Echelon Biosciences Inc.

ExoClean and ExoTracker

K-4300a, K-4300b, K-4300c, K-4300d (20 tests)

Support: echelon@echelon-inc.com

Description: Simple, quick protocols for labeling (ExoTracker) and isolating (ExoClean) extracellular vesicles (EVs) from biological samples*

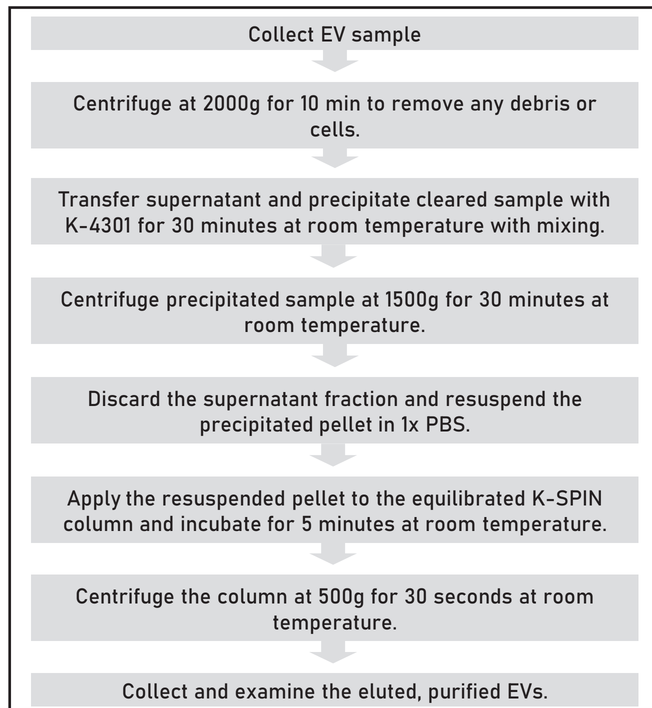
Materials Provided

Catalog #	Description	Quantity
ExoClean (K-4300a)		
K-4301	EV Precipitation Reagent	30 mL
K-SPIN	Microcentrifuge Columns	20 columns
N/A	2 mL sample tubes	40 tubes
ExoTracker - Membrane (K-4300b)		
K-4300a	ExoClean	1 kit
K-4302	EV Membrane Label	150 µg
ExoTracker - RNA (K-4300c)		
K-4300a	ExoClean	1 kit
K-4303	EV RNA Label	20 µL
ExoTracker - Total (K-4300d)		
K-4300a	ExoClean	1 kit
K-4302	EV Membrane Label	150 µg
K-4303	EV RNA Label	20 µL

Additional Materials Provided by User:

- Cells, tissue sample, or EV source
- Table top centrifuges
- 1x PBS
- Ultrapure lab-grade water (MQ H2O)
- Fluorescence microscope capable of imaging 488 and 555 nm ex. channels

ExoClean Quick Protocol



Storage: Upon receipt, store ExoClean (K-4300a) at 4°C. EV Membrane Label (K-4302) and EV RNA Label (K-4303) should be stored at -20°C and protected from light. All reagents are stable for at least 6 months under these conditions.

* This protocol has been validated with EVs from cell culture and serum samples. Other sample and tissue types are possible but have not been internally validated by Echelon Biosciences (EBI).

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Background

Extracellular vesicles (EVs) comprise a family of cell and tissue derived vesicles that are broadly classifiable as 1) exosomes, 2) microvesicles, and 3) apoptotic bodies. Despite being initially characterized as cellular garbage, EVs have been observed in nearly all biologic fluids and tissues and have rapidly gained scientific attention due to their ability to ferry protein and nucleic acid cargo between cells. Furthermore, EVs present a breadth of possibilities for both the development of clinical diagnostics and for EV-based drug and therapeutic delivery.

Kit Design

The three subtypes of EVs can be grossly distinguished based on size. In line with this, the initial standard for EV separation and isolation was differential centrifugation. Subsequent techniques took adavantage of the physiochemical properties of EVs either by using polymer-based precipitation or size-exclusion chromatography to isolate EVs. However, these techniques all have significant drawbacks in terms of either 1) time, 2) cost, or 3) potential for sample contamination. The ExoClean kit (K-4300a) from Echelon Biosciences (EBI) reduces all three of these constraints by using a simple two-step method of precipitation followed by secondary column-based purification. The ExoTracker kits (K-4300b-d) further enhances the experimental utility of purified EVs by allowing for the incorporation of membrane and RNA-specific labels. EVs can therefore be labeled, purified, and tracked within recipient cells or tissue in a time and cost-effective manner.

