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ExoMir™ PLUS Kit Manual

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The *ExoMir™ PLUS Kit is intended for laboratory use only, unless otherwise indicate. ExoMir™ is a Trademark of Bioo Scientific Corporation (BIOO).*

Note: Periodically, optimizations and revisions are made to the components and instruction manual. Therefore, it is important to use the manual included with the kit. If you need further assistance, you may contact your local distributor or BIOO at techsupport5@biooscientific.com.

GENERAL INFORMATION

Application

This kit is designed for the fractionation and RNA extraction from large, medium, and small membrane-bound particles in cell-free fluids. Large particles include cells and apoptotic bodies; medium particles include so-called microvesicles, and smaller particles include exosomes. Examples of cell-free fluids that can be processed with this kit include blood serum, urine, and cell culture media. The ExoMir-PLUS kit differs from the ExoMir kit in having a third filter, which has a pore size of 700 nanometers (nm) and is used as the Top filter to trap the largest particles. Passing the sample through the 700 nm filter avoids the need to carry out an initial low-speed centrifugation of the sample to eliminate intact cells whose RNA content could obscure the RNA from the smaller particles. Including the 700 nm filter may also reduce clogging problems and allow higher sample volumes to be processed. The ExoMir-PLUS kit is especially useful for allowing the initial sample processing steps to be carried out “in the field”, in situations where large-capacity centrifuges are not available.

Recent research has highlighted the role of small membrane-bound particles such as exosomes and microvesicles, as intercellular mediators of biological information (see references). Particles of various types and sizes are shed from solid tissues, leukocytes, and platelets, and find their way into the circulation and other bodily fluids such as urine and saliva. Exosomes, microvesicles, and other membrane-bound microparticles are also shed into the culture media by eukaryotic cells propagated in vitro. Shed microparticles have been shown to transfer active mRNA and microRNA between cells. Release of microparticles may increase in number and type during pathological conditions, especially malignancies. Traditional methods for recovering microparticles involve centrifuging liquid samples at increasing centrifugal force, to sequentially pellet the larger and smaller particles. To recover exosomes, ultracentrifugation of samples at relative centrifugal force of at least 100,000 x g for several hours or more is required.

Product Description

The ExoMir™ PLUS kit uses an alternative approach, in which samples are passed over syringe filters to capture small membrane-bound particles. The filters are then separated and flushed with an RNA extraction reagent to lyse the captured particles and release their contents. In the ExoMir-PLUS procedure, samples are passed over 3 filters connected in series, where the Top filter has the largest pore size of approximately 700 nm, the Middle filter has a pore size of about 200 nm, and the Bottom filter has the smallest pore size of approximately 20 nm. The liquid samples are aspirated or poured into standard syringes of size appropriate for the volume of liquid to be processed.

After the sample has passed through, the filters are disconnected and separately flushed with BiooPure™-MP to lyse the captured particles and release their contents. BiooPure-MP is a single-phase RNA extraction reagent containing guanidinium thiocyanate and phenol, which has been optimized to provide maximal recovery of the low-mass amounts of RNA in microparticles. Recovery of RNA is further improved by using the inert co-precipitant (linear acrylamide) included in the kit.

The kit also contains Proteinase K, which can be used in an optional sample pre-treatment step to minimize filter clogging and non-specific signal from RNA not associated with particles. The benefit of including the Proteinase K treatment depends on the type and volume of the sample. In general, the enzymatic sample treatment is useful for samples having high protein content, such as blood serum, especially when a relatively large volume (> 10 mL) is processed. The Proteinase K treatment may also be beneficial for pre-treating large volumes (greater than ~ 30 mL) of samples with lower protein concentration, for example conditioned media from cultured cells. The Proteinase K treatment also typically reduces the RT-qPCR signal of target microRNAs from the pre-filtered and post-filtered/flow-through samples. This RNA may be nonspecifically associated with extracellular proteins (see refs 13 and 15). The microRNA signal in pre-filtered and post-filtered samples varies according to the microRNA being detected.

Kit Contents, Storage and Shelf Life

The kit contains sufficient components to treat and fractionate 10 samples of cell-free fluids, using 3 filters for each sample, and to extract RNA from all filters. BiooPure-MP can be purchased separately (cat # 5301-05) to use for processing additional samples such as pre-filtered or post-filtered cell-free fluids.

