Rapid, pure, and concentrated purification of extracellular vesicles from biofluids

EVs isolated with Capturem spin columns:

- Exhibit higher consistency and purity than those isolated with ultracentrifugation
- Are enriched for exosome protein markers and do not express non-exosomal, contaminant proteins
- Contain sufficient RNA content for downstream RT-qPCR and NGS analysis
- Display classical morphology, as shown by transmission electron microscopy (TEM)

Introduction

Despite their small size, extracellular vesicles (EVs), such as exosomes, 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, can pull down non-exosomal proteins and nucleic acids, and suffers from low yield. More recently, precipitation solutions have been utilized to simplify EV isolation protocols, but precipitation-based techniques are often inconsistent, with low yield and reduced purity. Thus, there is a strong need for a method that rapidly isolates EVs without compromising purity or yield.

We have developed the Capturem Extracellular Vesicle Isolation Kit (Mini) to solve these challenges. This kit is comprised of novel membranes conjugated to a lectin-based EV-binding compound to selectively isolate EVs. These membranes have been chemically modified to have increased surface area, which allows higher binding capacity while still 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 (Figure 1). These columns can rapidly enrich EVs from biological fluids for downstream proteomic, genomic, and transcriptomic analyses.

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

Results

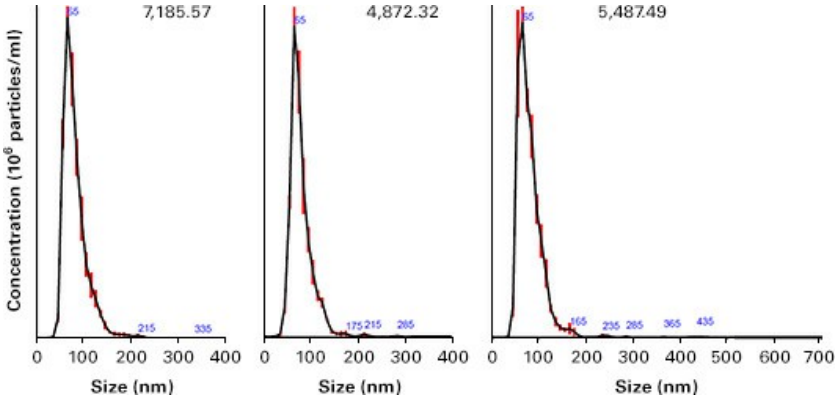
Capturem EV isolation yields pure EVs more consistently than ultracentrifugation, in under 30 minutes

Ultracentrifugation suffers from several limitations. First, additional debris and larger-sized particles can be pulled down during isolation. Second, fragile EVs can be damaged at high speeds, resulting in decreased yields. Third, removing the supernatant without disturbing the small EV pellet can be technically challenging and difficult to replicate (Jeppesen 2014). In contrast, the Capturem EV isolation kit provides rapid, pure, and consistent isolation of EVs.

To compare, EVs were isolated from 500 µl of plasma using either the Capturem Extracellular Vesicle Isolation Kit (Mini) or ultracentrifugation, in three replicates. Isolated EVs were subjected to nanoparticle tracking analysis (NTA) for visualization of size distribution (Figure 2). Capturem-isolated EVs exhibited a narrower, more-enriched yield of exosomes, with an average particle size of 81 nm and a D90 value of 110 nm. Conversely, ultracentrifugation-isolated EVs were larger, with an average particle size of 135 nm and a D90 value of 203 nm. Additionally, the particle sizes isolated from ultracentrifugation were more variable than those from Capturem isolation. This indicates that Capturem-isolated EVs were a much more distinct and pure population, and within size ranges commonly associated with EVs.

