuantStudio (Thermo Fisher Scientific).

7 EV miRNA purified with the Capturem kit can be used in downstream NGS

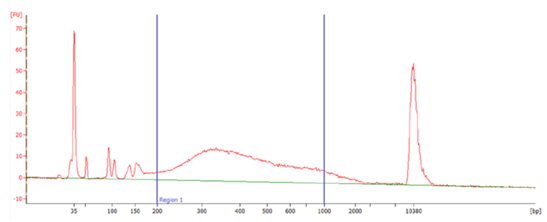


Figure 7. Profiling EV miRNA by NGS. EVs were isolated using the Capturem EV isolation kit. RNA was then extracted from these EVs and used as input for NGS analysis with SMARTer® Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (Pico v2; Takara Bio, Cat. # 634411). The bioanalyzer trace above shows the profile for the cDNA output from the Pico v2 kit, used as input for Illumina® next-generation sequencing.

8 Isolation of EVs from different biofluids

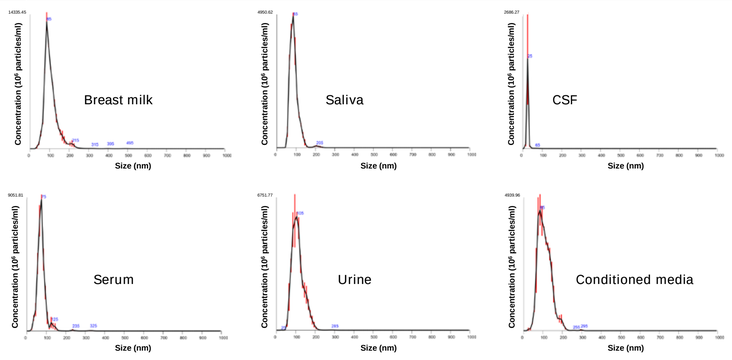


Figure 8. Nanoparticle tracking analysis (NTA) of EVs isolated from various biofluids by the Capturem EV kit.

Conclusions

The Capturem Extracellular Vesicle Isolation Kit (Mini) consists of spinnable affinity columns containing novel, modified nylon membranes conjugated to a lectin-based, EV-binding compound, thus providing a unique solution for the isolation of concentrated, highly pure EVs. The benefits of this system make it a powerful tool for a wide range of research settings.

- **Simple**—Capturem EV isolation can be done in a normal benchtop centrifuge at room temperature
- **Rapid**—isolation protocol is complete in <30 min, protecting the EVs from possible loss of activity
- **Reproducible**—innovative Capturem technology minimizes bias and captures EVs with high reproducibility
- **High purity**—both western blot and fluorescent NTA prove the Capturem EV isolation kit produces EVs with higher purity compared to ultracentrifugation
- **High yield**—Capturem EV isolation mini columns yield up to ~10^{10} exosomes/prep and maxi columns yield up to ~10^{11} exosomes/prep
- **Compatible**—eluted EVs are directly compatible with physical particle characterization such as NTA, protein analysis and RNA analysis
- **Flexible**—kit can be used for isolation of EVs from multiple biofluid and cell culture media

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