ExoTracker Kit Notes

1. The method described outlines EV labeling in cell culture using HEK 293 cells (Figure 1). The ExoClean and ExoTracker kits can be used with other cell culture systems, but other cell types have not been internally validated by EBI.
2. The EV Membrane Label (K-4302) contains a standard red fluorophore conjugate and can be imaged with standard filters set at approximately 590/615 nm ex/em.
3. The EV RNA Label (K-4303) contains a standard green fluorophore conjugate and can be imaged with standard filters set at approximately 488/525 nm ex/em.
4. The EV Membrane Label can be resonstituted in MQ H_2O or standard physiological buffers such as PBS at neutral pH. Buffers of pH >9.0 or <4.0 will result in degradation of the label.
5. Once resuspended, the EV Membrane Label is stable for at least 6 months at -20°C and can tolerate at least one freeze-thaw cycle. The EV Membrane Label should be aliquoted and stored at -20°C to aviod multiple freeze-thaw cycles. Storage at 4°C should be limited to no more than 3 days.
6. Labeling of cells for production of labeled EVs should be conducted with serum-free (SFM) or EV-depleted media (EV-) in order to maximize incorporation of the label into cells.
7. Cells can tolerate the EV Membrane Label and EV RNA Label for at least 16 hours.
8. The duration of labeled EV production post-cell labeling is dependent on the experimental endpoint and should be independently determined by the user.

ExoClean Kit Notes

1. This method outlines EV isolation from conditioned culture supernatnat (CCS). ExoClean was validated with both CCS from HEK 293 cells and human serum (Figure 2). The ExoClean kit can be used with other culture media and biological fluid samples, but have not been validated by EBI.
2. The microcentrifuge columns (K-SPIN) are shipped dry and should be hydrated prior to use. The column resin is compatible with most standard denaturing and chaotropic agents, reducing agents, and detergents including: urea, guanidinium chloride, β-ME, DTT, Triton X-100 and SDS.
3. It is recommended to use buffers with 50 mM ionic strength or higher with the microcentrifuge columns, although MQ H_2O is sufficient for the hydration step.
4. The EV Precipitation Reagent (K-4301) and microcentrifuge columns are stable up to 1 week at either 4°C or 37°C and can tolerate at least one freeze-thaw cycle. The EV Precipitation Reagent displays some phase-separation with excessive storage at 37°C, however this can be corrected with vortexing and does not affect the activity of the reagent.
5. In the event that the microcentrifuge columns need to be stored post-hydration, it is recommended to store the columns in a physiologically relevant buffer such as PBS.
6. Re-use of the microcentrifuge columns is not recommended.
7. The method described here is designed for media volumes from 6 and 12 well culture plates seeded at ~2.5 x 10^5 cells/mL. However, a single column can tolerate as much as 5 mL of CCS or 1 mL of serum.

Protocol for the labeling and purification of EVs

Please read this entire section and the assay notes section before beginning the assay. For ExoClean, skip Section 1 and proceed to Section 2.

Section 1 - Labeling

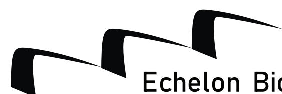
For labeling RNA, proceed to Step 6. For membrane labeling or dual labeling, proceed with Step 1.

EV Membrane Label

1. Reconstitute the EV Membrane Label with 110 µL 1x PBS to obtain a 1 mM stock and keep on ice protected from light. Excess, unused reagent can be aliquoted and stored at -20°C.
2. Aliquot 1 mL of SFM or EV- media (assay note #6) per well of cells to be labeled and add 5 µL of reconstituted EV Membrane Label (Step 1) per mL of media. Mix gently via pipette.
3. Aspirate media from cells and wash once with 0.5-1 mL of SFM or EV- media then apply 1 mL of the media-diluted EV Membrane Label (Step 2) per well.
4. Incubate the cells at 37°C, 5% CO_2 for 1 hour.
5. Following incubation, aspirate the media and wash the cells once with 0.5-1 mL SFM or EV- media.
    a. If labeling only with the EV Membrane Label, remove the wash and add complete media. Incubate for minimum 2 hours under basal conditions for labeled EV production and proceed to Section 2 for EV purification.
    b. For dual labeling, proceed to Step 6.