Capturem

A

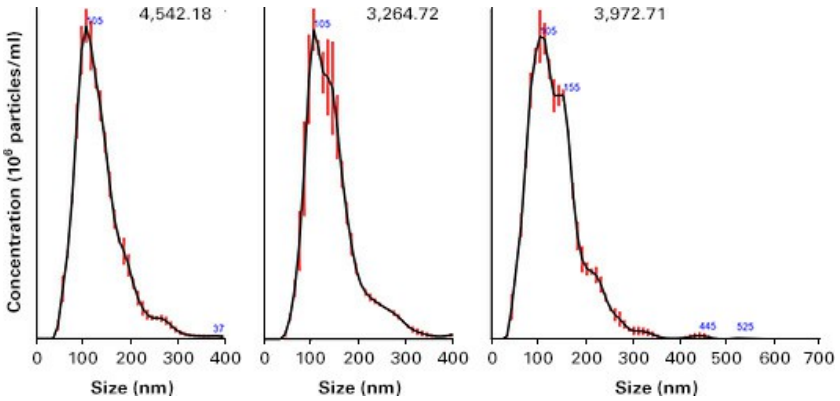


B

Size	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**

Ultracentrifuge

C



D

Size	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**

Figure 2. EVs isolated with the Capturem kit were shown to be a concentrated, purer population of EVs than those purified via ultracentrifugation. NTA was performed on EVs isolated by the Capturem EV isolation kit or ultracentrifugation. **Panels A and C.** The calculated size distribution for each method is shown as a mean (black line) with standard error (red lines). **Panels B and D.** Particle size statistics are shown for preparations from each isolation method.

This experiment was repeated in triplicate, using two different operators, starting with 500 µl from the same starting plasma source in order to compare particle yield variability. Notably, Capturem-isolated EV yields were highly consistent (2.61×10^9 vs. 2.60×10^9), whereas ultracentrifugation yields were much more variable (4.11×10^9 vs. 1.98×10^9). This variability could lead to significant workflow challenges as well as data interpretation difficulties.

Tests were also run to examine the ability of Capturem technology to isolate EVs from a range of other biofluids, including breast milk, saliva, cerebrospinal fluid (CSF), serum, urine, and conditioned media from cells (Figure 3). The Capturem kit was able to isolate EVs from all of these samples, thus demonstrating its usefulness for work with a variety of sample types.

