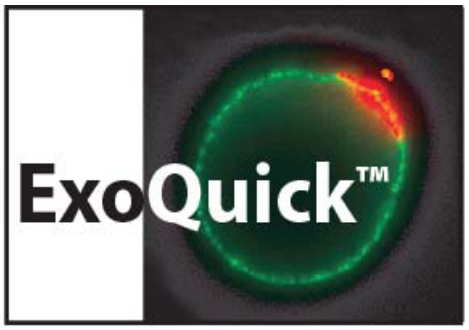


ExoQuick™ Exosome Precipitation Solution

Cat. # EXOQ5A-1
Cat. # EXOQ20A-1

User Manual

Store kit at 4°C on receipt

A limited-use label license covers this product. By use of this product, you accept the terms and conditions outlined in the Licensing and Warranty Statement contained in this user manual.

ver. 4-2011-07-11

Contents

List of Components

Item	Catalog #	Reactions
ExoQuick exosome precipitation solution (20 ml)	EXOQ20A-1	300 reactions
ExoQuick exosome precipitation solution (5 ml)	EXOQ5A-1	75 reactions

The ExoQuick™ kits are shipped at room temperature or on blue ice and should be stored at +4°C upon receipt. Properly stored kits are stable for 1 year from the date received. The reaction size is based on using 250 µl serum for exosome isolation. Examples of precipitating exosomes from various biofluids can be seen in the Table below.

Bio-fluid	Sample volume	ExoQuick volume
Serum	**250 µl**	**63 µl**
Ascites fluid	**250 µl**	**63 µl**

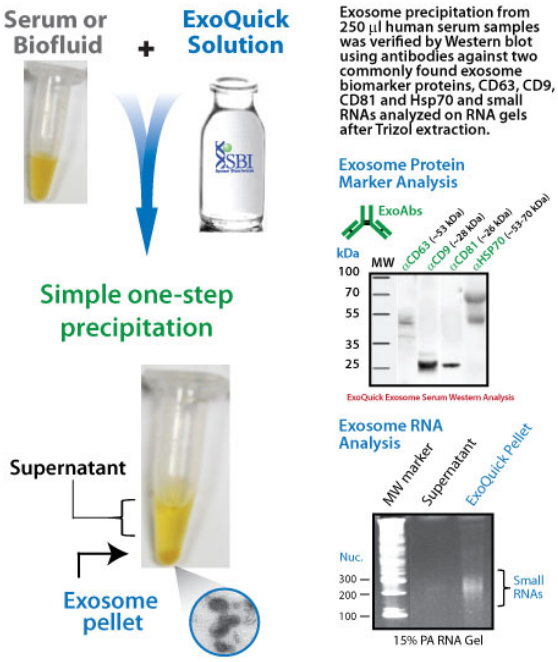
To isolate exosomes from tissue culture media or urine, we recommend using the ExoQuick-TC reagent (cat# EXOTC10A-1 or EXOTC50A-1) which is a distinct formulation from the original ExoQuick reagent detailed in this manual.

ExoQuick Exosome Precipitation

I. Overview

Exosomes are 40 –150 nm membrane vesicles secreted by most cell types in vivo and in vitro. Exosomes are found in blood, urine, amniotic fluid, malignant ascite fluids and contain distinct subsets of microRNAs depending upon the tumor from which they are secreted. SBI's ExoQuick exosome precipitation reagent makes microRNA and protein biomarker discoveries simple, reliable and quantitative. Enrich for circulating exosomal microRNAs with ExoQuick™ and accurately profile them using SBI's SeraMir™ qPCR arrays.

* No time-consuming ultracentrifugation
* Less expensive than costly antibodies and beads
* More effective than any other method
* Use as little as 100 µl of serum or bio-fluid



II. Exosome Precipitation Protocol

Isolate exosomes with ExoQuick

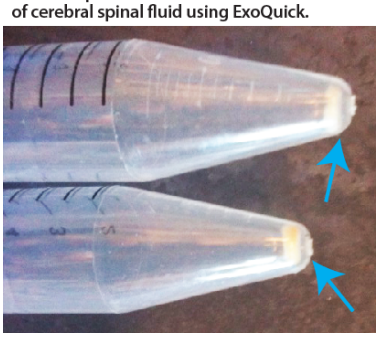
1. Collect biofluid and centrifuge at 3000 × *g* for 15 minutes to remove cells and cell debris.
2. Transfer supernatant to a sterile vessel and add the appropriate volume of ExoQuick Exosome Precipitation Solution to the bio-fluid. Some examples are shown in the Table below. Mix well by inverting or flicking the tube.

