diagenode

A Hologic Company

ExoIP kits

ExoIP kit: immunocapture for efficient exosome isolation

Cat. No. **C28010001** (ExoIP Composite kit)
C28010002 (ExoIP CD9 kit)
C28010003 (ExoIP CD63 kit)
C28010004 (ExoIP CD81 kit)
C28010005 (ExoIP EpCAM kit)

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Content

Introduction

ExoIP kits are the appropriate solution to **isolate** and **enrich exosomes** from diverse biological fluid samples and avoid contamination from vesicules or aggregates.

Exosomes are extracellular small vesicules of 30-100 nm constitutively released under certain stimuli in biological fluids including blood, urine, saliva, breast milk, culture medium of cell cultures, etc. Exosomes contain various molecular constituents of their cell of origin, including proteins and RNA. Circulant exosomes can potentially act as cargo messenger and transfer molecules from one cell to another via membrane vesicle trafficking. Exosomes are suitable for a variety of downstream applications including RNA analysis, protein analysis, etc.

ExoIP kits consist of **immunoprecipitation method** using magnetic capture beads coupled with antibodies that recognize exosome surface antigens for an optimal pure and high quality of exosome isolation.

5 exosome isolation kits were developed to optimize serum, plasma and cell culture supernatant exosome isolation:

- ExoIP CD9 kit
- ExoIP CD63 kit
- ExoIP CD81 kit
- ExoIP EpCAM kit
- ExoIP Composite Kit

Advantages of Diagenode's ExoIP kits:

- **Highly pure exosome population obtained after isolation**: ideal for RNA and non-coding RNA analysis (microRNA) protein analysis, Western blot, ELISA, flow cytometry, and more!
- **Ready to use, high-quality**: includes antibody-conjugated beads and optimized BSA-free buffers
- **Sample diversity**: serum, plasma, and cell culture supernatant
- **Highly validated with multiple methods**: Western blot, electron microscopy, RT-qPCR, dynamic light scattering, and flow cytometry

Kit materials components

- **Magnetic capture beads** of 3µm diameter coupled with anti-CD63 antibodies which recognize exosome surface CD63 antigens.
- **Washing/dilution buffer** which helps remove unwanted adsorbed biological material on the exosome-bead complex for low-protein sample (e.g. cell culture supernatant).
- **Treatment buffer** that decreases the non-specific binding on the magnetic bead for high-protein sample (e.g. plasma)
- **Exosome elution buffer** to recover fully intact and functional exosomes after immunocapture.

ExoIP kit components	Amount	Storage
Magnetic capture beads	2 ml	2-8°C
Treatment buffer	30 ml	
Washing/dilution buffer	60 ml	
Elution buffer	1.5 ml	

ExoIP kits	Magnetic capture beads
ExoIP CD9	CD9 capture beads
ExoIP CD63	CD63 capture beads
ExoIP CD81	CD81 capture beads
ExoIP EpCam	EpCAM capture beads
ExoIP Composite	Mixture of CD9, CD63, CD81 and EpCAM capture beads

Required equipments

- Diagenode's CaptEV precipitation reagent (Cat. C28030001 + C28030002)
- 0.2 µm filtration unit (e.g. syringe filter + syringe)
- Magnetic stand for 2.0 ml tube
- Vortex shaker
- Buffer for resuspending bead-exosome complex to process in downstream analyses (e.g. PBS)
- Centrifuge and micro-centrifuge
- PCR-grade micro-tubes : 1.5 ml + 2.0 ml
- 15 ml tubes + 50 ml tubes
- Pipetus + serological pipettes (10 – 20 ml)
- Micro-pipettes + corresponding tips: 10µl – 200 µl – 1000 µl
- Gloves
- Crushed ice

Protocol

Step 1: Cell culture guidance

The cell line which has been used as a model for the characterization of exosome preparations generated by ExoIP kits is HEK 293T. The cell culture was conducted in traditional cell culture flasks. The possibility of switching to a bioreactor culture is of great advantage since it can possibly enrich the exosome content of the culture supernatant and thus allowing the final user to process lower volume and even maybe, bypassing the pre-enrichment operation. In this case, we will recommend using the bioreactors from Integra.

The cell culture is actually the critical point and cannot be resumed by a standardization.