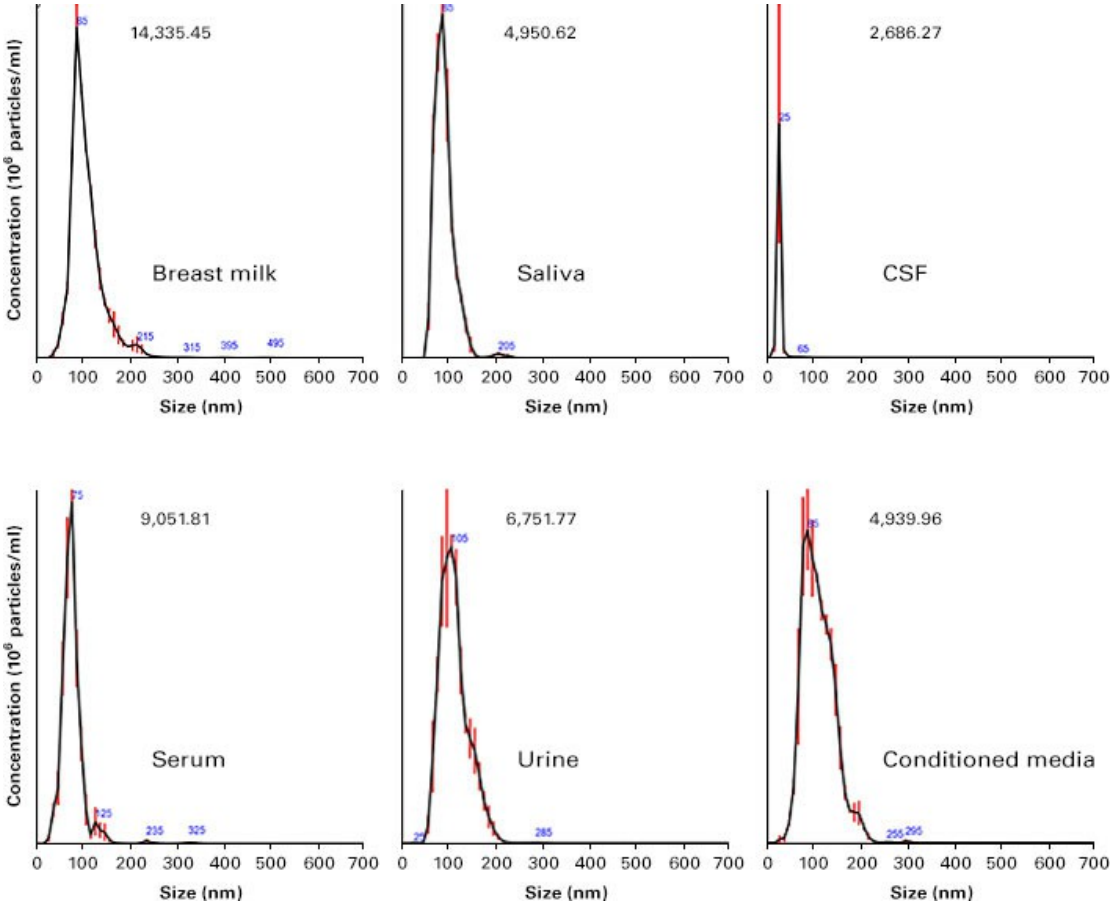


Figure 3. Successful isolation of EVs from different biological fluids. NTA of EVs isolated from breast milk, saliva, CSF, serum, urine, and conditioned media with the Capturem EV kit. Calculated size distribution for each preparation is depicted as a mean (black line) with standard error (red lines).

Conventional NTA can sometimes overestimate EV numbers, because broken particles, protein aggregates, or other debris can register as false

positives. Using a fluorescent dye that only crosses intact membranes can give more accurate insight into the quality of the isolated sample. Therefore, a final assessment of EV concentration was performed using fluorescent NTA (fNTA). Capturem-isolated EVs, ultracentrifuge-isolated EVs, and liposomes (positive control) were labeled with the fluorescent dye and subjected to fNTA analysis (Figure 4). The positive controls exhibited good concordance between NTA and fNTA methods, with 93% of the NTA particles exhibiting fluorescence. However, ultracentrifugation-isolated EVs were highly contaminated with non-EV particles, exhibiting only 20% EV fluorescence. In contrast, Capturem-isolated EVs demonstrated more than 2X this enrichment, with over 45% EV fluorescence. Thus, Capturem columns consistently provide a pure, intact, and concentrated population of EVs in less than 30 minutes.

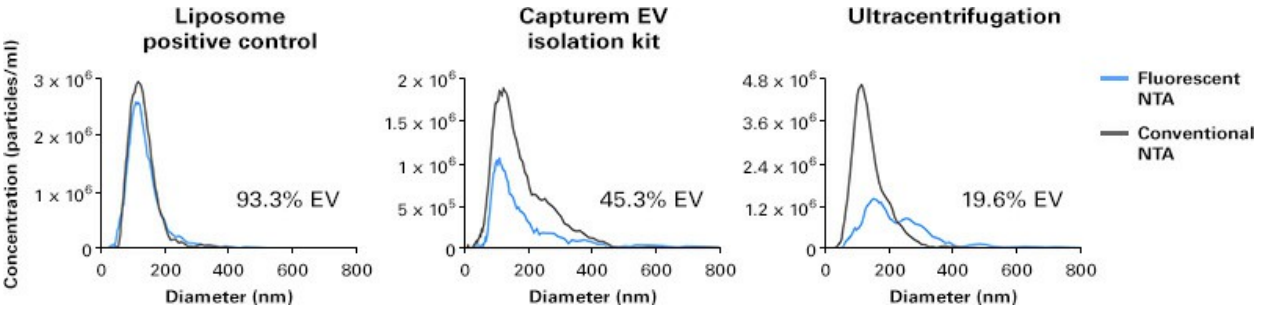


Figure 4. Capturem-based isolation of EVs provides a purer, more intact population of EVs than ultracentrifugation, as demonstrated by fluorescent NTA. A proprietary fluorescent dye was allowed to react specifically with the surface of intact vesicles, followed by quantification via fluorescent microscopy. The graphs above show the liposome positive control, EVs isolated using the Capturem EV isolation kit, and EVs isolated via ultracentrifugation. Analysis was performed by Alpha Nano Tech.

Capturem-isolated EVs exhibit classical exosomal protein markers without the presence of any contaminating proteins

EVs can express a variety of protein markers, and a consensus set has yet to emerge. However, it has generally been agreed upon that EVs should contain at least one tetraspanin (CD9, CD63, or CD81), as well as one biogenesis marker (alix). Additionally, isolates should be free of contaminating proteins, such as the endoplasmic reticulum protein calnexin, as well as any sample-dependent contaminating proteins, like albumin from blood samples.

