User Guide

Exo-spin™ midi columns

Exosome Purification Kit

For cell culture medium, saliva, urine and blood plasma/serum

Cat EX04

Protocol Version 5.5

Contents

Exo-spin™ midi columns Exosome Purification Kit

Product components

EX04-5 Exo-spin™ midi columns kit (5 columns)

- 5 x Exo-spin™ midi columns with waste collection tubes
- 1 x User Guide

EX04-20 Exo-spin™ midi columns kit (20 columns)

- 20 x Exo-spin™ midi columns with waste collection tubes
- 1 x User Guide

For all kits, large volume (15 ml or 50 ml) centrifuge tubes, PBS, and 1.5 ml microcentrifuge collection tubes are not supplied.

General exosome isolation information

A. Notes on cell culture

Fetal bovine serum (FBS) contains a large number of exosomes. Exosome-free FBS should be used in cell culture experiments, which can be obtained commercially. Alternatively, Vivaspin® 20 100kDa MWCO Polyethersulfone (GE Healthcare) or Amicon® Ultra-15 Centrifugal Filter Unit (Millipore) can be used to efficiently remove exosomes from FBS diluted 1:1 with PBS.

The number of exosomes that are obtained from a cell culture sample will vary depending on a variety of factors. These include the specific cell line, the length of time the cells are exposed to the medium, and cell density. Cancer cell lines may produce higher numbers of exosomes than non-transformed cell lines.

B. Notes on blood samples collection

Sample collection and handling prior to purification may have a significant impact on the quality of purified exosomes (Witwer *et al*., 2013). Sera can contain many platelet-derived exosomes released after clot formation. Use of heparin-based anticoagulants is discouraged because of possible effects on downstream applications (e.g. PCR).

Platelet-derived exosomes may be released from platelets by the physical forces associated with the blood sampling procedure. Standardization of sampling site, needle gauge (wider may be better), and other variables is recommended. To ensure the sample is not contaminated by skin fibroblasts, it has been suggested that the first few milliliters of drawn blood should be discarded.

Collected blood should be handled gently and processed rapidly (within 30 minutes of drawing).

C. Proteomic analysis

Precipitants can interfere with mass spectrometry analysis and so precipitation should not be used when purifying exosomes if mass spectrometry is to be performed. In such cases, an alternative concentration method should be used instead of precipitation prior to using the Exo-spin™ columns. To maximize the numbers of exosomes that can be purified from cell culture media, devices such as the CELLine Classic bioreactor flask (Sigma) can increase the concentration of exosomes in media by up to 8-fold.

Product information

Exo-spin™ technology combines Precipitation and Size Exclusion Chromatography (SEC), making it superior to techniques that rely solely on precipitation which result in co-purification of large amounts of non-exosomal proteins and other material as well as carry over of the precipitant.
However, EX04 kit only contains SEC columns as the precipitation step is not required for some applications (e.g. mass spectrometry downstream application and/or blood samples).

Isolated exosomes may be used in a variety of downstream applications including DNA and RNA studies, as well as in functional *in vitro* and *in vivo* exosome assays.

This kit has been developed to process different sample types:

- 75 to 500 ml starting volume per column for cell culture medium, saliva, urine, and other low-protein biological fluids
- 1 ml starting volume per column for blood samples (sera and plasma)

For more information in our exosome isolation range, a selection guide is available page 6.

Table. Exo-spin™ selection guide.

Cell culture medium **Saliva** **Urine** **Human breast milk*** **Cerebrospinal fluid*** **Any low protein biological fluids** (1x10^9 particles/ml)			**Blood samples** (plasma and sera)†		
Sample starting volume (per Exo-spin™ column)	**Isolation method**	**Exo-spin™ kit**	**Sample starting volume** (per Exo-spin™ column)	**Isolation method**	**Exo-spin™ kit**
<1 ml to 50 ml	Precipitation + SEC *(Centrifugation)*	EX01	**Sera**		
<1 ml to 75 ml	Precipitation + SEC *(Fractionation by gravity flow)*	EX05	≤100 µl	SEC *(Centrifugation)*	EX03
75 ml to 500 ml	Precipitation + SEC *(Fractionation by gravity flow)*	EX04	≤150 µl	SEC *(Fractionation by gravity flow)*	EX05
			<100 µl to 500 µl	Precipitation + SEC *(Centrifugation)*	EX02
			1 ml	SEC *(Fractionation by gravity flow)*	EX04
			Plasma		
			≤100 µl	SEC *(Fractionation by gravity flow)*	EX05
			<100 µl to 250 µl	Precipitation + SEC *(Centrifugation)*	EX02
			1 ml	SEC *(Fractionation by gravity flow)*	EX04

* For cerebrospinal fluid and human breast milk samples, validated protocols are available for EX01 only.
† Highly concentrated exosome samples (e.g. 1x10^{12} particles/ml) other than blood can also be used.