The shelf life of the *ExoMir™* PLUS kit is 12 months when stored properly.

Kit Contents	Amount	Storage
Syringe Filter Assembly	10 each of three-filter assemblies	20-25°C
Proteinase K (lyophilized)	10 mg	-20 ºC
Proteinase K Resuspension Solution (contains 50% glycerol)	1.0 mL	-20 ºC
BiooPure™-MP RNA Isolation Reagent	30 mL	4 ºC
Co-precipitant (inert linear polyacrylamide) (18 µg/µL)	0.3 mL	-20 ºC
RNase-free water	7.5 mL	20-25°C
RNA Resuspension Solution (0.1 mM EDTA in nuclease-free water)	1.8 mL	20-25°C
60 mL syringe	1	20-25°C
20 mL syringe	1	20-25°C
3 mL syringe	1	20-25°C

Required Materials Not Provided

- 1-Bromo-3-Chloropropane (BCP); suggested source: Sigma Life Science (cat #B9673). Store at room temperature. Chloroform may be used as an alternative to BCP.
- Additional Luer Lock syringes of appropriate size, depending on sample volumes to be filtered
- Additional 3 mL syringes used to flush the filters with BiooPure-MP (optional)
- Waste collection tubes to catch filtrate (for example, 15 mL or 50 mL plastic tubes) (optional)
- 1.5 mL microfuge tubes
- P-1000, P-200, P-10 pipettors and tips; note, small-bore tips are required for last step
- Isopropyl alcohol
- Ethanol (may be denatured with 5% isopropanol)
- Vortex mixer
- Microcentrifuge capable of at least 14,000 x g or 12,000 rpm
- Cabinet incubator at 37°C for Proteinase K treating samples (optional)
- Heat block at 65°C for solubilizing RNA at end of procedure (optional but recommended)

Warning

BiooPure-MP reagent in the ExoMir-PLUS kit contains phenol so be sure to wear lab coat, gloves, and safety goggles during the procedure. See MSDS for more information related to precautions and hazards associated with use of the BiooPure-MP reagent. The MSDS is available on-line at http://www.biooscientific.com/up/BiooPureMSDS.pdf

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SAMPLE PREPARATION

Pretreatment of sample with Proteinase K (optional)

This step is useful for minimizing clogging when filtering relatively large volumes (> 12 mL) of cell-free fluids that are viscous and/or contain high concentration of protein; a primary example of this type of sample is blood serum. Serum samples less than ~12 mL in volume can usually be filtered completely without this treatment, but RNA yields may be increased slightly by including it. The enzymatic treatment may also be useful for reducing clogging problems when filtering very large volumes (greater than about 30 mL) of less-viscous solutions, for example conditioned media recovered from mammalian cells cultured *in vitro*. The tendency for conditioned media samples to clog the filters varies according to type of cells, growth media, and density of cell growth; in some cases, Proteinase K treatment may be beneficial for conditioned media samples of less than 30 mL, or may not be needed for samples over 30 mL.

1. Add 100 µL of Proteinase K solution to the liquid sample and mix by inversion or gentle vortexing. In general, up to 20 mL of serum or 40 mL of tissue culture media can be treated.

2. Incubate prep at 37ºC or room temperature for 30 minutes – 1 hour.

EXOMIR™ PLUS KIT PROTOCOL

Reagent Preparation

- Prepare Wash Solution (75% ethanol) by adding 22.5 mL of ethanol to the bottle containing 7.5 mL of RNase-free water, provided with the kit, and mix thoroughly. Store at room temp.

- Dissolve the Proteinase K, which is supplied as 10 mg of lyophilized powder, in the 1 mL of Proteinase K Resuspension Solution provided with the kit. Vortex briefly and pop-spin, and store the reagent at -20ºC after use. The Proteinase K concentration after dissolving is 10 mg/mL.

Particle Capture

1. Load sample into syringe
   Standard sample volumes are 10 mL – 40 mL of conditioned media or 4 mL – 12 mL of blood serum or 20 – 60 ml of urine. Maximum volumes of other types of samples should be determined empirically. The sample may be loaded into the syringe either by aspiration (inserting the open end of the syringe into the container of sample and retracting the plunger) or by removing the plunger, attaching the filter stack, pouring the sample into the barrel of the syringe, then re-inserting the plunger. Use a syringe with a Luer-lok fitting, of appropriate size for the volume of sample to be filtered.
   Note: to extract RNA from the pre-filtered sample or from the post-filtration flow-through, mix 0.2 ml (200 µl) of the cell-free fluid with 1 ml BiooPure-MP. Samples may be stored at -20°C until ready to process. Additional BiooPure-MP (cat # 5301-05) may be purchased from Bioo Scientific.