Capturem-isolated EVs and ultracentrifugation-isolated EVs were analyzed by Western blot using antibodies specific to CD63, CD9, alix, calnexin, and albumin (Figure 5). Tetraspanins were detected in EVs from both isolation methods, but the biogenesis marker alix was not detected in ultracentrifuge-isolated EVs. This suggests that these EVs were less intact or damaged, as the internal protein cargo was not detected. Furthermore, ultracentrifuge-isolated exosomes exhibited high levels of non-EV proteins calnexin and albumin, indicating significant protein contamination in the isolate. Subsequent quantification demonstrated that ultracentrifugation resulted in the presence of contaminating proteins at a level 21X higher than what was observed in the Capturem isolation, which removes these contaminants during the flowthrough and wash steps. Thus, Capturem isolation of EVs provides a more pure and intact population than ultracentrifugation and does not contain as many contaminating, non-EV proteins.

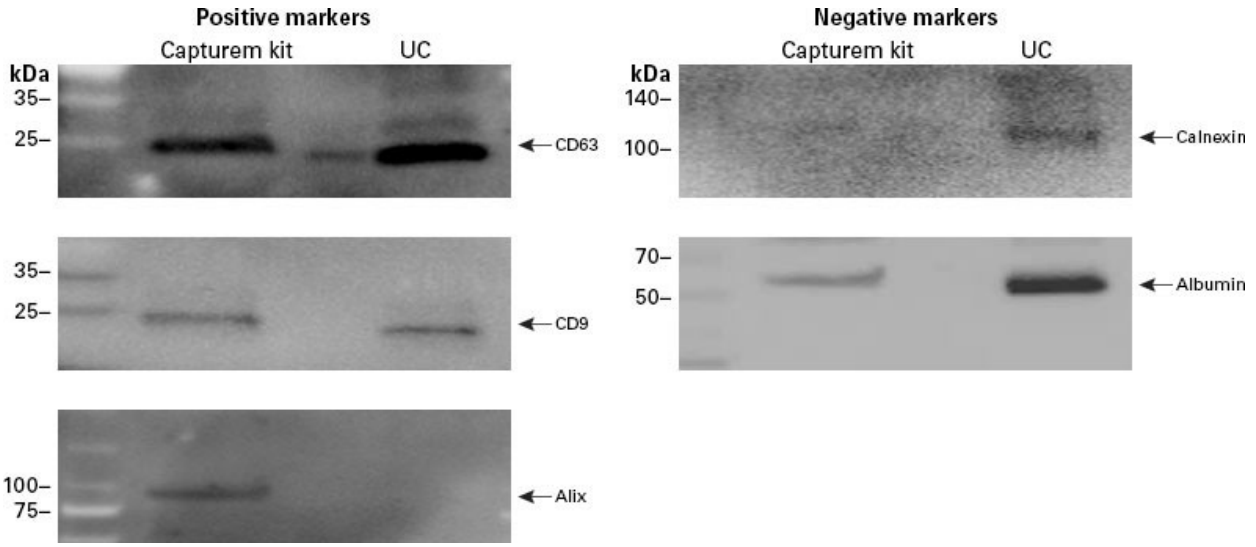


Figure 5. Capturem-isolated EVs present with common tetraspanin and biogenesis markers, with much less contaminating protein compared to

ultracentrifugation (UC). Western blotting shows that the preparation from the Capturem kit contains higher levels of exosome-specific markers CD63, CD9, and alix (positive markers), and lower levels of the non-exosomal protein calnexin and carryover protein albumin (negative markers), as compared to the UC preparation.

RNA yield from Capturem-isolated EVs is sufficient for downstream RT-qPCR or NGS analysis

Many research and clinical studies rely on the use of nucleic acids in EVs as potential biomarkers to study and track disease. These biomarkers are typically studied and quantified using qPCR or NGS techniques. Thus, EV isolates must provide sufficient nucleic acid starting material for subsequent analyses.

miRNA was isolated from Capturem- and ultracentrifugation-isolated EVs using NucleoSpin miRNA Plasma and quantified using a Bioanalyzer to generate the RNA profile (Figure 6). Notably, RNA yield was ~100 pg when using Capturem-isolated EVs from 50 µl of plasma, while 300 µl of plasma was required to obtain a comparable yield from ultracentrifugation.