*Note when precipitating exosomes from plasma, the resulting pellet may be difficult to resuspend due to precipitated fibrin and other fibrinogens. For plasma, please refer to the Appendix for additional preparation steps.

Incubation Time	Bio-fluid	Sample volume	ExoQuick volume
30 minutes	Serum*	250 µl	63 µl
Overnight	Ascites fluid	250 µl	63 µl

3. Refrigerate overnight (at least 12 hours). The tubes do not need to be rotated during the incubation period.
4. Centrifuge ExoQuick/biofluid mixture at 1500 × *g* for 30 minutes. Centrifugation may be performed at either room temperature or 4°C with similar results. After centrifugation, the exosomes may appear as a beige or white pellet at the bottom of the vessel.

Exosome pellets obtained from 10 ml of cerebral spinal fluid using ExoQuick.



5. Aspirate supernatant. Spin down residual ExoQuick solution by centrifugation at 1500 × *g* for 5 minutes. Remove all traces of fluid by aspiration, taking great care not to disturb the precipitated exosomes in pellet.
6. Resuspend exosome pellet in 1/10 of original volume using sterile or nuclease-free water. If the pellet is difficult to resuspend, add slightly more water to the pellet to further dilute the salt.

III.

A. Using Precipitated Exosomes for RNA Extraction

For RNA extraction, we recommend following the protocol outlined in the SeraMir Kit user manual as shown here (Catalog #: RA800A-1, RA805A-1, RA806A-1, RA810A-1, and RA820A-1).

Exosome Isolation and Lysis

1. Thaw serum sample on ice
2. Combine 500µl serum + 120 µl **ExoQuick**
3. Mix well by inversion three times
4. Place at 4ºC for 30 minutes
5. Centrifuge at 13,000 rpm for 2 minutes
6. Remove supernatant, keep exosome pellet
7. Add 350 µl **LYSIS Buffer** to exosome pellet and vortex 15 seconds
8. Place at room temperature for 5 minutes (to allow complete lysis)
   *--- optional--- add 5*µl *of SeraMir control RNA spike-in (cat#RA805A-1)*

exoRNA Purification

9. Add 200µl of 100% **Ethanol**, vortex 10 seconds
10. Assemble spin column and collection tube
11. Transfer all (600µl) to spin column
12. Centrifuge at 13,000 rpm for 1 minute (check to see that all flowed through, otherwise spin longer)
13. Discard flow-through and place spin column back into collection tube
14. Add 400µl **WASH Buffer**

15. Centrifuge at 13,000 rpm for 1 minute
16. Repeat steps 13 to 15 once again (total of 2 Washes)
17. Discard flow-through and centrifuge at 13,000 rpm for 2 minutes to dry (IMPORTANT !)

exoRNA Elution

18. Discard collection tube and assemble spin column with a fresh, RNase-free 1.5ml elution tube (not provided)
19. Add 30µl **ELUTION Buffer** directly to membrane in spin column
20. Centrifuge at 2,000 rpm for 2 minutes (loads buffer in membrane)
21. Increase speed to 13,000 rpm and centrifuge for 1 minute (elutes exoRNAs)
22. You should have recovered 30-40µl exosome RNA

The yield of RNA from isolated exosomes is different depending on the starting biofluid or the type of cells that were grown in culture. Different cell types secrete varying levels of exosomes. For serum, the level of RNA isolated from 500 µl is usually in the 500ng range and can be measured using a Agilent Bioanalyzer or a NanoDrop Spectrophotometer.

B. Using Precipitated Exosomes for Protein Extraction

ELISA analysis

SBI offers three ELISA kits (Catalog#: ExoELISA-63, ExoELISA-9, ExoELISA-81) for fast and quantitative analysis of well-characterized exosomal protein markers**: CD63, CD9** and **CD81**.

1. If frozen, thaw culture media or urine sample on ice
2. Combine 10ml sample + 2ml **ExoQuick-TC**
3. Mix well by inversion three times
4. Place at 4ºC for overnight (at least 12 hours)
5. Centrifuge at 1500 × *g* for 30 minutes
6. Remove supernatant, keep exosome pellet
7. Centrifuge at 1500 × *g* for 5 minutes to remove all traces of fluid (take great care not to disturb the pellet)
8. Add 200 µl **Exosome Binding buffer** to exosome pellet and vortex 15 seconds
9. Place at room temperature for 5 minutes (to allow complete lysis)
10. Exosome protein is now ready for immobilization onto micro-titer plate.

Please refer to the ExoELISA manual for the complete protocol.