EV RNA Label

6. Thaw the EV RNA Label at room temperature and protect from light. Excess reagent can be aliquoted and stored at -20°C.
7. Aliquot 1 mL of SFM or EV- media (assay note #6) per well of cells to be labeled and add 1 µL of the EV RNA Label (1 mM stock) per mL of media. Mix gently via pipette.
8. Aspirate media from cells and wash once with 0.5-1 mL of SFM or EV- media then apply 1 mL of the media-diluted EV RNA Label (Step 2) per well.
9. Incubate the cells at 37°C, 5% CO_2 for 20 minutes.
10. Following incubation, aspirate the media and wash the cells once with 0.5-1 mL SFM or EV- media.
11. Remove the wash and add complete media. Incubate for minimum 2 hours under basal conditions for labeled EV



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production and proceed with Section 2 for EV purification.

Figure 1

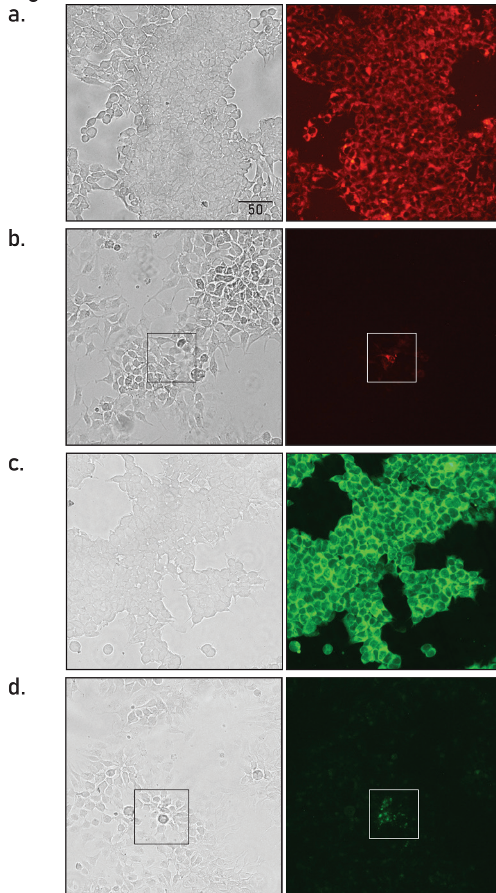

Figure 1 - Tracking labeled EVs purified from cells treated with EV Membrane Label and EV RNA Label.
EV Membrane Label (K-4302) and EV RNA Label (K-4303) were applied to HEK cells as described in Section 1 of the kit protocol. Cells were incubated for 12 hours post-labeling prior to EV harvesting. EVs were collected and purified as described in Section 2 of the kit protocol and applied to unlabeled HEK cells for 2 hours prior to fixation and imaging.
a) Brightfield and red channel images of cells treated with EV Membrane Label. b) Brightfield and red channel images of cells treated with labled EVs harvested from treated cells in (a). c) Brightfield and green channel images of cells treated with EV RNA Label. d) Brightfield and green channel images of cells treated with labled EVs harvested from treated cells in (c). Black and white boxes indicate regions of labeled EV recipient cells.

Section 2 - Purification

1. Centrifuge the sample containing EVs at 2,000g for 10 minutes at room temperature in a swinging arm or bucket centrifuge.
2. Recover the supernatant and transfer to a clean tube.
3. Add EV Precipitation Reagent to the cleared supernatant in a 1:4 ratio, i.e. for every 1 mL of supernatant, add 0.25 mL of EV Precipitation Reagent.
4. Incubate at room temperature for 30 minutes with gentle mixing.
5. Centrifuge the EV Precipitation Reagent-supernatant mixture at 1,500g for 30 minutes at room temperature in a swinging arm or bucket centrifuge.
6. Carefully remove the supernatant by pipette leaving as little as possible behind.
7. Resuspend the precipitated pellet in PBS or buffer of choice. We recommend 50 µL of buffer per every 1 mL of starting material.
8. Keep the resuspended pellet on ice until microcentrifuge columns are ready.
9. Tap the columns gently to ensure that the resin is settled. Remove the red caps and place the columns into 2 mL microcentrifuge tubes. To each microcentrifuge column, add 750 µL of MQ H2O to hydrate the column.
10. Let stand for 1 minute at room temperature and then centrifuge at 2,000g for 1 minute at room temperature in a fixed angle microcentrifuge.
11. Equilibrate the column with 500 µL of PBS or the same buffer used to resuspend the precipitated pellet.
12. Centrifuge at 2,000g for 1 minute at room temperature in a fixed angle microcentrifuge.
13. Repeat steps 11 and 12 twice (3 times total).
14. Following the final equilibration wash, add the resuspended pellet to the column and allow it to completely absorb. To increase the final volume of the flow through containing EVs, layer the column with an additional 50-100 µL of PBS or the same buffer used to resuspend the precipitated pellet.
15. Place the column in a new centrifuge tube. Centrifuge the column at 500-1000g for 30-60 seconds at room temperature in a fixed angle microcentrifuge. The flow through from this step contains EVs that are ready for downstream applications and analysis.