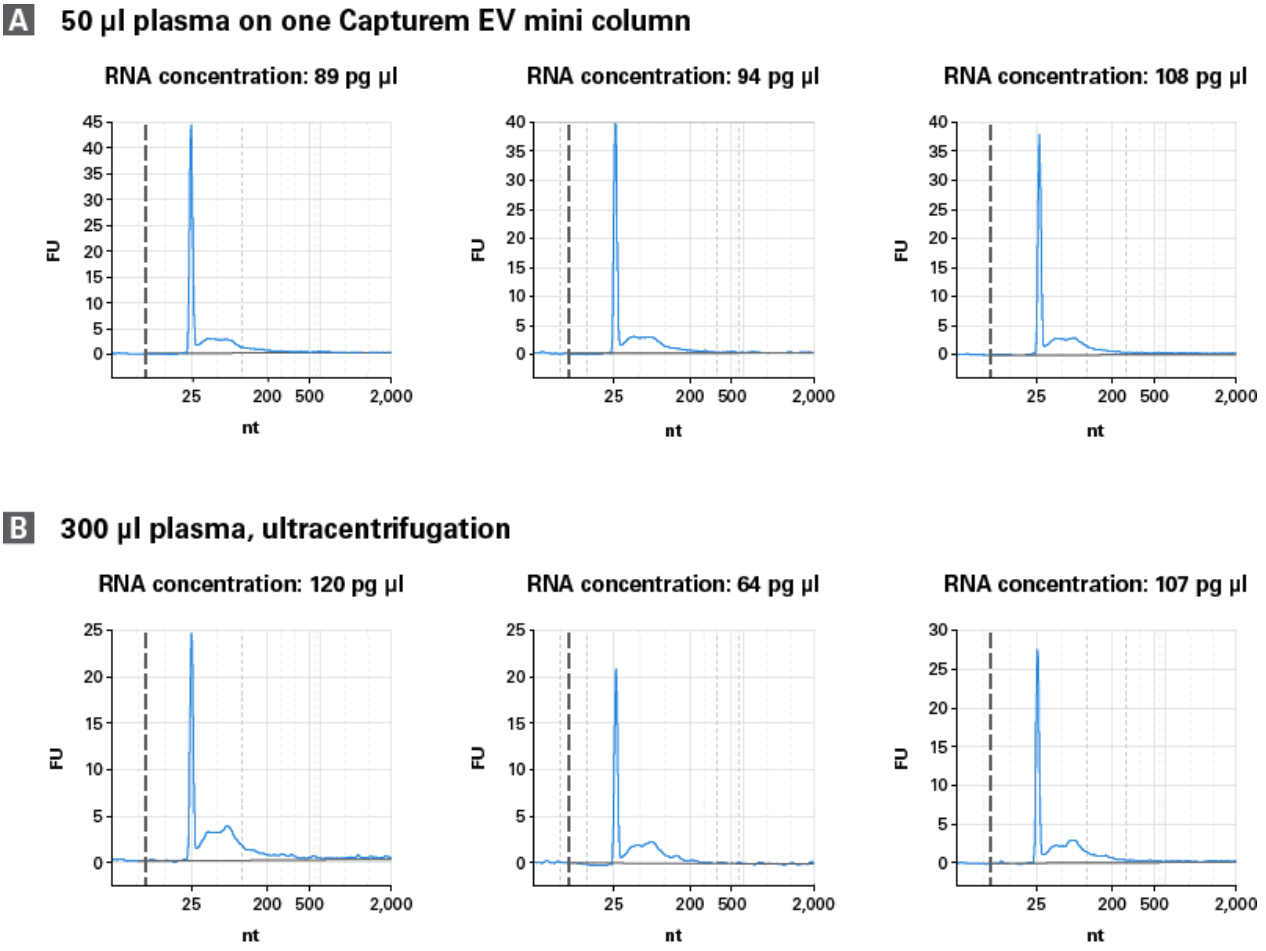
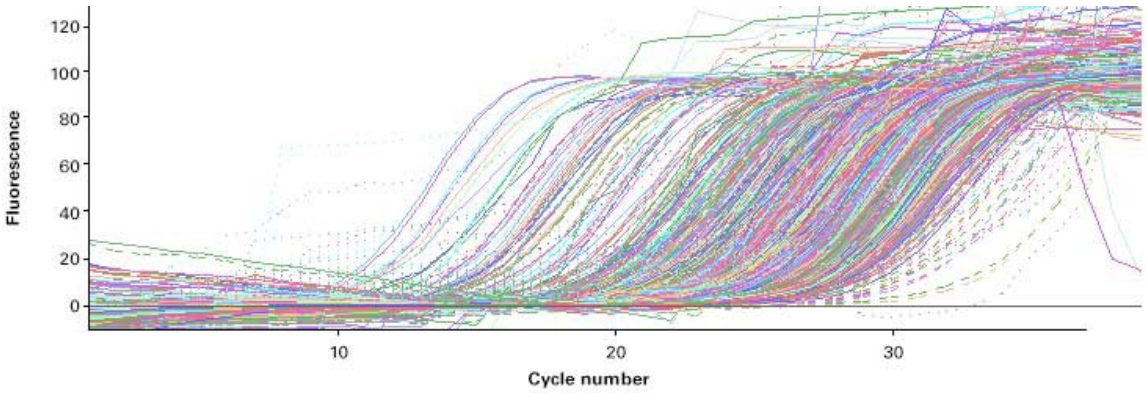