Western blot analysis

For Western blotting analysis, we recommend resuspending the exosome pellet in **1XRIPA buffer**[1] with the appropriate protease inhibitor cocktail.

SBI offers a Western blot antibody detection kit (Catalog# ExoAB-KIT-1) which includes four exosomal marker antibodies: **CD63, CD9, CD81, HSP70** and a Goat anti-Rabbit IgG HRP conjugated secondary antibody specifically tested for use in exosomal protein analysis.

Cat#	Description	Size
EXOAB-CD9A-1	Anti-CD9 Antibody (rabbit anti-human) with goat anti-rabbit HRP secondary antibody	25 ul
EXOAB-C63A-1	Anti-CD63 Antibody (rabbit anti-human) with goat anti-rabbit HRP secondary antibody	25 ul
EXOAB-CD81A-1	Anti-CD81 Antibody (rabbit anti-human) with goat anti-rabbit HRP secondary antibody	25 ul
EXOAB-Hsp70A-1	Anti-Hsp70 Antibody (rabbit anti-human) with goat anti-rabbit HRP secondary antibody	25 ul
EXOAB-KIT-1	ExoAb Antibody Kit (CD9, CD63, CD81, Hsp70 antibodies, rabbit anti-human) with goat anti-rabbit HRP secondary antibody	25 ul each
EXOEL-CD9A-1	Exosome ELISA Complete Kit (CD9 detection)	96 reactions
EXOEL-CD63A-1	Exosome ELISA Complete Kit (CD63 detection)	96 reactions
EXOEL-CD81A-1	Exosome ELISA Complete Kit (CD81 detection)	96 reactions

Exosome Isolation and lysis

1. If frozen, thaw culture media or urine sample on ice
2. Combine 10 ml sample + 2 ml **ExoQuick-TC**
3. Mix well by inversion three times
4. Place at 4ºC for overnight (at least 12 hours)
5. Centrifuge at 1500 × *g* for 30 minutes
6. Remove supernatant, keep exosome pellet
7. Centrifuge at 1500 × *g* for 5 minutes to remove all traces of fluid (take great care not to disturb the pellet)
8. Add 200 µl **RIPA buffer**[1] to exosome pellet and vortex 15 seconds
9. Place at room temperature for 5 minutes (to allow complete lysis)
10. Add **Laemmli buffer**[2] (with Beta-mercaptoethanol) and heat at 95ºC for 5 minutes.
11. Chilled on ice for 5 minutes before loading onto gel
12. Perform standard SDS-PAGE electrophoresis and Western transfer onto PVDF membrane
13. Block with 5% dry milk in Tris Buffered Saline + 0.05% Tween (TBS-T) for 1 hour
14. Incubate blot overnight at 4°C with SBI's exosome specific antibody (e.g. CD9) at 1:1000 dilution (5% dry milk in TBS-T)
15. Wash 3X with TBS-T
16. Incubate one hour at room temperature with SBI's Goat anti-Rabbit-HRP antibody at 1:20,000 dilution (5% dry milk in TBS-T)
17. Wash 3X with TBS-T
18. Incubate blot with chemi-luminescence substrate and visualize on film or other imaging equipment

[1] 1X **RIPA buffer** contains:
- 25mM Tris-HCl pH 7.6
- 150mM NaCl
- 1% NP-40
- 1% sodium deoxycholate
- 0.1% SDS

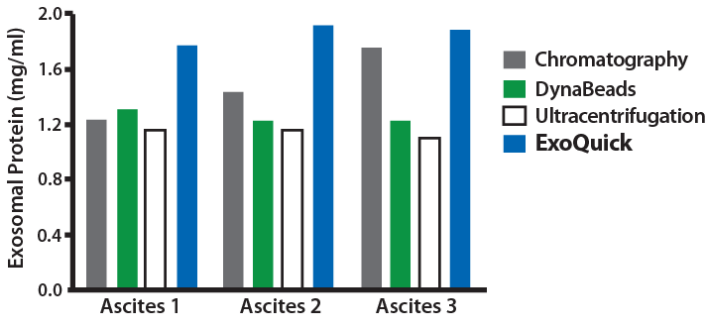
[2] 2X **Laemmli buffer** contains:
- 4% SDS
- 20% glycerol
- 10% 2-mercaptoethanol
- 0.004% bromphenol blue

0.125 M Tris-HCl pH 6.8

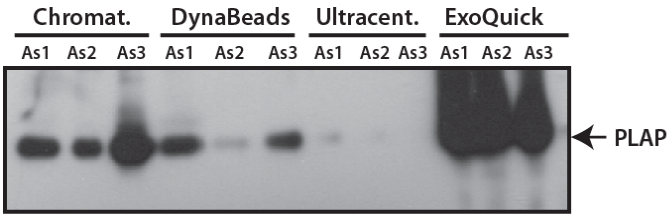
IV. Example Data and Applications

1. Protein Yield from Exosomes precipitated with ExoQuick versus other Extraction Methods

a.



b.