Protocol for purification of intact exosomes using Exo-spin™ midi columns

Supplied Exo-spin™ columns are pre-equilibrated with ultra-pure water containing 20% ethanol. The column matrix should be re-equilibrated with PBS prior to use.

A maximum sample volume of 500 ml may be used per column. For blood samples, the maximum sample volume is 1 ml per column. For larger sample volumes, use multiple columns per sample. **Please note that purchasing Exo-spin™ Buffer (cat EX06-30 (30 ml) or EX06-250 (250 ml)) is required for processing the aforementioned maximum volume of starting material for non-blood sample.**

All centrifugation steps can be performed at room temperature or 4°C unless otherwise specified.

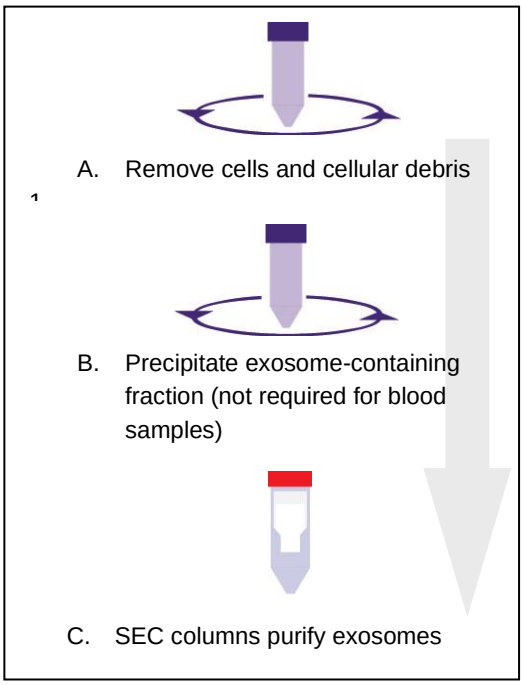

Figure. Protocol overview.

A. Prepare starting sample

Cell culture media, urine, saliva or blood sera

1. Transfer 75 – 500 ml of starting sample or 1 ml of blood sera to a microcentrifuge (not supplied with kit) tube and spin at 300 × *g* for 10 minutes to remove cells.

2. Transfer supernatant to a new microcentrifuge tube and spin at 16,000 × *g* for 30 minutes to remove any remaining cell debris.

Blood plasma

Adapted from Welton *et al.*, 2015.

1. Centrifuge blood plasma at 6000 x *g* for 10 minutes.

2. Filter the resulting platelet-free plasma through a 0.22 µm syringe filter. Separate sample into ≤1 ml aliquot.

B. Precipitate exosome-containing fraction (not required for blood sera/plasma, proceed directly to Part C)

3. Transfer supernatant to a new microcentrifuge tube and add Exo-spin™ Buffer in a 2:1 ratio (for example, add 50 ml of Exo-spin™ Buffer to 100 ml supernatant).

4. Mix well by inverting the tube and incubate at 4°C for at least 1 hour.
   Alternatively, the sample may be incubated overnight at 4°C. This may generate a small increase in exosome yield.

5. Centrifuge the mixture at 16,000 x *g* for 1 hour.

6. Carefully aspirate and discard the supernatant.
   Do not allow the sample to dry as this may cause damage to exosomes.

7. Resuspend the exosome-containing pellet in 1 ml of PBS. If the pellet does not readily resuspend, reduce the amount of starting material.

C. Exo-spin™ midi column preparation

8. Prepare the Exo-spin™ midi column prior to application of your sample.
   a. Remove the screw cap and discard the preservative buffer from the top of the column. Remove the outlet plug and replace the Exo-spin™ midi column into the waste collection tube provided. To prevent drying of the column bed, proceed to the next step immediately.

   b. Equilibrate the column by sequentially adding 2 x 10 ml of PBS and allow the column to drain under gravity. **Do not centrifuge.**

D. Purification of exosomes – high-resolution fractionation

9. When the column has been equilibrated, fit the column onto a clamp-stand, with a 1.5 ml microcentrifuge tube underneath the column for high resolution fraction collection.

10. Carefully apply 500 µl of the exosome-containing sample to the top of the column (filtered blood sera from step 2 or resuspended exosome-containing pellet in PBS from step 7). Collect the fraction in the 1.5 ml microcentrifuge tube by gravity and label the tube as Fraction 1.