2. Pass the sample through the filter stack
   Attach the inlet side of the top filter (marked with a red dot) to the outlet of the syringe if it was not already attached in Step 1. The filters should be tightened enough to ensure the connection is secure. Position the assembly over a reservoir to collect the flow-through if desired.

   Gently depress the plunger to pass the media through the filters. The sample should flow through

at a rate of ~ 2-3 drops per second. If the filters clog, as indicated by slowed flow rate (< 1 drop per second), and excessive force being required to depress the plunger, do not attempt to force more sample through the filter.

3. Remove Filters
   After the sample has been filtered, remove the filter disks from the syringe and separate them by gently pulling them apart. The filter disks contain particles captured from the liquid sample.

4. Remove residual fluid from the filters
   After removing and separating the filters, separately re-attach each filter to the syringe with the plunger of the syringe retracted to the level of ~ 3 mL, then gently depress the plunger to force the residual drops of fluid out of each syringe filter. There should be a visible clearing of the residual fluid from the top chamber of the filter housing. Complete removal of residual fluid from the filter disks is important to minimize the volume of aqueous phase recovered during the RNA extraction step, which will allow the RNA precipitation step to be carried out in a single 1.5 mL tube.

Lyse Captured Particles to Release RNA

Note: Wear safely goggles during this step!

1. Remove the plunger from a 3 mL syringe, attach the syringe barrel to a filter with captured particles, and load the syringe with 1 mL of BiooPure-MP.

2. Position outlet of the filter disk over a 1.5 mL microfuge tube, then re-insert the plunger and slowly depress it to flush the filter with the BiooPure-MP reagent. Tilt the assembly if needed to allow the entire surface of the filter to contact the reagent. When the filter is saturated with reagent, slightly pump the plunger up and down several times to aid in complete mixing of the reagent with the trapped particles on the filter, then depress the plunger completely to recover all the BiooPure-MP lysate into the 1.5 mL tube.

   Note: The filter will remain green even after thorough recovery of the BiooPure-MP lysate.

3. Repeat these steps to process the remaining filter disks. The preps may be stored at -20ºC at this step.

RNA Extraction

1. Add 100 µL of 1-Bromo-3-Chloropropane (BCP) to the lysate. Alternatively, use 200 uL of chloroform. Vortex vigorously for ~ 20 seconds to create an emulsion of the lysate with the BCP and store the preps at room temp for ~5 minutes before proceeding to the next step. Note: If the preps were frozen prior to beginning this step, thaw at room temperature before adding the BCP.

2. Centrifuge preps for 15 minutes at 12,000 rpm, preferably at 4ºC.

3. Remove aqueous phase (the colorless top phase) to fresh 1.5 mL tubes. The volume of aqueous phase is usually ~450 – 550 µL from filter samples and ~550 – 650 µL from pre-filtered or flow-through samples. Do not remove more than 0.75 mL of aqueous phase per 1.5 mL tube, since this would not allow sufficient space for isopropanol precipitation in the subsequent step. Avoid removing material from the interface or lower organic phase; however, the aqueous phase can be removed almost completely at this step since the amount of material at the interface is typically low compared to extractions from solid tissue.

4. Add 3 μL of the co-precipitant provided with the kit to the aqueous phase and vortex thoroughly, It is important to vortex thoroughly (~5 seconds) to completely disperse the co-precipitant. If desired, pop-spin the preps (spin briefly in microfuge) to remove liquid from around rim of tube before opening it in the next step.

5. Add isopropanol equal to or slightly greater than the volume of aqueous phase and vortex thoroughly. This can generally be approximated as 0.55 mL of isopropanol. Store prep at -20ºC for at least 1 hour to precipitate the RNA. Note, this is a stopping place; storage can be extended to overnight or longer if desired.

6. To recover the RNA, centrifuge the prep for 15 - 20 minutes at maximum speed (at least 12,000 rpm) in a microcentrifuge, preferably at 4ºC.