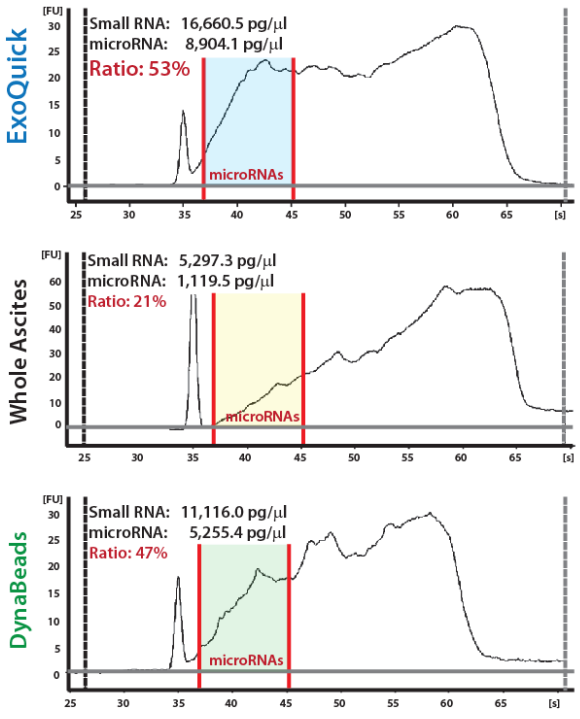


Data courtesy of Dr. Douglas Taylor, Univ. Lousiville, KY.

a. The quantity of protein was determined by the Bradford microassay method (Bio-Rad Laboratories) using BSA as a standard.

b. Proteins from each exosome isolate were standardized to the original sample volume and equal volumes were applied per lane of a 12.5% SDS-PAGE gel. Western immunoblotting was performed to analyze the presence of the specific marker protein, placental alkaline phosphatase (PLAP). The SDS-PAGE gel was transferred to a nitrocellulose membrane, the membrane blocked for 1 hour at room temperature with non-fat dried milk, and probed overnight at 4°C with primary antibody. The bound immune complexes were visualized by enhanced chemiluminescence (ECL, Amersham Life Sciences) and quantitated by densitometry (Un-Scan-it Software, Silk Scientific Corp).

2. MicroRNA Yield from Exosomes precipitated with ExoQuick versus other Extraction Methods



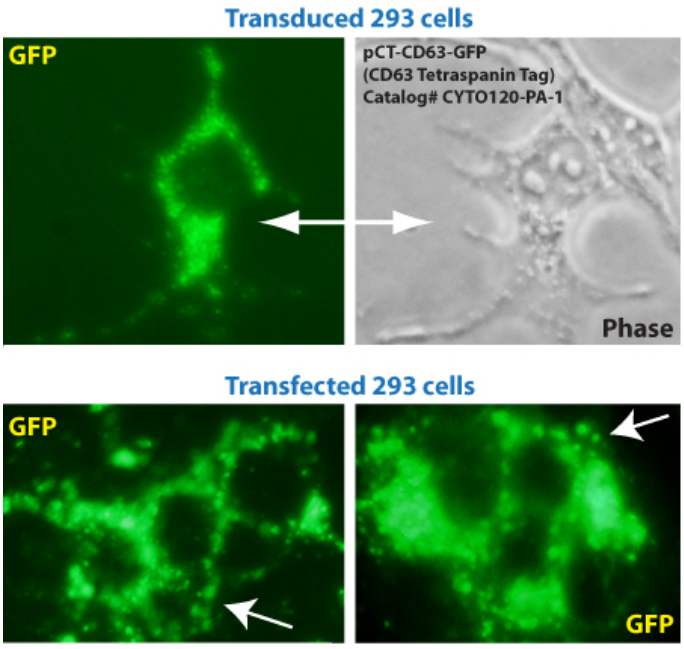
Agilent Bioanalyzer data courtesy of Dr. Douglas Taylor, Univ. Lousiville, KY.