11. Place a new 1.5 ml microcentrifuge tube underneath the column. Apply 500 µl fraction of the sample to the top of the column and collect by gravity. Label the tube as Fraction 2.

12. Place a new 1.5 ml microcentrifuge tube underneath the column. Apply 500 µl of PBS to the column and collect the fraction by gravity. Label the tube as Fraction 3.

13. Repeat this process with PBS a further 21 times, until 24 fractions of 500 µl each have been collected in total.

    The vast majority of the exosomes will elute between fractions 7 and 12. These fractions can be pooled to obtain a 3 ml fraction which contains highly pure exosomes. If a higher

yield is desired, 4 further fractions can be combined to obtain a 5 ml fraction (containing fractions 7–16).

As an alternative to steps 9 – 13, a quick collection protocol can be performed:

9. When the column has been equilibrated, carefully apply the 1 ml of exosome-containing sample to the top of the column (filtered blood sample from step 2 or resuspended exosome-containing pellet in PBS from step 7). Place the column into the waste tube and allow the column to drain under gravity.

10. Apply 2 ml of PBS to the top of the column. Allow the PBS to drain under gravity into a waste tube and discard.

11. Place the column into a new 50 ml collection tube. Add a further 3 – 5 ml of PBS and allow the PBS to drain under gravity to collect the exosome-containing eluate.
Use 3 ml to maximize exosome purity or 5 ml to maximize exosome yield.

Storage

Upon receipt, store Exo-spin™ midi columns at 4°C.

Correctly stored components are stable for at least 6 months following purchase.

Related products

Related products	Product description	Product code
Exosome detection	**Exosome antigen antibodies**	EX201, EX202, EX204, EX203
	TRIFic™ detection assay	EX101, EX102, EX103
Exosome tracking	**ExoFLARE™ tracking assay**	EX301, EX302, EX303, EX304, EX305, EX306
Nanoparticle Tracking Analysis (NTA) size profiling service	**ZetaView NTA Particle Analysis Service**	ZV-1 and ZV-12

TRIFic™ detection assay

The TRIFic™ exosome assay is similar to an ELISA, however, there are some significant differences. Unlike an ELISA, there is no enzymatic reaction. Rather, the target is directly detected with a Europium label. TRIFic™ exosome assays deliver clear, consistent, and quantitative data from purified or unpurified samples, including direct measurement of exosomes from plasma in a convenient 96-well format. TRIFic™ exosome assays are available for widely-used markers of exosomes, the tetraspanin proteins CD9, CD63 and CD81.

ExoFLARE™ tracking assay

ExoFLARE™ utilizes a combination of a FLARE (FLuorescence Activating Response Element) protein tag together with a pro-fluorophore dye. Neither the protein nor dye exhibit fluorescence in isolation. However, when the protein binds to the dye, it causes a change in structure which results in fluorescence. The dye and protein form an unstable bond with a continuous turnover of the dye, resulting in sustained fluorescence without the levels of photo-bleaching associated with fluorescent proteins (i.e. GFP). This enables ExoFLARE™ to be monitored for extensive periods to allow tracking of dye movement.

NTA size profiling service

Exosome characterization service for analysis of particle size and particle concentration using the ZetaView instrument from Particle Metrix.

Troubleshooting

My sample does not elute from the column.

- Ensure that the outlet plug has been removed from the base of the column.

My sample contains a lower amount of exosomes than expected.

- Ensure that the column does not dry out during the procedure.
- Adhere to the volumes indicated for sample addition to the column. If the sample volume is too small, the exosomes will be retained within the column.
- Ensure that precipitation of the exosome-containing pellet is performed for at least 1 hour at 4°C, and that the Exo-spin™ Buffer has been properly mixed with the sample.
- Exosome yield is dependent on a variety of factors, particularly the type of biological fluid used as starting material. If media is used, the amount of exosomes present will vary widely depending on the cell line and the length of exposure (conditioning) of cells to the media.

My sample has no measurable exosomes.

- This is most likely caused by complete drying out of the column causing loss of functionality. Ensure the columns are kept hydrated at all times.

I do not have a high-speed centrifuge.

- Increase the time of centrifugation by calculating the ratio of the recommended speed to the speed of your centrifuge. For example, if the protocol recommends to spin at 16,000 x *g* for 30 minutes, for a centrifuge with a maximum speed of 9,500 x *g*: 16000/9500=1.68 and 1.68*30 mins = 50.4 minutes.

References

- Witwer *et al*., Journal of Extracellular Vesicles 2: 10.3402/jev.v2i0.20360 (2013).
- Welton *et al.*, Journal of Extracellular Vesicles 4: 10.3402/jev.v4.27269 (2015).

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