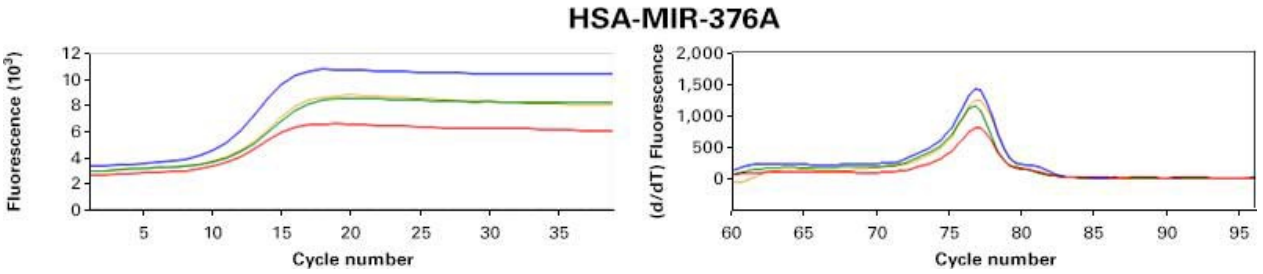
Figure 6. High and consistent RNA content in EVs isolated by the Capturem EV isolation kit. Representative Bioanalyzer profiles of RNA isolated from the same donor. **Panel A.** The Capturem EV miniprep was performed with just 50 µl of plasma and yielded ~100 pg of RNA. **Panel B.** In contrast, ultracentrifugation was performed with 300 µl of plasma. The peaks at 25 nt are internal standards.

Next, isolated RNA from Capturem-isolated EVs was screened for miRNAs (Figure 7). RNA was first reverse-transcribed to cDNA using PrimeScript RT Master Mix (Perfect Real Time) and then preamplified using Takara PreAmp Master Mix. The resulting cDNA pool was assayed for miRNA using a 1,200 miRNA target screen on the SmartChip Real-Time PCR System. 108 miRNAs were detected, and the top eight hits were verified off-chip. miR-548w, miR-496, miR-744-3p, miR-660-3, miR-3167, and miR-376A-3p had the highest copy numbers in the isolated samples, and many have been previously identified to be enriched in plasma-isolated EVs. This analysis shows that Capturem-isolated EVs contain sufficient RNA yield for downstream RT-qPCR.

Amplification curves from SmartChip RT-qPCR

A





Verify hits off-chip (QuantStudio)