The RNA quality and yield was accessed using a GeneQuant II. Small RNAs were analyzed with the Agilent 2100 Bioanalyzer Lab-on-a-Chip instrument system (Agilent Technologies), using the Agilent Small RNA chip and reagent kit. Approximately 100ng of isolated total RNA in 1µl was applied to each run. The manufacturer's recommended protocol was strictly followed to obtain Bioanalyzer profiles for the size range 6 to 150 nucleotides (nt). The profiles were calibrated for size (nt) using the small RNA ladder supplied with the kit, containing markers of 20, 40, 60, 80, and 150 nt in size, as reference. The instrument software quantitated the peak area between 0 and 150 nt as small RNA region, the area within 10 to 40 nt as microRNA region, and provides percentages of miRNA detected for each sample.

4. Activity Assays: Track Exosomes using Cyto-Tracers

SBI has created a line of lentivector-based Cyto-Tracers™ that utilize GFP-fusion proteins to mark cellular compartments, organelles, vesicles and structures to enable more long-term and more in-depth experimentation. The Cyto-Tracers can be used in transfections as well as packaged into virus to create stable GFP tracer cell lines in primary cells, tumor cell lines and stem cells.

The Tetraspanin CD63 protein is a common biomarker for exosomes. With the pCT-CD63-GFP construct you can make you cells of interest secrete exosomes that glow green for downstream functional delivery studies (Cat. # CYTO120-PA-1).

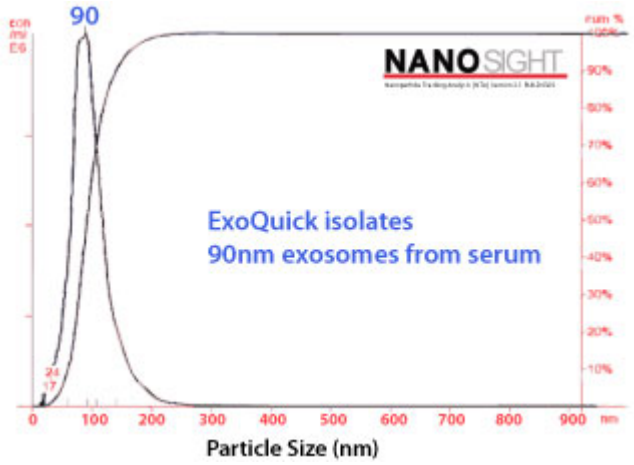


1. NanoSight

The NanoSight LM10 instrument is based on a conventional optical microscope and uses a laser light source to illuminate nano-scale particles within a 0.3 ml sample introduced to the viewing unit with a disposable syringe. Enhanced by a near perfect black background, particles appear individually as point-scatterers moving under Brownian motion.
The image analysis Nanoparticle Tracking Analysis (NTA) software suite allows users to automatically track and size nanoparticles on an individual basis. Results are displayed as a frequency size distribution graph and output to spreadsheet.

ExoQuick serum exosome analysis

Normal human serum from 50 pooled samples was used. Only 250ul serum was combined with 63ul ExoQuick to pellet the exosomes in 30 minutes. The exosome pellet was resuspended in 100ul PBS, diluted 1:10,000 and visualized on the NanoSight LM10 instrument. The analysis shows that ExoQuick isolated 90nm exosomes with a recovery of 2.74 x 10^12 particles/ml.



Data acquisition and analysis

These experiments were done in collaboration with Particle Characterization Laboratories, Inc (PCL).

V. Appendix

Pacific Hemostasis Thromboplastin D (Thermo cat# 10-0356)

1. Use ½ amount of Thromboplastin D in Pacific Hemostasis' protocol.
2. Squirt in Thromboplastin D reagent rapidly into plasma sample to mix thoroughly
3. Incubate at 37°C for 15 minutes.
4. Spin at 10,000 rpm at RT for 5 minutes (microfuge)
5. Save supernatant = "serum-like" sample, discard coagulated pellet
6. Use "serum-like" supernatant with ExoQuick to isolate exosomes as stated in ExoQuick protocol (use 25ul ExoQuick per 100ul coagulated plasma supernatant).

VI. References

As featured in: **Exosome Isolation for Proteomic Analyses and RNA Profiling** Douglas D. Taylor, Wolfgang Zacharias and Cicek Gercel-Taylor, Serum/Plasma Proteomics, Methods in Molecular Biology, 2011, Volume 728, Part 4, 235-246, (PDF) »

Tae Hoon Lee, Esterina D'Asti, Nathalie Magnus, Khalid Al-Nedawi, Brian Meehan and Janusz Rak. Review: Microvesicles as mediators of intercellular communication in cancer—the emerging science of cellular 'debris'. Seminars in Immunopathology DOI: 10.1007/s00281-011-0250-3. (PDF) »

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VII. Technical Support

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Technical Support: tech@systembio.com
Ordering Information: orders@systembio.com

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