7. Use a P-1000 to remove supernatant fluid down to level of ~30 μL. Do not aspirate all the fluid, as this runs the risk of losing the pellet of RNA. Small pellets are usually but not always visible at this step. Decanting the supernatant fluid instead of aspirating at this step runs the risk of losing the RNA pellet.

8. Wash the pellet with 0.8 mL of Wash Solution (75% ethanol). Vortex briefly but thoroughly enough to dislodge the pellet and ensure that the Wash Solution comes into contact with the entire inner surface of the tube.

9. Centrifuge for 10 minutes at maximum speed (at least 12,000 x g), preferably at 4ºC.

10. Carefully remove supernatant down to level of ~ 10- 20 μL. To minimize chance of aspirating the pellet, exercise care when removing the bottom portion of supernatant that lies directly above the pellet.

11. Re-spin tubes at ~10,000 x g for ~ 5 seconds to collect all residual fluid at bottom of tube. Orient tubes with hinges facing outward.

12. Use a fine-bore pipet tip (e.g. a P-10 tip) to completely remove all residual fluid from the bottom of the tube, taking care to avoid aspirating the pellet. A small white pellet should be visible.

13. Resuspend pellet in 50 μL of RNA Resuspension Solution provided with the kit. After adding the solvent, vortex briefly and pop-spin to recover fluid at bottom of tube. The RNA may be resuspended in as little as 25 μL to maximize the concentration.

14. Incubate the prep in 65ºC heat block for ~ 5 minutes to completely solubilize the RNA, then re-vortex and pop-spin. Store the RNA at -20ºC.

ADDITIONAL INFORMATION

Quantitation/Analysis of RNA

The RNA concentrations as determined by analysis on a Nanodrop 2000 are usually in the range of 30 – 150 ng/ µl (for pellets resuspended in 30 – 50 µl). For reasons that are not clear, the Abs 260/280 ratios from material recovered from the filters, as well as from the pre-filtered sample and post-filter flow-through fraction, are lower than expected, usually ranging from ~1.4 – 1.5. The RNA concentration from the pre- and post-filter samples is often similar to that recovered from the filters, as determined by Nanodrop analysis, however the microRNA signals from material recovered from the filters are usually higher by ~50 - 1000 fold, as determined by qRT-PCR, compared to the corresponding pre-or post-filtered samples. For some microRNAs, a more substantial proportion of the signal is observed in the pre- or post-filtered sample. These results are consistent with those reported in refs 13 and 15. Pretreatment of samples with RNase and Proteinase K prior to filtration usually leads to at least a several-fold reduction in microRNA signal from pre-or post-filtered samples, with much less if any reduction in signal from RNA trapped on the filters. Samples may be treated before filtration with 3 µg/mL of RNase A (not included with the kit) at 37ºC for 30 min – 1 hr, followed by treatment with Proteinase K as described in the protocol. The extent to which RNase A / Proteinase K pretreatment reduces microRNA signal in the pre-and post-filtered samples may vary according to the target microRNA being detected.

The mass amount of RNA recovered from exosomes and other microparticles is too low to visualize by ethidium staining on agarose gels. The RNA can sometimes be detected by capillary electrophoresis on the Agilent Bioanalyzer, depending on the amount and type of sample. The RNA lacks the typical 18S and 28S ribosomal RNA peaks seen in RNA extracted from cells and tissues; on a standard Agilent chip, the RNA migrates as a broad peak centered around 100 bases. Since microparticles are derived from the cytoplasmic cell compartment, they are not expected to contain appreciable amounts of genomic DNA.

RT-qPCR analysis of RNA

The best way to confirm recovery of RNA from microparticles is by reverse transcription-polymerase chain reaction (RT-PCR) or by RT followed by quantitative PCR (RT-qPCR). An example of data from an experiment designed to detect several microRNAs using the RT-qPCR stem-loop microRNA assays from Applied Biosystems/Life Technologies is shown in Table 1. The microRNA targets were detected using 2.5 µL of input RNA, corresponding to ~17% of the RNA recovered, in a protocol that was modified from that in the Applied Biosystems/Life Technologies instruction manual as described below.

Detection of microRNAs in human urine from particles trapped on ExoMir-PLUS filters, using RT-qPCR. In this experiment, 60 mL of a human spot urine sample was filtered through the 3- filter stack, without prior enzymatic treatment. Values shown in the table below are average Ct (Cycle threshold) values of duplicate qPCRs from a single RT, except when values differed by more than 1 Ct, in which case both values are shown. Duplicates were less than 1 Ct apart except for the pre-filtered and negative control samples. ND = not detected after 40 cycles.