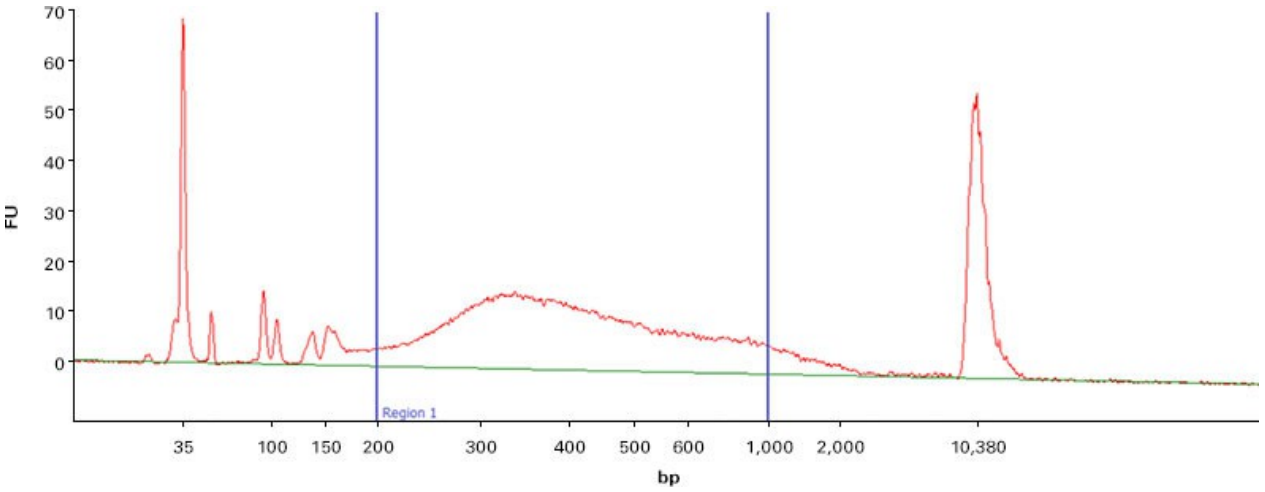
B

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 7. SmartChip RT-qPCR miRNA analysis of RNA purified from Capturem-isolated EVs. Panel A. RNA isolated from EVs using the Capturem isolation kit was reverse transcribed, preamplified, and then 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*.

Finally, we created an NGS library from Capturem-isolated EVs using the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian. A Bioanalyzer trace demonstrated a good, uniform library was created and subsequent sequencing demonstrated good gene coverage (Figure 8). Two transcripts with the highest number of reads were *ACACB* and *CUL9*, both of which have previously been published to be present in EVs. Thus, NGS libraries can be created and sequenced using Capturem-isolated EVs.

A



B

	Uniquely mapped	Nonspecifically mapped	Mapped	%of total mapped
Gene transcripts (total)	102,447	46,194	148,641	66.59
Intronic	88,373	36,645	125,018	56.01
Exonic	14,074	9,549	23,623	10.58
Intergenic	67,805	6,778	74,583	33.41
Total	170,252	52,972	223,224	100.00

Figure 8. Profiling the transcriptome of Capturem-isolated EVs by NGS. RNA extracted from Capturem-isolated EVs was used as input for stranded NGS analysis. **Panel A.** This Bioanalyzer trace shows the profile for the cDNA output from the NGS library preparation kit. **Panel B.** Sequencing alignment metrics from Illumina NGS, using the cDNA in Panel A as input. 0.1% of reads were from rRNA and 0.0% of reads were from mitochondrial RNA.

Morphological characterization of Capturem-isolated EVs by TEM and immunoelectron microscopy

Capturem-isolated EVs were also subjected to TEM and immunoelectron microscopy (Figure 9). Capturem-isolated EVs displayed classical EV morphology and were consistent in size and shape with those isolated using ultracentrifugation. Furthermore, EVs were positive for CD63 surface expression, as visualized by CD63 immunogold labeling. As these criteria show, Capturem-isolated EVs exhibit the correct size, shape, and surface proteins expected for EV morphological analysis.

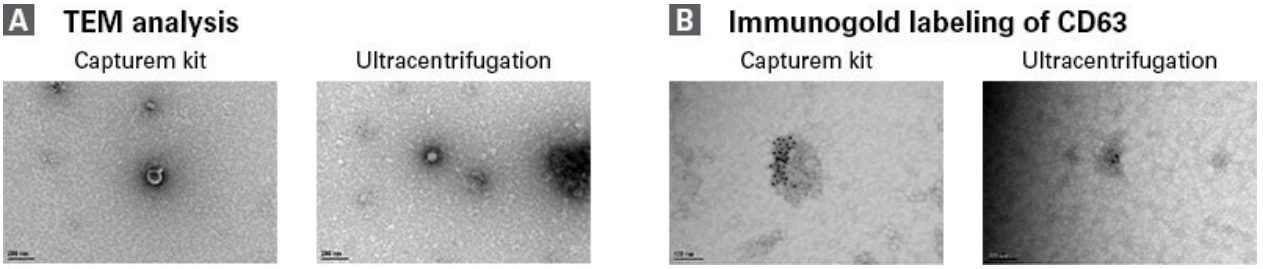


Figure 9. Capturem-isolated EVs display classical size, morphology, and CD63 expression on the surface. TEM analysis and immunogold labeling of EVS isolated by the Capturem EV isolation kit or ultracentrifugation. **Panel A.** Negative staining using uranyl acetate. Representative images of EVs isolated from the same donor. **Panel B.** EVs 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 Nano Tech.

