EXOTEST ®

SAMPLE PREPARATION PROTOCOLS

EXOTEST assay is validated for quantification and characterization of exosomes purified from different biological samples.

Here we list examples of exosome purification protocols used for preparation of both standards and processing of unknown samples.

Exosome purification from cell culture supernatants (modified from Thery et al, 2006)

Conditioned medium for exosome preparation and analysis should be collected from 80-90% confluent cells of interest. Fresh medium is typically added to cell culture 24-48 hours before supernatant harvesting. Supernatant collected in sterile conditions, with protease inhibitors added at 1:1.000 concentration, should be processed fresh, if possible. Otherwise, it should be pre-cleared by centrifugation (as shown in following steps 1 to 4) and either stored at +4°C for up to max 4 days or kept frozen at -20°C for longer periods. Noteworthy, long term storage and freezing of cell supernatants can diminish the yield of thereof obtained exosomes significantly. Strictly avoid repeated thaw and freeze cycles.

1. Pre-cool table top centrifuge to +4˚C. Collect supernatant from cell flasks and pour into 50 ml Falcon tubes. Store supernatant on ice until table top centrifuge has cooled down.
2. Centrifuge supernatant at 300 g for 10 minutes at +4˚C.
3. Pour supernatant into new Falcon tubes and centrifuge again at 1.200 g for 15 minutes at +4˚C.
4. Pour supernatant into new Falcons tubes and centrifuge at 10.000 g for 30 minutes at +4 ˚C.
5. Carefully transfer supernatant into ultracentrifugation tubes. Balance the tubes using 1xPBS, if necessary.
6. Ultracentrifuge at 200.000 g for 1 hour at +4 ˚C.
7. Remove the supernatant by pouring it off in case of swinging rotors (note: for swinging rotors the pellet will be set on the bottom of the tube) or, in case of fix angle rotors, by aspirating supernatant from opposite side of the pellet (note: for fix angle rotor, pellet will appear on the tube wall at the outer side of the rotor).
8. If some supernatant was left after 10.000 g centrifugation (step 4), you can add it to the same ultracentrifuge tubes (after step 7) and ultracentrifuge again at 200.000 g for 1 hour at +4˚C. Remove the supernatant as described above (step 7).
9. Fill the ultracentrifuge tubes with ice cold 1xPBS and ultracentrifuge at 200.000 g for 1 hour at +4˚C in order to wash the pellet. Remove the supernatant as described above (step 7).
10. Add 50 µl of PBS into the tube and vortex the pellet for 30 seconds. Re-suspend pellet thoroughly by pipetting. If you want to pool pellets from several tubes, transfer the same re-

suspended 50 µl into the next tube and repeat vortexing and re-suspend pellet thoroughly by pipetting. Perform these actions on ice.
11. Transfer exosome suspension into low binding protein tubes.
12. It is recommended to use exosome purified from the sample immediately while fresh. Otherwise add 0,01% NaN_3 and store at +4˚C for max 3 days, or freeze at -20˚C or -80˚C for longer periods. Note that prolonged storage and freezing can lead to some loss of exosomes from the sample.
13. Use Bradford method and BSA standards to measure the protein concentration of the sample (optional).

Exosome purification from human plasma (modified from Thery et al, 2006)

Blood samples collected in heparin or EDTA containing vessels are centrifuged for 10 minutes at 1.000 g within 30 minutes from collection. Obtained plasma is supplemented with protease inhibitors at 1/500 concentration and either assayed immediately or stored at -20°C until use. Strictly avoid repeated thaw and freeze cycles.

1. Thaw plasma samples on ice and pre-cool bench micro-centrifuge to +4˚C.
2. Centrifuge 1 ml of plasma sample at 300 g for 10 minutes at +4 ˚C.
3. Transfer supernatant in a new tube, add one volume of 1xPBS and centrifuge at 1.200 g for 20 min at +4˚C.
4. Carefully transfer supernatant in a new tubes without pellet contamination and centrifuge at 10.000 g for 30 min at +4˚C.

Note: Thus obtained plasma supernatants are considered a pre-cleared unfractioned plasma that can be assayed as such by EXOTEST, 100 µl per well.

5. Carefully collect supernatant, transfer it into disposable ultracentrifuge tubes and ultracentrifuge at 110.000 g for 2 hours at +4 ˚C.
6. Discard the supernatant. You can pour it off and remove remaining droplets carefully with a pipette.
7. Re-suspend the pellet in 100 µl of cold 1xPBS (pay attention to the expected position of the pellet, for fixed angle rotors it is on a side tube wall while for swinging rotors it is on the tube bottom), vortex for 30 seconds and pipette thoroughly. Bring the volume to 1,5 ml with cold PBS and filter into new tube using 0,22 µm syringe filter.
8. Ultracentrifuge again at 110.000 g for 1 hour at +4 ˚C.
9. Discard the supernatant and carefully dry the pellet by removing residual droplets and leave tubes upside down on filter paper.
10. Re-suspend the pellet in 100 µl of ice cold 1xPBS by vortexing for 30 seconds and thoroughly pipetting. This volume will be sufficient for 2 wells of an ELISA plate.
11. It is recommended to use exosome purified from the sample immediately while fresh. Otherwise, add 0,01% NaN3 and store at +4C for max 2 days, or freeze at -20˚C or -80˚C for longer periods. Note that prolonged storage and freezing can lead to a significant loss of exosomes from the sample.