Sample	miR-16	miR-204	miR-223
RNA from Top filter	21.66	27.51	24.98
RNA from Middle filter	28.46	22.99	30.75
RNA from Bottom filter	35.88	25.67	29.40
RNA from pre-filtered sample (0.2 ml)	33.75 / 36.07	33.18 / ND	34.47 / 35.69
Negative control (input was 1.5 µL of no-RNA-added RT reaction)	38.04 / ND	36.61 / ND	37.95 / ND

Modified Protocol for RT-qPCR miRNA analysis using Life Tech assay

1. Reverse Transcription step: (total reaction volume = 7.5 µL). Assemble first 5 components as a separate Master Mix for each microRNA. Aliquot 5 µL of Master Mix to each 0.5 mL reaction tube, then add 2.5 µL of sample RNA to each tube and mix well. Include a negative control with 2.5µl water instead of sample RNA.

Component	Volume per 7.5 µL Reaction	Source
10X RT buffer	0.75 µL	Bioo Scientific (cat# 521002)
Nuclease-free water	1.5 µL	Bioo Scientific (cat # 801001)
4dNTP mix, 10 mM each	0.75 µL	Bioo Scientific (cat # 370601)
MML-V Reverse Transcriptase (200 units/µL)	0.5 µL	Bioo Scientific (cat # 521001)
microRNA RT stem-loop primers	1.5 µL	Applied Biosystems TaqMan microRNA assays
Sample RNA	2.5 µL	from ExoMir filters or pre-filtered or post-filtered sample

2. Incubate reactions in thermalcycler as follows: 30 minutes at 16ºC / 30 minutes at 42ºC / 5 minutes at 85ºC/ 5 minutes at 4ºC / soak at 18ºC).

3. qPCR step (20 µL each reaction):

Component	Volume per 20 µL Reaction	Source
RT reaction	1.5 µL	Above reaction
TaqMan microRNA Assay	1 µL	Applied Biosystems Forward and Reverse PCR primers and the FAM-labeled TaqMan probe specific for each microRNA target
Nuclease-free water	13.5 µL	Bioo Scientific (cat # 801001)
Hot DuroTaq™ 5x qPCR Mix (Rox™ Free)	4 µL	Bioo Scientific (cat #370401)

Assemble reactions and run following real-time profile:
Hold 10 min at 95 ○ C, then cycle 40X: 95 ○ C for 10 sec / 60 ○ C for 60 sec.

TROUBLESHOOTING

Filter clogs during sample filtration step

Recommended Actions:

a. Carry out optional Proteinase K pretreatment as described in Protocol.

b. Reduce sample volume.

c. Make sure there is no air bubble between the liquid sample and the filter stack. To remove an air bubble, retract the plunger to aspirate the bubble up into the syringe, then remove the filter stack and expel a few drops of sample, leaving fluid in the outlet of the syringe. Then reattach the filter stack being sure that the fluid column is continuous with the inlet of the Top filter and that no air is introduced.

d. Filter the sample through each filter separately, starting with the Top, then the Middle, and then the Bottom filter. Save the flow-through from the Top and Middle filters for processing through the subsequent filter(s).

e. If sample was blood plasma, consider using serum instead of plasma. To use serum, collect blood without anticoagulant and allow blood to clot, then centrifuge to fractionate sample into cellular components and non-cellular serum, and remove serum for use. Serum is depleted of blood clotting factors, which may help to avoid clogging. Serum samples that are turbid usually indicate presence of high concentration of lipids, and these may not be suitable for filtration, even after Proteinase K treatment.

Volume of Aqueous Phase Exceeds 0.75 mL

(Volume of aqueous phase at removed exceeds 0.75 mL, so that the RNA cannot be precipitated in single 1.5 mL tube)

Recommended Action: Completely remove residual fluid from the syringe filter disks before flushing with BiooPure-MP, as described in the protocol. Precipitation of the RNA in 2 mL tubes is not recommended because the shape of the tube does not taper to a point, so the RNA/co-precipitant does not form a visible pellet; also, removing the last traces of supernatant fluid from 2 mL tubes without disturbing the RNA is difficult.

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