Here is some guidance about how cell culture can drastically change the exosome secretion:

- The exosome secretion depends directly of the cell type considered. (For example, it is of common knowledge that endothelial, dendritic and immune cells are cell types abundantly secreting exosomes)
- The medium chosen for the cell culture may influence the exosome secretion of the cells since exosomes are a way of intercellular communications. This communication may change if the growing environment is changing itself.
- The time of secretion obviously play a role in the quantity of exosomes that will be recovered when harvesting the conditioned culture media. Usually, in literature it is described a secretion time varying from 24h to 72h.
- For the secretion period during the cell culture, FBS depleted from extracellular vesicules (EV's) has to be used in the culture medium. Either culture medium without any serum at all or culture medium complemented with serum but depleted from EV's. The depletion of the serum from extracellular vesicles is usually performed by ultracentrifugation.

The following depletion protocol was used to eliminate EV's out of the Fetal Bovine Serum (FBS):

- Ultracentrifuge: Optima L-90K (Beckman Coulter); Rotor: SW32
- Centrifugation 18 hours (overnight) at +4°C with a speed of 110.000g
- The serum has first to be diluted in culture medium in order to decrease its inherent viscosity that may hamper the exosomes depletion by centrifugation Ð pelleting efficiency. A dilution factor of 2.4 was used for our depletion protocol (50 ml of serum in 120 ml complete medium).
- After the depletion by ultracentrifugation, the resulting serum diluted in medium is sterilized through a 0.2 µm filter.

Step 2: Exosome pre-enrichment

The pre-enrichment step is done with the Diagenode's CaptEV reagent. This reagent has been declined for two major applications:

- CaptEV for cell culture supernatant (Cat. No. C28030001)
- CaptEV for blood serum and plasma (Cat. No. C28030002)

The CaptEV reagent allows recovering exosomes and other extracellular vesicles from a sample in a much smaller volume prior immunocapture. The aim of this pre-enrichment is to increase the exosome concentration for a particular sample and to already eliminate a significant portion of contaminants to facilitate subsequent purification such as ExoIP immunocapture.

For serum and plasma-based sample, pre-enrichment could be omitted because it may already contain enough exosomes but, nevertheless, it is strongly advised to perform such a precipitation because it can greatly help further purification in eliminating unwanted components within the sample.

Particular attention needs to be paid for cell culture-based which may not contain quantity of exosomes since some cell lines may be poor at extracellular vesicles secretion.

For this, two important factors have to be considered before performing the concentration:

1. Do my cells secrete a lot of extracellular vesicles? If not, pre-enrichment needs to be done.
2. Do I need a lot of exosomes for the downstream analyses that I plan to perform? If yes, pre-enrichment needs to be done.

Moreover, this reagent has been especially designed to work prior immunocapture because it contains a co-precipitant which will help further exosomes capture on the beads.

However, this does not exclude in any way the use of the precipitation reagent prior other purification technique as the co-precipitant can be easily eliminated.

Protocol for exosomes precipitation from cell culture supernatant with the CaptEV reagent:

- Pipette cell culture supernatant out of the culture flasks
- Centrifuge the supernatant at 3000g for 30 minutes at room temperature to get rid of floating cells and large debris
- Carefully aspirate the supernatant without disturbing the pellet and filtrate through 0.2 µm to eliminate large vesicles.

When filtering, be careful not to apply an excessive pressure on the filter since it may break large vesicles in smaller debris which may flow through. Typically, if using a syringe-based device, push slowly the plunger to process the sample and stop if a resistance is appearing. This surely means that the filter is clogged and that a new one has to be used.

- Mix the 0.2 µm filtered supernatant with one volume of CaptEV reagent (sample : precipitation reagent, 1:1).
- Incubate under mild agitation for 1 hour at room temperature or overnight at +4°C.
- Centrifuge the sample for 30 minutes at 1500g at room temperature
- Discard the supernatant and resuspend the pellet in a desired volume of PBS (100 – 1000 µl)

We advise to first let the pellet sit with PBS for 5 minutes on ice, the time for it to rehydrate. Then, pipette slowly up and down until complete resuspension.

- In next steps process the enriched exosome sample through immunocapture.

Protocol for exosomes precipitation from serum and plasma with the CaptEV reagent:

- Mix the appropriate volume of serum/plasma with one volume of CaptEV reagent (sample : precipitation reagent, 1:1).
- Incubate under mild agitation for 1 hour at room temperature or overnight at +4°C.
- Centrifuge the sample for 30 minutes at 1500g at room temperature.
- Discard the supernatant and resuspend the pellet in a desired volume of PBS (100 – 1000 µl)

We advise to first let the pellet sit with PBS for 15 minutes on ice, the time for it to rehydrate. Then, pipette slowly up and down until complete resuspension.

