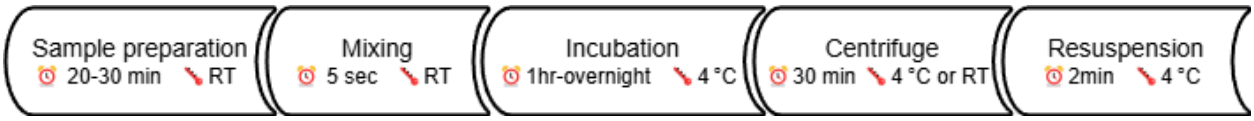
EV Isolation Protocol

This protocol describes the isolation of EVs from cell culture supernatants (conditioned medium) using the precipitation method. A full-size isolation reagent will be sufficient for processing up to 200mL of a sample.

Important notes before starting: To ensure that isolated exosomes originate from your cells of interest, culture the cells with exosome-depleted fetal bovine serum (FBS), because normal FBS contains extremely high levels of exosomes that will contaminate the cell-derived exosomes. Some cell lines can be grown for up to 12 hours in media without FBS.

Experiment Quick Guide



Prepare Sample

1. To obtain a cell-free specimen with low contamination, centrifuge the samples at 500 × g for 5-10 min to pellet cells.

2. Transfer the supernatant into a new tube, and centrifuge at 3000 × g for 15-20 min to pellet debris and aggregates.

3. Transfer the supernatant into a new tube prior to storage or immediate isolation. It is also highly recommended to centrifuge previously spun, frozen samples once more after thawing to remove cryoprecipitates. We recommend using a pipette to transfer supernatant instead of pouring it out.

- **This protocol is a starting point for general EV isolation from tissue culture medium. For the enrichment of exosomes (30-150 nm) only, we recommend a filtration step using a 0.2 µm syringe or spin-top filter before starting the precipitation protocol.*

EV Isolation Procedures

1. Collect cell culture media or biofluid, clear cell debris and aggregates by centrifugation following the steps as instructed above.

2. The final supernatant is transferred to a sterile tube or a conical tube. Gently mix the EV isolation reagent (avoid vortexing as it will cause foaming). Add the ¼ volume of EV isolation reagent to the supernatant. Reaction volume can be scaled accordingly. Close the tube and vortex for at least 5 seconds until a homogeneous solution is formed.

Sample Volume (mL)	EV Isolation Reagent (mL)	Reaction Tube
1 mL	0.25 mL	1.5-2.2 mL tube
10 mL	2.5 mL	15 mL conical
40 mL	10 mL	50 mL conical

- **Due to the high viscosity of the reagent, you typically need to pipette at a slower speed and pause before finishing every aspiration or dispense. This gives the liquid more time to smoothly move into and out of the tip. The alternative technique is reverse pipetting, by which you aspirate more than you need, dispense the desired amount slowly, and discard a smaller post-dispense volume.*

3. Incubate for at least 60min (can be extended to overnight) at +4°C.

- **Do not rotate or mix during the incubation period, and the tube should remain upright.*

4. Centrifuge sample mixture at 3000 × g (adjust according to the following table) for 30 minutes. Centrifugation may be performed at either room temperature or +4°C with similar results. After centrifugation, the EV may appear as a beige or white pellet at the bottom of the vessel.

Sample volume	Recommended tube size	Centrifuge speed
<1.5 mL	1.5–2.2 mL microcentrifuge tubes	10,000 g
1.5-10 mL	15 mL conical tubes	3000g
10-40 mL	50 mL conical tubes	3000g

- **We do not recommend starting volumes below 1000 µL. Working with smaller volumes will reduce precipitation efficiency.*

5. Aspirate supernatant. Spin down residual solution by centrifugation for 1 minute. Remove all traces of fluid by aspiration, taking great care not to disturb the precipitated exosomes in the pellet.

- **Sometimes EV pellets are not visible (e.g., exosome pellets from low starting volume of samples). A fixed-angle rotor will smear the pellet along the side of the tube, while a swinging-bucket rotor will pellet the exosomes at the bottom of the tube.*

6. Resuspend the pellet using exosome-depleted PBS or other specified buffers according to your downstream application.

Starting Cell Culture Media	Resuspension Volume
10 mL	25–100 µL
100 mL	100 µL–1 mL

- **The resuspension buffers must be filtered through 20nm to remove all the retention particles or aggregates.*

7. For protein or RNA isolation directly from the EV pellets, skip step 6 and follow the instructions for extractions.

8. The extracted EVs may be stored at 2–8°C for no more than a week. It is highly recommended to store isolated EVs/ exosomes at -20 °C or -80 °C for long-term storage. For RNA profiling, we recommend using the pelleted EVs immediately rather than freezing them for future use.

- **Multiple freeze-thaw cycles can cause damage to EVs and reduce their numbers. If multiple applications or thaws will be used for analysis, then aliquot the resuspension into multiple tubes to minimize the freeze/thaw cycle.*