Standard Operating Procedure

Midi Kit (Cat no. 01-MIDI)

40 isolation columns

Store at 4°C prior to use

Gentle, Efficient, Timely

GET EVs

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Summary: The GET EVs Isolation Kit works by separating EVs based on their affinity to achieve the lowest thermodynamic state, a biophysical principle known as the Aqueous Two-Phase Separation[1-4] (ATPS). Therefore, when EVs are mixed with two different phases/polymers (Dextran and PEG), EVs will preferentially accumulate in the lower DEX phase. Since the DEX phase volume is smaller than the starting volume, two benefits arise: 1) an enrichment of EVs and 2) depletion of non-EV entities, such as serum proteins. This kit can be combined with the Midi Enrichment Kit (Cat no. 02-MIDI) to achieve further rounds of enrichment.

You will need:

- Phosphate Buffered Saline (PBS)
  - Sterility and quality up to user
- Serological pipettes and pipettor
- Micropipette tips (wide bore if possible) and a P1000 micropipette
- Benchtop centrifuge that will accommodate 15 mL tubes (refrigerated centrifuge is optional)
- Vortex Machine

Abbreviations:

ATPS	Aqueous Two-Phase Separation
CM	Conditioned Media
DEX	Dextran
LP	Lower Phase
EV	Extracellular Vesicle
PEG	Polyethylene Glycol
PBS	Phosphate Buffered Saline
UP	Upper Phase
Prep	Preparation

Procedure:

1. **Place a single individual tube <u>upright</u> in any rack of your choice.**
   - This tube will contain a 1.8 mL mixture of the two polymers (DEX and PEG).
2. **Take your sample that contains the EVs of interest and dilute with PBS to make a final volume of <u>10.2 mL PBS</u>.**

- Deviating from this total volume will affect the possibility of the two phases (DEX and PEG) from properly separating/forming the two phases after the centrifugation step (Step #X).

3. **Take the diluted sample from Step #2 and add it into the 15 mL tube. Vortex for 1 minute. If you do not have a vortexer, mix with a pipette/micropipette. This will lead to a total of 12 mL in the tube. If there are visible clumps at the bottom of the tube, keep mixing until they are gone.**
4. **Centrifuge at 200 x g's for 15 minutes at room temperature (22-25°C).**
5. **Phase separation will form after centrifugation. This will appear as two phases: an *Upper Phase* containing PEG and a *Lower Phase* containing DEX and the isolated EVs. The Lower Phase will be approximately 1.2 – 1.5 mL. The Upper Phase (PEG phase) will be approximately 10.5 – 10.8 mL. The Lower Phase will appear turbid.**

   If you do not have a centrifuge, you can let the tube sit upright and wait for the phase separation occur naturally. In this way, it can take 30-45 min on average for the phase separation to form. Overnight separation can also be done which can improve EV isolation efficiency. **[Note]: The two phases are fragile after phase separation. Handle the tube gently and avoid mixing between the two phases. If any mixing occurs, centrifuge again.**

6. Set the pipettor speed from medium to low suction. With a 10 mL serological pipette, carefully remove the Upper Phase. Stop aspirating at around 0.5 mL above the interphase.

   [Note]: While aspirating, always keep the tip of serological pipette right beneath the liquid surface of PEG.

   [Note]: Avoid aspirating and disturbing the interphase.

7. With a P1000 micropipetter, carefully place the micropipette tip into the lower phase. Wait for 5 seconds until the interphase settles back. Then, slowly aspirate approximately 1.0 – 1.2 mL of the Lower Phase and then transfer into a clean 1.5 mL microcentrifuge tube.

   [Note]: Avoid disrupting and acquiring either the interphase or PEG phase when collecting the DEX phase.

8. If you wish to enrich the EVs further, please proceed to Procedure for Midi Enrichment Kit on page 4. If not, please proceed to Step #9.

9. Add 10 µL of Reagent A into the microcentrifuge tube containing the Lower Phase and its EVs.

10. Incubate for 15 minutes at room temperature (22-25°C).

    Incubation at any temperature +4°C to 37°C will not affect the quality of EVs. It will only affect the time needed for the reaction to reach completion. Incubating at lower

temperatures will require a longer reaction time. There is no benefit to incubating at 37°C unless specifically required by the user.

11. **Store the isolated EVs in -80°C for downstream analysis/analyses.**

If you find that you actually want to further enrich or purify your EV prep, please proceed to the **Procedure for Midi Enrichment Kit**. **This requires purchase of an additional kit (Cat no. 02-MIDI).**

End of Protocol

Procedure for Midi Enrichment Kit

Use this kit for a second round of ATPS which will achieve higher fold enrichment and further purify the EVs from protein contaminants (i.e. IgG, albumin, etc.).

12. Place a single individual tube upright from this kit in any rack of your choice. This tube is different from the Tube in Step #1 because it already has ~10 mL of PEG.
13. Transfer 1.2 mL of the Lower Phase from Step #8 into the tube from Step #12. This will lead to a total of 12 mL in the system.
14. Vortex for 1 minute. If you do not have a vortexer, mix with a pipette/micropipette. If there are visible clumps at the bottom of the tube, keep mixing until they are gone.
15. Centrifuge at 200 x g's for 15 minutes at room temperature (22-25°C).

16. Phase separation will form after centrifugation. This will appear as two phases: an *Upper Phase* containing PEG and a *Lower Phase* containing DEX and the isolated EVs. The Lower Phase will be approximately 0.35 – 0.4 mL. The Upper Phase (PEG phase) will be ~11 mL. The Lower Phase containing the enriched and further purified EVs may appear turbid.

17. Set the pipettor speed from medium to low suction. With a 10 mL serological pipette, carefully remove the Upper Phase. Stop aspirating at around 0.5 mL above the interphase.

    [Note]: While aspirating, always keep the tip of serological pipette right beneath the liquid surface of PEG.

    [Note]: Avoid aspirating and disturbing the interphase.

18. With a P1000 micropipetter, carefully place the micropipette tip into the lower phase. Wait for 5 seconds until the interphase settles back. Then, slowly aspirate approximately 0.4 – 0.42 mL of the Lower Phase and then transfer into a clean 1.5 mL microcentrifuge tube.

    [Note]: The entire DEX phase needs to be aspirated and some of the Upper Phase can also be isolated. This is because some EVs will also be trapped in the interphase during the second round of ATPS.

19. Add 10 µL of Reagent A into the microcentrifuge tube containing the Lower Phase which contains the further enriched and purified EVs.

20. Incubate for 15 minutes at room temperature (22-25°C).

    Incubation at any temperature +4°C to 37°C will not affect the quality of EVs. It will only affect the time needed for the reaction to reach completion. Incubating at lower temperatures will require a longer reaction time. There is no benefit to incubating at 37°C unless specifically required by the user.

21. Store the isolated EVs in -80°C for downstream analysis/analyses.

End of Protocol