In next steps process the enriched exosome sample through immunocapture.

Step 3: Immunocapture

Advices

Use fresh samples prior immunocapture since freezing may damage the surface integrity of the vesicles and decrease the binding efficiency.

Always prefer a long incubation period at +4°C. Optimal binding efficiency can be reached between 21 to 24 hours incubation. It is important to use 2 ml tube for proper mixing conditions during the incubation period.

Immunocapture recommendations

Sample	Dilution buffer – Treatment buffer (*)	Capture beads (**)
100 -1000 µl • Pre-enriched conditioned culture media (CCM) • Biofluids	In a 1:1 ratio with sample (100 -1000 µl)	Most of the applications (***): 100 µl
		Flow cytometry: 50 µl
		RT-qPCR/ NGS: 100 – 500 µl

(*) We recommend substituting dilution buffer with treatment buffer for high-protein sample (e.g. plasma).

(**) This is a recommended volume for a standard usage. It should be adapted according target abundance and application.

(***) Applications for which ExoIP has been validated: Western Blot, size measurement (DLS, TRPS,...), protein quantification, Transmission Electron Microscopy (TEM), Immuno-Gold Labelling (IGL).

Protocol

According to the recommendations table above, process between 100 to 1000 µl of sample into ExoIP immunocapture.

Samples can be pre-enriched conditioned culture media (CCM) or biofluids.

- In a 2 ml tube, add the appropriate volume of capture beads as indicated above (immunocapture recommendations).
- Put the tube on a magnetic stand and wait for the beads to completely separate from the aqueous phase.
- Discard the beads supernatant.
- Take the dry beads off the magnetic stand and add between 100 to 1000 µl of sample.
- Add in the same proportion (1:1), dilution buffer or treatment buffer to the sample.

  We recommend the use:

  - dilution buffer for pre-enriched cell culture supernatant;
  - treatment buffer for biofluids.
- Incubate the sample at +4°C for an extended period of 21 - 24 hours.
- Process the immunocaptured exosomes according to the following downstream options.

Step 4: Downstream processing of the sample

After the immunocapture step, the samples can be recovered by a quick spin. A pre-centrifugation is preferred before the magnetic capture of the beads since it will greatly reduce the risks of losing beads in the supernatant and reduce the time to attract all the beads dispersed in the aqueous medium to the magnet.

A quick high speed centrifugation (9000g during 30'' at room temperature) seems to be a good way to pellet all the beads at the bottom of the tube prior magnetic capture.

Two washing steps of 200 µl with the wash/dilution buffer are good to clean-up the preparation after the immunocapture. It is not necessary to perform more washing steps as this can be a source of potential beads loss.

When the beads pellet is properly washed, the exosomes are then ready to be processed either for elution, lysis/ extraction or flow cytometry analysis (Figure 1)

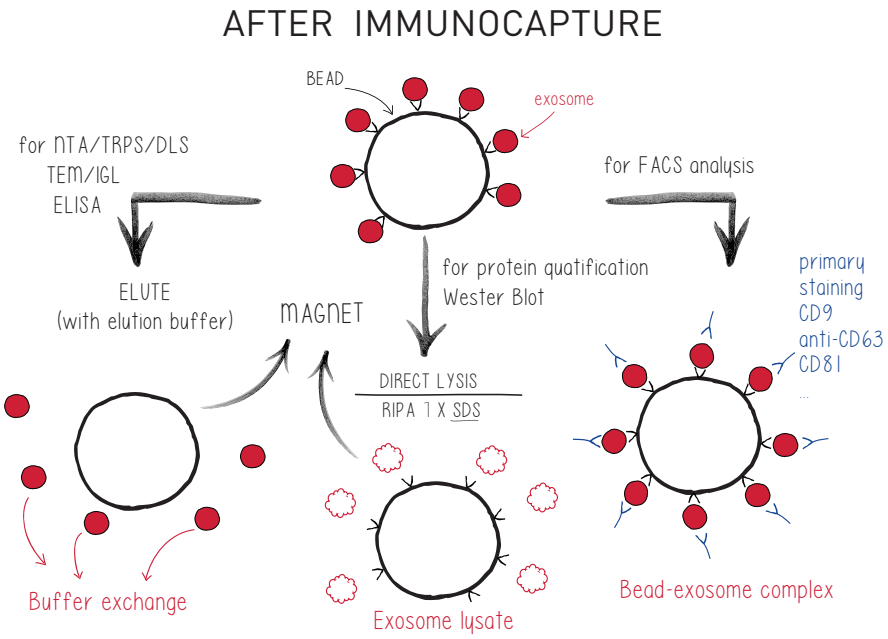

Figure 1. A cartoon showing three possibilities to process exosomes after the ExoIP.