Figure 2

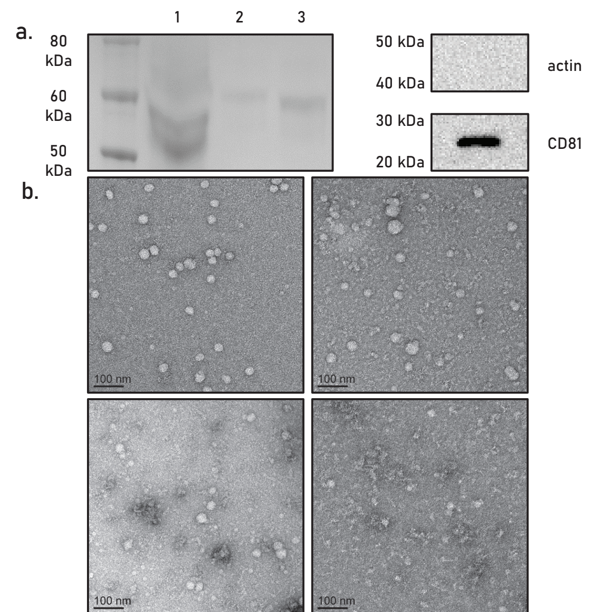

Figure 2 - EV purification from culture media and serum.
a) left - coomassie stained gel of 1 - preceipitated pellet, 2 - column flow through, and 3 - column retentate from EV Precipitation Reagent (K-4301) and microcentrifuge columns (K-SPIN). Right - negative and positive control western blots for actin and CD81 from the column flow through following EV Precipitation Reagent and microcentrifuge column purification. b) Electron micrograph images of EV fractions. Top, left - Serum EVs from the column flow through following EV Precipitation Reagent and microcentrifuge column purification. Top, right - HEK cell EVs from the column flow through following EV Precipitation Reagent and microcentrifuge column purification. Bottom, left - HEK EVs after EV Precipitation Reagent alone. Bottom, right - material retained on the microcentrifuge column following EV purification (column retentate).

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References:

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2. Li P, Kaslan M, Lee SH, Yao J, Gao Z. Progress in Exosome Isolation Techniques. Theranostics. 2017;7(3):789-804.
3. Deregibus MC, Figliolini F, D'Antico S, Manzini PM, Pasquino C, De Lena M, et al. Charge-based precipitation of extracellular vesicles. Int J Mol Med. 2016;38(5):1359-66.

Related Products

Products	Catalog Number
EV Kits	
ExoClean	K-4300a
ExoTracker – Membrane	K-4300b
ExoTracker – RNA	K-4300c
ExoTracker - Total	K-4300d
Lipid-Protein Interaction Tools	
PIP Strip, PIP Array	P-6001, P-6100
Membrane Strip, Membrane Array	P-6002, P-6003
PIP Beads	P-B00S, P-B003a P-B045a, P-B345a
PolyPIPosomes	Y-P000, Y-P003 Y-P045, Y-P039

Products	Catalog Number
Lipid Quantification Assays	
PI(3)P Mass ELISA	K-3300
PI(3,4)P2 Mass ELISA	K-3800
PI(4,5)P2 Mass ELISA	K-4500
PIP3 Mass ELISA	K-2500s
Lipids	
PI(3)P	P-3008, P-3016
PI(4)P	P-4008, P-4016
PI(5)P	P-5008, P-5016
PI(3,4)P2	P-3408, P-3416
PI(3,5)P2	P-3508, P-3516
PI(4,5)P2	P-4508, P-4516
PI(3,4,5)P3	P-3908, P-3916

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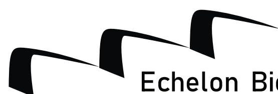

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