Conclusions

Research and clinical studies could greatly benefit from technology that provides rapid and simple isolation of EVs, such as exosomes, from various biofluids—especially as it concerns methods that generate pure, concentrated EV samples with enough yield for subsequent proteomic, genomic, and transcriptomic analyses. We have designed the Capturem Extracellular Vesicle Isolation Kit (Mini) to meet these high standards, enabling researchers to consistently obtain purely concentrated EVs. Furthermore, we have demonstrated that the RNA yield from these EVs is sufficient for downstream RT-qPCR and NGS analysis. These tools can be used for rapid analysis of samples for clinical research and can enable greater discovery and diagnostic potential for a variety of diseases.

Methods

EV isolation

EVs from the same donor were used for both ultracentrifugation and the Capturem EV kit. Isolation of EVs using the Capturem Extracellular Vesicle Isolation Kit (Mini) was performed as per the user manual. For ultracentrifugation, precleared biofluid supernatant was centrifuged at 100,000*g* for 1.5 hr at 4°C using an Optima MAX-E Ultracentrifuge (Beckman Coulter) to collect EVs. The EV pellet was then washed in PBS and further ultracentrifuged at 100,000*g* for 1.5 hr at 4°C. The final EV pellet was resuspended in 1 ml of PBS and stored at –80°C.

Analysis

For reliable NTA measurement, we used an appropriate EV sample dilution that yields approximately 20–100 particles in the field view. Optimized video capture setting parameters were used with a screen gain set to 2 or 3 and camera level set to 14 or 15. The chosen tracking settings were a screen gain between 8–10 and a detection threshold of 2 or 3. Three 30-second videos were recorded on the Malvern Panalytical NanoSight LM10 and then analyzed using tracking software.

For Western blotting, each lane was loaded with 5 µg of total protein, as measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). For TEM analysis, negative staining was performed with uranyl acetate, and representative images were taken of preparations from each method. For immunoelectron microscopy, EVs were stained with 10 nM of gold-conjugated anti-CD63 antibody, followed by uranyl acetate counterstaining. Analysis was performed by Alpha Nano Tech.

RNA was extracted from isolated EVs using the NucleoSpin miRNA Plasma kit and analyzed with the RNA 6000 Pico Kit (Agilent). Bioanalyzer profiles were generated for RNA extracted from EVs isolated either from 50 µl of plasma input for the Capturem EV kit or 300 µl of plasma input for ultracentrifugation.

RT-qPCR

RNA was extracted from Capturem-isolated EVs, as described above, and cDNA was generated using PrimeScript RT Master Mix (Perfect Real Time). Resulting cDNA was preamplified with Takara PreAmp Master Mix. Analysis was performed using the SmartChip Real-Time PCR System. The top eight assays were tested off-chip using QuantStudio (Thermo Fisher Scientific).

NGS

RNA (extracted from Capturem-isolated EVs, as described above) was used as input for the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian. The resulting cDNA was used as input for Illumina sequencing.

References

Jeppesen, D. K., *et al*. Comparative analysis of discrete exosome fractions obtained by differential centrifugation. *J. Extracell. Vesicles* **3**, 25011 (2014).

Related Products

Cat. #	Product	Size	License	Quantity	Details
635741	Capturem™ Extracellular Vesicle Isolation Kit (Mini)	20 Minipreps		*	

The Capturem Extracellular Vesicle Isolation Kit (Mini) provides a complete solution for the simple and rapid isolation of extracellular vesicles, like exosomes, from plasma and other small volumes of biological fluids. The kit includes 20 single-use, disposable pre-clearing columns; 20 single-use, disposable isolation columns; as well as equilibrium, wash, and elution buffers. Extracellular vesicles are isolated using specially designed Capturem spin columns containing a lectin-based binding compound which is not antibody-based.

Documents	Components	Image Data	Resources

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