A. Exosome elution:

For nanoparticles size analysis (NTA, TRPS, DLS), electron microscopy (TEM, IGL) and ELISA.

The exosome-bead complex has been washed two times with 200µl of wash/dilution buffer and is ready for the exosome elution procedure.

- Add 25-50 µl of exosome elution buffer to the beads pellet. Mix gently by pipetting (do not vortex).
- Incubate the beads without mixing for 3 minutes at room temperature.
- Place the tube on the magnetic stand for about a minute and transfer the supernatant to a new tube.
- Immediately proceed to exchange the exosome elution buffer with wash/dilution buffer or your preferred buffer (e.g. PBS). The recommended buffer exchange is a diafiltration protocol using Amicon 0.5 10kDa columns.

Load the eluted exosomes properly diluted with PBS 1X to a final volume of 500 µl on the Amicon column.

Operate centrifugation cycles at 14 000g at room temperature until a dilution factor of 50X is reached for the elution buffer:

25 µl elution buffer (one sample) + 475 µl PBS 1X : 20X dilution

Centrifuge 5min at 14 000g RT Ð concentrate = 74 µl

74 µl + 426 µl PBS 1X Ð dilution factor of 6.76X Ð 20*6,76 = 135,2X for the initial amount of elution buffer Ð above 50X dilution : exosomes are safe !

Centrifuge one last time to reach to desired volume of concentrate.

- Recover manually by pipetting the concentrate containing the exosomes.

Note: Exosome elution buffer contains a denaturing agent. The exosome elution buffer may interfere with downstream

applications unless diluted to 20-100 times or exchanged with wash/dilution buffer or your preferred buffer.

Wash/dilution buffer contains a synthetic polymer to reduce adsorption of exosomes on tubes and buffer exchange devices. However, the polymer may disturb certain downstream analysis of the exosomes.

B. Exosome direct lysis on the beads:

For protein quantification and Western Blot.

For one sample:

- Add 10-15 µl of lysis buffer (*) + protease inhibitors to the beads pellet.
- Incubate 15 minutes at +4°C.
- Recover the supernatant containing the lysed exosomes.
- Process it directly for downstream analysis or store it at -80°C until further use.

(*) lysis buffer: we recommend using RIPA buffer with a special SDS concentration of 2% to efficiently extract lipid raft domains which are detergent resistant components.

C. Flow cytometry analysis of the exosome-bead complex:

The exosome-bead complex has been washed two times with 200µl of wash/dilution buffer and is ready for flow cytometry analysis.

- Resuspend the exosome-bead complex in 100µl PBS (0.2 µm filtered).
- Add to your convenience the appropriate volume of staining antibody (fluorochrome conjugated antibody) to the 100µl exosome-bead complex.
- Incubate on a rotating wheel for 1 hour at room temperature in a dark place.
- Wash the exosome-bead complex two times with 300 µl PBS (0.2 µm filtered).
- Resuspend the exosome-bead complex in a final volume of 300 µl PBS (0.2µm filtered) and keep it on ice until flow cytometry processing.

RNA extraction of the exosome:

For RT-qPCR, NGS,...

The exosome-bead complex has been washed two times with 200µl of wash/dilution buffer and is ready for RNA extraction.

For the total RNA extraction of the exosomes, we recommend to follow, with a slight modification, the miRNeasy protocol from QIAGEN.

- Resuspend the exosome-bead complex in 100 µl PBS.
- Add 5 µg of linear polyacrylamide (LPA, GenElute SIGMA- 56575) to the sample.
- Process the polyacrylamide-containing sample through the miRNeasy protocol and start with 700 µl QIAzol extraction.

Be aware that protein interphase may not be visible after QIAzol extraction.

We recommend loading two times the extracted RNA on the miRNeasy column at step (7) in order to maximize RNA binding to the membrane.

- After miRNeasy extraction/purification, evaluate the RNA profile of your exosome sample with the RNA 6000 Pico kit on Agilent's 2100 BioAnalyzer.
- Process the RNA immediately into your preferred application or store at -80°C.

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